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Exposure assessment of fishes to a modern pulp and paper mill effluents after a black liquor spill

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Abstract Conjugated resin acids (RAs) in fish bile are considered a sensitive chemical indicator of exposure to pulp and paper mill effluent, and were used in this study to monitor the post-spill situation of a lake area (Southern Lake Saimaa) contaminated by black liquor discharged from a mill in June 2003. From the exposure perspective of populations of wild roach and perch, which were studied for their bile RAs at four time periods (July 2003, September 2003, May 2004, July 2004), the exceptional event passed in 2 months or less. Perch had lower concentration of RAs in bile than roach in all sampling areas and all times. Besides the current emissions present in the water column, part of the exposure status of the roach population to RAs seemed to derive from historically contaminated sediments. In order to test this hypothesis, a laboratory experiment with perch and roach, along with three teleosts (rainbow trout, brown trout and whitefish), was conducted. The species were simultaneously exposed for 7 days to RAs (23 μg/l). We calculated a perch/roach-ratio to investigate the difference in origin of exposure between perch and roach populations in the wild and in the laboratory. One year after the spill, the perch/roach-ratio of bile RAs was 0.25 (CV 25%) at 1 km from the mill. This is in contrast to that found under the sole waterborne conditions (0.44; CV 24%), supporting the idea that sediments serve as an additional source of RAs in roach. Additionally, bioconcentration factor log $BCF_{bile(RA)}$ was calculated to assess hepatobiliary performance and the capacity to excrete RAs in fish.

Keywords Bile · Bioconcentration · Black liquor · Brown trout · Perch · Rainbow trout · Resin acid · Roach · Sediment · Whitefish · Wood industry

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Introduction

On 27 June 2003, there occurred an unscheduled discharge of black liquor (910 t) from a pulp and paper mill in Southern Lake Saimaa (SE Finland), which exceeded its environmental permit conditions. This event was preceded by accidental releases of soft soap from June 16 to 21 and on June 23, which did not exceed the mills environmental permits. The spills occurred during start-up of the mill after major repair operations and the midsummer stoppage, just before many Finns were starting their summer holidays at thousands of summer cottages. Saimaa Water Protection Agency, a local environmental monitoring association, began a hydrological follow-up 9 days after the first spill. In addition to the dramatic decrease in lake water quality, there were large fish kills (mainly small perch) in the area that local people use for the purpose of recreational and minor commercial fishing. These consequences attracted a lot of attention and a debate in the local and national media until late autumn. As a result of the activities of the local inhabitants, city administration, and regional environmental authorities, the public prosecutor was led to consider preferring charges, primarily on the grounds of the mill exceeding the environmental permit (Korjonen-Kuusipuro et al. 2004). By law (Finnish Environmental Protection Act 4 §), the responsibility for remedying a contaminated site rests with the polluter. This study started 4 weeks after the last and largest discharge, 2 days after a request was received from the company to investigate and monitor the post-spill situation in Southern Lake Saimaa. The question to be answered concerned the fish populations and for how long they were exposed to an elevated degree to chemical constituents due to the spills.

Resin acids (RAs) are still one of the major pollutants in treated modern pulp and paper industry effluents, with concentrations ranging from 40 to 100 µg/l (Johnsen et al. 1995; Verta et al. 1996; Leppänen and Oikari 1999). Although the amounts of toxic compounds in effluents have decreased during the last two decades (Karels et al. 2001), minor or major accidental spills may happen, such as those in June 2003 in Southern Lake Saimaa (Korjonen-Kuusipuro et al. 2004). In monitoring the post-spill situation, knowledge of the hepatobiliary excretion of RAs in fish was applied. We noticed however, that there was insufficient knowledge on interspecies differences in the excretion of RAs in bile, despite that the species concerned have been used widely in research and monitoring situations.

In kraft pulping, under normal operations, black liquor is recovered after cooking wood chips in alkaline conditions. Wood extractives, such as resin acids, fatty acids, and wood sterols are part of the black liquor, which is normally combusted. Recoveries of tall oil and pulping chemicals are also conducted (Sjöström 1993). Spills of black liquor can result from overflows, leaks from process equipment, or from deliberate dumping by operators to avoid a more serious accident in a mill (USEPA 2002). Technically, however, the majority of black liquor spills are diverted to holding ponds and bled back to the plant's treatment systems before any discharge into the environment (Hodson et al. 1997).

Earlier studies on the uptake and metabolism of RAs and related organic acids in fish have demonstrated that they are mostly metabolized in the liver to glucuronic acid conjugates (90–98%), and excreted into the bile (Kobayashi 1979; Oikari et al. 1984). The concentration of RA conjugates in bile can be up to 110,000 times higher than that in the surrounding water (Oikari et al. 1984; Johnsen et al. 1995; Meriläinen et al. 2007). It thus appears that the analysis of bile conjugates is an extremely sensitive and highly practical tool for investigating the contaminants of BKME in waters. It can detect RAs in the water at concentrations as low as tens of nanograms per liter (Meriläinen et al. 2007).

Studies on the metabolism of RAs and the effects of pulp and paper industry effluents containing them have been made among several fish species (Oikari et al. 1984; Johnsen et al. 1995; Soimasuo et al. 1998; Meriläinen et al. 2007), including perch and roach (Karels and Niemi 2002). The species used in this study (perch, roach, brown trout, rainbow trout and whitefish) were selected on the basis of their previous use for monitoring BKME effects and on their feeding habits and ecological relevance to the Fennoscandian environment (Koli 1998). Roach, a benthic fish, feeds typically on sediment-associated detritus, mollusks, and other invertebrates (Rask 1989; Koli 1998), and may thus be exposed to compounds that are reversibly sorbed to sediment particles, as opposed to the perch, which is a species that prefers to occupy the upper parts of the water column and feeds on fish and invertebrates. Depending on age and competitive status, however, perch may feed also benthic invertebrates (Rask 1986; Koli 1998; Horppila et al. 2000).

In this study we investigated the exposure of fish populations after an accidental release of black liquor from a pulp mill. Additionally we validated the field data by a laboratory experiment in which relative levels of bile RAs in perch, roach, rainbow trout, brown trout, and whitefish were investigated under strictly comparable conditions to a mixture of RAs. We also investigated the possible metabolic differences of RAs in five fish species, and calculated ratios that can be used to extrapolate exposure to RAs between species. The ratios can be used to estimate water concentrations on the basis of fish bile concentrations of RAs. To account for the presence of several individual RAs, we grouped them into pimaric (pimaric, sandaracopimaric and isopimaric acids) and abietic-type (dehydroabietic, abietic, neoabietic and palustric acids) RAs on the basis of their structural characteristics (Sjöström 1993).

Materials and methods

The research area and the black liquor spill

The study area was Southern Lake Saimaa, in southeast Finland (Fig. 1). The mean depth of water is 14 m and the lake area 150 km^2. In 2003, the pulp and paper mill, operated since 1897, produced 670,000 t of mechanical pulp and 550,000 t of soft- and hardwood-based kraft pulp. The treated effluent characteristics in June 2004 were the following (UPM Kaukas, Research Center): flow was 107,800 m^3/d, pH 8, sodium 409 mg/l, COD (chemical oxygen demand) 263 mg/l, and AOX (adsorbable organic halogen) 2.5 mg/l. The average concentrations of total nitrogen and phosphorus were 4.5, and 9.1 mg/l, respectively.

The mill complex also includes a sawmill. To improve the water quality, a pumping station was built in 1936 replacing ca. 40 m^3/s water from the clean area of the lake to the watercourse upstream of the mill, causing a net flow in the study area from west to northeast, and thereby diluting the effluents (Fig. 1). Measurements of sodium, an inert effluent tracer, suggest a theoretical concentration of 3.5% BKME in the mixing zone, which is within one km of the outlet (Soimasuo et al. 1995). No municipal effluents are discharged into the same area of the lake.

The black liquor spill extended 5 km to the north-east in Southern Lake Saimaa after 10 days, and 15 km after 20 days (Fig. 1), impairing the water quality considerably but temporarily (Korjonen-Kuusipuro et al. 2004). Moreover, the spill caused a large aerobically degrading organic load to the down-stream lake system, decreasing the oxygen concentration from 10–11 mg/l to less than 3 mg/l for a period of 2 weeks (Korjonen-Kuusipuro et al. 2004). The water quality characteristics in the study areas before and after the spill in May 1996, March 1997, May 2003 (pre-spill), September 2003 (3 months after the spill), and May 2004 (11 months after the spill) are given in Table 1.

The question arose as to how long fish populations in Southern Lake Saimaa were exposed to elevated levels of black liquor relative to normal releases of BKME. Follow-up of the post-spill situation was conducted by RA analyses of bile from two species of wild fish, perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*). Bile samples were collected at four time points: the first was 26 days after the spill, July 2003 (Period I: 24 July–6 August 2003); second was at 2 months and 3 weeks, in September 2003 (Period II: 9–22 September 2003); the third was at 11 months, in

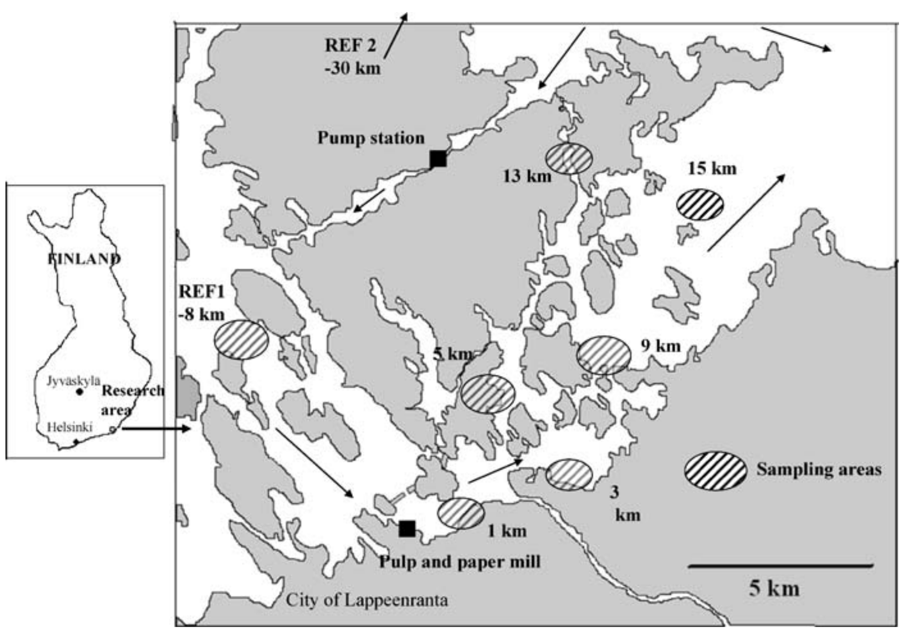

Fig. 1 The sampling areas of perch and roach in Southern Lake Saimaa, Finland in 2003–2004. Areas Ref 1 and Ref 2 are reference; areas 1–15 km located downstream from the pulp and paper mill with unscheduled spill in June 2003. Ref 2 is located 30 km north, outside the map

Table 1 Lake water quality in May 1996 (Karels and Oikari 2000), March 1997 (Karels et al. 2001), May 2003, September 2003, and May 2004 in reference areas 1 and 2, and in selected exposure areas (1, 2 and 5 km; see Fig. 1), in Southern Lake Saimaa

Sampling area	Sampling period	pH	Oxygen (mg/l)	Oxygen saturation (%)	Colour (mgPt/l)	Turbidity (FTU)	Na (mg/l)	COD, Mn (mg/l)	Total P (μg/l)	Total N (μg/l)
Ref 1	May 1996	7.1	12.6	103	35	0.4	3.9	7.4	11.0	390
	March 1997	6.8	13.0	91	35	0.2	3.0	6.0	4.0	560
	May 2003	7.1	11.3	104	25	0.9	6.6	6.2	7.8	383
	September 2003	7.3	8.9	93	25	1.5	6.6	6.6	15.5	310
	May 2004	7.2	11.0	98	24	0.6	4.2	5.8	6.5	365
Ref 2	May 1996	6.9	12.1	96	38	0.2	4.3	6.8	7.0	430
	March 1997	6.7	12.1	86	36	0.2	5.2	7.1	7.0	400
	May 2003	7.0	12.1	99	23	0.2	3.1	5.5	4.8	364
	September 2003	7.1	9.6	92	24	0.5	3.2	6.0	4.3	373
	May 2004	7.1	12.4	99	25	0.3	3.3	5.8	5.2	364
1 km	May 1996	7.0	11.0	94	85	1.5	9.7	10.3	23.0	545
	March 1997	6.8	11.5	85	50	0.8	19.6	11.8	13.0	643
	May 2003	7.3	10.0	95	45	1.5	28.8	13.0	16.0	500
	September 2003	7.0	5.7	61	50	3.0	19.3	11.5	39.0	503
	May 2004	7.3	8.8	83	49	1.9	1.9	6.5	19.8	580
2 km	May 2003	7.4	9.6	93	50	1.5	29.5	13.8	16.5	548
	September 2003	7.2	7.4	78	50	2.4	18.8	11.3	35.3	500
	May 2004	7.4	9.4	90	50	1.4	23.3	11.5	16.5	505
5 km	March 1997	6.7	11.1	79	59	0.5	13.1	11.5	11.0	518
	May 2003	7.5	11.3	103	38	1.0	17.3	9.7	13.5	458
	September 2003	7.5	8.4	88	50	2.4	16.0	10.5	37.0	475
	May 2004	7.5	11.1	100	38	0.9	12.6	8.2	12.8	405

Data are from Saimaa Water Protection Inc., Lappeenranta, and are means of water column values from 1 m to near bottom.

May 2004 (Period III: 13–24 May 2004); and the fourth was at 12 months after the spill, in July 2004 (Period IV; 3–14 July 2004). Fish were caught in sampling periods I and II with a Nordic net and in periods III and IV with a weir. In periods III and IV, the fish were caged at the catching sites for 2 days to obtain a higher volume of bile fluid. Two areas—Ref 1, ca. 8 km upstream from the discharge area, and Ref 2, ca. 30 km upstream—were taken as background reference, and six areas contaminated by BKME receiving emission from the mill (1, 3, 5, 9, 13, and 15 km; Fig. 1).

Bile samples were frozen in liquid nitrogen and kept at −80°C. For the RA analyses, individual samples or subsamples from 2 to 10 animals (equal volumes from each) were pooled for one analysis. Free and conjugated fractions were extracted and analysed together (Oikari et al. 1984) using gas chromatograph–mass spectrometer (GC–MS).

Experimental animals and their acclimatization

Hatchery-reared juvenile 1-year-old brown trout (*Salmo trutta* m. lacustris) from Hanka-Taimen Oy (Hankasalmi, Finland), rainbow trout (*Salmo gairdneri*), and whitefish (*Coregonus lavaretus*) from the Finnish Game and Fisheries Research Institute (Laukaa, Finland), were studied in March 2005. The average length and weight of the brown trout was 12.7±SD 1.5 cm and 20.8±SD 7.4 g. The length of the rainbow trout and whitefish were 12.9±SD 1.3 cm and 13.4±SD 0.9 cm, and weight 21.1±SD 6.6 and

16.1±SD 3.7 g, respectively. Perch (*P. fluviatilis*) and roach (*R. rutilus*) were caught with a weir in Lake Konnevesi, Central Finland, an unindustrialized area, and transported to the laboratory in less than 1 h. The average lengths of the perch and roach were 14.7±SD 1.6 cm and 17.0±SD 1.1 cm, and their weights 30.3±SD 10.7 g and 41.8±SD 10.4 g, respectively. All species were transported in a plastic (PE) package partially filled with gaseous oxygen, placed in fish tanks (3°C), and the temperature was adjusted gradually to 10°C over 3 days.

Before the experiments, the fish were acclimatized in continuously changing unchlorinated active carbon filtered municipal water (pH 7.7±0.1, temperature 10.0±0.5°C) for 2 weeks. During acclimatization, water oxygen saturation was over 80%, and the light: dark cycle 12:12, the same as in the hatchery and during the exposures to RAs. All the fish, including the perch and roach, were fed on alternate days with pellet food (Nutra SvEv 1.5, Rehu Raisio Co., Raisio, Finland) *ad libitum* with a ration approximately 0.5% of fish biomass. Accumulated faeces and possible excess food were removed from the aquaria every other day. Feeding was stopped 4 days before the experiment, and during the exposure the fish were not fed.

Exposure settings and sampling

A mixture of eight RAs, a Polish wood rosin (Hercules Co., Wilmington, USA) containing 95.5% of RAs, was used for the exposures. A stock solution with 1,200 mg/l wood rosin was prepared by dissolving rosin in ethanol (purity 99.6%), and diluting first with a small volume of 1 M NaOH and then with 0.1 M NaOH, and finally with distilled water. The pH of the rosin stock was 9.5 and it did not affect the pH of the experimental waters when added to the aquaria. The initial concentration in the two aquaria with the added RAs, done 12 h before the transfer of animals, was 60 µg/l. Fish were transferred gently in water, avoiding any stress. In order to prevent any possible aerial contamination by RAs, the control experiments were conducted in a separate room. At the start, 10 randomly chosen fish per species were placed in two identical all-steel tanks with standing preaerated water (volume 2,160 l, 10.0±0.2°C), 50 fish in total (0.6 g fish/l at the beginning), providing an approximate biomass loading of 0.4 l/g/d. On the fourth day, half of the water volume was changed. During the experiment, one whitefish died but otherwise no abnormal behaviour was observed. Water temperature, oxygen and pH levels were measured daily.

Samples were taken after 7 days. Fish were randomly netted from a tank and stunned with a blow to the head. Fish weight (nearest 0.1 g) and length (1 mm) were recorded. The fish were dissected, and the gallbladder with the bile separated was frozen in liquid nitrogen (LqN), and stored at −80°C. Water samples were collected before and after the exposure period, and daily for a combined sample to represent the average of the entire exposure time. Waters were stored in glass bottles at −20°C. Additionally, the fish food was analysed for RA concentration.

Resin acid analyses

Water samples were analyzed by GC–MS (modified from Oikari et al. 1984) for the concentrations of seven RAs. Samples (0.5–1 l) were evaporated by a vacuum evaporator (Heidolph WB2000, Germany) to a volume of 50–100 ml, and extracted three times in a separating funnel with hexane–acetone (3:1 v/v) from acidified (pH 3.5) solution. Extracts were evaporated with vacuum evaporator and under nitrogen stream, and the substances silylated with BSTFA +1% TMCS (Regis Technologies Inc, Morton Grove, USA) at 70°C for 50 min. Two internal standards, *cis*-10-heptadecenic acid (purity >99%, Sigma Chemicals Co., St. Louis, Mo, USA) and tricosanoic acid (purity >99%, Fluka Chemie, Buchs, Switzerland), added to samples in methyl *tert*-butyl ether (MTBE) before extraction, were used. HPLC grade acetone, MTBE and hexane were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland).

Fish bile samples were analyzed for the total concentrations of conjugated and free RAs (Oikari et al. 1984; Meriläinen et al. 2007). The conjugated fraction was hydrolyzed (0.5 M KOH in ethanol, 70°C, 2 h) and extracted from acidified (pH 3.5) sample together with the free fraction by hexane–acetone (3:1 v/v). The combined extracts were dried under a nitrogen stream, and the RAs silylated, diluted with pyridine (GC grade, Roth (Karlsruhe, Germany) and analysed. Fish food was analysed for total RAs by freeze drying the pellets, homogenizing them, and extracting with hexane–isopropanol

(2:1 vol/vol) as described in Leppänen and Oikari (1999). The extracts were dried under a nitrogen stream, and the substances silylated, diluted with pyridine and analysed. For the analysis, a HP 6890 gas chromatograph (Hewlett Packard, Waldbronn, Germany), equipped with HP-5 capillary column (25 m, 0.2-mm I.D., 0.33 μm phase thickness) and a HP 5973 MS detector (Hewlett Packard, Palo Alto, CA, USA), was used with splitless mode (2.00 min) and an injection volume of 1.0 μl. Quantification of RAs were performed by raising the temperature from 100°C (maintained for 1.5 min) to 180°C (maintained for 15 min) at the rate 6°C/min and further to 290°C (maintained for 13 min) at the rate 4°C/min.

The recovery of analyte after hydrolysis and extraction was 92–127% (three replicate procedures). Limit of detection (LOD) of the method for individual RAs was 0.05 μg/l for the water analysis, 0.01–0.05 μg/ml for the bile analysis, depending on the compound, and 0.05 μg/g dw for the fish food analysis. The determination of LOD and limit of quantification (LOQ) was done from three replicate measurements from the same sample. LOQ was determined by multiplying the concentrations obtained from LOD with SD, and considering analytical background from the analytical process, was 0.5 μg/l for the water analysis, 0.5 μg/ml for the bile analysis, and 0.5 μg/g dw for the fish food analysis. The concentrations of RAs in fish bile samples were presented in units of concentration per volume in order to compare our result to previous studies conducted in Southern Lake Saimaa area without unit conversion.

The bioconcentration factor (BCF_{bile}) was calculated to assess hepatobiliary performance and the capacity to excrete RAs in fish. It was calculated by dividing the concentration of RAs in the bile by the average concentration of RAs in the water. $BCF_{bile(RA)}$ denotes the bioconcentration of all conjugated plus free RAs, and, for instance, $BCF_{bile(DHAA)}$ that of conjugated and free dehydroabietic acid (DHAA). Additionally, the results from the laboratory exposures were used to estimate the ambient concentrations of RAs in Southern Lake Saimaa 2003–2004. The average concentrations of bile total RAs obtained for each sampling area in Southern Lake Saimaa and the $BCF_{bile(RA)}$ of perch was used in the calculations.

For the statistical analyses all the data were first assessed for normality and homogeneity of variance. The bile RAs were compared with Student's *t* test, and one-way analysis of variance (ANOVA) followed by Tukey's HSD test. The significance of differences was set at $p<0.05$. In the tables and figures, mean and standard deviation (SD) are given. A coefficient of variance (CV %), calculated dividing the standard deviation by the mean measurement, was determined for the perch/roach-ratios. Statistical analyses were performed with SPSS software (Statistical Product Service Solutions, Chicago, IL, US).

Results

Monitoring of fish populations: post-spill

Water oxygen levels decreased immediately after the accident (down to 3 mg/l), which may partially explained the fish kills in the area 3 km from the mill. Normal oxygen level (>90% of saturation) was restored after 1 month. Other consequences of decreased water quality were restrictions on lake water usage in households, swimming at the nearby beaches, and consumption of contaminated fish in the spillage area. This was one reason for the decision to monitor the post-spill exposure situation of fish populations in Southern Lake Saimaa. Monitoring began 27 days after the accidental release of the black liquor.

Fish populations in the Southern Lake Saimaa area are subject to continuous low-level exposure to BKME derived RAs, due to the normal operation of the pulp mill and the current environmental permits. The flow from the pumping station prevents the dispersion of effluent into reference area 1 (−8 km; Fig. 1). Reference area 2, located outside of the area affected by the mill (−30 km), is probably the environment least affected by pulp mills in Southern Lake Saimaa. No visible effects of the black liquor spill in June 2003 were observed in this area. In reference area nearest to the mill (−8 km), in all the sampling periods from July 2003 (1 month post-spill) to July 2003, the total concentrations of RAs in the bile were less than 5 μg/ml in perch and 7 μg/ml in roach with no significant differences between them (Student's *t* test, $p>0.05$). In reference area 2, the RA concentrations in perch bile were only 1.2 μg/ml in July 2003, remaining within the same range in July 2004 (2.7 μg/ml). In roach, the bile RA concentrations were 4.2 μg/ml and 3.3 μg/ml in July 2003 and

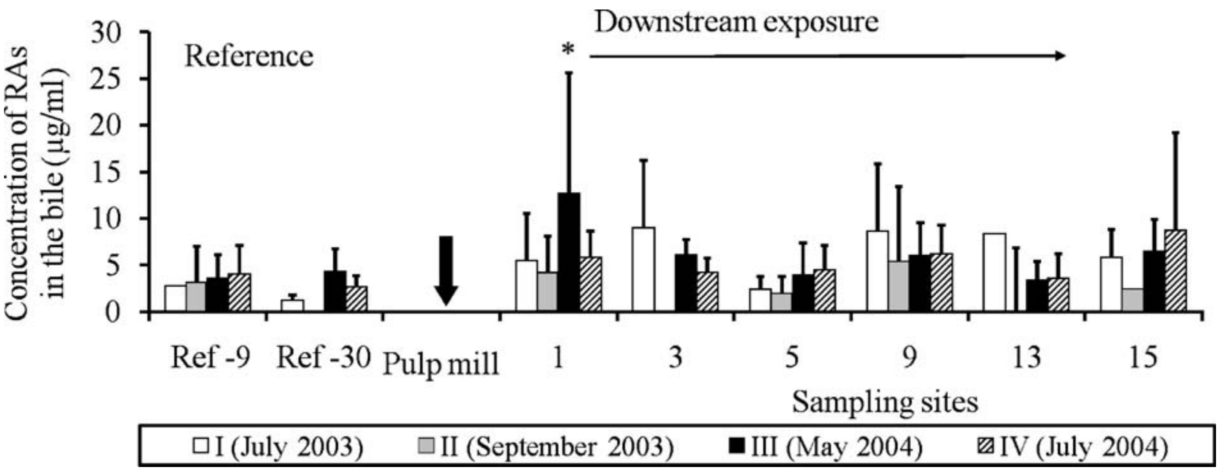

Fig. 2 Total resin acid concentrations (µg/ml) in the bile of perch in Southern Lake Saimaa from July 2003 to July 2004. There were two reference areas (see Fig. 1), and six areas downstream of the pulp and paper mill, discharging regular effluents and the spill of black liquor in June 2003. Values are means ± SD, *asterisk* denotes statistically significant difference compared to respective reference areas ($p<0.05$)

July 2004, respectively. In both reference areas, DHAA was the most abundant RA (54% for perch and 49% for roach) in fish bile (Figs. 2 and 3).

Downstream from the pulp and paper mill 'Kaukas,' the total concentration of RAs in perch decreased with distance from the mill in all the sampling periods (I–IV). In the sampling area 1 km downstream from the mill, the total RA concentration in perch bile averaged 5.5 µg/ml in July 2003, and remained the same in July 2004 (5.7 µg/ml) (Fig. 2). The situation was similar in the other sampling areas for RA concentrations in perch bile measured in July 2003 and July 2004. However, the highest exposure to RAs in perch 1 km from the mill occurred in May 2004 (mean: 12.7 µg/ml), right after the spring mixing of the water column. At no other post-spill monitoring period was any impact observed on perch populations in terms of increased exposure to RAs which could be contributed to the previous black liquor spill (Fig. 2). The statistical differences between the reference and exposure areas are given in Fig. 2 (Student's t test, $p<0.05$). No significant differences between the sampling periods were found in perch (Student's t test, $p>0.05$), although RA concentrations showed a decreasing

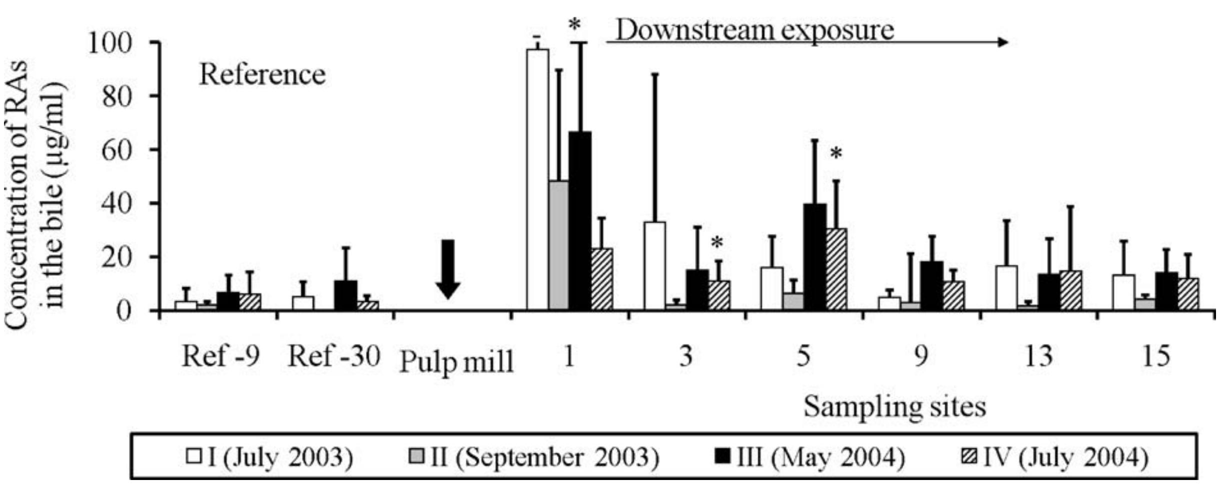

Fig. 3 Total resin acid concentrations (µg/ml) in the bile of roach in Southern Lake Saimaa from July 2003 to July 2004. There were two reference areas (see Fig. 1), and six areas exposed to the pulp and paper mill effluents, in addition to the spill of black liquor in June 2003. Values are means ± SD, *asterisk* denotes statistically significant difference compared to respective reference areas ($p<0.05$)

trend of exposure in perch bile in the area 3 km downstream from the mill (Fig. 2). In perch at 1 km downstream from the mill in September 2003, the total RA concentration in the bile was on average 94% lower than in roach.

In roach, in the areas 1 and 3 km from the mill, the highest concentrations post-spill were observed in July 2003 (1 km: 97 µg/ml; Fig. 3). In July 2004, the RA concentrations were 76–66% less in the same areas, indicating a significant decrease in the exposure of roach during 1 year (Fig. 3). More precisely, after July 2003, RA concentrations in roach bile decreased in all the sampling areas in September 2003 and increased some (38–590%) in spring 2004, apparently due to the annual mixing of the water column. The trend towards decreased exposure was clearest at 1 km from the mill. Statistical differences between reference and exposure areas are given in Fig. 3 (t test, $p<0.05$).

As in the reference areas, DHAA was the most abundant RA in the bile of perch and roach in all the areas contaminated by BKME and during all the sampling periods (I–IV). The proportions in perch were 22–62% (I), 36–96% (II), 36–92% (III) and 43–63% (IV), and those in roach: 52–62% (I), 46–97% (II), 36–76% (III) and 33–71% (IV).

In conclusion, Southern Lake Saimaa had recovered from the accidental release of black liquor by September 2003, at the latest. This is based on the results that the RA concentrations in perch bile, downstream from the pulp mill in September 2003, were in the same range as those in reference areas in June 2004. Although best shown by perch, this conclusion is also supported by the observations on roach. Even though the concentrations in roach were higher than in perch, the bile RA concentrations of both species can be assumed 'normal' permit-based exposures taking into account the presence of the pulp and paper mill industry in the area. Altogether 198 RA bile analyses in 423 perch and 210 analyses in 340 roach were performed to obtain a comprehensive overview of the fish populations in the area.

Exposure of fishes to waterborne RAs

Maintenance of experimental conditions

The composition of the wood rosin stock contained: 8.7% (w/w) pimaric, 1.8% sandaracopimaric, 3.4% isopimaric, 22.6% palustric, 33.4% dehydroabietic, 24.4% abietic, and 5.8% neoabietic acid (Fig. 4). At the beginning of the experiments, the composition of

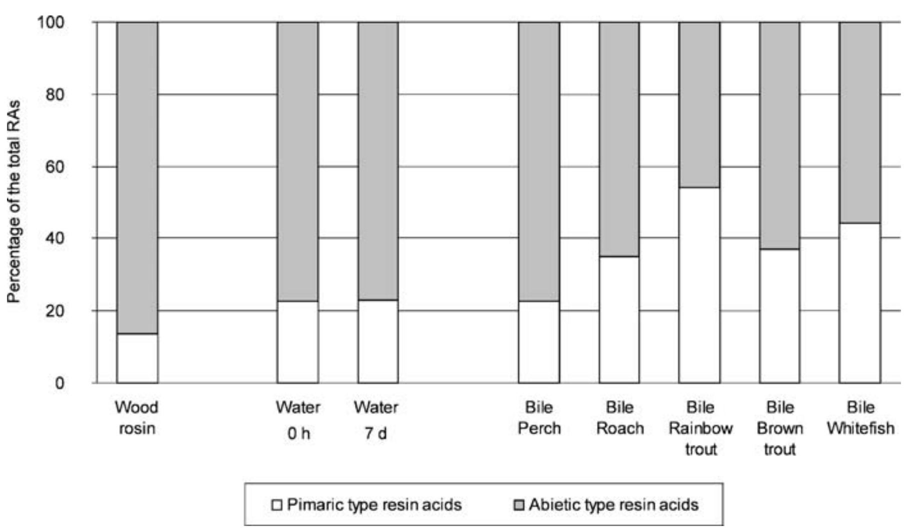

Fig. 4 Percentage of summed pimaric-type (pimaric, sandaracopimaric, and isopimaric acid) and abietic-type (dehydroabietic, abietic, neoabietic, and palustric acid) resin acids in the wood rosin stock, in the exposure water at the beginning of the laboratory experiment, during the exposure (combined sample of 7 days; total concentration averaged 23 µg/l)), and in the bile of perch, roach, brown trout, rainbow trout and whitefish when exposed for 7 days to this RA mixture in water. Values are means of three analyses. Limit of quantification (LOQ) was 0.5 µg/l for water analysis, and 0.5 µg/ml for bile analysis

RAs in the water was similar to that in the wood rosin, i.e. DHAA predominated (Fig. 4). Over the exposure period, the pimaric- and abietic-type ratios remained fairly constant, at around 23:77 (Fig. 4). The fish food did not contain any RAs over the LOQ (0.5 μg/g dw), although traces of dehydroabietic acid were found. Since there were no RAs in the control water, the source of background concentrations in the bile derived from the food, and possible exposure to RAs in the previous living location.

In the exposures, the initial water concentration of RAs was 60 μg/l in both replicates, decreasing to an average of 23 μg/l over the entire 7-day experiment. Water change on the fourth day increased the concentration by 80% compared to the pre-change situation. The reason for the fluctuation, besides absorption into the fish, might have been microbial degradation, isomerization (Morales et al. 1992), or adsorption to fecal remains. With regard to the concentrations of individual RAs over the entire period, the most abundant was DHAA (62% of total RA). While abietic-type RAs were predominant in the ambient water (66%) and in the bile (56–77%) of most species, concentrations of pimaric-type RAs slightly predominated in rainbow trout bile (54%).

Accumulation of RAs in fish bile

In the case of the control fish kept in clean water, three determinations of bile RAs from each species were performed. Perch showed the lowest average concentration of all the species, 1.4±SD 0.2 μg/ml, compared to 2.7±SD 1.3 μg/ml in roach. For brown trout the average concentration was 2.1±SD 0.3 μg/ml, for rainbow trout 2.5±SD 0.5 μg/ml, and for the whitefish 15±SD 12 μg/ml (Fig. 5). Once again, DHAA was the most abundant RA, and was highest in brown trout (86% of the total RAs) followed by rainbow trout (79%). For perch, roach, and whitefish, DHAA accounted for about 46% of the total RAs (Fig. 5).

The results of the two replicate experiments were similar, and so the bile analyses were combined. The lowest concentrations of total RAs were detected in perch bile, 660±SD 310 μg/ml (Fig. 5). The sum of seven RAs revealed a concentration of 1,500±SD 760 μg/ml in roach bile, 1,670±SD 380 μg/ml in brown trout bile, and 1,650±SD 450 μg/ml in rainbow trout bile. As in the control animals, the whitefish (4,330±SD 1,080 μg/ml) showed highest

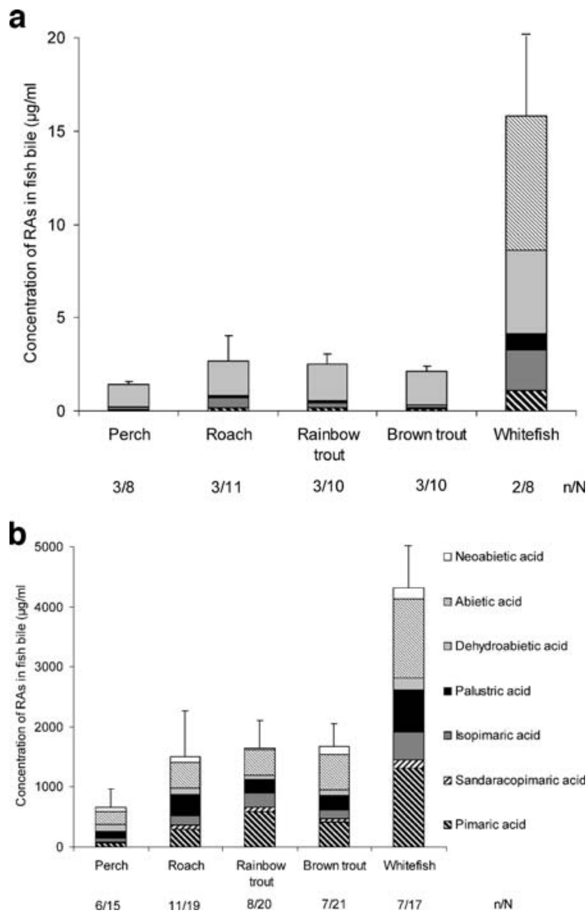

Fig. 5 Mean concentration of total RAs (sum of dehydroabietic, abietic, pimaric, isopimaric, palustric, sandaracopimaric and neoabietic acid) in the bile of five species (μg/ml) in (a) control exposure (no added RAs) and (b) exposure to RAs (average total concentration 23 μg/l). Values are means ± SD. Symbol *n*/*N* depicts the number of analyses (*n*) and the total number of animals in them (*N*). Limit of quantification (LOQ) was 0.5 μg/ml for bile analysis

bioconcentration ability of all five species. The perch/roach-ratio of bile RAs on uptake from water averaged 0.40 (Table 2).

Since the rainbow trout is a worldwide model species, we wanted to estimate ratios that can be used as conversion factors in interspecies extrapolation, e.g. in monitoring situations. The ratios in Table 2 were calculated between rainbow trout and other species with respect to their RA concentrations in bile. When using the overall average values in water over the 7-day experiment (23 μg/l), the ratio was lowest in perch (0.4), close to one in roach and brown trout (0.9 and 1.0), and highest in whitefish (2.2) (Table 2).

Table 2 Average ratio of RA concentration in fish bile compared to rainbow trout (taken as 1.00)

	Perch/rainbow trout	Roach/rainbow trout	Brown trout/rainbow trout	Whitefish/rainbow trout	Perch/roach
Pimaric acid	0.12	0.51	0.71	2.23	0.23
Pimaric-type RAs	0.23	0.77	0.76	2.32	1.12
Dehydroabietic acid	1.68	1.50	1.28	2.69	0.45
Abietic-type RAs	0.75	1.20	1.55	3.23	0.62
Total RAs	0.40	0.91	1.01	2.61	0.44
(Variation %)	(13.0)	(13.9)	(6.3)	(6.8)	(24.3)

Values of perch, roach, brown trout and whitefish compared to rainbow trout were calculated on basis of their RA concentration in bile when simultaneously exposed for 7 days to RAs (total concentration 23 µg/l in average). Additionally, perch/roach-ratios from the same exposure are shown (Fig. 5). A coefficient of variation for total RAs is shown in parenthesis.

With respect to individual RAs in bile, abietic-type RAs predominated in the bile of perch and roach (77 and 65%), with pimaric acid the most abundant in these two species (32 and 29%) (Fig. 5). In bile of brown trout and whitefish, abietic-type RAs predominated (63 and 56%), with abietic acid the most abundant RA in brown trout (36% of the total) and pimaric acid in whitefish (33% of the total). In contrast to brown trout, pimaric-type RAs predominated in the bile of rainbow trout (54%), with pimaric acid the most abundant (35%). It is noticeable that palustric acid, an abietic-type RA, was present in bile (e.g. 17% for perch, and 23% for roach), and contributed to the dominance status of abietic-type RAs regardless of the fact that pimaric acid was often the most abundant RA.

The average total concentration of water RAs (23 µg/l) was used to calculate the log $BCF_{bile(RA)}$ values for each species (Table 3). Due to the large numbers, the results are shown as logarithms. The mean log $BCF_{bile(RA)}$ varied from 4.45 to 5.27 between species (Table 3). The lowest degree of individual RAs was for DHAA, 3.84–4.04, and the highest for pimaric acid, 4.23–5.51.

Post-spill reconstruction of ambient RA concentrations in 2003 on the basis of bile metabolites

The values of $BCF_{bile(RA)}$ of total RAs (Table 3) obtained from the laboratory experiment were used to estimate the RA concentration in the water in Southern Lake Saimaa after the black liquor spill, in July 2003 sampling, with particular reference to perch, this species having more access to the water compartment than roach (Table 5). First, in the reference areas (Ref 1 and 2) the estimated concentrations of RAs in water were less than 0.50 µg/l. The result show that one km from the pulp mill the water RA concentrations contributed to be elevated in July 2003 (4.07 µg/l) after the black liquor spill still, but had decreased to 0.96 µg/l by July 2004.

Sediment as an origin of RAs

The annual peak concentrations of RAs in fish bile in spring led us to consider that BKME-contaminated sediment served as a source of RAs, due to resuspension of its surficial layer into the water compartment. As discussed earlier, perch and roach

Table 3 Average bioconcentration factors of resin acids (RAs) $BCF_{bile(RA)}$ in the bile of perch, roach, rainbow trout, brown trout, and whitefish exposed to waterborne RAs (average concentration 23 µg/l) in a 7-day laboratory exposure

Resin acid	Perch	Roach	Rainbow trout	Brown trout	Whitefish
Pimaric acid	4.23	4.87	5.16	5.01	5.51
Pimaric-type RAs	4.70	5.22	5.33	5.21	5.70
Dehydroabietic acid	3.84	3.79	3.61	3.72	4.04
Abietic-type RAs	4.35	4.55	4.47	4.66	4.98
Total RAs	4.45	4.81	4.86	4.86	5.27

Seven RAs were grouped into pimaric-type (pimaric, sandaracopimaric, and isopimaric acids) and abietic-type (dehydroabietic, abietic, neoabietic, and palustric acids) RAs. The values are expressed as log BCF_{bile} of the sum of total RAs relative to RAs in water.

Table 4 Average perch/roach-ratios of RA concentrations in the bile of perch and roach in Southern Lake Saimaa from July 2003 to July 2004

Sampling area	I July 2003	II September 2003	III May 2004	IV July 2004
1 km	0.06 (130)	0.09 (81)	0.19 (84)	0.25 (25)
3 km	0.27 (130)	n.a. (n.a.)	0.40 (28)	0.38 (25)
5 km	0.15 (42)	0.30 (69)	0.10 (53)	0.15 (35)
Laboratory exposure (water only)	0.40 (24)			

The nearest areas (1–5 km) are exposed to the pulp and paper mill effluents, in addition to the spill of black liquor in June 2003 (Fig. 1). Additionally, values from water-only exposure in laboratory (average ambient concentration of RAs 23 μg/l) in laboratory are shown (Fig. 5). A coefficient of variation (%) is shown in parenthesis.
n.a. data not available

have largely different living habitats and ways of foraging. In the great majority of fish in any sampling area downstream to the pulp mill, the bile concentrations of RAs were much lower in perch than in roach. We assumed two reasons for this; the species differences in biliary excretion, and unidentified sources of RAs in the environment. The first was based on the perch/roach-ratios of bile RAs (Table 4) and the second on the results of the laboratory experiment (see "Exposure of fishes to waterborne RAs"). Since perch can be assumed to accumulate its RAs mainly via water, the following suggestions may be formulated. First, increased uptake from sediment can follow if higher concentrations of RAs exists in the sediment, as documented at 1 and 3 km from the mill, i.e. 700 and 500 μg/g dw, respectively (Lahdelma and Oikari 2005). In July 2004 the RA concentration in the water was assumed to be on the normal operational level, and the relative exposure of sediment to roach to be the highest. Moreover, the reliability of the perch/roach-ratio was higher in July 2004 than in June 2003 due to low variance (Table 4), indicating the highest possible sediment uptake of roach. Perch/roach-ratios were 0.25 (CV 25%) in the 1 km area, and 0.38 (CV 25%) in the 3 km area. Secondly, the uptake from sediment by roach is zero when this species is exposed to water only. For this simple reason, areas with higher sediment RA concentrations (Leppänen and Oikari 1999; Lahdelma and Oikari 2005) are the most likely potential source of the high RAs in roach bile. Therefore, before further considerations of sediments as a source of RAs in roach in particular, an experiment in which water was the sole source and branchial uptake the predominant route of RA, was

Table 5 Retrospectively estimated water concentrations of RAs (μg/l) in Southern Lake Saimaa

Sampling Area (km from the mill)	I July 2003	II September 2003	III May 2004	IV July 2004
Ref 1 (−8 km)	0.26	0.30	0.34	0.38
Ref 2 (−30 km)	0.12	n.a.	0.41	0.26
1 km	0.52	0.40	1.21	0.55
3 km	0.86	n.a.	0.58	0.40
5 km	0.23	0.19	0.37	0.43
9 km	0.82	0.51	0.57	0.59
13 km	0.80	n.a.	0.32	0.34
15 km	0.55	0.23	0.62	0.83

The values are calculated on the basis of RA concentrations in the bile of perch (Fig. 2) in sampling areas Ref 1 and 2, and 1–15 km, and bioconcentration factor log BCF_{bile} of the total RAs from Table 3 for perch. The RA concentrations were calculated for all sampling periods of post-spill monitoring (I: July 2003, II: September 2003, III: May 2004, IV: July 2004).
n.a. data not available.

conducted in the laboratory (see "Accumulation of RAs in fish bile"). As a result, the perch-roach-ratio was 0.44 (CV 24%), demonstrating equal exposure of both species to waterborne RAs. When comparing the laboratory water-only based ratio to those obtained in the field in July 2004, the results suggest that the lower the latter ratio, the greater the difference between perch and roach in RA concentrations, indicating that roach may have an additional origin of exposure to RAs, plausibly sediment-derived RAs.

Discussion

Assessment of exposure

The number of unscheduled non-permit spills in the pulp and paper industry may be on the rise globally. For example, in Canada, spills increased from an average of 98 per year during 1984–1989 to an average of 354 per year during 1990–1995 (Environment Canada 1998). Similarly, the total number of spills from pulp and paper mills in Finland increased from 29 to 59 during 1986–1990 (Ettala and Rossi 1992). However, tighter legislation and an improved environmental culture within the industry has affected in the increased number of reported spills. In Southern Lake Saimaa, the accidental release that occurred first (June 16–23, 2003) were within the mill's environmental permits, but those containing more black liquor (June 27–30, 2003) exceeded it. On the other hand, the pulp and paper industry in Finland has performed well in its permit-based discharges recently. During the last 15 years, although the production of pulp, paper, and board in Finland has increased by 50%, solid emissions and biological oxygen demand (BOD) have decreased significantly, e.g. BOD_7 load from 90,000 t/a in 1990 to 10,000 t/a in 2004 (Finnish Forest Industries Federation 2005). This illustrates improvements in production technology and effluent treatment that have taken place in mills, but may also reflect the fall in the number of accidental spills (Sarlin et al. 1999).

The area investigated, Southern Lake Saimaa, has been studied widely before (Oikari 1986; Soimasuo et al. 1995; Karels et al. 2001). Therefore the pre-accidental situation of fish exposure to RAs and the effects of the pulp and paper mill in question on the fish community and populations in the area were known. The monitoring of perch and roach started 1 month after the spill at the company's request. Because the bile reflects the exposure status of fish to RAs over the past 24–48 h (Oikari et al. 1984), the results in this study serve as a biomarker of exposure to RAs over the last 2 days before each sampling period, not just that of a random moment of time, as is the case when collecting water samples. Importantly, the results of the bile analysis represent the bioavailable fraction of RAs. Thus, we hoped to use the results of this study to assess the recovery of fish populations in Southern Lake Saimaa area after the black liquor spill.

The levels of RAs in modern BKME fall within the range 40–100 μg/l (Johnsen et al. 1995; Verta et al. 1996; Leppänen and Oikari 1999), and in black liquor 240–400 g/l depending on the feedstock (Sjöström 1993). However, some studies have reported concentration higher than these due to wider range of analyzed resin acids (van den Heuvel et al. 2002). Over the last two decades, the concentrations of RAs in water 1–8 km downstream from the mill in Southern Lake Saimaa have been low, within the range 0.6–2.1 μg/l in May 1996 (Leppänen et al. 1998) and 0.5–2.4 μg/l in July 2004 (Lahti 2006). According to the RA levels in perch bile, estimates of ambient RA concentrations in the downstream areas from the mill (1–3 km) were in the range 0.4–0.9 μg/l in July 2003 (Table 5). This is well in accordance with the random values in water in the pre-spill situation, when BKME emissions were under permit levels (Leppänen and Oikari 1999). When comparing the BKME-contaminated areas and reference areas in July 2003, the water RA concentration was three times higher at 1 km distance from the mill. The estimated water RA concentrations decreased by 30% from July 2003 to September 2003 close to the mill (1 km), but settled back to the same level in July 2004 as they had been 1 year before. In the majority of the downstream fish-sampling the situation was similar, except in the areas 5 and 15 km from the mill, where the estimates increased from July 2003 to July 2004. This may indicate an alternative source of RAs in these areas. However, the overall results confirm the assumption that the water RA concentration was in the same range as the permit-based pre-spill situation (June 2004) even 1 month after the black liquor spill.

Resin acids have been deposited and released from the sediments in Southern Lake Saimaa area downstream from the Kaukas pulp and paper mill for decades. Some 15 years ago, sediments 1 km from the mill contained high concentrations of RAs (1,600 μg/g dw, Holmbom et al. 1992). More recently (1997–2004), the sediments RAs concentrations have been in the range 1,300–1,500 (Leppänen and Oikari 1999; Lahdelma and Oikari 2005; Lahti 2006), i.e. no significant recovery has followed.

In studies performed in other freshwater areas contaminated by the pulp and paper mille effluents, a clear distance relationship has been observed when using metabolites of fish bile (Tavendale et al. 1996). RA concentrations of 3,000 μg/g bile have been measured in rainbow trout exposed to 10% pulp mill effluent for 50 days (Lindesjöö et al. 2002). When comparing the results of the fish bile RAs in this study to those of previous studies made in the Southern Lake Saimaa area, it is evident, that even in July 2003, 1 month after the black liquor spill, the bile RAs concentrations in perch and roach were lower than under the normal operation of the factory in February to March 1997. Then the bile RA concentrations of perch and roach were 10–30 times higher than those in the reference areas (Karels et al. 2001).

It is noticeable that all the previous results were obtained under normal operational circumstances, and that the environmental permit levels in terms, for instance, of COD were not exceeded during those studies. July 2003 and July 2004 are most comparable time points since they reflect the situation in the area at the same season and in 2004 under strictly normal mill operating conditions. By comparing these time points, we can conclude that Southern Lake Saimaa area had recovered from the black liquor spill by July 2003. In the case of the black liquor spill in Southern Lake Saimaa in 27–30 June 2003, the permit levels were exceeded. However, after numerous investigations by government authorities, in response to concern about environmental effects among locals, the charges were dropped. Eventually at the end of 2004, the state prosecutor did not find enough evidence to prefer charges, as the spill had occurred in three occasions, which were considered separate permit exceeding situations instead of one more severe spill. Additionally, there was no evidence that any of the mill workers or the company itself had acted intentionally or negligently in breach of the Finnish water legislation or penal code.

Species differences in excretion of resin acids

The species for this study were chosen because of their prior use in field monitoring and relevance to the Nordic environment. For example, perch and roach have been studied in Southern Lake Saimaa, as these are the most common species in the area (Karels and Niemi 2002). The native freshwater species perch, roach, brown trout, and whitefish were chosen for the laboratory exposure together with the common benchmark, rainbow trout. Some species, such as rainbow trout, perch, and brown trout, prefer at advanced age to feed on small fish and invertebrates from nonbenthic origing (Rask 1986; Koli 1998; Horppila et al. 2000). Probably the most pelagic species, coregonid whitefish, feeds on zooplankton (Koli 1998). Roach, a benthic fish, feeds typically on sediment-associated invertebrates (Rask 1989; Horppila et al. 2000), and may thus be exposed to compounds that are reversibly sorbed to sediment particles.

Wood rosin is representative of the RA composition of pulp industry effluents. The initial concentration 60 μg/l of RAs was chosen to demonstrate a realistic situation in the effluents of modern pulp and paper mill or the waters adjacent to it. Since, from the physiological point of view, the lowest observed effective concentration (LOEC) of DHAA is around 20 μg/l (Oikari et al. 1984), the proportion of 33% in rosin is appropriate as it is equal to 20 μg/l of DHAA of total RAs. In addition, we aimed to produce RA concentrations in fish bile without any severe physiological effects. Instead of presenting the results for seven RAs separately, we grouped them into pimaric- and abietic-type RAs. Additionally, as examples of individual RAs, the results of pimaric acid and DHAA, the predominant RAs, are presented. In the stock solution, the pimaric-type comprised 14% and the abietic-type 86% of total RAs.

The concentrations of RAs during the experiments decreased to 23 μg/l despite the low biomass ratios, as in a previous study (Meriläinen et al. 2007). Most importantly, although the original level of RAs set in the beginning of the 7-day experiment changed, the concentrations were identical for the five species, allowing direct comparison between species.

The ratios of pimaric- and abietic-type RAs changed slightly from water to bile. In water, abietic-type acids were predominant (77%) whereas in the bile of most fish species it was lower (56–77%). However, in rainbow trout the proportion of pimaric-type acids increased by as much as 54%. The same phenomenon was observed in our previous study with brown trout (Meriläinen et al. 2007), but to an even higher extent. It was explained by the lower water solubility of pimaric-type RAs than dehydroabietic acid, isopimaric acid being the least soluble (Peng and Roberts 2000).

According to Brumley et al. (1998), the feeding status of fish at the time of sampling influences the measurement of bile metabolites of organic compounds. Since the laboratory exposures were conducted in identical conditions, bias from differences in physical or chemical conditions can be excluded. Nutritionally, the fishes were treated rather similarly, i.e. kept unfed for the last 3 days. Regarding to dynamics of bile production, the gallbladder of the rainbow trout refills 48–72 h after feeding has stopped (Förlin and Wachtmeister 1989), which also proved to be the case in this study. Although the perch and roach used in the experiment were taken from the wild, they were kept under laboratory conditions for 2 weeks, and were fed with same food as the other species. Thus we harmonized the bile accumulation into the bladder by keeping all fish unfed for 4 days before the start of the exposure. Therefore the hepatobiliary excretion of RAs was strictly due to species differences in this function, not their feeding status or living habitats in the aquatic environment.

When comparing the bile concentrations of RAs between the five species, whitefish had the highest concentrations, seven times higher that of perch, which was the lowest. Moreover, roach, brown trout and rainbow trout had two to three times the bile concentrations of RAs found in perch. According to Karels et al. (2001), who studied an area near the same pulp and paper mill as in the present study, the concentration in bile was 20% higher in roach than in perch.

Can sediments contribute to the exposure of fish to RAs?

The laboratory exposure was conducted after the field monitoring to clarify the reason for the different levels of RAs in the biles of perch and roach. Since roach always had a higher RA concentration in the bile and thus higher log $BCF_{bile(RA)}$ values than perch, we considered the possibility that a part of the exposure status of roach to RAs may derive from sediment origin. Perch are assumed to accumulate RAs primarily via the water compartment. One possibility to assess this numerically would be through the introduction of perch/roach-ratios, from both the field and laboratory experiments. Even though roach bioconcentrated over two times more RAs in the bile than perch in the simultaneous laboratory exposure to RAs (average perch/roach-ratio 0.40), expressing solely a species difference, wild roach in Southern Lake Saimaa, e.g. at 1 km from the mill, had 18 times higher bile RA concentrations than perch. Since high concentrations of RAs exists in the top 5 cm of the sediment at 1 and 3 km from the mill (Leppänen and Oikari 1999; Lahdelma and Oikari 2005; Lahti 2006), we suggest that a significant fraction of the RA exposure of roach derived from sediments contaminated by RAs. In respect to our hypothesis, in July 2003 after the black liquor spill, the highest RA concentration of water of all the sampling periods was in the area nearest to the mill where fish were mainly exposed via water. By June 2004, the water RA levels had decreased, but simultaneously increased exposure by sediment in the 1 km area followed. This situation can be compared to the water-only exposure situation in the laboratory exposure to RAs. In June 2004, the perch/roach-ratios 1 km from the pulp mill were lower than those in the laboratory exposures, possibly indicating an additional exposure to roach of RAs. Additionally, in May 2004 RA concentrations in fish bile showed a slight increase, which may be partially due to sediment exposure, and upwelling of contaminants from the lake bottom during the annual mixing of the water column. The same phenomenon had been observed earlier in May 1996, when the perch/roach-ratio was around 0.30 in the 1 km area, indicating possible exposure to roach of sediment (Karels and Oikari 2000). However, in winter 1997, the perch/roach-ratio was around 0.8 at 1 km from the mill (Karels et al. 2001), indicating similar exposure of the two species. The reason for the higher ratio during winter may be a significant stratification of the BKME layer at the bottom of the lake in Southern Lake Saimaa downstream from the mill during that season (Seppälä 1986), exposing both perch and roach equally to RAs in the effluent layer. Moreover, after the annual mixing of the water column in spring, including the BKME layer, the exposure of roach to sediment increases. In conclusion, the perch/roach-ratio may indicate an additional source of exposure of roach to RAs in the 1 km area downstream from the mill. We suggest that the use of this ratio may be useful in monitoring situations (Table 4).

Use of bile metabolites in exposure assessment

In exposure assessment, the existence of bile metabolites is evidence of bioavailability and the involvement of the excretory system. Since the concentrations of RAs in bile are up to 110,000 times higher than in the ambient water (Statham et al. 1976; Oikari et al. 1984; Brumley et al. 1998; Meriläinen et al. 2007), metabolites offer an effective tool to assess the level and origin of RAs and, thus, of BKME discharged regularly by environmental permit as well as due to an unscheduled spill, such as a spill of black liquors. With respect to the RAs in the bile of brown trout, recently studied in more detail (Meriläinen et al. 2007), it is possible to measure the exposure status of fish to RAs in time and concentration-dependent manner. Moreover, the method detects RAs in fish bile when the water concentrations are in the range of tens of nanograms per liter, levels that are common in wide lake areas adjacent to modern pulp and paper mills (Leppänen et al. 1998). We sought to harmonize the differences in biliary excretion of RAs between species by using the benchmark species, the rainbow trout. We propose that the ratios can be used as conversion factors in interspecies extrapolation, and for comparison of laboratory and field conditions. When the results from this study are combined with the previous ones, estimation of ambient RA concentrations can be performed with the aid of several species. However, this must be done with caution due to the possible differences between sexes, annual fluctuations and feeding status (Brumley et al. 1998; Jobling et al. 2002; Tyler et al. 2005), among other factors.

Conclusions

This study showed that RAs in fish bile can be used as effective biomarkers of exposure to pulp and paper mill effluents. Bile analyses are extremely sensitive and relatively quantitative as a tool for investigating water-related contamination by BKME and related emissions. When, in June 2003, 970 m^3 of black liquor was accidentally released into Southern Lake Saimaa, elevated concentrations of RAs in the bile of perch and roach were observed for a few weeks. The levels had decreased to the pre-spill situation by late July 2003, around 1 month post-spill. A clear relation, similar to that noted in previous years, between RA concentrations in fish bile and the distance from the mill was seen throughout the monitoring period (over 390 days). Additionally, differences between five fish species allow comparison and harmonization of exposure from one species in relation to another, reference species, e.g. the rainbow trout, possibly providing better toxicological knowledge. We conclude that by analyzing RAs in fish bile, it is possible to use them quantitatively as biomarkers of exposure under ecologically realistic circumstances.

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