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INDUCTION OF EROD ACTIVITY IN HEPA-1 MOUSE HEPATOMA CELLS AND ESTROGENICITY IN MCF-7 HUMAN BREAST CANCER CELLS BY EXTRACTS OF PULP MILL EFFLUENTS, SLUDGE, AND SEDIMENT EXPOSED TO EFFLUENTS

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Abstract—Extracts of effluents and sludges from the primary and secondary clarifiers of an activated sludge treatment plant at a Finnish bleached kraft pulp and paper mill were analyzed in two cell bioassays. Total dioxin-like activities were determined by measuring the induction of ethoxyresorufin-*O*-deethylase (EROD) activity in Hepa-1 mouse hepatoma cells. Estrogenicity was studied by measuring luciferase activity in MCF-7 ERE-luc, which are MCF-7 human breast cancer cells stably transfected with an estrogen-responsive element linked to a luciferase promoter. Sediments collected near the pulp mill and from other sites in Lake Saimaa as well as fillets of whitefish exposed to effluents were examined and EROD activity was determined for complex mixtures of compounds extractable with dichloromethane (DCM) from the pulp mill effluent or compounds collected by semipermeable membrane devices (SPMDs) from the same effluent. Extracts of effluents, particulates, SPMDs, and sludges all caused measurable EROD induction. Because the induction potencies of the total DCM extract and the fraction of neutral compounds were similar, it was concluded that most of the EROD induction in pulp mill effluents was due to neutral compounds. Sediment collected from the vicinity of the pulp mill had greater EROD-inducing potency compared to that of the control sites from the same lake. Induction of EROD potencies of muscle extracts of whitefish exposed to diluted effluents were not significantly greater than those of control fish exposed to dilution water only. All extracts contained some estrogen agonist activity when tested in the MCF-7 bioassay.

Keywords—Ethoxyresorufin-*O*-deethylase Hepa-1 Estrogenicity Pulp mill Effluent

INTRODUCTION

Effluents from bleached kraft mills contain numerous substances that can cause adverse effects to biota. There are reports of physiological and reproductive disorders and increased activity of mixed-function oxygenase (MFO) enzyme systems in fish in the vicinity of pulp mills [1]. Bleached kraft pulp mill black liquor has been reported to exhibit significant estrogenic activity [2].

Several hundred identifiable compounds can be released to the environment from pulp bleaching. In addition, there are a number of unidentifiable compounds. A list of 200 compounds with toxic potential that had been identified by the beginning of the 1980s has been compiled [3]. Terpenes, aromatics, resin acids, and fatty acids are examples of classes of compounds released to the environment through pulp and paper mill activities [4–6].

Previously chlorinated compounds formed during chlorine bleaching of pulp have caused significant adverse effects on aquatic biota near pulp mills. For example, chlorinated phenolics are acutely toxic [7]. Other chlorinated compounds such as polychlorinated dibenzo-*p*-dioxins (PCDD) and dibenzofurans (PCDF) can cause chronic effects that have been described as endocrine disruption. In addition, there are chlorinated compounds for which toxic potencies are unknown. The use of chlorine in the bleaching of pulp has been reduced markedly during recent years, which has minimized the amount of chlorinated compounds entering receiving waters. Nonchlorinated compounds, such as phytoestrogens that originate from wood, however, can also be of concern because they function as xenoestrogens [8].

The great number of compounds with their possible interactions makes it difficult to assess which compounds are most harmful and the overall toxicity of effluents. One way to approach this problem is to study extracts of pulp mill effluents by in vitro assays. These bioanalytical techniques are adaptable to a number of situations because they employ a number of measurement endpoints, including simple toxicity to specific receptor-mediated responses. Dioxin-like compounds bind to the aryl hydrocarbon (Ah) receptor (dioxin receptor) and cause toxic responses and induction of MFOs [9]. For instance, dioxin-like compounds induce ethoxyresorufin *O*-deethylase (EROD) activity that has been used in mammalian cell lines to measure toxic potency of environmental extracts [2,10,11]. Similarly, the total estrogenic potency of compounds acting through the estrogen receptor (ER) can be measured in in vitro bioassays such as MCF-7 human breast cancer cells [2].

The main objective of this study was to develop a rapid and inexpensive monitoring method to assess 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TEQs) in pulp mill samples. The Hepa-1 mouse hepatoma cell line was selected as an in vitro bioassay. The main focus was on effluents, but other samples like sludge, sediment, and fish were examined as well. The effluent and sludge samples were from

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an activated sludge treatment plant of a Finnish ECF-bleached (elementally chlorine-free) kraft pulp and paper mill. Selected sample extracts were also investigated for estrogenicity in MCF-7-luc human breast cancer cells because we wanted to see whether the sample extracts contained estrogen agonists.

The effluents used in this study had previously been analyzed for the concentrations of resin acids and chlorinated phenols [12]. Mean concentrations of chlorophenolic compounds in the primary clarifier effluent and secondary clarifier effluent (final effluent) from the activated sludge treatment plant were 1.7 and 0.5 μg/L, respectively, and those of resin acids 1.1 and 0.05 mg/L, respectively. Because compounds present in pulp mill emissions are often present at concentrations less than instrumental method detection limits, liquid–liquid extraction with dichloromethane (DCM) [6] was selected to isolate compounds from effluents to investigate ecotoxiclogical potential of compounds that are released from pulp mills to the environment.

Solid-phase extraction (SPE) and semipermeable membrane devices (SPMDs; triolein-containing polyethylene tubes) were tested as other possible isolation methods for measurements in the Hepa-1 bioassay. C_{18} SPE cartridges that have trifunctional sorbent have been developed to be used for isolation of hydrophobic species such as polynuclear aromatic hydrocarbons from aqueous environmental samples [13]. Both SPE and SPMDs have been used to accumulate inducers of MFOs from pulp mill effluents [14,15]. The SPMDs can be used to assess the bioavailability of lipophilic contaminants in water to aquatic organisms and have been used in several studies to concentrate lipophilic pollutants from water [16–20]. Accumulation of compounds into SPMDs is not identical to those in aquatic organisms; on the other hand, SPMDs are not affected by the toxicity of compounds and can be used to collect pollutants at very contaminated sites [20].

MATERIALS AND METHODS

Sample material included kraft mill effluents, sludge, sediment, and fish (Table 1). Effluents and sludge were from the activated sludge treatment plant of a pulp and paper mill located in southeast Finland near Lake Saimaa. This integrated bleached kraft pulp and paper mill discharges about 120,000 m^3/d of effluents to the southern part of Lake Saimaa. The mill produces 440,000 t/a ECF bleached kraft pulp, 142,000 t/a mechanical pulp, and 420,000 t/a low and medium weight coated (LWC, MWC) paper. The bleaching sequence of hardwood pulp is OD–Eo–D–Ep–D (O = oxygen delignification; D = chlorine dioxide; Eo = caustic extraction and addition of oxygen; Ep = caustic extraction with addition of peroxide) and that of softwood pulp D–Eop–D–E–D.

Pulp mill effluent

Effluents taken from the primary clarifier (PE) and secondary clarifier (SE) of the activated sludge treatment plant of the pulp and paper mill were analyzed. Effluents from the biological treatment plant were collected as four 5-d average composite samples (I–IV) for exposure experiments with whitefish at the Department of Biological and Environmental Sciences, University of Jyväskylä [12]. Samples for the present study were taken from the original delivery tank on the last day of 7-d exposure experiments and were stored at −20°C in brown glass bottles.

One liter of effluent was acidified to pH 2, then 5 ml of saturated NaCl solution was added, and the sample was extracted with 100 ml of DCM by shaking for 15 min. The layers were separated and the organic layer was centrifuged in 50-ml tubes to break the emulsion. The water layer was reextracted with 50 ml of dichloromethane. The organic portions were combined, dried with Na_2SO_4 and divided into two equal parts.

Table 1. Sample data

Sample description/date	Code	Sample size[a] (g)
Effluent (April 1996)		
Primary clarifier	Effluent = PE I Particulate = PEP I	0.4288
Primary clarifier	Effluent = PE III Particulate = PEP III	0.3448
Secondary clarifier[b]	Effluent = SE I Particulate = SEP I	0.4649
Secondary clarifier[b]	Effluent = SE III Particulate = SEP III	0.8013
Sludge (May 1996)		
Primary clarifier	PS	8.859
Secondary clarifier[c]	SS	4.266
Sediment (June 1996)		
Lake Saimaa,[d] Ref 1	REF 1	16.187
Lake Saimaa,[e] Ref 2	REF 2	21.514
Lake Saimaa,[f] Site 1	Site 1	16.838
Kymijoki River	Myllykoski	10.00
Detroit River	122	10.00
Whitefish (April 1996)		
Dilution water	CTRF	106.48
Primary clarifier	PEF	107.37
Secondary clarifier	SEF	105.95

[a] Weights for effluent, sludge, and sediment samples are dry weight of solids; weights for whitefish are wet weight; lipid-% were 0.54, 0.61, and 0.68 for CTRF, PEF, and SEF, respectively.
[b] Final effluent emitted to Lake Saimaa.
[c] Return sludge.
[d] REF 1 = upstream reference, 8 km upstream from the mill.
[e] REF 2 = 35 km from the mill.
[f] Site 1 = 1 km downstream from the mill.

Acidic and phenolic compounds were removed from one part based on the method presented by Schwantes and McDonough [21]. The DCM extract was shaken with 100 ml of 0.5 M $NaHCO_3$ to remove acidic compounds and the layers were separated. The organic extract was further shaken with 100 ml of 0.5 M NaOH for the removal of phenolic compounds. After this step, the residual DCM extract contained mainly only neutral compounds. For the measurement of EROD activity, both the original extract (DCM extract = A) and the purified extract ($DCM_{neutral}$ = B) were dried over anhydrous Na_2SO_4, concentrated by rotary evaporator to 1 ml, and transferred into a microvial with hexane (3 × 0.5 ml). The extract was concentrated by evaporating with nitrogen gently to dryness, and 200 μl of iso-octane was added.

Some effluents were also enriched using SPE cartridges developed for environmental samples by the Waters company. The enrichment method was performed as proposed by Waters [13]. An aliquot of 100 ml of acified effluent (pH < 2) was passed 5 to 10 ml/min through a Waters Sep-Pak tC_{18} Cartridge (WAT036810) that had been conditioned with 20 ml of DCM and 20 ml of methanol before use. After the effluent had passed through, the cartridge was eluted with 15 ml of DCM. According to preliminary experiments no EROD activity was observed in an additional 10-ml fraction of DCM. After col-

lection, the eluate was dried over anhydrous Na_2SO_4 and concentrated to the final volume of 200 μl as described above.

Semipermeable membrane devices

Semipermeable membrane devices were obtained from the University of Jyväskylä. The SPMDs were 86-cm-long, 2.5-cm-wide, 73.2 ± 1.7-μm-thick low-density polyethylene lay-flat tubes (Cope Plastics, Fargo, ND, USA) that contained 0.8 ml of 99% pure triolein (C_{18}:1,*cis*-9; Sigma Chemical, St. Louis, MO, USA). The SPMDs had been stored in a closed glass jar in a freezer (−18°C) before use because SPMDs might be contaminated and triolein can come out from the tube at room temperature (A.L. Rantalainen, personal communication). In the exposure experiment with whitefish, two SPMDs were installed vertically in the outlet of each steel fish tank and were exposed to pulp mill effluents for 4 weeks. Each of four effluents (I–IV) was allowed to flow through the same tank for 7 d in turn. The SPMDs were exposed to purified regular tap water (control), undiluted and diluted (3.5 vol-%) PEs and diluted (2.3, 3.5, and 7 vol-%) SEs. After the exposure period, SPMDs were stored frozen (−18°C) until analyzed.

Before dialysis, each SPMD was cleaned with a brush in distilled water and rinsed with 1 M HCl solution, distilled water, and isopropanol. The SPMDs were dialyzed twice with hexane (130 ml) in a brown glass bottle for 24 h. The hexane extracts were combined, concentrated by rotary evaporator to 1 ml, and transferred into microvials with hexane (3 × 0.5 ml). Hexane was gently evaporated by nitrogen flow and residue was dissolved in 200 μl of iso-octane. One hundred microliters of this extract was purified on a 1-g column of silica gel (Kieselgel 60, 70–230 mesh, Merck, Darmstadt, Germany) deactivated with 5% H_2O. The sample was introduced onto the column, and impurities were washed away with hexane by collecting 1 ml of hexane. Then a 12-ml fraction was collected with a mixture of hexane:DCM (1:1, v/v). This second fraction was evaporated gently to dryness, and 100 μl of iso-octane was added.

Particulate material isolated from effluents

Suspended particulate material was collected from the material that was left after exposure experiments at the bottom of the delivery tanks containing original effluents from the pulp mill. The sample was stored in a freezer at −18°C until analyzed. Before analyses, the material was filtered using glass microfiber filters (GF/A and GF/F Whatman, Clifton, NJ, USA) to isolate particles >0.7 μm. Air-dried particles were extracted in a Soxhlet system with DCM for 24 h. The extract was concentrated by rotary evaporator to 1 ml and was transferred into a microvial with hexane (3 × 0.5 ml). Hexane was evaporated gently off by nitrogen flow, and 200 μl of iso-octane was added. One hundred microliters of this extract was purified on silica gel as described above.

Sludge

Sludge was obtained from the PE and the SE of the activated sludge treatment plant of the pulp mill. Sludges were stored in refrigerator (+4°C) for 1 week before analyses. As a pretreatment step, sludge was centrifuged to remove excess water and air-dried. Dried material was weighed into a prewashed extraction thimble, and the sample was extracted in a Soxhlet apparatus with DCM for 24 h. The extract was concentrated similarly as particulate material extracts and the residue was dissolved in 1,000 μl of iso-octane. An aliquot of the extract was purified on activated charcoal. This cleanup removed nonplanar compounds from the extract and was performed as described earlier [22].

Sediment

Sediments analyzed were surface sediments collected from the bottom (depth 6 m) of the Southern Lake Saimaa, where there are pulp-bleaching activities in several locations. Three sites were investigated: an upstream reference site (REF 1; 8 km from the mill), a reference site located upstream and remote from pulp and paper mills (REF 2; 35 km from the mill), and a site near the pulp and paper mill (site 1; 1 km from the mill). The freeze-dried sediment sample was extracted in a Soxhlet apparatus with DCM for 24 h. The extract was concentrated similarly as the particulate material extract. The residue was dissolved in 1,000 or 3,000 μl of iso-octane. An aliquot of Lake Saimaa extracts was purified on activated charcoal. In addition, an aliquot of the extract of Lake Saimaa sediment from site 1 was analyzed for the concentration of TEQ after the sulfuric acid cleanup.

Fish

Whitefish (*Coregonus lavaretus* s.l. L.) were exposed in steel tanks receiving uncontaminated dilution water (activated carbon-filtered tap water of City of Jyväskylä) or diluted effluents from a bleached kraft pulp mill. Each tank contained 15 fish that were exposed with diluted effluents from PE and SE of the activated sludge treatment plant. Extracts of whitefish muscle fillets from the following treatment groups were selected for measurements of EROD activity: control, 7 vol-% SE, and 3.5 vol-% PE.

For analyses, 2- to 3-g samples of lateral muscle from each fish were combined to obtain one analysis sample from each tank. Each composite sample was homogenized and freeze-dried. The dried sample was weighed into a prewashed extraction thimble, and the thimble was extracted in a Soxhlet system with a mixture of petroleum ether:acetone:hexane:diethyl ether (18:11:5:2; v/v/v/v) for 6 h. The extract was concentrated to 1 ml and was transferred with hexane (3 × 2 ml) into a glass tube. The volume was adjusted to 8 ml with hexane, and the extract was divided into two parts. A 100-μl subsample was taken from one part to measure gravimetrically the lipid content of the extract by evaporating hexane away under nitrogen stream. Both parts of the extracts were purified by shaking with concentrated sulfuric acid to remove most of the lipids. Residues were then concentrated with nitrogen, reduced to dryness, and 200 μl of iso-octane was added. One part was analyzed for the concentration of TEQ after the sulfuric acid cleanup, and the second was analyzed after an additional cleanup on activated charcoal.

Mouse Hepa-1 cells

Hepa-1 mouse hepatoma cells (Hepa-1c1c7 subclone) [23,24] were obtained from the University of Kuopio. The bioassay used was developed by modifing the Invitox protocol for measuring CYP1A1-inducing potency in the Hepa-1 mouse cells [25] and the procedure of Sanderson et al. [26] for the H4IIE rat hepatoma cell line bioassay. We used a similar kind of procedure with the H4IIE cells to measure toxic loads of white-tailed sea eagle sample extracts [27]. Briefly, Hepa-1 cells were cultured in flasks (Corning, 75 cm^2) in a humidified CO_2 incubator (Sanyo, MCO-17A) at 5% CO_2/air and at >90% relative humidity for 7 d until they reached near confluency.

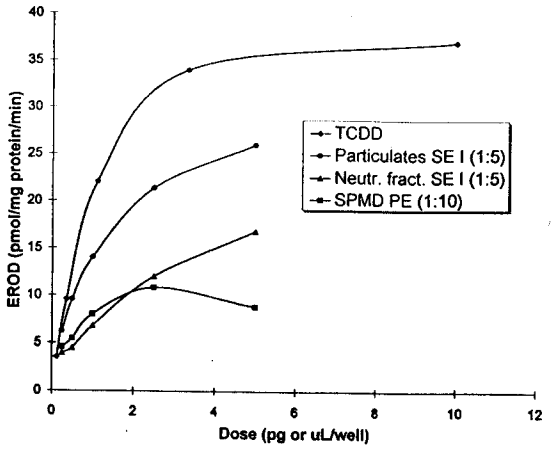

Fig. 1. Dose–response curves for the potency of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and those of the neutral fraction of a secondary clarifier effluent (SE I), particulates isolated from a secondary treatment effluent (SE I), and an SPMD dialysate of primary clarifier effluents (PE).

Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco 52100-021), which was prepared from DMEM base supplemented with $NaHCO_3$ and sterilized by filtration. Before use, the medium was supplemented with fetal bovine serum (FBS; Gibco 10106-078) and penicillin/streptomycin (Gibco 15140-021) solutions. Twenty-five milliliters of FBS and 4.5 ml of penicillin/streptomycin was added per 400 ml of culture medium. For the bioassay, the medium was removed and cells were washed twice with phosphate-buffered saline (PBS). Cells were trypsinized with 4 ml of trypsin-EDTA (Gibco 3540027) for 5 min in the incubator. Six milliliters of medium was added to the cell suspension and cell densities were determined. Live cells were counted visually from a 100-μl aliquot mixed with 10 μl of 0.1% trypan blue solution. Culture plates (96-well; Corning) were seeded with 250 μl of cell suspension at a density of 4×10^4 cells/ml. A new flask was seeded by adding a drop of the cell suspension to a flask containing 25 ml of medium.

Cells grown on the culture plate for 1 d were dosed with 5 μl of TCDD (0.0083–2.0 pg/μl) solutions in iso-octane or test extracts dissolved in iso-octane. The TCDD (Wellington Laboratories) was kindly provided for this study from the Department of Chemistry at the University of Jyväskylä. Five replicates were prepared for five different concentrations of TCDD. Five dilutions were prepared from each sample extract and each dilution was analyzed as three replicates. Three samples were analyzed on one plate. The EROD activity in Hepa-1 mouse cells was measured 3 d after dosing using Fluoroskan Ascent (Labsystems) according to the procedure presented by Sanderson et al. [26]. Briefly, Hepa-1 cells were washed with buffer, frozen at −80°C for 15 min, and reagents were added. Buffer and ethoxyresorufin were added first, then NADPH (50 μl of 0.5 mM), and after 60 min, the reaction was stopped by adding fluorescamine in acetonitrile. Fluorescence of resorufin was measured using an excitation/emission wavelength of 530/590 nm and total proteins by using a wavelength of 400/460 nm, respectively. Resorufin and BSA (bovine serum albumin) standard curves were measured in each bioassay. The dose–response data were transformed to produce Woolf plots in order to calculate slopes. The concentrations of TEQs for samples were obtained by dividing the slope of the dose–response curve of the sample with that of the TCDD standard curve.

Human MCF-7 cells

The MCF-7 cells stably transfected with an estrogen-responsive element linked to a luciferase reporter (MCF-7 ERE-Luc) were obtained from Dr. Michel Pons. The MCF-7 ERE-Luc cells were cultured in dishes (Corning, 100 mm) in a humidified CO_2 incubator at 5% CO_2/air and at >90% relative humidity until they reached ~80% confluency. Cells were grown in a 1:1 mixture of DMEM:Ham's F-12 nutrient medium (Sigma D-2906), supplemented with $NaHCO_3$, 1 μg/ml insulin, 1 mM sodium pyruvate, and either defined FBS (for routine culturing; Hyclone) or charcoal-stripped FBS (for treatment and exposure period; Hyclone). For the bioassay, 96-well culture plates (Packard Instruments) were seeded with 250 μl of cell suspension at a density of 6×10^4 cells/ml.

After 24 h, the medium was changed to a medium from which most of the estrogen had been removed by charcoal stripping the FBS, and the cells were dosed with either no treatment (blanks), 17β-estradiol (E_2), solvent only, or the various samples to be tested. Cells were dosed in triplicate with 1.25 μl of E_2 in ethanol (0–150 pM final concentration) or 1.25 μl of test extracts dissolved in isooctane. Solvent controls for ethanol and isooctane were not significantly different from blanks. Cell viability, luciferase activity, and protein content were measured 3 d after dosing. To determine cell viability, cells were washed with PBS, then a Live/Dead® kit (Molecular Probes) was utilized according to the manufacturer's instructions, and fluorescence was measured by a Millipore Cytofluor 2300 fluorescence measurement system. To prepare the cells for the luciferase assay, cells were washed with PBS, then 50 μl of PBS with 0.1 g/L $CaCl_2$ and 0.1 g/L $MgCl_2$, and 50 μl of LucLite® reagent (Packard Instruments) was added to the cells for 5 min at room temperature. Light production, a measure of luciferase activity, was determined with a Dynatech ML 3000 luminometer at 30°C. Protein content was determined by adding 50 μl of 1.07 mM fluorescamine in acetonitrile to 100 μl of cell lysate and measuring fluorescence at an excitation/emission wavelength of 400/460 nm. Estrogenic activity of each sample was determined by comparison to the E_2 standards. Statistical significance between samples and controls was determined by performing a two-tailed Student's *t* test.

RESULTS AND DISCUSSION

Activity of EROD and TEQs

The Hepa-1 mouse cells proved to be quite sensitive in assessing TEQs of analyzed sample extracts. Full dose–response curves for TCDD, which was used as a reference material, were achieved by exposing cells to doses ranging from 0.04 to 3.3 pg TCDD per well. The 50% effective dose ED50 for EROD induction by TCDD was approximately 1 fmol/well with a detection limit 0.1 fmol of TCDD per well. Some example dose–response curves of TCDD and samples are presented in Figures 1 and 2. When we converted the doses that caused the maximal induction to sample sizes, we got the following results. Two milliliters of effluent, 1 to 5 mg of dried particulate material, 10 mg of dried sludge, 0.2 to 10 mg of dried sediment (depending on the degree of contamination), and 1 g of dried muscle tissue of whitefish as an extract in 5 μl of iso-octane was enough to cause an induction comparative to 0.3 to 1 pg of TCDD.

Because not all extracts induced EROD activity to the same maximal level as TCDD, the results of the present study give semiquantitative estimates of EROD induction potencies ex-

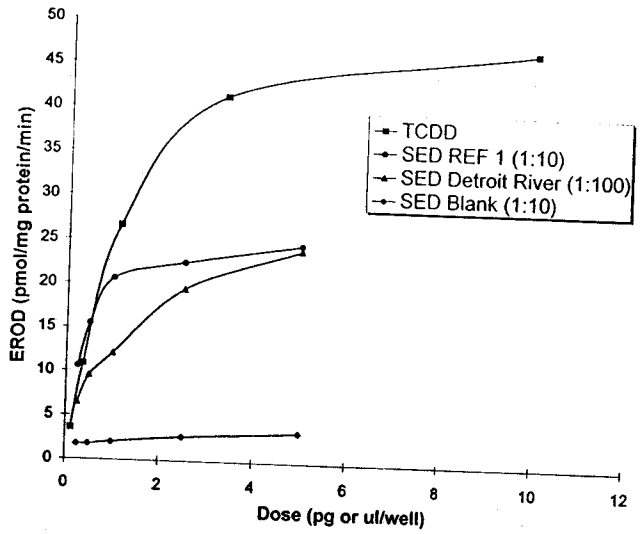

Fig. 2. Dose–response curves for the potency of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and those of a sediment blank, a sediment from a reference site in Lake Saimaa (REF 1), and a sediment from the Detroit River.

pressed as TEQ in pulp mill-related samples. When the maximum activity of a sample extract does not reach that of TCDD, the ED50 of the extract might be determined as too low and thus the TEQ of the sample will be overestimated. The interaction of compounds can affect EROD induction potencies of sample extracts that are complex mixtures of numerous compounds.

Effluents

DCM extracts. The TEQs measured in the DCM extracts of effluents were between 0.6 and 2 ng TEQ/L (Table 2). Concentrations of TEQs in SE, after removing phenolic and acidic compounds, were similar to those of the total extracts. Therefore, it seems that neutral compounds could be responsible for most of the EROD activity in extracts of pulp mill wastewaters entering into the aquatic environment. We cannot be absolutely sure that our procedures completely removed phenolic and acidic compounds from neutral ones because we did not analyze the phenolic/acidic fraction. Due to activated sludge treatment the amount of acidic and phenolic compounds were less in SEs compared to those in PEs. In the case of PEs, neutral compounds constituted about half of the TEQ.

C_{18} cartridges. The results obtained with Waters Sep-Pak cartridges for TEQs of effluents were considered unreliable because measurable EROD activity, which was most probably due to impurities in cartridges, was observed in the blanks. Washing the cartridge with 20 ml of DCM followed with 20 ml of methanol seemed to be insufficient to remove these impurities. Only PE III showed EROD activities that were clearly greater than the blank. The concentration of TEQ in this effluent (3.1 ng/L) was greater than that obtained for the DCM extract of the same effluent.

Table 2. The TCDD equivalents (pg TEQ/L) in effluents of a bleached kraft pulp mill

Effluent	TEQ (pg/L)	
	Neutral fraction	Total extract
Primary clarifier (PE I)	730	2,040
Primary clarifier (PE III)	550	1,270
Secondary clarifier (SE I)	1,220	1,470
Secondary clarifier (SE III)	560	680

Table 3. The TCDD equivalents (pg TEQs) in SPMDs exposed to effluents of a bleached kraft pulp mill

Effluent	TEQ (pg)
Control (CTR)	490
Primary clarifier (PE)	6,190
Primary clarifier (PE 3.5 vol-%)	3,600
Secondary clarifier (SE 7.0 vol-%)	2,440
Secondary clarifier (SE 2.3 vol-%)	680

SPMDs. Extracts isolated from SPMDs also showed measurable EROD activity (Table 3). The results of SPMDs are given in pg, which is the total concentration of TEQ of compounds collected by SPMDs during 4 weeks exposure. Because the amount of water passing SPMDs is unknown, these results cannot be compared directly with the results of effluents. Concentrations of TEQs from SPMDs were measured after silica gel purification because nonvolatile material was observed in hexane after the dialysis of SPMDs. The dose–response curves obtained for SPMD extracts had low maximal induction, possibly because they are compounds that have interactions with each other. We observed in an earlier study that there are possibly interactions between compounds that can affect the potency of EROD induction. For example, no EROD activity could be measured for the total extract of salmon, whereas EROD activity was measurable in the planar fraction [27]. The results of sampling with SPMDs are preliminary but show that SPMDs could be used for monitoring concentrations of TEQs in pulp mill effluents when exact TEQs are not needed. This technology should be used as a preliminary screening to determine if subsequent, more detailed analyses are warranted.

Particulates. Concentrations of TEQs in particulate material isolated from effluents ranged from 1.5 to 7.4 ng/g dry weight (dw) and those in sludge between 1.5 and 2.1 n/g dw (Table 4). Similarly to the TEQs in the DCM extracts and SPMD dialysates, higher TEQs were measured for the particulate material of PEs than those in the particulate material isolated from SEs. This shows that the active sludge treatment removes part of the compounds with dioxin-like activity. Concentrations of TEQs in sludges were similar before and after purification on activated carbon, which isolates the planar frac-

Table 4. The TCDD equivalents (pg TEQ/g dry weight) in particulate material isolated from primary and secondary clarifier effluents and sludge

Sample	TEQ (pg/g, dry weight)	
	Total extract	Activated carbon fraction 2[a]
Particulate		
Primary clarifier (PEP I)	7,430	ND
Primary clarifier (PEP III)	7,030	ND
Secondary clarifier (SEP I)	2,810	ND
Secondary clarifier (SEP III)	1,510	ND
Sludge		
Primary clarifier (PS)	1,480	1,550
Secondary clarifier (SS)	2,070	1,990

[a] ND = not determined.

Table 5. The TCDD equivalents (pg TEQ/g dry weight) in sediments

Sample	TEQ (pg/g dry weight)		
	Total extract	Activated carbon fraction 2[a]	Sulfuric acid cleanup[a]
Lake Saimaa (REF 1)	1,300	1,120	ND
Lake Saimaa (REF 2)	750	1,180	ND
Lake Saimaa (Site 1)	8,470	6,990	830
Kymijoki River (Myllykoski)	11,900	ND	ND
Detroit River (122)	18,500	ND	ND

[a] ND = not determined.

tion from nonplanar compounds. This indicates that compounds responsible for induction of EROD activity could be planar that bind to the Ah receptor.

Sediment

Concentrations of TEQ in the total extract and concentrations of TEQ of the fraction containing the planar compounds extracted from the sediment collected adjacent to a pulp mill in the Southern Lake Saimaa (site 1) were about 8.4 and 7.0 ng/g dw (Table 5). Two other sediments (REF 1 and REF 2) from Lake Saimaa contained lesser concentrations of TEQs (0.8 and 1.3 ng/g dw), which indicates that these sites have received lesser loads of contaminants. Increased EROD induction had been observed in livers of whitefish caught from near site REF 2 in 1995 [12].

Sediment from site 1 seems to be quite contaminated because the concentration of TEQs in this sediment was not much less than that of a sediment from the Kymijoki River and only half that of a sediment from the Detroit River, Michigan, USA. The Kymijoki River has been polluted by PCDDs and PCDFs [28] and the Detroit River by a number of contaminants including polycyclic aromatic hydrocarbons [29,30]. All sediments analyzed here contained greater concentrations of TEQs than the value 500 pg/g dw, which is the criterion for TEQ-contaminated soil in Finland [31].

According to our preliminary results with site 1 sediment, sulfuric acid cleanup possibly removes about 90% of the compounds that cause EROD activity in DCM extracts of sediments. The concentrations of TEQ in the total extract of sediment site 1 purified with concentrated sulfuric acid (TEQ_{Pur}) was an order of magnitude less than that of the unpurified total extract (TEQ_{Tot}). The concentration of TEQ_{Pur} for sediment site 1 (1.1 ng/g dw) is similar to the concentration of TEQ of PCDDs and PCDFs ($TEQ_{PCDD/PCDF}$) in the Kymijoki River sediment [28]. The $TEQ_{PCDD/PCDF}$ was calculated from instrumental analyses of the purified extract by converting the concentrations of toxic PCDDs and PCDFs to TEQ using international toxic equivalency factors. Concentrations of toxic PCDDs and PCDFs in the Kymijoki River sediment were measured after cleanup with sulfuric acid and fractionation on activated carbon and basic alumina columns [28]. In the case of the sediment from the Kymijoki River, which is heavily polluted by hepta- and octachlorinated PCDFs due to earlier production of chlorophenol-based wood preservative Ky-5 [28], toxic PCDDs and PCDFs probably constitute the major portion of the TEQ, which are resistent to sulfuric acid cleanup. In sediments from Lake Saimaa near pulp mills, compounds that originate from pulp bleaching are most likely responsible for the TEQ observed.

Fish

Concentrations of TEQs in total extracts of whitefish muscle samples were between 10 and 21 pg TEQ/g fresh weight (Table 6). Fish exposed to PEs had greater concentrations of TEQ than those exposed to SEs (biologically treated). This is consistent with the concentrations of TEQs in effluents themselves; the PE had greater concentrations of TEQ than did the SE. Because the TEQs of the exposed fish muscle samples after carbon column cleanup did not differ significantly from those of the control fish exposed to clean dilution water only, the accumulation of TEQ from effluents by fish muscle was small. Apparently, little of the TEQ are bioavailable. Concentrations of TEQs in muscle tissue probably reflect background contamination of these fish, via their food, rather than due to exposure to effluent.

As in the case of sediments near the pulp mill, concentrations of TEQs in extracts of fish without sulfuric acid cleanup could be expected to be greater because sulfuric acid removes part of the compounds responsible for EROD induction. Toxic PCDDs, PCDFs, and PCBs could partly be responsible for the TEQ observed in the planar fraction of fish extracts.

Estrogenicity

Ten sample extracts and three blanks were investigated for the presence of estrogen agonists by the use of MCF-7-Luc human breast cancer cell line bioassay (Fig. 3). Greater estrogen agonist activity was observed in the total DCM extract of the SE I A than in the total DCM extract of the PE III A. The neutral fraction of the PE was less potent than the total extract. The estrogenic activity of SE I A was between the activity caused by 5 and 15 pM (final concentration; 1.25–3.75 fmol/well or 0.34–1.03 ng/well) of 17β-estradiol (E_2). The blanks of sediment and SPE cartridges caused similar ERE-mediated luciferase activity as that of the medium. The effluent blank caused slightly greater activity than the medium, however this difference was not statistically different.

Estrogenic activity was also observed in extracts of PE

Table 6. The TCDD equivalents (pg TEQ/g fresh weight) in extracts of juvenile whitefish exposed to primary clarifier effluent, secondary clarifier effluent, or regular tap water

Whitefish exposed to	TEQ (pg/g fresh weight)	
	Total extract	Activated carbon fraction 2
Regular tap water (CTRF)	12	8
Primary clarifier effluent (PEF)	21	7
Secondary clarifier effluent (SEF)	10	9

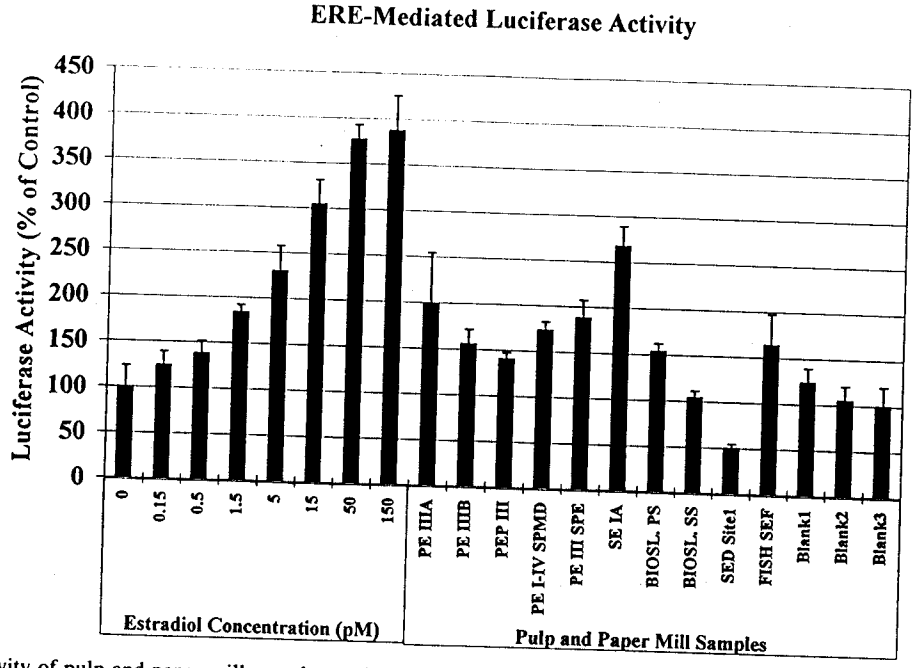

Fig. 3. Estrogenic activity of pulp and paper mill samples as determined in MCF-7 ERE-Luc cells. Results are expressed as mean ± 1 standard deviation (n = 3) as a percentage of control. Abbreviations used for the pulp and paper mill samples are the same as in Table 1 with the following modifications: A = dichloromethane (DCM) total extract; B = neutral fraction; Blank1 = effluent blank; Blank2 = blank for particulate material, sludge, and sediment; and Blank3 = SPE blank.

obtained by SPMD and SPE. The amount of estrogen agonists in the SPE extract was greater than in the DCM extract, because the ERE-mediated luciferase activities in both extracts were similar even though the SPE extract was more dilute. The purified extract of muscle tissue of whitefish exposed to the SE also showed some estrogenic activity; however, it is unclear what the contribution would be for endogenous fish hormone in this sample. Estrogenicity could not be measured in the sediment from the vicinity of the pulp and paper mill (site 1), because the sediment extract was too toxic to MCF-7 cells (Fig. 4). In addition, no estrogenicity was observed in secondary clarifier sludge SS.

While the estrogen agonists in these samples have not been identified, previous research clearly shows that pulp mill effluents contain phytoestrogens and other compounds that reduce gonadal steroid biosynthesis, gonad size, number of lymphocytes, and reproductive capacity in fish [32–34]. In addition, surfactants and plasticizers such as alkyl phenol ethox-

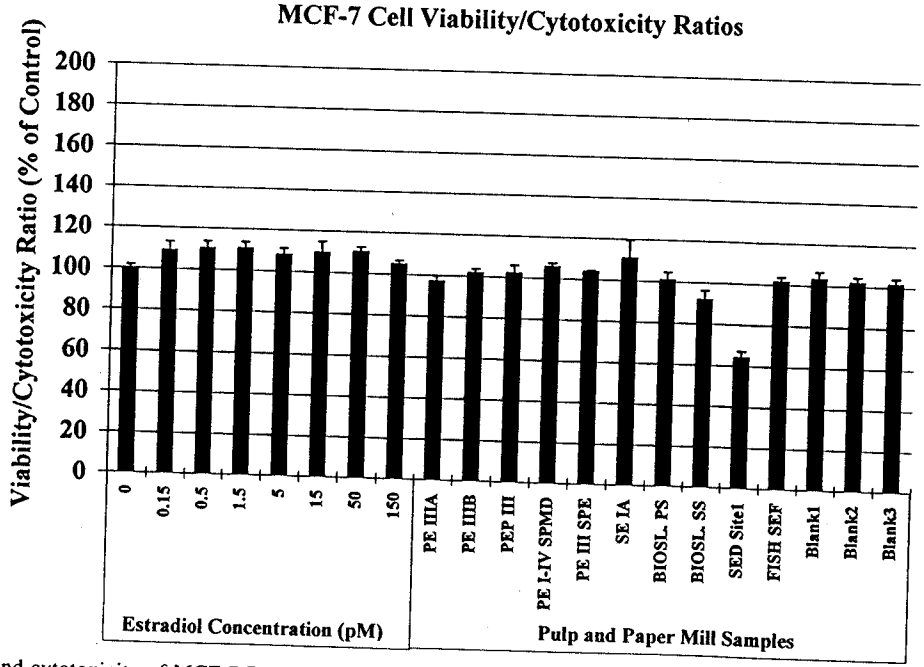

Fig. 4. Cell viability and cytotoxicity of MCF-7 ERE-Luc cells after exposure to pulp and paper mill samples. Cell viability and cytoxicity was measured as described in the Materials and Methods. Results are expressed as mean ± 1 standard deviation (n = 3) as a percentage of control. Abbreviations used for the pulp and paper mill samples are the same as in Figure 3.

ylates and phthalates, which are ubiquitous in aquatic environments, have been reported to exhibit estrogenic activity [35,36].

CONCLUSIONS

These results demonstrate that both dioxin- and estrogen-like activities can be detected in pulp and paper mill-related samples using the Hepa-1 and MCF-7 ERE-luc cell bioassays, respectively. Our results are similar to those reported by other researchers [2,15,37,38]. Further fractionation studies, however, will be necessary to identify the active compounds in the samples. The results of this study demonstrate that bioassays are reliable, sensitive, inexpensive, rapid, and specific bioanalytical tools to complement conventional chemical analyses for screening of environmental samples for estrogen- and dioxin-like activity.

The presence of estrogen agonists observed in this study may partly explain reproductive disorders observed in fish in the vicinity of pulp mills [32–34]. Interestingly, levels of estrogen agonists were greater in the effluents from the secondary clarifier than the primary clarifier but, other than pulp mill-related compounds (possibly partly originating from the contamination of the sample extract in the laboratory), could contribute to the estrogenicity in the SE. While the estrogen agonists in these samples have not been identified, it is likely that these samples contain phytoestrogens and possibly other compounds, such as alkyl phenol ethoxylates and phthalates, which have all been reported to exhibit estrogenic activity [35,36].

The presence of dioxin-like activity observed in this study suggests that compounds originating from pulp mills could be responsible for significant EROD induction potencies in sediments receiving effluents from pulp mills. If one wants to determine the exposure of fish to pulp mill effluents, measurements of EROD activity of liver tissues better describe the exposure level than analyses of accumulated xenobiotics in muscle tissue [12]. In the case of the sediment from the Kymijoki River, PCDDs and PCDFs probably constitute the major load of TEQ of organic pollutants due to earlier production of chlorophenol-based wood preservative Ky-5 [28]. In sediments from Lake Saimaa near the pulp and paper mill, compounds that originate from pulp bleaching are most likely responsible for the TEQ. Specifically, toxic PCDDs, PCDFs, and PCBs are likely to be responsible for part of the TEQ of the planar fraction of fish extracts.

From a practical point of view, extraction of isolated particulate material in a Soxhlet apparatus is easier than the liquid–liquid extraction of effluent water in a separatory funnel by a solvent for the determination of toxic loads and estrogenicity of effluents. The formation of emulsion often complicates the extraction in a separatory funnel. Compounds responsible for TEQ in effluents probably tend to bind to particles in water; therefore, the determination of TEQ extracts from the particles gives a better idea of toxicity and induction potencies. Another possibility for isolation of compounds in effluents for measurement of dioxin-like activity is SPE. The SPE with multiple sample handling from effluents is even easier than extraction of particulate material. There are, however, possible interference due to impurities in SPEs, and cartridges should be cleaned well before use. One major advantage of SPE is that several samples can be handled simultaneously without sample contamination using vacuum manifolds, thereby decreasing the time spent on sample preparation.

For estrogenicity studies, too, SPE could be the best isolation method of the three methods tested because the SPE sample had the highest levels of estrogen agonists and because the SPE blank had no estrogenic activity. According to our study, SPMDs are also effective at collecting compounds originating from pulp mills responsible for both estrogen- and dioxin-like activity and could be used to monitor levels of estrogenic chemicals and TEQs in the vicinity of pulp mills.

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