BIOLOGICAL RESEARCH REPORTS FROM THE UNIVERSITY OF JYVÄSKYLÄ

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A Biomarker Approach

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston vanhassa juhlasalissa
lokakuun 10. päivänä 1997 kello 12.

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UNIVERSITY OF JYVÄSKYLÄ

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Publishing Unit, University Library of Jyväskylä

URN:ISBN:978-951-39-9070-1
ISBN 978-951-39-9070-1 (PDF)
ISSN 0356-1062

Jyväskylän yliopisto, 2022

ISBN 951-39-0063-0
ISSN 0356-1062

Jyväskylä University Printing House, Jyväskylä
and ER-Paino, Lievestuore 1997

ABSTRACT

Markus Soimasuo
The effects of pulp and paper mill effluents on fish: a biomarker approach
Jyväskylä: University of Jyväskylä, 1997, 59 p.
(Biological Research Reports from the University of Jyväskylä,
ISSN 0356-1062; 60)
ISBN 951-39-0063-0
Yhteenveto: Kalan biomarkkerivasteet selluloosa- ja paperiteollisuuden jätevesien vaikutusten osoittajina
Diss.

Physiological and biochemical biomarkers were studied in juvenile whitefish (*Coregonus lavaretus* L. s.l.) experimentally exposed to effluents from the pulp and paper industry. During 1991-1993, whitefish were caged in the recipient (Southern Lake Saimaa, Finland) of a bleached kraft pulp and paper mill. In 1992, the mill changed its processes and chlorine dioxide for the bleaching and activated sludge treatment was introduced. A comparative study (1993) showed considerably decreased effluent constituents in lake water. Thus, the bile accumulation of chlorophenolics (CPs) was only 0.5 %, and resin acids (RAs) 2 %, of that in 1991. Compared to the reference, whitefish liver 7-ethoxyresorufin *O*-deethylase (EROD) activity was 13-fold (1991) and 2-fold (1993) 3 km from the mill. The levels of plasma immunoglobulin M and blood hemoglobin were decreased in the exposed fish before, but not after, the mill renewals. Other parameters measured remained unchanged. The laboratory exposures (1992 and 1996) simulating real effluent concentrations confirmed the field observations well. In addition, 17β-estradiol and testosterone were significantly decreased in fish exposed in the laboratory (1996). During 1995-1996 the area was extended to cover the whole of Southern Lake Saimaa, which has four pulp and paper mills. CPs and RAs accumulated at low levels in fish bile. EROD activity was 2- to 4-fold at sites 1-6 km from all the mills, whereas the cytochrome P450 1A1 (CYP1A1) gene was expressed only near one mill. The reproductive steroids, IgM, glucose or lactate were not changed, whereas vitellogenin gene was induced near one mill. However, the results indicate site specific inconsistency in the mixing and dispersion of effluents within the area, hindering the comparison of the mills. The selected biomarkers proved feasible and relevant in quantifying the effluent exposure and effects on fish, both in the laboratory and in the field. This study showed that the modernized mill processes and the advanced effluent treatment substantially reduced the load of harmful effluent constituents, diminishing biological impact on the receiving aquatic environments.

Key words: Pulp and paper mill effluent; biomarker; biotransformation; cytochrome P450; CYP1A1; EROD activity; whitefish.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by Roman numerals I-V.

I Reino Soimasuo, Ilmari Jokinen, Jussi Kukkonen, Tiina Petänen, Tiina Ristola & Aimo Oikari (1995). Biomarker responses along a pollution gradient: Effects of pulp and paper mill effluents on caged whitefish. Aquat. Toxicol. 31: 329-345.

II Reino Soimasuo, Tuula Aaltonen, Mikko Nikinmaa, Jukka Pellinen, Tiina Ristola & Aimo Oikari (1995). Physiological toxicity of low-chlorine bleached pulp and paper mill effluent on whitefish (*Coregonus lavaretus* L. *s.l.*): A laboratory exposure simulating lake pollution. - Ecotox. Environ. Safety 31: 228-237.

III M.R. Soimasuo, A.E. Karels, H. Leppänen, R. Santti & A.O.J. Oikari. Biomarker responses in whitefish (*Coregonus lavaretus* L. *s.l.*) experimentally exposed in a large lake receiving effluents from pulp and paper industry. - Arch. Environ. Contam. Toxicol. (in press).

IV M.R. Soimasuo, J. Lappivaara & A.O.J. Oikari. Validation of field exposure of fish and role of activated sludge treatment of BKME by a laboratory simulation. - Env. Toxicol. Chem. (submitted).

V Pirkko Mellanen, Markus Soimasuo, Bjarne Holmbom, Aimo Oikari & Risto Santti. Differential expression of vitellogenin gene and CYP1A system in the liver of juvenile whitefish (*Coregonus lavaretus* L. s.l.) exposed to effluents from three pulp and paper mills. (manuscript).

In addition, some data from Petänen et al. (1996) and unpublished data is presented.

Abbreviations

Ah receptor	aryl hydrocarbon receptor
ALAT	alanine aminotransferase
AOX	adsorbable organic halogen
ASAT	aspartate aminotransferase
BKME	bleached kraft mill effluent
CF	condition factor
CP	chlorophenol
CTMP	chemi-thermomechanical pulp
CYP 1A1	cytochrome P450 1A1
DHAA	dehydroabietic acid
DTPA	diethylenetriaminepentaacetic acid
ECF	elemental chlorine free
EDTA	ethylenediaminetetraacetic acid
EOX	extractable organic halogen
EROD	7-ethoxyresorufin *O*-deethylase
GST	glutathione S-transferase
GW	groundwood
HW	hardwood
IgM	immunoglobulin M
LDH	lactate dehydrogenase
MO	monooxygenase
mRNA	messeger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NTP	nucleoside triphosphate
PAH	polyaromatic hydrocarbon
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzo-*p*-furan
PCR	polymerase chain reaction
PGW	pressured groundwood
PROD	pentoxyresorufin *O*-deethylase
RA	resin acid
RBC	red blood cell
SW	softwood
2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCF	totally chlorine free
TEQ	toxic equivalent
TMP	thermomechanical pulp
UDP-GT	uridine-5´-diphospho glucuronosyltransferase

1 INTRODUCTION

Chemical pulping and bleaching produces highly complex mixtures of organic substances varying in both chemical structure and molecular weight distribution (Kringstad & Lindström 1984). In consequence, during the last few decades, concerted efforts have been made to investigate the chemical characteristics and environmental fate of discharges associated with the pulp and paper industry. A substantial portion of the research has been directed at adverse biological effects observable in fish and aquatic biota (Owens 1991).

Generally, assessment of the environmental quality of an aquatic ecosystem includes the measurements of contaminant level in sediment or water (environmental monitoring). However, in order to provide a more complete assessment of the health of the aquatic environment, the exposure analysis alone is not feasible. Consequently, biological effect monitoring by determining the early alterations in animals is also necessary for a reliable examination of aquatic contamination. Recently, growing attention has been paid to the biomarker concept as a powerful tool in ecotoxicology, providing information about the exposure of an animal to xenobiotics as well as the sublethal effects arising from such exposure. A variety of biological effects, including structural, physiological and biochemical responses in fish, have been associated with subchronic exposure to discharges from the pulp and paper industry (Lindström-Seppä & Oikari 1989, Södergren et al. 1989, Hodson et al. 1992). Thus, in addition to conventional testing, an alternative integrative approach using selected biomarkers in surrogate animals has been recommended (Peakall & Shugart 1993). In the present biomarker approach selected physiological and biochemical responses in fish were emphasized as the biomarkers of exposure and effect of effluents from the pulp and paper industry.

Induction of cytochrome P450 -dependent monooxygenases (MO) in fish liver has been one of the most distinct physiological responses caused by unbleached and bleached pulp mill effluents (Södergren et al. 1988, Lindström-

Seppä & Oikari 1989, 1990a, 1990b, Munkittrick et al. 1992). Consequently, MO induction has been considered as a useful biomarker of exposure to pulp and paper mill effluents, although the ultimate significance of the induction is largely unknown (Kloepper-Sams & Benton 1994). Recent studies have suggested that compounds other than chlorinated dioxins (PCDDs) and furans (PCDFs) present in mill effluents are obvious inducing compounds (Munkittrick et al. 1992, van den Heuvel et al. 1995). Evidently, inducing agents are moderately hydrophilic, most likely planar, polyaromatic hydrocarbons (PAHs) even without chlorine substitution (Burnison et al. 1996), and more readily metabolized in fish than PCDDs/Fs.

The effects of effluents on fish reproduction, including reduced gonadal size, increase in the age to maturation and reduced reproductive steroid levels in blood have been observed in several studies (McMaster et al. 1991, 1992, 1996, Munkittrick et al. 1992, 1994). Certainly, being one of the most meaningful responses in fish to pulp mill discharges, reproductive functions deserve special attention from the ecotoxicological perspective. Although the detailed mechanisms of the endocrine disruptions are still unknown, some constituents existing in pulp mill effluents, including β-sitosterol, are able to cause hormonal changes in fish (MacLatchy et al. 1994, Servos et al. 1994).

Caging techniques offer several advantages in aquatic toxicology (Oikari & Kunnamo-Ojala 1987). Of these, a knowledge of the history of the studied fish, the precise site and the duration of exposure are important. Also, the selection of a desired species and its particular developmental stage and genetic background are possible and decrease the variability seen in wild populations studied in the field (Lindström-Seppä & Oikari 1990b). On the other hand, when compared to laboratory experiments, field studies may suffer from the fact that environmental conditions, e.g., temperature, oxygenation etc., can in addition to the concentration of effluent, vary between the sampling sites, influencing the responses in animals. These physical-chemical water qualities can be controlled in laboratory experiments, enabling a more accurate evaluation of dose-response relations of the physiological effects to effluent. Thus, in conjunction with ongoing field studies, controlled laboratory-based studies were carried out in a close integration.

In recent years the pulp and paper industry has introduced several alterations in the internal manufacturing processes and external facilities for wastewater treatment (Axegård et al. 1993). It is evident that many of these technological alterations in pulp and paper mills have resulted in improved quality of wastewaters with a substantially lower impact on the aquatic ecosystems (Landner et al. 1994). The alterations, including modified cooking, oxygen delignification, substitution of chlorine dioxide for elemental chlorine, and biological treatment, are reported to have a direct beneficial impact on effluent quality, as well as on the environment receiving the chemical loads (Oikari & Holmbom 1996).

2 OBJECTIVES

The main objective of the research for this thesis was to detect possible adverse effects in fish exposed to effluents from the wood processing industry using suitable physiological and biochemical biomarkers. In addition, further purposes of the research were:

- to assess the utility of fish biomarker responses for ecotoxicological studies and biomonitoring (I, III and V)
- to study the spatial extent, as well as the magnitude, of biomarker responses over the effluent receiving areas (I, III and IV)
- to investigate the effects of the altered mill processes on fish biomarker responses (I, II, III, IV)
- to compare fish biomarkers in response to different effluent quality in varied receiving environments (III)
- to investigate the usefulness of the fish caging technique for ecotoxicological studies and biomonitoring (I and III), and
- to validate field observations by laboratory-based simulations (II and IV).

3 MATERIALS AND METHODS

3.1 Study areas and pulp and paper mills

A water area (approx. 50 km^2) located in the western part of Southern Lake Saimaa, S.E. Finland, receiving effluent (approximately 120,000 m^3 d^{-1}) from an integrated bleached kraft pulp and paper mill (Lappeenranta, Finland) was used in the ECOBALANCE-project in 1990 (Soimasuo et al. 1992), 1991 (I) and 1993 (Petänen et al. 1996) (Fig. 1, upper part). During the ESAITOX-project in 1995 (III) and 1996 the area covered the whole of Southern Lake Saimaa (609 km^2) with four pulp and paper mills discharging 330,000 m^3 d^{-1} of biologically and 55,000 m^3 d^{-1} of chemically treated effluents into the lake. For easier comparison of the data obtained, the large water area was divided into five subareas: reference area I (main upstream reference), reference area II (additional reference) and the effluent receiving areas A, B and C (Fig. 1, lower part). The four mills studied during the research in 1995/1996 were as follows: the bleached kraft pulp and paper mill in Lappeenranta (referred as mill A), a bleached kraft pulp mill in Joutseno (mill B), a bleached kraft pulp, paper and cardboard mill (mill CI) and an unbleached pulp and cardboard mill (mill CII) both in Imatra. All the mills employed secondary treatment (activated sludge process) for pulp effluents. Moreover, mills CI and CII have separate chemical treatment plants for effluents from paper and cardboard (mill CI) and cardboard (mill CII) production. The characteristics of the mills are presented in Table 1.

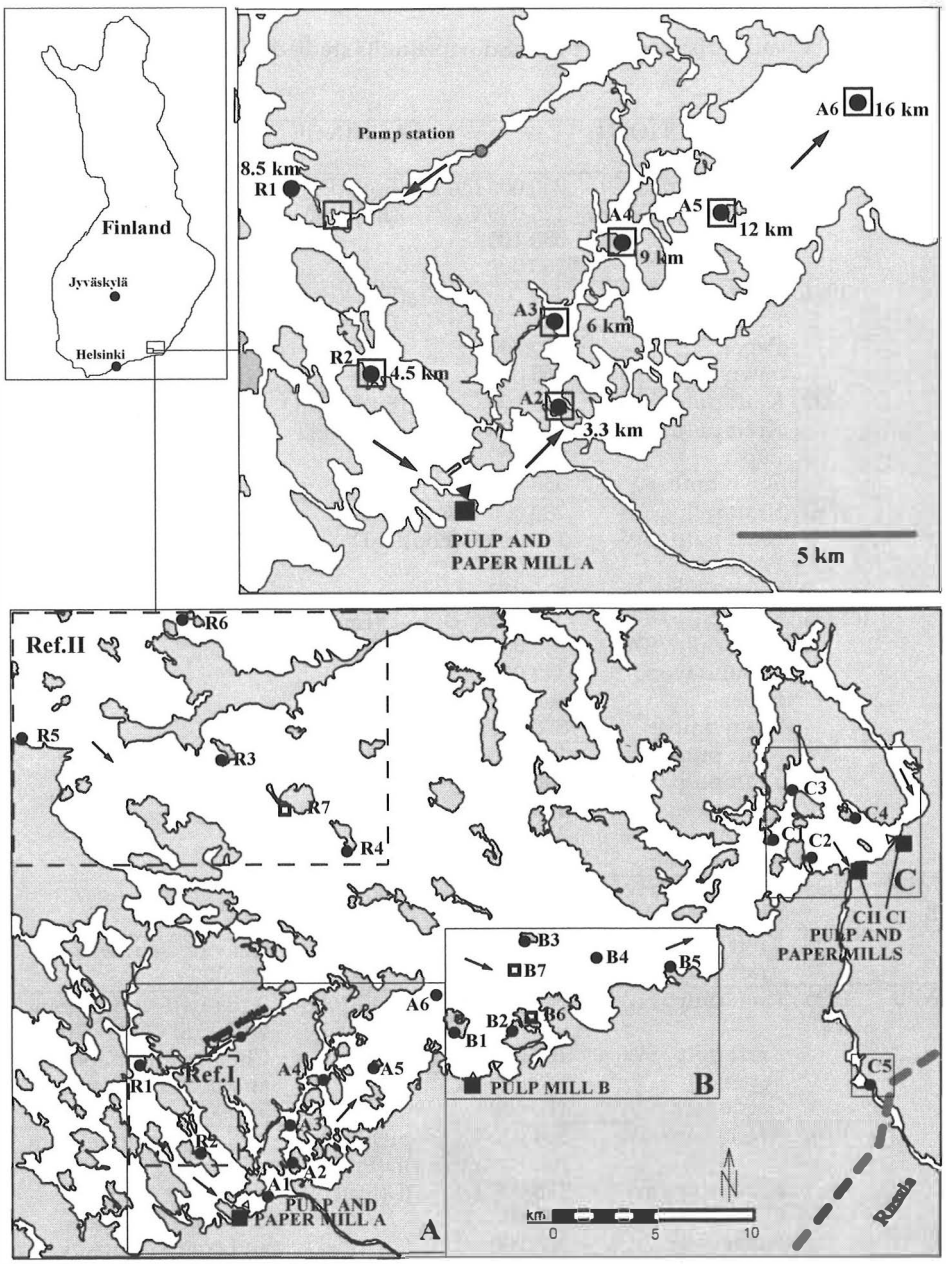

FIGURE 1 The research areas in Southern Lake Saimaa, S.E. Finland. Upper map: The western part of Southern Lake Saimaa (subarea A), which was used during the ECOBALANCE -project in 1990-1993. The open squares indicate the sites in 1991 and the black dots the research sites in 1993. Lower map: The large water area (>600 km^2) was used during the ESAITOX-project in 1995-1996. The recipient subareas A, B and C are indicated by solid lines and the reference subareas (Ref. I and Ref. II) by dashed lines. The black dots indicate the research sites both in 1995 and 1996. The squares stand for the additional sites used in 1996. Sites R3, R4 and B3 were not in use in 1996.

TABLE 1 Characteristics of the pulp and paper mills studied in the research for this thesis.

MILL	YEAR	PRODUCTION $t y^{-1}$		BLEACHING[1]	TREATMENT/ VOLUME $m^3 d^{-1}$
Mill A	1990	Kraft pulp/HW[2]	250 000	C_4/D_{96}(Eo)D(Ep)D	Aerated lagoons/
		Kraft pulp/SW[3]	125 000	C_{46}/D_{54}(Eo)DED	135 000
		Paper	380 100		
		Sawn timber	[4]360 000		
	1991	Kraft pulp/HW	240 000	C_5/D_{95}(Eo)D(Ep)D	Aerated lagoons/
		Kraft pulp/SW	115 000	C_{43}/D_{57}(Eop)DED	124 000
		Paper	380 700		
		Sawn timber	265 000		
	1992	Kraft pulp/HW	255 000	C_6/D_{94}(Eo)D(Ep)D	Aerated lagoons[5]/
		Kraft pulp/SW	122 000	C_1/D_{99}(Eop)DED	120 000
		Paper	377 000		Activated sludge[6]/
		Sawn timber	355 800		129 000
	1993	Kraft pulp/HW	280 000	D(Eo)D(Ep)D	Activated sludge/
		Kraft pulp/SW	140 000	D(Eop)DED	125 000
		Paper	419 000		
		Sawn timber	376 000		
	1995	Kraft pulp/HW	212 000	OD(Eo)D(Ep)D	Activated sludge/
		Kraft pulp/SW	259 000	D(Eop)DED	121 000
		Ground wood	142 000		
		Paper	466 000		
		Sawn timber	370 000		
	1996	Kraft pulp/HW	226 000	OD(Eo)D(Ep)D	Activated sludge/
		Kraft pulp/SW	190 000	D(Eop)DED	110 000
		Ground wood	135 000		
		Paper	391 000		
		Sawn timber	364 000		
Mill B	1995	Kraft pulp/HW	21 000	DoO/OD(Ep)D	Activated sludge/
		Kraft pulp/SW	316 000	DoO/OD(Ep)D	70 000
	1996	Kraft pulp/HW	0		Activated sludge/
		Kraft pulp/SW	306 600	DoO/OD(Ep)D	70 000
Mill CI	1995	Kraft pulp/HW	374 000	DE(Eop)D(Ep)D	Activated sludge, joint
				DE(Ep)D(Ep)D	with mill CII/145 000
		Kraft pulp/SW	168 000	DE(Eo)D(Ep)D	Chemical for paper
		Paper	283 000		and board effluents/
		Cardboard	480 000		55 000
	1996	Kraft pulp/HW	364 000	DE(Eop)D(Ep)D	Activated sludge, joint
				DE(Ep)D(Ep)D	with mill CII for pulp
		Kraft pulp/SW	175 000	DE(Eo)D(Ep)D	effluents/ 148 000
		CTMP [7]	43 000		Chemical for paper
		Paper	206 000		and board effluents/
		Cardboard	487 000		50 000
Mill CII	1995	Kraft pulp	129 000	Unbleached	Activated sludge, joint
		Paper	15 000		with mill CII. Chemical
		Cardboard	210 000		for paper/board effluents
	1996	Kraft pulp	133 000	Unbleached	Activated sludge, joint
		Paper	14 000		with mill CII. Chemical
		Cardboard	201 000		for paper/board effluents

[1] C = chlorine, D = chlorine dioxide, E = caustic extraction, Eo = caustic extraction with addition of oxygen, Ep = caustic extraction with addition of peroxide, Eop = caustic extraction with addition of oxygen and peroxide, O = oxygen delignification, O/O = two-stage oxygen delignification; [2] hardwood; [3] softwood; [4] $m^3 y^{-1}$; [5] until April 1992; [6] implemented in May 1992; [7] chemi-thermo mechanical pulp

3.2 Sampling of lake waters and mill effluents

Lake water samples were collected as composites from the water column at 0, 1, 2, 3 and 4 m in 2.5 L glass bottles at each site four times during the course of the study in May-June (I, III). All samples were kept frozen (-20 °C) prior to the analysis. For the laboratory experiments (II, IV), composite effluent samples were collected in 1 m^3 polyethene containers, transported to the laboratory and stored at 12 °C for a maximum of 4 days before use. Evaluation of the exposure conditions to components in pulp and paper mill effluents was based on chlorophenolics (CPs), resin acids (RAs), and adsorbable organic halogens (AOX) in the lake water samples. Moreover, water sodium (Na^+) concentration was used as an effluent tracer indicating theoretical dilutions within the experimental areas.

3.3 Experimental caging of fish

In the field experiments, immature (1-year old), hatchery reared whitefish (*Coregonus lavaretus* L. s.l.) obtained from the Central Fish Culture and Fisheries Research Station for Eastern Finland, at Enonkoski, Finland were used. The plankton feeding strain of whitefish originated from the River Pielisjoki, Finland. Fish were transported from the hatchery to the experimental area in polyethylene bags filled with oxygenated water at a temperature of about 5 °C. During transportation the bags were chilled with ice. Twelve to fifteen fish (mean weight 32 g, range 8 g) were exposed for about 30 d in oval-shaped 250-litre cages (diameters 50 x 70 x 70 cm) made of steel wire and polyester net construction on the bottom, at a depth of about 4-5 m. The average fish density in a cage was 1.2 kg per m^3.

In 1990 (Soimasuo et al. 1992), eight contaminated sites downstream and two reference sites upstream from mill A were used, whereas in 1991 (I) and 1993 (Petänen et al. 1996) five downstream and two upstream sites were monitored (Fig.1). In 1995 (III) and 1996, the large study area included five subareas with a total of 22 different research sites (Fig. 1).

3.4 Exposure setup of laboratory simulations

Laboratory experiments simulating the exposure conditions in the lake contaminated by effluent from pulp and paper mill A in Lappeenranta were carried out in 1992 (II) and 1996 (IV). Exposure concentrations were set on the basis of effluent volume (vol./vol.) and water sodium concentration. Three out of four concentrations (3.5, 2.3 and 1.3 vol.-%) corresponded to distances of about 3.3, 9 and 16 km from the mill sewer discharging to the lake. In addition, a double concentration (7 vol.-%) relative to the calculated theoretical maximal

concentration in the lake was used. Moreover, a 3.5-vol.% pre-treated effluent (PTE) concentration was used in the laboratory experiment in 1996 (IV). As in the field studies, hatchery-reared juvenile whitefish (*Coregonus lavaretus* L. *s.l.*) were used for the laboratory exposures. In 1992 (II), fish (2- -year old) obtained from the Central Fish Culture and Fisheries Research Station for Eastern Finland, at Enonkoski, Finland (River Pielisjoki strain) were used, while fish exposed in 1996 (IV) were obtained from the Finnish Game and Fisheries Research Institute's Fish Culture Research Station, at Laukaa, Finland (Lake Rautalampi strain). The fish were randomly distributed among five identical all steel tanks (540 liters), 12-15 fish being put into each, and exposed for 30 d in flow-through conditions (1 L min^{-1} = approx. 1 L fish g^{-1} day^{-1}) at 12±0.5 °C. During the experiments, fish were fed daily with fish fodder in an amount approximately equivalent to 1 % of the fish biomass. The photoperiod was 12 h : 12 h (light:dark). The general characteristics of the aerated experimental waters (temperature, O_2, pH) and their dilution flow volumes were monitored daily.

3.5 Chlorophenolics and resin acids in bile and in water

The free and conjugated chlorophenolics (CPs) and resin acids (RAs) in the bile of whitefish were analyzed by gas chromatography (GC) using a modified method described by Oikari & Ånäs (1985), and described in more detail in papers I, II (CPs) and II, III and IV (CPs and RAs). CPs in water samples were analyzed by GC with a modified method originally described by Voss et al. (1981) and described in detail in papers III and IV. The method for RAs in water is described in paper III.

3.6 Fish sampling and assays of biotransformation enzymes

Both in the field and the laboratory experiments fish were sampled using similar procedures, described in detail in papers I and II. In the field, the fish were sampled in a laboratory on the research vessel Muikku. The entire sampling procedures for each fish required approximately 6-8 min.

The preparation of microsomes is described in detail in paper I. In paper I and II, the activity of 7-ethoxyresorufin *O*-deethylase (EROD) was determined fluorometrically (Shimazu spectrofluorometer RF-5000) from the microsomal fraction using resorufin as an internal standard (Burke & Mayer 1974). The cuvette mixture consisted of buffer (0.1 M Tris-HCl, pH 7.6) and ethoxyresorufin (0.5 mM) at 20 °C. For the assay 25 µL of microsomes was used (500 µg protein) and the reaction initiated with 20 µL NADPH (5 mM). Microsomal proteins were measured using a Folin-Ciocalteu method (Lowry et al. 1951) with bovine serum albumin as a standard. In papers III, IV and V, induction of hepatic MO was measured fluorometrically as the activity of EROD and pentoxyresorufin *O*-

deethylase (PROD), according to the method given by Burke et al. (1985), adapted for the microplate format (Labsystems Ascent microplate fluorometer). Microsomes (200 μg protein well^{-1}) were incubated in 100 mM potassium phosphate buffer of pH 8, 2.5 μM ethoxyresorufin or 5 μM pentoxyresorufin (Sigma Chemical Co.), and 0.5 mM NADPH (Sigma Chemical Co.) in a final volume of 200 μL. Fluorescence (excitation 530 nm, emission 584 nm) was recorded at 30 s intervals for 4 min at 20 °C. Microsomes from rainbow trout injected with β-naphthoflavone (50 mg kg^{-1}) were used as a positive control for the EROD and PROD assays. The protein concentration of microsomes was measured with a Bio-Rad DC Protein Assay Kit, using bovine serum albumin as a standard.

Microsomal uridine-5´-diphòspho glucuronosyltransferase (UDP-GT) was determined spectrometrically using *p*-nitrophenol as a substrate (Hänninen 1968). Cytosolic glutathione S-transferase (GST) was measured according to Habig et al. (1974) with 1-chloro-2,4-dinitrobenzene as a substrate. Microsomal (UDP-GT) (I, II) and cytosolic (GST) (I, II) proteins were measured using the Folin-Ciocalteu method (Lowry et al. 1951) with bovine serum albumin as a standard.

3.7 Plasma and blood measurements

Plasma lactate was measured with Boehringer test kits: L-lactic acid 139084 UV-method (I and II), L-lactic acid 256 773 (III) and plasma glucose GOD-Perid method 124010 (I and II) and GOD-Perid method 124036 (III and IV). Plasma lactate dehydrogenase activity (I) was determined by an UV-method based on NAD/NADH absorbance difference (Boehringer LD 191353) and serum aspartate aminotransferase (I) by Boehringer ASAT 191337. Plasma testosterone and 17β-estradiol concentrations were determined using Fenzia Enzyme Immunoassay test kits (EIA, Orion Diagnostica, Finland) and read with a plate reader (Labsystems EMS Reader MF) at 405 nm (III and IV). Red blood cell (RBC) sodium concentration was measured directly from the packed pellet by atomic absorption (Hitachi AAS) (II). The method used to measure the concentration of whitefish plasma immunoglobulin M (IgM) is described in paper I. Hemoglobin (Hb) was measured spectrophotometrically using the cyanmethemoglobin method (I,II,III and IV). For the red cell nucleoside triphosphate (NTP) concentration (II), red cell pellets were taken from liquid nitrogen, weighed, and deproteinized with 0.6 M perchloric acid. NTP was determined enzymatically as described by Albers et al. (1983) using a Transcon 101 fluorometric analyzer. The concentration of sodium in red blood cells was measured by atomic absorption spectrometer (Hitachi AAS). For production of the liver vitellogenin and CYP1A1 cDNAs (V), Northern blot analysis, purification and quantification of polymerase chain reaction (PCR) products were carried out as described in Mellanen et al. (1996).

3.8 Statistics

In papers I and II, the comparison between the exposed and the reference groups for all the parameters measured were performed by one-way ANOVA followed by Tukey's HTD test. In papers III, IV and V, monooxygenase activity, levels of reproductive steroids and bile accumulation of compounds in the different exposure groups were compared to the reference group using a nonparametric Kruskal-Wallis test. The blood and plasma parameters were first log-transformed and compared using one-way ANOVA followed by Tukey's HTD test (III, IV, V). For examining correlations, linear regression analyses were performed. To meet statistical demands, all data was first assessed for normality and homogeneity of variance. The significance level (denoted by an asterisk *) was set to $p<0.05$. The statistics were performed using SYSTAT® (I, II) and SPSS® (III, IV, V) software.

Kriging is a method estimating the value of a spatially distributed variable at a given point from known adjacent values while applying the interdependence expressed in the variogram. The kriging involves the construction of a weighted moving average equation including knowledge of the spatial covariance between the estimation point and sample points within the range of interaction. While other linear unbiased estimators exist, the kriging method as a minimum variance estimator minimizes the variance of the estimation errors. It is possible to allow kriging to alter the original measurement for better smoothing (smaller estimation error). However, in this thesis the interpolations have been forced to pass through the measured values at the data points, not just near them. It should be noticed that kriging, or any other interpolation method, does not produce reliable estimates at distant areas with no data point. Additionally, in these estimations, the effect of islands has been ignored. Kriging interpolations (Cressie, 1993) of the spatially distributed variables EROD and CPs in Southern Lake Saimaa were performed by Variowin (2.01) and Surfer (6.03) softwares.

4 RESULTS

4.1 Exposure conditions in receiving lake areas

Subarea A in 1991 and 1993. Before the new bleaching processes and the activated sludge as the secondary treatment were introduced in April in 1992, a distinct concentration gradient of several effluent constituents including CPs, RAs and AOX could be seen in the receiving lake area (I). Due to the process alterations (April 1992), the concentrations of CPs were reduced by 96 % and AOX by 80 % in the study sites downstream of the mill (Fig. 2). In 1991, according to sodium (Na^+) in lake waters, the effluent volume percent (vol.-%) showed a distance related trend in the receiving waters, going from about 3.8 vol.-% at 3.3 km to 1.2 vol.-% at 16 km from the mill (I). Physical-chemical features of the lake water at the research sites in 1991 and 1993 are given in Table 2.

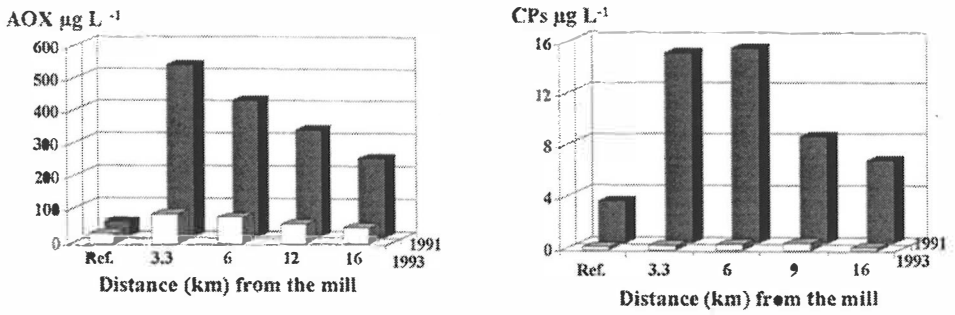

FIGURE 2 The concentrations of adsorbable organic halogens (AOX) and chlorophenolics (CPs) in lake water from the research sites upstream (Ref.) and downstream from mill A in 1991 (I) and 1993 (Kaplin et al. 1997).

TABLE 2 Water physical-chemical parameters at different research sites at the end of the field experiments in 1991 (I) and 1993 (Petänen et al. 1996, Kaplin et al. 1997 for TOC in 1991 and 1993) in the western part of Southern Lake Saimaa (area A).

Parameter		pH		Na^+ mg L^{-1}		Cond. µS cm^{-1}		TOC[1] mg L^{-1}		Temp. °C		O_2 mg L^{-1} / sat. %	
Year		1991	1993	1991	1993	1991	1993	1991	1993	1991	1993	1991	1993
Site	(km)[2]												
R1	-8.5	7.7	7.0	n.m.	n.m	58	54	n.m	n.m.	15.9	11.7	10.5/95	10.5/96
R2	-4.5	7.4	7.0	2.9	2.9	56	54	6.9	7.1	16.2	12.1	9.8/89	10.4/97
A2	3.3	6.9	7.0	13.1	11.9	154	93	22.9	9.3	16.0	13.3	4.8/46	8.0/76
A3	6	6.9	7.0	12.9	10.8	148	93	11.5	9.1	15.4	13.3	5.8/55	8.5/81
A4	9	7.0	n.m.	n.m.	8.0	141	n.m.	n.m.	n.m.	13.8	12.7	7.9/76	9.9/94
A5	12	7.2	7.2	8.9	8.0	120	99	9.4	8.6	13.5	11.9	9.8/88	10.7/99
A6	16	7.3	7.0	6.8	6.1	89	81	8.1	8.0	12.8	10.4	10.4/90	11.0/99

[1] total organic carbon; [2] distance from the mill; n.m. not measured.

Large research area in 1995 (Fig. 1). The concentration of lake water AOX in reference area I (sites 1 and 2 upstream from mill A) was 25 µg L^{-1}, whereas the highest AOX value in the mixing zone of mill A was 140 µg L^{-1} (1 km from the mill), in area B (2 km) 50 µg L^{-1} and, in area C 160 µg L^{-1} (2 km) (III).

The concentrations of chlorophenolic compounds in waters collected from the research sites were low, often approaching the analytical detection limit of approximately 0.1 µg L^{-1}. The mean CP concentration at reference site 2 was 0.2 µg L^{-1}, while the highest concentration of CPs in area A was 1.2 µg L^{-1} (3.3 km from mill A), the levels decreasing with distance. Considerably lower concentrations of CPs were observed in the vicinity of mill B (0.2 µg L^{-1}, 2 km), while in area C, the highest concentration of CPs near mill CI (2 km) was 1.5 µg L^{-1}. The concentrations of resin acids in lake water columns were low, often just near or below the detection level (<0.5 µg L^{-1}) along the large area. Consequently, no data is presented.

Compared to subarea A, where the maximum effluent concentration was found 1 km from the mill (4.6 vol.-%), considerably higher effluent dilutions based on Na^+ concentrations were observed at the study sites of subarea B (0.9 vol.-% at 2 km from mill B) and in subarea C (2.4 vol.-% at a distance of 2 km from mill CI), suggesting substantially lower exposure of the fish to effluents in areas B and C. Consequently, the results indicate site specific inconsistency in the mixing and dispersion of wastewaters within the large study area. At the beginning of the caging, the water temperature close to the cages (5 m) was around 3.6 °C and the mean temperature was 13.6 °C (range 5 °C) at the end of the caging period.

4.2 Exposure of fish to effluent constituents

Subarea A in 1991 and 1993. The exposure of whitefish to different effluent constituents was assessed with the aid of accumulated chlorophenolics

(chlorophenols, chloroguaiacols and chlorocathecols) (I, II, III, IV) and resin acids in the bile (II, III, IV), as well as with CPs and extractable organic halogens (EOX) in the lipid adjacent to the intestinal tract (I, II).

In 1991, the concentrations of total CPs in the bile of whitefish were 55-fold in the vicinity of pulp and paper mill A, compared to the upstream reference concentration of 10 $\mu g\ mL^{-1}$ (I). The amount of bile CPs gradually decreased in proportion to the distance from the effluent source. The most abundant CPs in the bile and gut lipids was 345-CG (64%), a chlorophenolic compound considered to have an origin related to chlorine bleaching (Paasivirta et al. 1988). Similar to the bile CPs, a clear body burden gradient was seen in the accumulation of CPs and in the EOX levels in the gut lipids (I).

A substantial decrease in the accumulation of the bile CPs was observed in the laboratory exposure in 1992 (II), at the time when mill A replaced almost all the chlorine in the bleaching by chlorine dioxide. Additionally, the concentrations did not reveal a distinct dose-response relationship and were not significantly different from the values in the control fish. Compared to the field exposure of 1991 (I), the bile CPs were at the level of about 0.5-1% in the laboratory simulation. Similar result was also seen in the field conditions in 1993 (Petänen et al. 1996), where the bile CPs decreased by 99 % compared to the levels at the same sites in 1991.

Large research area in 1995. The concentrations of free and conjugated CPs in the bile showed low exposure to chlorophenolic substances, although the CPs were still measurable at each site (III). The bile accumulation of CPs in 1995 was 0.5 % that of 1991 (III). A markedly lower mean value of the bile CPs was detected also in fish kept in the reference area in 1995 (0.29 $\mu g\ mL^{-1}$) compared to the levels in 1991. Compared to the upstream references (mean), an approximately 3-fold increase in the bile CPs was measured downstream from mill A in 1995. In the vicinity of mills B and C, the bile CP levels were considerably lower than in area A (III). The spatial interpolations (Kriging) of the CPs in the bile in the receiving waters of mill A in 1991 and 1995 are presented in Fig. 3.

The concentrations of RAs in the bile of whitefish varied considerably between the research areas in 1995 (III). In areas A and C, RA concentrations in the vicinity of the mills were nearly at the same level (150 $\mu g\ mL^{-1}$), while a markedly lower accumulation was observed near mill B. In the laboratory simulation of 1992 (II), at the time when mill A was still employing aerated lagoons as the secondary treatment, the bile accumulation of RAs was about 50-fold compared to the field study in 1995 (III) and the laboratory exposure in 1996 (IV). In the laboratory simulation in 1992 (II), the sum of free and conjugated RAs in the bile was maximally 460-fold in the exposed fish (7 vol.-% BKME) compared to the control, while in 1996 the accumulation was only 7-fold (IV). Dehydroabietic acid (DHAA) (40-64 %), pimaric and isopimaric acids (28%) and abietic (17%) acids were always the dominating RAs in the bile of exposed whitefish.

4.3 Liver monooxygenase induction in whitefish in field

4.3.1 EROD activity

Subarea A. The long-period background of EROD activity averaged 3.8 pmol min.$^{-1}$ mg prot.$^{-1}$ (S.D. 1.1, n=55 analysis, pools of 140 fish) at reference sites 1 and 2 (I, Petänen et al. 1996, III). Since these reference sites year after year showed equal EROD activity, the data from these two was handled as one entity. In 1991, a clear gradient of EROD activity was seen in exposed whitefish in the recipient of mill A (I). At the nearest study site (3.3 km) from the mill, EROD activity was 53 pmol min.$^{-1}$ mg prot.$^{-1}$, i.e. 13-fold compared to the mean reference value (4 pmol min.$^{-1}$ mg prot.$^{-1}$). The statistically significant activity of EROD reached as far away as 12 km from the effluent source and at the most distant station (16 km) a trend toward increased EROD activity was still observable. In 1993, at the nearest study site, a 2.2 -fold EROD activity (9.8 pmol min.$^{-1}$ mg prot.$^{-1}$) relative to the reference was measured (Petänen et al. 1996). In all, in 1993 EROD activity was about 20 % and in 1995 4-10 % of the activity in 1991 in the area from 3.3 to 12 km from the mill. Kriging interpolations of EROD activity in research area A in 1991 and 1995 are presented in Fig. 4.

Large research area. Compared to the appropriate reference sites (Ref. I), significant differences ($p<0.05$) were measured in whitefish EROD activity at six of the total 22 sites along the large research area in Southern Lake Saimaa in 1995 (III). A statistically significant EROD activity was measured in whitefish exposed 1 km (four-fold) and 5.8 km from mill A (two-fold). Relationships between the liver EROD activity of whitefish and the calculated effluent concentration in the field exposures in the effluent receiving waters of the pulp and paper mill A in 1991 (I) and 1996 (unpublished data) are given in Fig. 5. In addition to the elevated EROD activity in the vicinity of mill A, significant activity was also measured 2 km and 6 km from mill B (2.2-fold in both) and at 2 km from mill CI (1.9-fold). Overall, as a general pattern, the highest EROD activities were found at the sites nearest the mills. Unexpectedly, however, a three-fold EROD activity compared to the reference was found in fish caged at the distant background site 22. The activity was at about the reference level in 1996 (unpublished data). The correlations between liver EROD activity and the body residues and ambient water compounds are presented in Table 3.

4.3.2 PROD activity

No statistically significant differences were observed in liver PROD activity in subareas A, B and C compared to the main reference area (Ref. I). However, in respect of EROD activity, increased PROD activities were measured in the vicinity of the mills (III), although there was no significant correlation between the liver activities of EROD and PROD (r^2 =0.064 for whole data, n=115).

TABLE 3 The correlations between the liver EROD activity and the body residues of whitefish as well as selected ambient water constituents in the field experiments in 1991 (I), 1993 (Petänen et al. 1996) and 1995 (III) in Southern Lake Saimaa and in the laboratory exposures with effluent from mill A in 1992 (II) and 1996 (IV).

Study	Field (A[1])			Laboratory			Field (A)			Field (A)			Field (L[2])			Laboratory		
	1991			1992			1993			1995			1995			1996		
	r^2	p<	n	r^2	p<	n	r^2	p<	n	r^2	p<	n	r^2	p<	n	r^2	p<	n
Water:																		
AOX[3]	0.84	0.05	5	0.91	0.05	4	0.71	0.10	5	0.27	0.50	6	0.10	0.50	20	0.48	0.50	5
CPs[4]	0.86	0.05	5	0.29	0.10	4	<0.01	1.00	6	0.07	0.10	5	<0.01	0.10	16	0.84	0.05	5
RAs[5]	0.78	0.10	5	n.a.			n.a			0.85	0.05	6	0.02	0.50	16	0.99	0.01	5
Bile:																		
CPs	0.84	0.01	15	0.15	0.10	12	0.31	0.50	7	0.04	0.10	6	0.01	0.10	20	0.15	0.50	5
RAs	n.a.			0.87	0.01	12	0.11	0.50	7	0.04	0.10	6	0.01	0.10	19	0.85	0.05	5
Lipid:																		
CPs	0.88	0.01	9	0.43	0.05	12	n.a.			n.a.			n.a.			n.a.		
EOX[6]	0.63	0.01	24	0.29	0.10	23	n.a.			n.a.			n.a.			n.a.		

[1] subarea A; [2] large research area; [3] adsorbable organic halogens; [4] chlorophenolics; [5] resin acids; [6] extractable organic halogens; n.a. not analyzed

4.3.3 CYP1A1 mRNA expression

Whitefish hepatic CYP1A1 mRNA expression (IV, V) exhibited a statistically significant increase (5.5-fold, $P<0.05$) in two sites immediately downstream from mill A compared to the reference area. At the sites more distant from the mill, it was still possible to measure a 2.4-fold elevation. At other study sites, whitefish CYP1A1 mRNA lay at approximately similar levels to the references. No correlation was found between CYP1A mRNA and EROD activity within all the sites ($r^2=0.05$). However, the comparison is not fully applicable, as CYP1A1 mRNA concentrations were determined from individual fish, whereas liver EROD was measured from the pools of fish livers.

4.3.4 Monooxygenase activity in laboratory simulations

There was a strong dose-related induction of liver EROD activity of whitefish exposed to effluent from mill A in the laboratory-based exposures in 1992. At 3.5 vol.-% effluent treatment, i.e. the concentration of effluent actually found in the field at around 3 km from the mill, the induction was 12-fold, while at 7 vol.-% concentration EROD activity was 18-fold compared to the control (II). In 1996, secondary treated effluent from the same mill revealed a 2-fold relative EROD activity ($p<0.05$) in fish exposed to 3.5 and 7 vol.-% effluent concentrations (IV). While EROD induction remained low in fish exposed to secondary treated effluent, an 11-fold induction was observed in fish exposed to 3.5 % pre-treated effluent. Dose response regressions between the liver EROD activity and the concentrations of effluent in the laboratory experiments in 1992 and 1996 are given in Fig. 6.

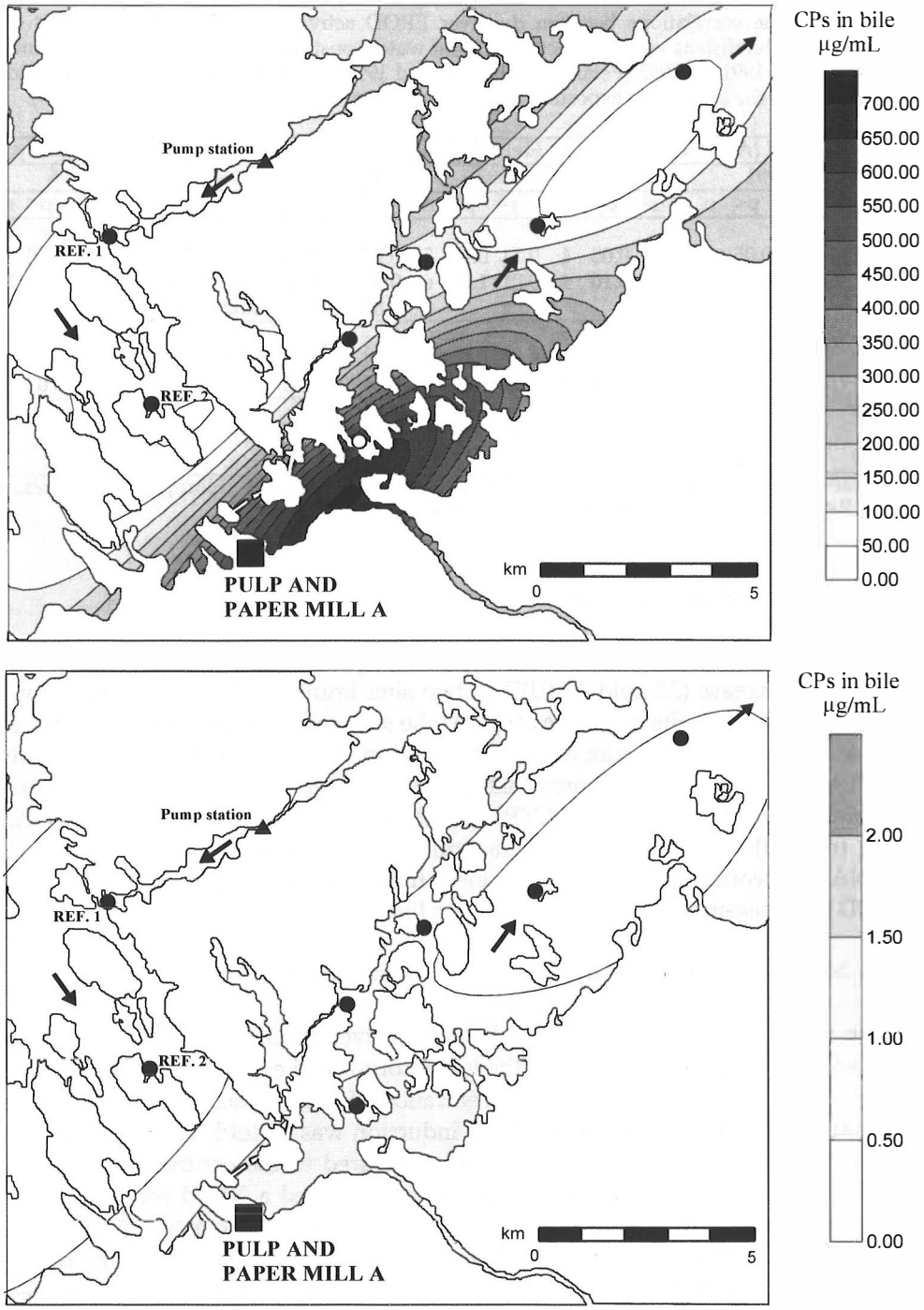

FIGURE 3 Kriging interpolation, given as contours of the chlorophenolic (CP) accumulation to the bile of whitefish exposed in the receiving waters of mill A in 1991 (upper) and 1995 (lower). The black dots indicate the caging sites in the area. The gray scales used in the graphs are not comparable between 1991 and 1995. Data from I and III.

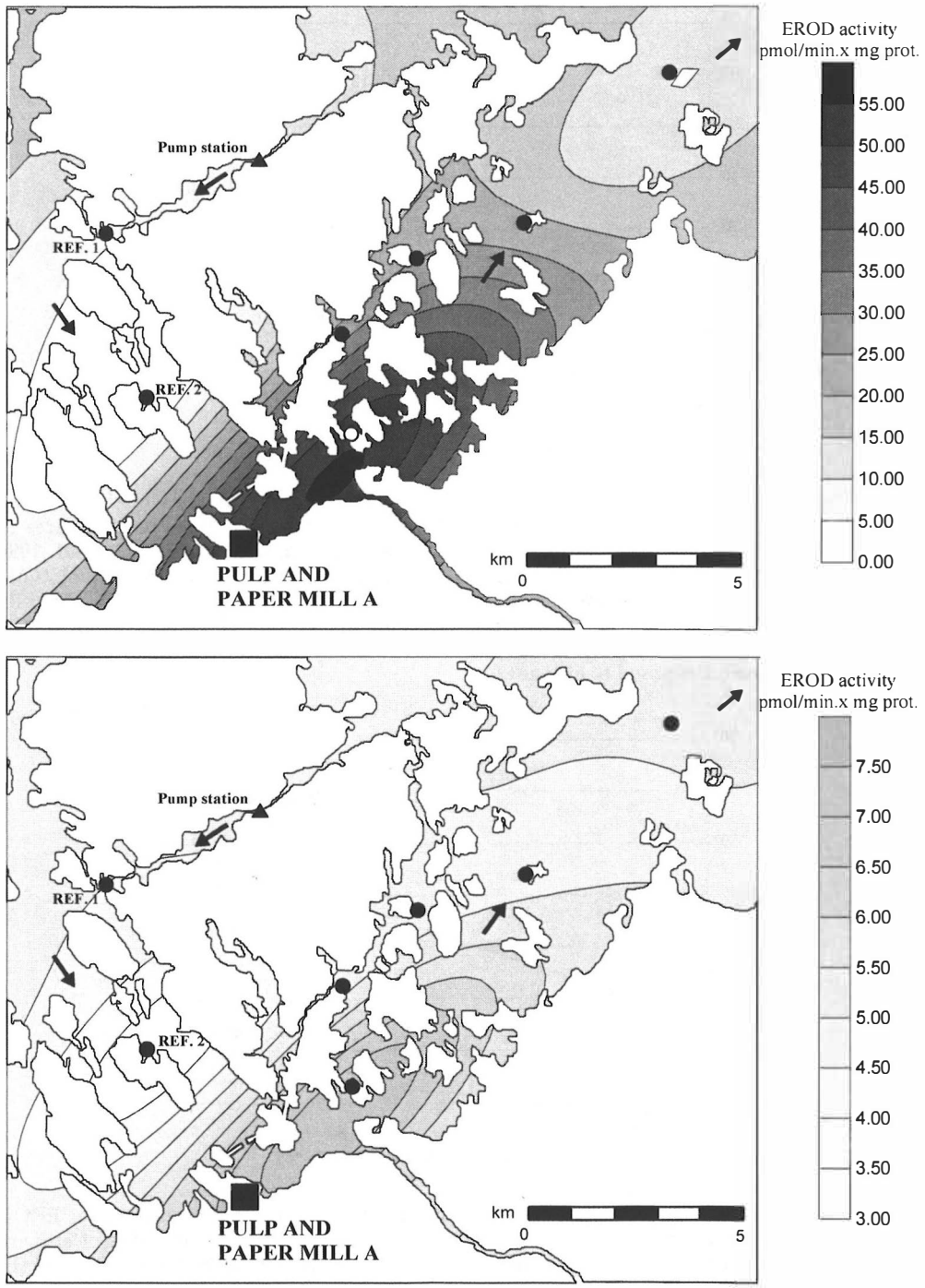

FIGURE 4 Kriging interpolation, given as contours of the liver EROD activity of whitefish exposed in the receiving waters of mill A in 1991 (upper) and 1995 (lower). The black dots indicate the caging sites in the area. The gray scales used in the graph are comparable in 1991 and 1995. Data from I and III.

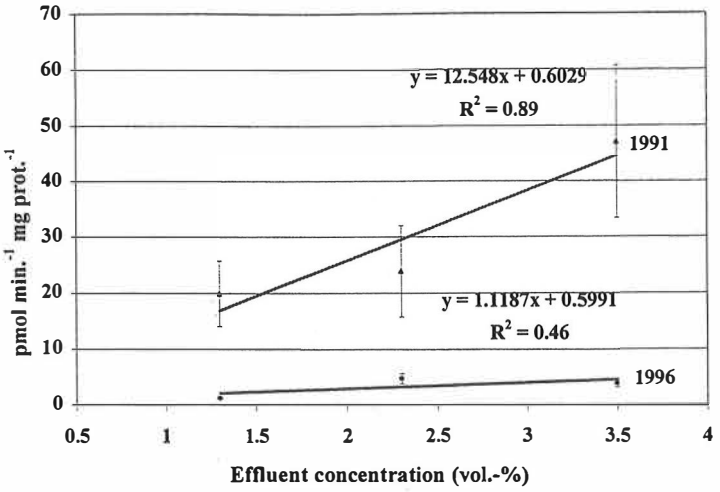

FIGURE 5 Relationship between the liver EROD activity (mean ±SD) of whitefish and the calculated effluent concentration in the field exposures in the recipient area of pulp and paper mill A in Southern Lake Saimaa in 1991 (I) and 1996 (unpublished data). The effluent concentrations of 1.3, 2.3 and 3.5 % (vol./vol.) correspond to distances of about 16, 9 and 3.3 km, respectively, from the mill. The regression equations are given in the graph. The mean value of the reference (3.8 pmol min.$^{-1}$ mg prot. $^{-1}$) is subtracted from the values of the groups exposed to effluent.

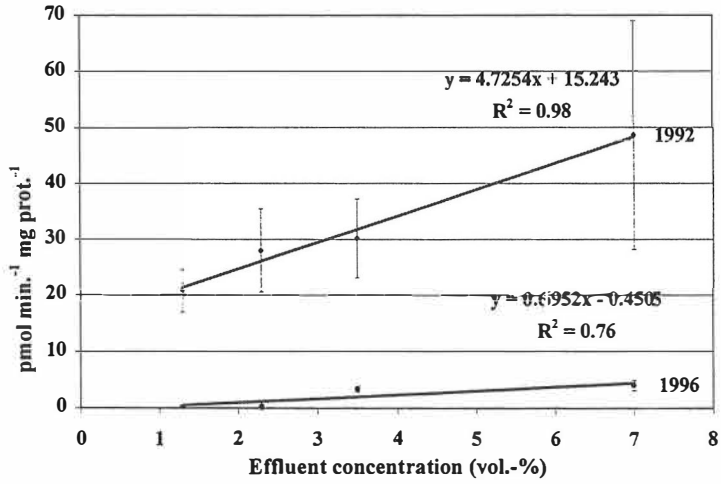

FIGURE 6 Dose response regressions showing the relationship between the liver EROD activity (mean ± SD) of exposed whitefish and the concentrations of effluent from pulp and paper mill A in the laboratory-based experiments in 1992 (II) and 1996 (IV). Whitefish were exposed for 30 d to effluent at concentrations of 1.3, 2.3 and 3.5 % (vol./vol.), corresponding to distances of about 16, 9 and 3.3 km in the effluent receiving subarea of mill A. Moreover, an additional concentration (7 vol-%) was used in the laboratory. The mean value of the reference (3.8 pmol min.$^{-1}$ mg prot. $^{-1}$) is subtracted from the values of the groups exposed to effluent. Data from I and III.

Similarly to EROD, liver PROD activity was significantly increased in fish exposed to 3.5 % (1.6-fold) and 7 % (1.8-fold) secondary treated effluent and 3.5 % pre-treated effluent (9-fold), compared to the control. Additionally, hepatic EROD and PROD inductions exhibited a high correlation (r^2=0.85, $p<0.01$, n=85) when all the data was combined, whereas the PTE group with the highest EROD activity exhibited a statistically nonsignificant relationship (r^2=0.22, p=0.1, n=15).

4.4 Activity of liver conjugation enzymes: UDP-GT and GST

The activity of whitefish liver UDP-GT was not significantly ($p>0.05$) changed, although a tendency toward increased activity was observed when fish were exposed in the field (I). On the contrary, in the laboratory simulation (II) the activity of UDP-GT was significantly reduced by 34 % at 7 % effluent concentration compared to the control. At the higher dilutions, the activity was also decreased, but not statistically significantly. In 1993, after the process alterations in the mill, no significant changes were observed in UDP-GT either. Neither were significant changes seen in the activity of cytosolic GST in the field (I, Petänen et al. 1996) nor in the laboratory exposure (II).

4.5 Plasma immunoglobulins (IgM)

In 1991, immunoglobulin M levels were decreased by 42-11 % ($p<0.05$) in fish caged at 6 km up to 16 km from mill A, respectively, compared to the reference level (I). However, an apparent exception was observed at 3.3 km from the mill, where the mean IgM was not different from the reference. In the field experiment in 1993 (Petänen et al 1996), no differences in IgM levels were observed at the effluent receiving sites of mill A compared to the reference. In the large study area in 1995 (III), whitefish IgM tended to be lower 6-12 km downstream from mill A, although the change was not significantly different from the reference level. Additionally, a 28 % decreased IgM level was found also in whitefish exposed near mill CI.

Whitefish exposed to effluent from mill A at concentrations of 1.3, 2.3, 3.5 and 7 % in laboratory conditions, exhibited 33-46 % decreased IgM concentrations in the exposed groups compared to the controls (II). By contrast, in the laboratory in 1996 whitefish IgM remained unchanged at all concentrations of secondary treated (activated sludge) effluent, as well as at 3.5% untreated effluent from the same mill (IV).

4.6 Hematological effects

Whitefish blood hematocrit did not vary at different sites in the receiving area of mill A in 1991 (I), nor in the large area covered whole Southern Lake Saimaa in 1995 (III). Similarly, the laboratory experiment in 1996 (IV) showed unchanged hematocrit, whereas in the laboratory in 1992 (II) decreased hematocrit in the exposed fish was observed. Whitefish blood hemoglobin (Hb) was significantly reduced 9 km and 12 km from the effluent source in the receiving waters of mill A compared to the control in 1991 (I). Similarly, blood Hb was reduced at all concentrations, when fish were exposed to effluent from mill A in the laboratory in 1992 (II). In 1995, blood hemoglobin also showed significant differences in areas B and C. Evidently, however, the changes in Hb were not effluent related, since the reference sites also showed some alterations (III). In the second laboratory exposure to effluent from mill A in 1996, whitefish Hb remained unchanged at all the treatment groups (IV).

The concentrations of plasma glucose did not change in whitefish exposed to effluent of mill A at different sites in the field in 1991 (I) and in the laboratory in 1996 (IV). By contrast, decreased plasma glucose was observed in exposed fish in the first laboratory study (II), while in the large area in Southern Lake Saimaa in 1995 (III), glucose was significantly elevated at 14 out of 22 sites. Similarly to the plasma glucose, the concentrations of whitefish plasma lactate were unchanged in fish exposed in the effluent receiving area of mill A in 1991 (I) and in the laboratory in 1996 (IV). Reduced plasma lactate was measured in whitefish exposed in the laboratory to effluent from mill A in 1992, as well as in whitefish at seven sites in the field experiment in the large research area in 1995 (III).

In the laboratory in 1992, the concentrations of NTP in erythrocytes were significantly lower, while erythrocyte sodium was significantly higher in fish exposed to the different concentrations of effluent from mill A, compared to the control (II).

The activity of plasma lactate dehydrogenase (LDH) in whitefish did not change in the different caging sites in the field in 1991 (I) or 1993 (Petänen et al 1996), while in the laboratory conditions the activity was reduced in the exposed groups. In the same laboratory exposure, plasma activity of aspartate aminotransferase (ASAT) was significantly reduced (28-40 %) in all exposed groups. In the field experiment in 1993, ASAT was similar at both the downstream and upstream sites. As a consequence, the results indicate that no gross membrane leakage had developed in the liver or other organs due to exposure to BKME.

4.7 Reproductive steroids and vitellogenin expression

The concentrations of the reproductive steroids, 17β-estradiol and testosterone, were measured in juvenile whitefish in the field in 1995 (III) and in the laboratory

experiments in 1996 (IV). In the field, increased estradiol concentrations were seen 1 km (76%) and 3.3 km (43%) from mill A compared to the reference. On the other hand, decreased plasma estradiol concentrations (63 %) were observed near mill B, whereas at the other sites plasma estradiol levels were similar to the reference. The concentrations of plasma testosterone showed no significant differences at the different exposure sites compared to the reference area. All in all, great variability was observed within and between the sites in the plasma reproductive steroids.

In contrast to the field experiment, the exposure to both 3.5% secondary and pre-treated effluent from mill A decreased 17β-estradiol concentrations by 37 % ($p<0.05$) and by 20 %, respectively and testosterone concentrations by 41 % and by 40%, respectively in the laboratory-based exposures in 1996 (IV).

Compared to the mean references 1 and 2, a 20-fold ($p<0.05$) expression of vitellogenin mRNA was observed in 1995 in area B at sites B1, B2 (14-fold) and B4 (9-fold), as well as at site A6 (3-fold) in area A (V).

4.8 Condition and mortality of fish

The body condition factor (CF) of fish did not vary among the different exposure sites in the field (I, Petänen et al. 1996, III). However, in the laboratory simulation (II), a significantly elevated CF was observed in all the exposed groups compared to the control. Later, in 1996 the CF remained unchanged in the laboratory (IV). In the field, the mortality of the exposed fish was low in 1991 (I) and 1993 (Petänen et al. 1996), whereas an elevated mortality occurred in 1995 (III). In the laboratory experiments (II, IV), no mortality was recorded in the exposed or control fish.

5 DISCUSSION

5.1 Pulping and bleaching: an overview

For producing pulp, wood can be processed either mechanically or chemically. Chemical pulping includes the treatment of wood chips with strong chemical solutions under conditions where most of the lignin dissolves out from the wood material, after which the fibers are washed and possibly bleached (Sjöström 1993). Being the most common way to produce wood pulp, kraft (sulfate) pulping deserves special attention. Compared to the sulfite process the kraft process provides some advantages. First, the inorganic chemicals can be recovered, reducing discharges and improving the energy balance of a mill. Second, when the spent pulping liquor is evaporated and the solids (inorganic salts and lignin residues) are burned, the system generates enough energy for the whole pulp mill.

All mills involved in this study produced kraft pulp, either bleached (A, B and CI) or unbleached (CII). However, the mills differed somewhat in terms of the production volumes, the wood furnish used and the effluent volumes (Table 1). Moreover, mills A and CI were integrated mills that were also producing groundwood (A), paper (A and CI), sawn goods (A) and cardboard (CI).

In mechanical pulping, the wood material is refined or ground with refiners to separate the cellulose fibers. The resulting mechanical pulps, including groundwood (GW) or pressured groundwood (PGW), thermomechanical pulp (TMP) and chemi-thermomechanical pulp (CTMP), differ substantially in respect of their properties as well as the production requirements. Similar to kraft pulp bleaching without chlorine or chlorine dioxide (TCF), the bleaching of mechanical pulp, normally made by the peroxide process, requires the chelating agents EDTA or DTPA. In consequence, the use of chelating agents by mill A in

bleaching groundwood pulp was also detected in the receiving water of the mill (Sillanpää & Oikari 1996).

After pulping, dark pulp can be bleached in a sequence of electrophilic and nucleophilic reactions. A so-called "conventional bleaching sequence" might be CEHDED, where C stands for chlorine (Cl_2), E for caustic extraction (NaOH), H for hypochlorite and D for chlorine dioxide (ClO_2). In modern processes, however, chlorine is totally replaced with chlorine dioxide and the sequence may include a prebleaching stage with oxygen (O-stage). The alkaline stage may be reinforced by oxygen and/or peroxide (p). Thus, a modern bleaching sequence might be ODEopDEpD. All the mills involved in this study in 1995 and 1996 performed elemental chlorine free (ECF) pulp bleaching.

5.2 Implications of changed mill processes on effluent quality

5.2.1 New manufacturing processes

During the last few years, the pulp and paper industry has introduced several alterations in the manufacturing processes and wastewater treatment all over the world (Axegård et al. 1993). In the development of the bleaching processes, the input of chlorine in the first bleaching stage was first reduced using lower multiple chlorination and later in the 1980s by replacing elemental chlorine by chlorine dioxide. Consequently, ECF bleaching has substantially reduced the formation of different chloroorganic constituents and total organically bound chlorine in effluents. In this study, the process renewals in mill A, including replacement of chlorine by chlorine dioxide, nearly eliminated chlorinated phenolic compounds (reduction 98 %) and considerably reduced the levels of the bulk parameter of organic chlorine (AOX) by 87 % in treated mill effluent (Oikari & Holmbom 1996, Kaplin et al. 1997). As a consequence, the better quality of the discharging effluent has been reflected in a profound improvement in the quality of the receiving waters of the mill. It is apparent that the use of chlorine dioxide instead of chlorine in bleaching results in more oxidation processes than substitutions when reacting with lignin, particularly with its aromatics (Robinson et al. 1994). Additionally, decreased chlorination of organic materials reduces the persistence, accumulation and toxicity of these components. The formation of polychlorinated dibenzo-*p*-dioxins and -furans (PCDD/PCDF) is avoided or practically eliminated by low multiple chlorination or by using chlorine dioxide in the first bleaching stage. Consequently, only trace amounts of PCDDs/Fs, near the detection limits, have been measured in the final effluents from modernized mills (Berry et al. 1989).

In addition to substituting elemental chlorine by chlorine dioxide in bleaching, several alterations in pre-bleaching techniques, such as extended or otherwise modified cooking and oxygen delignification, have been implemented in mills. Oxygen delignification in softwood pulping has reduced the kappa number, the amount of residual lignin in pulp, from about 30-35 to 20 compared

to the conventional kraft cooking processes. Furthermore, the combination of extended cooking and oxygen delignification is able to reduce the kappa number to around 10 (Strömberg et al. 1996). Effective recovery of lignin influences the discharges, as the reduced amount of residual lignin enters the bleaching process, diminishing the use and the costs of bleaching agents. Moreover, when burned, residual lignin releases more energy for the pulp mill, affecting the energy balance of the mill. The combination of an extended cooking stage together with oxygen delignification, ECF bleaching and secondary treatment has been found to be essential in reaching low AOX loads, resulting in discharges as low as <0.2 kg Cl ton^{-1} softwood pulp (Axegård et al. 1993, Strömberg et al. 1996).

5.2.2 Effects of biological treatment on effluent quality

Aerobic secondary treatment of wastewaters by aerated lagoons or by the activated sludge process have been most widely used in the pulp and paper industry to date. In addition, chemical processes are also applied in some mills for paper effluents. Effluent treatment together with modified manufacturing processes have considerably decreased levels of the conventional pollutants in effluents, although moderate variation due to different internal factors of the mills has still been observed (Strömberg et al. 1996).

Initially, the objective of a biological treatment has been to remove organic carbon, usually measured as BOD, COD and suspended solids in wastewaters. In the case of mill A, the average removal efficiency of the biological stage of mill A has been 68% for COD, 97% for BOD_7 and 62 % for AOX in 1995 (Data supplied by UPM-Kymmene Kaukas Mill, The Environmental Research Laboratory). In addition to diminishing mill constituents above, implementation of the activated sludge treatment with the process changes reduced resin acids by 94 % and fatty acids by 85 % in the mill (A) effluent compared to the levels before the changes (Kaplin et al. 1997). However, the concentrations of the wood-derived sterols, like the main sterol β-sitosterol, has remained nearly unchanged since the mill alterations. Further, the effluent treatment by the activated sludge process decreased the amount of CPs by about 60 %. A somewhat higher removal of CPs (72%) and almost complete removal of RAs (99.6%) by the biological stage of the treatment was observed when pre-treated and following secondary treated effluent (mill A) were compared in connection with the laboratory experiment (IV). These results indicate a high effectiveness of the biotreatment of wastewaters with the activated sludge process in removing these effluent components.

In 1995 (III), all the mills studied employed secondary treatment by the activated sludge process for pulp effluents. Moreover, mills CI and CII possessed a chemical treatment plant for effluents for paper and cardboard production. Evidently due to older treatment facilities (built in 1986) the efficiency of the biotreatment in mill B resulted in discharges of wood extractives per unit of produced pulp from the mill being substantially higher compared to the other mills (V). However, new treatment facilities in mill B have been implemented in November 1996.

5.3 Mill effluents and exposure conditions

5.3.1 Substances in pulp and paper effluents

Effluents from chemical and mechanical wood processing contain a combination of highly complex mixtures of inorganic as well as high and low molecular weight organic substances. A large proportion of the organic substances in kraft pulp mill effluent, including chlorinated or unchlorinated residual lignin, can be found in the high molecular weight fraction (HMW >1000 kD, Pellinen & Salkinoja-Salonen 1985, Dahlman et al. 1993). Due to its highly complex structure, chlorolignin has not been well characterized up to now. Owing to the similarities of chlorolignin from industrial processes and naturally chlorinated humic substances, it is often difficult to separate these. Chlorolignin, lignin and natural humic material considerably absorb lipophilic substances in an aquatic environment, affecting the bioavailability of these substances to aquatic animals (Kukkonen 1992).

The low molecular weight (LMW) organic fraction present in kraft mill effluents is usually divided into three separate classes: carboxylic acids, phenolic compounds and neutral substances (Paasivirta 1991). In addition to phenolic compounds usually originating from lignin (Dahlman & Mörck 1993) and neutral compounds, the LMW fraction includes other chemical classes, such as lipophilic wood extractives, viz. resin acids, fatty acids, sterols and triterpene alcohols. Additionally, all these compounds can be found with various degrees of chlorination, as well as in an unchlorinated form (Kringstad & Lindström 1984). Nowadays, the very toxic PCDDs/Fs are formed only in trace quantities in ClO_2 bleaching (Berry et al. 1989).

Chlorophenolic compounds formed during chlorine bleaching have been one of the most attractive groups of pulp and paper mill discharges. As the introduction of new bleaching technologies dramatically decreased the formation of chlorophenolics (Dahlman et al. 1993), considerable reduction of their ecotoxicity is expected. The low chlorophenolic concentrations were typical also in the effluents from the mills in this study. Resin acids, a group of diterpene acids extracted from wood material during the pulping process, have been found to be the most important group of acutely toxic chemicals in pulp and paper discharges (Holmbom & Lehtinen 1980). In addition to resin acids, there are several wood-derived polycyclic compounds, including triterpenoids (e.g. petulinol), sterols and phenolic constituents like lignans and stillbenes (Sjöström 1993).

The concentrations of resin acids, fatty acids and sterols varied substantially among effluents from mills A, B and C studied (III). It is plausible that the variability depends on a whole array of factors, in addition to the wood species. These include storage time and conditions of the wood raw material and to some extent the process stages of the mill (Strömberg et al. 1996).

5.3.2 Dilution and dispersion of effluents

In 1991, before the new bleaching process and the activated sludge plant were introduced in mill A, a distinct concentration gradient of effluent constituents including CPs, RAs and AOX was seen. Additionally, the effluent tracer sodium revealed a clear decreasing trend in effluent plume in the lake. Thus, effluent concentrations were calculated as being from 3.8 vol.-% at 3.3 km to 1.2 vol.-% at 16 km from the mill (I). Consequently, the dispersion and dilution conditions of effluent proved comparable to those in the late 1980s in the same receiving area (Lindström-Seppä & Oikari 1989, 1990a, 1990b).

In 1995, the concentrations of CPs, RAs and AOX still showed a moderate distance dependence along the receiving waters (III), although the concentrations of the marker constituents were substantially lower than those observed prior to the modifications at the mill (I).

Comparing the areal dispersion and dilution of effluents in the whole Southern Lake Saimaa, large differences were observed between the subareas (III). Thus, in 1995, considerably lower effluent volumes were observed in subarea B and C at the site nearest to mill B (0.9%) and mill CI (2.4%) compared to mill A (4.6 %, 1 km). Consequently, considerable local and possibly also temporal variability in the actual exposure of fish to effluent constituents can be suggested between the subareas. These results assume a site specific inconsistency in the mixing and dispersion of wastewaters in all the subareas. Apparently, the time of the experiment in spring - early summer, coinciding with the spring overturn, may explain part of the variation observed. Thus, the characteristics of the effluents, as well as the recipient area hydrology are important site-specific factors to be considered when assessing the fate and effects of effluent components in the large lake ecosystem. The site specific factors have been suggested as substantially limiting the development of generalized models of the fate and effects of effluents and their constituents (Gifford 1995).

5.4 Fish biomarkers - utility in ecotoxicological research

In common use the term biomarker is characterized as a biochemical, physiological or histological indicator of exposure or effect caused by xenobiotics at organismal or suborganismal levels (Huggett et al. 1992). The rationale behind the biomarker approach is that living organisms integrate exposure to contaminants in their environment and respond in some measurable and predictable way. The biomarker approach has recently received considerable attention in ecotoxicology as a new and potentially powerful and informative tool for detecting and documenting exposure to, susceptibility and the effects of environmental contamination.

Physiological responses of organisms to aquatic contaminants take place at the level of the whole organism (e.g. respiration, growth, reproduction), in organs and tissues (organosomatic indices) or at cellular levels (biochemical or

cytochemical). In respect of the impact on aquatic animals, normally a distinction is made between whole-organism and within-organism effects and tests. Whole-organism tests include life cycle, partial life cycle and life stage assays, whereas within-organism tests involve studies of biochemical and physiological responses. However, divisions of the groups are necessarily artificial as responses often act at more than one level.

Several criteria have been suggested as necessary for assessing the suitability of a physiological response to be used as a measure of the condition of an animal subjected to environmental stress and pollution. According to Mayer and coworkers (1992) the criteria for the selection and development of useful biomarkers for individual-level responses are as follows:

> The biomarker should respond in a dose- or time-dependent manner to the toxicant so the magnitude of the exposure or effect can be determined.
>
> The biomaker response should have biological significance related to effects on the growth, reproduction or survival of the individual, the population, and ultimately the well-being of the community. Only biomarkers that can be linked to important biological processes and for which changes can be interpreted should be used.
>
> Sufficient sensitivity of the biomarker to the contaminant should exist. The response should be measurable with precision and easily detectable above the biological variability.
>
> The biomarker should be relatively easy to measure, allowing quantification of multiple individuals in the laboratory and/or field preferably without the use of expensive equipment, complicated procedures or high running costs.
>
> The variability due to other factors (season, temperature, sex, weight, and handling) should be understood and within acceptable limits.

These criteria will assist in biomarker selection, study planning, implementation and data interpretation, although not all of these criteria need to be fulfilled for a biomarker to be useful. To be of the greatest use, biochemical and physiological biomarkers of exposure and effects should be applicable in the field, which may raise several tasks regarding the use and application of biomarkers. There is a need for increased understanding of the basic biochemistry of the organisms including "normal" physiological ranges of the animal with the statistical confidence in determining that the change is out of the normal range (Mehrle and Mayer, 1980). It is also critical to differentiate between the acclimation and toxic effects in an organism. Exposure to a low-level stress may cause an alteration in a biomarker parameter due to an acclimation response by the organism. However, increased stress with toxicity effects may either increase a known biochemical response when the organism attempts further acclimation or decrease, representing exhaustion of the acclimation response. Thus, it can be difficult to statistically distinguish between the normal and the acclimated state or particularly between the acclimated range of response and only a slightly increased response in the toxic range. However, appropriate selection of the biomarkers has been suggested as

a resolution of this dilemma (Mayer et al.1992). Additionally, variability in the measurements of responses due to organismal, environmental and methodological reasons may be a problem in environmental toxicology. Biomarkers can succeed only when these variability issues are resolved. Since little can presently be done to reduce the organismal variability, proper selection of biomarkers, research on experimental protocols and factors affecting variability, and application of appropriate statistical methods have been suggested for reducing the variability of biomarker responses (Adams et al, 1985).

The objective of the biomarker approach in this thesis research included documenting exposure related to the pulp and paper industry by estimating the spatial extent of bioavailable constituents with various degrees of physiological or biochemical responses related to the exposure. In conjunction with ongoing field studies, controlled laboratory studies were carried out in a close integration. Although the choice of the biomarkers used in this research was based on the criteria presented above, the measurements carried out in earlier studies in the area were preferable. No attempt either was made in this research to predict the individual responses in fish population or community levels. Additionally, the criteria for the measurements without expensive equipment, complicated procedures or high running costs are often difficult to meet.

5.5 Technique of fish caging

Studying fish by means of caging has proved to have many advantages relative to collecting feral fish: there is a precisely known exposure site and duration and fish material with a known developmental stage and genetic background (I). The type of whitefish used in this research was a plankton and seston feeder able to manage in cages during a one-month exposure, thus allowing subchronic experiments. It has been demonstrated that juvenile whitefish can be caged for several weeks in an unstressed condition in the cages used in this research (Oikari & Sillanpää 1993). By placing cages at varying distances from the single source of effluent and by comparing biochemical, physiological and body burden responses to constituents of the effluent, it was possible to demonstrate a cause and effect relationship, together with a magnitude and a spatial extent of the responses.

5.6 Validation of field responses of fish by laboratory simulations

In the field studies it is generally impossible to control environmental factors like temperature, oxygen concentration, levels of contaminants etc., which affect the responses in animals. These physical-chemical qualities of water can be controlled in laboratory conditions, enabling a more accurate dose-related evaluation of the physiological effects of the effluent. Therefore, field data must be validated with

appropriate laboratory studies in which pollution-unrelated environmental correlates can be avoided. On the other hand, although it is possible to expose fish under controlled laboratory conditions to complex mixtures or individual constituents, the total effects on feral or experimentally exposed fish cannot be determined by laboratory experiments alone.

The effluent concentrations chosen for the exposures in the laboratory (II, IV) were environmentally relevant and representative of those existing in the receiving area of the mills (I, Petänen et al. 1996, III). Additionally, in order to predict the margin of safety of ecotoxicological risks, a lower level of effluent dilution (7%) was also studied. Altogether, in regard to the exposure of fish to the effluent constituents in the field, the present laboratory simulation was quite comparable with the field circumstances.

5.7 Evidence of exposure of fish to effluent constituents

5.7.1 Biliary accumulation of effluent components

Several investigations have demonstrated that effluent components, including selected chloroorganics and wood-derived extractives, may accumulate in bile and in other fluids or tissues of fish (Oikari & Kunnamo-Ojala 1987, Kierkegaard & Renberg 1988, Lindström-Seppä & Oikari 1989, Söderström & Wachtmeister 1992). Actually, the results indicate that these chemical metabolic markers serve as one of the most sensitive indicators of the aquatic exposure of fish to pulp and paper effluents. As a consequence, the bioaccumulation of chlorophenolics and resin acids in bile may be used as biomarkers of internal dose and actual exposure of the animals to effluent constituents.

Previously, up to the 1990s a clear-cut distance-related response was observed in the levels of CP and RA metabolites in whitefish bile in the lake contaminated by mill A (Oikari & Kunnamo-Ojala 1987, Lindström-Seppä & Oikari 1989, 1990a, I). In relation to these chemical markers of effluent, the exposure of fish to effluents has been very low since the mill alterations compared to the previous studies (III, IV). Consequently, the bile accumulation of CPs in 1993 and 1995 was only 0.5 - 1 % that of 1991. In the vicinity of mills B and C (1995) CP levels in the bile were somewhat lower than in area A, mainly reflecting the higher dilution of effluents in these subareas.

A drastic decrease in organochlorines such as chlorinated phenolics was observed after replacing chlorine with chlorine dioxide in the bleaching. Consequently, the low ambient concentrations of CPs were reflected as low biliary accumulation of CPs in fish in the laboratory conditions (II), a result which was conspicuous also in the field in the recipient of the same mill (Petänen et al. 1996, III). In contrast to CPs, resin acids still accumulated up to high levels in the bile of fish exposed to "low-chlorine" BKME in the same experiment (II). This is because of substitution of chlorine dioxide for chlorine in the bleaching does not alter the total RA emissions. Thus, the RA load remained comparable to earlier

studies, with potential risks to fish health. Later, after introducing the activated sludge treatment, a prominent decrease also in wood-derived extractives in the mill effluent was observed, also resulting in a very low accumulation of resin acids in the bile of fish both in the field (III) and in the laboratory (IV). Today, the biliary accumulation of CPs and RAs in exposed fish is nearby negligible in environmentally relevant effluent concentrations (i.e. <5 vol-%). In addition, the rapid biliary metabolism of CPs and RAs suggests only minor effects on fish.

5.7.2 Other body burden parameters

Extractable organic halogens (EOX or EOCl) can be used as a measure of the total load of organochlorines in fish tissues. However, the chlorinated constituents of EOX have been only partially identified (Wesén et al. 1992b). Whatever the nature of EOX, this parameter, as well as CPs in lipids, exhibited a relatively sensitive occurrence in this study. In the laboratory, EOX concentrations of 20 - 30 $\mu g\ g^{-1}$ lipid were found in whitefish exposed to effluent from mill A in concentrations of 1.3-7 % (II). Highly variable EOX levels, up to 1200 $\mu g\ g^{-1}$ lipid, have been observed in fish near forest industry plants (Södergren et al. 1988, Wesén et al. 1992a). Apparently, the variable EOX levels in fish are due to an array of factors that include pulping, bleaching and treatment processes, flow conditions and the natural background of organohalogen compounds, as well as the species examined. Consequently, EOX residues should always be evaluated in relation to a local reference level.

5.8 Fish cytochrome P450 induction as a biomarker of exposure

5.8.1 Field observations

A striking feature of the biotransformation enzymes is the fact that their activities can be enhanced following the treatment of animals with chemicals. In general, the enhanced activity results from an increase in the rate of synthesis of the biotransformation enzymes. Therefore, this process has been termed enzyme induction, an event requiring *de novo* protein synthesis.

The induction of a cytochrome P450 enzymes occurs through a series of molecular events, which finally lead to the synthesis of a protein with specific catalytic activity and which thus facilitates the metabolism of the toxic chemical (Stegeman 1993). Two major products of these steps, the P450 protein or its mRNA transcript, are routinely analyzed by catalytic or immnunochemical methods to determine the status of the cytochrome P450 detoxifying enzymes in an organism exposed to toxicants.

In fish, the induction of cytochrome P450 dependent monooxygenases through the cytosolic aryl hydroxylase receptor (AhR, Nebert & Gonzalez 1987) generally indicate the possibility of exposure to a wide variety of polycyclic and chlorinated-aromatic hydrocarbons (PAHs, HAHs, PCBs, Kleinow et al. 1987).

Also, almost all pulp and paper mill effluents have been observed to have the potency in some degree to induce cytochrome P450 -dependent monooxygenases. Hence, MO induction is one of the most consistently observed responses in fish exposed to pulp and paper effluents in the field and in the laboratory (Andersson et al. 1988, Lindström-Seppä & Oikari 1989, 1990a, Södergren 1989, Lindström-Seppä et al. 1992, Munkittrick et al. 1992, 1994, Ahokas et al. 1994). However, although the MO induction is an useful biomarker of exposure to pulping effluent, the ultimate biological significance of the induction is still largely unknown (e.g. Kloepper-Sams & Benton 1994, Munkittrick et al. 1994).

There are several factors, both exogenous and endogenous in the organism and its environment, which may affect the interpretation of data generated by the various methods that analyze the cytochrome P450 system (Goksøyr & Förlin 1992). In addition to the toxic response of the animal to environmental stress, essential facts are that certain inducers can be inhibitors of catalytic activity (e.g. some PCB congeners) and that normal physiological states of the animal produce endogenous compounds such as steroids, which in turn may modulate catalytic activity (Jiminez et al. 1990, Monosson et al. 1991, Oikari & Jimenez 1992).

During recent years, alterations in manufacturing processes and wastewater treatment in mills have resulted in an altered quality of discharges, leading to substantially lower impact also in fish (Landner et al. 1994). Apparently, the reduced potency of effluents for MO induction also reflects these improvements. Consequently, according to an extensive Canadian study (Martel et al. 1996) only one third of secondary treated effluents from several mills caused a more than two-fold EROD induction in rainbow trout at 10 % effluent concentration, when compared to the control. Consistent with the recent studies in Southern Lake Saimaa (III, V), only a low induction potency of effluent from pulp and paper mills is predominant to date. On the other hand, varying effluent concentrations, the structures of specific chemicals related to production and treatment processes, as well as many physiological and toxicological factors, may still considerably affect the levels of MO induction (Burnison et al. 1996). The threshold concentration for MO induction has been found to vary considerably (Williams et al. 1996), although there is no distinct relation in MO inductions regarding different pulping, bleaching and treatment processes (Martel et al. 1996).

Earlier studies showed inhibitory or suppressed catalytic function of CYP1A1 of fish caged near the pulp and paper mill (mill A) in Lake Saimaa, apparently due to hepatotoxic effects or inactivation of the activity by compounds existing in effluent (Lindström-Seppä & Oikari 1989, Soimasuo et al. 1992, Lindström-Seppä et al. 1993).

In the reseach for this thesis, the monooxygnase induction in whitefish was monitored as the activity of EROD (I, II, III, IV, V), PROD (III, IV) and CYP1A1 mRNA expression (V) as a measure of exposure to pulp and paper mill effluents. A marked reduction of induction was noted in the receiving waters of mill A, where a 80-90 % decrease of EROD activity was measured in caged whitefish after the shift to elemental chlorine free (ECF) bleaching and the implementation activated sludge treatment in the mill (I, Petänen et al. 1996, III, Fig. 7). Interesting

is the fact that the basic EROD induction potency remained unchanged in fish, although the amount of ClO_2 was increased in the bleaching (former sequence $C_{5\text{-}45\%}$ / $D_{55\text{-}95\%}$, later $C_{1\text{-}5\%}$ / $D_{95\text{-}99\%}$) (I, II). However, Martel et al. (1993) has reported that MO inductions cannot be eliminated by replacing molecular chlorine with ClO_2. It is also observed, that chlorine is not essential for MO induction in fish exposed to BKME (Lindström-Seppä et al. 1992, Hodson 1996).

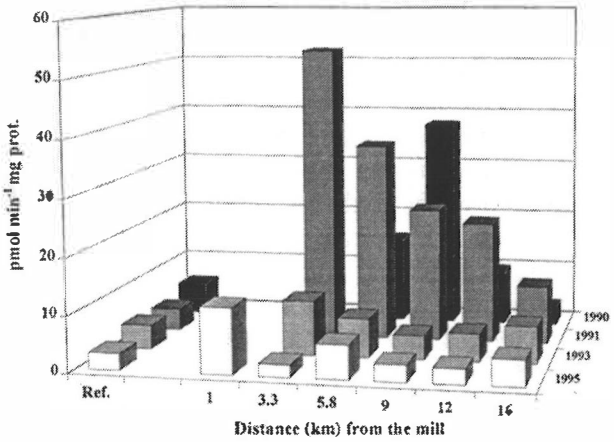

FIGURE 7 Liver EROD activity (mean, pmol $min.^{-1}$ mg $prot.^{-1}$) of whitefish experimentally exposed at different distances from an integrated pulp and paper mill (A) in Southern Lake Saimaa in 1990 (Soimasuo et al. 1992), 1991 (I), 1993 (Petänen et al. 1996) and 1995 (III). The nearest site (1 km) was used only in 1995. In 1990, all exposed fish died at site 3.3 km from the mill.

In the experimental exposure of whitefish by caging, great variability was observed in EROD activity and CYP1A mRNA expression between the different effluent receiving subareas (III, V). Apparently, some of the variability observed was due to different dilutions and dispersions of wastewaters in each mill case. However, there are similarities among the mills: all of them produce bleached kraft pulp (soft- and hardwood) with 100 % ClO_2 substitution and each mill employs a modern treatment for its effluent. On the other hand, one mill (B) produced only pulp, while the others are integrated mills producing both pulp and paper. Nevertheless, the effluent flow rate from the mill was only about half of that from the other mills, some effluent parameters (suspended solids, COD, N, P, AOX) being equal to those of the other mills. Additionally, the levels of wood derived compounds *e.g.* sterols and resin acids, were considerably higher in the effluent of mill B (V). Thus, the effluent quality together with the unequal dilution and dispersion of effluents during the field experiment seemed to cause variability in MO induction.

5.8.2 Laboratory validations

A prominently higher potency for MO induction in whitefish was observed when fish were exposed to untreated effluent compared to the following effluent treated by the activated sludge process (IV). The resultant MO induction in fish exposed to the treated effluent was one fifth of that caused by pre-treated effluent, indicating that the biotreatment of wastewaters with the activated sludge process effectively decreased the induction potency. Before the process and treatment changes implemented in the mill (I), the potency for the MO induction of the final effluent was comparable to the induction in fish exposed to the pre-treated effluent in the laboratory study in 1996 (IV). In the first laboratory validation in 1992 (II), before the activated sludge treatment (*i.e.* aerated lagoons as secondary treatment) a prominent EROD induction was observed in exposed fish, although the mill used the bleaching sequence with a high chlorine dioxide substitution (98-99%). Similarly, aerated lagoons were not successful in removing MO induction in a Canadian study either (Munkittrick et al. 1992). In accordance with our studies over the 1990s in Southern Lake Saimaa it has been shown that optimized or enhanced secondary treatment could substantially reduce, but apparently not abolish, the EROD-inducing potential of the pulp mill effluent (Schnell et al. 1993, Hodson 1996, Kloepper-Sams 1996). Consequently, as Martel and coworkers (1996) have suggested, more work is required to understand the real role of secondary treatment on effluent quality. Since 1993, MO inducing potency of the final effluent of mill A has been observed to remain at approximately the same level, after which the mill has introduced only small alterations in its processes up to 1996.

5.8.3 Expression of CYP1A1 gene

In the study in Southern Lake Saimaa (V), CYP1A mRNA expression and EROD activity did not correlate. Variable inducers together with water temperature may be partly accounted for the discrepancy between the expression of CYP1A gene and EROD activity, since these factors have been indicated to influence the translation and stability of CYP1A1 mRNA (Kloepper-Sams & Stegeman 1992, Pesonen et al. 1992). However, Campbell et al. (1996) observed fish (juvenile Chinook salmon) exhibiting a dose-dependent increase in hepatic CYP1A1 mRNA levels in response to environmentally relevant concentrations of treated BKME.

5.8.4 Characteristics of monooxygenase inducers in effluents

Polychlorinated dibenzo-*p*-dioxins and furans (PCDDs/Fs), the most potent inducers of CYP1A1 known in fish, were previously thought of as the major inducing constituents in BKME (Hodson et al. 1992, Kloepper-Sams & Benton 1994). However, some recent findings suggest that compounds other than PCDDs and PCDFs are dominant in CYP1A induction to date. Fish exposed to BKME have shown short-lived rapidly declining induction, indicating a ready

metabolism of affecting chemicals in fish, which is in contrast to the characteristical long-term induction (for weeks or months) of CYP1A in fish exposed to PCDDs/Fs (Munkittrick et al. 1992, 1994, Bankey et al. 1995). Also unbleached effluent with apparently negligible PCDD/F levels induced MO in fish (Lindström-Seppä et al. 1992). Moreover, liver MO activity is not always parallel with the toxicity equivalents (TEQs) calculated from PCDDs and PCDFs (van den Heuvel et al. 1995, 1996). Recently, moderately hydrophobic, probably planar, aromatic PAHs with low chlorine substitution have been suggested as compounds causing CYP1A induction in fish exposed to BKME (Burnison et al. 1996). The compounds include an alkyl substituted phenanthere (retene), which has been observed to induce EROD in rainbow trout (Parrott et al. 1994). Retene has also been detected in effluent as well as in sediment near one mill (A) (Koistinen et al. submitted a). Lignin derivatives or humic material liberated and leached from cellulose both in pulping and bleaching have been proposed as the primary source of MO inducers in BKME (Williams et al. 1996, Burnison et al. 1996).

One of the main aims in this research was to monitor the differences in the spatial extent and the magnitude of the monooxygenase responses due to the mill differences (III, V) and the changes in the mill processes over time (I,II,III,IV). Consequently, the individual compounds responsible for MO induction, or for other manifestations of exposure or effects, were beyond the scope of this monitoring. However, in some joint studies individual compounds in regard to pulp and paper mills, as well as their possible effects on HEPA-1 cells, have been investigated (Koistinen et al. submitted a, b). Although AOX, CPs, RAs or fatty acids are not the contributors to EROD induction (Lindström-Seppä & Oikari 1990, Hodson et al. 1992, Williams et al. 1996, Hewitt et al. 1996), some chlorophenolic derivatives have been found to induce EROD (Hewitt et al. 1996) and the main inducers in black liquor may be degradation products or wood extractives (Hodson 1996, Hodson et al. 1997). High correlations were found between EROD activity and the ambient water AOX, CPs and RAs, as well as the bile CPs and RAs when fish were exposed to effluents in 1991 (I). After the mill alterations, the same correlations were substantially lower (III, IV).

5.9 Liver conjugating enzymes

Hepatic UDP-GT activity indicated no response upon exposure to BKME in the field (I). However, the laboratory simulation showed a significant inhibition (by 34%) of UDP-GT at the highest BKME concentration (7%), and the same tendency was also obvious at lower concentrations (3.5, 2.3 and 1.3%). Similar inhibition of UDP-GT has been observed previously in whitefish exposed to the same receiving waters (Lindström-Seppä & Oikari 1989). A probable cause for inhibition of UDP-GT is exposure to resin acids in BKME (Mattsoff & Oikari 1987). In all, the results of this research support previous results, which have

demonstrated an inconsistency of UDP-GT response in BKME exposed fish (Oikari & Kunnamo-Ojala 1987, Lindström-Seppä & Oikari 1989, 1990a).

Glutathione S-transferases (GST) include a group of conjugating enzymes, which are important in metabolism and the elimination of several electrophilic compounds. The absence of GST induction seen in the field (I) and laboratory (II) is in accordance with other observations made in Southern Lake Saimaa. However, like glucuronidation, glutathione conjugation may also be decreased in fish exposed to components of BKME (Oikari et al. 1988, Hodson et al. 1992). One compounding factor is the possibility of differential effects on various GST isoforms (George 1994), but further isoenzyme specific studies are needed.

5.10 Reproductive steroids and vitellogenin mRNA as biomarkers

Several studies have consistently revealed the effects of pulp and paper mill effluents on fish reproductive functions. Exposed fish exhibit reduced circulating levels of reproductive hormones, reduced gonadal growth, increased age to sexual maturation, and reduced expression of secondary sexual characteristics (McMaster et al. 1991, 1992, 1996, Van Der Kraak et al. 1992, Munkittrick et al. 1992, 1994, Van den Heuvel et al. 1995). However, in contrast to the studies made on the effects of BKME on mature fish, significant changes were not observed in circulating reproductive steroids in juvenile whitefish exposed in the receiving areas in Southern Lake Saimaa (III). A better differentiation might be achieved by sexing the whitefish into two subgroups, which, however, is not possible by external appearance, but a histological analysis was necessary. On the other hand, the laboratory simulation with whitefish (IV) and a field study with perch and roach (Karels et al., in press) revealed significantly depressed levels of the reproductive steroids in juvenile whitefish in the vicinity of one pulp and paper mill (A). Thus, further studies are needed to clarify the contradictory results obtained thus far in caged juvenile whitefish. Although detailed mechanisms of the endocrine disruptions are still largely unknown, some components existing in pulp mill effluents, possibly including e.g. β-sitosterol, may cause hormonal changes in fish (MacLatchy et al. 1994, Servos et al. 1994,). A recent review on the *in situ* assessment of pulp mill effluents on reproductive parameters indicated that 8 of the 10 fish populations showed increasing age to sexual maturity and 4 of the 6 species studied had reduced gonad size, recorded from 14 of the 24 reported cases (Sandström et al. 1996). In this study the CYP1A activity and the levels of the sex steroids did not correlate, which is consistent with several other reports failing to show direct correlation between induced MO activity and the hormone levels (Servos et al. 1992, Van Der Kraak et al. 1992, Gagnon et al. 1994a, 1994b, Kloepper-Sams & Benton 1994, Munkittrick et al. 1994, Swanson et al. 1994).

Vitellogenin gene was expressed to a greater extent than in the controls only in subarea B (V), in the vicinity of mill B discharging the highest amount of wood-derived compounds such as resin acids and sterols (V). β-sitosterol, abietic- and

dehydroabietic acids and debarking effluent have been observed to induce the vitellogenin gene in juvenile rainbow trout (Mellanen et al. 1996).

5.11 Immunological responses

The vertebrate immune system is under the control of complicated physiological regulation and is also affected by environmental factors (Anderson 1990). Humorally mediated immunity can be assessed by quantifying the amounts of circulating antibodies, for instance IgM. Up to now, however, immunological biomarkers have not been very much used to assess the effects of toxic compounds, present in effluents from the forest industry. However, subchronic stress associated with BKME has been observed to effect on hormonal balance and further, the immunosystems in fish (Jokinen et al. 1995).

In this study, reduced IgM levels were observed in whitefish caged near the mill in the recipient lake water (I) and in fish exposed in the laboratory (II). Thus, contamination by BKME may have changed the endocrine function in fish, and consequently have caused immunosuppression in fish. Cortisol has been observed to cause immunosuppression also in fish (Bennett & Wolke 1987, Kaattari & Tripp 1987). A decreased level of immunoglobulins may increase the susceptibility of fish to bacterial and parasitic infections. In addition, compounds of BKME may have both direct and indirect effects on the hemopoietic system and lymphoid organs, hindering development and maturation of lymphocytes. Immunoglobulin production may also be disturbed at the level of macrophages, other regulatory cells or soluble mediators e.g. interleukins and other cytokines. However, the mechanisms causing the reduced antibody levels due to BKME in this study remain obscure. The observed variation of plasma IgM may reflect the complexity of the exposure conditions or the immune system itself.

The studies conducted in IgM in the receiving waters and with effluents from mill A after the mill alterations did not show any immunological effects in fish (III, IV). The changes in the bleaching process together with the installation of the secondary treatment system abolished the decrease of plasma immunoglobulin M.

5.12 Other physiological parameters

A common effect of pulp and paper mill effluents on the hematology of fish has been a reduction in red blood cell (RBC) number or hemoglobin concentration (McLeay 1973, Oikari et al. 1988). This was also observed in the field study in 1991 (I) and especially in the laboratory in 1992 (II). The decreased blood hemoglobin may result from increased breakdown of red blood cells. RBC breakdown is caused *in vitro* by resin acids (Bushnell et al. 1985, Mattsoff & Nikinmaa 1987). The mechanism by which resin acids cause red cell breakdown is not quite clear,

but resin acids appear to increase the ionic permeability of cell membranes (Isomaa et al. 1986). Consequently, cellular sodium concentration will be elevated, as observed in the laboratory (II) and in an earlier field study on rainbow trout (Lindström-Seppä & Oikari 1990a). Altered RBC sodium concentration indicates a possible disturbance in cellular acid-base regulation (Nikinmaa 1992). An increase in red cell sodium concentration will increase cellular energy consumption, whereby the cellular NTP concentration becomes reduced (II). Furthermore, it appears that resin acids reduce the energy production (oxygen consumption) of fish erythrocytes (Bushnell et al. 1985). Together, the reduced energy production and increased energy demand will ultimately lead to breakdown of the cell. Similar changes, i.e. an increase in RBC sodium concentration and a decrease in cellular NTP concentration, would also be caused by hypoxic conditions (Soivio et al. 1980, Nikinmaa et al. 1987). Hypoxia, however, is unlikely in our whitefish since the oxygen level of water was kept at a high level by aeration in the laboratory. Similarly, low-oxygen conditions were not observed in the field, either. The changes in blood hemoglobin observed in 1995 (III) were not effluent related, because Hb also showed differences between the reference sites. This variability may reflect changes in ambient physical conditions like water temperature (Nikinmaa 1990). In all, blood hemoglobin has not been affected in whitefish in the recipient areas since 1993.

The activities of blood plasma aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and lactate dehydrogenase (LDH) have been used as biomarkers of cell or tissue damage in mammalian and fish toxicology. Many heavy metals and organic compounds can increase the plasma activities of these indicators of cell membrane damage (Versteeg & Giesy 1986, Oikari & Jimenez 1992). Lehtinen et al. (1992) reported elevated values of ASAT and ALAT values in rainbow trout exposed to certain types of pulp mill effluents. The groups exposed to phytosterols in water and food and softwood BKME showed the most conspicuous changes in this respect. In this thesis study, however, the levels of ASAT, ALAT and LDH in blood plasma were not significantly affected in fish exposed to effluents (I, II) indicating that tissue damage had not occurred.

Disturbances in metabolic dysfunctions including carbohydrate metabolism have previously been observed in fish exposed to BKME (Anderson et al. 1988). According to the observations made in this research (I , II, III, IV, V), the adverse effects on fish energy metabolism are not obvious in fish exposed to pulp and paper mill effluents. The condition factor (CF) of exposed whitefish did not vary among different groups (I, II, III, IV, V), and resembled the findings of an earlier experiment on rainbow trout (Lindström-Seppä & Oikari 1990a). All in all, the general gross physiological parameters, including those related to energy metabolism or the condition of the fish, showed no disruptions or disturbances in fish.

6 CONCLUSIONS

The chemical quality of an aquatic environment can be monitored by measuring the contaminant level in the water, sediment or biota (known as chemical exposure). However, in order to assess the biological effects of chemical stressors as well as the status of the aquatic ecosystem, the chemical monitoring assessing is not relevant by itself. Consequently, biological effect monitoring, i.e. the measurements of the early responses of animals, is essential for a reliable examination of potential ecological risks in aquatic environment to be made.

One of the primary aims of the research for this thesis was to select a suite of relevant biomarkers in the biological assessment of the receiving aquatic environments of the forest industry. It is evident that measuring a single biomarker in environmental field studies is not sufficiently reliable. Thus, an integrated approach, using selected biochemical and physiological parameters in fish, was used in this study. Induction of the cytochrome P450 dependent monooxygenases, measured as EROD activity, was one of the most sensitive markers in whitefish subchronically exposed to pulp and paper mill effluents in the field and in the laboratory. Some of the biomarker responses in whitefish, including monooxygenase activity and immunoglobulin response were significantly reduced after the mill alterations i.e. changes in the bleaching and effluent treatment. Moreover, the body residues and the biliary accumulation of chlorophenolics and resin acids were considerably lower after the renewals. With the current knowledge, however, it is not possible to simply extrapolate biomarker responses in individual animals observed in this study to the levels of populations, communities or ecosystems.

When assessing a status of water pollution with biomarkers, the experimental conditions have to be chosen carefully, since many external factors (e.g. season, water temperature, hatching period, water salinity, age and sex of the fish, diet, food availability, etc.) may have a confounding effect, for example, on the activities of biotransformation enzymes.

According to the present study, the modernized mill processes with their advanced wastewater treatment technologies have substantially reduced the load and ecotoxicity of harmful constituents present in pulp and paper mill effluents. As a consequence, diminished biological effects on the aquatic environment were followed, although some risks relative to the health and fitness of fish may still exist.

Whitefish proved to be an excellent species for field as well as laboratory experiments, responding to subchronic exposure to pulp and paper mill effluents in a dose-dependent fashion. This was seen in the laboratory-based experiments and as gradient-related responses in the field. The stress-free or low-stress caging technique, optimized for whitefish, permits subchronic experiments without supplemental feeding or other maintenance procedures. Studying fish by means of caging has proved to have many advantages relative to collecting feral fish, including a precisely known exposure site and duration and fish material with a known developmental stage and genetic background. By placing cages at varying distances from point sources of effluent and by comparing biochemical, physiological and body burden responses to effluent constituents, it was possible to demonstrate causal relationships between organismic exposure and biological responses even in a large spatial extent.

Compared to the field exposure, the responses in the laboratory simulations were in good accordance with the field data. And more importantly, the results of the laboratory experiments, which simulated a real aquatic environment in terms of effluent concentrations downstream from a large bleached kraft pulp and paper mill, confirmed that the biochemical and physiological responses observed earlier in the field were the results of pulp and paper mill effluents as such. The selected series of biomarkers proved to be feasible and relevant in quantifying the exposure to and effects on fish, both in the laboratory and in the field.

Acknowledgements

This study was carried out at the Karelian Institute, Section of Ecology, University of Joensuu and at the Department of Biology, University of Joensuu during the years 1991-1994 and at the Department of Biological and Environmental Science, University of Jyväskylä during the years 1995-1997. I am indebted to these institutions for providing excellent working facilities.

I would like express my warmest thanks to my supervisor, Prof. Aimo Oikari for his excellent guidance in the field of aquatic ecotoxicology. His endless support, encouragement and constructive criticisms has been of great importance throughout these years.

My sincere thanks belong to Prof. Heikki Hyvärinen, Assoc. Prof. Jussi Kukkonen and Dr. Jukka Pellinen for support, co-operation and valuable advice during the years in University of Joensuu.

Also, I wish to thank Prof. Bjarne Holmbom, Åbo Akademi University, Prof. Mikko Nikinmaa, University of Turku and Prof. Risto Santti, University of Turku.

I want to thank my nearest co-workers in Jyväskylä Mr. Aarno Karels, M.Sc., Mr. Jarmo Lappivaara, M.Sc. and Mr. Harri Leppänen, M.Sc.. Also thanks belong to Mrs. Tuula Aaltonen, M.Sc., Dr. Jaana Kostinen, Ms. Tiina Petänen, Phil.Lic. and Dr. Ilmari Jokinen and Mr. Riku Suutari. In addition, many persons have been involved in this study, which I would like to acknowledge.

I greatly acknowledge Doc. Pirjo Lindström-Seppä, University of Kuopio, and Dr. Peter V. Hodson, Queen's University, Kingston, Ontario, Canada, for their extremely valuable suggestions and criticisms of this thesis study.

I wish to thank the personnel of the forest companies involved for contribution to many practical arrangements for the studies. Special thanks belong to Mr. Esa Simpura and Mr. Ilkka Westergren, UPM-Kymmene Kaukas.

I wish to thank Mr. Leigh Plester, M.Sc. for checking the language.

Finally, I would like to thank Pirjo, my family and friends for their support during this work.

This study was financially supported by the Academy of Finland and the Maj and Tor Nessling Foundation.

YHTEENVETO

Kalan biomarkkerivasteet selluloosa- ja paperiteollisuuden jätevesien vaikutusten osoittajina

Selluloosa- ja paperiteollisuus on edelleen merkittävä vesistöjen kuormittaja, huolimatta jätevesien kiintoaineen ja happea kuluttavan orgaanisen aineksen sekä orgaanisten klooriyhdisteiden päästöjen oleellisesta vähenemisestä. Jätevesien sisältämien yhdisteiden kemialliset ominaisuudet, määrä ja vaihtelevuus sekä biologisten järjestelmien monimutkaisuus vaikeuttavat jätevesien eri biologisille organisaatiotasoille kohdistuvien vaikutusten arviointia, mikä edelleen vaikeuttaa myös puunjalostusteollisuuden jätevesien riskinarviointia.

Tämä väitöskirjatutkimus on osa laajaa, Etelä-Saimaalla toteutettua ekotoksikologista tutkimuskokonaisuutta. EKOTASE-hankkeessa (v. 1990-1993) kohteena oli valkaistua sulfaattiselluloosaa ja painopaperia valmistavan tehtaan alapuolinen vesistönosa. Vuonna 1992 tehdas siirtyi selluloosan valkaisussa klooridioksidin käyttöön alkuainekloorin sijaan ja otti käyttöön aktiivilietemenetelmän jätevesien puhdistuksessa. Myöhemmin myös eräitä muita prosessimuutoksia, esimerkiksi happidelignifiointi on otettu käyttöön. ESAITOX-hankkeessa (1995-1996) tutkimusalueena oli koko Etelä-Saimaa (yli 600 km^2), jonka ympäristössä sijaitsee neljä selluloosa- ja paperitehdasyksikköä, edellä mainittu tehdas mukaanlukien.

Tutkimuksessa mitattiin sellu- ja paperiteollisuuden jätevesille altistettujen siikojen (*Coregonus lavaretus* L. s.l.) subletaaleja fysiologisia ja biokemiallisia vasteita, jotka liittyvät kalan vierasaine- ja energiametaboliaan, lisääntymiseen sekä immunologiaan. Valittujen biomarkkereiden avulla arvioitiin sekä kalojen altistuminen jätevesien sisältämille yhdisteille että niiden mahdolliset haittavaikutukset. Kaloja altistettiin jätevesille sumputtamalla tehtaiden alapuolisilla vesialueilla sekä puhtailla vertailualueilla. Altistukset toistettiin myös laboratorio-olosuhteissa vastaanottavan vesistön jätevesipitoisuuksia mukaillen. Tutkimuksessa selvitettiin myös valmistus- ja puhdistusprosessien vaikutuksia kaloihin, samoin kuin eri tehtaista peräisin olevien jätevesien vaikutuksia ja mahdollisia vaste-eroja. Lisäksi tutkimuksessa kiinnitettiin huomiota biomarkkereiden soveltuvuuteen kaloihin kohdistuvien vaikutusten arvioinnissa, vasteiden alueelliseen jakautumiseen sekä käytetyn sumputustekniikan soveltuvuuteen.

Kalan kudoksista, elimistön nesteistä tai makromolekyyleistä voidaan määrittää niihin kerääntyviä yhdisteitä tai metaboliitteja, jotka ovat osoituksena altistumisesta vierasaineille. Tutkimuksessa selvitettiin lähinnä valkaisun yhteydessä syntyvien orgaanisten klooriyhdisteiden sekä eräiden puun sisältämien uuteaineiden, kuten hartsihappojen kerääntymistä kalan sappeen. Jäte- ja järvivedessä havaittiin kloorifenolisten yhdisteiden oleellinen väheneminen (98 %) klooridioksidiin siirtymisen ja jätevesien aktiivilieteprosessin ansiosta. Vastaavasti aktiivilietepuhdistus alensi jäteveden hartsihappopitoisuuksia 95 %. Muutosten jäl-

keen kalojen sappeen kerääntyi vain 1-2 % mainittuja yhdisteitä verrattuna ajankohtaan ennen muutosta. Kalojen biotransformaatioentsyymien, kuten maksan sytokromi P450 -järjestelmään kuuluvan 7-etoksiresorufiini O-de-etylaasin (EROD) aktiivisuutta on pidetty eräänä herkimmistä osoituksista kalan altistumisesta polyaromaattisille hiilivedyille (PAH). Tutkitun tehtaan lähellä (3.3 km) altistettujen kalojen EROD-aktiivisuus oli ennen prosessimuutoksia 13-kertainen ja muutosten jälkeen kaksinkertainen, puhtaan alueen kaloihin verrattuna, mikä osoittaa jätevesialtistuksen merkittävästi pienentyneen. Laboratorioaltistuksen perusteella havaittiin myös EROD-aktiivisuuden oleellinen aleneminen vasta, kun kloori oli kokonaan korvattu klooridioksidilla valkaisussa. Ennen tehtaan prosessimuutoksia veren immunoglobuliini- (IgM) ja hemoglobiinipitoisuudet olivat merkittävästi alentuneet altistetuilla kaloilla. Myös punasolujen nukleositrifosfaatti (NTP) -pitoisuus oli alentunut lisääntyneen energiankulutuksen osoituksena, kun taas punasolujen natriumpitoisuus oli altistusryhmissä kohonnut solukalvojen ioniläpäisevyyden lisääntymisen vuoksi. Sukupuolihormonien, estradiolin ja testosteronin, havaittiin alentuneen altistetuissa kaloissa vielä tehtaan prosessimuutosten jälkeen. Entsyymimittausten (ASAT, ALAT, LDH) perusteella kudosvaurioita ei kuitenkaan voitu osoittaa. Vaikka sellu- ja paperiteollisuuden jätevesien on havaittu vaikuttavan kalojen energia-aineenvaihduntaan, tämän tutkimuksen perusteella ei yksiselitteisiä vaikuttavia syitä energia-aineenvaihdunnan häiriöihin voitu osoittaa.

Koko Etelä-Saimaan alueen tutkimus osoitti verraten vähäistä, edellä kuvatun tehtaan prosessimuutosten jälkeisten tasojen suuruista yhdisteiden kerääntymistä kalan sappeen. Samoin maksan EROD-aktiivisuus oli koko alueella muutosten jälkeisten arvojen suuruisia, keskimäärin kaksinkertaisia tehtaiden lähellä (2-3 km) vertailualueeseen nähden. Sukupuolihormonien, immunoglobuliinien tai glukoosin tasot eivät altistuneilla kaloilla oleellisesti poikenneet vertailukaloista. Mahdollista estrogeenisten yhdisteiden olemassaoloa osoitti kalan lisääntymisessä oleellisen proteiinin, vitellogeniinin, induktio kaloissa lähellä tehdasta, jonka jätevedet sisälsivät runsaasti puun uuteaineita, kuten puusteroleja. Eri tehtaiden jätevesien aiheuttamien vasteiden vertailua haittasi kuitenkin jätevesien sekoittumisen ja leviämisen erot tehtaiden lähistöllä.

Tutkimuksessa käytetyt biomarkkerivasteet osoittivat selvästi metsäteollisuusjätevesien aiheuttaman kalojen altistumisen sekä eräitä jätevesien aiheuttamia haittavaikutuksia. Jätevesien todettiin edelleen sisältävän yhdisteitä, jotka saattavat vaikuttaa haitallisesti eliöiden keskeisiin biologisiin toimintoihin, kuten lisääntymiseen. Tutkimus osoitti kuitenkin myös sen, että nykyaikaiset selluloosan ja paperin valmistusmenetelmät yhdistyneenä edistyneeseen jätevesien puhdistustekniikkaan vähentävät huomattavasti haitta-ainekuormitusta järvessä, vähentäen näin myös vastaanottavan vesistön biologisia, ja mahdollisesti myös ekologisia vaikutuksia.

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ORIGINAL PAPERS

I

Biomarker Responses along a Pollution Gradient: Effects of Pulp and Paper Mill Effluents on Caged Whitefish

Soimasuo, R.M., Jokinen, I., Kukkonen, J., Petänen, T., Ristola, T. & Oikari, A.O.J.

Aquatic Toxicology 31: 329-345, 1995

https://doi.org/10.1016/0166-445X(94)00082-2

II

Physiological Toxicity of Low-Chlorine Bleached Pulp and Paper Mill Effluent on Whitefish (*Coregonus lavaretus* L. *s.l.*): a Laboratory Exposure Simulating Lake Pollution

Soimasuo, R.M., Aaltonen, T., Nikinmaa, M., Pellinen, J., Ristola, T. & Oikari, A.O.J.

Ecotoxicology and Environmental Safety 31: 228-237, 1995

https://doi.org/10.1006/eesa.1995.1068

III

Biomarker Responses in Whitefish (*Coregonus lavaretus* L. s.l.) Experimentally Exposed in a Large Lake Receiving Effluents from Pulp and Paper Industry

Soimasuo, M.R., Karels, A.E., Leppänen, H.,Santti, R. & Oikari, A.O.J.

Archives of Environmental Contamination and Toxicology (in press)

https://doi.org/10.1007/s002449900287

IV

Validation of Field Exposure of Fish and Role of Activated Sludge Treatment of BKME by a Laboratory Simulation

Soimasuo, M.R., Lappivaara, J. & Oikari, A.O.J.

Environmental Toxicology and Chemistry (submitted)

https://doi.org/10.1002/etc.5620170722

V

Differential Expression of the Vitellogenin Gene and CYP1A1 System in the Liver of Juvenile Whitefish (*Coregonus lavaretus* L. *s.l.*) Exposed to Effluents from Three Pulp and Paper Mills

Mellanen, P., Soimasuo, M.R., Holmbom, B., Oikari, A.O.J. & Santti, R.

(manuscript)

https://doi.org/10.1006/eesa.1999.1782