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# Detection of Estrogenic Activity from Kraft Mill Effluents by the Yeast Estrogen Screen

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**Abstract** Estrogenic activity of kraft pulp mill effluents (*P. radiata*, *E. globulus* and mixed −50% *E. globulus* and 50% *P. radiata*) was evaluated by the yeast estrogen screen assay. The estrogenic activity values were relatively low, ranking between 1.475 and 0.383 ng/L of EE2 eq. (Estrogenic equivalent of 17 α-ethynylestradiol), where the highest value corresponds to the *E. globulus* effluent and the lowest value to the *P. radiata* effluent. Analysis by solid phase extraction (SPE) and gas chromatography—mass spectrometry (GC-MS) of chemical compounds present in all three effluents detected at least five major groups of organic compounds, corresponding to fatty acids, hydrocarbons, phenols, sterols and triterpenes. Comparison of analytical and biological data suggests that sterols could be the cause of the estrogenic activity in the evaluated effluent.

**Keywords** Pulp mill effluents · Extractives · Estrogenic activity · SPE-extraction · GC-MS

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Effluent discharges from kraft pulp mill have been identified as a potential source of endocrine disruption activity in aquatic ecosystems (Hewitt et al. 2006; Orrego et al. 2009). Low concentrations of extractives, such as resin acids (2 μg/L) and sterols (10 μg/L), known to be present in kraft effluents, contributed to the chronic toxicity to which the fish were exposed (Hewitt et al. 2006), producing alteration in the sexual steroid level in fish plasma and the diminishment of their reproductive adaptation (Larsson et al. 2002).

When estrogenic effects of kraft mills effluent has been evaluated in invertebrates (*Daphnidae*) (Xavier et al. 2005) and by the yeast estrogenic screen (YES) assay (Fernandez et al. 2007; Hamm et al. 2006), compounds like chlorocimenes, retene, abietanes, sterols, among others, have been identified as being putatively responsible for the estrogenic activity (Xavier et al. 2005). The concentration of these compounds in effluents may vary depending on both the raw material and the technology used in kraft pulp production. Reported values range from 0.5 to 7.0% w/w in effluents from *Pinus radiata* to 0.2–3.5% w/w in effluents from *Eucalyptus globulus* (Gutiérrez et al. 1999).

The in vitro YES assay is a screening tool used to evaluate the potential endocrine disruption activity of a given substance or environmental sample. This assay is based on a *Saccharomyces cerevisiae* recombinant yeast strain containing an expression plasmid for the human estrogen hormone receptor (ERE) and an appropriate reporter of β-galactosidase (*lacZ*) (Routledge and Sumpter 1996). The association between YES assays and gas chromatography (GC-MS) is a powerful scheme for the detection, identification and quantification of compounds with estrogenic activity released into the environment by human action (Quirós et al. 2005; Céspedes et al. 2005).

In this work, we evaluate the estrogenic activity of effluents from three kraft mills operating with different raw materials: *Pinus radiata*, *Eucalyptus globulus* and a combination of both at approximately the 50%. These effluents were treated where produced (secondary treatment in all cases) prior to discharge into rivers. Our objective is to evaluate the environmental risk of these effluents as endocrine disruptors with the combination of the YES assay and GC-MS analysis.

## Materials and Methods

Three samples were obtained from three local kraft pulp mills that process *Pinus radiata*, *Eucalyptus globulus* and mixed (50% *Pinus radiata* and 50% *Eucalyptus globulus*) as raw material using an ECF (elementary chlorine free) bleaching system. The samples were taken after secondary treatment and stored in the dark at 4 ± 1°C.

Chemical oxygen demand (COD) and biological oxygen demand ($BOD_5$) were measured following standard methods (APHA-AWWA-WPCF 1985). Total nitrogen (TN), total phosphorous ($PO_4^{-2}$-P) and nitrogen like ammonia ($NH_4^+$-N) were measured by means specific kit espectrocuant NOVA-60 of Merck. The total phenolic compounds (UV phenols) 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 into concentration using a calibration curve with phenol as standard solution. Samples were membrane-filtered (0.45 μm). Color was measured at wavelengths of 440 in a 1 × 1 cm quartz cell using a model Spectronic Unicam UV–Visible Series Genesys™ 10.

A *Saccharomyces cerevisiae* recombinant strain was provided by Dr. J.P. Sumpter from GLAXO Research and Development Limited (Brunel University). The yeast estrogen screen (YES) assay was performed according to Routledge and Sumpter (1996). Effluents were diluted and aliquots (10 μl) of each concentration were transferred to a 96-well optically flat bottom microtiter plate. The samples dissolved in absolute ethanol were allowed to evaporate. Aliquots (200 μl) of the seeded assay medium and the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) were dispensed to each sample. Each plate contained at least one row of blanks as well as a standard curve. This assay uses 17-α-ethynylestradiol (EE2) (54.48 μg/L to 26.61 ng/L) as standard and of a sample's estrogenicity is expressed as 17-α-ethynylestradiol equivalents (EE2 eq., ng/L). The plate was placed in an incubator (Thermo Areus B6) for three days at 30°C. Trays were then analyzed spectrophotometrically at 570 nm and 630 nm using a plate reader (ELx 800). Data were corrected for yeast turbidity and constitutive Lacz Z expression observed in the samples and controls according to the equation described by Nakama et al. (2007). The effective concentration $EC_{50}$ of EE2 was 0.09 ng/L, calculated by dose–response curves.

Extraction techniques with reverse phase C-18 (non-polar) cartridge were used to analyze organic compounds from treated pulp mill effluents (Conceição et al. 2002). About 500 mL samples of each effluent were filtrated through Whatman Binder-free Glass microfiber filter (type GF/C: 4.7 cm in diameter and 0.4 μm particle retention). After that, each sample was extracted with solid-phase extraction (SPE) using ACCUBOND ODS C-18 reverse phase cartridges previously conditioned with two volumes (ca. 12 mL) of milli-Q water volumes and one methanol, ethyl acetate and *n*-hexane. Runs were performed at 4 mL min/L (two cartridges were prepared for each sample). The effluent's composition was analyzed from one of the cartridges previously eluted with two of methanol volumes, two ethyl acetate volumes and two *n*-hexane volumes.

The extracts obtained by SPE were evaporated using a gentle nitrogen current and then finally reconstituted and analyzed by gas chromatography mass detection (GC-MS) in Agilent gas chromatograph 7890A with Agilent 5975°C detector (Avondale, PA. USA) equipped with a HP 5MS column (0.25 mm diameter and 0.25 μm thickness). Oven conditions were as follows: Initial temperature of 100°C, rising at 8°C/min to final temperature of 250°C. Injector temperature 250°C and mass detector was used at 300°C. Mass detector was used in a SCAN mode with a range of scanning between 100 and 400 amu. Post acquisition analyses were performed using the extracted ion tools.

## Result and Discussion

Table 1 shows the physicochemical characteristics of the three kraft mill effluents. In all cases, pH was maintained inside the range of the neutrality (7.1–7.7), which is considered to be ideal value for the survival and development of most aquatic organisms. Effluents showed low $BOD_5$ values (16.0–34.0 mg/L), whereas COD values ranged between 135.5 and 213.7 mg/L.

The $BOD_5$/COD ratio, indicative of the non-biodegradable fraction, ranged from 0.079 to 0.18. This fraction corresponded mainly to recalcitrant compounds, like phenolic compounds, and was associated to color. As reported previously by Chamorro et al. (2005), high color values were associated with the presence of high molecular weight (>10,000 Da) phenolic compounds (Vidal et al. 2001).

Values of estrogenic activity for the kraft mill effluents are shown in Table 2. It is remarkable the highest estrogenic activity is observed with the eucalyptus effluent. In

**Table 1** Physicochemical characteristics of the effluents

| Parameter (units) | *P. radiata* | *E. globulus* | Mixed |
|---|---|---|---|
| pH | 7.2 ± 0.2 | 7.1 ± 0.1 | 7.7 ± 0.1 |
| COD (mg/L) | 213.7 ± 8.3 | 135.5 ± 3.7 | 202.0 ± 9.0 |
| $BOD_5$ (mg/L) | 34.0 ± 11.0 | 25.0 ± 2.8 | 16.0 ± 4.0 |
| Total phenolic (mg/L) | 204.2 ± 17.0 | 177 ± 3.1 | 164.0 ± 8.0 |
| Color (unit 1×, 1 cm) | 0.2 ± 0.3 | 0.2 ± 0.1 | 0.4 ± 0.1 |
| Nitrogen ($NH_4^+$–N) (mg/L) | 0.5 ± 0.3 | 0.4 ± 0.2 | 0.7 ± 0.1 |
| Total nitrogen (TN) (mg/L) | 1.7 ± 0.1 | 1.1 ± 0.1 | 1.9 ± 0.1 |
| Total phosporuos ($PO_4^{-2}$–P) (mg/L) | 0.8 ± 0.3 | 0.5 ± 0.1 | 0.9 ± 0.2 |

*COD* chemical oxygen demand, *$BOD_5$* biological oxygen demand: mixed: 50% *P. radiata* and 50% *E. globulus*

**Table 2** Estrogenic activity of effluents, and EE2 eq. determinate by YES assay

| Effluents | EE2 eq. (ng/L) |
|---|---|
| *Pinus radiata* | 0.383 ± 0.127 |
| *Eucalyptus globulus* | 1.475 ± 0.169 |
| Mixed | 0.849 ± 0.072 |

*EE2 eq.* estrogenic equivalent of 17 α-ethynylestradiol, mixed: 50% *P. radiata* and 50% *E. globulus*

any case, the data indicate weak estrogenic activity in the studied effluents in contrast with previous results for eucalyptus effluents (42–83 ng/L EE2 eq., Fernandez et al. 2007). We propose that adequate effluent treatment may reduce very significantly the total estrogenic load actually discharged into the environment (see Céspedes et al. 2005).

Fractionation of the effluents resulted in a complete loss of the estrogenic activity for *P. radiata* and mixed effluents (not shown). In contrast, all three fractions (methanol, ethyl acetate and hexane) from *E. globulus* effluent showed significant estrogenic activity (60%, 25% and 5% of the total activity, Fig. 1). These results are consistent with previous reports in a similar study (Orrego et al. 2009; Fernandez et al. 2007), and suggest a mild non-polar nature of the putative estrogenic compounds present in the effluent.

Figure 2 shows a characterization of the different effluents studied in this work by SPE and GC-MS. In the three kraft mills effluents, the predominant classes of extractives were hydrocarbons, fatty acids, sterols, phenols and triterpenes. The first group consisted in hydrocarbons, particularly abundant in the *E. globulus* effluent (0.599 μg/L). Hydrocarbon concentrations were 0.341 μg/L in *P. radiata* effluent and 0.154 μg/L in the mixed effluent. These compounds are very frequently detected in *P. radiata* and *E. globulus* samples, and are produced by incomplete hydrolysis of waxes (Gutiérrez et al. 1999). Fatty acids were found mainly in *P. radiata* effluent (0.349 μg/L) followed by mixed (0.139 μg/L) and *E. globulus* (0.043 μg/L) effluents. Sterol compounds were more abundant in the *E. globulus* and mixed effluent (0.087 and 0.020 μg/L, respectively) (Fig. 3).

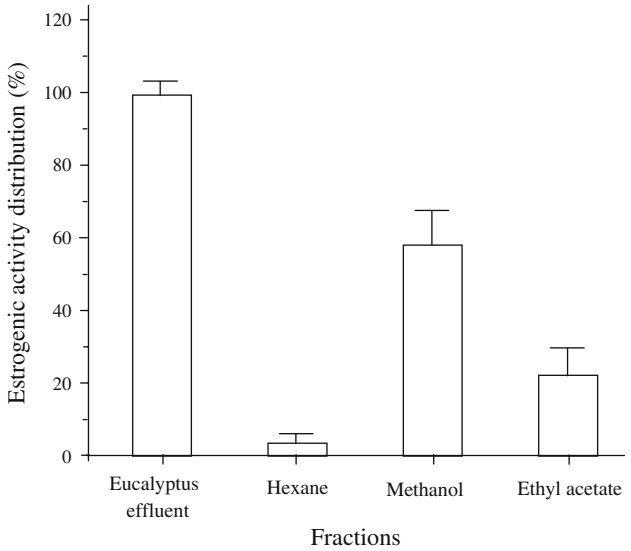


**Fig. 1** Percentage of estrogenic activity of *E. globulus* effluent fractioned by solid-phase extraction (SPE C-18)

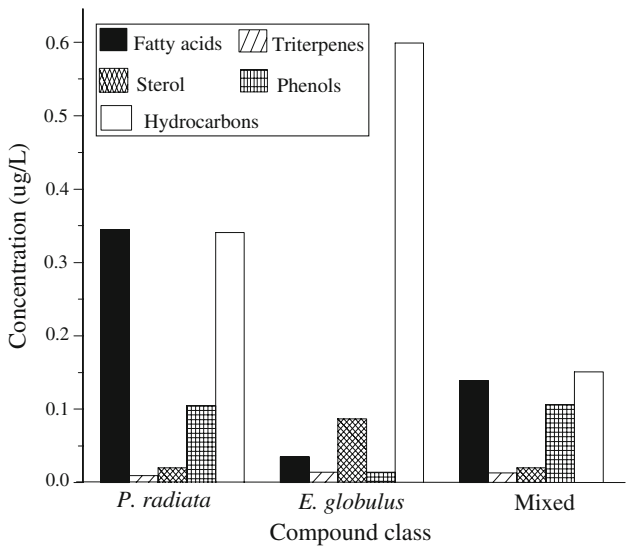


**Fig. 2** Class compound found in effluents of cellulose, fatty acids, triterpenes, sterol, phenols and hydrocarbons

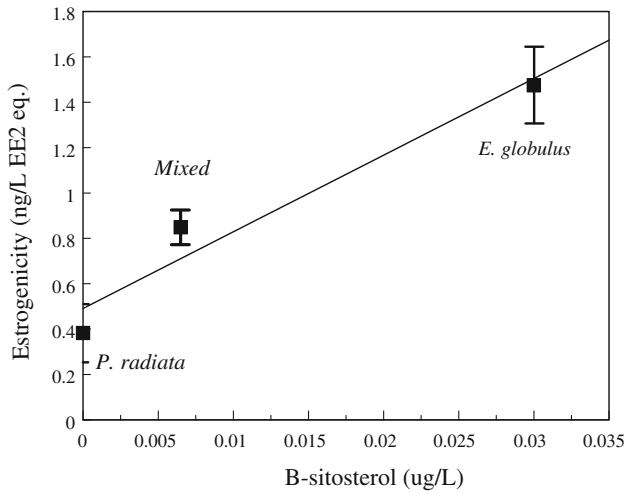


**Fig. 3** Relationship of the $\beta$-sitosterol concentration with the observed estrogenic activity on the *P. radiata*, mixed and *E. globulus* effluents

Table 3 shows specific compounds measured in each effluent and fraction. When comparing these values with the observed estrogenic activity of the different effluents, sterols emerge as likely candidates for the estrogenic activity, including $\beta$-sitosterol, stigmastanol and squalene, among others. These compounds are particularly abundant in the *E. globulus* effluent, the one showing the highest estrogenic activity, and their distribution in the different fractions correlated with their observed estrogenicity. $\beta$-sitosterol has been shown to induce feminization in fish (Mellanen et al. 1996; Honkanen et al. 2005; Wartman et al. 2009).

The presence of endocrine disruptors in wastewater has turned into a topic of world interest due to their putative impact on the environment. Hitherto, there is no consensus among scientists on the best screening methods for determining endocrine disruption in the aquatic environment. In this paper, we showed the utility of the YES assay for estrogenic activity determination in complex mixtures as well as the usefulness of its association with the CG-MS system for identification and characterization of specific compounds in the kraft pulp mill effluent.

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**Table 3** Sterols and triterpenes fractionated by means of SPE-C18 methanol, ethyl acetate and hexane

| Effluent | Compounds<br>*Sterols and triterpenes* | Fractions (µg/L) | | |
|---|---|---|---|---|
| | | Methanol | Ethyl acetate | Hexane |
| *P. radiata* | Cholesterol | 0.0168 | ND | ND |
| | Stigmasterol | 0.0032 | ND | ND |
| | Dihidroxy pregnanolone | 0.0229 | ND | ND |
| | Cholest-5-en-3-ol (3 beta) | ND | 0.0117 | ND |
| | 20-(alpha-20 S) 13,21 cyclo-18 *n*-pregnol | 0.1150 | ND | ND |
| | Hydroxy-dehydroabietic methyl ester | ND | ND | 0.062 |
| | Squalene | ND | 0.0040 | ND |
| | 1,2 benzeno dicarboxylic di tridecyl ester | ND | ND | 0.0034 |
| | Lupanol acetate | ND | 0.0210 | ND |
| *E. globulus* | $\beta$-sitosterol | 0.0167 | 0.0144 | ND |
| | Epoxy pregnanediol-dione | ND | 0.0104 | ND |
| | Cholesterol | ND | 0.0312 | ND |
| | Stigmastan-3,5,22-trien | ND | 0.019 | ND |
| | Squalene | ND | 0.0278 | 0.0032 |
| | Stigmastanol | 0.021 | ND | ND |
| *Mixed* | Stigmastanol | 0.0049 | 0.0929 | ND |
| | Cholesterol | 0.0029 | ND | ND |
| | Stigmasterol | 0.0059 | ND | ND |
| | $\beta$-sitosterol | 0.0065 | ND | ND |
| | Cholesta 3,5 diene | ND | 0.0039 | ND |
| | Cholesta 2,5 diene-4 ona | ND | 0.0005 | ND |
| | Stigmastan-3,5 diene | ND | 0.0014 | ND |
| | Preg-9 (11)9-en-20-one 3,6-dihydro | 0.022 | ND | ND |
| | (3 beta 5 alpha, 6 alpha) squalene | ND | 0.0031 | 0.0215 |

*ND* no detected, mixed: 50% *P. radiata* and 50% *E. globulus*

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