Effects of phytosterols contained in pulp mill effluent on *D. magna* and *D. obtusa*

Claudia Regina Xavier (1), José Becerra (2), Víctor Hernández (2), Soledad Chamorro (1) and Gladys Vidal (1).

(1) Environmental Science Center EULA- Chile. (2) Natural Products Chemistry Laboratory, Faculty of Natural and Oceanographic Science. University of Concepción. P. O. Box 160-C. Concepción- Chile. (e-mail: glvidal@udec.cl)

Extractives constituents of bleached kraft pulp mill (BKPM) effluents are focused as sources of toxicity. Phytosterols are among the compounds present in wood extractives. Effluents with phytosterols concentrations as low as 0.26- 1.0 mg/L have reported chronic toxicity. β-sitosterol and stigmasterol have been detected in treated BKPM effluents. Daphnids like *D. magna* have used for testing acute and chronic toxicity of effluents and pure compounds. The aim of this work is to evaluate the acute toxicity of BKPM effluent and phytosterols by using *D magna* and *D. obtusa*.

BKPM effluent from a local industry was used in the assays. The effluent was characterized regarding pH, Chemical Organic Demand (COD), Biological Organic Demand (BOD_5), phenolic compounds (UV_{215}), color (VIS_{440}), and phytosterols contents. The acute toxicity at 24 - 48 h to female *D. magna* and *D. obtusa* (< 24 h old) were evaluated for BKPM effluent and β-sitosterol & stigmasterol (0.3 and 1.0 mg/L). The lethal concentration (LC_{50}) was calculated using the Probit and the Spearman-Karber methods. COD, BOD_5, phenolic compounds (UV_{215}) and color (VIS_{440}) of effluent were performed according to Standard Methods. Phytosterols contents were measured by CG-MS, after dichloromethane extraction. COD and BOD_5 concentrations were 881 ± 24.3 mg/L and 300.5 ± 9.44 mg/L, respectively. Concentrations of β-sitosterol (0.333 ± 0.028 mg/L) and stigmasterol (0.069 ± 0.014 mg/L) were measured in the effluent. Acute toxicity of the effluent was obtained to *D. magna* (LC_{50} 48 h 42.08 ± 6.5 %), however not toxicity was detected on *D. obtusa*. Moreover, no toxicity was detected by daphnids (LC_{50} 24 h> 100 %) in β-sitosterol or stigmasterol separately. Conversely, 0.3 mg/L of β-sitosterol & stigmasterol concentrations were toxic to *D. magna* (LC_{50} 48 h 20.48 ± 3.4 %). Stigmasterol on BKPM effluent has high toxicity than β-sitosterol. Together they were more toxic than separately.

Keywords: Pulp mill effluent, phytosterols, acute toxicity, *D. magna*, *D. obtusa*.

INTRODUCTION

Pulp mills generate high quantities of effluent, about 60 m^3/ton of pulp. Improvements on bleaching processes had a positive impact in the quality of the BKPM effluent, specially regarding to its toxicity. However, the main source of BKPM effluent toxicity is the extractives constituents released during cooking and debarking process. Most of pulp mills recover the extractives in a separate process. However, the recovery process are not effective, Because of this, part of these compounds are transferred to the treatment plant (Rod´ko et al. 1996, Thompson et al. 2001).

Resin acid, long chain fatty acid, phenols, terpens, phytosterols and triglycerides are the main wood extractives. Softwood (SW) like pines has more extractives (0.5- 7.0 %) than hardwood (HW) (0.2- 3.5 %). BKPM effluents from SW have high resin acid content (0.1 and 12 mg/L). Whereas those from HW are more abundant in phytosterols (0.02 to 3.4 mg/L). Figure 1 shows phytosterols concentration according to the kind of wood used as raw material in BKPM (Hewitt et al. 1996, LaFleur 1996, Mellanen et al. 1996, Strömberg et al. 1996, Verta et al. 1996, Cook et al. 1997, Kukkonen et al. 1999, Mattsson et al. 2001, van den Heuvel et al. 2002).
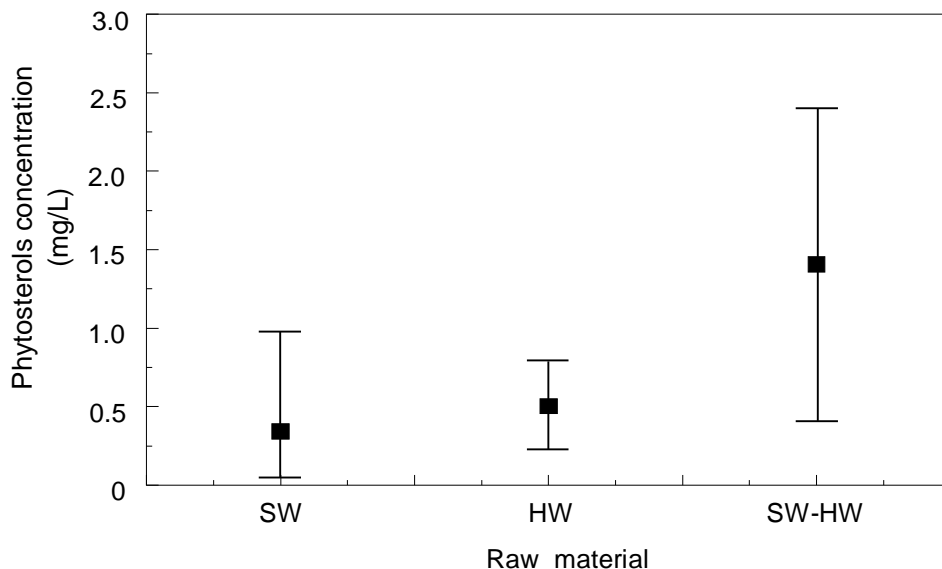
1

Figure 1. Phytosterols concentration in kraft mill effluent according to the raw material: softwood (SW), hardwood (HW), and combination of softwood and hardwood (SW-HW).

β-sitosterol, stigmasterol, campesterol, β-sitostanol, stigmastanol and campestanol are the main phytosterols contained on BKPM effluents (Figure 2 a) (Verta et al. 1996, Cook et al. 1997, Jenkins et al. 2001, van der Heuvel et al. 2002). Phytosterols concentration on BKPM treated effluent could achieve 1.3 mg/L, where more than 50 % correspond to β-sitosterol (Karels et al. 2001, Mattsson et al. 2001, Larsson et al. 2002, van den Heuvel et al. 2002, Kostamo and Kukkonen 2003). Because of their structure like cholesterol (Figure 2 b) (an important substrate to steroid hormone production), phytosterols have been related to endocrine disruption on fishes. Phytosterols concentrations as low as 0.26- 1.0 mg/L could cause this effect on fishes (Munkittrick et al. 1992a,b,c, Gagnon, et al. 1994, Munkittrick et al. 1994, Gagnon et al. 1995, Lehtinen 1996, McMaster et al. 1996, Lehtinen et al. 1999, van den Heuvel et al. 2002).
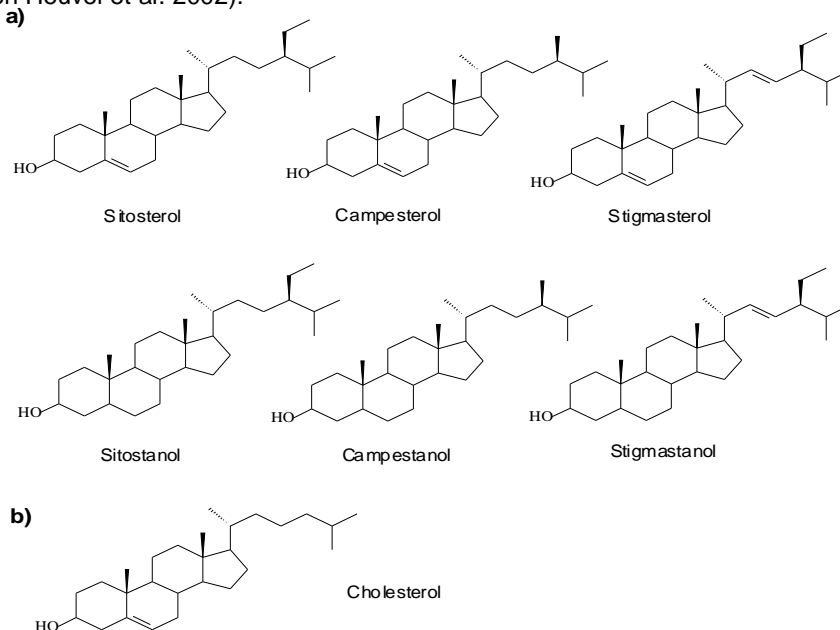

Figure 2. a) Main phytosterols found on BKPM effluents. b) Cholesterol structure.

Daphnids (like *Daphnia* spp. and *Ceriodaphnia* spp.) are used as indicator of acute and chronic toxicity; because of their easy and inexpensive culture and testing (Peng and Roberts 2000). Specifically, *Daphnia magna* has been used for detecting acute toxicity of BKPM effluents. Indeed, untreated BKPM 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 reproduction is still present even after biological treatment of BKPM effluents (Martel et al. 2004).

Peng and Roberts (2000), verified that the toxicity of resin acids on *D. magna* was correlated inversely with their solubility. But pure compounds are not adequate to determine toxicity, because there are interactive toxic effects. However the individual toxicity data may be valuable in estimating toxicity of whole effluent. Studies employing isolated phytosterols were not found, and it should be interesting to assess their contribution for BKPM effluent acute toxicity. The aim of this work is to evaluate the acute toxicity of BKPM effluent and phytosterols by using *D. magna* and *D. obtusa*.

MATERIALS AND METHODS

ECF BKMP effluent sample was obtained from a local industry. The effluent was primary treated in a settler tank to reduce fiber and total solids. Sample was transported on ice in insolated cooler to the laboratory and stored in the dark at 4 ± 1 °C.

β-sitosterol (Carbiochem), stigmasterol (Sigma) and cholesterol (Carbiochem) were employed. The effluent characteristics were verified by measuring COD and BOD_5, color, total phenols, phytosterols concentration and acute toxicity on *Daphnia magna* and *Daphnia obtusa*.

Female *D. magna* and *D. obtusa* 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 (*D. magna*). While, *D. obtusa* receives suspension of baker's yeast, trout chow and alfalfa with an equivalent carbon content of 3.6 mg C/L on Monday and Wednesday, and 5.4 mg C/L on Friday. A culture medium was changed before feeding and neonates were removed from a culture within 24 h (U.S.EPA 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).

Acute toxicity assessment were done by exposing female *D. magna* and *D. obtusa* neonates (< 24 h old), during 24 - 48 h to: (i) BKPM effluent, (ii) BKPM effluent plus β-sitosterol and stigmasterol, (iii) water plus β-sitosterol and stigmasterol. BKPM effluent was filtrated through 0.45 μm membrane, and its pH was adjusted to 7.0 ± 0.5 before bioassays. Phytosterols were used separately and on mixture. They were sonicated during 10 hours in quantities to produce added concentrations of 0.3 and 1.0 mg/L. 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 BKPM 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 dissolved concentration (OD), pH and conductivity were measured at the beginning and end of each test. The 24 and 48 h mean lethal concentration were calculated using the Probit and the Spearman-Karber methods, as appropriate (Cooman et al. 2003).

COD and BOD_5 were measured according to Standard Methods (APHA 1985). 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). The measurements of COD, BOD_5, VIS_{440}, UV_{215} were done after membrane (0.45 μm) filtration. The OD was measured using a WTW Oxycal 323B oxygen electrode. Phytosterols concentration was determined by CG-MS in a HP 5890 chromatograph with mass selective detector HP5972 (limits of detection of 1 μg/L). After dichloromethane extraction and by using cholesterol as internal standard (Cook et al. 1997, Khan and Hall 2003).

PDF created with pdfFactory trial version www.pdffactory.com

RESULTS AND DISCUSSION

Table 1 shows the physico-chemical characteristics of BKPM effluents. The COD/BOD_5 ratio (2.9) indicates that high concentrations of recalcitrant compounds are present in kraft mill 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. β-sitosterol and stigmasterol were determined (Table 1). Total phytosterol concentration (0.40 ± 0.03 mg/L) agree with the values determined for SW kraft mill effluents (0.39 ± 0.67 mg/L) (Strömberg et al. 1996, Cook et al. 1997, Mattsson et al. 2001). Although phytosterols concentration in this effluent was not very high as those concentrations found in SW-HW kraft mill effluent (Figure 1). It was in the range to produce endocrine disruption on fishes (0.26 - 1.1 mg/L) (Munkittrick et al. 1992a,b,c, Gagnon et al. 1994, Munkittrick et al. 1994, Gagnon et al. 1995, McMaster et al. 1996, Lehtinen et al. 1999, van den Heuvel et al. 2002). Because of this, dispersions of β-sitosterols and stigmasterol (0.3 and 1.0 mg/L) separately and mixed were prepared on water to assess their toxicity. β-sitosterols and stigmasterol toxicity were also assess by dispersion on BKPM effluent (added concentration 0.3 and 1.0 mg/L). The final concentration of these compounds on BKPM effluent were about: (i) 0.64- 1.34 mg/L (β-sitosterol), (ii) 0.37- 1.07 mg/L (stigmasterol) and (iii) 1.0- 2.4 mg/L (β-sitosterols and stigmasterol).

Table 1. Characteristics of ECF BKPM effluent.

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 (UV_{215}) (mg/L)	271.9 ± 14.2
β-sitosterol (mg/L)	0.333 ± 0.028
Stigmasterol (mg/L)	0.069 ± 0.014
Phytosterols (mg/L)	0.402 ± 0.03
Color (VIS_{440}) (1x1 cm)	0.41 ± 0.01

BKPM effluent does not has acute toxicity on *D. magna* during the first 24 h (LC_{50} 24h, > 100%). However, after 48 h of exposure it displayed acute toxicity (LC_{50} 48 h = 42.08 ± 6.5 %). This value means the concentration that causes the mortality to 50 % of daphnids exposed to the effluent. In Chile there are not toxicity regulations for effluent discharges. However, the Canadian Pulp and Paper Effluent regulations requires at least 50 % survival of *D. magna* exposure to an undiluted effluent, to compliance toxicity quality (Kovacs et al., 2004). Thus, the assayed BKPM effluent was not toxic by *D. magna* the first 24 h, whereas at 48 h exposure, it does not carry out which international ecotoxicity regulations.

Figure 3 shows the acute effects of β-sitosterol and stigmasterol on *D. magna*. As is possible to see in Figure 3, the toxicity of separated compounds (a-d) on water were higher than on BKPM effluent. The acute toxicity on *D. magna* was not depending on the concentration of β-sitosterol or stigmasterol (a-f). Besides, exposure time had an important effect on acute toxicity to separated compounds. Although, no toxicity was observed for 24 h exposure on BKPM effluent, after 48 h exposure it was toxic. Moreover BKPM effluent with extra stigmasterol (c-d) was more toxic than BKPM effluent with β-sitosterol (a-b). The mixture of β-sitosterol and stigmasterol on water or effluent had high acute toxicity (e-f), when it was compared to separate compounds toxicity. Nevertheless, the toxicity of mixed compounds was high in effluent media (LC_{50} 48 h = 20.48 ± 4.3 %) than in water (LC_{50} 48 h = 45.29 ± 13.0 %) (e-f).
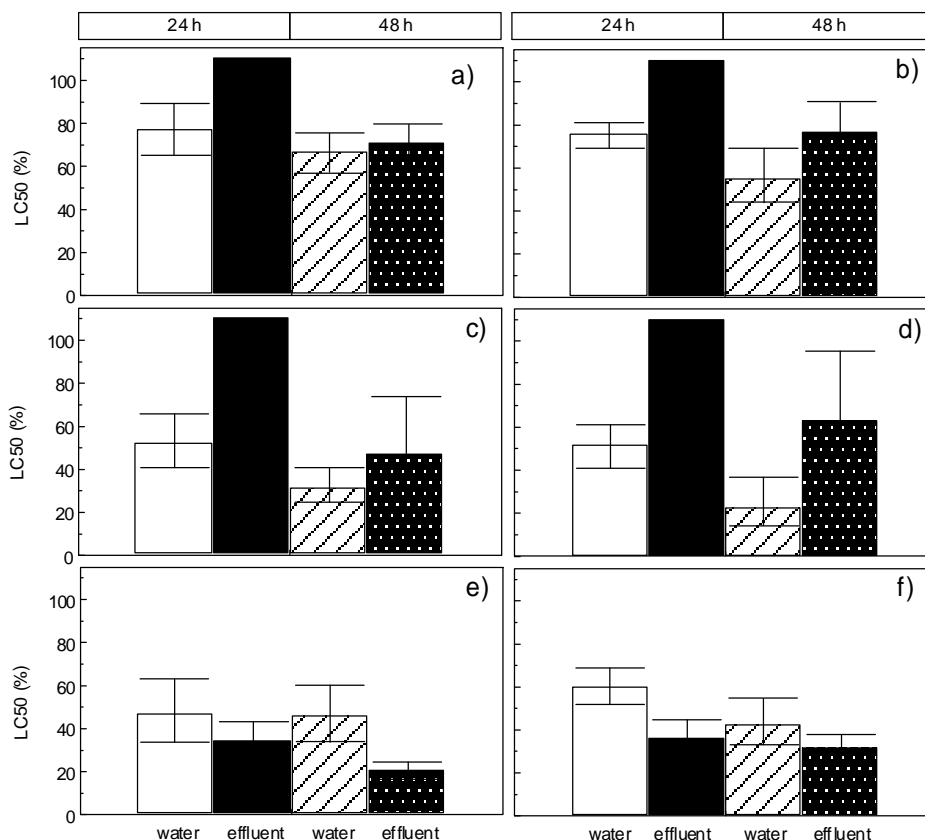
4

Figure 3. *D. magna* acute toxicity (LC_{50}) on water and on BKPM effluent dispersion of : a) β-sitosterol 0.3 mg/L, b) β-sitosterol 1.0 mg/L, c) stigmasterol 0.3 mg/L, d) stigmasterol 1.0 mg/L and e) β-sitosterol and stigmasterol 0.3 mg/L, f) β-sitosterol and stigmasterol 1.0 mg/L.

D. obtusa were less sensitive to BKPM effluent, because no acute toxicity was detected at 24 h or 48 h (LC_{50} 24h, 48h > 100%). The same result was obtained for 0.3 and 1.0 mg/L of β-sitosterol at 24 or 48 h (Figure 4 a-b). However, stigmasterol (0.3- 1.0 mg/L) added into BKPM effluent produce acute toxicity especially after 48 h (Figure 4 c-d). Furthermore, the toxicity on *D. obtusa* was higher on water than on BKPM effluent (Figure 4 a-f).

The acute toxicity for both *D. magna* and *D. obtusa* was higher on stigmasterol dispersion than on β-sitosterol media. The mixture of β-sitosterol and stigmasterol had high acute toxicity than separated compounds. The presence of BOD_5 on the effluent (Table 1), indicated the presence of biodegradable substances, which daphnids could used as food. It could help to understand lowest toxicity on BKPM effluent. But also is possible that *D. magna* and *D. obtusa* consume non-biodegradable substances. Because they become colored during exposition time and they could grow on BKPM effluent even after biological treatment. When the BOD_5 removal was higher than 98 % (unpublished data).
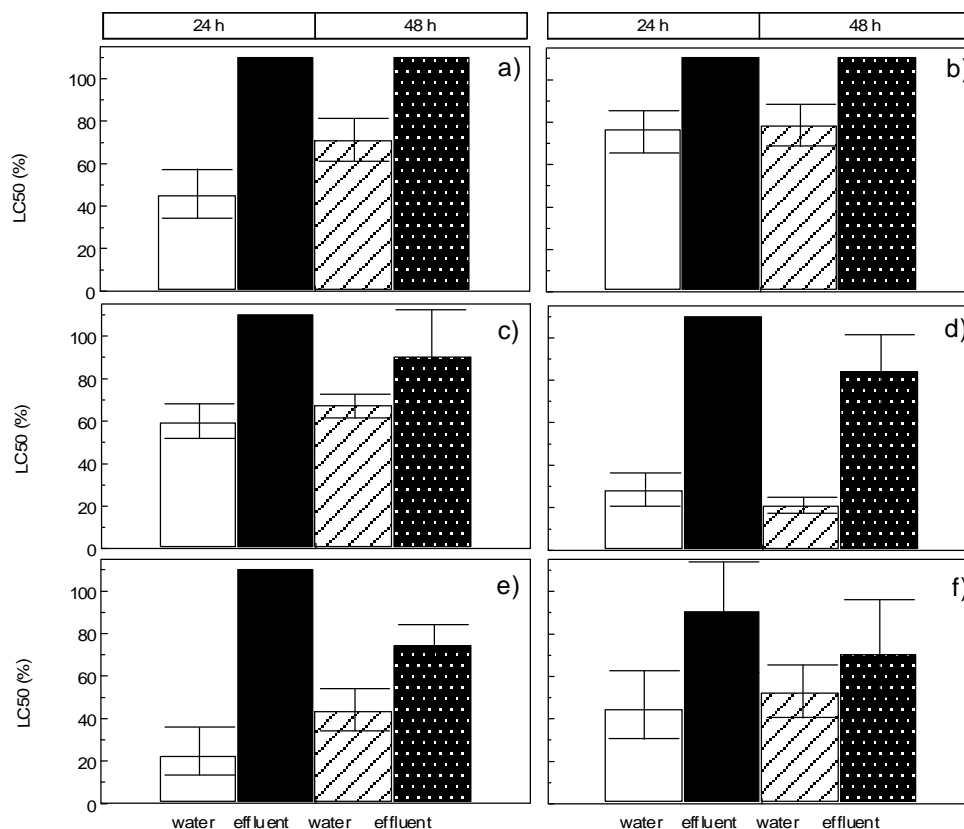
5

Figure 4. *D. obtusa* acute toxicity (LC$_{50}$) on water and on BKPM effluent dispersion of: a) 0.3 mg/L of β-sitosterol, b) 1.0 mg/L of β-sitosterol, c) 0.3 mg/L of stigmasterol, d) 1.0 mg/L of stigmasterol and e) 0.3 mg/L of β-sitosterol and stigmasterol, f) 1.0 mg/L of β-sitosterol and stigmasterol.

Mortality slopes to *D. magna* and *D. obtusa* are shown on Figure 5, where is possible to observe that *D. obtusa* was not affected by any concentration of BKPM effluent exposure (Figure 5 d). Indeed, *D. obtusa* shows no significant mortality until 48 h exposure to (6.25- 25 %) of BKPM effluent or BKPM effluent plus β-sitosterol and stigmasterol 0.3- 1.0 mg/L (Figure 5 d-f). Despite, *D. magna* was more sensitive even in low concentration (12.5- 25 %) of β-sitosterol and stigmasterol added to pulp mill effluent (Figure 5 b-c). As it was saw before (Figure 3 and 4) phytosterols concentrations do not affect the toxicity. Because they are insoluble in water media and they are only slightly soluble on BKPM effluent. Phytosterols are expected to have a log Kow > 5 (Hewitt et al., 2000), which indicate they are very hydrophobic. Due to this, when the BKPM effluent dispersion of β-sitosterol and stigmasterol 1.0 mg/L, was diluted (25%) reaching about 0.3 mg/L of β-sitosterol and stigmasterol concentration (third points on Figure 5 c and f). The number of dead daphnids was lower than the expected for a 100 % dispersion of β-sitosterol and stigmasterol on BKPM effluent (0.3 mg/L) (last point on Figure 5 b and e). This data suggest that phytosterols concentration does not influence the whole toxicity of the BKPM effluents. But, phytosterols addeed on BKPM effluent enhances its toxicity, making it not able to accomplish with international ecotoxicity regulations (Figure 5 b-c).
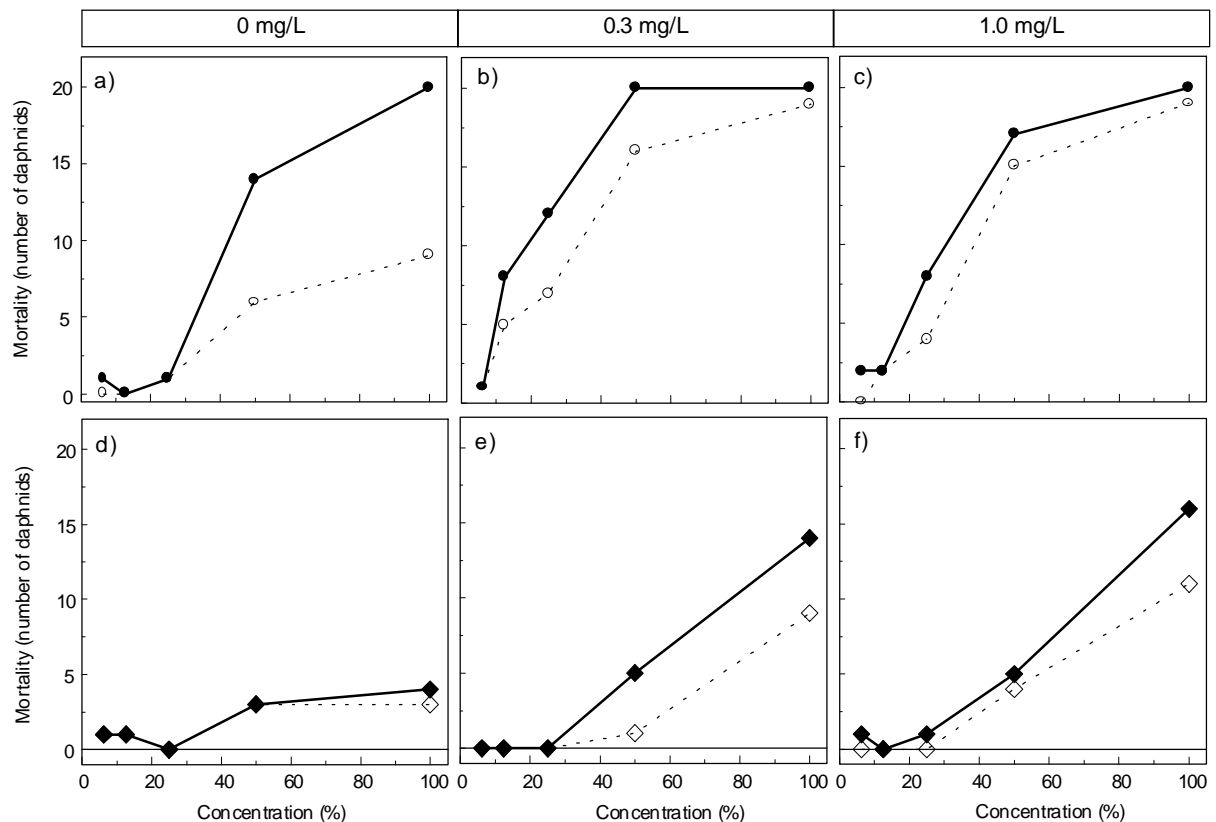
PDF created with pdfFactory trial version www.pdffactory.com

0 mg/L	0.3 mg/L	1.0 mg/L

Figure 5. Mortality slopes 24 h (······) - 48 h (——) to *D. magna* (a-c) and *D. obtusa* (d-f) exposed to: (0 mg/L) - BKPM effluent, (0.3 mg/L) - BKPM effluent + β-sitosterol and stigmasterol 0.3 mg/L, (1.0 mg/L) - BKPM effluent + β-sitosterol and stigmasterol 1.0 mg/L.

CONCLUSION

Stigmasterol on BKPM effluent has high toxicity than β-sitosterol. Together they were more toxic than separately.

ACKNOWLEDGEMENTS

REFERENCES

APHA-AWWA-WPCF. 1985. Standard methods for examination of water and wastewater, 16th edition. Washington.USA.

Cook DL, LaFleur L, Parrish A, Jones J, Hoy D. 1997. Characterization of plant sterols from 22 US pulp and paper mills. *Water Sci Technol* 35:297-303.

Cooman K, Gajardo M, Nieto J, Bornhardt C, Vidal G. 2003. Tannery wastewater characterization and toxicity effects on *Daphnia* spp. *Environ Toxicol* 17:45-51.

Diez MC, Castillo G, Aguilar L, Vidal G, Mora ML. 2002. Operational factors and nutrient effects on activated sludge treatment of *Pinus radiata* kraft mill wastewater. *Biosource Technol* 83:131-138.

Gaete H, Larrain A, Bay-Schmith E, Baeza J, Rodriguez J. 2000. Ecotoxicological assessment of two pulp mill effluent, Biobio River Basin, Chile. *Bull Environ Contam Toxicol* 65:183-189.

Gagnon MM, Bussieres D, Dodson JJ, Hodson PV. 1995. White sucker (*Catostomus commersoni*) growth and sexual maturation in pulp mill-contaminated and reference rivers. *Environ Toxicol Chem* 14:317-327.

Gagnon MM, Dodson JJ, Hodson PV. 1994. Ability of BKME (bleached kraft mill effluent) exposed white suckers (*Catostomus commersoni*) to synthesize steroid hormones. *Comp Biochem Physiol* 107:265-273.

Hewitt LM, Carey JH, Dixon DG, Munkittrick KR. 1996. Examination of bleached kraft mill efluent fractions for potencial inducers os mixed funtion oxygenase activity in rainbow trout. 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 Beach, FL, USA, pp. 79-94.

Hewitt LM, Parrot JL, Weells KL, Calp MK, Biddiscombe S, McMaster ME, Munkittrick KR, van der Kraak G. 2000. Characteristics of ligants for the Ah receptor in hepatic tissues of fish exposed to bleached kraft mill effluent. *Environ Sci Technol* 34:4327-4334.

Jenkins RJ, Angus RA, McNatt H, Howell WM, Kemppainen JA, Kirk M, Wilson EM. 2001. Identification of androstenedione in a river containing paper mill effluent. *Environ Toxicol Chem* 20:1325- 1331.

Karels A, Markkula E, Oikari A. 2001. Reproductive, biochemical, physiological, and population responses in perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) dowstream of two elemental chlorine-free pulp and paper mills. *Environ Toxicol Chem* 20: 1517-1527.

Khan ZM, Hall ER. 2003. Ocurrence and removal of plant sterols in pulp and paper mill effluents. *J Environ Eng Sci* 2:17-26.

Kostamo A, Kukkonen JVK. 2003. Removal of resinic acids and sterols from pulp mill effluents by activated sludge treatment. *Water Res* 37:2813- 2820.

Kovacs T, Gibbons S, Naish V, Voss R. 2004. Survey of pulp and paper mill effluents for their potencial to afect fish reproduction. In Borton DL, Hall TJ, Fisher RP, Thomas JF, eds, *Pulp and Paper Mill Effluent Environmental Fate Effects*. DEStech Publications, Lancaster, PA, USA, pp 294-303.

Kukkonen JVK, Punta E, Koponen P, Paranko J, Leppänen H, Holopainen IJ, Hyvärinen H. 1999. Biomarker responses by crucian carp (*Carassius carassius*) living in a pond of secondary treated pulp mill effluent. *Water Sci Technol* 40:123-130 .

LaFleur 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 Beach, FL, USA, pp. 21-31.

Larsson DG, Kinnberg K, Sturve J, Stephensen E, Skön M, Förlin L. 2002. Studies of masculinization, detoxification, and oxidative stress responses in guppies *(Poecilia reticulata)* exposed to effluent from a pulp mill. *Ecotoxicol Environ Saf* 52:13-20.

Lehtinen KJ. 1996. Biochemical responses in organisms exposed to effluents from pup produccion: are they related to bleaching? 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 Beach, FL, USA, pp. 359-368.

Lehtinen KJ, Mattsson K, Tana J, Engström C, Lerche O, Hemming J. 1999. Effects of wood-related sterol on the reproduction egg survival and off-spring of brown trout (*Salmo trutta lacustrus L.*). *Ecotoxicol Environ Saf* 42:40-49.

Martel P, Kovacs T, Voss R. 2004. Survey of pulp and paper mill effluents for their potencial to afect fish reproduction. In Borton DL, Hall TJ, Fisher RP, Thomas JF, eds, *Pulp and Paper Mill Effluent Environmental Fate Effects*. DEStech Publications, Lancaster, PA, USA, pp 78-91.

Mattsson K, Lehtinen KJ, Tana J, Härdig J, Kukkonen J, Nakari T, Engström C. 2001. Effects of pulp mill effluents and restricted diet on growth and physiology of rainbow trout (*Oncorhynchus mykiss*). *Ecotoxicol Environ Saf* 49:144-154.

McMaster ME, van der Kraak GJ, Munkittrick KR. 1996 . An epidemological evaluation of the biochemical basis for steroids hormonal depresión in fish exposed to industrial wastes. *J Great Lakes Res* 22:153-171.

Mellanen P, Petänen T, Lehtimäke J, Mäketä S, Bylund G, Holmbom B, Mannila E, Oikari A, Santti R. 1996. Wood-derived estrogens: Studies *in vitro* with breast cancer cell lines and *in vivo* in trout. *Toxicol Appl Pharmacol* 136:381-388.

Munkittrick KR, McMaster ME, Portt CB, van der Kraak GJ, Smith IR, Dixon DG. 1992a. Changes in maturity plasma sex steroid levels, hepatic mixed function oxygenase activity, and the presence of external lesion in lake whitefish (*Coregonus clupeaformis*) exposed to bleached kraft mill effluent. *Can J Fish Aquat Sci* 49:1560-1569.

Munkittrick KR, van der Kraak GJ, McMaster ME, Portt CB 1992b. Response of hepatic MOF activity and plasma sex steroids to secondary treatment of bleached kraft pulp mill effluent and mill shutdown. *Environ Toxicol Chem* 11:1427-1439.

Munkittrick KR, van der Kraak ME, McMaster ME, Portt CB. 1992c. Reproductive dysfunction and MOF activity in three species of fish exposed to bleached kraft mill effluent at Jack Bay, Lake Superior. *Water Pollut Res J Can* 27:439-446.

Munkittrick KR, van der Kraak GJ, McMaster ME, Portt CB, van der Heuvel MR, Servos MR. 1994. Survey of receiving water environmental impacts associated with discharges from pulp mill. II. Gonad size, liver size, hepatic EROD activity and plasma sex esteroid levels in white sucker. *Environ Toxicol Chem* 13:1089-1101.

NCh 2083- Norma Chilena Oficial (1999). Bioensayos de toxicidad aguda mediante la determinación de la inhibición de la movilidad de *Daphnia magna* o *Daphnia pulex* (Crustacea, Cladocera. Instituto Nacional de Normalización, 1st edition, Chile.

Peng G, Roberts JC 2000. Solubility and toxicity of resin acid. *Water Res* 34:2779- 2785.

Priha MH. 1996. Ecotoxicological impacts of pulp mill effluents in Finland. 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 Beach, FL, USA, pp. 637-650.

Rod´ko IY, Scott BF, Carey JH. 1996. Analysis of pulp mill black liquor for organosulfur compounds using CG/atomic emission detection. 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 Beach, FL, USA, pp. 195-202.

Strömberg L, Mörck R, de Souza F, Dahlman O. 1996. Effects of internal process changes and external treatment on effluent chemistry. 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 Beach, FL, USA, pp. 3-19.

Thompson G, Swain J, Kay M, Forster CF. 2001. The treatment of pulp and paper mill effluent: a review. *Biosource Technol* 77:275-286.

U.S. Environmental Protection Agency. 1993. Methods for measuring the acute toxicity of effluents snd receiving waters to freshwater and marine organisms, EPA- 600/4-90-027F, Cincinnati, OH.

van den Heuvel MR, Ellis RJ, Tremblay LA, Stuthridge TR. 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 Beach, FL, USA, pp. 41-51.