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Effects of pulp and paper mill effluents on the reproductive and physiological status of perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) during the spawning period

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Reproductive and physiological parameters were studied during the spawning period in perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) downstream of two pulp and paper mills and reference sites in southern Lake Saimaa, Finland. Plasma 17β-estradiol (E_2) and testosterone (T) concentrations in female perch and roach decreased during the spawning period. At one of the mill sites, plasma E_2 and T concentrations in pre-spawn female and male perch and male roach were significantly higher. As compared with the reference sites, liver ethoxyresorufin *O*-deethylase (EROD) activity in exposed perch and male roach was 29%–91% higher, and for exposed female roach it was 53%–79% lower. In contrary to perch, EROD activity in male roach was higher than in females. Resin acids concentration in the bile of exposed fish (0.2–2.0 µg ml^{-1}) was higher than at the reference sites, whereas chlorophenolics concentration was similar (0.4–0.7 µg ml^{-1}). Results indicate that exposure to pulp and paper mill compounds still exists, although much lower than before implementation of ECF bleaching and activated sludge wastewater treatment at the mills. Effects of pulp and paper mill effluent on reproduction of spawning fish were inconclusive.

1. Introduction

It has been demonstrated that fish exposed to pulp and paper mill effluents evoke a variety of sublethal effects (Owens 1991, Sandström 1996). This can lead to fish population and community changes which are often associated with eutrofication and toxic compounds of the effluent (Neuman & Karås

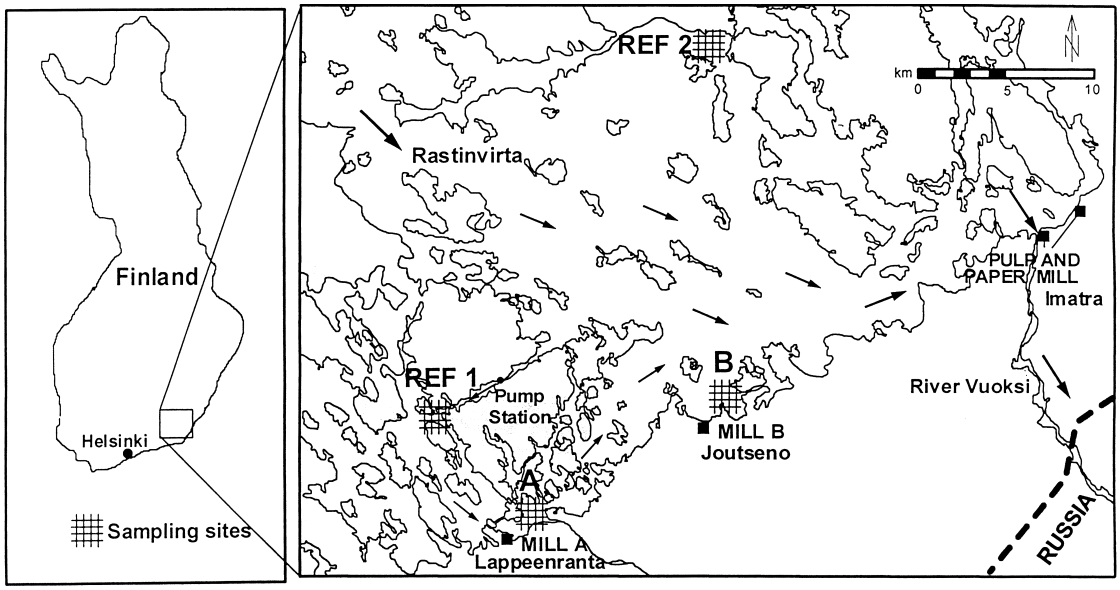

Fig. 1. The study area and the fish population sampling sites in southern Lake Saimaa, Finland. Arrows indicate the direction of the water flow in the area. The pump station keeps the water flow steady in the area receiving effluent from mill A.

1988, Hakkari 1992). For example, salmoniform fishes like lake trout, whitefish and vendace have disappeared from many Finnish waters receiving pulp and paper mill effluents, whereas certain other species, like perch and roach have survived (Hakkari 1992).

Recent studies, however, have demonstrated the effects of pulp and paper mill effluent on reproduction in fish. Decreased steroid hormone levels and gonad size were demonstrated in fish exposed to pulp and paper mill effluents (McMaster *et al.* 1991, 1992, Munkittrick *et al.* 1991, 1994, Gagnon *et al.* 1994, Sandström 1996, Van Der Kraak *et al.* 1998). The compounds responsible for the reproductive effects, nevertheless, have still not been identified. At first, it was suspected that persistent organochlorines caused these reproductive effects, but now it seems that wood derived compounds such as sterols, lignans, stilbenes and resin acids are responsible for the observed effects (Mellanen *et al.* 1996, Van der Kraak *et al.* 1998). However, some studies have not shown any critical reproductive effects (Van Der Kraak *et al.* 1998).

Since 1991, an intensive case study has been conducted at southern Lake Saimaa, in connection to major technological changes in the pulp and paper industry. After the introduction of elemental chlorine free (ECF) bleaching technologies and activated sludge treatment of effluents at the mills in the early 1990s, the exposure of fish to pulp and paper mill effluent compounds was dramatically decreased (Oikari & Holmbom 1996).

The interpretation of results of field studies is, however, often hindered by many seasonal, site and fish population specific factors (e.g., hydrology and dilution, effluent quality fluctuations, water temperature, age, sex and state of reproduction of the fish, diet, and fish migration patterns). In order to ascertain the possible effects of pulp and paper mill effluent on reproductive and other biochemical and physiological markers in feral fish, we suggested that more detailed information on natural levels and seasonal variations needed to be collected. Therefore in 1995, we started a 2-year field survey on perch and roach populations at southern Lake Saimaa, a lake contaminated by pulp and paper mill effluents since 1897. The main objectives of this research were at first to document natural levels and seasonal variations of reproductive parameters (e.g., steroid hormones, fecundity, gonad and egg size) and the liver cytochrome P450 1A enzyme during different stages of the reproductive cycle. Secondly, to measure the effects of modern pulp and paper

mill effluents on reproductive and other biochemical and physiological markers in feral fish. Three survey seasons, spring, fall and winter were chosen according to the annual reproductive cycle of perch and roach. Results of early vitellogenic perch and roach (autumn 1995) which showed decreased steroid hormone levels in pulp mill effluent exposed fish were already described in Karels *et al.* (1998). The results during the spawning period of perch and roach (spring 1996) are reported in this study.

2. Materials and methods

2.1. Study sites and research area

Southern Lake Saimaa (609 km^2) is a large oligotrophic lake, in southeast Finland. At the present time, three pulp and paper mills discharge their effluents into the lake (Fig. 1). Perch and roach were collected 1 to 2 km downstream from pulp and paper mill A and pulp mill B and at two reference sites (Fig. 1). The reference sites, located ca. 8 and 30 km upstream of the mills, were considered not to be influenced by the point sources of the pulp and paper mill effluents (Soimasuo *et al.* 1998). As the treated municipal effluents of the cities of Lappeenranta and Joutseno are not discharged into the lake, the pulp and paper mills are the primary source of contamination (chemicals, nutrients and log-floating) in the research areas. Water quality characteristics at the study sites in May–June 1996 are given in Table 1. A pump station, which displaces water from the clean area of Lake Saimaa to the watercourse upstream of mill A, causes a net water flow in the study area of mill A from west to north-east. As a result, the lake water passes the outlet point of mill A with a flow of about 40 m^3 s^{-1}, diluting its effluents. Measurements of sodium, an inert effluent tracer, suggests a theoretical concentration of approximately 3%–4% of pulp and paper mill effluent in the mixing zone, within 1 km of the outlet. The dilution ratio of the effluent of mill B is more variable, but measurements of sodium indicate concentrations from 1%–2% of pulp and paper mill effluent at mill site B.

2.2. Characteristics of the industrial processes and their effluents

Representative data on mill A and B effluents are given in Table 2. In 1996, mill A produced fully bleached hardwood and softwood pulp (ratio 45:55, total ca. 1 400 t d^{-1}) and paper (ca. 1 300 t d^{-1}). The factory also includes a saw mill and a chemical factory producing crude and refined tall oil as well as sterol products. Between 1 and 10

Table 1. Lake water quality in May 1996 at reference sites (REF 1 and 2) and mill sites (Mill A and B) in southern Lake Saimaa. Data are from the Saimaa Water Protection Association Inc., Lappeenranta, and are means of water column values from 1 m to near bottom (n.a = not analysed).

Parameter	REF 1	REF 2	Mill A	Mill B
pH	7.1	6.9	7.0	7.3
Oxygen (mg l^{-1})	12.1	12.1	11.0	10.7
Oxygen saturation (%)	103	96	94	91
Conductivity (mS m^{-1})	5.6	6.0	9.8	15.0
Colour (mgPt l^{-1})	35	38	85	70
Turbidity (FTU)	0.35	0.24	1.49	0.77
Na (mg l^{-1})	3.9	4.3	9.7	15
COD, Mn (mg l^{-1})	7.4	6.8	10.3	7.3
Total Resin acids(µg l^{-1})	1.0[a)]	0.1[a)]	n.a.	1.0[a)]
Chlorophyl *a* (µg l^{-1})	3.4[b)]	2.0[b)]	5.7[b)]	8.4[b)]
Total P (µg l^{-1})	11	7	23	40
Total N (µg l^{-1})	390	430	545	455

[a)] Data from Leppänen *et al.* (1998)
[b)] Mean from May and June.

May 1996, the pulp mill was shut down for a maintenance revision. Before 1992, mill A used mixed chlorine and chlorine dioxide bleaching by a D/$C_{(5\%–45\%)}$(EO)DED sequence. Since 1992, the company discontinued using molecular chlorine, and currently uses chlorine dioxide for bleaching by a sequence OD(EO)D(EP)D for hardwood and D(EOP)DED for softwood pulp (C = chlorine, D = chlorine dioxide, E = caustic soda, O = oxygen, P = peroxide). Before 1992, effluents from mill A were treated in aerated lagoons. Since April 1992, effluents have been treated in an activated sludge treatment plant.

In 1996, mill B produced fully bleached softwood pulp (total ca. 841 t d^{-1}) and used chlorine dioxide bleaching by a Do O/O D E D sequence (Do = chlorine dioxide extraction with oxygen). Before 1986, mill B effluents were treated in aerated lagoons, but thereafter, effluents have been treated in an activated sludge plant.

2.3. Sampling of fish populations

Eurasian perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) are abundant in the research areas. Both species are relatively non-migratory, so their conditions are assumed to reflect the quality of the environment at their catching sites. Fish were caught during the spawning period between 3–30 May 1996. All fish were caught with wire traps (1 cm square mesh) in the littoral zone at approximately 1 to 2 m depth. Traps were set at suggested spawning sites, e.g., close to submerged branches, twigs or aquatic vegetation. Captured fish were kept in the water, then gently removed from the traps, and placed into 200 litre cages to recover for 24 hours prior to sampling. The cages (1 cm square mesh, made of nylon), separate for perch and roach, were placed at about 2-m depth in order to minimize temperature variations and other possible disturbances. Depending on the size of animals, no more than 10 to 15 fish were held in one cage (max. 1 fish/13.5 litre). Capture stress was assumed to be equal at all sites.

For sampling, fish were stunned with a blow to the head, blood (200–500 µl) was drawn from caudal vessels into 1 ml heparinized syringes, placed on ice and centrifuged within 30 min for 3 min (12 000 × g) for plasma separation. Fish total weight (to the nearest 0.1 g) and fork length (in mm) were measured, and the bile (10–15 µl/fish) aspired from gall bladders using a hypodermic needle. The liver was dissected free and weighed to the nearest 0.1 g. Plasma, bile and liver tissue samples were immediately stored in liquid nitrogen for later analyses. Sex, gonad weight (to the nearest 0.1 g) and the stage of spawning were determined for each fish. Three stages of spawning were detected: pre-spawn, spawning and post-spawn. In the pre-spawn stage, milt or eggs do not run with a slight pressure on the belly. At the spawning stage, eggs and milt run with slight pressure. At the post-spawn stage, the ovary of the female is empty; males that still delivered some remains of milt after pressure of the abdomen were also considered as post-spawn. Ovaries from pre-spawn fish were preserved in 10% buffered formalin and later sub-sampled for egg counts. The relative fecundity (number of eggs per fish wet weight) of pre-spawn female fish was calculated to reduce the bias due to age and size of the fish. The entire sampling procedure for each fish required about 10 minutes. Opercular bones of perch and roach were taken for age determination. The gonad free condition factor (CF) was calculated as CF = 10^5 × (total weight (g) – gonad weight

Table 2. Average mill effluent characteristics in May 1996. Data are the mean from daily measurements. Analyses were conducted by the environmental laboratories of UPM-Kymmene Inc. Kaukas, Lappeenranta and UPM-Kymmene Inc. Joutseno Pulp, Joutseno (n.a. = not analysed).

Parameter	Mill A	Mill B
Flow (m^3 d^{-1})	99 000	57 300
Colour (mgPt l^{-1})	1083	n.a.
Conductivity (mS m^{-1})	191	n.a.
pH	8	8
Suspended solids (t d^{-1})	1.7	1.3
Na (mg l^{-1})	326	470
COD (t d^{-1})	29.8	36.3
BOD_7 (t d^{-1})	0.6	1.7
AOX (t d^{-1})	0.27	0.20
Total Chlorophenolics (µg l^{-1})	8.20[a)]	7.60[a)]
Total Resin acids (µg l^{-1})	94[a)]	659[a)]
Phytosterols (µg l^{-1})	214[a)]	875[a)]
Total N (kg d^{-1})	293	320
Total P (kg d^{-1})	20	66

[a)] Data from Leppänen *et al.* (1998)

(g))/fork length^3 (mm). Liver (LSI) and gonad somatic indices (GSI) were calculated as LSI or GSI = 100 × tissue weight, (g)/(total weight (g) – gonad weight (g)). Thus, in calculations of CF, LSI and GSI total body weight was adjusted for gonad weight to minimize the bias due to variations in sexual maturation.

2.4. Analytical methods

Plasma concentrations of E_2 and T were measured with E_2 and T enzyme immunoassay test kits of Orion Diagnostica, Finland. The plates were read with a plate reader (Labsystems EMS Reader MF V2.7-0, Finland) at 405 nm. Recoveries were checked by internal additions of fish plasma and steroid standards. The detection limit for T was 0.2 nmol l^{-1} and for E_2 0.04 nmol l^{-1}. Plasma calcium (Ca^{2+}) concentration was measured by Boehringer Mannheim GmbH test kit No 1553 593.

The 7-ethoxyresorufin *O*-deethylase (EROD) activity of liver microsomes was measured fluorometrically according to the method of Burke *et al.* (1985), adapted for the microplate format. Microsomal fractions were prepared as described in Karels *et al.* (1998). Liver samples of approximately equal weight from one to four fish, of equal sex and spawning condition, were used for one analysis. Positive controls for EROD analysis were liver samples from rainbow trout (*Oncorhynchus mykiss*) dosed by i.p. injection with 100 mg kg^{-1} β-naphtoflavone (BNF) in corn oil. Protein concentration of the microsomes was measured with a Bio-Rad DC protein assay kit, with bovine serum albumin as standard.

Total (conjugated and free) chlorophenolics (CPs) and resin acids (RAs) in the bile were measured using published procedures (Oikari & Ånäs 1985, Hemming *et al.* 1992). The following phenolics were quantified: 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, pentachlorophenol, 3,4,5-trichloroguaiacol, 4,5,6-trichloroguaiacol and tetrachloroguaiacol. The resin acids analysed were pimaric, isopimaric, levopimaric, sandaracopimaric, abietic, dehydroabietic, palustric and neoabietic acid. Samples of approximately equal volume from two to seven fish were combined for one analysis. Chlorophenolics and resin acids were quantified by gas chromatography (GC), using a Perkin-Elmer Autosys XL instrument equipped with electron capture detection and a 25 m NB-54 capillary column (i.d. of 0.32 mm).

For statistical analyses sexes were treated separately. All data were first assessed for normality and homogeneity of variance. Length and weight were compared with one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Data were $\log_{10}$-transformed where appropriate. Although CF, LSI and GSI are used for presentation purposes, estimates of condition, liver size, gonad size, fecundity and egg size were compared by analysis of covariance (ANCOVA), with site as a fixed factor. The adjusted body weight (body weight – gonad weight) was used as a covariate to eliminate possible effects of altered gonad weight. The non-parametric Kruskall-Wallis test was used to compare age, steroid hormones, calcium, liver EROD, bile chlorophenolics and resin acids data between sites. The significance of all tests was set at $p < 0.05$. In the tables and figures, mean and the standard error of the mean (SEM) are given. Correlations with two-tailed significance were determined with the non-parametric Spearman Rank Correlation Coefficient. Statistics was performed with SPSS software (Statistical Product Service Solutions, Chicago, IL, US).

3. Results

3.1. Spawning of perch and roach

Between 3 and 30 May 1996, a total of 195 perch and 114 roach were sampled. Perch and roach were actively spawning and densities of fish were high at the study sites. The spawning peak period for perch was between 7 and 15 May and for roach between 20 and 27 May. Male perch and roach entered the spawning grounds 1 to 2 weeks before the majority of female fish. The duration of the spawning period of perch lasted several weeks, whereas that of roach peaked for a few days only. The first spawned egg strands of perch were observed on 5 May at mill site A, 10 and 11 May at mill site B and reference site 1, and on 23 May at reference site 2. Water temperatures at 1 meter depth on these days at these sites were 5.0, 5.0, 5.1 and 5.9 °C, respectively. The majority of male perch (> 97 %) were in the spawning stage.

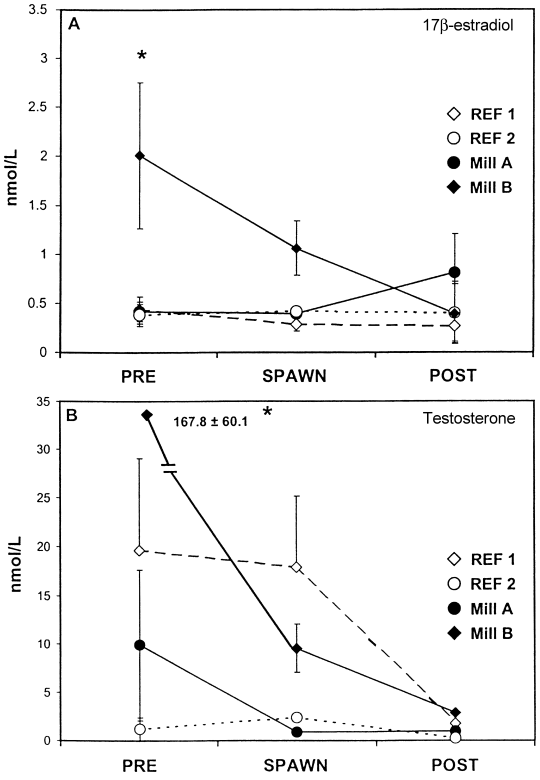

Fig. 2. Plasma 17β-estradiol (A) and testosterone (B) concentrations in female perch at reference sites and mill sites in southern Lake Saimaa in May 1996. Fish were classified as pre-spawn (PRE), spawning (SPAWN) and post-spawn (POST). Values are the mean ± SEM. Statistical difference from reference site 1: * = $p < 0.05$. Sample sizes for pre, spawn and post-spawn perch at the study sites were, respectively: REF 1 (Fig. 4A): 17, 3, 4; REF 1 (Fig. 4B): 19, 4, 4; REF 2: 2, 1, 5; Mill A: 25, 2, 2; Mill B: 18, 6, 5.

The first spawning female roach were observed on 19 May at mill site A, 24 and 25 May at mill site B and reference site 1 and 27 May at reference site 2. Water temperatures at 1 meter depth on these days at these sites were 8.7, 10.9, 10.0, and 9.0 °C, respectively. The majority of male roach (> 95 %) were in the spawning stage.

We started sampling before the spawning peak periods of the fish, immediately after the melting of the ice in the first week of May. Perch was sampled at all study sites and roach was sampled at the mill sites and reference site 2. We were logistically unable to sample roach at reference site 1, because the spawning (and catching) of roach occurred in a very short period of time.

3.2. Length, weight, age, condition factor and relative liver size

Random sampling of adult spawning populations indicated some differences in animal sizes and age between the study sites (Table 3). Perch at reference site 1 and mill site A were smaller than at the other two sites. Perch at mill site A was younger compared to the other sites. Remarkably, perch older than 4+ years did not occur in the catch at mill site A. The condition of perch and roach was the same among sites (ANCOVA, $p > 0.05$). The liver size of male perch and female roach at both mill sites was higher compared to the references (ANCOVA, $p < 0.05$).

3.3. Relative gonad size and fecundity

Because numbers of pre-spawn female roach were too small, statistical comparisons between sites were only made for pre-spawn female perch at reference site 1 and mill sites A and B (Table 4). The gonad size and relative fecundity of pre-spawn female perch was the same among sites (ANCOVA, $p > 0.05$). A comparison between gonad size of male fish was not made because the majority (> 97%) of the sampled fish were in the spawning stage, when milt is running with a slight pressure on the belly, making relative gonad size values unreliable.

3.4. Plasma steroid hormones

3.4.1. Female fish

Plasma steroid hormone concentrations are given at three reproductive stages (*see* material and methods): pre-spawn (PRE), spawning (SPAWN) and post-spawn (POST). In general, plasma E_2 and T concentrations in female perch (Fig. 2) and female roach (Fig. 3) showed, despite the variability, a decreasing trend during the spawning period. Because sample sizes were occasionally limited for some of the reproductive stages, comparisons between sites were only made for pre-spawn female perch at reference site 1 and mill sites A and B (Fig. 2; sample size = 19, 25 and 18,

respectively). Plasma E_2 and T concentrations in pre-spawn female perch at mill site B were significantly higher compared to reference site 1 (Kruskall-Wallis, $p < 0.05$).

3.4.2. Male fish

In male perch, plasma E_2 and T concentrations were significantly different between reference sites (Table 5). Plasma hormone concentrations in male perch and roach at mill site B were higher than at the reference site(s) (Kruskall-Wallis, $p < 0.05$).

3.5. Plasma calcium concentration

We measured plasma calcium as an indirect marker of VTG, a calcium binding lipophosphoprotein (Matty 1985). In female fishes, VTG is the egg yolk precursor protein synthesised in the liver in response to circulating estrogens like E_2. It is transported in the blood to the ovary (Matty 1985). Within sites, plasma calcium was similar among spawning stages (Table 6). Plasma calcium was higher in female than in male fish ($p < 0.05$). As compared with the reference site, male roach at mill sites A and B had significantly lower plasma calcium concentrations ($p < 0.05$).

Table 3. Number of samples taken (*n*), length, weight, age, condition factor (CF) and liver somatic index (LSI) of female and male perch and roach collected in spring 1996, 1–2 km downstream of pulp and paper mills A and B, and at reference sites 1 and 2. Site differences of length and weight were tested with ANOVA, age with nonparametric Kruskall-Wallis test and CF and LSI with an analysis of covariance. In a row, within species and sex, differences between sites ($p < 0.05$) are denoted with letters.

	Site	*n*	Length (cm)	Weight (g)	Age (years)	CF	LSI
Perch							
Females	REF 1	27	16.0 ± 0.5^{a}	48.4 ± 6.6^{a}	5.3 ± 0.3^{a}	0.84 ± 0.02^{a}	1.14 ± 0.05^{a}
	REF 2	8	18.6 ± 1.5^{b}	90.8 ± 39.9^{ab}	7.1 ± 1.7^{a}	0.98 ± 0.06^{a}	1.28 ± 0.09^{a}
	Mill A	29	14.2 ± 0.3^{c}	33.2 ± 1.7^{c}	3.4 ± 0.1^{b}	0.86 ± 0.01^{a}	1.21 ± 0.05^{a}
	Mill B	29	17.9 ± 0.4^{b}	69.0 ± 5.6^{b}	5.9 ± 0.4^{a}	0.94 ± 0.02^{a}	1.30 ± 0.05^{a}
Males	REF 1	30	14.9 ± 0.5^{a}	38.2 ± 6.5^{a}	4.9 ± 0.4^{a}	0.93 ± 0.02^{a}	1.08 ± 0.07^{a}
	REF 2	11	17.0 ± 0.8^{b}	51.1 ± 8.3^{b}	5.2 ± 0.4^{a}	0.94 ± 0.05^{a}	0.96 ± 0.10^{a}
	Mill A	31	14.3 ± 0.2^{a}	30.7 ± 1.3^{a}	3.8 ± 0.2^{b}	0.99 ± 0.01^{a}	1.36 ± 0.06^{b}
	Mill B	30	16.3 ± 0.3^{b}	48.8 ± 3.2^{b}	4.9 ± 0.3^{a}	1.03 ± 0.02^{a}	1.44 ± 0.06^{b}
Roach							
Females	REF 2	19	20.1 ± 0.4^{a}	101.2 ± 7.6^{a}	8.8 ± 0.5^{a}	1.12 ± 0.02^{a}	1.13 ± 0.04^{a}
	Mill A	10	18.6 ± 0.4^{b}	75.6 ± 6.8^{b}	8.6 ± 0.7^{a}	1.10 ± 0.04^{a}	1.27 ± 0.09^{b}
	Mill B	17	19.1 ± 0.3^{b}	91.1 ± 6.1^{a}	8.8 ± 0.5^{a}	1.12 ± 0.02^{a}	1.56 ± 0.13^{c}
Males	REF 2	22	17.6 ± 0.3^{a}	62.8 ± 3.0^{a}	7.0 ± 0.3^{a}	1.10 ± 0.01^{a}	1.49 ± 0.07^{a}
	Mill A	11	17.3 ± 0.3^{a}	60.9 ± 3.6^{a}	9.0 ± 0.6^{b}	1.11 ± 0.02^{a}	1.54 ± 0.05^{a}
	Mill B	35	17.5 ± 0.2^{a}	65.0 ± 2.3^{a}	7.9 ± 0.3^{b}	1.13 ± 0.01^{a}	1.50 ± 0.04^{a}

Table 4. Number of samples taken (*n*), gonad somatic index (GSI), egg size and relative fecundity (number of eggs per gram wet weight of fish) of pre-spawn female perch at mill sites and reference site 1 in spring 1996. Values are the mean ± SEM. Site differences were tested using analysis of covariance. In a row, differences between sites ($p < 0.05$) are denoted with letters.

Site	*n*	GSI	Egg size (mm)	Relative fecundity
REF 1	19	30.9 ± 1.0^{a}	1.41 ± 0.05^{a}	135 ± 11^{a}
Mill A	25	35.3 ± 1.7^{a}	1.54 ± 0.03^{b}	116 ± 9^{a}
Mill B	18	25.7 ± 1.1^{a}	1.38 ± 0.05^{a}	134 ± 11^{a}

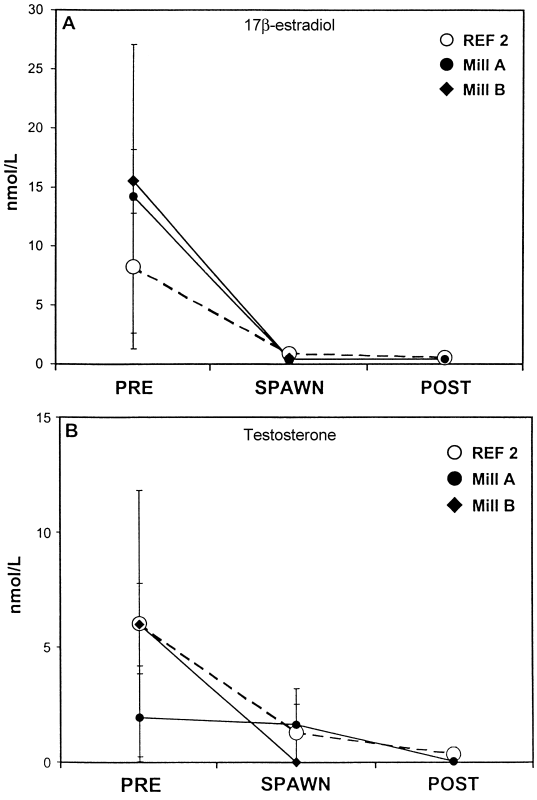

Fig. 3. Plasma 17β-estradiol (A) and testosterone (B) concentrations of female roach at reference sites and mill sites in southern Lake Saimaa in May 1996. Fish were classified as pre-spawn (PRE), spawning (SPAWN) and post-spawn (POST). Values are the mean ± SEM. Mill sites were not statistically different from reference site 2: $p > 0.05$. Sample sizes for pre, spawn and post-spawn roach at the study sites were, respectively: REF 2: 4, 6, 9; Mill A: 2, 3, 5; Mill B: 15, 2, 0.

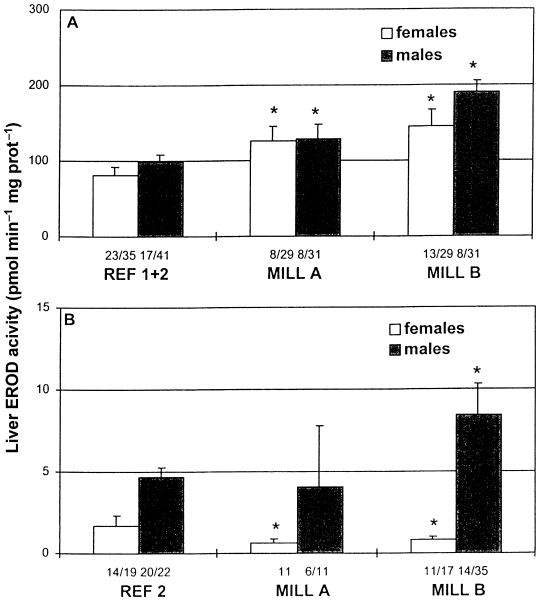

Fig. 4. Liver EROD activity (pmol min^{-1} mg prot^{-1}) of female and male perch (A) and female and male roach (B) in southern Lake Saimaa in May 1996. Number of pooled samples analysed/number of animals, are given below each pair of columns. Values are the mean ± SEM. EROD in perch at the mill sites was compared with combined reference sites 1 and 2, and roach with reference site 2. Statistical difference from reference site(s): * = $p < 0.05$.

3.6. Liver ethoxyresorufin *O*-deethylase activity

Within sites, liver EROD activity was the same among the three spawning conditions. Therefore, data of pre-spawn, spawn and post spawn fish were combined for statistical analysis. EROD activity

Table 5. Plasma 17β-estradiol and testosterone concentrations (nmol l^{-1}) and number of samples taken (n) of spawning male perch and roach at mill and reference sites in spring 1996. Values are the mean ± SEM. Site differences were tested with the Kruskall-Wallis test. In a row, differences between sites ($p < 0.05$) are denoted with letters.

Species	Site	17β-estradiol	n	Testosterone	n
Perch	REF 1	0.42 ± 0.07^a	30	48.53 ± 6.79^a	30
	REF 2	0.20 ± 0.04^b	10	4.20 ± 2.11^b	10
	Mill A	0.39 ± 0.09^a	30	45.76 ± 7.77^a	31
	Mill B	0.85 ± 0.10^c	28	88.80 ± 9.60^c	30
Roach	REF 2	0.23 ± 0.03^a	22	4.26 ± 1.36^a	22
	Mill A	0.17 ± 0.02^a	11	3.30 ± 0.90^a	11
	Mill B	0.41 ± 0.02^b	35	8.14 ± 2.04^b	35

of perch at the reference sites was the same ($p > 0.05$) and data were combined for increased statistical power. As compared with the reference, EROD activity was 29%–91% higher in exposed female and male perch near both mills and in male roach at mill site B ($p < 0.05$). EROD activity in exposed female roach, on the contrary, was 53%–79% lower than the reference ($p < 0.05$). In contrast to perch, male roach showed 3 to 11-fold higher EROD activity than females (Fig. 4). Interestingly, as a species characteristic, the EROD activity of perch was up to 30 times that of roach.

3.7. Chlorophenolics and resin acids in the bile

In order to associate biological effects to the pulp and paper mill effluents, the exposure was assessed by measuring the concentrations of chlorophenolics (CPs) and resin acids (RAs) in the fish bile. The concentrations of CPs in the bile of perch and roach were the same among sites (Fig. 5). On the other hand, RAs concentrations in the bile of exposed perch and roach was 3–124 times that of the reference ($p < 0.05$).

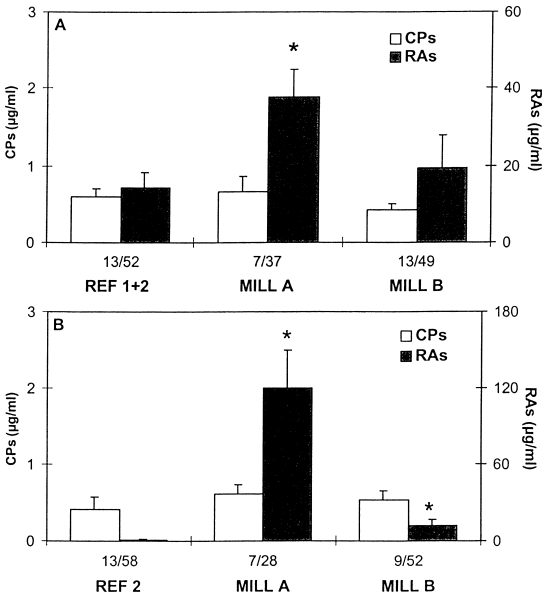

Fig. 5. The sum of conjugated and free chlorophenolics (CPs) and resin acids (RAs) in the bile (µg ml^{-1} bile) of perch (A) and roach (B) in southern Lake Saimaa in May 1996. Number of pooled samples analysed/number of animals, are given below each pair of columns. Values are the mean ± SEM. EROD in perch at the mill sites was compared with combined reference sites 1 and 2, and in roach with reference site 2. Statistical difference from reference site: * = $p < 0.05$.

4. Discussion

4.1. Somatic indices

Although measurements of CF and LSI are crude and prone to the effects of non-pollutant factors (e.g., season, disease, biological rhythms), they may serve as a first tier screen indicative of exposure and effect (Mayer *et al.* 1992). In the present study, the condition of fish exposed to pulp and paper mill effluents was not affected. In a review of Sandström (1996), covering 25 reported CF

Table 6. Plasma total calcium concentrations (mmol l^{-1}) and number of samples taken (*n*) of female and male perch at mill and reference sites in spring 1996. Values are the mean ± SEM. Site differences were tested with the Kruskall-Wallis test. In a row, differences between sites ($p < 0.05$) are denoted with letters.

Species	Site	Plasma calcium			
		Females	*n*	Males	*n*
Perch	REF 1	2.50 ± 0.10^{a}	27	1.76 ± 0.09^{a}	30
	REF 2	3.20 ± 0.34^{b}	8	2.21 ± 0.17^{b}	11
	Mill A	2.70 ± 0.09^{c}	29	2.00 ± 0.07^{b}	31
	Mill B	3.12 ± 0.17^{b}	29	2.07 ± 0.05^{b}	30
Roach	REF 2	3.06 ± 0.16^{a}	19	2.39 ± 0.08^{a}	22
	Mill A	2.70 ± 0.20^{a}	10	2.10 ± 0.09^{b}	11
	Mill B	2.78 ± 0.13^{a}	17	2.20 ± 0.07^{b}	35

estimations, a higher CF in fish exposed to pulp and paper mill effluents was noted in 12 studies (sometimes, however, only in one sex) and a lower CF only in two studies. The CF may provide information on energy reserves and may be affected if food is limited or if food consumption is impaired due to stressors.

A higher LSI, as measured in exposed male perch and female roach in the present study, can be associated with elaboration of cellular structures such as the endoplasmatic reticulum for protein synthesis (Andersson *et al.* 1988), or with a high carbohydrate diet resulting in carbohydrate storage (Dixon and Hilton 1985). An increased LSI has often been observed in fish exposed to various types of pulp and paper mill effluents (Andersson *et al.* 1988, McMaster *et al.* 1991, Huuskonen & Lindström-Seppä 1995, Karels *et al.* 1998).

4.2. Reproductive biology and physiology

In the present study, the act and timing of spawning of perch and roach was not severely affected at the pulp mill sites. Fish at the mill site A, however, tended to spawn earlier than at the other sites, most likely because of the slightly higher water temperatures downstream of mill A.

The gonad size and fecundity in pre-spawn perch was similar among sites. In the review of Sandström (1996), no effect on gonad size was measured in 9 studies and a decrease in 14 studies on fish exposed to pulp and paper mill effluents. In the relatively few studies on the effects of pulp and paper mill effluents on fecundity, exposed fish showed contradictory results (Sandström 1996).

In the present study, plasma E2 and T levels in female fish decreased during the spawning period. This has been observed earlier in many other fish species (Liley & Stacey 1983, Matty 1985).

In the present study, as compared with the references, plasma E_2 and T concentrations in pre-spawn perch and spawning male roach at the mill site B were higher, and in perch and roach at mill site A similar. These observations are in contradiction with the results of an earlier study at southern Lake Saimaa which showed lower plasma E_2 and T levels in early vitellogenic perch and roach downstream of mill A (Karels *et al.* 1998). Similarly, several other studies have shown that exposure to pulp and paper mill effluents can lead to reduced levels of sex steroid hormones in fish (McMaster *et al.* 1991, 1992, Munkittrick *et al.* 1991, 1992, 1994, Gagnon *et al.* 1994, Van der Kraak *et al.* 1998). Some other studies, however, reported no differences in steroid hormone concentrations of fish exposed to pulp mill effluents (Van der Kraak *et al.* 1998). The large variability in steroid hormone concentrations between and within sites in the present study was probably caused by the large physiological and endocrine temporal instability during the spawning period.

Steroid hormone concentrations in perch and roach were neither correlated with length and weight nor with the exposure biomarkers liver EROD activity and bile conjugates. Although cause–effect relationships are difficult to establish, it was remarkable that the effluent of mill B, compared to mill A, showed the highest concentrations of phytosterols, of which 71% β-sitosterol (Leppänen *et al.* 1998). Phytosterols, like β-sitosterol have demonstrated alterations on the reproductive status in a number of fish species (Mellanen *et al.* 1996, Van Der Kraak *et al.* 1998).

Plasma calcium was used to measure indirectly possible changes in plasma VTG. Results indicate that exposure to pulp and paper mill effluents had no effect on calcium, and thus VTG, levels of fish.

4.3. Liver mixed function oxygenase responses

Measurement of the liver EROD activity is a common method to examine the catalytic activity of cytochrome P450 1A (CYP 1A), an important mixed function oxygenase (MFO) isoenzyme catalyzing phase 1 reactions of xenobiotic compounds. A higher liver EROD activity has been the most consistent response observed in fish exposed to pulp and paper mill effluents (Hodson 1996, Sandström 1996). Inducers of CYP 1A isolated from ECF pulp and paper mill effluents exhibit properties of PAHs, but their full identity has remained unclear (Hodson 1996). Tentative candidates, are retene and other related microbial PAH-like metabolites derived from resin acids, which are

present in and bioavailable from sediments contaminated by pulp and paper mill effluents (Billiard *et al.* 1999, Leppänen & Oikari 1999).

In the present study, EROD activity was higher in perch at both mill sites and in male roach at one of the mills. In an earlier study at mill site A, a higher EROD activity was also reported in early vitellogenic roach, but it was similar in perch (Karels *et al.* 1998). In the present study, however, a reduced EROD activity was measured in exposed female roach. A decrease in MFO activity was observed earlier in fish exposed to pulp and paper mill effluents (Lindström-Seppä & Oikari 1989, Jiminez *et al.* 1990, Huuskonen & Lindström-Seppa 1995). Male roach in the present study showed higher EROD activity than females. It was earlier demonstrated that EROD activity is higher in male than in female fish, and represents a sensitive, short-term indicator of exposure to pulp and paper mill effluents (Ahokas *et al.* 1976, Lindström-Seppä 1985, Jiminez *et al.* 1990, Munkittrick *et al.* 1994). EROD induction in fish in this study could be related to contaminants from the water phase (including suspended particles) as well as to sediment contamination. It is, however, difficult to determine how the 10 days' mill shutdown of mill A, which overlapped the first week of the present study, has affected the EROD activity in perch and roach. Munkittrick *et al.* (1992), for instance, showed that EROD activity in longnose sucker recovered within 2 weeks after a mill shutdown. EROD activity in perch at mill site A, however, was higher than the reference sites, indicating that EROD activity was not recovered due to the 10-days shutdown of the mill.

EROD activity was not correlated with length, weight, LSI, GSI, steroid hormone concentrations or spawning stage in the perch or the roach populations.

4.4. Assessment of exposure by bile chlorophenolics and resin acids

In order to show more conclusively the connection of ecological and ecotoxicological effects in feral fish, demonstration of ongoing exposure to pulp and paper mill effluent-related components is necessary. Bile conjugates of CPs and RAs, serving this purpose, were used as biomarkers of exposure to pulp and paper mill effluent. Previous studies have shown that these biomarkers serve as sensitive indicators of exposure of fish to pulp and paper mill effluent (Oikari & Ånäs 1985, Söderström & Wachtmeister 1992, Oikari & Holmbom 1996). In the present study, higher RAs concentrations in the bile of the fish at the mill sites were indicative of exposure to pulp and paper mill effluent. Earlier studies showed that as a result of the process changes at mill A in 1992, CPs and RAs concentrations in the bile of caged and feral fish have decreased considerably until 1996 (Oikari & Holmbom 1996, Soimasuo *et al.* 1998, Leppänen *et al.* 1998, Karels *et al.* 1998). In the present study, bile CPs levels in perch and roach were similar among sites. Whitefish caged between 15 May and 19 June 1996, 1 km downstream of the discharge point of mill A and B, however, showed bile CPs concentrations 2 to 4 times that of the references (Leppänen *et al.* 1998). The clearance half-rate time of CPs in the bile of fish is in the order of 3–4 days (Oikari *et al.* 1999). This indicates that factors like the hydrological variability near mill B and the shutdown of mill A between 1 and 10 May in 1996, partially overlapping the sampling, could have reduced the exposure of feral fish to CP compounds.

5. Conclusions

Results of bile conjugates and liver EROD activity in perch and roach at the mill sites indicate that exposure to pulp and paper mill compounds still exists, although much lower than before the implementation of ECF bleaching and activated sludge wastewater treatment at the mills in the early 1990s. Plasma steroid hormone levels of E_2 and T in female perch and roach decreased during the spawning period and were higher in fish at mill site B. Cause and effect relationships, however, are inconclusive because of many natural environmental and biological factors affecting the endocrine regulation of spawning fishes. Therefore, the spawning period of fish is probably not preferred to study effects of pollutants on sex steroid hormones and other reproductive parameters, unless the determination of the spawning stage of the fish can be conducted very precisely from large enough number of animals.

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