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Chlorinated Phenolics and Their Conjugates in the Bile of Trout (*Salmo gairdneri*) Exposed to Contaminated Waters

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With spent liquors from the bleaching of kraft pulp a wide variety of chlorinated organic substances are discharged into the environment (Holmbom 1980; Voss et al. 1980). Of these compounds chlorinated phenolics are the most toxic to fishes and other aquatic organisms. Interestingly, an increase in the number of chlorine atoms in phenolic molecules from one to five results in a hundredfold rise in the acute lethal toxicity to fishes (Voss et al. 1980). Generally speaking, at a given water pH, the differences in toxicity of substituted phenolics seem to be mostly accountable for their different lipophilities (Saarikoski and Viluksela 1982).

With the exception of pentachlorophenol (PCP) there is very little information on the metabolic fate of various chlorophenols and other related phenolics, characteristic of bleached kraft pulp mill effluents (BKME), in the fish body. Biliary excretion after glucuronide conjugation seems to be a general detoxication mechanism for PCP in fish (Kobayashi 1979). In the rainbow trout no other PCP metabolite other than glucuronide has been detected in the bile (Glickman et al. 1977). In the liver of goldfish, on the other hand, both pentachlorophenyl -β- glucuronide and pentachlorophenylsulphate are synthetized (Kobayashi 1977). Also in this species, however, only the glucuronide is secreted in the bile the sulphate being eliminated branchially and renally (Kobayashi 1979). Sulphate conjugation has been demonstrated in the soluble fraction of goldfish liver *in vitro* also for some other phenols (e.g. 2,3,4,6-tetrachlorophenol, 2,4,6- and 2,4,5-trichlorophenols, and 2,4-dichlorophenol; Kobayashi 1977). In this study we investigated whether glucuronidation and sulphatation products are detectable in the bile of rainbow trout exposed to several chlorinated phenolics in concert or to actual BKME.

The physiological rationale of glucuronide and sulphate conjugation is to provide the hepatobiliary anion carrier with a suitable form of the xenobiotic molecule (Levine 1978; Chambers and Yarbrough 1976). The concentration of xenobiotics as their conjugates in the

gallbladder bile is therefore greatly amplified in respect to their concentration in the blood plasma or in the surrounding water (Statham et al. 1976). This phenomenon can be of great utility as a sensor of biologically detectable contamination of the aquatic environment by BKME pollutants.

MATERIALS AND METHODS

Hatchery reared rainbow trout (Salmo gairdneri Richardson; Savon Taimen Inc.) were transported to the laboratory aquaria and allowed to recover there for at least 4 days before the start of experiments. During acclimatization to their new surroundings trout were fed daily ad libitum with pelleted fodder. The water O_2 concentration was kept at 8-9 mg/L, and a seasonal photoperiod was maintained using partially covered windows. There were three experimental groups (Tables 1-3), the principal difference between them being the quality of their background water. In all the experiments a flow-through system was arranged in the polythene aquarium (300 or 500 L) to deliver a test water mixture to the fish.

Nine juvenile trout (110-145 g; Table 1) were exposed for 30 days to 2% (v/v) solution of biologically treated BKME. Grab samples of BKME were collected 2 or 3 times per week, the sampling period covering 3-6 hours. The total concentrations of chlorinated phenolics in the undiluted BKME varied between 90 and 120 ug/L (3 analyses), the corresponding level analysed from the test aquarium being between 2.95 and 4.56 ug/L (four 3-day composite samples analysed according to Holmbom 1980). The actual dilution of BKME was followed using water sodium analyses. During the exposure (loading density about 1.2 g/L per day), conducted in September-October, the water temperature was gradually lowered from 15-16 ^{o}C to 11-12 ^{o}C. Water pH was 6.7 and its hardness 20 mg $CaCO_3$/L. The diluent water used was taken from a location upstream relative to the mill (southern Lake Saimaa, SE Finland). Apart from the last 4 days before sampling, the trout were fed a ration of 0.5% fish biomass twice a day. Samples from 3 fish were pooled for one analysis. In the second experiment, ten trout (120-145 g; Table 2) were similarly exposed to BKME, but only for 10 days at a concentration of 0.6% (v/v) BKME. However, the water temperature remained at 14-16 ^{o}C. Bile samples from all the fish were pooled for one (triplicate) analysis.

In the third experiment 4 trout (250-450 g, all $\varphi\varphi$; Tables 3 and 4) were exposed for 6 days to a 7:10:4 mixture (w/v) of 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and PCP, respectively. The average total concentration was 150 ug/L. The animals studied were acclimatized in the dechlorinated tap water, used as the diluent in this experiment, for 3 weeks. The water temperature was 11-13 ^{o}C, its pH 7.3-7.4 and its hardness about 80 mg $CaCO_3$/L. The fish were not fed during the exposure period.

At the end of the exposure, the fish were stunned by a blow to the head, and a blood sample collected from the caudal vessels with a heparinized syringe. The blood was centrifuged immediately. Bile was then aspirated from the gall bladder with a hypodermic needle. The samples were frozen in liquid nitrogen and stored at -20 ^{o}C until analysed.

For determination of the chlorinated phenolics in bile (volume 0.5 -1.5 mL) and plasma (vol. 1.5-2.5 mL) samples, the techniques outlined in our previous paper was followed (Oikari et al. 1984a). Samples were acidified to a pH of 3.5-4 with diluted H_2SO_4 and the internal standard (2,6-dibromophenol, p.a.; 2-10 ug/mL) was added. Extraction was performed with three 3 mL portions of n-hexane: acetone (3:1 v/v, both redistilled) yielding a fraction of the "free" substance. The extract was evaporated to dryness in a flow of N_2 and silylated by 25 uL BSTFA (=bis(trimethylsilyl)-trifluoroacetamide, Merck; 1 h, 60 ^{o}C). The silylated sample was then analysed on a Varian 6000 GC equipped with 30m/0.3 mm i.d. capillary column coated with SE-30. The oven temperature was programmed at 80 to 270 ^{o}C (4 ^{o}C/min), and the signal from the ^{63}Ni-EC detector was automatically integrated. The integrated peak areas were further calculated into sample concentrations, by using appropriate correction factors for the different chlorophenols relative to the internal standard.

For determination of the total conjugated phenolics, after the initial extraction, the sample was further treated with an alkaline solution (0.5 M KOH in 90% ethanol at 70 ^{o}C). Before hydrolysis the internal standard (100-500 ug/mL) was again added. Preliminary experiments showed that the 3 h hydrolysis time we used as the standard gave quantitative recoveries. Finally, the sample was acidified to a pH of 3.5 and the chlorinated phenolics extracted and analysed as previously described.

The amounts of glucuronide acid conjugates of different phenolics was analysed from another aliquot (0.2-0.6 mL diluted to 1 ml with d.w.) of the first extraction residue. Besides the sample the incubation cocktail included 2 mL acetate buffer (0.3 M, pH 3.8) and 1 mL β-glucuronidase solution (3000 units/mL; Sigma type L-II). Similarly, for determination of sulphate conjugates, a third portion of the first extraction residue was incubated in the presence of sulphatase (8 units/mL; Sigma type VIII) in acetate buffer (pH 5) for 24 h at 37 ^{o}C. Controls without the addition of enzymes were included in each case. The liberated phenolics were extracted, derivatized and measured as described above.

RESULTS AND DISCUSSION

In this study we investigated phase II metabolites (conjugates) in the bile of trout by hydrolysing them with a strong base and with specific enzymes. It is important to remember, however, that certain chlorinated phenolics characteristic to BKME may become bio-

Table 1. Concentrations (ug/mL) of free and conjugated chlorinated phenolics in the bile of rainbow trout exposed for 30 days to 2 % v/v solution of biologically treated kraft pulp mill effluent.

Phenolic compound	Concentration in bile Free	Conjug.	Percentage conjugated
2,4,6-trichlorophenol	0.07 ± 0.03 #	40.8 ± 12.6	99.8
2,3,4,6-tetrachlorophenol	0.15 ± 0.03	19.4 ± 6.2	99.2
3,4,5-trichloroguaiacol	ND	49.8 ± 4.8	100
4,5,6-trichloroguaiacol	ND	5.6 ± 0.5	100
3,4,5-trichlorosyringol	0.11 ± 0.03 #	24.1 ± 7.8	99.5
3,4,5,6-tetrachloroguaiacol	0.41 ± 0.15	63.9 ± 10.1	99.3
Pentachlorophenol	0.32 ± 0.12	7.8 ± 0.9	96.1
Total (7 compounds)	1.06 ± 0.34	211 ± 40	96.1 - 100

Mean ± SD, n = 3 sample pools from 9 fish
ND = not detectable (< 0.02 ug/mL)
# = includes one sample pool expressing ND

transformed before phase II metabolism (cf. Ahlborg et al. 1978). In case of metabolism of PCP in fish, at least, this seems not to happen (Glickman et al. 1977; Kobayashi 1979). As far as chlorinated guaiacols and other chlorinated phenols are concerned there are no relevant studies yet available. Further, as is typical for phenolics in BKME, the situation may become extremely complicated with respect to possible interactions both in the water and inside an organism (e.g. competition for a common carrier in hepatocytes). We tested the possibility of chlorinations/dechlorinations of BKME phenolics by treating our GC calibration mixture (6 phenolics) with KOH just as we did our samples, but no changes were observed. Therefore, our results (Tables 1-3) demonstrate that all major chlorinated phenolics, except the more hydrophilic chlorocatechols, are metabolically conjugated in the trout liver and excreted to the bile. To our knowledge, phase II metabolism of tri- and tetrachloroguaiacols has not been reported before.

In the bile of trout exposed for 30 days to a low concentration of microbiologically treated BKME, the degree of conjugation approached hundred percent (Table 1). The amount of conjugated PCP (96 %) corresponds well with earlier observations (Kobayashi 1977; Glickman et al. 1977), all other phenolics rank still higher. Thus, by liberating the conjugated phenolics with hydrolysis and analysing them with GC we have a tool hundreds of times more sensitive than the "direct" extraction of the sample. Furthermore, the concentration of free chlorophenols in the bile of rainbow trout is known to be higher than in some other tissues (Oikari et al. 1984 b).

Comparison of the amounts hydrolysed by KOH and β-glucuronidase (Tables 2 and 3) reveals that 68-90 % of the chlorinated phenolics measured in trout bile occur as glucuronides. This seems to be at

Table 2. Hydrolysis of bile-accumulated conjugates of chlorinated phenolics by KOH and by β-glucuronidase. Fish were exposed for 10 days to 0.6% v/v solution of biologically treated kraft pulp mill effluent*.

Phenolic compound	Total = alkaline hydrolysis	Hydrolysis by β-GU	Percentage glucuronides
2,4,6-trichlorophenol*	9.6	8.6	90
2,3,4,6-tetrachlorophenol	13.0	9.0	69
3,4,5-trichloroguaiacol	37.0	28.0	75
4,5,6-trichloroguaiacol	5.6	4.6	82
3,4,5-trichlorosyringol	9.5	6.5	68
3,4,5,6-tetrachloroguaiacol	19.1	13.0	68
Total (6 compounds)	93.8	69.7	68 - 90

* Pool of 10 fish, concentrations given as ug/mL

odds with earlier observations on rainbow trout (Glickman et al. 1977), which found the only conjugate of PCP in the bile to be the glucuronide. Results from our third experiment, with three chlorophenols (including PCP), support the idea that some other conjugate is also involved in the biliary excretion of the chlorinated phenolics typical of BKME. In fact it is logical to suppose, in a situation where the fish is simultaneously exposed to many chlorinated phenolics, that the synthetized sulphate anion (Kobayashi 1977) is also secreted in the bile.

Table 3 indicates that the separate hydrolysis with sulphatase yields quite well the amount of the nonglucuronide conjugation (the sum glucuronides + sulphates not being statistically different from the total). As there is a fairly big variation between the individuals, the present analysis does not conclusively exclude the possibility of additional types of conjugates.

The concentrations of free chlorophenols in the blood plasma were about half of those in the bile (Tables 3 and 4). These ratios are very much in accordance with earlier observations (Oikari et al. 1984 b). In relative terms, tetrachlorophenol being an intermediate reference, it can be noted that the level of trichlorophenol in the plasma is much lower (1/10) and that of PCP much higher (6-7 x) than that in the surrounding water. This is in accordance with their different lipophilities, i.e. with their different absorption rates from the water to the fish (Saarikoski and Viluksela 1982). On the other hand, the ratio of free substance in the plasma to its conjugate in the bile (Tables 3 and 4) indicates that the net efficiency of the excretion of 2,4,6-trichlorophenol from the plasma to the bile is much higher than that for PCP. Finally, the presence of chlorophenol conjugates in the plasma (Table 4) is in keeping with the idea that some of molecules conjugated in the hepatocytes re-enter the circulation and are then excreted either via the kidneys or the gills (Kobayashi 1979).

Table 3. Free and conjugated chlorophenols in the bile of rainbow trout. Fish were exposed for 6 days to a 7:10:4 (w/v) mixture of three phenols (CP-3 : CP-4 : PCP ; total concentration 150 ug/L).

Phenolic compound	Concentration ug/mL		Percentage conjugated	Percentage glucuronides	Percentage sulphates
	Free	Conjug.			
2,4,6-trichlorophenol	1.5 ± 0.58	1343 ± 259	99.9 ± 0.0	84 ± 10	9 ± 4
2,3,4,6-tetrachlorophenol	14.8 ± 6.5	1795 ± 530	99.1 ± 0.6	81 ± 4	25 ± 4
Pentachlorophenol	45.7 ± 13.0	1428 ± 551	96.5 ± 1.7	72 ± 6	25 ± 5

Mean ± SD, n = 4 fish

Table 4. Concentrations (ug/mL) of free and total conjugated chlorophenols in the plasma of rainbow trout exposed for 6 days to a 7 : 10 : 4 mixture of three phenols (total conc. 150 ug/L; cf. Table 3).

Phenolic compound	Free	Conjugated
2,4,6-trichlorophenol	0.7 ± 0.5	3.3 ± 2.3
2,3,4,6-tetrachlorophenol	9.7 ± 3.1	16.3 ± 13.7
Pentachlorophenol	26.0 ± 12.1	27.0 ± 17.0

Mean ± SD, n = 3 fish

Research on the toxicokinetics of complex mixtures of organic xenobiotics has been fairly unpopular up to the present time. However, we cannot avoid the reality that most industrial waste waters are of this type. In the present study we have showed that even at very low environmental concentrations of BKME (0.6 - 2% BKME ≦ 5 ug/L the phenolics measured) the phenolics absorbed still are almost wholly metabolized before hepatic disposition. The situation may be representative of that caused by the chronic aquatic pollution adjacent to kraft pulp mills.

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REFERENCES

Ahlborg UG, Larsson K, Thunberg T (1978) Metabolism of pentachlorophenol in vivo and in vitro. Arch Toxicol 40:45-53

Chambers JE, Yarbrough JD (1976) Xenobiotic biotransformation systems in fish. Comp Biochem Physiol 55C:77-84

Glickman AH, Statham CN, Wu A, Lech JJ (1977) Studies on the uptake, metabolism, and disposition of pentachlorophenol and pentachloroanisole in rainbow trout. Toxicol Appl Pharmacol 41:649-658

Holmbom B (1980) A procedure for analysis of toxic compounds in pulp and paper mill waste waters. Pap Puu 62:523-531

Kobayashi K (1977) Metabolism of pentachlorophenol in fishes. In: Rao KR (ed) Pentachlorophenol. Plenum Press, New York, pp 89-105

Kobayashi K (1979) Metabolism of pentachlorophenol in fish. In: Khan MAQ, Lech JJ, Menn JJ (eds) Pesticide and Xenobiotic Metabolism in Aquatic Organisms. ACS No 99, Washington DC, pp131-143

Levine WG (1978) Biliary excretion of drugs and other xenobiotics. Ann Rev Pharmacol Toxicol 18:81-96

Oikari A, Ånäs E, Kruzynski G, Holmbom B (1984 a) Free and conjugated resin acids in the bile of rainbow trout, Salmo gairdneri. Bull Environm Contam Toxicol 33:233-240

Oikari A, Nakari T, Holmbom B (1984 b) Sublethal actions of simulated kraft pulp mill effluents (KME) in Salmo gairdneri: residues of toxicants, and effects on blood and liver. Ann Zool Fennici 21:45-53

Saarikoski J, Viluksela M (1982) Relation between physicochemical properties of phenols and their toxicity and accumulation in fish. Ecotoxicol Environm Safety 6:501-512
Statham CN, Melanchon MJ, Lech JJ (1976) Biotransformation of xenobiotics in trout bile: a proposed monitoring aid for some waterborne chemicals. Science 193:680-681
Voss RH, Wearing JT, Mortimer RD, Kovacs T, Wong AL (1980) Chlorinated organics in kraft bleachery effluents. Pap Puu 62:809-814
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