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Cytochrome P450-Dependent Enzymes and Oxidant-Mediated Responses in Rainbow Trout Exposed to Contaminated Sediments

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Hatchery-reared immature rainbow trout (*Oncorhynchus mykiss*) were exposed to different concentrations (2 and 4 liters) of contaminated sediment taken from a site receiving unbleached pulp mill effluents. The fish were held in aquaria and sampled three times during an experimental period of 21 days. The monooxygenase activity, measured as the deethylation of 7-ethoxyresorufin (EROD activity), increased three- to fourfold in the exposed fish relative to controls. The increase was not dependent on exposure concentration. Cytochrome P450IA1, the EROD catalyst, demonstrated proportional induction in the 2-liter exposed fish. However, exposure to 4 liters sediment strongly induced P450IA1 and did not reflect EROD activity. This may suggest inhibition of P450IA1 activity by the amount of chemicals discharged from pulp mills. UDPglucuronosyltransferase increased at one stage of the experimental period, while glutathione *S*-transferase remained unchanged. Amounts of total glutathione in blood, liver, and muscle were slightly increased by exposure to contaminated sediments, but hepatic enzyme activities of superoxide dismutase and catalase were not affected. In conclusion, monooxygenase activities appear to be a sensitive tool in the monitoring of sediment toxicity.

INTRODUCTION

In Scandinavian countries the high loading of waters with chemicals discharged by pulp and paper mills is of general concern (Rosemarin *et al.*, 1990). The effluents discharged from those mills are complex mixtures of organic and inorganic compounds and biotreatment (i.e., mechanical or biological treatment) of the effluent determines its final toxicity. The principal constituents causing toxicity in fish are resin acids, fatty acids, chlorinated phenols, guaiacols, and catechols (Hutchins, 1979; Oikari *et al.*, 1985). These compounds have been known to have highly concentrated in tissues of various fish species (Oikari *et al.*, 1982; Rosemarin *et al.*, 1990). It is, therefore, important to monitor the physiological and biochemical responses in fish after exposure to those chemicals.

Several studies have focused on physiological and biochemical parameters to elucidate the influence of pulp and paper mill effluents on fish health. Marked alterations in blood cell status, plasma electrolytes, and carbohydrate metabolism have been noted in exposed fish in field or laboratory studies (Andersson *et al.*, 1988; Soivio *et al.*, 1988; Lehtinen *et al.*, 1990). Activities of cytochrome P450-dependent enzymes, particularly of monooxygenases, appear to be highly sensitive parameters for pulp and paper mill pollution studies (Förlin *et al.*, 1985; Munkittrick *et al.*, 1991) and are said to be the major biochemical tools used to measure the effects of pulp mill effluents. The activities of various monooxygenases (such as benzo(*a*)pyrene hydroxylase, 7-ethoxycoumarin *O*-deethylase, and 7-ethoxyresorufin *O*-deethylase) have been determined and all reflected a high tendency to be induced, but 7-ethoxyresorufin *O*-de-

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ethylase activity (EROD activity) appeared to be one of the most responding to pulp and paper mill effluent exposure (Lindström-Seppä and Oikari, 1989).

Increasing attention is now given to antioxidant mechanisms as biomarkers for the monitoring of environmental pollution (Mather-Mihaich and Di Giulio, 1991). Glutathione (L-γ-glutamyl-L-cysteinylglycine) is a tripeptide which is widely distributed in animal tissues and participates in detoxication reactions for xenobiotics and metabolism of numerous cellular compounds (Meister and Anderson, 1983). Xenobiotics or metabolites are conjugated to glutathione by the action of glutathione *S*-transferase to render them higher in water solubility and facilitate their excretion. As an antioxidant, glutathione plays a crucial role in the protection of cells against damage resulting from exposure to oxidizing environments, e.g., hyperoxia, or oxidizing agents formed in xenobiotic metabolism (Meister and Anderson, 1983; Deneke and Fanburg, 1989). The aquatic environment is a sink for numerous contaminants, which often produce oxyradicals during the course of their metabolism. The involvement of glutathione and antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, to counteract such prooxidant forces has been demonstrated in diverse studies (Thomas and Wofford, 1984; Mather-Mihaich and Di Giulio, 1991; Winston, 1991).

Most of the studies on biochemical responses have been carried out to investigate the effects of bleached pulp mill effluents. Only a few approaches have been made to study the responses to unbleached pulp mill effluents (Lindström-Seppä *et al.*, 1992; Pesonen and Andersson, 1992). The authors were, therefore, interested to examine the physiological and biochemical responses in fish exposed to contaminated sediments, taken from a site receiving unbleached pulp mill effluents. The main aim of the present study was to measure the toxicological effects in rainbow trout (*Oncorhynchus mykiss*) exposed to different concentrations of sediments, taken from a site receiving unbleached pulp mill effluents. The primary interest was to analyze the manner in which cytochrome P450-dependent enzymes were affected in the fish exposed to such sediments. Also, antioxidant-mediated responses were studied to evaluate them as tools in the monitoring of pollution caused by pulp mill effluents.

MATERIALS AND METHODS

Chemicals

Chemicals for laboratory analysis were obtained from the following sources: Epinephrine, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), NADPH, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), and uridine 5′-diphosphoglucuronic acid were purchased from Sigma Chemical Co. (St. Louis, MO); 7-ethoxyresorufin and resorufin from Pierce (Rockford, IL). All other chemicals were of analytical grade.

Contaminated Sediments

The paper mill, situated in Eastern Finland, produces different types of cardboard and unbleached papers by using birch as raw material. The applied production method is the ammonium-based sulfite process and the effluent undergoes mechanical and biological treatment (anaerobic process) before being discharged into the lake (Lake Kallavesi). The sediment was collected from the site of effluent discharge with an

Ekman bottom sampler within a radius of 15–20 m and stored at 6–8°C prior to use (3 days).

For the laboratory analysis 10 g of dry sediment was mixed with 100 ml of water and filtered. The samples were extracted and analyzed for resin acids and phenols according to the methods of Holmbom (1980) and Voss *et al.* (1980), respectively. For the quantitative determination known amounts of internal standards, stearinic acid and 2,3,6-trichlorophenol, were added. For the analysis a gas chromatograph–mass spectrometry (Hewlett–Packard 5890/5970) equipped with a capillary column [Hewlett–Packard-5/(25 m × 0.20 mm i.d.)] was used. The injector temperature of the gas chromatograph was 250°C; at the time of injection the temperature was 50°C rising at a rate of 5°C/min to a final temperature of 200°C.

Fish in the Laboratory Studies

Cultured 1+ year-old rainbow trout, *O. mykiss* (length, 22–27 cm; weight, 110–170 g), were obtained from Nilakkalohi Fish Farm (Tervo, Finland) and were transported in aerated basins to the University of Kuopio. The fish were held in glass fiber tanks (150 liters), supplied with unchlorinated tap water, and acclimated for 5 days before the start of the experiment in June 1991 (temperature, 11°C). The fish had been fed commercial diets, but were starved during the acclimating and experimental period in the laboratory.

Two 50-liter (blow area, 1600 cm^2) and two 60-liter (blow area, 2000 cm^2) aquaria were filled with 2 and 4 liters contaminated sediment (shortly drained through a cheese cloth), respectively. The sediment in each aquarium was covered with a cheese cloth (boiled in hot water prior to use) and an antiseptic metallic frame for stabilization. The aquaria were filled with dechlorinated tap water and left for a day. By the start of the experiment each aquarium contained 8 fish (randomly sampled) and 51 fish were kept as controls in the tanks. One additional 60-liter aquarium was prepared with 4 liters of sediment 7 days after the start of the experiment and filled with 7 fish from the control tanks, which were harvested at the end of the experiment. All aquaria were artificially aerated and one-third of their water was changed daily. Temperature (T), pH, and dissolved oxygen (DO) were measured daily: T, 10.5 ± 1°C; pH, 7.0 ± 0.5; and DO, 9.0 ± 1.0 mg/liter. The tanks were artificially aerated during the acclimation period, but aeration was stopped after excess amounts of oxygen (11–13 mg/liter) were measured, which still persisted for a prolonged period in the control tanks. Temperature and pH was in the same range as that described for the aquaria. The experiment lasted for 21 days and fish were sampled at Days 0, 3, 10, and 21.

Blood and Tissue Sampling

Blood was collected from the caudal vein with a sterile syringe and needle. The samples were prepared for total glutathione (TGSH) and glutathione disulfide (GSSG) analysis as described by Adams *et al.* (1983) and stored at −80°C for 2 weeks. The fish were killed with a blow to the head. The liver was removed, cleaned, weighed, cut into pieces, and stored in liquid nitrogen until preparation. A small amount of muscle was cut from the dorsal site, freed from the skin, and stored in liquid nitrogen.

Analytic Procedures

The biotransformation enzyme activities were analyzed from the microsomal and supernatant fraction. Microsomes were prepared within the next 4 days after sampling.

Liver samples were thawed at 4°C, put into ice-cold 0.25 *M* sucrose, and homogenized in a Potter–Elvehjem-type glass Teflon homogenizer. The homogenates were centrifuged at 10,000*g* for 15 min at 4°C in a Sorvall Instruments RC5C. For the isolation of the microsomal fraction the postmitochondrial supernatant solution was further spun at 105,000 in a TGA-65 ultracentrifuge (Kontron Zuerich) at 4°C for 60 min. Microsomes were resuspended in 0.25 *M* sucrose (containing 0.06 *M* Tris, 5 m*M* EDTA, and 20% glycerol), while 1 ml corresponded to 1 g of liver wet weight, and were homogenized with a Heidolph Laborruehrer (Type RZR1, Heidolph Elektro KG, Germany). Supernatants and microsomes were stored at −80°C for further analysis within the following weeks.

The microsomal activity of EROD was measured in a Shimadzu fluorometer RF-5001PC (Shimadzu Corp., Japan) after the method of Burke and Mayer (1974). Microsomal UDPglucuronosyltransferase (UDPGT) was determined by the method of Hänninen (1968) using 0.35 m*M* *p*-nitrophenol as aglycone and measuring the absorbance in a Shimadzu OPI-2 spectrophotometer (Shimadzu Corp.). Glutathione *S*-transferase (GST) from the liver homogenate was assayed according to Habig *et al.* (1974) with CDNB as substrate. The change in absorbance was monitored in a Perkin–Elmer Lambda 2 uv/vis spectrophotometer (Perkin–Elmer Corp., CT).

Measurement of rainbow trout cytochrome P450IA1 equivalents was performed as described previously (Kloepper-Sams *et al.*, 1987). Hepatic microsomes were pooled so that each sample contained equal amounts of microsomes from one to four fish. The samples were diluted with sample treatment buffer and 45 μg protein (15 μl) from each pool as well as standard scup microsomes with known P450IA1 content were taken and subjected to separation with SDS–polyacrylamide gel electrophoresis in a 6–14% acrylamide gradient gel. The proteins were electrophoretically transferred onto 0.2 μm nitrocellulose and incubated first with monoclonal antibody (MAb 1-12-3) to scup P450 (scup P450E), then with secondary antibody (goat anti-mouse IgG linked to alkaline phosphate (Bio-Rad). The nitrocellulose was exposed to color developer (NBT and BCIP) to visualize the P450 bands. The color development was quantified by video imaging densiometry (Master Scan, Scanalytics/CSPI, Billerica, MA). Cytochrome P450E equivalent values for the microsomal samples were determined from the integrated optical density of the microsomal proteins relative to the integrated optical density of the scup standard.

Superoxide dismutase and catalase activities were assayed spectrophotometrically from the supernatant fraction. The SOD activity was measured by the method based on the inhibition of the autoxidation of epinephrine (Misra and Fridovich, 1972). Catalase activity was assayed as described by Cohen *et al.* (1970). The protein contents of liver microsomes and supernatants were determined by using the Folin–Ciocalteau method (Lowry *et al.*, 1951) with bovine serum albumin as standard.

Total glutathione in blood plasma was analyzed after the method of Adams *et al.* (1983). For the total GSH determination in tissues, liver and muscle samples were prepared and assayed as described by Sen *et al.* (1992). For the analysis of GSSG, liver samples were prepared and determined by the method of Adams *et al.* (1983).

Data Analysis

The assumption of equal variances was tested by the Cochran's C test. With homogeneity in many variables ($P > 0.05$) the data were tested by the one-way analysis

of variance and the differences between groups were screened by the Duncan's multiple range test ($\alpha = 0.05$). With heterogeneity in many variables ($P < 0.05$) the data were tested with the nonparametric Kruskal–Wallis one-way analysis of variances and Mann–Whitney test with Bonferroni's correction.

RESULTS

Sediment Constituents and Fish

The calculated and estimated amounts of phenols and resin acids are listed in Table 1. The data indicate that large amounts of chemicals discharged from pulp mills, as dehydroabietic and abietic acid, but also phenol derivates were observed to be concentrated in the bottom sediments.

During the acclimation period some ($n = 5$) fish died and a high mortality ($n = 10$) among the control fish was still recorded during the first week of the experiment. Despite the removal of the aeration tubes the oxygen levels remained quite high in the beginning and appeared to have caused stress to the control fish, which may explain the mortality. Condition factor (CF) remained unchanged, which could be generally observed for the liver somatic index (LSI) (Table 2). CF and LSI did not appear to be sensitive indicators for the exposure to contaminated sediments. However, a significant increase in LSI toward controls was recorded once in the 2-liter exposed group on Day 10.

Cytochrome P450-Dependent Enzymes

Exposure of rainbow trout to contaminated sediments evoked great induction in the monooxygenase activities. While the deethylation of 7-ethoxyresorufin lowered in the control groups during the experimental period, EROD activity in the exposed groups was greatly enhanced when compared to controls. On Day 3, the activity was increased to almost fourfold to that of the control levels and was significantly different in the 4-liter exposure group (Fig. 1). But the EROD induction was discontinuous, as exposed groups had a range at similar levels as controls on Day 10. However, after

TABLE 1

CALCULATED AND ESTIMATED AMOUNTS OF RESIN ACIDS AND PHENOLS DETECTED IN CONTAMINATED SEDIMENTS TAKEN FROM A SITE RECEIVING UNBLEACHED PULP MILL EFFLUENTS

Resin acid	μg/g	Phenols	μg/g
Pimaric acid	0.86[a]	Phenoxyphenol	2.54[b]
Isopimaric acid	2.05[a]	Benzaldeyde	3.45[b]
Levopimaric acid	0.50[a]	Phenol	4.44[b]
Palustrinic acid	1.78[a]	Hydroxy–dimethoxyphenol	18.88[b]
Dehydroabietic acid	6.76[a]	Vanillin	22.16[a]
Abietic acid	6.94[a]	Methyl–hydroxy–benzaldehyde	27.20[b]
Neoabietic acid	ND	Methoxyphenol types	6–8[b]

Note. ND, not detectable.

[a] Calculated according to internal standards (stearinic acid or trichlorophenol).

[b] Estimated by comparing the peaks with vanillin.

TABLE 2

LIVER SOMATIC INDEX AND CONDITION FACTOR IN RAINBOW TROUT EXPOSED TO CONTAMINATED SEDIMENTS DURING A PERIOD OF 3 WEEKS

Parameter and group	Day 0	3	10	14	21
LSI					
C	0.89 ± 0.10[a] (8)[b]	0.90 ± 0.10 (8)	0.89 ± 0.07 (7)		0.87 ± 0.13 (11)
E 2 liters		0.93 ± 0.08 (4)	1.07 ± 0.21* (4)		0.98 ± 0.13 (8)
E 4 liters		0.93 ± 0.17 (4)	0.93 ± 0.12 (4)	1.00 ± 0.18 (7)	0.84 ± 0.10** (7)
CF					
C	0.92 ± 0.09 (8)	0.99 ± 0.12 (8)	0.87 ± 0.05 (7)		0.90 ± 0.05 (11)
E 2 liters		0.98 ± 0.05 (4)	1.16 ± 0.37 (4)		0.90 ± 0.07 (8)
E 4 liters		0.96 ± 0.06 (4)	0.99 ± 0.12 (4)	0.83 ± 0.08 (7)	0.93 ± 0.09 (7)

Note. LSI, liver somatic index (percentage of liver weight of total fish weight); CF, condition factor [W (g)/liter (cm)^3] × 100; C, controls; E 2 (4) liters, exposed to 2 (4) liters sediment.

[a] Mean ± SD.

[b] Number of fish (n).

* Significantly different from controls ($P < 0.05$).

** Significantly different from 4-liter exposed group (Day 14) ($P < 0.05$).

21 days a threefold induction could be observed in both exposure groups, which was statistically significant. The 2-liter exposed group on Day 21 was significantly different from that on Day 10. But in all cases, the increased activity could not be related to the exposure concentration.

Cytochrome P450IA1 gave a similar picture as that of EROD induction in the 2-liter exposed group. The amounts were increased on Days 3 and 21 in comparison to controls, but on Day 10 the levels were low. P450IA1 levels in the 4-liter exposure group were not proportional to the EROD activity during the experiment. On Days 3 and 10 the levels were higher than controls, but were strongly increased on Days 14 and 21. The 21-day exposure group was significantly different from controls (Fig. 2).

Glucuronidation was unchanged in the four control groups, while exposed groups demonstrated marked alterations during the experimental period (Fig. 3). In comparison to controls, the exposure groups differed in response on Day 3, with lower and higher activity in the 2- and 4-liter exposure groups, respectively. Thereafter, the responses in both exposure groups were increased on Day 10. The enzyme activity was significantly different in the 4-liter exposure group when compared to the controls. UDPGT activity was at similar levels as controls on the last day of exposure. Such strong alterations in the enzyme activity during the exposure period also led to significant changes in between groups (Fig. 3). No real change could be observed in the conjugation with glutathione with similar levels of GST activities in all groups (Table 3).

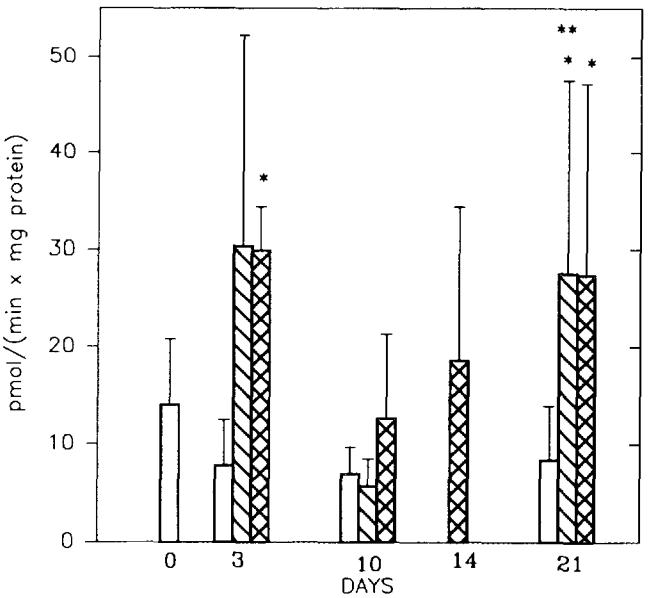

FIG. 1. Specific EROD activity in liver microsomes of rainbow trout exposed to contaminated sediments during an experimental period of 3 weeks. Groups: controls (empty bar), 2 liters sediment (hatched bar), and 4 liters sediment (crossed bar). Values represent mean ± SD; numbers of fish are the same as those denoted in Table 2. *Significantly different from controls of the same day. **Significantly different from fish of same group on Day 10 ($P < 0.05$).

Antioxidant Responses

A two-week exposure to different concentrations of sediment increased total GSH of blood plasma in most cases. In both exposure groups 33–52% elevations of TGSH could be recorded in comparisons to controls, but these data were not significantly different (Table 3). The hepatic TGSH levels strongly increased in the controls; by the end of the experiment the amount of TGSH was elevated by 92% compared to 0-controls (Fig. 4). But some changes could be observed in the exposed groups, which appeared to be dependent on the sediment concentration. The fish exposed to 2 liters sediment demonstrated increased levels on Days 3 (67%) and 21 (34%) relative to the controls of the same days. However, these data were not significantly different. Only same treatment groups were found to be significantly different from each other (Fig. 4). In the 4-liter exposure group similar GSH concentrations were measured as found in the controls. Liver GSSG concentrations were rather unchanged in all groups. However, increased amounts of GSSG were recorded once in the controls (Day 3) and in both exposure groups by the end of the experiment (Table 3).

Muscle TGSH concentrations in the controls were increased by 88% after 3 weeks. Total glutathione also increased in the exposed groups, being generally higher than controls. This effect was more pronounced in the 4-liter exposure group. This group demonstrated rather high TGSH levels on Days 10 (79%) and 14 (32%) when compared to the controls of the same days (Fig. 5), but these data were not significantly different.

Superoxide dismutase levels were low in the exposed groups and were significantly different from controls. The controls were very high at the beginning and the end of

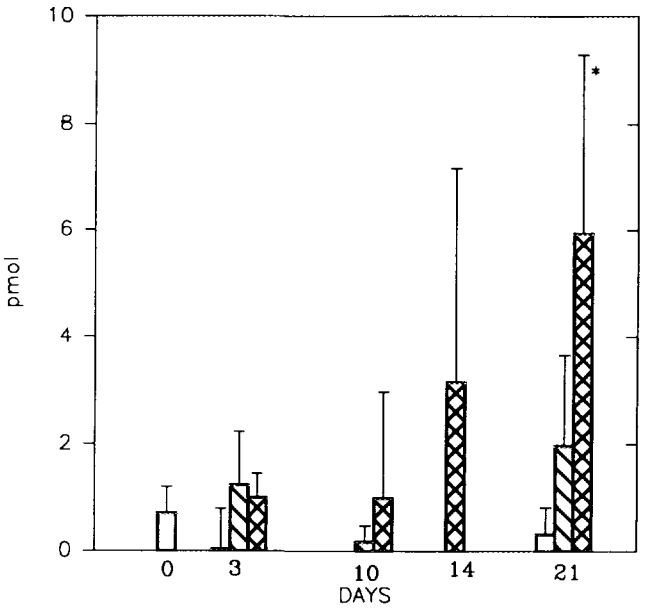

FIG. 2. Cytochrome P450IA1 equivalents in liver microsomes of rainbow trout exposed to contaminated sediments during an experimental period of 3 weeks. Samples were pools from 1–4 fish. *Significantly different from controls of the same day ($P < 0.05$). For further details see legend to Fig. 1.

the experiment. On Day 21, catalase levels were increased in all groups in comparison to 0-controls, but no difference could be observed between controls and exposed groups on that day (Table 4).

DISCUSSION

In Scandinavian countries increased attention has been given to study the impact of pulp and paper mill effluents on fisheries and aquatic ecosystems. Most experiments have been carried out to study the effects of bleached kraft mill effluents (BKME) on fish (Oikari and Lindström-Seppä, 1990; Lehtinen, 1990). Up to now, few approaches have been made to study the effects of unbleached pulp mill effluents on fish (Lindström-Seppä *et al.*, 1992; Pesonen and Andersson, 1992). The various compounds found in these complex effluent mixtures, such as resin acids and phenols, tend to accumulate in the sediments and can be found in considerable amounts, as seen in the GC–MS analysis (Table 1).

Fish exposed to sediments may obtain some food particles from it, as microbes are attached to sludge particles and certain invertebrates (worms, molluscs, or insect larvae) can inhabit the sediment. Chemicals can also be absorbed by the body through dietary intake, which may increase the responsiveness of, i.e., biotransformation, enzymes to pollutants.

The high mortality among the control fish during the acclimation period and the first week of the experiment may have been caused by the high oxygen levels recorded in the tanks. Although it seems unlikely that hyperoxia may cause mortalities to rainbow trout (Dr. Steve Perry, personal communication), the fish appeared stressed, as they

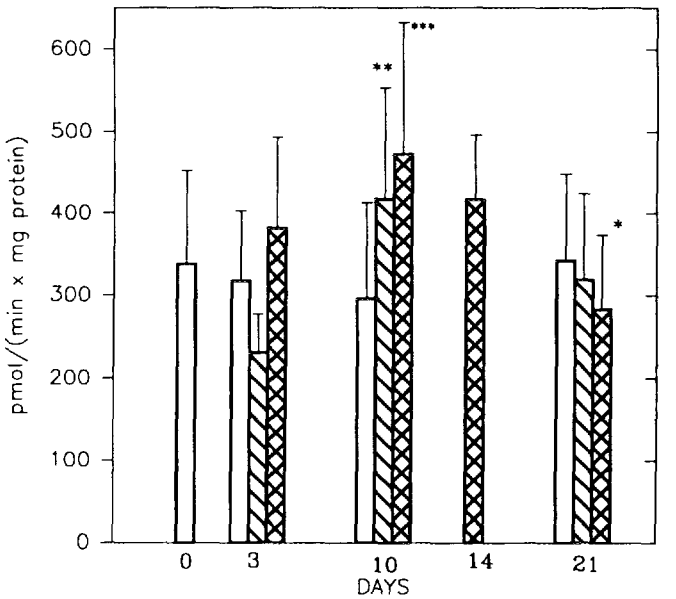

FIG. 3. Specific UDPGT activity in liver microsomes of rainbow trout exposed to contaminated sediments during an experimental period of 3 weeks. *,**Significantly different from fish of same group of previous days. *** Significantly different from controls ($P < 0.05$). For further detail see legend to Fig. 1.

demonstrated a change in behavior (swimming to the surface, slow motions). After the removal of the aeration tubes, the swimming motions were normal and no stress signs could be recorded. However, the real cause of these mortalities may remain enigmatic.

Cytochrome P450-Dependent Enzymes

EROD activity has been described as a very specific parameter to detect metabolic changes in fish exposed to BKME (Lindström-Seppä and Oikari, 1989). Also, in this experiment, EROD has proven to be well induced by pollutants discharged from unbleached pulp mill effluents, but the induction could not be related to exposure concentration (Fig. 1). Induction of EROD activity by diluted unbleached pulp mill effluents has previously been indicated in feral perch and rainbow trout, caged in waters downstream from the pulp mill, with a high induction at different distances (0.5, 3, 6, and 12 km) from the mill, particularly in winter, spring, and summer (Lindström-Seppä *et al.*, 1992). Also, the effect of unbleached pulp mill effluents has been studied in primary cultures of rainbow trout hepatocytes, indicating high EROD induction at lower effluent concentration, while higher effluent concentrations strongly inhibited EROD activities (Pesonen and Andersson, 1992).

Such observations may complicate the speculation regarding the identity of inducing components in pulp and paper mill effluents. Studies with simulated bleached pulp and paper mill effluents in the composition of a sulfate soap preparation, containing mainly resin, fatty acids, and added chlorophenols, have been conducted (Oikari *et al.*, 1988; Oikari and Lindström-Seppä, 1990).

TABLE 3

GLUTATHIONE *S*-TRANSFERASE, AMOUNT OF TOTAL GLUTATHIONE IN BLOOD PLASMA, AND HEPATIC GSSG CONTENT IN RAINBOW TROUT EXPOSED TO CONTAMINATED SEDIMENTS DURING 3 WEEKS

Parameter and group	Exposure day				
	0	3	10	14	21
GST					
C	248.2 ± 68.4[a]	257.8 ± 32.9	243.4 ± 19.0		265.5 ± 42.9
E 2 liters		206.7 ± 37.2	228.8 ± 34.1		245.2 ± 57.9
E 4 liters		222.6 ± 59.5	282.4 ± 86.6	238.0 ± 24.2	235.3 ± 19.8
Plasma TGSH					
C	53.1 ± 16.5	62.9 ± 44.9	58.1 ± 29.8		57.7 ± 16.1
E 2 liters		83.4 ± 30.6	80.8 ± 33.5		64.7 ± 29.1
E 4 liters		67.3 ± 21.4	88.4 ± 40.3	81.8 ± 29.8	62.9 ± 23.8
Liver GSSG					
C	63.9 ± 8.0	63.4 ± 11.9	91.3 ± 23.1		64.3 ± 10.2
E 2 liters		53.0 ± 4.2	59.3 ± 9.6		94.1 ± 24.7
E 4 liters		55.7 ± 5.9	80.3 ± 41.3	67.7 ± 6.5	96.4 ± 44.6

Note. Groups and number of fish are the same as those denoted in Table 2. GST, glutathione *S*-transferase specific activity in nmol/min × mg protein; TGSH, total glutathione content in blood plasma in μmol/liter; liver GSSG, concentration in nmol/g wet weight.

[a] Mean ± SD.

Induction of monooxygenase activity was always markedly lower (about 50–70% higher than controls) in these fish than in fish of the same species caged in waters receiving BKME (several-fold induction in comparison to controls). BKME contain a much more heterogeneous mixture of xenobiotic chemicals than do simulated pulp mill effluents and conclusions have been drawn that resin acids and chlorophenols are actually not responsible for monooxygenase induction and nonchlorinated compounds present in the sulfate liquor seem to be negligible as well. It was suggested that polychlorinated dibenzofurans and dibenzodioxins could cause such an induction of monooxygenases (Oikari and Lindström-Seppä, 1990).

The very complex liquors of pulp and paper mill effluents are not yet fully characterized in their containing constituents and the potential of certain synergistic, additive, or antagonistic effects of those chemicals remain unknown (Mather-Mihaich and Di Giulio, 1991). The decreased EROD activity in the second week of the present study may suggest an inhibitory effect of certain chemicals (Fig. 1). The response of cytochrome P450IA1, which is responsible for the EROD activity, gave a clearer picture in this regard. Exposure to lower sediment concentration (2 liters) indicated a very similar trend as the EROD activity, with discontinuous induction (Fig. 2). This mainly indicates that the catalytic activity of P450IA1 is fully expressed and reflects the EROD activity. However, this is not the same for the higher exposure group (4 liters), demonstrating strong and continuous increase of the EROD catalyst. The high P450IA1 levels, measured in this group, do not appear to reflect EROD levels and, therefore, raise the assumption that a higher concentration of certain chemicals found in the sediment may suppress the catalytic activity of P450IA1. Suppression of the catalytic activity of P450E, the representative of P450IA1 in winter flounder, through high

amounts of polychlorinated biphenyls (PCBs), had been observed by Elskus *et al.* (1989). This study revealed a complex relationship between levels of EROD activity, P450E, and tissue PCB concentration. Competitive catalytic inhibition of P450E by certain PCB congeners was shown in scup microsomes (Gooch *et al.*, 1989). Also, endogenous compounds may exert such an effect, as indicated in the results obtained by Goksøyr and Larsen (1991). Their results indicated that sex steroids inhibited P450IA1 protein in mature salmon, resulting in decreased EROD activity.

Conjugation Activity

The alterations recorded in the UDPglucuronosyltransferase activity during the run of the experiment (Fig. 3) support the differing results obtained in other exposure studies on this enzyme in different fish species. Exposure to diluted BKME changed UDPGT activity in piscine species by either inducing (Förlin *et al.*, 1985; Andersson *et al.*, 1988) or inhibiting it (Soivio *et al.*, 1988). An alteration in glucuronidation activity was recorded in vendace in a long-term exposure, with an inhibition in the first weeks (14–28 days) and a slight increase after several weeks (120 days) (Lindström-Seppä *et al.*, 1989). It is known that the chemical composition of the effluent can vary according to the process and wood material used, and the data obtained in the various studies may indicate that UDPGT is very sensitive to either quantitative or qualitative changes. On the other hand, no changes could be observed in the glutathione *S*-transferase activity (Table 3). Preliminary studies in the laboratory on rainbow trout exposed to unbleached pulp mill effluents give rise to the assumption that this enzyme may not be a useful tool in the monitoring of the pollution caused by pulp mill effluents.

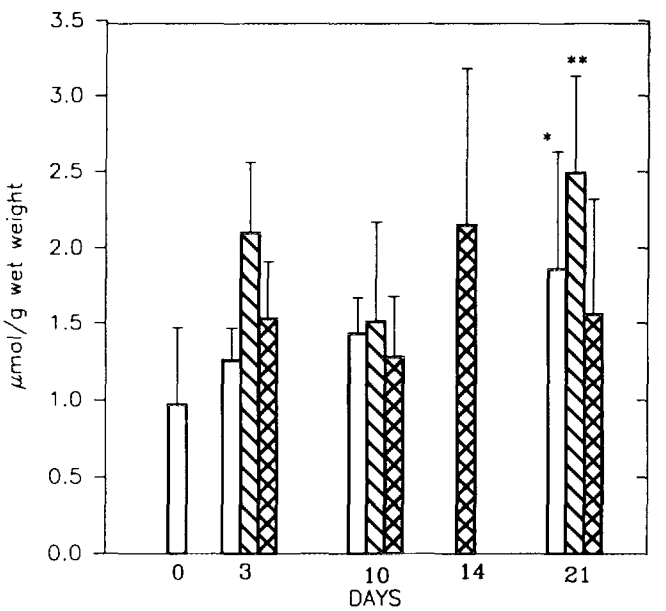

FIG. 4. Total glutathione content in liver of rainbow trout exposed to contaminated sediment during an experimental period of 3 weeks. *Significantly different from 0-controls. **Significantly different from fish of same group of previous day ($P < 0.05$). For further detail see legend to Fig. 1.

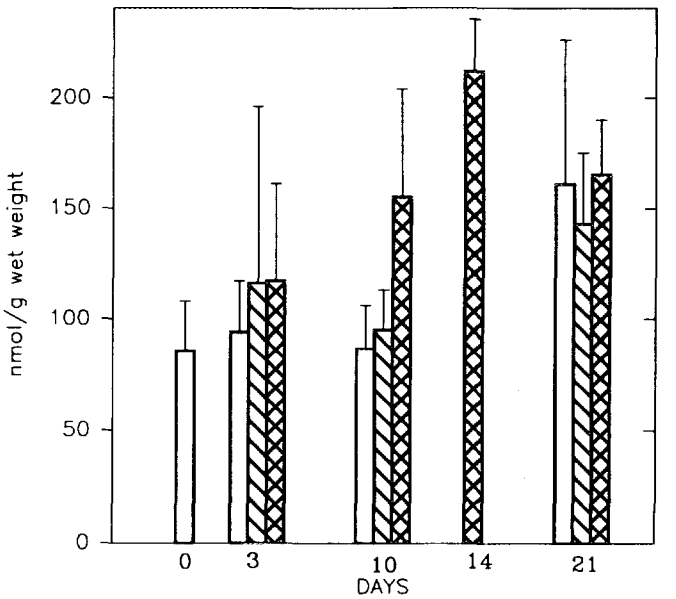

FIG. 5. Total glutathione content in muscle of rainbow trout exposed to contaminated sediments during an experimental period of 3 weeks. For further detail see legend to Fig. 1.

Oxidant-Mediated Responses

Through normal oxygen utilization in aerobic organisms, various reactive oxygen species (oxyradicals) are produced. The imbalance between the production of oxidants and the removal or scavenging of oxidants is called oxidative damage (Winston, 1991). The scavenging of oxyradicals is carried out by specially adapted enzymes as superoxide dismutases, catalase, and glutathione peroxidases. Nonenzymatic responses include glutathione, vitamin E, and ascorbic acid.

Only a few studies have examined oxidant-mediated responses after xenobiotic exposure in fish and the results of such studies have often been inconclusive (for review see Winston and Di Gulio, 1991). In channel catfish the oxidant-mediated effects were studied after exposure to mercaptan-containing compounds, but neither antioxidant enzymes nor reduced glutathione were affected (Mather-Mihaich and Di Gulio, 1986). However, exposure to complex mixtures appeared to exert some effect on nonenzymatic defenses in fish. Mullet exposed to metals and petroleum hydrocarbons demonstrated enhanced hepatic glutathione levels (Thomas and Wofford, 1984). Andersson *et al.* (1988) recorded elevations in the liver reserves of ascorbic acid in perch collected from sites polluted with BKME.

In the present study, total glutathione contents in plasma appeared to be slightly affected by the exposure to contaminated sediments (Table 3). The observed increase in TGSH of liver and muscle in the control fish (Figs. 4 and 5) may result from the high dissolved oxygen levels at the beginning of the experiment. The aquaria contained a lower amount of DO (8–10 mg/liter), since there was a higher organic loading causing a higher oxygen demand, while the tanks did not contain any sediment and exhibited (despite the removal of the artificial aeration) very high oxygen contents (11–13 mg/

liter). This incidence may give an additional hint that oxyradicals formed in hyperoxia may result in increased glutathione levels, as previously found in mammals (Deneke and Fanburg, 1989). Intracellular GSSG levels appeared to be only once affected by the hyperoxia in the controls (Table 3), but GSSG is generally expelled from the cells after formation.

Despite the problems of hyperoxia in the controls, moderate increases of TGSH could be observed in the liver and muscle of sediment-exposed fish. Also, hepatic GSSG levels were elevated in exposed fish at the end of the experiment. These data support the findings of other research teams. Oikari *et al.* (1988) reported elevated GSH levels in brown trout exposed to simulated pulp mill effluents. Similar results were obtained by Soimasuo *et al.* (1991), caging whitefish in a lake receiving BKME which demonstrated increased hepatic GSH levels during an experimental period of 12 weeks. Also, total glutathione levels in muscle were strongly elevated in rainbow trout exposed to different dilutions of unbleached pulp mill effluents (preliminary results of our laboratory). Such studies clearly indicate that glutathione is involved in the cellular protection against undesirable compounds in fish. Studies on primary cultures of rainbow trout hepatocytes indicated that unbleached pulp mill effluents have a more profound effect than BKME. GSH content was strongly decreased in hepatocytes exposed to unbleached effluents and could not be detected after exposure to very high effluent concentrations (Pesonen and Andersson, 1992).

In the present study, the antioxidant enzymes superoxide dismutase and catalase did not appear to be responsive to the exposure to contaminated sediments. SOD levels in the controls were higher than those in exposed fish (Table 4). It may be argued that sediment exposure decreased the activity of this enzyme. However, it is known that SOD catalyzes the reaction, $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. H_2O_2 is then further converted to H_2O and O_2 by catalase (Winston and DiGiulio, 1991). DO was very

TABLE 4

SUPEROXIDE DISMUTASE AND CATALASE ACTIVITIES IN RAINBOW TROUT EXPOSED TO CONTAMINATED SEDIMENTS FOR 3 WEEKS

Parameter and group	Day 0	Day 14	Day 21
SOD			
C	26.68 ± 8.02[a]		25.85 ± 10.18
E 2 liters			12.50 ± 5.96*
E 4 liters		18.92 ± 9.03	13.77 ± 4.04*
Catalase			
C	1.14 ± 0.28		1.67 ± 0.30**
E 2 liters			1.74 ± 0.41**
E 4 liters		1.63 ± 0.47**	1.81 ± 0.57**

Note. Groups and number of fish are the same as those denoted in Table 2. SOD, superoxide dismutase in U/mg protein; catalase, activity in k/mg protein.

[a] Mean ± SD.

* Significantly different from controls ($P < 0.05$).

** Significantly different from 0-controls ($P < 0.05$).

high in the control tanks; it may, therefore, be suggested that SOD levels in the controls were so high that more oxyradicals, such as $2O_2^-$, may have been produced. This may also explain the rise in catalase activities in the controls after 21 days. On the other hand, elevated catalase levels in exposed fish may have been caused by the exposure to contaminated sediments. In a study conducted by Mather-Mihaich and Di Giulio (1991) the oxidant responses of channel catfish after BKME exposure were tested. Only significant increases could be noted in the catalase activity (glutathione concentrations were strongly decreased in the exposed fish during the first days of the experiment), but no changes could be noted in the SOD and glutathione peroxidase activity.

The role of antioxidant enzymes as SOD and catalase still remains very inconclusive. The present study has shown that high dissolved oxygen levels in the water may interfere in the results, as oxyradicals are produced affecting antioxidant responses. DO must, therefore, always be recorded in the monitoring of such enzymes in either laboratory or field experiments. But, changes in glutathione concentrations appear more indicative for the involvement of antitioxidant responses in pulp mill effluent pollution studies. However, more research is needed to investigate the role of antioxidant responses in the monitoring of polluted sites.

CONCLUDING REMARKS

Chemicals of unbleached pulp mill effluents, which accumulate in the bottom sediment, have a strong inductive potential on the EROD activity and cytochrome P450IA1 in rainbow trout. A higher amount of chemicals, as found in the higher exposure concentrations, indicates a suppressive effect on the activity of the EROD catalyst. UDPglucuronosyltransferase also appears to be a responsive indicator in the pollution caused by unbleached pulp mill effluents. In the case of oxidant-mediated responses, glutathione concentrations appear to be generally affected by such pollutants, while the changes of antioxidant enzymes are not so obvious.

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