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Effects of Kraft Lignin and Chlorolignin on the Binding and Bioavailability of Benzo(a)pyrene to *Daphnia magna* Straus

J. Kukkonen[1] and A. Oikari[2]

[1]University of Joensuu, Department of Biology, P.O. Box 111, SF-80101 Joensuu, Finland and [2]Helsinki University of Technology, Laboratory of Environmental Protection, SF-02150 Espoo, Finland

Besides the natural dissolved organic materials (DOM), often referred as humic substances, there is another group of DOM, called chlorolignins, dominating in waters affected by pulp and paper industry effluents. Chlorolignins resemble natural DOM structurally, so that it is presently difficult to analyze chlorolignin and humic substances separately in environmental samples (Knuutinen et al. 1987). Chlorolignin is usually defined as the chlorinated product of lignin that has a molecular weight greater than 1000. It is a major byproduct in the bleaching of wood pulp with chlorine (Kringstad and Lindström 1984). In bleaching, the pulp is treated with chlorine chemicals sequentially with alkaline extraction between the chlorination stages. Residual lignin in pulp is chlorinated and oxidized in the bleaching process and dissolved in alkali. The characteristics of chlorolignin vary depending on the bleaching process and wood species. Chlorolignins are, for example, rich in carboxylic and hydroxyl groups and consequently are very hydrophilic (Sågfors and Starck 1988). Similar characteristics can also be found in humic substances (Stevenson 1982) and the most dominating difference is the higher chlorine content of chlorolignin.

At present, large quantities of chlorolignin are released to recipients, the load generally varying 1-3 kg of total organically bound chlorine per ton bleached pulp. However, very little is known about its environmental fate and effects. Chlorolignin itself is not considered harmful for aquatic life since its large, hydrophilic molecules may not penetrate cell membranes and therefore not enter the cells of living organisms. It has been shown that chlorolignin is not acutely toxic to *Daphnia magna* (Sågfors and Starck 1988). Additionally, it is worth noting that, like humic substances, chlorolignin is very resistant to microbial activity. For instance, the bacteria from aerated lagoon of a pulp mill were unable to degrade chlorolignin to any significant degree (Eriksson and Kolar 1985). On the other hand, it is reported that some low-molecular weight chlorinated compounds were formed from chlorolignin as products of their microbial

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Send reprint requests to Dr. J. Kukkonen at above address degradation (Neilson et al. 1983). Similar breakdown or dissociation has been seen also under sterile conditions (Eriksson et al. 1985). It is possible that the low-molecular weight compounds were not real degradation products of chlorolignin but were present in the chlorolignin preparations in some kind of associates as suggested by Paasivirta et al. (1990) and released to the water when concentration of free compound was low. Direct measurements about the ability of chlorolignin to bind organic xenobiotics, however, have not been reported.

The objectives of this study was to get some basic information on the ability of unchlorinated kraft lignin as well as chlorolignin preparations isolated from pulp mill effluents to bind benzo(a)pyrene (BaP), as a lipophilic neutral model xenobiotic, and whether this binding can affect the bioavailability of BaP to waterflies, *Daphnia magna*.

MATERIALS AND METHODS

Radiolabeled ^{14}C-benzo(a)pyrene (BaP; 52.0 mCi/mmol) was purchased from Amersham (UK). The stock was diluted in ethanol for further dilutions. Aqueous solutions of radiolabeled contaminant were prepared by addition of the stock carrier solution.

Indulin AT (Westvaco Co., CA, USA) was used as a model kraft lignin in the experiments. Indulin was dissolved in distilled water (1g/l) by adding a small volume of 0.1 N NaOH giving pH 10-11. Chlorolignin preparations were isolated from the bleached kraft mill effluent (BKME; Kaukas Ltd., Lappeenranta, Finland) which was an combined sample of 5 days collecting period. The effluent was collected before the external waste treatment plant of the mill. Wood used in the processes was pine and the effluent sample composed 36% of C/D stage, 32% of E_1 stage and 32% of E_2 stage. High molecular weight chlorolignin and lignin were isolated from the BKME by dialysing (mol. weight cut off 12000D, Medicell International Ltd.) the effluent and indulin AT solution against overflowing deionised water for 72 hours. Part of the effluent sample (1 l) was extracted two times with 100 ml hexane (p.a.) and once with 100 ml diethylether (p.a.) before the dialysis to maximize a removal of low molecular weight toxic compounds (Kringstad and Lindström 1984). During the dialysis 50% and 30 % of dissolved organic carbon (DOC) from the effluent and indulin AT, respectively, were lost. Elemental composition (C, H, N) of chlorolignin and lignin preparations were measured with Carlo Erba elemental analyzer (mod. 1106). Chlorolignin and kraft lignin preparations were also characterized by UV-VIS spectroscopy. The absorbance of the samples was measured at 250, 270, 365, 465 and 665 nm using a Hitachi U-2000 spectrophotometer and 10 mm quartz cuvette.

Artificial organic-free control water was made up of Milli-Q grade water (DOC $\leq$ 0.3 mgC/l) and the following reagent-grade salts: $CaCl_2 \cdot 2H_2O$, 58.8 mg/l; $MgSO_4 \cdot 7H_2O$, 24.7 mg/l; KCl, 1.1 mg/l; and $NaHCO_3$, 13.0 mg/l (Ca + Mg hardness = 0.5 mM, pH adjusted to 6.5). This control water was used in the Daphnia experiments and diluting down the chlorolignin and lignin preparations.

Equilibrium dialysis (McCarthy and Jimenez 1985) method was used to determine the partition coefficient (K_p) between BaP and the preparations. Five ml of a filtered (Nuclepore 0.22 μm) sample was added to a dialysis bag (Spectra/Por 6, mol. weight cutoff of 1000 daltons) and placed in a 200-ml glass jar containing an aqueous solution of ^{14}C-BaP (190 ml). Sodium azide (0.005%) was added to inhibit microbial activity. The jar was sealed with a teflon-lined cap and shaken in the dark at 20°C for 4 days. At least four replicate determinations were made. Equilibrium was confirmed in a parallel experiment using control water. Solutions inside and outside the dialysis bag was analyzed for ^{14}C-activity using scintillation cocktail (Luma Gel Safe, Lumac LSC, NL) and a liquid scintillation counter. The outside concentration (C_o) is the freely dissolved organic pollutant, while the difference between the inside and outside concentration (C_p) is the pollutant bound to organic matter in the bag. K_p was calculated as

$$K_p = C_p/(C_o \times DOC)$$

where DOC is the concentration of dissolved organic carbon (kg carbon/L) and K_p has the unit of l/kg.

For bioavailability measurements, ligninuous water samples (DOC = 22 mgC/l) were filtered (Nuclepore, 0.22 μm) and pH adjusted to 6.5 with 0.1 N NaOH or HCl. Aqueous concentration of BaP was 1 μg/l. *Daphnia magna* used in these experiments were approximately 6-8 days old and did not have eggs in the brood chamber. Before exposures, daphnids were held for 1 hr in the clean control water to clear their gut contents. Groups of ten *D. magna* were transferred to 200-ml glass beakers containing 100 ml of DOM sample and added ^{14}C-BaP. Four replicate determinations were made for each sample. Beakers were kept in the dark at 20°C. After 24 hr animals were removed from the water with a widemouthed pipet, collected on filter paper, briefly rinsed with 5 ml of distilled water, blotted dry, and weighed in two groups on a microbalance. The five animals were added to 5 ml of scintillation cocktail (Luma Gel Safe, Lumac LSC, NL) and analyzed for radioactivity. The radioactivity remaining in the exposure water was also determined. Each experiment included a parallel control experiment using organic-free control water. The results are reported as bioconcentration factors (BCF) calculated as the ratio of the concentration of the pollutant in the animals (nanogram per gram wet wt) and in the water at the end of the experiment (nanogram per millilitre), both calculated from the specific activities of the compounds. The fitting of

Table 1. Elemental composition of the kraft lignin and chlorolignin preparations. Percentages are on ash free basin.

Sample	C%	H%	N%	Cl%	Ash%
Indulin	64.8	5.0	1.5	0.01	3.8
Dialyzed Indulin	60.2	4.8	1.2	0.05	3.5
Chlorolignin	45.7	4.3	0.8	6.0	9.5
Extracted Chlorolignin	47.4	4.4	0.4	6.2	9.2

regression lines and statistical analyses (ANOVA) were performed using SAS software (SAS Institute Inc., NC, USA) on VAX-6420 computer.

RESULTS AND DISCUSSION

The elemental composition of kraft lignin (Indulin AT) and chlorolignin preparations is shown in Table 1. The major differences between indulin and chlorolignin preparations are the higher carbon content in indulin than in chlorolignin and chlorine in chlorolignin structure. The E_2/E_3 (A250/A365) and E_4/E_6 (A465/A665) ratios and the absorptivity at 270 nm (ABS_{270}) were analyzed for indulin and chlorolignin preparations at different DOC concentrations (Table 2). For Indulin samples E_2/E_3 ratio was 5.7-5.8 and for chlorolignin 4.1-4.2. In all four cases E_4/E_6 ratio decreased when DOC concentration increased (Table 2).

According to Chen et al. (1977), a high E_4/E_6 ratio is proportional to the degree of humification and to the molecular weight of humic substances, as measured by the colligative properties of material extracted from soil. They also showed that E_4/E_6 ratio is constant for a sample over a range of DOC concentrations over 100 mgC/l. However, with the kraft lignin and chlorolignin preparations we did not get a constant values (Table 2). Schnitzer (1977) found that the E_4/E_6 values for humic acids and fulvic acids extracted from soils, formed under widely differing conditions, are within the range of 3.8 to 5.8 and 7.6 to 11.5, respectively. According to De Haan (1983), fulvic acids from strongly humic and oligotrophic waters are characterized by a E_2/E_3 ratio of about four. The absorptivity of the sample at 270 nm [ABS_{270}; in units of L/mgC × cm × 10^3] reflects absorbance of pi-pi' transitions in substituted benzenes and most polyenes and have been related to the aromatic content of isolated soil humic acids (Gauthier et al. 1987). Judging by this information chlorolignins isolated from pulp mill effluent are much like fulvic acids having similar absorptivity as natural DOM in brown water humic lakes (Kukkonen and

Table 2. Spectroscopic characterizationof the kraft lignin and chlorolignin preparations. All absorbances were measured at pH 6.5 at given dissolved organic carbon (DOC) concentration.

Sample	DOC (mg/l)	E_2/E_3[a]	E_4/E_6[b]	ABS_{270}[c]
Indulin	22	5.77	8.00	27.41
	44	5.70	7.14	25.32
	110	-[d]	7.22	-
	220	-	6.33	-
Dialyzed Indulin	22	5.70	7.80	33.91
	44	5.71	8.13	32.19
	110	-	6.83	-
	220	-	6.66	-
Chlorolignin	22	4.18	11.20	36.55
	44	4.15	11.56	33.84
	110	-	9.39	-
	220	-	9.32	-
Extracted Chlorolignin	22	4.18	11.20	35.73
	44	4.11	10.60	33.95
	110	-	10.07	-
	220	-	9.75	-

a: E_2/E_3 ratio is the ratio of absorbances at 250 and 365 nm
b: E_4/E_6 ratio is the ratio of absorbances at 465 and 665 nm
c: absorptivity at 270 nm (units of l/mgC × cm × 10^3)
d: not measured, because of too dark color of the sample

Oikari 1991). The aromaticity of the kraft lignin preparation increases somewhat during a dialysis of the preparation. Extraction of the BKME before dialysis do not change the characteristics of the chlorolignin preparation.

The K_p values of BaP to lignin and chlorolignin preparations are shown in Fig. 1. The measured K_p values of BaP are about the same or slightly higher than reported for BaP to natural aquatic DOM (Kukkonen et al. 1989, Kukkonen and Oikari 1991, McCarthy et al. 1989). The kraft lignin has better binding capacity than chlorinated lignin from BKME. Based on this data it is hard to say whether this difference between kraft lignin and chlorolignins is due to chlorination of lignin which can change the binding

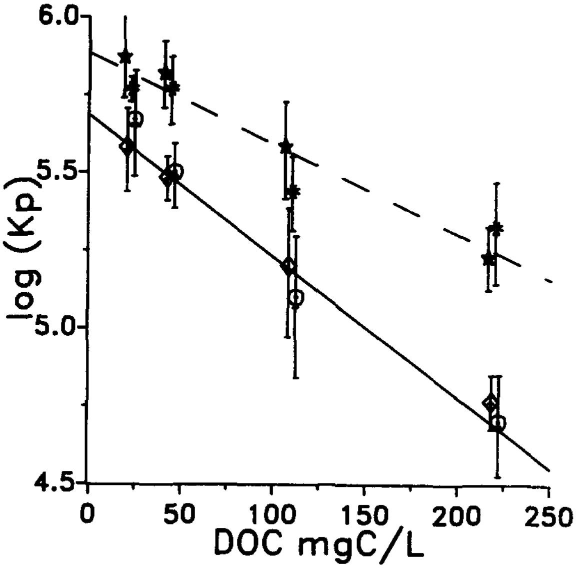

Figure 1. Partition coefficients (Kp ± SD, n = 4) of BaP to the kraft lignin and chlorolignin preparations as a function of DOC. * = Indulin AT, ★ = Dialysed Indulin, ⊙ = Chlorolignin, ◇ = Extracted chlorolignin.

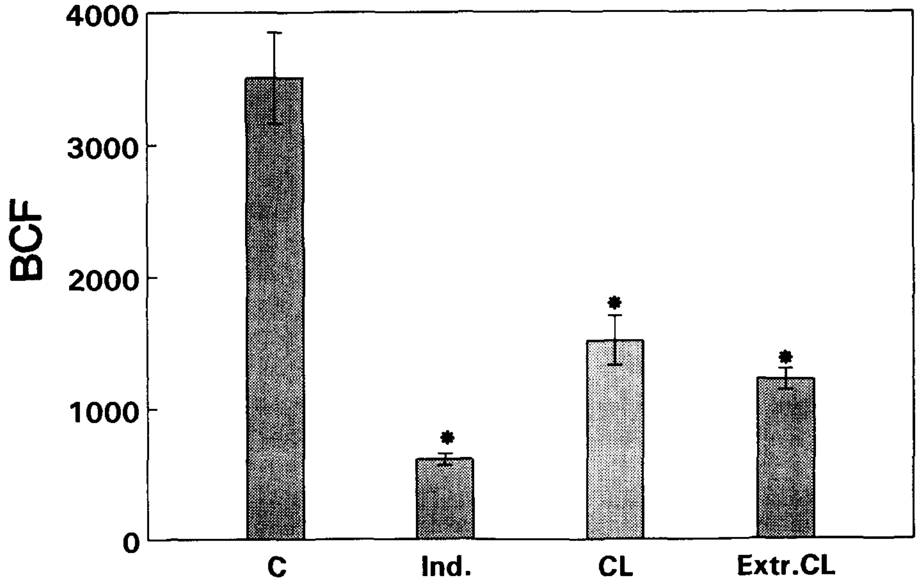

Figure 2. Bioconcentration factors (BCF ± SD, n = 4) of BaP to *Daphnia magna*. C = Control (DOC < 0.3 mgC/l), Ind. = Indulin AT, CL = Chlorolignin and Extr.CL = Extracted chlorolignin (for all preparations DOC = 22 mgC/l). * = Significantly ($p < 0.05$, ANOVA) different from the control.

capacity or to some other structural or chemical differences between preparations. Dialysis of Indulin AT or extraction of chlorolignin did not change significantly ($P>0.1$) the K_p's (Fig. 1). The concentration dependence of the K_p is also clear.

Compared to the organic-free (DOC < 0.3 mgC/l) control water the accumulation of BaP was reduced by 60% - 80% from waters containing kraft lignin or chlorolignin preparations at the concentration of 22 mgc/l (Fig. 2).

Remberger et al. (1986) showed that majority of polychlorinated guaiacols and catechols in sediments downstream from a pulp mill were chemically bound. Paasivirta et al. (1990) suggested binding of hydrophobic organic contaminants by dissolved chlorolignin and affecting their possible transport in the environment. The present research has extended previous observations by showing and measuring, at the first time, with one model compound the ability of chloroligning and kraft lignin to bind highly lipophilic xenobiotic and thus also affect the bioavailability of this compound to aquatic animals.

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