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A FISH HEPATOMA CELL LINE (PLHC-1) AS A TOOL TO STUDY CYTOTOXICITY AND CYP1A INDUCTION PROPERTIES OF CELLULOSE AND WOOD CHIP EXTRACTS

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ABSTRACT

Cytotoxicity and CYP1A induction properties of celluloses and wood chips were studied with a teleost liver cell line, PLHC-1. Cells were exposed to acetone extracts of celluloses produced using new bleaching techniques (elemental chlorine free, ECF; totally chlorine free, TCF) in two sulphate mills or without any bleaching (unbleached, UB) in a sulphite mill. In another set of exposures, celluloses (ECF and TCF bleached) and wood chips (from pine and birch) were collected from a sulphate mill, extracted with acetone, and the extracts used to treat the cells. After exposure, *O*-deethylation of 7-ethoxyresorufin (EROD, a measure of cytochrome P4501A (CYP1A) catalytic activity), and total protein content, a measure of cytotoxicity, were assayed. The presence of the CYP1A protein in the exposed cells was assessed by immunoblotting. The cellulose and wood chip extracts were able to cause both cytotoxicity and EROD induction in the PLHC-1 cells. In the exposures conducted with the material from three different mills, the celluloses made of birch were more cytotoxic and more potent inducers of EROD activity than were the celluloses of pine. Further, UB celluloses increased EROD activity and caused cytotoxicity at lower doses than material bleached with modern bleaching techniques. In the exposures made with material from one single mill, there were no clear trends between the celluloses made of pine or birch. Wood chips of pine, however, were more cytotoxic than wood chips of birch. Especially with pine wood chips, cytotoxicity interfered with the induction of EROD activity, thus complicating the evaluation of CYP1A induction. CYP1A protein content was not detected in cells exposed to extracts of celluloses or wood chips, possibly due to low amounts of protein available for the assay. Wood and pulp processing, like bleaching, may change the chemical composition of the raw material in a way that reduces the potency for biological effects of the final product, cellulose. This could explain why both UB celluloses and wood chips were more potent in the cells than ECF or TCF bleached celluloses. In this study the PLHC-1 cell line showed its potential for use in evaluating the biological activity existing in pulp and paper mill products and raw materials. The identity and source of the compounds that were able to affect the PLHC-1 cell line remain to be determined.

INTRODUCTION

The processing of wood into pulp and paper loads the aquatic environment with a complex discharge of compounds, such as extractives, organic acids, a range of sulfur-containing compounds, chlorinated phenolics, chlorinated neutral compounds, and chlorinated organic acids [1,2]. Hazardous chemicals can also be found in the products of the pulp and paper industry [3-6]. Recently, the use of elemental chlorine in wood pulping has been diminished or abolished. Therefore, the debate concerning biological effects of various chemicals produced in pulp and paper processing has been widened from chlorinated chemicals to others such as, e.g., wood extractives. Many of the biologically active compounds may have their origin in the wood material itself.

At a sublethal level, the effects of some hazardous chemicals, namely polycyclic aromatic hydrocarbons (PAHs) and compounds with a chemical structure similar to that of PAHs, can be monitored by measuring the induction of cytochrome P4501A (CYP1A) in exposed organisms. CYP1A induction in fish is mediated by a cytosolic *Ah* receptor and can be detected in several steps (mRNA, CYP1A protein, catalytic enzyme activity) [7-9]. Most of the previous programs in which CYP1A has been used to monitor the effects of pulp and paper industry on fish have been conducted *in vivo* with material in the field or in the laboratory [10-16]. Using *in vitro* techniques, the CYP1A-inducing effect of various pulp and paper mill effluents have been studied in primary hepatocyte cultures of rainbow trout [17].

So far, few cell lines containing an inducible CYP1A system have been developed from the liver of fish [18-20]. One of these, a PLHC-1 liver cell line, was initiated from hepatic tumors in the topminnow (*Poeciliopsis lucida*) [19]. The PLHC-1 cell line has potential for studying both CYP1A induction and inhibition in the cells as well as for detecting cytotoxicity [21-26]. Such studies have been conducted mainly with individual compounds. The response of PLHC-1 cell line in biomonitoring complex environmental samples is not well known, except in a few studies [e.g., 27-30].

Mammalian liver cell lines, such as the Hepa-1 mouse hepatoma cell line, have been used in biochemical screening of various fly ashes, including fly ashes of bleached sludge of kraft pulp mills [31,32], celluloses and wood-based materials used as bedding for laboratory animals [33,34] as well as river sediments [35]. In these studies the Hepa-1 cell line has been useful in comparing the toxicological and biochemical responses of various chemically complex samples.

The main aim of this study was to test whether the PLHC-1 cell line is sensitive enough to respond to compounds in the products and wood materials of the pulp and paper industry. Although recent changes in bleaching techniques have reduced the formation and discharge of chlorinated aromatic compounds such as dioxins and furans, some biological effects persist in fish exposed to pulp mill effluents [11,14]. Our hypothesis was that biologically active compounds may be present in the products made by new techniques, as well. Another aim was to compare the toxicological and enzyme-inducing properties of celluloses collected from various mills. To ensure that the differences between mills were not affecting the results, various celluloses and wood chips collected from one mill were also evaluated. To fulfill the objectives, PLHC-1 cells were exposed

to the acetone extracts of unbleached (UB) or bleached (elemental chlorine free = ECF, or totally chlorine free = TCF) celluloses and wood chips of pine and birch. After exposure, the CYP1A induction (EROD activity, CYP1A protein content) and cytotoxicity (total protein content) were assayed in the cells.

MATERIALS AND METHODS

Cell line, reagents and chemicals

The PLHC-1 cell line, derived from a hepatocellular carcinoma of topminnow *Poeciliopsis lucida* [19], was maintained in a humidified, 5% CO_2 atmosphere at 30°C in MEM, containing Earle's salts, non-essential amino acids, L-glutamine and 10% calf serum. Cells were grown as a monolayer in 94/16 mm dishes in 10 ml of medium or in 75 cm^2 flasks in 20 ml of medium and subcultured as described previously [22,29]. Reagents and chemicals were obtained as described previously [22,23,29].

Study material

The celluloses in the first experiment were collected from three different pulp and paper mills in Finland. Two of the mills (**A, B**) produced elementary chlorine free (ECF) or totally chlorine free (TCF) bleached pulp from either pine (softwood) or birch (hardwood) by a sulphate method. Mill **C** produced unbleached (UB) semipulp and cardboard from birch by a sulphite method.

Table 1. Identification of cellulose samples which were collected from three different mills.

mill	wood material	bleaching method	pulping process
A	birch	ECF[a]	sulphate
A	birch	TCF	sulphate
A	pine	ECF	sulphate
A	pine	TCF	sulphate
B	pine	ECF	sulphate
B	birch	ECF	sulphate
C	birch	UB	sulphite
C	birch + pure cardboard	UB*	sulphite

[a]ECF = elemental chlorine free; TCF = totally chlorine free; UB = unbleached; UB & UB* raw material different in part

In the second set of exposures, celluloses and wood chips were collected from one Finnish pulp and paper mill. Celluloses were either ECF or TCF treated. Pine and birch were used as raw materials.

Extractions of celluloses

Celluloses and wood chips were first cut into pieces (1 x 1 cm) or carved and weighed. The samples were dissolved in acetone overnight. The next day the samples were shaken twice (total time at least 2 h) at room

temperature. The composite samples were filtered and then evaporated with a rotatory evaporator. The samples were finally transferred into glass vials and evaporated into 1 ml or 0.5 ml volume under nitrogen flow. Final stock contained an extract from 60 g of cellulose or 40 g of wood chips in 1 ml of acetone. The control samples consisted of pure acetone and were treated the same way as the actual study material.

PLHC-1 bioassays

Determination of catalytic CYP1A induction after three days exposure in 94/16 mm dishes was conducted as previously described [29]. The cells (2 x 10^6 per dish) were first seeded in the dishes, and the medium was refreshed with medium containing test compounds/extracts on the next day. After three days exposure at 30°C, the cells were scraped from the dish, pelletted and stored at -80°C. The *O*-deethylation of 7-ethoxyresorufin (EROD) was measured from a sonicated cell lysate in a kinetic reaction with RF-5001PC spectrofluorometer (Shimadzu) as basically described in [36]. Measurement of cytotoxicity after three days exposure in 24-well plates also followed the procedures of [29]. The cells (2.5 x 10^5 per well) and the test extracts were introduced into the wells on the same day. After three days at 30°C, the living cells were dissolved with 0.05 M NaOH. Protein content in the sample obtained was assayed by the method of [37].

Experiments in 48-well plates were conducted as described earlier [22,23,29]. Catalytic CYP1A induction in the cells, which were exposed to the extracts of celluloses and wood chips for 24, 48 or 72 h, was first followed in a kinetic EROD reaction with a multiwell plate reader (Cytofluor 2300 fluorescence plate reader, Millipore). Total protein content was measured in each well after adding 100 µl of 1.08 mM fluorescamine (FA) in acetonitrile and incubating for 10 min at room temperature [38]. In this exposure it was observed that the metabolism of the acetone-extracted compounds in these samples was relatively rapid (seen as higher EROD activities in the cells exposed for 24 h than for 48 h or 72 h, not illustrated). Therefore, 24 h was selected as the exposure time for subsequent experiments. In these experiments, EROD activity and total protein content were measured simultaneously as endpoints [38]. The *O*-deethylation of 7-ethoxyresorufin was initiated with 7-ethoxyresorufin (final concentration 2 µM) in Na-phosphate buffer (0.05 M, pH 8.0) and the reaction was run for 10 min. The reaction was stopped with 150 µl of cold FA solution (150 µg/ml in acetonitrile) and the plate was allowed to sit for 15 min prior to the measurement with a plate reader (wavelengths for excitation and emission filters were 530 nm and 590 nm for EROD activity and 409 nm and 460 nm for protein content). Resorufin and bovine serum albumin standards were included on each plate [38].

Cells for measuring CYP1A protein content were dosed and treated in 48-well plates in a similar way as for EROD assay. Whole cell lysates were prepared by solubilizing the cells in 100 µl of sample treatment buffer [23]. CYP1A of PLHC-1 cells were measured as described by [39]. Protein (4.5-9.5 µg) from each sample and standards with known CYP1A content were subjected to separation with SDS polyacrylamide gel electrophoresis in a 12% acrylamide gel. The proteins were electrophoretically transferred onto nitrocellulose and incubated with monoclonal antibody (MAb 1-12-3) to CYP1A1 of scup (*Stenotomus crysops*) [40] and with secondary antibody (goat anti-mouse IgG linked to alkaline phosphatase, Bio-Rad). The results in nitrocelluloses

were further processed with VistaScan for Machindosh (version Vistascan v2.3.1. equipped with UMAX S-12 scanner).

Data were analysed in the programs of Jandel Scientific (SigmaPlot) and SPSS for Windows (Release 6.1.3).

RESULTS

Among the samples collected from three different mills, UB celluloses, made of birch and birch + pure cardboard by a sulphite process, were the most toxic for the cells. Compared to pure medium controls, these acetone-extracted samples showed a 97% reduction in total protein content at 300 mg/ml of cellulose (Fig. 1A). ECF bleached celluloses made of birch (sulphate process) reduced the total protein content by 80% at 300 mg/ml and by 45% at 210 mg/ml. TCF bleached cellulose made of birch (sulphate process) showed a slight toxicity at 300 mg/ml dose (12% of the total protein was diminished, compared to the control value). At the doses studied, samples made of pine did not show cytotoxicity.

Celluloses made of birch were the most potent inducers of EROD activity (Fig. 1B). At most, EROD activity in the cells treated with UB cellulose reached a peak value of 52±69 pmol/min/mg at the 120 mg/ml dose. This was 5 times less than the EROD activity of the positive control, 2 nM TCDD (262 pmol/min/mg). However, the cytotoxicity of UB celluloses partly interfered with the measurement of EROD activity in these samples, due to diminished content of total protein. Among the ECF and TCF bleached celluloses, a TCF bleached sample made of birch induced EROD activity most, reaching 30±15 pmol/min/mg at 300 mg/ml dose. An ECF bleached sample made of the same wood material induced EROD activity to 23±35 pmol/min/mg at 300 mg/ml. In contrast to the UB samples, EROD induction by the ECF and TCF bleached samples occurred at concentrations where little or no cytotoxicity was evident.

In the samples collected from one single mill, only wood chips reduced total protein content, compared to pure medium control (Fig. 2). EROD activity, however, was induced by both wood chips as well as by some celluloses (Figs. 2, 3). The most potent inducer of EROD activity seemed to be wood chips of pine (peak 74±33 pmol/min/mg at 40 mg/ml dose, 2.7 times less than 2 nM TCDD in this set of exposures; Fig. 2). However, especially in this sample the cytotoxicity interfered with the induction of EROD activity. Extracts of birch wood chips increased EROD activity to 49±26 pmol/min/mg at 200 mg/ml dose, although cytotoxicity interfered in the same way as with pine wood chips. This sample was, however, able to induce EROD activity to 17±4 pmol/min/mg at 100 mg/ml dose where total protein content was not diminished. In the samples from a line of birch pulp bleaching, TCF bleached cellulose induced EROD activity (peak 25±9 pmol/min/mg at 300 mg/ml dose, 8 times less than 2 nM TCDD) but ECF bleached cellulose did not. In the samples from a line of pine pulp bleaching, however, ECF bleached cellulose proved to be an inducer of EROD activity (peak 23±7 pmol/min/mg at 300 mg/ml dose), whereas TCF bleached cellulose was not (Fig. 3). However, none of the wood chips or celluloses was able to induce CYP1A content in the cells, at least to levels detectable with the studied amounts of protein (not illustrated).

The standard deviation (SD) of EROD activity varied from the respective mean value by over 100%, especially at those doses of study material that also showed cytotoxicity. The acetone-extracted material from wood chips or UB celluloses, in particular, was not easily dissolved into the medium when exposing the cells. This may have led to between experiment variability in the actual exposure concentrations as compared to nominal exposure concentrations, which may have caused the high variation at some doses between the results of parallel tests.

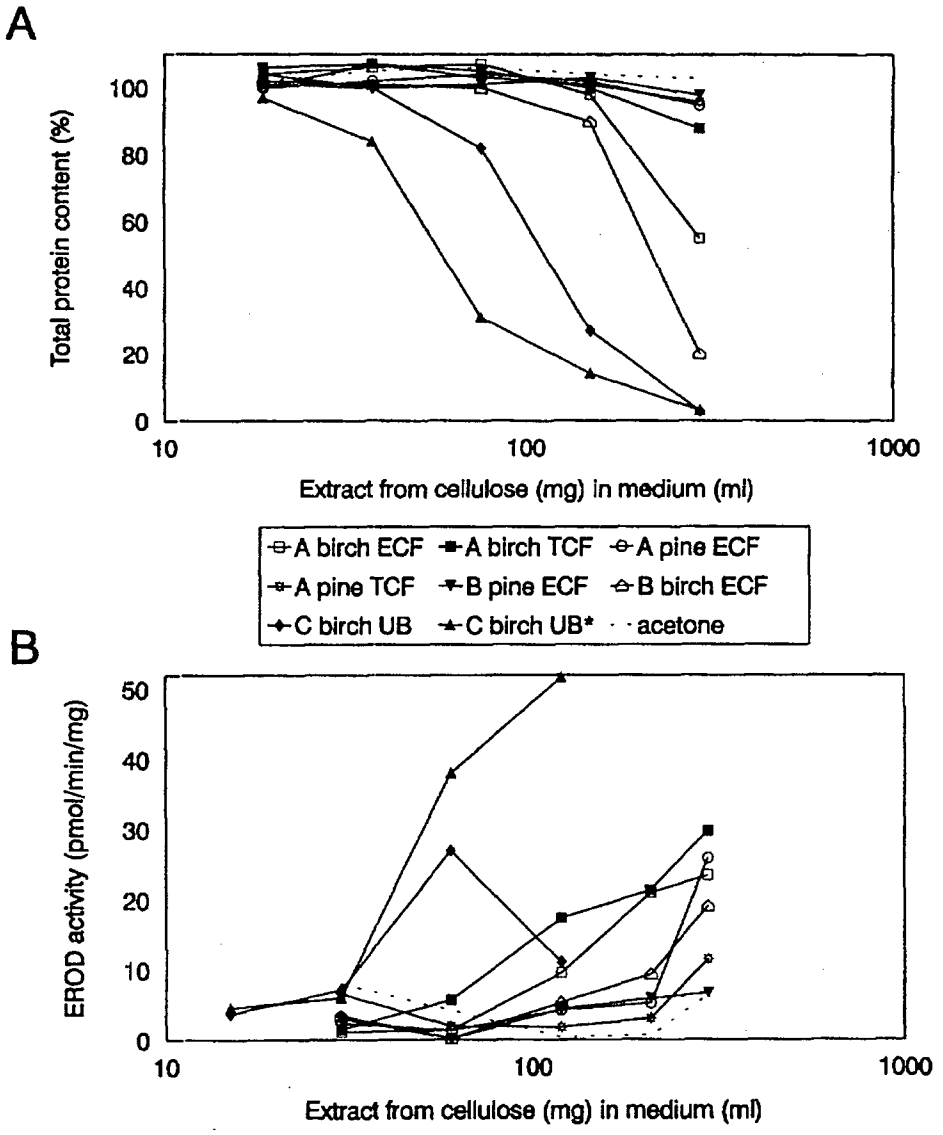

Fig. 1. A) Cytotoxicity as total protein content (%, untreated = 100%) and B) EROD activity (pmol/min/mg protein) in PLHC-1 cells after exposure to cellulose extracts. The cytotoxicity test lasted three days and was conducted in 24-well plates (3 different experiments). In the CYP1A induction test, the cells were exposed for three days in 94/16 mm dishes (1-3 different experiments for each dose). Celluloses were collected from three pulp and paper mills (A, B and C) and extracted with acetone. A positive control in the cytotoxicity test, 2,4-dinitrophenol, reduced the total protein content of the cells by 50% at 32 µg/ml (3 replicates). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, a positive control for CYP1A induction, increased EROD activity to 262±95 pmol/min/mg at 2 nM concentration (6 replicates).

Overall, SDs in cytotoxicity tests conducted for three days in 24-well plates were higher than those from tests conducted for one day in 48-well plates. In 24-well plates, the SD as a percentage of the mean value varied from 1 to 54% (except the results of UB celluloses, in which the range was 5-136%). In 48-well plates, the SD varied from 7 to 23% of the mean (except the results of pine wood chips with a range of 7-106%).

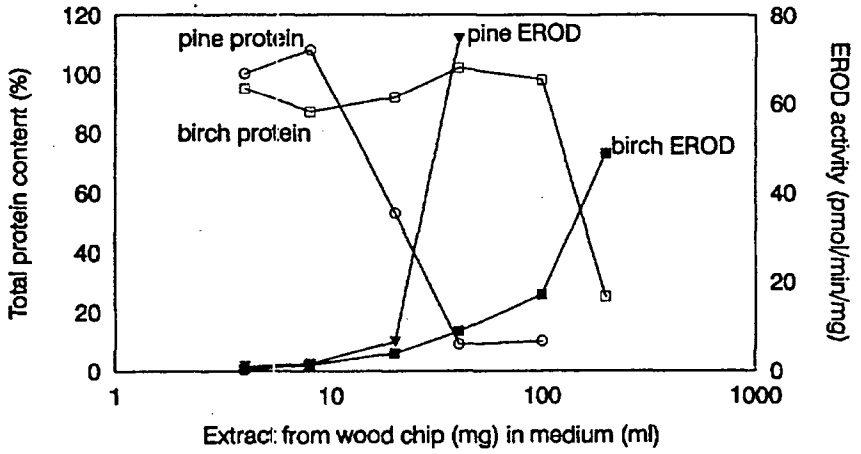

Fig. 2. Cytotoxicity (total protein content; %, medium = 100%) and EROD activity (pmol/min/mg prot.) in PLHC-1 cells exposed to wood chips of pine or birch which were used as raw materials in a pulp and paper mill. The study was conducted as one day exposure in 48-well plates (6 replicates; 2 plates with 3 wells each). The wood chips were extracted with acetone. Cells treated with an acetone control sample did not show cytotoxicity (not illustrated) or EROD activity exceeding 5 pmol/min/mg (see Fig. 3). EROD activity in cells exposed to 2 nM TCDD was 198±26 pmol/min/mg (24 replicates).

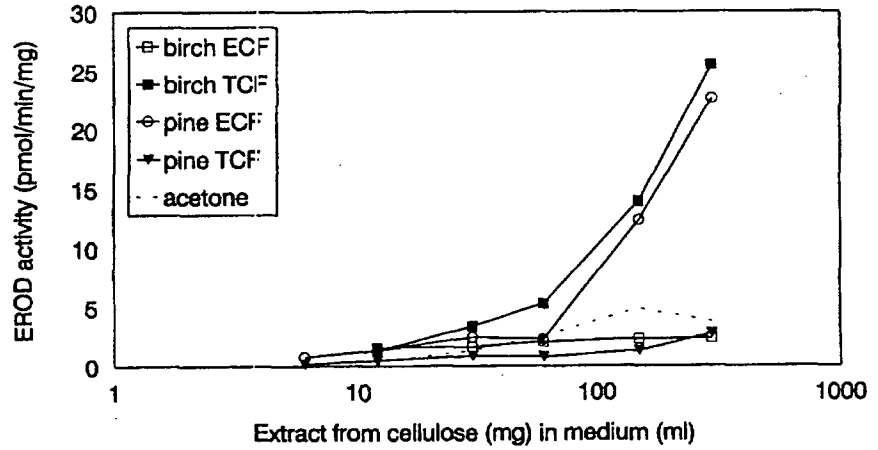

Fig. 3. EROD activity (pmol/min/mg prot.) in PLHC-1 cells exposed to celluloses collected from one mill. None of the doses of cellulose extracts decreased total protein content in the cells more than 21% when compared to untreated samples. For further information see Fig. 2.

DISCUSSION

A teleost liver cell line (PLHC-1) was used to biomonitor the cellulose and wood chip extracts from pulp and paper mills. This was done in order to evaluate the use of the cell line in monitoring the biological properties of chemically complex mixtures of materials. This was also done as part of a study to detect the distribution of toxic as well as biologically active components of various modern pulp processes. After exposure to the extracts made from the celluloses and wood chips, both cytotoxicity and/or EROD activity could be observed in the PLHC-1 cells.

In the experiments conducted with the celluloses from three various pulp and paper mills, the celluloses made of birch were more cytotoxic than the celluloses of pine. Hardwood celluloses were also more potent in inducing CYP1A. However, these trends were not precisely seen in the different celluloses collected from one single mill. A TCF bleached cellulose of birch was a more potent EROD inducer than the respective cellulose of pine; ECF bleached celluloses in this mill, however, seemed to behave more like corresponding raw materials. Thus, it cannot be determined from our study whether birch or pine as a raw material for celluloses would contain more hazardous or biologically active compounds.

There were no clear differences between the effects of ECF and TCF bleached celluloses, either. Because the celluloses used in this study were ECF or TCF bleached or totally UB, the compounds formed during the bleaching with elemental chlorine could not have caused the CYP1A induction and toxicological effects in the cells. Thus, it was not determined in this study whether the bleaching with elemental chlorine would have been critical in increasing toxicological and biochemical properties of the cellulose. Further, the UB celluloses caused cytotoxicity and induction of EROD activity at lower concentrations than the ECF or TCF bleached samples did. This may imply that bleaching of the cellulose could reduce some biological effects. In any case, it is clear from our studies that even wood materials that have not been processed through bleaching can contain compounds that induce CYP1A, in agreement with previous studies [33,34]. Moreover, the PLHC-1 cell line can be used to detect such inducers.

The cytotoxicity of especially UB celluloses and wood chips began at doses where also EROD activity was observed. EROD induction is often detected at sublethal concentrations. This was the case in a study in which dichloromethane-extracted sediments began to show cytotoxicity at doses where EROD activity had reached its peak and started to decline [29]. The material used in the present study evidently contained compounds that were highly toxic for the cells. We did not study the composition of the chemicals in the acetone extracts. However, organic solvents such as ethanol, acetone and dichloromethane are known to extract some of the wood constituents, although usually representing only a minor proportion of all possible compounds. Wood extractives removed in this manner include terpenes and terpenoids, fats, waxes, phenols, lignans, stilbenes, flavonoids, tannins and various other compounds. The content and composition of extractives may vary among wood species, like softwoods containing all classes of terpenes but hardwoods mainly higher terpenes [41].

In our study, acetone-extracts of pine wood chips were more cytotoxic for the PLHC-1 cells than extracts of

birch wood chips. The comparison of these samples was not clear when studying CYP1A induction, due to strong interference of cytotoxicity and lack of detectable CYP1A content. However, at least wood chips of birch showed a weak induction of EROD activity at doses where total protein content was not diminished. In any case, pine wood chips showed effects at lower doses than birch wood chips. The effects of wood material in *in vitro* test system have been previously studied with mammalian Hepa-1 cells [33]. After exposure to acetone-extracts made from different wood chips, pine proved to be the most potent inducer of CYP1A; all together softwoods were more potent than hardwoods. When the cytotoxicity of these wood chips was measured, the softwoods and alder were more toxic than aspen. Further, pine induced AHH activity in the Hepa-1 cells at doses about one order of magnitude lower than those which induced EROD activity in the PLHC-1 cells [33, the present study). Thus, though the pine samples investigated in these studies were not the same, mammalian Hepa-1 cells may be more sensitive for CYP1A inducing chemicals than teleost PLHC-1 cells. This can be also observed when EC50 values for CYP1A induction of TCDD are compared in these cell lines. The EC50 for induction of EROD activity in the Hepa-1 cells was about 10 pM [42] whereas it was 130 pM in the PLHC-1 cells [23]. However, the degree of induction can be extremely high in the PLHC-1 cells, due to low CYP1A activity in vehicle controls. For example, in a study of [23] EROD activity of untreated or DMSO-treated cells was undetectable (<1 pmol/min/mg) whereas it was highly induced (up to 150 pmol/min/mg) in the cells exposed to TCDD. According to [42] the extent of EROD induction in the Hepa-1 cells was 36-fold.

To get an overview about the routes and endpoints of the chemicals and their biological effects in a pulp and paper mill it is necessary to study different materials. In wood and/or pulp processing the biologically active compounds can be released into, e.g., the products and/or effluents. The possible role of natural products in the wood material in causing physiological effects in effluents has been proposed in recent laboratory and field studies with fish [14,43]. Further, effluents from processing of different wood materials have been shown to affect fish differently. The effluents produced during the pulping of pine in a kraft mill induced more EROD activity in fourhorn sculpin (*Myoxocephalus quadricornis*) than the effluents of birch pulping did [10]. This was explained as being due to the presence of less potent toxic compounds in the bleached kraft mill effluents from the birch pulp line. According to another study [16], hardwood pulping generated black liquour that was less potent than that from softwood. Compounds inducing EROD activity may have been natural wood extractives associated with wood resins rather than compounds created by the digestion of lignin. It has been also suggested that compounds causing EROD induction in fish are moderately hydrophobic and are likely planar, aromatic PAHs with a low degree of chlorine substitution [44]. This was evidenced by studying the chromatographic properties of the chemicals. There is also evidence that the PLHC-1 cells could be used in studying the biological effects of especially process waters in a pulp and paper mill [30] as well as, to some extent, lake waters [28].

It has been postulated that the studies that use a lower organizational level, such as cultured cells, cannot be used in assessing the ecological risk at a higher organizational level, such as individuals or population [45]. Furthermore, in liver cell lines the effects of absorption, distribution and extrahepatic metabolism are eliminated

as compared to the whole animal. In addition, when complex mixtures of pollutants are studied in any kind of test system the interactions among various compounds are not always additive; they may be nonadditive, thus showing, e.g., potentiation or antagonism. In spite of these potential disadvantages, *in vitro* studies with liver cell lines are promising for comparing different samples. They also serve as a prescreening method for *in vivo* experiments. In addition, because the cells can be rapidly and easily exposed to several types of samples (e.g., extracts of wood, cellulose, sediment and waste water), the cell lines could be used to detect the routes of hazardous chemicals in industrial processes and in the aquatic environment. In this way it would become easier to pinpoint where the biologically active pollutants originate from, where they are finally released and how they are modified during the industrial processes or in nature.

In conclusion, our studies introduced the use of PLHC-1 fish cell line in monitoring the toxic and enzyme-inducing effects of cellulose and wood chip extracts. Our findings suggest further use of *in vitro* bioassays in evaluating potential environmental risks of hazardous compounds.

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