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Sublethal actions of simulated kraft pulp mill effluents (KME) in Salmo gairdneri: residues of toxicants, and effects on blood and liver

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Rainbow trout were exposed for 3, 11 and 30 days to sulphate soap preparation (KME-Sa), the composition of which resembles that of unbleached kraft pulp mill effluents. Test concentrations of KME-Sa corresponded to 0.3, 0.15 and 0.08 times that of 96h LC50, respectively. An additional experiment was conducted with KME-Sa supplemented with four chlorophenols which occur in bleached kraft pulp mill effluents (KME-Sa+CP).

In all experiments high concentrations of resin acids and chlorophenols were detected in the bile. Since, in the 30-day experiments, the concentration of resin acids in the blood plasma was the same as in the bile, the possible concentrating step of free acid across the hepatocytes cannot be very steep. Of the chlorophenols, however, free 2,3,4,6-tetrachlorophenol accumulated to a level 20 times higher in the bile than into the plasma. Residues of resin acids in gill tissues were much lower than those in the brain. Of the different acids, however, dehydroabietic acid seemed to be largely excluded, in comparison with its plasma level, from the central nervous system of trout.

Blood analyses revealed the most severe physiological consequences in trout exposed for 11 days at 0.15 x 96h LC50. Significant decreases in blood haematocrit, plasma protein concentration and condition factor were detected in these fishes whereas their leucocrit value was increased. Of these alterations none was observed in the 3-day experiment to KME-Sa or in the 30-day experiment to KME-Sa+CP, but 30-day exposure to KME-Sa caused a significant decrease in the plasma protein level.

The liver activity of UDP-glucuronyltransferase was significantly reduced in all the experiments by an average of 24 to 40 %. Hyperbilirubinaemia was followed only in the 11-day group, but decreased "direct"/total ratios of plasma bilirubin also indicated impaired glucuronidation of this substance in the 30-day experiments. The relative weight of the liver (LSI) was notably increased in the 11-day experiment, whereas in fish exposed for 30 days to KME-Sa+CP the LSI was decreased.

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1. Introduction

In animal toxicology knowledge of the most sensitive organ system affected by a chemical is often the primary goal of research. In ecotoxicological investigations the responses of ectothermic animals and their organs are almost always modified by external abiotic factors. Therefore, e.g. when industrial waste waters are tested with aquatic animals, a great deal of the variability in the results arises from the quality of the dilution waters used. On the other hand, due to the versatility of an effluent cocktail itself, versatile toxicological effects may also be invoked in test animals. Discovery of the single most probable target organ, e.g. in fish, in such a chaotic situation is usually almost impossible.

The acute lethality of kraft (sulphate) pulp mill effluents (KME) to salmonids is known to vary on both daily as well as an hourly basis (Chandrasekan et al. 1978). Chemical data on KME have shown that most of this variation is due to different concentrations of major toxicants such as the resin acids and chlorinated phenolics (Holmbom & Lehtinen 1980, Rogers 1973). For this reason, the use of authentic KMEs in etiological studies is

unjustified because their chemical composition may vary from one experiment to another. Preservation of a single large sample of KME for long-term exposures may also detoxify it to a variable degree. These facts complicate, of course, any conclusions drawn on sublethal effects and modes of action for an average KME. To avoid these problems the use of a "model effluent", a sulphate soap concentrate composed of the main toxic compounds of KME (termed KME-Sa), was started in a series of experiments. This paper presents the results from acute and subacute exposures of rainbow trout to KME-Sa. Residues of toxic compounds in trout and their effects on the blood and liver, systems strongly affected by the poisons in KME (Walden & Howard 1977, McLeay & Brown 1979, Oikari et al. 1983), were studied first.

2. Material and methods

Rainbow trout (*Salmo gairdneri* Richardson) were obtained from the stock of Laukaa Fish Culture Research Station in central Finland. Before the experiments, conducted in late March-early May, the fish were allowed to acclimatize for at least two weeks in dechlorinated Helsinki City tap water (pH 7.3-7.4) at 11-13 °C. They were fed *ad libitum* once a day with pelleted food (Ewos), and an L:D rhythm of 12:12 h (08.00-20.00 L) was applied using artificial light in the aquarium room. Water 0_2 concentration was kept close to air saturation, its conductivity was 24 mS/m total hardness 80 mg $CaCO_3$/l and total ammonia concentration below 0.5 mg/l (for other parameters cf. Oikari et al. 1983).

Two experiments were conducted:

Experiment I

Three groups of juvenile (1— year old) rainbow trout, weighing 24±5 g and with a length of 13.9±0.9 cm ($\bar{x}\pm SD$), were exposed at 12.5 °C in 200 l glass aquaria to KME-Sa. The measured total resin acid concentration in the test waters corresponded to 0.3 x 96h LC50 for trout exposed for three days and to 0.15 x 96h LC50 for 11 days (2 and 4 analyses, respectively). The third aquarium, containing no poison, served as control. Other conditions in the aquaria were kept the same as before the experiment. Exposures were of the flow-through type (3.5 l/g fish/day; 10-11 fish/aquarium).

The percentage composition (analysed according to Holmbom 1977) of the sulphate soap preparation is given in Table 1. In terms of total resin acids the measured 96h LC50 of this KME-Sa to the same size rainbow trout was 0.53 mg/l (95 % confidence limits 0.43-0.66 mg/l), which corresponds to 1.9 mg dry substance/l. This value for resin acids is about the same as determined for resin acids in real unbleached KME (Leach & Thakore 1977, Holmbom & Lehtinen 1980).

Experiment II

Three groups of rainbow trout (1+year old; 8-10 fish/aquarium), weighing 132±40 g (length 24.4±2.7 cm) and of both sexes, were exposed at 10 °C in 300 l polythene aquaria for 30 days. The first group of fish received KME-Sa (Table 1) with a toxicity corresponding to about 0.08 x 96h LC50. The second group received 20 % less KME-Sa but received an addition of four chlorophenols (2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol = CP-4, and pentachlorophenol = PCP; all of grade "purissimum") in a ratio of 1:6:10:1 by weight (termed KME-Sa+CP). Analysis of the test water, according the Holmbom (1980), showed their weight ratio to be 1:10:1.5:10, i.e. the relative concentration of PCP in particular had increased. The total concentration of chlorophenols remained at around 23 ug/l, and the approximate toxic proportion of KME-Sa+CP was calculated to equal 0.08 x 96h LC50. These experiments were also of the flow-through type (0.6 l/g fish/day), and the controls were treated in the same way but without toxicants. Throughout the exposures, like the group exposed for 11 d in Expt. I, the trout were fed about 0.5 % of the fish weight per day. The uneaten food was siphoned from the aquaria within one hour of feeding.

Sampling

Feeding was stopped four days before sampling, and one day before this each fish was allowed to swim into a restrainer (Oikari et al. 1983). The fish and the restrainer were removed from the aquarium, and fish immobilized by stunning with a blow on the head. Total weight was quickly recorded to the nearest 0.1 g, and a blood sample aspirated into a heparinized syringe by puncturing the tail vessels (Expt. I) or the heart (Expt. II). The blood was mixed thoroughly and the haematocrit (Hct) and leucocrit (Lct) determinations were performed immediately (McLeay & Gordon 1977, Oikari et al. 1983). The rest of the blood was centrifuged for 1 min with a Beckman Microfuge B and the plasma was separated for later analysis. The total length was then measured (to the nearest mm), the body cavity opened, and the bile collected by puncturing the gall bladder. Both the plasma and the bile were stored (3-4 h) in the dark at 4 °C until analysed for bilirubin.

The liver was detached, weighed to the nearest mg, divided, and subsamples frozen and stored in liquid nitrogen (Eppendorf tubes vol. 1.5 ml). The same part of the liver was always chosen for a given analysis. Primary lamellae from three gill arches on both sides were removed and stored in liquid nirogen. Finally the skull was opened with a knife and the whole brain removed for analysis. All these steps were conducted at 5-7 °C and it took about 10 min to prepare each fish.

Analytical techniques

Residues of free resin acids and chlorophenols in the fish tissues and body fluids were analysed according to the lines of our previous papers (Holmbom 1980, Oikari et al. 1980, Oikari & Holmbom 1981). Frozen samples were first freeze-dried to a constant weight, ground in a mortar with sodium sulphate acidified with a drop of 10 % sulphuric acid, and transferred to extraction thimbles (10 x 50 mm): internal standard was added and the mixture extracted with n-hexane in a small-scale Soxhlet apparatus. The extraction time was 5 h, corresponding to 150 extraction cycles. The internal standard solution used contained 2,6-dimethylphenol, 2,6-dibromophenol, heptadecanoic acid (17:0), tetracosane, and tricosanoic (23:0) acid in the proportions 4:1:4:1:2, respectively. The amount of standards added varied from 5 to 20 µg/g sample dry weight. One portion of extract was methylated with diazomethane and analysed isothermally at 187 °:c with a Varian 2100 GC equipped with a 32 m/0.3 mm i.d. glass capillary column coated with BDS (Fl detector). Concentrations of resin acids were calculated against the internal standards of 17:0 acid or 23:0 acid. Another portion was silylated with BSTFA (20 µl, 1 h, 60 °C) and chlorophenols analysed with a GC equipped with a ^{63}Ni-EC detector. The column used was a 30 m/0.3 mm i.d. glass column coated with SE-30 and operated at a column temperature of 80-270 °C, 4 °C/min. Peak areas were integrated with a Perkin-Elmer Sigma 10 data system. Corrections for various chlorophenols with respect to the internal standard (dibromophenol) were achieved from parallel runs of a known chlorophenol mixture. Finally, all concentrations were calculated back on the wet weight basis.

The concentration of bilirubin in plasma and bile was determined by the sulphanilic acid reaction using a commercial test kit (Boehringer Mannheim CmbH No. 123 919). The "direct" (presumably glucuronic acid conjugated) bilirubin was measured by deleting caffeine from the assay. The activity of UDP-glucuronyltransferase (UDP-GT, EC 2.4.1.17) was assayed from the homogenate at 25 °C using p-nitrophenol as substrate (Hänninen 1966, Oikari et al. 1983). The plasma protein concentration was determined according to Lowry et al. (1951), using bovine serum albumin as standard. The liver somatic index (LSI) is expressed as the weight of the liver as a percentage of the whole fish weight, and the condition factor (CF) of the trout was calculated from the fish weight and length:

$$CF = 100 \times W(g)/L(cm)^3.$$

3. Results

3.1. Residues of toxicants in bile and plasma

Only the bile samples of the fish of Expt. I were analysed for their concentrations of "free", hexane extractable, resin acids (Table 2). Somewhat more of the total resin acids had accumulated in the bile of the trout exposed for 11 days at 0.15 x 96h LC50 than in those kept for three days at 0.3 x 96h LC50 of KME-Sa. The abietic acid, which was also the dominant acid in the soap preparations tested (Table 1), displayed the highest concentrations in the bile. A certain picture of the concentrations of resin acids relative to the water surrounding the fish can be obtained, even though the water concentrations during the experiments were not monitored very frequently. Therefore, in this study, we have opted to discuss the "apparent bioconcentration factor" (BCF = conc. in fish/conc. in water) instead of "true BCF". The calculation of apparent BCF for total free resin acids in the bile of the Expt. I trout revealed a value of 1400 for the higher (0.3 x 96h LC50) test concentration of KME-Sa and 2800 for the lower (0.15 x 96h LC50). Actually these are clearly higher values of BCF than reported previously for pure acids or their mixtures (Oikari et al. 1983). They also indicated that a steady-state was not reached during the three days of Expt. I, but they seem to show that all individual resin acids were concentrated as such into the trout bile.

On the other hand, data in Table 3 show that after both 30-day exposures (Expt. II) the bile contained much less resin acids than in the more acute Expt. I (Table 2). Although the size/age of fish can have an influence, the difference in results between Expts. I and II may be due to the development of more efficent biliary excretion of resin acids when more time is allowed to elapse after the application of poisons. The apparent BCFs of 800–1000, which were clearly lower than in the 11-day experiment, support this possibility. A comparison of blood plasma and bile values may hint at the existence of an "uphill" transfer, in this case for free resin acids, across the liver glandular tissue. The present data (Table 3) show that values not much higher than 1.0 were found in the bile/plasma (B/P) ratio. The highest B/P

Table 1. Percentage composition of the sulphate soap preparations (KME-Sa, A. Ahlström Co., Finland) used in Experiments I (3 and 11 days) and II (30 days).

Compound/parameter	Expt. I	Expt.II
Pimaral	0.37	0.47
Secodehydroabietic	0.33	0.35
Pimaric	2.61	2.55
Sandaracopimaric	0.48	0.50
Levopimaric	2.50	1.52
Palustric	3.91	3.82
Isopimaric	1.62	1.67
Abietic	6.85	7.62
Dehydroabietic	3.41	3.53
Neoabietic	3.32	3.47
Total resin acids	25.0 %	25.0 %
Palmitic (16:0)	1.37	0.90
Stearic (18:0)	0.43	0.25
Oleic (9-18:1)	13.66	11.50
Linoleic (9, 12-18:2)	22.69	16.10
Pinolenic (5, 9, 12-18:3)	6.25	4.45
Conjugated linoleic	2.24	2.28
Conjugated pinolenic	1.28	1.53
Eicosatrienoic (20:3)	2.37	2.03
Behenic (22:0)	0.50	0.44
Other fatty acids	0.75	0.65
Total fatty acids	51.5 %	40.1 %
Lignin material Sodium Oxyacids Neutral compounds	23.1 %	34.4 %

Table 2. Concentrations of resin acids (μg/ml, mean of two pooled samples) in the bile of rainbow trout exposed for 3 and 11 days to a sulphate soap preparation (KME-Sa, Expt. I in Table 1), simulating unbleached effluents from kraft pulp mills. Approximate toxicity fractions were 0.3 and 0.15 x 96h LC50, respectively. No resin acids were detected in control fish. n = number of fishes.

	3 days $n = 7$	11 days $n = 10$
Pimaric	30	32
Sandaracopimaric	5.5	3.5
Levopimaric + palustric	11	21
Isopimaric	21	24
Abietic	78	91
Dehydroabietic	24	29
Neoabietic	4.5	6.5
Total	174	206

Table 3. Concentrations of resin acids (μg/ml or μg/g wet weight, mean ± *SD* of 3 pooled samples) in fluid and tissue samples collected from rainbow trout exposed for 30 days to KME-Sa and KME-Sa+CP (Expt. II). In both cases the approximate toxicity fraction was 0.08 x 96h LC50. + = detected but less than 0.1 μg/ml or μg/g wet weight, ND = not detected. *n* = number of fishes. For further details, see "Materials" and Tables 1 and 2.

	KME-Sa				KME-Sa+CP			
	Plasma	Bile	Gill	Brain	Plasma	Bile	Gill[1]	Brain
n	10	11	10	9	11	11	7	10
Pimaric	9.7± 0.3	7.1± 7.0	3.4±0.8	18.2± 3.3	13.4± 2.1	9.6± 6.2	+	11.8± 7.3
Sandaracopimaric	3.2± 1.9	1.9± 1.8	+	1.4± 1.0	2.6± 0.6	2.4± 1.8	+	+
Levopimaric + palustric	1.8± 1.2	2.0± 1.0	+	+	5.1± 1.2	7.6± 4.1	+	+
Isopimaric	3.6± 1.4	5.6± 4.9	1.5±1.4	4.0± 1.6	4.7± 1.7	3.5± 1.7	+	+
Abietic	8.3± 3.7	9.6±10.4	+	22.6±15.4	12.4± 5.6	11.8± 6.1	+	9.8± 5.5
Dehydroabietic	5.6± 4.5	5.0± 3.1	5.2±4.6	ND	10.2± 4.7	5.4± 2.6	+	+
Neoabietic	2.5± 2.1	+	+	ND	1.7± 0.7	1.1± 0.9	+	+
Total	33.0±16.0	27.6±27.0	8.9±5.6	46.1±15.2	50.4± 8.5	41.3±22.8		22.1±12.9

[1]) Two pooled samples.

ratio was 1.56 for isopimaric acid in the 30-day KME-Sa experiment, but most B/P ratios were close to or below 1.0. Considering an average for all resin acids, a value of 0.83 was found in both 30-day experiments. It therefore seems plausible that in subacute exposures free resin acids are not at all or only weakly concentrated across the hepatocellular step.

Analyses of trout exposed to different chlorophenols in Expt. II (KME-Sa+CP; Table 4) indicate that some of these, especially 2,3,4,6-tetrachlorophenol (B/P ratio = 20), may be secreted as such from plasma to bile. On the other hand, the B/P values of CP-2 and CP-3 are close to unity and they may be passively distributed between the blood and the excretory bile. The apparent BCF in the plasma varied from 250 (PCP) to 750 (CP-4), whereas in the bile it ranged from 400 (CP-3) to 15 000 (CP-4). The values for CP-4, however, are very approximate because the water concentration approached the lowest level at which analysis can be considered reliable (CP-4 in water varied from 1 to 2 ug/l). With respect to CP-4 we must also remember that particularly its proportion in the test water was changed most (cf. Materials), it possibly being absorbed from water more rapidly than other chlorophenols (the chlorination number of chlorophenols may also change, of course, in the water body itself).

Table 4. Concentrations of chlorophenols (μg/ml or μg/g wet weight, mean ± *SD* of three or (gill) two pooled samples) in rainbow trout after 30-day exposure to a sulphate soup preparation supplemented with four chlorophenols (KME-Sa+CP). The approximate toxicity fraction was 0.08 x 96h LC50. + = detected but less than 0.05 μg/g wet weight. *n* = number of fishes. For details, see "Materials" and Tables 1-3.

	Plasma	Bile	Gill	Brain
n	11	11	7	10
CP-2	1.3±1.2	1.8±1.2	3.2± 2.2	+
CP-3	4.0±3.0	4.1±0.9	1.6± 0.0	+
CP-4	1.2±0.3	24.0±6.1	5.6± 5.5	+
CP-5	2.6±2.2	18.8±6.1	4.8± 4.4	+
Total	8.8±6.2	48.7±13.0	14.3±13.5	

3.2. Residues of toxicants in gills and brain (Expt. II)

The concentrations of resin acids in trout gills in both experiments were markedly lower than in the blood plasma (Table 3). Tissue concentrations were much higher in the brain than in the gills, indicating easy entrance of free resin acis into the central nervous system (CNS). There were certain differences, however, as the abietic and pimaric acids were concentrated most into the CNS, whereas dehydroabietic acid was largely excluded. In Expt. II the apparent BCF values of abietic and pimaric acids into the brain were around 5200 and 9400, respectively.

Chlorophenols largely seem to behave differently from resin acids (Table 4), especially as only negligible amounts of chlorophenols were traced in the CNS whereas their concentrations in the gills generally exceeded the levels in the blood plasma. Analyses of chlorophenols in gills and water revealed an apparent maximum BCF of around 3400 for CP-4.

3.3. Sublethal effects on blood and condition of fish

The results in Table 5 show dramatically that within 11 days the trout were seriously affected at

Table 5. Effects (mean ± *SD*) of 3-day and 11-day exposures (Expt I) of trout to a sulphate soap preparation (KME-Sa) simulating unbleached effluents from kraft pulpmills. Approximate toxicity fraction 0.3 and 0.15 x 96h LC50, respectively. Number of fishes in parenthesis. Exposed groups are compared (Student's t test) to the controls. For details, see Tables 1 and 3.

Parameter	Control	3 days	11 days
Haematocrit %	34.4±3.6 (10)	33.3±2.3 (8)	24.7±3.2*** (8)
Leucocrit %	0.59±0.10 (10)	0.58±0.12 (8)	0.94±0.24*** (8)
Hct/Lct-ratio	58±13 (10)	60±16 (8)	30±13*** (8)
Plasma proteins g/l	37.8±2.7 (7)	38.2±4.8 (10)	13.5±3.0*** (3)
CF index	0.93±0.04 (11)	0.91±0.04 (10)	0.84±0.04*** (10)

Table 6. Effects (mean ± *SD*) of 30-day exposures (Expt. II) of trout to a sulphate soap preparation, as such (KME-Sa) and after supplementation with four chlorophenols (KME-Sa+CP). Approximate toxicity fraction 0.08 x 96h LC50. Number of fishes in parenthesis. For details, see Tables 1,3 and 5.

	Control	KME-Sa	KME-Sa+CP
Haematocrit %	26.5±3.8 (7)	24.6±5.2 (10)	30.3±4.2 (8)
Leucocrit %	0.63±0.21 (7)	0.76±0.30 (10)	0.66±0.27 (8)
Hct/Lct-ratio	46±16 (7)	36±12 (10)	55±26 (8)
Plasma proteins g/l	30.7±2.4 (6)	25.7±3.0** (9)	30.8±2.7 (9)
CF index	0.89±0.11 (8)	0.85±0.12 (10)	0.88±0.08 (9)

Table 7. Effects of 3-day and 11-day exposures (Expt. I) of trout to a sulphate soap preparation (KME-Sa) simulating unbleached effluents from kraft pulp mills. For further details see legend to Table 5.

	Control	3 days	11 days
LSI %	0.88±0.06 (11)	0.92±0.07 (10)	1.24±0.21*** (8)
Liver UDP-GT mU/g prot	223±18 (9)	160±19*** (10)	134±13*** (8)
Plasma bilirubin Total mg/l	2.1±0.5 (11)	1.6±0.4 (7)	5.0±0.7*** (5)
Bile bilirubin Total mg/l	298±129 (4)	202±20 (3)	215±23 (3)

Table 8. Effects of 30-day exposures (Expt. II) of trout to a sulphate soap preparation as such (KME-Sa) and after supplementation with four chlorophenols (KME-Sa+CP). For further details see legend to Table 6.

Parameter	Control fish	KME-Sa	KME-Sa+CP
LSI %	1.03±0.13 (8)	0.95±0.13 (10)	0.87±0.12* (9)
Liver UDP-GT mU/g prot	263±34	199±39** (10)	168±26*** (8)
Plasma bilirubin Total mg/l	2.8±0.4 (8)	2.3±0.8 (10)	3.0±0.8 (9)
Direct/total	0.69±0.26 (5)	0.31±0.14*** (9)	0.22±0.14*** (7)
Bile bilirubin Total mg/l	417±243 (4)	400±174 (6)	416±177 (8)

the test concentration corresponding to 0.15 x 96h LC50. On the other hand, after three days at a concentration double this no statistically significant differences were observed. In the 11-day experiment Hct was reduced by about a quarter and Lct increased by almost 60 %. As a result the proportion of leucocytes to erythrocytes was doubled ($P<0.001$). These parameters were not, however, changed in either of the 30-day exposures (Table 6). Plasma total protein concentration was markedly decreased in the 11-day and 30-day exposures to KME-Sa (Tables 5 and 6) whereas the addition of four chlorophenols to KME-Sa abolished this response.

Measurement of the condition factor (CF) of the trout exposed to KME-Sa for 11 days revealed that the fish had become much thinner, possibly through consumption of body energy stores. In the other groups the CF did not change significantly (Tables 5 and 6).

3.4. Sublethal effects on liver

Despite the fact that the CF was reduced the relative weight of the liver (LSI) was increased by 40 % in the trout exposed for 11 days to KME-Sa (Table 7). This increase was largely due to hydration of liver tissue (Oikari & Nakari 1982). On the other hand, in the trout exposed to KME-Sa+CP for 30 days, LSI was reduced ($P<0.05$; Table 8), possibly indicating that no osmoregularoty problems had occurred in the liver but the chlorophenols may otherwize affect the metabolism.

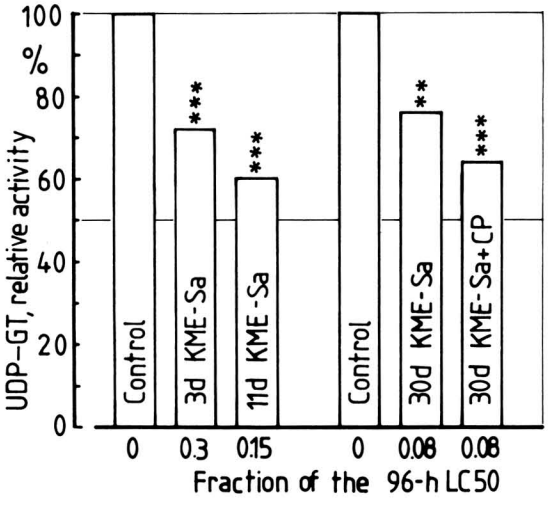

Fig. 1. Inhibition of liver UDP-glucuronyltransferase activity in rainbow trout exposed to sulphate soap preparations as such (KME-Sa), and after supplementation with four chlorophenols (KME-Sa+CP). The concentrations tested are related, estimated on the basis of water resin acid contents, to the fraction of the 96h LC50. Statistical differences between the means, as compared to the appropriate controls, are indicated by asterisks (*** $P<0.001$, ** $P<0.01$).

Inhibition of liver UDP-glucuronyltransferase was significant in all test groups (Tables 7 and 8), varying from an average of 24 to 40 % (Fig. 1). In the trout exposed to KME-Sa for 11 days, the plasma total bilirubin concentration was more than doubled, resulting in easily visible jaundice. On the other hand, in trout exposed for 30 days (Expt. II), no hyperbilirubinaemia was detected in the blood plasma, but the proportion of conjugated "direct" bilirubin was significantly ($P<0.01$) reduced (Table 8). We also measured the total bilirubin concentration in bile, but no statistically significant differences were detected (Tables 7 and 8).

4. Discussion

In this paper an attempt has been made to simulate an "average" Finnish kraft pulp mill effluent (KME), in particular the unbleached one, and its effects are compared to those obtained with true KMEs. An additional comparison with the effects of individual toxic compounds of KME, like those caused by resin acids and chlorophenols, has been made. The present results are also representative of the effects of occasional spills of black liquor and black liquor soap into the aquatic environment. In pulp mill this kind of spill may originate from the cooking and screening or evaporation departments.

4.1. Residues of toxicants

It has been stated that in acute exposures of rainbow trout to resin acids the highest tissue concentrations will be accumulated in liver (Oikari et al. 1982). This points to the importance of this organ in the excretion of resin acids. Fish liver normally contains high quantities of fatty acids which cause major problems in analysis of resin acids, especially if their concentration in liver tissue is low. Therefore in the present experiments no liver resin acids were determined but their plasma-bile relations were studied. In plasma and bile, as in gill brain tissues, the bulk of the fatty acids did not cause analytical problems with our technique.

With respect to the concentrations of free (hexane extractable) resin acids in fish plasma and bile the present results agree well with those reported before for rainbow trout exposed for different periods to KME (Oikari & Holmbom 1981). The apparent BCF values calculated are also close to those measured from trout exposed for 20 days to 2.5 % (vol/vol) KME. The bile/plasma (B/P) ratios in trout exposed to low concentrations of KME-Sa (Expt. II, Table 3), however, did not differ much from unity, whereas ratios from 4 to 10 have been revealed in subacute experiments with KME (Oikari & Holmbom 1981). The apparent BCF values calculated are free resin acids thus warrants further study.

Various chlorophenols, like PCP, absorbed into the fish will become conjugated to glucuronides and sulphates, e.g. in the liver. Only a small proportion, about 10 %, is excreted as free PCP (Kobayashi 1977). The glucuronide conjugate of PCP, in particular, was excreted through the biliary route. Of the other chlorophenols, to our knowledge, it is not known specifically how they are conjugated and excreted, but it is thought that they are treated much like PCP (Kobayashi 1977). Whatever the size of the free part of the total, our data (Table 4) suggest that the free CP-4 and PCP can become concentrated from the plasma into the bile. The gall bladder bile, however, is not fully representative of the canalicular excretion but, e.g. water reabsorption and hydrolysis of conjugates back to free aglycones are factors possibly weakening the above conclusion. In any case, a B/P ratio of 5 or more would be enough for the postulation of active excretion of free chlorophenols. Despite this, with respect to overall excretion, the conjugated proportion is clearly more essential (Kobayashi 1977; Holmbom, Oikari & Ånäs, unpubl.).

Fish gills are generally considered to be the primary route of absorption of aquatic toxicants

(Esser & Moser 1982). The existence of toxicants in gills, however, does not only tell us that the substances are being absorbed or excreted, but that they also represent the toxicant concentrations in gill tissue, affecting its various physiological functions. Examples of such functions are active uptake of electrolytes (Na^+, Cl^-) and volume regulation of respiratory cells in the gills. In the rainbow trout exposed to KME-Sa and KME-Sa+CP for 30 days, the concentration of resin acids in the primary lamellae of gills was only a quarter or less when compared to the corresponding plasma levels (Table 3). The ratio is about the same as that noted in an acute four-day exposure of trout to dehydroabietic acid (Oikari et al. 1982). The gill/plasma ratios of chlorophenols were not the same, with the exception of CP-3, as those of resin acids (Table 4). If we had measured the total instead of the free chlorophenols, different gill/plasma ratios would probably have been found (Kobayashi 1977).

When juvenile sockeye salmon *(Oncorhynchus nerka)* were acutely exposed to dehydroabietic acid (0.65 mg/l for five days) very high levels of this toxicant were detected in the brain tissue (Kruzynski 1979). The two-day exposure of rainbow trout to a wood rosin containing eight resin acids (1.4 mg/l) resulted in a total concentration of 82 μg/g brain tissue, which was double that in gill tissue (Oikari et al. 1982). The two 30-day experiments (Table 3) show that significant amounts, about 1/4–1/2 of the above level, of resin acids can also occur in the CNS during long-term exposures to KME. Pimaric and abietic acid, in particular, are heavily accumulated into the brain. On the other hand, chlorophenols were not detected in such high amounts in the CNS (Table 4).

At present there are only a few relevent studies that can be used for assessing the biological importance of the high toxicant levels analysed from the CNS. In one attempt, the rotary-flow technique developed by Lindahl (1978) was applied (Lehtinen & Oikari 1980). In perch *(Perca fluviatilis)* exposed to 2-4 % bleached kraft pulp mill effluent for 14 days the capacity to resist increasing torque of water current was significantly decreased. Impaired scores in this test can be related to intoxication of the CNS (Lindahl 1978). We have also measured the activities of brain acetycholinesterase from rainbow trout caged downstream from a Finnish pulp mill (theoretical effluent dilution about 5 % vol/vol), but no differences were observed (Oikari & Castren, unpubl.). Therefore it is clear that bleached KMEs do not exert their effects via mechanisms similar to those of carbamate and organophosphorus pesticides (Coppage & Braidech 1976, Rosic & Lomax 1974).

In cellular systems, generally only the free part of a substance is physiologically effective. Therefore we may assume that the free unconjugated resin acids and chlorophenols, as measured in the present work, are responsible for the sublethal effects evoked. The two toxicological aspects, levels of poisons and their physiological effects, have been combined in this work. Knowledge of this relationship can help us when assessing the biological consequences to fishes when only chemical data on toxicant residues are available.

4.2. Sublethal effects

In some ways the haematological responses caused by KME-Sa differ from those of pure resin acids. Haemoconcentration followed the acute exposure of sockeye salmon and rainbow trout to high sublethal concentrations of dehydroabietic acid (Kruzynski 1979, Oikari et al. 1983). Exposure to KME-Sa caused haemodilution (Table 6) in 11 days at 0.15 x 96h LC50. In coho salmon *(O. kisutch)* receiving KME at 0.33 x 96h LC50 for 25 days the haematocrit value was also decreased (McLeay 1973). In all, this effect can be considered disadvantageous to the fish as the oxygen carrying capacity of its blood may be reduced. We must remember, however, that several other factors such as intraerythrocytic pH and concentration of ATP can affect, by impairment or improvement, the oxygen loading in gills (Nikinmaa & Oikari 1982). Very little is known about these respiratory adjustments, especially with regard to long-term exposures.

The strong positive correlation between leucocrit (Lct) values and total leucocyte blood counts (McLeay & Gordon 1977) justifies the use of this "by-product of haematocrit" as a simple measure of leucopaenic or leucocytic reactions, e.g. in rainbow trout. Marked acute reductions in leucocyte counts or leucocrit values due to stress, inclubing exposure to sublethal concentrations of bleached KME, have been described (McLeay 1973, McLeay & Gordon 1977). On the other hand, there are indications that in subacute or chronic intoxications the leucocyte counts, especially those of neutrophils, will increase, e.g. in the coho salmon (McLeay & Brown 1974, McLay 1973). In our 11-day experiment Lct was significantly increased. The reaction may be indicative of the necessity of a strong immunological defence for the fish to survive (Corbel 1975) The leucocytic response possibly demands more time than is generally considered "acute", but below a certain level of KME-Sa no such response

will develop (Table 6).

Changes in the condition factor (CF) of fish may arise for several reasons. Its decrease (thinning of fish) may result from increased consumption of body energy reserves or may be due to other energetic reasons or nutritional deficiencies. On the other hand, if the CF increases, osmoregulatory problems in fresh water or accelerated anabolic growth or even physical inactivity may be involved. Changes in the relative weight of individual organs, e.g. the liver (LSI), may follow for similar reasons. In the present study, however, the trout exposed for 11 days at 0.15 x 96h LC50 displayed different responses in CF and LSI (Tables 5 and 7). Further analyses showed that osmoregulatory problems in the liver can precede those in the rest of the body (Oikari & Nakari 1982).

It now seems evident that serious disorders in trout liver metabolism will follow acute exposures to resin acids and KMEs (Oikari & Nakari 1982, Nikinmaa & Oikari 1982, Oikari et al. 1983). Besides the fact that liver UDP-glucuronyltransferase was inhibited in all test groups, it is worth noting that the activity was markedly decreased even after 30 days' exposure to relatively low concentrations of simulated KMEs (Fig. 1). As glucuronidations are involved in the regulation of many physiologically active substances (e.g. steroid hormones; Levine 1978), interference with their functions would be expected. Impairment of the metabolism and elimination of bilirubin (Tables 7 and 8), led acutely to jaundice. In a more subtle form, changed ratios of the free and conjugated ("direct") bilirubin were seen in the blood plasma. This was observable whether or not chlorophenols were added to KME-Sa (Table 8). In contrast to our 30-day exposures to KME-Sa, acute five-day exposure to dehydroabietic acid revealed that more conjugatd "direct" than "free" bilirubin had accumulated in the blood (Kruzynski 1979). The differences may be due to the impairment of the transcanalicular transport of bilirubin-glucuronide (Levine 1978), which may occur only in severe acute intoxication leading to jaundice. Lack of sufficient amounts of plasma prevented the measurement of this ratio from our short-term material (Table 7), but data from the bile support this hypothesis (Oikari & Nakari 1982).

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