# Sensitivity Study Comparing *Daphnia obtusa* (Kurz 1874) and *Daphnia magna* (Straus 1820) Exposure to Treated Kraft Mill Effluents, Diethylstilbestrol, and Androstenedione

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Kraft mill effluents treated by activated sludge show a reduction in acute toxicity. However, their discharges produce hormonal effects in an aquatic ecosystem, due to the sterols metabolites as androstenedione contained in the effluents. Daphnids bioassays are a powerful approach for determining toxicity. However, there are relative sensitivities depending on the species. The main objective of this study is to determine the sensitivity of *D. magna* and *D. obtusa* when exposed to kraft mill effluents, diethylstilboestrol (DES), and androstenedione (AED). The sensitivities were tested using acute bioassay exposed to kraft pulp mill. Moreover, the allometric growth rate of both daphnids affected by DES and AED regarding time were also evaluated for a period of nine days. Variation in the ratio between body length and body width – at the abdominal cavity – over time (*k* index) was evaluated. Results indicated that AED and DES compounds affected the allometric growth rate of daphnids. Specifically *D. magna* exhibited more sensitivity when it was exposed to kraft pulp mill.

*Key words: Kraft mill effluents; Diethylstilbestrol; Androstenedione; Daphnia obtusa; Daphnia magna; Sensitivity*

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## INTRODUCTION

Effluent discharges from the kraft pulp mill have been identified as a potential contaminant of aquatic environments (Belmonte *et al*. 2006; Vidal *et al*. 2007; Chamorro *et al*. 2010). Their compound nature is derived from the presence of several naturally occurring and xenobiotic compounds, which are formed and released during various stages of the process (Chamorro *et al*. 2005). Special attention has been directed toward plant sterols metabolites as androstenedione for being an active hormonally compound capable of inducing androgenic effects in organisms (Malaviya and Gomes 2008; Lambert and Edwards 2017). This compound has been found in kraft mill effluents, even after being treated by activated sludge (Chamorro *et al*. 2016a) at a concentration of 2.4 nM sediment near to discharge and 0.14 nM in the water column (Jenkins *et al*. 2001, 2003; Van den Heuvel *et al*. 2006).

Cosmopolitan *Daphnia magna* (Straus 1820) is widely used as an indicator for freshwater evaluation (Cooman *et al.* 2003; Xavier *et al.* 2005; Reyes *et al.* 2009), although *Daphnia obtusa* (Kurz 1874) is the principal species that survives in many

aquatic ecosystems, including the Chilean ecosystem. Previous evidence for chronic activity, such as comparative study of the sensitivity for *D. magna* and *D. obtusa*, show the effect of exposure to inorganic and organic compounds. Therefore, Gaete and Paredes (1996) and Silva *et al.* (2004) show that chlorophenol coming from the bleaching process of the kraft mill has an exposure effect that is 38% stronger for *D. obtusa* than for *D. magna*. On the other hand, chromium exposure studies for *D. magna* (Gordillo *et al.* 1998) and *D. obtusa* (Bulus and Ronco 1996; Silva *et al.* 2004) show that the effects are 44% and 63%, respectively.

Previous studies of *D. magna* show that fertility, growth rate, maturity, and sex ratio are affected by organic compounds with chronic estrogenic activity (Xavier *et al.* 2005; López *et al.* 2011; Chamorro *et al.* 2016b). Also, the allometric growth rate of *D. magna* can be affected by estrogenic compounds (Olmstead and LeBlanc 2000). The vertebrate estrogen agonist diethylstilboestrol (DES) was selected because it has been shown that these compounds reduce molt frequency in immature daphnids (Baldwin *et al*. 1995) and modify the allometry of this organism (Olmstead and LeBlanc 2000). The vertebrate androgen androstenedione (AED) was selected because it has been shown that this steroid elicits developmental toxicity in daphnids (LeBlanc 1999).

The objective of the present study is to determine the sensitivity of *D. magna* and *D. obtusa* when exposed to treated kraft mill effluents, DES and AED. Additionally, the allometric growth rate for both daphnids affected by DES and AED were evaluated over time.

## EXPERIMENTAL

Samples were obtained from a local kraft mill with an elemental chlorine-free (ECF) bleaching system. The kraft mill water samples were obtained after primary treatment, in which mainly the solids were removed. Effluent also was taken after secondary treatment by activated sludge with the hydraulic retention time of 2.0 h. Samples were stored in the dark at 4 ± 1 °C.

The aqueous samples were characterized in terms of chemical oxygen demand (COD) and biological oxygen demand (BOD) following Standard Methods (APHA-AWWA-WPCF 1985). Total phenolics compounds concentration was measured by UV absorbance in a 1-cm quartz cell at 215 nm, pH 8.0 (0.2 M $KH_2PO_4$buffer), and transformed to concentration using a calibration curve with phenol as standard solution. Aromatic compounds at (UV254) and lignin derived compounds (UV280) were assessed in a 1 cm quartz cell (Chamorro *et al*. 2005).

The β-sitosterol and stigmasterol were determined using a Hewlett-Packard HP 5890 (chromatograph with mass detector HP 5972) using a column Agilent (19091s-433 HP-MS 5% phenyl-methyl-siloxane, length 30 m, internal diameter 0.5 µm). The detection limit was fixed at 1 µg $L^{-1}$. The compounds were extracted from 100 mL of samples with 20 mL dichloromethane at a pH value of 7. Working analyses conditions were as follows: carrier gas, helium flow rate, 2 mL $min^{-1}$; injector temperature, 250 ºC; detector temperature, 280 ºC; and oven temperature, 325 ºC. A volume of 1 µL was injected to the GC-FID chromatograph. The solutions were prepared with stigmasterol (Sigma) using cholesterol (Carbiochem).

Female *D. magna* and *D. obtusa* were obtained from in-house cultures maintained according to guidelines given by USEPA (1993). The culture medium and bioassays were

conducted at 20 ± 2 °C in a photoperiod of 16 h light, 8 h dark, and maintained at a density of 20 to 30 daphnids $L^{-1}$. The culture medium was maintained at 250 ± 25 mg $CaCO_3L^{-1}$, and the pH ranged from 7.5 to 8.6 (NCh 2083 1999). The daphnids were fed with 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 $L^{-1}$ on Monday and Wednesday, and 10.8 mg C $L^{-1}$ on Friday. The culture medium was renewed three times weekly. The neonates were counted and removed from culture medium (24 h) (USEPA 1993).

The acute toxicity of influent and effluent on *Daphnia obtusa* and *Daphnia magna* (< 24 h old) was evaluated at 24 and 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 samples with different concentrations (6.25, 12.5, 25, 50 and 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 (NCh 2083 1999). The lethal concentration was calculated for the concentration of 15% at 24 and 48 h ($LC_{15}$) using the Probit method (USEPA 1993).

Chronic toxicity assessments were conducted by exposing female *D. magna* neonates (< 24 h old) over nine days. Ten replicates of 50 mL (each one containing one organism) were used for each sample. The deviation values were calculated from ten replicates for each sample. The culture medium was renewed every 2 d during the test. Dissolved oxygen concentrations, pH, and conductivity were measured at the beginning and end of each test (USEPA 1988). Hormonal compounds diethylstilbestrol (DES) (97% purity) and androstenedione (AED) (98% purity) were supplied by Sigma-Aldrich (Saint Louis, Missouri, USA). Three different concentrations were evaluated following Olmstead and LeBlanc (2000); for DES: C1 (0.75 µM), C2 (1.50 µM), and C3 (3.00 µM); and for AED: C1 (6.25 µM), C2 (12.50 µM), and C3 (25.00 µM). For each compound, a hormonal control containing ethanol (0.02%) (C) was prepared. Specifically, specimens were assessed on days 3, 6, and 9 the anatomical development in daphnids, because after 12 d there would be hormonal changes related to sexuality maturity (Terra and Gonçalves 2013). The organisms were examined using a microscope connected to a camera that recorded the images. Total body length (distance from the top of the head capsule to the base of the shell spine) and body width (measurement of the wider side of the body) were measured. Then, the relationship between body width divided by body length was obtained and expressed as the allometric growth rate (AGR) (Olmstead and LeBlanc 2000; Xavier *et al.* 2005) and calculated as shown in Eq. 1:

$$\text{AGR} = \frac{Body\ width}{Body\ lenght} \quad (1)$$

The rate of growth in body length to growth in body width over time was calculated as shown in Eq. 2 (Xavier *et al.* 2005):

$$k = \frac{\Delta\frac{Body\ length}{Body\ width}}{\Delta t} \quad (2)$$

Statistical processing involved first checking data for normality (Chi-squared test) and the homogeneity of variances (Bartlett's test). The result for allometric growth was analyzed by ANOVA using Statistica 5.1 Statsoft, 1998 (95% confidence intervals). The differences among treatments were assessed Dunnett's and Williams test in the statistical programme TOXSTAT (USEPA 1994; López *et al.* 2011).

Hormonal compound concentrations DES and AED were determined by CG-MS (Gas Chromatography - Mass Spectrometry) in a HP 5890 chromatograph (Hewlett-Packard, Wilmington, DE, USA) with mass selective detector HP5972 (Hewlett-Packard, Mississauga, Canada) (detection limit of 1 µg $L^{-1}$) (Xavier *et al*. 2005). Based on these concentrations, the method sensitivity was evaluated by virtue of the limit of detection (LOD) and limit of quantification (LOQ). The LOD was obtained from the AGR for DES and AED on daphnids of both species. The LOD and LOQ were calculated using Eqs. 3 and 4 (Armbruster *et al.* 1994; INMETRO 2003),

$$LOD = C + t\,s \quad (3)$$

$$LOQ = C + 10\,s \quad (4)$$

where $C$ is the average of the AGR on day 3 for the control (smaller expected measurement for control), $t$ is the Unilateral Student t value with 95% confidence and nine degrees of freedom, and $s$ is the standard deviation of the response (10 replicates).

The LODs for estrogenic compounds were 0.7560 and 0.6878 cm for *D. obtusa* and *D. magna*, respectively. Meanwhile, the LOD values for AED were 0.6598 and 0.8763 cm for *D. obtusa* and *D. magna*, respectively. The LOQs for DES were 0.9343 cm and 1.5705 cm for *D. magna* and *D. obtusa*, respectively. Therefore, the average LOQ for *D. magna* in the presence of AED was 1.919 cm, and that for *D. obtusa* was 1.0739 cm.

## RESULTS AND DISCUSSION

Table 1 shows the characteristics of the influent to wastewater treatment. The pH value was acidic 3.4 ± 0.17, characteristic of raw wastewater (Chamorro *et al*. 2009). COD and BOD values were 881.0 ± 24.3 and 300.5 ± 9.5, respectively. The COD/BOD ratio (2.9) indicates high concentrations of recalcitrant compounds, such as phenolic compounds, tannin, and lignin, among others (Vidal *et al*. 2007; Xavier *et al*. 2009). On the other hand, Table 1 shows the presence of β-sitosterol and stigmasterol with concentrations of 0.333 ± 0.028 and 0.069 ± 0.014 respectively.

**Table 1**. Characterization of Kraft Pulp Mill Influent

| Parameter | Value |
|---|---|
| pH | 3.4 ± 0.17 |
| COD (mg $L^{-1}$) | 881.0 ± 24.3 |
| BOD (mg $L^{-1}$) | 300.5 ± 9.5 |
| Total phenolics compounds (mg $L^{-1}$) | 271.9 ± 14.2 |
| β-sitosterol (mg $L^{-1}$) | 0.333 ± 0.028 |
| Stigmasterol (mg $L^{-1}$) | 0.069 ± 0.014 |
| Aromatic compounds (mg $L^{-1}$) | 6.69 ± 0.07 |
| Lignin byproducts | 5.90 ± 0.08 |

COD: Chemical oxygen demand; BOD: Biological oxygen demand

Table 2 shows the acute toxicity of influent and effluent of kraft mill wastewater treatment, assessed by lethal concentration 15% for *Daphnia magna* and *Daphnia obtusa*.

**Table 2**. Lethal Concentration of Kraft Pulp Mill by *Daphnia magna* and *Daphnia obtusa*

| Organism | Kraft pulp mill | |
|---|---|---|
| | Influent (%) | Effluent (%) |
| *Daphnia obtusa* $LC_{15}$ 24-h | 71.72 | >100 |
| *Daphnia obtusa* $LC_{15}$ 48-h | >100 | >100 |
| *Daphnia magna* $LC_{15}$ 24-h | 37.79 | >100 |
| *Daphnia magna* $LC_{15}$ 48-h | 20.58 | >100 |

$LC_{15}$: Lethal concentration predicted for 15%

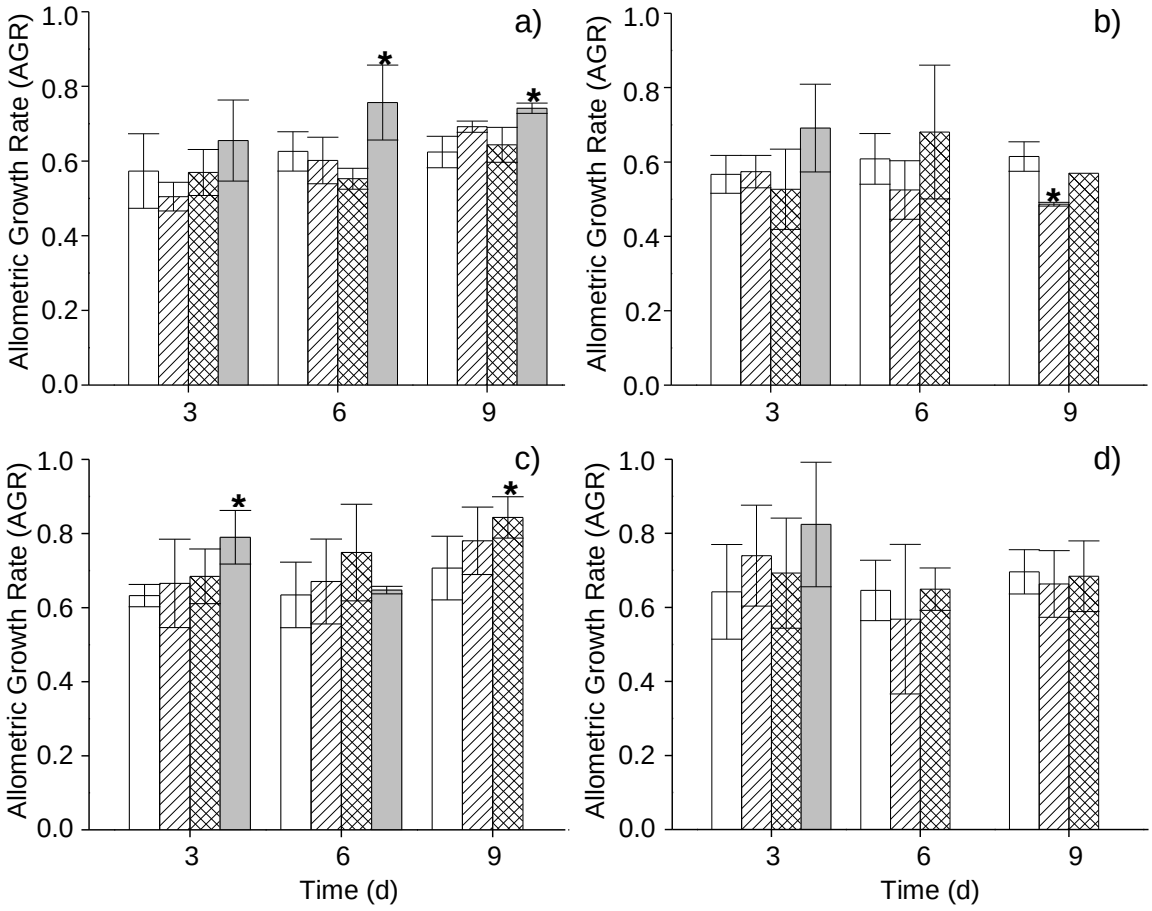


**Fig. 1.** Relationship between body width and body length (average + SD) in *Daphnia obtusa*: (a) diethylstilbestrol; (b) androstenedione. For *Daphnia magna*: (c) diethylstilbestrol; (d) androstenedione when exposed at concentrations C1 (▨), C2 (▩), C3 (■) and control (□).*Significantly different from the control, $p \leq 0.05$

Results demonstrated that influent of kraft mill wastewater treatment shows acute toxicity $LC_{15}$-24h for *D. magna* and *D. obtusa* at values of 71.72% and 37.79%

respectively, while $LC_{15}$-48h for *D. obtusa* toxicity was not demonstrated. By contrast, *D. magna* showed acute toxicity with a value of 20.58%. In this sense the different sensibilities are exposed. It was observed that activated sludge treatment removed 100% of the acute toxicity (Belmonte *et al*. 2006; Rosa *et al*. 2010).

Figure 1 shows the relationship between body length and body width of *D. obtusa* and *D. magna* exposed to DES and AED. *Daphnia magna* exposed to 0.75, 1.50, and 3.00 µM of DES showed an increase of 10% body width after nine days of exposure. However, *D. obtusa* showed a higher increase compared (17%) with *D. magna* with respect to the abdominal cavity for 3.00 µM of DES with an exposure time of nine days (data with respect to the control sample). These results agree with those of Olmstead and LeBlanc (2000) and Lopez *et al.* (2011). These effects can be explained because DES in concentrations higher than 0.2 mg $L^{-1}$ on *Daphnia magna* reduces the time and frequency of movements, inhibits fertility, and causes metabolic alterations of the steroid capabilities (Baldwin *et al.* 1995; Zou and Fingerman 1997; Brennan *et al.* 2006). Moreover, studies have shown the interaction probability of estrogenic compounds with the receptor responsible for molting and tissue differentiation in invertebrates (ecdiesteroides) (Baldwin *et al.* 1995; Zou and Fingerman 1997; Dinan *et al.* 2001; Brennan *et al.* 2006).

Variation in the proportion of body length to body width shows a reverse trend in relation to DES effects when daphnids were exposed to AED. Indeed, when *D. magna* was exposed to AED (6.25 µM), the abdominal cavity decreased by 15% after six days of exposure (value with respect to the control). For *D. obtusa* under similar conditions, the body width decreased by 13% and 19% for six and nine days of exposure, respectively (see Fig. 1).

Table 3 shows the rate between body length to body width for *D. magna* and *D. obtusa* (k) over time. When exposed to DES (0.75 µM), the k values were 0.0192 $d^{-1}$ and 0.0312 $d^{-1}$ for *D. magna* and *D. obtusa*, respectively, and the abdomen cavity showed more growth compared with the body length. In addition, the sensitivity of *D. obtusa* is 1.6 times that of *D. magna*.

**Table 3.** Comparison of *k* Values at Various Concentrations of DES and AED

| Treatment | Concentration (µM) | *k* ($d^{-1}$) | |
|---|---|---|---|
| | | ***D. magna*** | ***D. obtusa*** |
| **Diethylstilbestrol** | 0.00 | 0.0124 | 0.0085 |
| | 0.75 | 0.0192 | 0.0312 |
| | 1.50 | 0.0265 | 0.0123 |
| | 3.00 | - | 0.0144 |
| **Androstenedione** | 0.00 | 0.0090 | 0.0064 |
| | 6.25 | -0.0128 | -0.0146 |
| | 12.50 | -0.0014 | -0.0072 |
| | 25.0 | - | - |

On the other hand, *k* results were positive for DES exposition (estrogenic effect), but are negative when daphnids are exposed to AED (androgenic effect). In the case of AED, the *k* values showed that the rate of body length to abdominal cavity for daphnids

decreased over time with respect to the control (0.0090 to 0.0064 $d^{-1}$). These negative values indicate that the ratio body length/body width decreased over time, mostly because of the abdominal cavity dimensions, where *D. obtusa* was affected the most. These results agree with those found by LeBlanc (1999), who showed that the exposure of androstenedione embryos on daphnids resulting in developmental abnormalities was concentration-dependent. In previous works with bleached kraft pulp mill effluents (BKMEs), López *et al.* (2005) identified the effluent factors capable of modifying the body proportion of *D. magna*. Also, organic compounds contained in BKMEs, such as β-sitosterol and stigmasterol, may be contributing to the allometry. Moreover, phytosterols *per se* are responsible for 12.9% and 8.1% of the deviation from the natural shape of *D. magna*, while the BKMEs account for 25.6% to 27.8% of shape deviation (López *et al.* 2011; Chamorro and Vidal, 2014; Chamorro *et al.* 2016b).

## CONCLUSIONS

1. Acute toxicity based on $LC_{15}$-48h for kraft mill influent to wastewater treatment exhibited different sensibilities for daphnids.
2. DES and AED compounds affect the allometric grow rate of *D. obtusa* and *D. magna*. Moreover, the effect of DES and AED on daphnids was estrogenic and androgenic, respectively.
3. Consequently, the daphnid allometric growth rate under an estrogenic effect increased the abdominal cavity with respect to body length, while an androgenic effect produced the opposite effect.
4. *D. obtusa* was more sensitive than *D. magna* with respect to the effects of DES and AED exposure.

## ACKNOWLEDGEMENTS

This work was partially supported by Fondecyt Grant No. 1120664 and CONICYT/FONDAP/15130015.

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Article submitted: February 10, 2017; Peer review completed: April 13, 2017; Revised version received and accepted: July 14, 2017; Published: July 24, 2017.
DOI: 10.15376/biores.12.3.6558-6567