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Biomarker responses in fish exposed to effluent from bleached sulphite pulp production

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The effects of biologically treated effluents from bleached sulphite pulp production were studied using brown trout (*Salmo trutta*) as test organism. Continuous-flow exposures under laboratory conditions were conducted at two dilutions (1:40 and 1:400) for eight weeks, followed by a six-week recovery period. Physiological and biochemical biomarkers including liver histology, hematology and hepatic enzyme assays were used and the exposure was verified by analysing fish bile and fish tissue concentrations of resin acids and chlorophenols. The conjugated chlorophenols and resin acids levels in the exposed fish bile are considered as low. Only two significant responses were observed, both in liver function.The MFO associated EROD activity was induced parallel to lower liver glycogen levels. Otherwise, the changes in physiological parameters analysed were few as compared to reference fish. The small differences in physiological parameters between the reference group and the exposure group (1:40) after six-week recovery period show that the observed responses were reversible. The spectrum and pattern of results observed in this study are very much the same as seen in laboratory and field studies with bleached kraft mill effluents from modern mills.

Introduction

A large number of laboratory and field studies have been conducted in Scandinavia and North America to determine the effects of pulp mill effluents on the organism level (McLeay 1987, Owens 1991, Owens and Lehtinen 1996, Tana and Lehtinen 1996). Many studies on pulp mill effluents have focused on "within-organism effects", such as physiology and biochemistry. Despite the profound alterations in the industry, there is still evidence of several physiological changes in organisms; however the ecological significance of these changes is unknown. Physiological and

biochemical measurements have been referred to as biomarkers and, in the majority of the studies, fish have been used as test organisms. Limited knowledge is available on fish's physiological responses to effluents from production of sulphite pulp as compared with bleached kraft mill effluents from mechanical pulp production (Johnsen *et al*. 1995). One of the reasons might be the small production of bleached sulphite pulp world-wide (Reeve 1996).

Sulphite whole mill effluents contain the same resin acids, fatty acids and chlorophenols that are found in kraft mill effluents. However, the toxic responses caused by bleached kraft mill effluents (BKME) from modern mills are generally very low, and show no variation related to the actual release of organically bound chlorine (AOX) (Tana and Lehtinen 1996). This indicates that, at least in this category of mills, the chlorinated organics in the effluents do not contribute significantly to the toxicity of the effluents. The levels of chlorinated organics are in general lower in effluents from modern kraft pulp mills (Strömberg *et al*. 1996) than in effluents from old generation sulphite pulp mills (McLeay 1987). Similarly, the resin acid concentrations reported for modern BKME are generally lower than those found in biotreated bleached sulphite mill effluents (BSME) (McLeay 1987). However, information is very scarce concerning physiological responses in fish exposed to effluents from modern sulphite pulp mills.

The aim of this work has been to study sublethal physiological responses and uptake and elimination of resin acids in fish exposed to biotreated whole mill effluent from bleached sulphite pulp production. The sublethal effects were studied using freshwater exposure at environmentally relevant dilutions and a reference with brown trout (*Salmo trutta*) as the test organism.

Table 1. Effluent characteristics (in mg l^{-1}).

Total suspended solids TSS	86 (52–157)
Volatile suspended solids (540°C) VSS	62 (22–141)
Chemical oxygen demand COD_{Cr}	1 730 (1 150–3 100)
Adsorbable organic halogens AOX	14.2 (8.3–22.7)
Resin acids	0.04

Material and methods

Effluent characteristics

The mill studied produces different grades of elementary chlorine free (ECF) bleached calcium sulphite pulp from spruce (*Picea abies*). The spent cooking liquor evaporation condensates and the effluents from the bleach plant undergo biological treatment. This secondary treatment comprises an anaerobic stage followed by an aerobic stage, resulting in an overall 70% reduction of the dissolved organic material calculated as the chemical oxygen demand COD_{Cr}. The extractives are reduced by 90%. The mean values for the characteristics of the treated effluent are given in Table 1.

Test procedure

The experiment took place in September–December 1995. Fish were exposed indoors in 1 m^3 polyethylene pools (water volume 700 l) at the mill water station. The pools received sand filtered fresh water pumped from the river upstream the mill, with a continuous flow of 3.2 l min^{-1}. Effluent was sampled twice a week and transferred to the study site and kept at +4 °C. The effluent was continuously dosed into the inflow tubes of each pool by means of peristaltic pumps. Juvenile brown trout (*Salmo trutta*) were used as test organisms and 50 individuals (mean weight 117 ± 19 g) were placed randomly in each testpool and allowed to acclimatize for two weeks prior to the exposure. The fish were exposed to the diluted effluent for eight weeks in one high dose group (dilution 1:40), one low dose group (dilution 1:400) and one clean water reference group. The fish biomass was 8 g l^{-1} at the beginning of the experiment. The fish were fed a commercial fish feed. The feeding rate was 1% of the fish's weight per day. A 12:12h light:dark regime was maintained throughout the experiment. The fish (n = 25) in the different exposure groups were sampled after an eight-week exposure period. For growth measurements all 50 individuals in each exposure group were weighed after the exposure period. After the exposure period the remaining

high dose fish (n = 25) were placed in reference water for a six-week recovery period. In the high dose and reference groups, there was an additional sampling after the recovery period. Before the exposure started samples (n = 10) were taken for measurements of liver enzyme (EROD and UDP-GT) activities and liver glycogen and lipid contents.

Sampling

After exposure, the fish were caught individually with a dip net and stunned with a blow on the head. Blood was collected from caudal vessels with a disposable syringe using crystalline ammonium heparin as an anticoagulant. Blood hematocrit (packed red blood cell volume) was determined immediately with a Compur Microspin hematocrit centrifuge. The leucocrit (packed white blood cell volume) value was determined from the same sample. For blood hemoglobin measurement, 10 µl of blood was placed in a test tube containing 2 ml of hemoglobin reagent. The rest of the blood sample (1–2 ml) was centrifuged, and 500 µl plasma was placed in a plastic tube and frozen on dry ice. Frozen samples were kept on dry ice until analysis.

After blood sampling, both the length and the weight of the fish were measured. The body cavity was opened, the sex determined, and the bile sampled using a 2 ml disposable syringe. Bile from four fish was pooled, frozen, and stored on dry ice for later analysis of conjugated chlorophenolics, resin and fatty acids, as well as other extractives. After the bile sampling the liver was weighed, cut into pieces, placed in Eppendorf tubes and frozen in liquid nitrogen for later analysis of enzyme activities and different metabolic parameters. The liver sample for enzyme analysis was taken from the central (ventral) part of the liver. Fresh liver samples for histological analysis were taken from the first ten fish in each group. For liver histology, the peripheral dorso-posterior (5 × 5 mm) liver tissue sample was fixed in Bouin's solution for 24 hours and transferred to 70% ethanol. The samples were kept in ethanol before dehydration and embedding in paraffin and sectioning (5 µm). Tissue samples were stained with hematoxylin-eosin. Sampling time for each fish was less than four minutes after capture.

Effluent samples were taken as four two-week composite samples during the exposure period.

Analytical methods

The effluent samples were adjusted to pH 9 and resin acids were extracted, with methyl, *tert*-butyl ether (MTBE); 8-abietenic acid was used as a recovery standard. The resin acids in the extracts were derivatized and quantified by means of GC analysis according to the method described by Ekman and Holmbom (1989).

The determination of conjugated chlorophenolis, resin acids and other extractives in bile was based on a modified method (Lehtinen *et al*. 1992) originally described by Oikari and Ånäs (1985).

Liver-somatic index (LSI) and condition factor (CF) were calculated according to standard formulae (Wheatherley and Rogers 1978). Fish growth in each group was measured as mean weight increase from the beginning of the experiment to the end of the exposure period and after recovery ([mean weight at start] – [mean weight at end]/[number of individuals]).

The analyses of plasma aspartate aminotransferase (ASAT) and lactatedehydrogenase (LDH) were made using a Corbas Mira autoanalyzer. Xenobiotic transformation was studied by measuring the activity of the enzyme 7-ethoxyresorufin-0-deethylase (EROD), as part of the mixed-function oxidase (MFO) system, and the conjugation enzyme UDP-glucuronosyltransferase (UDP-GT) (Lindström-Seppä 1990). Liver glykogen and liver lipids were measured using standardized method as described by Soivio and Virtanen (1980).

Finally, liver tissue preparations were examined in a light microscope. Different cell types were quantified using a method by Chalkley (1943), later described by Mitchell *et al*. (1973). The microscope was equipped with a micrometer scale in the eyepiece containing a grid of squares with a wall length of 0.5 mm. Analysis was performed under 500× magnification so that 16 ran-

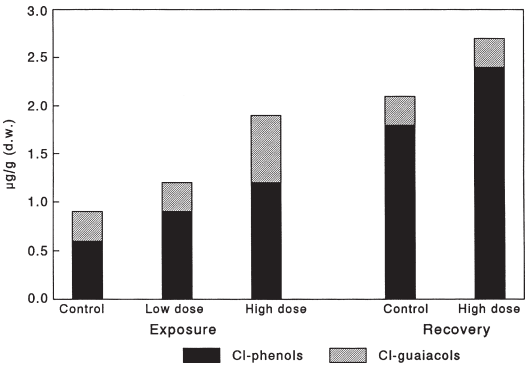

Fig. 1. Conjugated chlorophenols in the bile from brown trout after eight weeks exposure to treated sulphite pulp mill effluent and after a six-week recovery period.

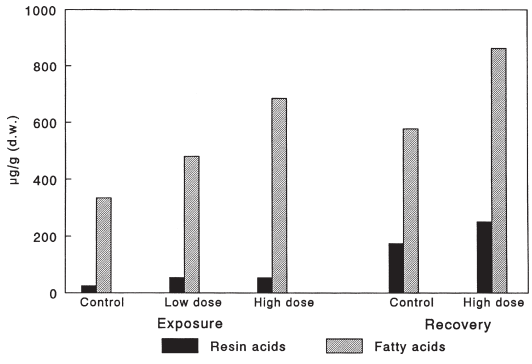

Fig. 2. Conjugated resin and fatty acids in bile samples from brown trout following eight weeks of exposure to treated sulphite pulp mill effluent and after a six-week recovery period.

dom squares per microscopic field were observed. Every square was regarded as a "hit" and the cell content of the square was determined. A total of 400 squares per fish were analysed. The liver cells were analysed for normal, necrotic, binuclear, degenerated and other structures.

Statistical comparison of the data between control and exposed groups was performed using one-way analysis of variance followed by Student's *t*-test.

Results

The temperature in the test pools decreased from 15 °C to 6 °C due to influent stream temperature during the exposure period and was constant (6 °C) during the whole recovery period. The oxygen saturation was measured daily and stayed at a constant level of 80% during the experiment.

Total resin acid concentration of the tested effluent varied between 0.01 and 0.05 mg l^{-1}, with an average of 0.04 mg l^{-1} (detection limit 0.01 mg l^{-1}).

Bile conjugates

The level of exposure of brown trout to effluent-specific contaminants was determined by analysis of conjugated chlorophenols and resin acids in bile samples. The results obtained after eight weeks of exposure and the following six-week recovery period are shown in Figs. 1 and 2.

A relationship between the chlorophenolic conjugate level and the effluent exposure was observed (Fig. 1). The resin acid levels presented in Fig. 2 after an eight-week exposure period showed no dose response. Dehydroabietic acid (DHAA) and isopimaric acid (IP) were the most predominant resin acids. After the recovery period, both chlorophenolic and resin acid conjugates had increased in the fish bile of both control and high dose groups. The increase in chlorophenolic conjugates depended mainly on the increase in 2,3,5-trichlorophenol and pentachlorophenol. The DHAA and IP were still the most predominant resin acids.

Concentrations of conjugated fatty acids were higher in the bile of effluent-exposed fish as compared to control fish and showed a dose-response relationship (Fig. 2). After the recovery period an increase in the fatty acid concentration of fish bile could also be observed.

Plant sterols, such as β-sitosterol, sitostanol, campesterol and campestanol, from the fish bile were also analysed, but no differences between the test groups after exposure or recovery periods were observed.

Physiological status of brown trout

No mortality was observed in the experimental groups during the experiment. Results from the morphological studies are presented in Table 2.

No statistically significant differences could

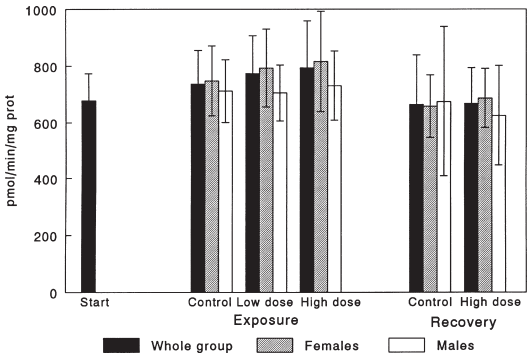

Fig. 3. The activity of UDP-glucuronosyltransferase (UDP-GT) (mean ± S.D.) in the liver of brown trout following eight weeks exposure to treated sulphite pulp mill effluent and after a six-week recovery period.

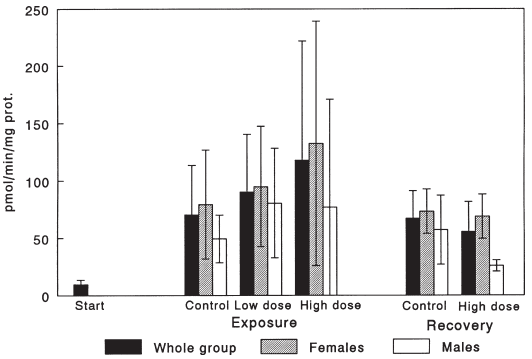

Fig. 4. The activity of 7-ethoxyresorufin 0-deethylase (EROD) (mean ± S.D.) in the liver of brown trout following eight weeks exposure to treated sulphite pulp mill effluent and after a six-week recovery period.

be observed in the morphological parameters between the control group and the exposure groups. According to the condition factor (CF), the exposure did not affect the condition of fish. However, there was a small but not statistically significant difference in growth between the control and the high dose group during the recovery period (Table 2).

From a statistical point of view, no significant deviation from the control values was found in the hematological parameters measured from the effluent-exposed fish (not illustrated). The mean hematocrit value (Hct) for each group was within 30%–33%, and blood hemoglobin concentrations varied between 1.44 and 1.46 mmol l^{-1} in the different experimental groups. The mean leucocrit (Lct) value after the exposure and the recovery periods was the same (0.6%) in all experimental groups.

Enzyme measurements included serum levels of the tissue enzymes ASAT and LDH and the liver enzymes EROD and UDP-GT. No change from the control values was found in the serum enzymes (not illustrated). Neither was the activity of the liver UDP-GT influenced by the exposure to the tested effluent (Fig. 3).

The mean level of enzyme activity of the MFO-associated transformation enzyme EROD seemed to be slightly higher in the effluent-exposed fish as compared to the control fish (Fig. 4) However, due to the great variation within the effluent-exposed groups, these differences between control fish and exposed fish were not statistically significant. After the recovery period the EROD activity of the exposed fish had decreased to the same level as for the control fish. A time dependent increase in the EROD activity was also observed in the control group during the expo-

Table 2. Length, weight, liver somatic index (LSI), condition factor (CF) (mean ± S.D.) and individual mean growth of brown trout after eight weeks exposure to treated sulphite mill effluent and after a six-week recovery period.

	Control	Exposure		Recovery	
		Low dose	High dose	Control	High dose
Length (cm)	24.8 ± 1.4	25.0 ± 1.6	25.1 ± 1.7	25.6 ± 2.0	26.3 ± 1.3
Weight (g)	165 ± 29	167 ± 38	171 ± 39	176 ± 48	191 ± 38
LSI (%)	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.2	1.2 ± 0.1
CF	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Growth (g)	1.0	1.0	1.1	0.6	1.0

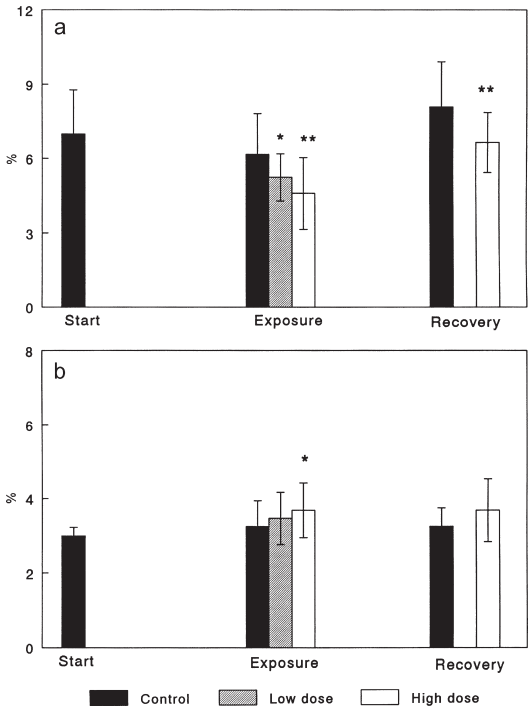

Fig. 5. Liver glycogen and lipid levels (mean ± S.D.) in brown trout after eight weeks exposure to treated sulphite pulp mill effluent and after a six-week recovery period. — a: glycogen, — b: lipids. Statistical significance (*t*-test) relative to control * = $p < 0.05$, ** = $p < 0.01$.

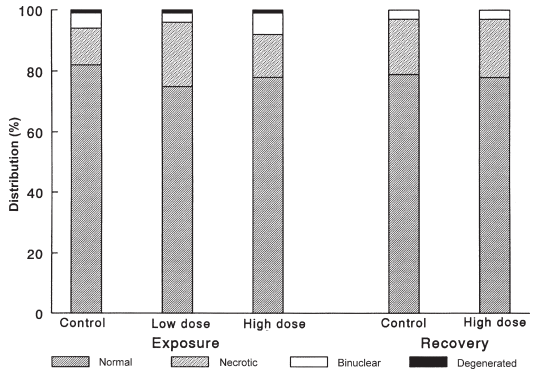

Fig. 6. Quantitative liver histological analysis of brown trout after eight weeks exposure to treated sulphite mill effluent and after a six-week recovery period. The different parts of the bars describe the average percentage of cell structures counted from a total of 400 cells per analyzed fish.

sure period.

Gender-related differences in the EROD activity were seen within the different groups (Fig. 4). The males seemed to have lower EROD activities as compared to the females. During the recovery period the EROD activity of the control female group stayed at a constant level, whereas the activity of the males increased slightly. The EROD activity decreased in both females and males in the group previously exposed to a high dose (1:40) of the tested effluent. After the recovery period the EROD activity of the female fish in control and exposure groups was at the same level, but the males in the exposure group had lower EROD activity as compared to control group males. The activities of the conjugation enzyme UDP-GT did not show the same kind of differences between sexes as those observed for EROD (Fig. 3).

Significantly decreased levels of liver glycogen were observed in both exposure groups (Fig. 5). The glycogen levels were lower in the exposed fish as compared to the control group also after the six-week recovery period (Fig. 5a). Statistically higher liver lipid levels (Fig. 5b), occurred in the high dose group both after eight weeks exposure but after a six-week recovery period the difference was no longer statistically significant. No differences between the sexes, neither of the liver glycogen nor the lipid levels, could be observed in either of the experimental groups.

The histological analyses of liver showed no clear structural differences between the experimental groups (Fig. 6). The percentage of different cell types studied was the same in fish from the control group and the high dose group after exposure and recovery periods. After recovery in clean water no degenerated cells were observed in the livers of the studied fish. Fish in the low dose group had a nine percent higher number of necrotic cells in the liver than the control fish. On the other hand, the precentage of binuclear cells was lower in the low dose group.

Discussion

When assessing the potential environmental hazards of pulp mill effluents, the individual compounds' ability to produce toxic responses in various aquatic species, communities and ecosystems is analysed. The potency of whole mill effluents

or fractions of such effluents to produce biological effects is also assessed, and dose-response relationships should, in principle, be established. This requires a good description of the exposure conditions. In order to assist the industry in their process development, the analysis should preferably result in an identification of the compounds or process steps responsible for inducing the observed effects. In this respect, the knowledge is very limited regarding effluents from mills producing sulphite pulp, probably due to the modest production of sulphite pulp compared to bleached kraft pulp.

In the present study analysis of conjugated chlorophenols and resin acids in fish bile was used to describe the exposure of brown trout to the tested treated sulphite pulp mill effluent. The background levels of fish bile conjugates of chlorophenols and resin acids in fish from uncontaminated waters are about 1–10 and 50 μg g^{-1} dry wt., respectively (Tana *et al.* 1988, Oikari and Holmbom 1986, Landner *et al.* 1994). Similar levels have also been observed in control fish from mesocosm studies with bleached kraft mill effluents (Lehtinen *et al.* 1993, Tana *et al.* 1994). In the present experiment the levels of fish bile conjugates of chlorophenols and resin acids in all test groups after eight week exposure were at the levels that may be considered as natural background levels. The studied mill in the present experiment uses 100% substitution of elementary chlorine with chlorine dioxide in the bleach plant. Previous studies with effluents from kraft mills using the same chlorine dioxide substitution (100%) have shown that bile levels of conjugated chlorophenols were at the control fish level (Lehtinen and Tana 1996, Tana and Lehtinen 1996).

The effluent concentration of resin acids was 40 μg l^{-1}, giving nominal resin acid concentrations of 0.1 μl in the low dose and 1.0 in the high dose pool, respectively. Based on the observed bile concentrations (55 μg g^{-1} d.w.) in the effluent-exposed fish, the calculated concentration factor (log CF) in this study was below 1 (conversion factor from bile dry weight to wet weight approx. 6.7). This is very low compared to concentration factors between 5 and 7 observed in experiments with effluents from a newsprint mill (Johnsen *et al.* 1997). The increase in levels of resin acids and also of chlorophenols in both experimental groups during the six-week recovery period suggests changes in the river water quality. The reasons for this increase are unclear. Plausible explanations might be the increased land runoff from the drainage area (Asplund 1992). No heavy rains were recorded during the recovery period, however.

Brown trout exposed to the treated sulphite pulp mill effluent showed no macroscopic lesions or parasitic infestation. According to the condition factor (CF) and the liver somatic index (LSI), the exposure had no effects on the condition or liver size of the fish. During the recovery period the growth within the control group decreased compared to the previous eight-week exposure period. The decreased growth is a natural consequence of decrease in water temperature during the recovery period. However, in the high dose group, the growth was higher than in the control group during this period. This indicates some delayed exposure based effects on the growth. Changes in growth indicate changes in energy metabolism, and such responses have been seen in earlier studies with bleached kraft pulp mill effluents (Lehtinen *et al.* 1993, Lehtinen and Tana 1996). Previously, responses of this kind have been hypothetically connected with anabolic stimulation (Lehtinen 1990).

The studied hematological parameters (Hct, Hb) showed no differences between the control fish and the exposed fish. The hematological values were all within the natural range observed for salmonids in experimental systems (Monfelt *et al.* 1991). Therefore it can be concluded that the effluent exposure did not affect the oxygen-carrying capacity of fish. The leucocrit (Lct) values were also the same in all the experimental groups. The analysed tissue enzymes ASAT and LDH from the blood plasma showed no deviation from the control values.

The induction of the liver enzyme EROD has been considered a biomarker for pulp mill effluent exposure, as well as for exposure of chlorinated compounds (Södergren 1988). EROD induction has been observed at numerous BKME sites in both feral and caged fish and also in several laboratory experiments (Tana and Lehtinen 1996). EROD induction has also been observed in feral and caged fish in receiving waters of unbleached and bleached sulphite mills (Lindström-Seppä *et*

al. 1992, Monfelt and Grahn 1990), as well as in studies with primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes (Pesonen and Andersson 1992). On the other hand, in an earlier study (Ahokas *et al*. 1976), a reduced *in vitro* MFO capacity has been observed in pike (*Esoc luxius*) exposed to water containing mixed effluents from a mill producing both sulphate and sulphite pulp. In the different studies, induction values have ranged from none up to 30-fold (Tana and Lehtinen 1996).

In the present study, effluent exposure caused an increase in the activity of the liver enzyme EROD, but the activity in the exposed fish was reduced to the control fish level after a recovery period. These results indicate that the treated sulphite mill effluent contains EROD inducing substances. Martel *et al*. (1994) and Schnell *et al*. (1993) demonstrated that an important source of MFO inducers among bleached kraft mill waste streams was weak black liquor, reinforcing the conclusion that chlorine is not an essential ingredient for induction. Hodson (1996) also concluded that chlorine is not an essential component for compounds causing MFO induction. However, labile inducers remain in effluents and they share the properties of PAHs. They are found in aqueous, particulate and dissolved organic carbon phases, they are moderately hydrophobic, and they appear readily metabolised by fish. This opinion is strongly supported by the results in the present study where the effluent is from a mill using 100% substitution of elementary chlorine by chlorine dioxide in the bleaching. At the same time, no elevated levels of chlorophenolis were detected in the bile of the exposed fish. The resin acids in the tested effluent cannot explain the observed EROD induction, even though the levels of conjugated resin acids increased during the recovery period, along with a decrease in the enzyme activity.

The overall increase in the EROD activity including control fish during the exposure period is season dependent (Koivusaari *et al*. 1981, Lindström-Seppä 1990), and the large variation within the groups may depend on the nutritional status of fish in combination with the fact that fish of this kind behave in a hierarchial manner in each pool (Mattson *et al*. 1994). In a test system like the present with a constant feeding rate, the stronger individuals get more food causing differences in the individual nutritional status within the test group. The increase in standard deviation in the exposed fish may also be a consequence of natural variation in the ability of fish to respond (Hodson 1996). Differences between sexes have also been found in other studies (Johnsen *et al*. 1997, Jimenez *et al*. 1990), indicating that the impact of environmental, physiological and toxicological factors on MFO responses must be understood in order to properly interpret the biomarker response.

The activity of the other liver enzyme analysed, UDP-GT, showed no differences between the control fish and the exposed fish. The activity of this enzyme was at the same level as in previous studies, using effluents from kraft pulp or mechanical pulp production as pollutants (Lehtinen *et al*. 1993, Lehtinen and Tana 1996, Johnsen *et al*. 1995). The decreased liver glycogen in the effluent-exposed fish as compared to the control fish indicates a higher use of primary energy for carbohydrate and general metabolism. Changes in the liver clycogen as well as in the liver lipid levels are indications of the ability of the fish to respond to increased energy demands without loosing their regulatory capability. The changes were within the normal range (Monfelt *et al*. 1991) and did not seem to be affecting the liver size of the fish.

The liver histological analysis of the exposed fish showed no significant changes as compared to the control fish. Fish in the low dose group showed a somewhat higher number of necrotic cell (9%) than the other experimental groups. Necrosis was similar in the control and high dose groups, both after exposure and after the recovery period. In previous studies with mechanical pulp mill effluents (Johnsen *et al*. 1995, 1996) and with bleached kraft mill effluents (Lehtinen *et al*. 1993), structural changes in the liver have been much more conspicuous. According to the results from this study it could be anticipated that the tested biologically treated sulphite mill effluent did not contain components of high enough concentrations to cause changes in the liver structure.

Conclusions

The concentrations of conjugated chlorophenols and resin acids in fish bile were low and the physiological responses in the exposed fish were small. The only significant responses observed in the liver function were the induction of the enzyme activity of the MFO-associated EROD and the parallel lower liver glycogen levels. The small differences in physiological parameters between the control group and the high dose group after a 6-week recovery period show that the observed responses were reversible. The spectrum and pattern of results observed in this study are very much the same as seen in the laboratory and field studies with bleached kraft mill effluents from modern mills. In this respect the effects caused by effluents from the production of sulphite pulp cannot be regarded as different from effects of bleached kraft mill effluents. Gender-related differences should be taken into account in the interpretation of biomarker responses.

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