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Biomarker responses in female rainbow trout exposed to untreated and secondary treated whole mill effluent from production of TCF-bleached sulphate pulp

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Juvenile, female rainbow trout (*Oncorhynchus mykiss*) were exposed for 8 weeks to 1:400 dilutions of treated and untreated totally chlorine free bleached kraft mill effluents (BKME). Individual growth, feed consumption, bile conjugates, liver enzyme 7-ethoxyresorufin-*O*-deethylase (EROD), liver histology, liver glycogen, muscle lipid, hematology parameters and plasma 17β-estradiol levels were measured. Compared with the controls, no significant effects on growth, feed consumption, plasma 17β-estradiol and EROD were observed. A significant positive relationship between growth and plasma 17β-estradiol and muscle lipid content was found in fish exposed to treated effluent. The fish bile sterol levels did not correlate with the sterol levels analyzed directly from effluent samples. The treated effluent exposed fish excreted more cholesterol through bile than control fish. Livers of fish exposed to treated effluent had lower glycogen content and muscle lipid content than control fish. Only minor changes in liver structure and hematology of the BKME exposed fish were observed. Other studies has shown phytosterols to inhibite intestinal cholesterol absorbtion and this might explain why a higher liver energy metabolism was observed in fish exposed to treated effluent.

Introduction

During the past years, considerable development on pulp bleaching and effluent treatment technology has occured. In the pulp bleaching process, elementary chlorine has been substituted by elementary chlorine free (ECF) pulp bleaching using 100% chlorine dioxide or totally chlorine free (TCF) bleaching by using oxygen delignification, peroxide and ozone in the bleaching process in combination with extended cooking. In a modern kraft mill designed for ECF or TCF bleaching, the discharge of polychlorinated phenolics, dioxins or furans is not a matter of concern (Tana and Lehtinen 1996). Instead, non-chlorinated compounds such as resin and fatty acids, plant sterols and other wood extractives, as well as chelating agents are currently attracting the majority of interest. Several studies on metabolic and endocrine effects on fish exposed to pulp mill effluents have been published (Munkittrick *et al.* 1994, McLatchy and Van Der Kraak 1995, Soimasuo *et al.* 1995, Tremblay *et al.* 1995, Mellanen *et al.* 1999, Tremblay and Van Der Kraak 1999). As metabolism and steroid hormones are intimately connected, it has been hypothetically assumed that disturbances in the metabolism or the synthesis of body steroids could result in growth- and/or reproductional disturbances in fish (Lehtinen 1990, Ashfield *et al.* 1998). In a recent study it was shown that phytosterols occurring in the wood raw material affected fish reproduction and offspring development (Lehtinen *et al.* 1999). The exposure of the parental fish to phytosterols caused increased egg mortality, decreased weight of newly hatched fish larvae and stimulated growth after the yolk-sac stage. No appreciable effects on hematological parameters between unexposed and exposed fish were observed, an indication that conventional parameters, used in ecotoxicological studies on pulp mill effluents since the early 1980s, may not detect significant population regulating mechanisms on fish (Lehtinen *et al.* 1990, 1999).

The purpose of this work was to study potential metabolic and hormonal effects on female fish exposed to a total, untreated and active sludge treated pulp mill effluent from modern bleached TCF sulphate production.

Material and methods

Tested effluents

The pulp mill studied produces ECF (elementary chlorine free) and TCF (totaly chlorine free) bleached pulp in campaigns. During sampling in

Table 1. The production conditions during the effluent sampling and the specific load from the active sludge treated effluent at 48 m^3 Adt^{-1} (Air dry tonnes of pulp).

Production		1255 (Adt^{-1})
Brightness		86.1 (% ISO)
Kappa	— from digestion	32–36
	— after O_2 delignification	15–16
Washing loss, after O_2-stage		25.8 (kg COD (Chemical Oxygen Demand Adt^{-1})
TCF-bleaching sequence		(O)-A(Q)-EOP-PZQ-P (O = oxygen delignification; A(Q) = enzyme stage; EOP = alkalistage with oxygen and peroxide; PZO = peroxide ozone oxygen stage; P = peroxide)
Chemical use in the TCF bleaching		44 kg H_2O_2 Adt^{-1}
		28 kg NaOH Adt^{-1}
		1.35 kg DTPA Adt^{-1}
Water use in the bleach plant		20 m^3 Adt^{-1}

Specific load from the active sludge treated effluent

COD_{Cr}	12.90 kg Adt^{-1}
BOD_7 (Biological Oxygendemand)	0.10 kg Adt^{-1}
Suspended material	0.53 kg Adt^{-1}
AOX (Adsorbable Organohalogens)	0.03 kg Adt^{-1}

September 1998, the mill was producing TCF-bleached softwood pulp. Prior to sampling, a 2.5 day ECF-bleached pulp campaign had ended. The production conditions during the effluent sampling is presented in Table 1.

The effluent was collected on two occasions as follows:

i) Mechanically treated effluent, also containing small amounts of community sewage water (0.03%) from a small 1200 people town, before the effluent flowed into the aeration basin and before it mixed with the return sludge. The effect of sewage water is ignored in the interpretation of the results.
ii) Biologically treated, postsedimented effluent.

The process conditions in the activated sludge treatment plant were stable during the sampling period. The COD-reduction within the biological section of the treatment plant was 62%–66% while the BOD_7-reduction was 98%–99%. The active sludge composition was, according to microscopical judgement, normal. The oxygen level in the aeration stage was relatively high (2–3 mg O_2 l^{-1}), which is typical for modern active sludge treatment plants. The corresponding specific load from the active sludge treated effluent at an effluent flow of 48 m^3 Adt^{-1} is presented in Table 1

The effluent was analyzed by GC-MS method for extractives (fatty and resin acids and sterols), and organic- and suspended material and nutrients. (Table 2).

The chloride concentration in the biologically treated effluent was 75–88 mg l^{-1}, which indicates that some ECF effluent was left in the treatment plant, although TCF-pulp had been produced for 2.5 days. Also, the AOX analysis verifies this as the concentrations were higher in the secondary treated effluent compared to pre-treated effluent.

The sampled effluent was stored at 4 °C in 1 m^3 tanks. To study the chemical stability of the effluents, the chemical composition was analyzed from frozen (–20 °C) effluent samples, frozen immediately after sampling and compared with chemical results analyzed from 4 °C effluents dosed and sampled at the end of the experiment. The chemical analysis showed that the effluent stored at 4 °C generally had lower concentrations of resin acids and chlorinated phenols compared to frozen (–20 °C) effluent samples (data not shown). However, some fatty acids and sterols showed higher concentrations in the +4 °C effluent than in the frozen effluent, which might be an indication of breakdown of e.g. steryl esters (data not shown).

The effluents also contained some larger

Table 2. The content of fatty and resin acids, sterols, organic material and nutrients tested in untreated and treated whole TCF BKME. The reduction% is calculated.

	Untreated	Treated	Reduction%
Fatty acids (µg l^{-1})	586	59	90
Resin acids (µg l^{-1})	662	1	99
Sterols (µg l^{-1})	652	22	97
COD_{CR}	860	270	69
BOD_7	170	2	99
Susp.	96	11	88
TOC*	318	114	64
AOX** (µg l^{-1})	270	570	(+111)
Nitrate-Nitrite (mg l^{-1})	0.02	0.02	
Ammonium-N (mg l^{-1})	0.04	0.05	25
Tot-N (mg l^{-1})	12	4.2	65
Phosphate-P (mg l^{-1})	0.20	0.07	65
Tot-P (mg l^{-1})	1.0	0.23	77

* analyzed at Åbo Akademi, Institute of Forest Product Chemistry.
** analyzed at KCL-laboratory

unidentified components, one possible resin acid (only before treatment) and one group of components which eluted in the same region as sterols, i.e. relatively large molecules. This group of molecules seemed to be very stable and occurred in the same concentrations both before and after the treatment. One of the peaks could, according to its mass spectre, be 3,5-bis(1,1-dimethyl)-4-hydroxy benzene propanoin acid octadecyl ester.

Experimental set-up.

The fish exposure occurred indoors at the Finnish Environmental Research Groups Baltic Sea laboratory in Nagu, SW Finland. Juvenile, female, rainbow trout (*Oncorhynchus mykiss*) (1-year-old 105.5 ± 31.1 g [mean ± SD]), transported from a local fish farm (under veterinary medical supervision) to the laboratory were used. An all female population was used to eliminate any variation arising from the intersex differences. Fifteen fish were randomly each placed into nine, 500-l polyethane pools, equipped with a continuous flow (2.8 l min^{-1}) of brackish (6‰) water. The water in each pool was gently aerated.

The test fish were acclimatized to the laboratory conditions for one week, after which they were individually marked with the Panjet method (Johnstone 1981). During the marking, the fish were anaesthetized with benzocaine (4%) after which the length and weight was measured and a color code was marked on the abdominal skin of fish. Alcian blue was used as the marking color. After marking, the fish were further acclimatized for 10 days before effluent dosage was started. The fish were fed *ad libitum* daily during the whole experiment and the amount of feed given to each basin was noted. The feeding of the fish was terminated 4 days before sampling.

The fish were exposed to one dilution (1:400) of the tested effluents for 8 weeks, from 8 September to 3 November 1998. The 1:400 dilution has commonly been used and represents environmentally realistic exposure of fish in lake and coastal areas approximately 1 km outside the pulp mill. The tested effluents were directly pumped from the 1-m^3 tanks with membrane pumps (Prominent) to the respective test pools. Each treatment group consisted of 3 separate, randomly chosen, replicate pools. The experiment consisted of the following treatment groups: Control, Untreated and Treated (biologically) effluent exposure with 3 replicates and 15 fish in each replicate.

The water temperature dropped during the experiment from 15.0 °C to 9.7 °C and the water oxygen content varied between 8 and 9 mg l^{-1} (90% saturation).

Due to a pump failure, the water supply stopped for 12 hours during a weekend three weeks after the dosage start of effluent. This pump failure led to the loss of one control and one treated effluent-exposed replicate. In one of the untreated effluent exposed replicate, two fish died due to the pump failure. The growth and almost all other parameters differed significantly compared to the two other replicates within the treatment. Therefore, in the results section, two replicates from Control, Untreated and Treated have been used.

Sampling

After 8 weeks of exposure, the fish were caught individually with a dip net and stunned with a blow to the head. Blood was collected from the caudal blood vessels with a heparinized disposable syringe. Blood smears for differential cell counts of white blood cells and immature red blood cells were made on glass microscope slides. The blood sample was centrifuged (10 000 g, 5 min) after which the blood plasma was transferred to an Eppendorf tube which was frozen in liquid nitrogen for later sex hormone (17β-estradiol and testosterone) analysis. After blood sampling, both the length and the weight of the fish were measured. The body cavity was opened and the bile was sampled with a 2-ml disposable syringe. Bile from four fish was pooled, frozen (–20 °C), and stored for later analysis of conjugated chlorophenolics, resin acids, as well as other extractives. After the bile sampling, the liver was preparated out and weighed, cut into pieces, placed in Eppendorf tubes and frozen in liquid nitrogen for later analysis of enzyme activities and glycogen content. The liver sample

for enzyme analysis was taken from the central (ventral) part of the liver. Fresh liver samples for histological analysis were taken from the first ten fish in each group. For liver histology, a peripheral dorso-posterior (5 × 5 mm) liver tissue sample was fixed in Bouin's solution for 24 hours and transferred to 70% ethanol. The samples were kept in ethanol before dehydration and embedding in paraffin and sectioning (5 µm). Tissue samples were stained with hematoxylin-eosin. One muscle sample for lipid determinations was taken from the left dorsal muscle from a spot just under the front of the dorsal fin. Sampling time for each fish was less than four minutes after capture.

Parameters studied in this experiment are presented in Table 3.

During the sampling, macroscopic changes to fish skin, gills, fins and the interior organs were checked for parasites and clinical disease symptoms. No parasites and clinical diseases could be observed.

Analytical methods

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

The determination of conjugated chlorophenolics, resin acids and other extractives in bile was based on a modified method (Lehtinen and Tana 1992) orginally described by Oikari and Ånäs (1985).

Liver-somatic index (LSI = liver wet weight (g)/somatic weight (g) × 100) and condition factor (CF = total wet body weight (g) × 100/length^3 (cm)) were calculated according to standard formulae (Weatherley and Rogers 1978). Fish growth in each group was measured as percentage of (%) individual growth (% growth = [(weight at end) – (weight at start)] × 100). Feed coefficient (FC) was calculated as feed (g)/wet weight gain (g).

Xenobiotic transformation was studied by measuring the activity of the enzyme 7-ethoxyresorufin-*O*-deethylase (EROD), as part of the mixed-function oxidase (MFO) system (Hodson *et al.* 1991). Liver glycogen and muscle lipids were measured using standardized methods as described by Soivio and Virtanen (1980).

Liver tissue preparations were examined by with a light microscope. Different cell types were quantified using Chalkley's (1943) method, later described by Mitchell *et al.* (1973). The microscope was equipped with a micrometer scale in the eyepiece containing a grid with 16 randomly marked squares. The analysis was performed under 500× magnification so that

Table 3. Morphometry, metabolism, endocrinological and hematological status describing parameters studied (MCHC = mean cell hemoglobine concentration; MCH = mean cell hemoglobine; MCV = mean cell volume).

Morphometry	Metabolism	Sex hormone levels	Hematology
Length, cm	Liver glycogen,%	17b-estradiol, pmol l^{-1}*	Hematocrite (Hct)%
Total Weight, g	Muscle lipid,%		Red Blood Cells (RBC)%
Somatic weight, g			Hemoglobine (Hb)%
Liver weight, g			MCHC
Liver somatic index (LSI),%			MCH
Liver structure (%)			MCV
Condition factor (CF)			ImRBC %
Growth (individual),% day $^{-1}$			White blood cells (WBC)
Feed consumption, g			Lymphocytes (Lymp) %
			Granulocytes (Gran) %
			Trombocytes (Trom) %

* analyzed by Biomedical Centre at the Swedish University of Agriculture (SLU), Uppsala, Sweden.

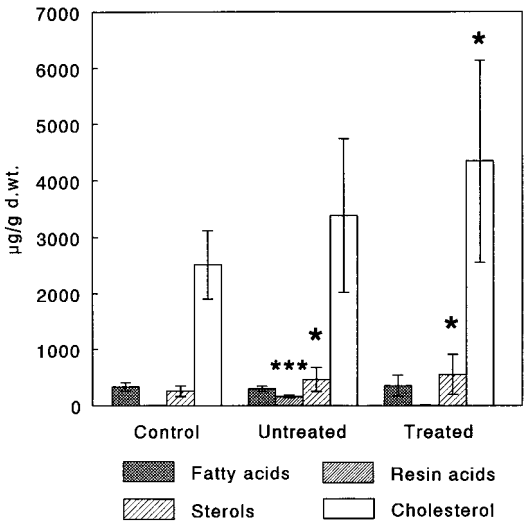

Fig. 1. Concentrations of fatty and resin acids and sterols (µg g $^{-1}$ d.wt.; mean ± SD) and cholesterol (µg g^{-1} d.wt.; mean ± SD) analyzed in fish bile exposed to an untreated and treated total chlorine free BKME for 8 weeks. n = 6; * = $p < 0.05$; *** = $p < 0.001$.

all the 16 marked squares were regarded as a "hit" and the cell content of the square was determined. A total of 200 squares per fish were analysed from six fish per treatment. The liver cells were analysed for normal, necrotic, binuclear, degenerated and other (sinusoids, bile ducts and blood vessels) structures.

Hormone assays

Plasma levels of estradiol and testosterone were analyzed at the Biomedical Centre at the Swedish University of Agriculture (SLU), Uppsala with a radioimmunoassay (RIA) kits (Diagnostic Products Corp., Los Angeles, United States) (3–180 pmol l^{-1}). The estradiol kit has been modified and validated for fish estradiol at SLU. Extrapolation of standardcurve was used for higher values. Testosterone was analyzed according to the original description.

Statistics

ANOVA-test was used to check if there were any significant differences between the replicated within each treatment. All percentual data was arcsin transformed before computations. If no significanses within the treatment were found and the variances were homogenous, the replicates were pooled. The pooled means were statistically tested using t test (if homogenous variance) or Mann-Whitney U-test. The individual growth in gram was tested against plasma 17β-estradiol concentration and muscle lipid concentration with linear regression and the regression was tested with t test. Homogenity was tested with F test.

Results

Bile analyses

The bile was analyzed for extractive content i.e. fatty and resin acids and sterols. The fatty acid levels showed no statistical significant levels as compared with the control. The resin acid content in bile from fish exposed to untreated effluent (161 ± 24 µg g^{-1} [mean ± SD]) was significantly ($p < 0.001$) higher as compared with the levels (only 6 ± 1 µg g^{-1}) in control fish bile. Significantly ($p < 0.05$) higher (2 fold) sterol levels were observed in the bile of fish exposed to untreated (465 ± 211 µg g^{-1}) and treated (550 ± 354 µg g^{-1}) effluent (Fig. 1) as compared with the control (257 ± 93 µg g^{-1}) levels. Sitosterol and campesterol were the dominating sterols in the bile. Highest cholesterol levels ($p < 0.05$) were observed in bile from treated (4346 ± 1792 µg g^{-1}) exposed fish as compared with the control (2510 ± 607 µg g^{-1}).

Morphometry

Compared with the control, no statistically significant differences in bodyweight, individual growth, condition factor and feed coefficient could be observed in the exposed fish (Table 4). Fish exposed to treated effluent had the smallest growth and the highest feed coefficients. The liver somatic index (LSI) was significantly ($p < 0.05$) lower for the fish exposed to untreated effluent and nearly significant ($p = 0.067$) for fish exposed to treated effluent.

Hematology

Significantly higher numbers of red blood cells (RBC) were observed in fish exposed to untreated ($p < 0.001$) and treated pulp mill effluents (Table 5). Also, for the calculated indexes mean cell hemoglobin (MCH) and mean cell volume (MCV), significantly ($p < 0.01$ and $p < 0.05$ respectively) lower values were observed for the treated effluent exposed fish.

The white blood cell picture was affected in fish exposed to untreated effluent and showed statistical significantly higher numbers of white blood cells (WBC) and trombocytes (Tromb) ($p < 0.05$ and $p < 0.01$ respectively; Fig. 2).

Metabolism, liver structure, and muscle lipid content

The liver glycogen content was significantly ($p < 0.05$) lower in fish exposed to treated pulp mill effluent as compared with the control. Also, the muscle lipid content was significantly ($p < 0.001$) lower in fish exposed to treated effluent as compared with the control (Table 6).

The mean activity of the enzyme activity of MFO associated, cytocrome P_{450} enzyme, 7-ethoxyresorufin-*O*-deethylase, EROD showed no statistically significant differences as compared with the control levels (Table 6).

Histological examination of livers from the different treatment groups showed only minor changes in liver structure, such as sporadic necrosis and binucleated cells, although the analyzed parameters were significantly different as compared to the control fish (Fig. 3).

Sex steroid levels

No statistically significant differences in the plasma concentration of 17β-estradiol between effluent exposed and control fish were seen. However, the

Table 4. Morphometrical parameters in juvenile rainbow trouts exposed for 8 weeks to untreated and treated whole TCF BKME. (CF = condition factors; LSI = liversomatic index; FC = feed coefficients, * = $p < 0.05$). $n = 30$.

	Control	Untreated	Treated
Length (cm)	26.2 ± 2.6	26.2 ± 2.0	25.3 ± 3.7
Weight (g)	215.5 ± 78.0	214.9 ± 53.2	199.5 ± 84.0
Som. Weight (g)	192.4 ± 65.4	188.9 ± 45.3	177.8 ± 76.1
Growth (%)	105.3 ± 53.4	111.5 ± 36.9	93.0 ± 57.9
CF	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.2
Liver Weight (g)	3.0 ± 1.1	2.7 ± 0.9	2.6 ± 1.2
LSI (%)	1.4 ± 0.2	1.3 ± 0.2 *	1.3 ± 0.3
FC	1.3 ± 0.2	1.2 ± 0.1	1.4 ± 0.2

Table 5. Hematology parameters in juvenile rainbow trouts exposed for 8 weeks to untreated and treated whole TCF BKME. (Hct = hematocrite; RBC = red blood cells; Hb = hemoglobin; MCHC = mean cell hemoglobin concentration; MCH = mean cell hemoglobin; MCV = mean cell volume; ImRBC = immature red bloodcells; * = $p < 0.05$; ** = $p < 0.01$). $n = 30$.

	Control	Untreated	Treated
Hct (%)	33.6 ± 4.2	33.6 ± 4.2	34.0 ± 4.37
RBC (%)	1.28 ± 0.12	1.39 ± 0.18 *	1.41 ± 0.16 **
Hb (%)	1.11 ± 0.13	1.11 ± 0.17	1.17 ± 0.13
MCHC (%)	3.37 ± 0.57	3.52 ± 0.66	3.29 ± 0.45
MCH	0.87 ± 0.13	0.85 ± 0.08	0.79 ± 0.08 **
MCV	26.3 ± 4.0	24.7 ± 4.2	24.3 ± 2.8 *
ImRBC	1.38 ± 0.60	1.48 ± 0.2	1.26 ± 0.61

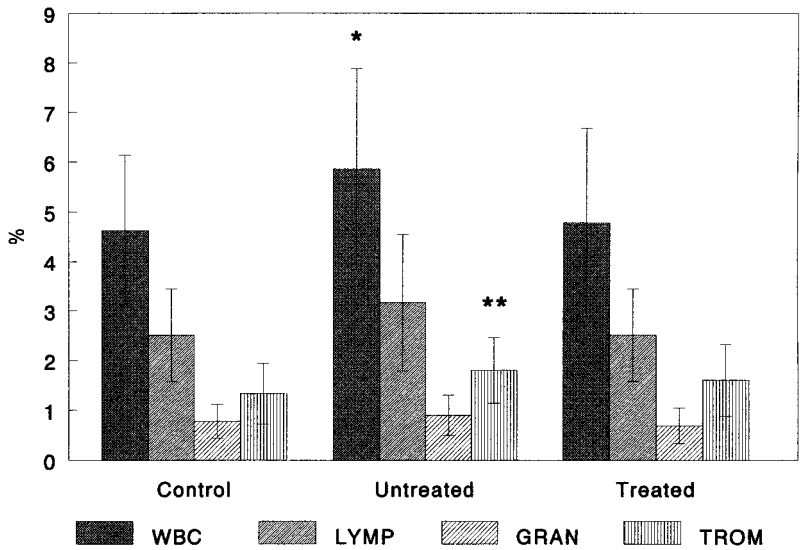

Fig. 2. White blood cells (%; mean ± SD) in fish exposed to an untreated and treated total chlorine free BKME for 8 weeks. WBC = white blood cells; Lymp = lymphocytes; Gran = granulocytes; Trom = thrombocytes. $n = 30$; * = $p < 0.05$; ** = $p < 0.01$.

variation within groups was large. (Fig. 4). The testosterone levels were under the detection limit.

Regression analysis with individual growth

A significant positive correlation was found between individual growth and 17β-estradiol levels ($p < 0.01$) as well as muscle lipid concentration ($p < 0.01$) in fish exposed to treated effluent (Fig. 5a and b). Correlations were not found in the control groups nor in the untreated effluent exposed groups.

Discussion

The fish bile was analyzed for fatty and resin acids and sterols which occur naturally in the wood raw material and which are biologically active compounds. The resin acid concentrations were elevated in fish exposed to untreated effluent. Compared with similar, previous studies (Johnsen *et al.* 1995, 1998, Lehtinen and Tana 1992, Lehtinen *et al.* 1993), the bile resin acid concentrations found in this experiment were only moderately elevated. Excretion of sterols via bile was higher in fish exposed to treated effluent and the bile sterol levels did not correlate with the levels analyzed directly from the treated and untreated effluents. The main portion of phytosterols in the bile consisted of campesterol and sitosterol. The phytosterols are known to be biologically active and affect the endocrinological status of different fish species (Hegrenes 1999, Lehtinen *et al.* 1999, MacLatchy and Van Der Kraak 1995). The plant sterols β-sitosterol and β-sitostanol are known to inhibit cholesterol absorbtion through the intestinal wall (Heinemann *et al.* 1991). In an experiment made on human volunteers, sitosterol infusion caused

Table 6. Liver and muscle metabolism describing parameters in juvenile rainbow trouts fish exposed for 8 weeks to untreated and treated whole TCF BKME. * = $p < 0.05$; *** = $p < 0.001$). $n = 30$.

	Control	Untreated	Treated
Liver glycogen (%)	6.93 ± 2.4	6.80 ± 2.8	5.56 ± 1.9 *
EROD (pmol mg prot^{-1} min^{-1})	34.49 ± 17.3	37.50 ± 22.6	32.15 ± 17.9
Muscle lipid (%)	1.10 ± 0.6	0.86 ± 0.4	0.64 ± 0.3 ***

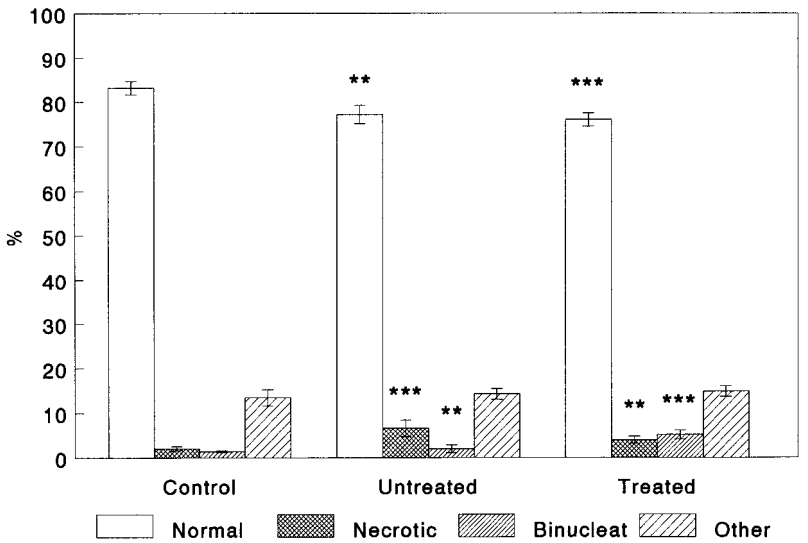

Fig. 3. Liver structure (%; mean ± SD) of fish exposed to an untreated and treated total chlorine free BKME for 8 weeks. n = 6. ** = p < 0.01; *** = p < 0.001.

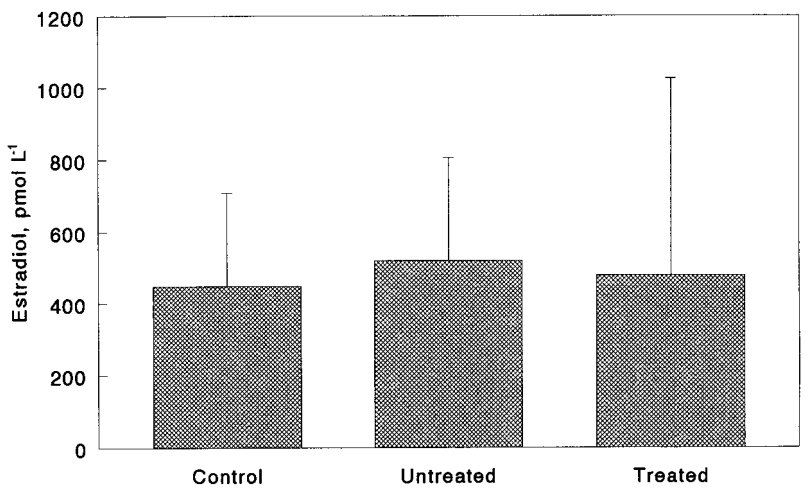

Fig. 4. Plasma content of 17β-estradiol (pmol l^{-1}; mean ± SD) of juvenile female rainbow trout exposed to an untreated and treated total chlorine free BKME for 8 weeks. n = 30.

an overall 50% cholesterol absorption decline, whereas sitostanol infusion caused a reduction of cholesterol absorption by almost 85% (Heinemann *et al.* 1991). Diet and bile excretion are the two major cholesterol sources in the intestine. To compensate for a lower cholesterol absorbtion in the intestine, the liver must synthesize more cholesterol, which is excreted via the bile. The fish exposed to treated effluent had significantly higher bile cholesterol levels compared to the control fish. Simplistically described, the treatment of a pulp mill effluent is degradation of compounds by bacteria under optimal conditions. Bacteria naturally occurring in water are able to transform and metabolize phytosterols into new, bioactive compounds (Denton *et al.* 1985), therefore in this study it cannot be ruled out that compounds have been transformed by bacteria in the treatment plant as well as within the test organism since rainbow trout drink water when existing in brackish water. Significantly lower glycogen contents in fish livers exposed to treated effluent may be a sign of a higher metabolism in the liver. The histological examination of the livers revealed no severe damage in the effluent exposed fish, as has been observed in previous studies with pulp mill effluents (Johnsen *et al.* 1995, 1998),

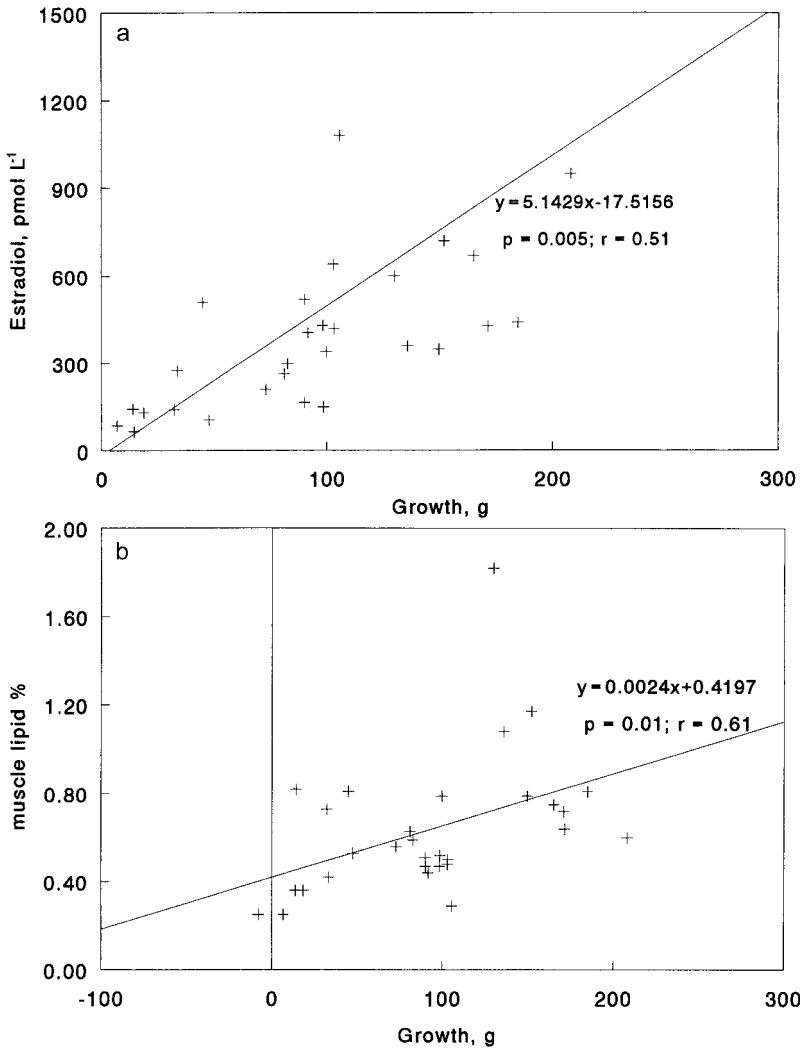

Fig. 5. — **a**: Regression between individual growth (g) and plasma 17β-estradiol (pmol l^{-1}) in fish exposed to treated total chlorine free BKME for 8 weeks. — **b**: Regression between individual growth (g) and muscle lipid content (%) in fish exposed to treated total chlorine free BKME for 8 weeks. n = 30.

only sporadic necrotic cells and binucleated cells occurred. The observed changes in the liver structure can be interpreted as a sign of an overall higher liver metabolism.

The growth can be simplified as energy intake minus energy consumed, based on a balanced energy budget of the assimilated energy and energy used in respiration (Ursin 1979). Changed growth can be the result of physiological stress caused by the physical chemical environment (pH, oxygen content or temperature) or xenobiotics present in the water. Physiological stress could alter enzyme and hormonal levels in fish which could cause cell or tissue damage which further could affect the ability to produce a vital off-spring (Lehtinen 1990). In this study, the individual growth was not statistically affected; only a slight decrease in growth compared to the control was observed in the groups exposed to treated effluent. Significant stimulated or inhibited growth has been observed in newly hatched fish fryes exposed to pulp mill effluents (Lehtinen 1989, Mattsson 1992) and phytosterols (Mattsson 1992, Lehtinen *et al.* 1999). It has been speculated that phytosterols occurring in the effluent could affect fish metabolism through affecting the endocrinological mechanisms, thus affecting growth and reproduction status (Tremblay and Van Der Kraak 1999).

Significantly lower muscle lipid levels in the

treated effluent exposed fish is a sign that energy reserves were depleted due to a higher energy metabolism. The advantage of using individually marked fish in longterm exposures is clearly shown in this experiment. Although not statistically significant, differences compared to the control were found when comparing the plasma 17β-estradiol concentrations, a significant positive correlation between individual growth increase and 17β-estradiol and muscle lipid content was found in fish exposed to treated effluent. Crandell and Gall (1993) found a strong positive relationship between body weight and early maturity in individually tagged rainbow trout. A hypothesis that fish strive for maturation as early as possible, but that physical and physiological factors are slowing down the process seems to hold true for the majority of fish species. The maturation process is growth-dependent and completion of the process depends on environmental factors such as temperature and day length. The process may be reversed every year if the environmental conditions are unfavorable. Whether the maturation process is reversed or not depends on the individual's available energy resources during certain critical periods of the year. This mechanism, which is based on enough parental energy being available in order to support the progeny, influences both the primary reproduction processes as well as the vitality and survival of the larvae during the first winter period. In case of low energy reserves, reproduction will not take place. Rainbow trout is known to be a hierarchial fish species (Peters and Schwarzer 1985) and feeding hierachies within groups, due to changed energy metabolism, have been observed (Jobling and Koskela 1996). In our experiment it seems as if the increased energy metabolism in fish exposed to treated pulp mill effluent led to a competition for the feed and thereby the establishment of feeding hierarchies within these groups. The dominant fish grew more quickly and had higher plasma 17β-estradiol and muscle lipid concentrations. Janz *et al.* (1997) reported increased plasma 17β-estradiol, but not induction of hepatic ethoxyresorufin-*O*-deethylase activity in a feral prespawning white sucker (*Catostomus commersoni*) population exposed to BKME (bleached kraft mill effluent). The detoxification enzyme EROD was not affected by effluent exposure in this study either.

In similar long-term 8-week experiments with individually marked juvenile, female, rainbow trout exposed to pulp mill effluents from modern ECF and TCF bleaching processes, no effects on growth were observed either (Mattsson *et al.* unpubl. data); it may be that 8 weeks may not be enough time for growth effects to occur. However, it seems that fish exposed to treated TCF bleached pulp mill effluent need more energy to maintain a similar growth as controls, i.e. they have a higher energy demand. (K. Mattsson unpubl. data). In this study, fish groups exposed to treated TCF bleached pulp mill effluent had the highest feed coefficient.

The observed changes in the red blood cell picture were small, only about a 10% difference for RBC between the controls and the treated effluent exposed groups. Physiologically a 10% difference in red blood cell numbers may be of no significance for respiratory gas exchange to function properly, since the levels of hemoglobin were unaffected. The fact that the total number of white blood cells (WBC) and trombocytes was higher in untreated exposed fish could be a sign of some infection or tissue damage. In the histological examination a slightly higher amount of sporadic necrotic cells was observed in the liver of fish exposed to untreated effluent.

Conclusions

No toxicological or hormonal effects were observed from exposure of rainbow trout to a modern total chlorine free (TCF) bleached kraft mill effluent (BKME).The hematological changes were small and lack physiological meaning. However, more physiological effects on fish exposed to secondary treated than untreated whole mill effluent were observed. Fish exposed to the treated effluent had a higher liver and muscle metabolism accompanied by higher levels of sterols and cholesterol levels in bile. Inhibition of cholesterol absorption through the intestinal wall and compensatory cholesterol synthesis by the liver to be excreated via the bile is given as a possible explanation. The higher energy metabolism led to competition for feed

and establishment of feeding hierachies within the treated effluent exposed groups. Individual marking of fish is strongly recommended in longterm, sublethal exposure of fish.

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