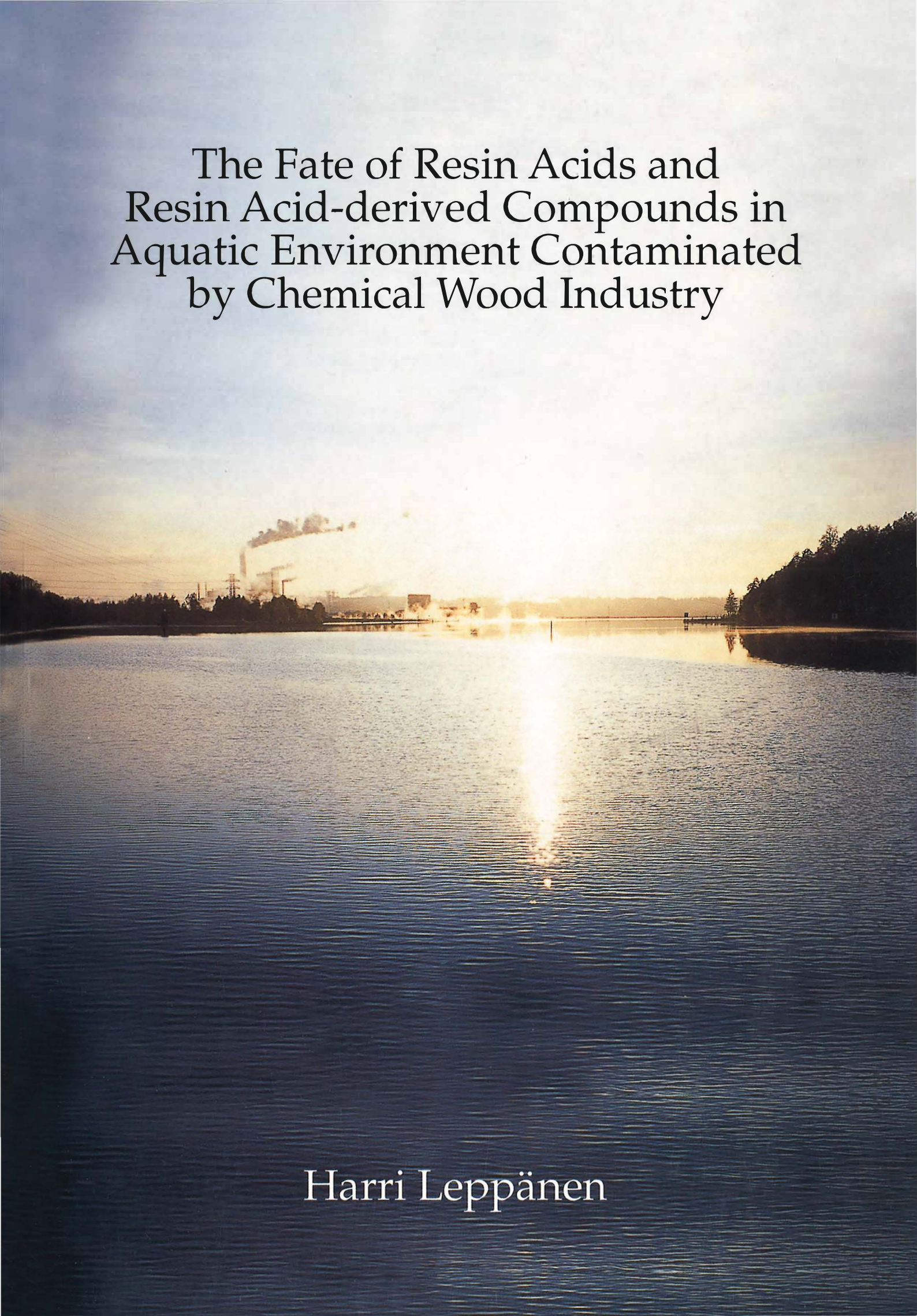
The Fate of Resin Acids and Resin Acid-derived Compounds in Aquatic Environment Contaminated by Chemical Wood Industry



Harri Leppänen

JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 80

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 1999

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Editors
Jukka Särkkä
Department of Biological and Environmental Science, University of Jyväskylä
Kaarina Nieminen
Publishing Unit, University Library of Jyväskylä

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ABSTRACT

Leppänen, Harri
The fate of resin acids and resin acid-derived compounds in aquatic environment contaminated by chemical wood industry
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Yhteenveto: Hartsihappojen ja hartsihappoperäisten yhdisteiden ympäristökohtalo kemiallisen puunjalostusteollisuuden likaamissa vesistöissä
Diss.

Because of the transition of pulp manufacturing technology from chlorine bleaching to elemental chlorine free and total chlorine free bleaching processes, more attention has been drawn to wood-derived and other non-chlorinated compounds in treated discharges from pulp mills. The occurrence of resin acids and resin acid-derived base neutrals, particularly retene (7-isopropyl-1-methylphenanthrene) in sediments and sedimenting particles in lake areas polluted by mill effluents was studied in this work. At Southern Lake Saimaa substantial concentrations of these substances were observed in sedimenting particles (highest concentration of retene and resin acids 54 and 1500 μg/g d.w., respectively). High concentrations were also observed in sediments of both industrial areas studied (at Southern Lake Saimaa 1600 and 1500 μg/g d.w. of retene and resin acids, respectively; at Lake Lievestuoreenjärvi 3300 and 1100 μg/g d.w.). In an anaerobic incubation of a sedimental microbial consortium spiked with dehydroabietic acid, the amount of dehydroabietic acid decreased and the amount of retene increased at 24 °C, indicating the biotransformation of the former to the latter. At 4 °C no changes were observed over a one year of incubation. Under aerobic conditions the concentrations of both retene and dehydroabietic acid decreased. In order to assess the bioavailability of the lipophilic, apparently particle-bound retene to fish we analysed bile samples of feral perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) caught downstream of the pulp mill sewer in an area with high concentrations of retene in the sediment and from the reference area upstream from the mill. Retene was found in the bile of roach, but not in the bile of perch caught downstream of the mill, indicating that retene is bioavailable to benthic fish species. We also studied the utility of fish bile analysis in experimentally exposed whitefish (*Coregonus lavaretus*) to assess the exposure of fish to elemental chlorine free bleached, biologically treated pulp and paper mill effluents and evaluated the most suitable compounds for tracers. The results revealed that the bleaching-derived chlorinated phenolics still are the most suitable tracers of exposure to bleached kraft mill effluent.

Key words: Aerobic; anaerobic; bile; biotransformation; BKME; resin acids; retene; sediment.
H. Leppänen, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FIN-40351 Jyväskylä, Finland

Author´s address

University of Jyväskylä
Department of Biological and Environmental Science, P.O. Box 35, FIN-40351, Jyväskylä, Finland
Email hjleppan@dodo.jyu.fi

Supervisor

Professor Aimo Oikari
University of Jyväskylä
Department of Biological and Environmental Science, P.O. Box 35, FIN-40351, Jyväskylä, Finland
Email aoikari@dodo.jyu.fi

Reviewers

Professor Max M. Häggblom
Department of Biochemistry and Microbiology
Lipman Hall, Cook College
Rutgers, The State University of New Jersey
76 Lipman Drive, New Brunswick, NJ 08901-8525
USA

Dr. Trevor R. Stuthridge
New Zealand Forest Research Institute
PAPRO Division
Private Bag 3020, Rotorua
New Zealand

Opponent

Dr. Rainer Ekman
Process Chemistry Group
Åbo Akademi University
Biskopsgatan 8
FIN-20500 Turku,
Finland

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is a summary and discussion of the following articles and manuscripts, which are referred to by their Roman numerals in the text:

I Harri Leppänen & Aimo Oikari (1999). Occurrence of retene and resin acids in sediments and fish bile from a lake receiving pulp and paper mill effluents. – Environ. Toxicol. Chem. 18: 1498 – 1505.

II Harri Leppänen & Aimo Oikari (1999). Retene and resin acid concentrations in sediment profiles of a lake recovering from the exposure to pulp mill effluents. - J. Paleolimnol. (submitted).

III Harri Leppänen, Jussi Kukkonen & Aimo Oikari (1999). Concentration of retene and resin acids in sedimenting particles collected from bleached kraft mill effluent receiving lake. – Water Res. (in press).

IV Harri Leppänen & Aimo Oikari (1999). The biotransformation of dehydroabietic acid under anaerobic and aerobic conditions at different temperatures by a sedimental microbial consortium. – Environ. Sci. Technol. (submitted).

V Harri Leppänen, Sanna Marttinen & Aimo Oikari (1998). The use of fish bile analyses as exposure biomarkes to pulp and paper mill effluents. – Chemosphere 36: 2621 – 2634.

VI Harri Leppänen & Aimo Oikari (1999). Chemical markers of exposure to pulp and paper mill effluents in aquatic animals – a review. – Environ. Toxicol. Chem. (submitted).

ABBREVIATIONS

BKME	bleached kraft mill effluent
EOCl	extractable organic chlorine
EOX	extractable organic halide
CPs	chlorophenolics
DHA	dehydroabietin
DHAA	dehydroabietic acid
ECD	electron capture detector
ECF	elemental chlorine free
FID	flame ionization detector
GC	gas chromatography
GC-MS	gas chromatography - mass spectrometry
LOEC	lowest observed effective concentration
MFO	mixed function oxygenase
MTBE	methyl *tert*-butyl ether
OC	organic carbon
PAH	polyaromatic hydrocarbon
PCDDs	polychlorinated dibenzo-p-dioxins
PCDFs	polychlorinated dibenzofurans
TCF	total chlorine free
THR	tetrahydroretene

1 INTRODUCTION

The pulp and paper industry in all major producing countries is in 1990s largely different than earlier. The most significant changes concerning the well-being of aquatic life in receiving waters have been the employment of ECF and TCF bleaching techniques and biological wastewater treatment systems (Oikari & Holmbom 1996). Even after these changes in the pulping and bleaching processes, residual ecotoxicological effects linger on. For example, effects that were formerly believed only to be connected to bleached pulp mill effluents, have also been observed in receiving waters of unbleached pulp mills (Hodson 1996).

Resin acids, diterpene compounds occur naturally in many softwood species such as spruce and pine (Sjöström 1993), are commonly found in pulp and paper mill effluents (Zender et al. 1994). Typical concentrations of total resin acids in lake water downstream of a biologically treated pulp and paper mill discharge approximate a few $\mu g\ L^{-1}$ (Kaplin et al. 1997). These compounds are lipophilic, which causes them to adsorb to suspended solids during biological wastewater treatment (Holmbom et al. 1992; Liu et al. 1996) and in receiving aquatic environments (Holmbom et al. 1992). Because of this, elevated concentrations of resin acids in sediments have been found several kilometers downstream of pulp and paper mill wastewater discharges (Lee & Peart 1991; Judd et al. 1995). Presently the information about the biodegradation of resin acids is limited. However, aerobic bacterial strains which grow on various resin acids have been isolated and characterized (Mohn 1995; Mohn et al. 1996; Wilson et al. 1996). Also some fungi can transform resin acids, but none have been shown to degrade them (Kutney et al. 1981a, 1981b, 1982; Brush et al. 1994; Wang et al. 1995). The anaerobic fate of resin acids is poorly studied. Sierra-Alvarez et al. (1990) indicated that resin acids were poorly removed through anaerobic wastewater treatment. In an anaerobic sediment incubation at 25 °C only a 50 % decrease in the total resin acid concentration was observed after 264 days of incubation (Tavendale et al. 1997a). Additionally many resin-derived compounds were formed. Abietic type resin acids are believed to be microbially transformed to retene, 7-isopropyl-1-methylphenanthrene, under anaerobic conditions (Osborne 1991; Tavendale et al. 1997b; Fig. 1).

Recently retene has drawn attention as a possible biologically significant effector in aquatic life in areas contaminated by pulp and paper mill effluents. Retene is a polynuclear aromatic hydrocarbon (PAH) that has been found in sludges from aerated wastewater stabilisation basins of pulp and paper mills (Zender et al. 1994; Koistinen et al. 1998) and in sediments downstream of pulp and paper discharges (Tavendale et al. 1995; Judd et al. 1995; Judd et al. 1996). Since retene is hydrophobic (log K_{ow} ~ 6) (Burnison et al. 1996) it is likely also adsorbed to suspended solids during biological wastewater treatment and in aquatic receiving environments (Karickhoff et al. 1979; Means et al. 1980; Schwarzenbach & Westal 1981). Recent studies on embryo-larval stages of zebrafish and rainbow trout showed that subchronic exposure to retene causes exposure-related increases in the prevalence of (chemically induced) blue sac disease, a disease originally described as being due to persistent chlorinated compounds, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Billiard et al. 1999). A common factor for retene and dioxin-exposed fish is prolonged induction of MFO activity due to either continued exposure (retene) or chemical persistence (dioxin) (Fragoso et al. 1998; Billiard et al. 1999). Thus far, based on several effects, the threshold concentration approximates 16 - 32 µg/L of retene, but even lower concentrations were observed to cause fin necrosis and opercular loss, which might cause death of larval fish due to subsequent bacterial or fungal infection (Billiard et al. 1999). In developing embryos of Pacific herring the LOEC value for edemic yolk sac and pericardium approximates 10 µg/L (Oikari 1999).

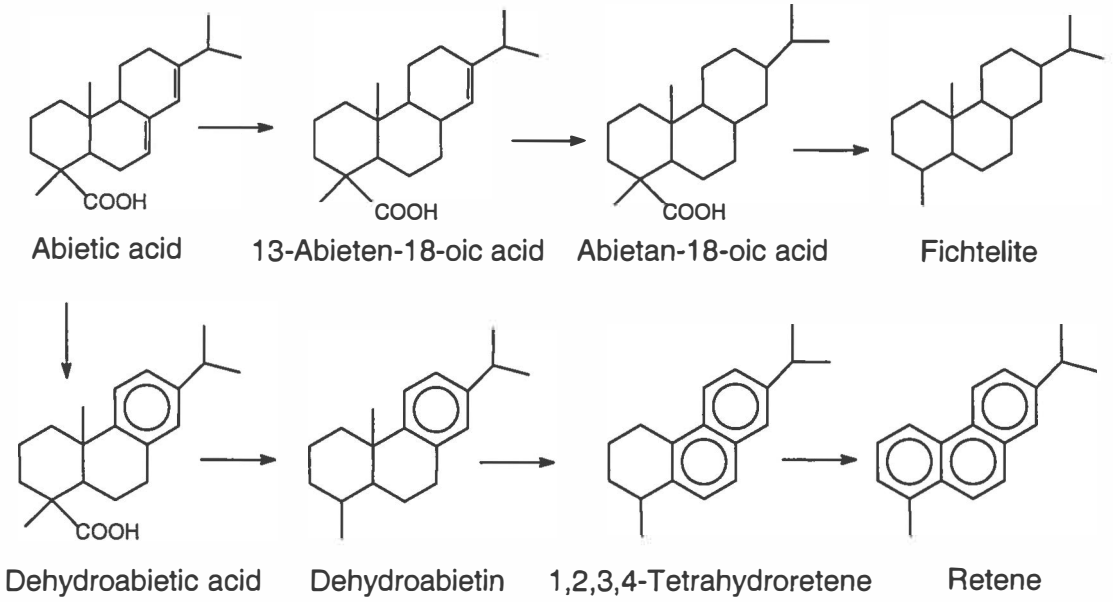

FIGURE 1 The biotransformation pathways proposed for abietic acid under anaerobic conditions (Osborne 1991).

To connect the observed ecotoxicological effects with the exposure to pulp and paper mill effluents, or to the transformation products of compounds present in effluents, exposure biomarkers are needed. Tissue residues and biliary metabolites of compounds present in BKME, or biotic or abiotic transformation products of these compounds, have been used as exposure biomarkers to aquatic animals (Table 1., VI and the literature cited therein). For instance, fish exposed to resin acids typically show increased levels of their metabolites in the bile. Besides TCF technology, which yields nonchlorinated process residues, chlorinated derivatives may still serve as monitoring tools for effluents from pulp and paper mills using ECF bleaching.

TABLE 1 Summary of the suitability of tissues or body fluids of some species for monitoring exposure markers of pulp mill effluents on the basis of literature reviewed (VI). + = rated suitable in the study, - = rated unsuitable.

Species	Marker	Tissue or fluid	Suitability	Bleaching	Ref.
Freshwater mussel	EOCl, CPs	Soft tissues	-	Cl_2,	Mäkelä et al. 1992
Whitefish, mountain whitefish	EOCl, CPs	Filet, bile	+	Cl_2	Owens et al. 1994; Soimasuo et al. 1995
Mountain whitefish	EOCl	Bile	+	Cl_2	Swanson et al. 1996
Mountain whitefish	EOCl	Bile	-	Cl_2, ECF	Swanson et al. 1996; Owens et al. 1994
Lake mussel	CPs, aromatic chlorohydrocarbons[1]	Lipid	+	Cl_2, ECF	Herve et al. 1988; Herve 1991; Herve et al. 1996; Rantio et al. 1996
Four-horned sculpin, pike, eel, flounder, burbot	Chloroanisoles, -veratroles	Liver, filet	+	Cl_2	Neilson et al. 1984; Paasivirta et al. 1987
Carp, crab, White sucker,	PCDDs, PCDFs	Liver, filet	+	Cl_2	Hodson et al. 1992; Whittle et al.1993; Ahokas et al. 1994; Servos et al. 1994

continued

Rainbow trout	Resin acids, CPs	Blood plasma	-	Cl_2	Oikari et al. 1980; Oikari & Kunnamo-Ojala 1987
Roach, rainbow trout, whitefish	CPs	Bile	+	Cl_2, ECF	Oikari 1986; Oikari & Kunnamo-Ojala 1987; Lindström-Seppä & Oikari 1990a; Soimasuo et al. 1995; Soimasuo et al. 1998;V
Whitefish, mountain whitefish, longnose sucker	Resin acids, fatty acids	Bile	-	Cl_2, ECF	Owens et al. 1994; Fraikin et al. 1996; Soimasuo et al. 1998; V
Perch, roach, rainbow trout	Resin acids	Bile	+	Cl_2	Oikari 1986; Oikari & Kunnamo-Ojala 1987
Goldfish	Transformed resin acids[2]	Bile	+	ECF	Tavendale et al. 1996

[1] polychlorocymenes, polychlorocymenenes, alkylpolychloronaphthalenes, alkylpolychlorobibenzyls and alkylpolychlorophenanthrenes

[2] abietanic, 13-abietanic and secodehydroabietic acid

2 OBJECTIVES

In this work we studied the environmental fate of resin acids and resin acid-derived compounds in aquatic environments. Given the lipophilic nature of the targeted chemical species the work focuses on the interactions of the constituents with sedimenting particulate material (III) and recipient sediments (I,II). Occurrence of the biotransformation processes in sediments was assessed by studying the correlations between concentrations of various compounds in sediment profiles (I,II) and in sedimenting particles (III). To validate the field observations of the biotransformation processes, aerobic and anaerobic laboratory incubations of sediments containing the compounds of interest were conducted (IV). The exposure of biota to these compounds and other compounds present in BKME was evaluated by fish bile analysis (I,V). The utility of chemical markers in assessment of biotic exposure was fully reviewed (VI).

The main objectives of this work were:

- To provide information on the fate of resin acids and resin-derived neutral compounds, such as retene, THR and DHA in polluted aquatic environments by measuring environmental concentrations and by so help to assess the risk that these compounds might cause in areas receiving pulping effluents (I,II,III).
- To evaluate the exposure of feral fish populations to retene by using fish bile analysis (I).
- To characterize and experimentally validate the biotransformation and degradation of resin acids and base neutral compounds (DHA, THR, retene and fichtelite) by sediment microbes under anaerobic and aerobic conditions (IV).
- To evaluate the utility of bile analyses as exposure biomarkers to biologically treated pulp and paper mill effluents after transition of chlorine based bleaching of pulp to ECF technology and to find most suitable compounds for BKME tracers (V,VI).

3 MATERIALS AND METHODS

3.1 Study areas, pulp and paper mill characteristics and their history

The sediment sampling (I), collection of sedimenting particles (III), conduction of fish caging experiments (V) and the sampling of feral fish (I) were implemented at Lake Saimaa. Lake Saimaa is the largest lake in Finland with the basin area of 4380 km^2. The average depth is 17 metres and the hydrological retention time is approximately 4.3 years (Granberg 1985). The study area, the southern part of this lake - the Southern Lake Saimaa -, has a water area of 609 km^2 (Fig. 2). As a large water body with several subareas, the hydrology of Southern Lake Saimaa varies making the estimation of the retention time difficult. However, it has been estimated that the water flow from Mill A to Päihänniemi (site 9) takes 57 days. (P. Laine, personal communication). In 1996 this area received approximately 330 000 $m^3 d^{-1}$ of biologically and 55 000 $m^3 d^{-1}$ of chemically treated effluents from several mills: an integrated bleached kraft pulp and paper mill (Mill A), a bleached kraft pulp mill (Mill B), a bleached kraft pulp, paper and cardboard mill (Mill C1), and an unbleached pulp and cardboard mill (Mill C2). Since mills C1 and C2 discharge from the same effluent pipe, they are jointly called Mill C. Southern Lake Saimaa has received pulp and paper mill effluents for decades: pulping in Lappeenranta (Mill A) began in 1897, in Joutseno (Mill B) in 1908 and in Imatra (Mill C1) in 1935. The chlorine bleaching at Mill A began in 1964, at mill B in 1969 and in 1950s at mill C. By the end of 1992 all mills had employed ECF bleaching. In 1992 Mill A employed activated sludge wastewater treatment system and by 1997 all mills used similar systems.

TABLE 2 Distances from the sampling sites to the mills investigated in 1996 - 1997 at Southern Lake Saimaa.

	Upstream references					Downstream sites					
Site	1	2	10	22	23	3	4	5	6	7	8
dist. (km)	8.5	4.5	16.7	28.4	35.8	1.0	3.3	5.8	9.0	12.0	2.0
mill	A	A	B	B	C	A	A	A	A	A	A

	Downstream sites											
Site	9	11	12	13	14	15	16	17	18	19	20	21
dist. (km)	16.0	2.6	1.3	3.8	5.3	7.2	9.6	16.0	4.9	6.2	2.4	14.4
mill	A	B	B	B	B	B	B	B	C	C	C	C

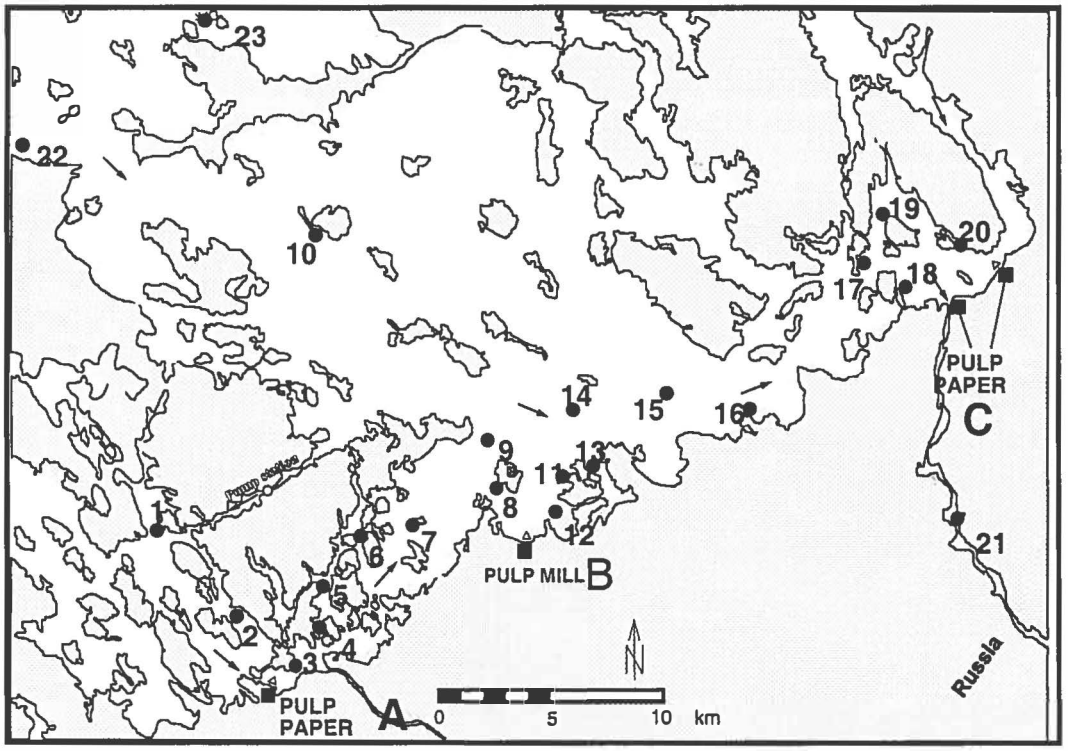

FIGURE 2 The study area in Southern Lake Saimaa. The arrows represent the directions of lake water flow in the area. The pump station keeps the water flow in the eastern basin of the lake (downstream of Mill A) steady. • denote the sampling sites (I,III,V).

Lake Lievestuoreenjärvi represented a lake recovering from exposure to sulphite pulp mill effluents (II). Lake Lievestuoreenjärvi is located in Central Finland, 20 km east of the city of Jyväskylä. The area of the lake is 40.5 km^2, water volume 413.3 million m^3 and the average depth 10.2 m (Vesihallitus 1980). The theoretical retention time is almost 4 years. From 1927 to 1985 the lake re-

ceived effluents from a mill producing bleached pulp by the sulphite process. The effluent discharges resulted in decreased oxygen concentrations in the deep water layers (Meriläinen 1986). According to standard monitoring data, the condition of the lake has improved after the mill closed down in 1985 (Finnish Environment Institute 1999). The reference site at lake Kuusvesi, a pristine oligotrophic water body, is located about 20 km northeast from the mill.

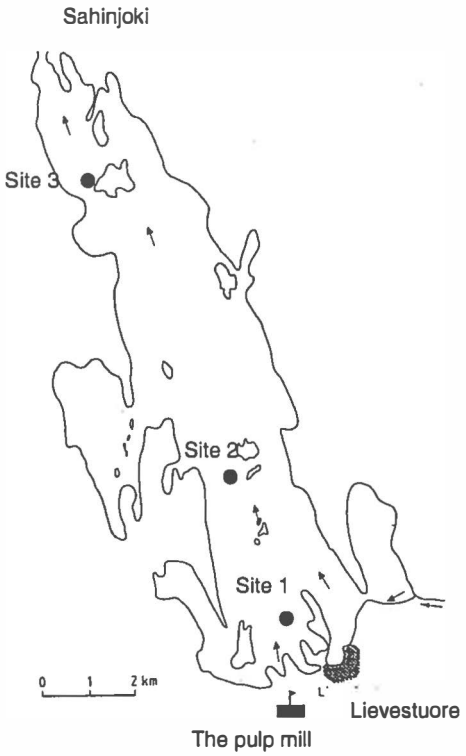

FIGURE 3 The study area in Lake Lievestuoreenjärvi. • denote the sampling sites (II). The reference site was located about 20 km northeast from the mill in Lake Kuusvesi. The small arrows represent the direction of the waterflow in the lake.

3.2 Sampling

Sediments

Sediment samples from Southern Lake Saimaa (I) were collected with a Kajak corer from seven sites affected by pulp and paper mill effluents and from two upstream reference sites (Fig. 2). Water depth at the sampling sites varied from 7 to 15 m. The samples were taken from transportation bottoms with active sedimentation and resuspension. Each duplicate sediment core was divided into five subsamples: 0 - 5 cm, 5 - 10 cm, 10 - 20 cm, 20 - 30 cm and > 30 cm (Fig. 4).

The total length of sediment column ranged between 40 and 55 cm. The distance between the two samplings was approximately 20 m. Samples were freeze-dried and homogenized.

At Lake Lievestuoreenjärvi (II) two or three sediment columns were obtained from each site with a Limnos-corer. The sediment samples were taken from deep, profundal areas where sediment accumulated undisturbed. The water depths were 23 meters (site 1), 70 meters (site 2) and 20 meters (site 3, Fig. 3). At lake Kuusvesi, the reference site, the sample was taken from a deposition area, at a depth of 22 meters. The sediment columns from the effluent exposed sites (sites 1,2 and 3, Fig. 3) were divided into the following 9 subsamples: the uppermost, oxic layer representing the post-pollution period; the following mixed oxic / hypoxic layer; the polluted, sulphide-rich section of the sediment column (7 – 30 cm long), divided into 6 portions equal in size, each portion representing approximately 10 years accumulation; and 5 cm of the sediment column beneath the sulphide-rich portion, representing the situation before the mill was started (Fig. 4). The sediment columns from the reference site were divided into similar subsamples, with the 10-year slices dated by the ^{210}Pb method. The corresponding subsamples from each site were pooled together to represent the average sediment quality of the layers. Samples were freeze-dried and homogenized.

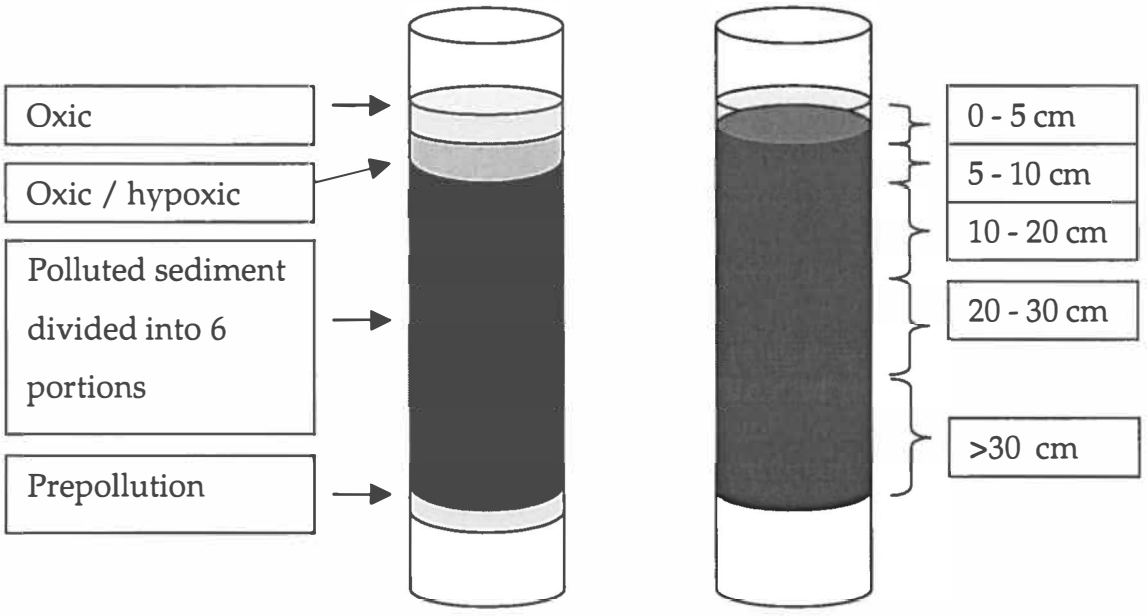

FIGURE 4 The subsamples obtained from the sediment cores from Lake Lievestuoreenjärvi (left) and Southern Lake Saimaa (right).

Sedimenting particles

Sedimenting particles from Southern Lake Saimaa (III) were collected with sediment traps which were suspended two meters above the bottom in areas where overall water depth was 12 - 15 m. Samples were collected from 5 sites affected by pulp and paper mill discharge and two reference sites. The collection period was 30 days in May - June 1991, 1995, and 1996. The collected samples were freeze-dried and weighed within 3 days of collection. The sediment traps and collection technique are further described in Kukkonen et al. (1996).

Lake waters and mill effluents

Lake waters from Southern Lake Saimaa were sampled twice during the 30 - day whitefish caging period in 1996 (V). Samples were collected at each site as composites from the water column at 0, 1, 2, 3, and 4 meters. Mill effluents were collected daily with an autosampler and combined into one-week samples in each mill. The one-week composite samples were combined in the laboratory to one sample which represented the average quality of mill effluent and gave information about the average condition of the BKME receiving area during the experimental exposure period (V).

Sampling of feral fish

Feral perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) from Southern Lake Saimaa (I) were captured with hook and worm or with a fish trap at approximately 1 - 2 m depth from reference site 2 and from a site affected by pulp and paper mill effluents from Mill A (site 3, see Fig. 2) (I). Once recovered in holding cages, the fish were sampled on the spot within 24 - 48 hours. Bile samples were placed in liquid nitrogen, transferred to the laboratory and placed in a deep freezer (- 80°C) (Karels & Oikari 1999).

Whitefish caging and sampling

1-year old, hatchery reared, juvenile coregonid whitefish (*Coregonus lavaretus* L. s.l., plankton feeding species) were exposed to BKME by caging them at various distances from the mills (V). The exposures took place in 250-litre cages at a depth of 4 - 5 meters and lasted for 30 days in May - June, 1996. Each cage had twelve fish with an average weight and length of 40 g ± 10.7, 16.5 cm ± 1.3. Bile samples were taken in the laboratory of R/V Muikku and stored in liquid nitrogen before placing in a deep freezer (-80°C) where the samples were kept until analysis. The fish caging technique is further described in Soimasuo et al. (1998).

3.3 Actions of the sedimental microbial community

The sediment used in the incubations (IV) was collected from Site 3 (Fig. 2) downstream of Mill A at Southern Lake Saimaa in fall, 1997. Three subsamples were combined from the top 10 – 15 cm of sediment. To facilitate the detection of changes and to avoid toxic concentrations, the contaminated sediment was diluted with a reference sediment collected from an unpolluted site (Lake Palosjärvi, Central Finland).

The sediment inoculum was added to a methanogenic mineral salts medium (Alder et al. 1993), supplemented with vitamins and trace elements (modified from Hurst 1997) to form a 10 % (v/v) sediment slurry. The slurry was sealed in serum bottles with CO_2 / N_2 (30 / 70 %) added as the headspace gas. The samples were spiked with methanol, and DHAA or retene in methanol to the final concentrations. Sterile controls prepared in the same way were autoclaved twice (Tavendale et al. 1997a). Bottles were incubated statically in the dark for 52 weeks, either at 4 °C or at 24 °C. Cultures were mixed by shaking once a week. All incubations were carried out in duplicate.

The aerobic incubations were carried out similarly, but the headspace of the bottles was flushed with air once a week by opening the bottle. Sterile controls were prepared in the same way and autoclaved twice, but were not flushed with air. Bottles were incubated in dark at 24 °C for 52 weeks without any mixing of solid material.

3.4 Analytical methodology

Resin acids and base neutrals in sediments and sedimenting particles

Sediments and sedimenting particles were extracted using a method modified from Tavendale et al. (1995) (I,II,III). Freeze-dried sediment or sedimenting particles were extracted for 24 h with hexane : isopropanol (2:1, v/v) in an automatic Soxhlet-extraction apparatus. Heptadecanoic acid and biphenyl, androstane (I,III) or anthracene–d_{10} (II) were used as internal standards. After extraction, the samples were evaporated to ca. 5 mL, made up to 50 mL with hexane and further extracted with K_2CO_3 solution.

The hexane extracts were dried and reduced to 1 mL. The residue was then introduced to Al_2O_3 (4% deactivated): SiO_2 (1 : 2, w/w) mini-column preeluted with hexane. The sample was first eluted with hexane and this fraction discarded. Retene and other base neutral compounds were eluted with dichloromethane. This fraction was evaporated to 0.5 mL with a stream of nitrogen and analysed with GC-MS.

The pH of the carbonate extract was adjusted to 9 and extracted with dichloromethane in a mechanical horizontal shaker. The extract was evaporated to dryness and the residue dissolved to 1 mL of MTBE. The sample was silylated and analysed for resin acids with GC-MS. The recoveries obtained were sa-

tisfying and are presented in paper I. The compounds were analyzed using spectral scanning (*m* / z 40 - 500) and quantified from the resulting total ion current (TIC) chromatogram. Base neutrals were quantified using a response factor of 1 : 1 relative to retene. Resin acids for which no standards were available (isopimaric and sandaracopimaric acids) were quantified using a response factor of 1 : 1 relative to DHAA.

Resin acids and base neutrals in the incubation experiment

The aquous phase from the incubation bottle (IV) was transferred to an extraction vessel, the pH adjusted to 9.5. and extracted with dichloromethane. The extract was divided into two Kimax tubes, evaporated to dryness and redissolved with hexane and MTBE for base neutrals and resin acid analyses, respectively. By redissolving the residue with hexane for base neutral analyses the background noise of the GC-MS analyses was reduced. The sample was silylated for resin acid analysis. Heptadecanoic acid and androstane were used as internal standards.

The sediment slurry remaining in the incubation bottle was dried with anhydrous sodium sulphate and extracted with dichloromethane in a mechanical horizontal shaker. The extract was divided into Kimax tubes, evaporated to dryness, and redissolved with hexane or MTBE for base neutrals and resin acid analyses, respectively. The sample was silylated for resin acid analysis. The amount of the compounds in the aqueous phase and in the dried slurry were reported jointly as the total amount of the compounds per bottle. The samples were analysed with GC-MS as described above for sediment samples. The comparison between the Soxhlet extraction method described in paper I and the extraction method used for the incubated samples is presented in paper IV.

Methane analysis

Methane concentration in the headspace of the anaerobic incubations was measured weekly with a GC equipped with FID. The column used was PE-Alumina, length 30 m, inner diameter 0.53 mm. The sample from each serum bottle was obtained through the stopper with a gas-tight syringe.

Fish bile analyses

Resin acids in the bile samples of caged whitefish and feral perch and roach were analysed by a method modified from Oikari et al. (1984). Conjugated resin acids were released by alkaline hydrolysis and the extractions were performed with MTBE. CPs in bile were analysed by a method modified from Hemming & Holmbom (1992). Conjugated CPs were released by acidic hydrolyses and the extractions were performed with hexane. The methods are described in more

detail in (V). Free and conjugated compounds were analysed separately, but generally summed for data presentation.

Resin acids and fatty acids were methylated and quantified with heptadecanoic acid as the internal standard. Retene and other base neutrals were analysed in the same extract as resin and fatty acids and quantified with heptadecanoic acid as the internal standard (I). Cholesterol was silylated and also quantified with heptadecanoic acid as the internal standard (V). CPs were acetylated with acetic anhydride and quantified with 2,6-dibromophenol as the internal standard.

Resin acids, fatty acids and cholesterol were analysed using GC equipped with a FID, CPs with a GC equipped with an ECD and retene and other base neutrals with GC-MS.

Lake waters and mill effluents

CPs, fatty and resin acids and phytosterols from lake water samples and mill effluent samples (V) were analysed according to Hemming & Holmbom (1992) and Örså & Holmbom (1994). Water samples were methylated for fatty and resin acid analyses, acetylated for CPs analyses and silylated for sterol analyses. Free and bound CPs were analysed separately according to Voss et al. (1981) and Paasivirta et al. (1992). Fatty and resin acids and phytosterols were analysed with GC equipped with FID and CPs with GC equipped with ECD. Water sodium (Na^+) concentration was used as an effluent tracer within the experimental area. The data was provided by the monitoring program conducted by the Saimaa Water Protection Association Inc., Lappeenranta, Finland.

4 RESULTS AND DISCUSSION

4.1 Base neutrals and resin acids in sedimenting particles collected from a lake polluted by BKME

Suspended particulate matter affects the fate and cycling of many hydrophobic low molecular weight constituents. The cycling of such materials in the aquatic environment can be a two stage process: relatively rapid settling of constituents associated with particulate matter and incorporation into the bioturbated layer of surface sediments. Subsequently these constituents are available for reintroduction into the water column or food web, possibly on seasonal to decadal time scales, through resuspension (Eadie & Robbins 1987) and uptake by benthos (Landrum 1989; Landrum & Robbins, 1990; Kukkonen & Landrum 1994). To evaluate the significance of the sedimenting particulate matter on the fate of resin acids and resin acid-derived compounds in the recipient the concentrations in the sedimenting material, and the sedimentation rates of these compounds were determined in the study area at Southern Lake Saimaa. The impact of the employment of activated sludge wastewater treatment system and ECF bleaching in the pulp mill on the sedimentation of these compounds was also assessed.

The results suggest that the sedimenting particles must be considered as a significant route of resin acids and base neutrals from water to sediment and also as an ecotoxicologically important potential route of these contaminants to biota (III). The highest concentration of resin acids observed was 1470 μg/g d.w. of sedimenting particles (site 20, Fig. 2) and the highest concentration of retene 54 μg/g d.w. (site 4, Fig. 2). In treated effluents of mills A and C the concentrations of resin acids were similar in 1995 and 1996 (38 - 179 μg/L), while the concentrations in the effluent from mill B were higher (560 - 1200 μg/L) (Mellanen et al. 1999, V). This indicates that most of the material trapped at site 20 was resuspended from bottom sediment. The concentrations of other base neutrals in the particles in areas exposed to mill effluents were in range of < 0.2 – 37 μg/g d.w. DHAA was the most abundant resin acid at all sites. The concentrations of retene and resin acids in sedimenting particles showed a distance

related derease downstream of Mill A. In the upstream reference areas (sites 1 and 2, Fig. 2) the concentrations of base neutrals and, with one exception, resin acids were clearly lower than in BKME exposed areas. Also the OC content was higher in particles collected from BKME exposed areas than reference areas, indicating the influence of mills as sources.

By comparing the samples of sedimenting material collected prior to and after the conventional aerated lagoon wastewater treatment system of the mill was modernized with an activated sludge plant, and ECF bleaching had replaced chlorine bleaching, it can be seen that these technology changes have decreased the sedimentation of retene and resin acids by up to 70 % (Fig. 5, III). However, the relative contributions of the two technology changes cannot be quantified since both took place between the initial and final study periods.

The OC content in sedimenting particles was higher than that of the transport bottom sediments from the same site (I,III). The sediment traps may have collected not only currently formed sedimenting material but also resuspended material. This was demonstrated by Meyers et al. (1984), who found that the great majority of the material in a sediment trap placed 1 meter above the bottom in Lake Michigan may be resuspended sediment. They suggested that the concentration of OC can be higher in resuspended material than in the few top centimeters of sediment, because resuspended material contains a high proportion of fine-grain low-density particles, and organic matter is preferentially associated with these materials. Also the concentration of retene in the sedimenting particles was higher than in surface sediment, whereas the concentration of resin acids were generally higher in sediment than in sedimenting particles (I,III). This is most likely related to the lipophilicity of compounds: retene, being more lipophilic (Stuthridge & Tavendale 1996; Brumley et al. 1997) associates more tightly with resuspending material than resin acids. Other possibilities include preferential resuspension of deeper sediments where higher retene concentrations prevail (e.g. via bioturbation). The composition of resin acids in sedimenting particles was similar to the composition in the top five centimeters in the sediment.

The high concentrations of resin acids and retene observed in sedimenting particles indicated rapid partitioning onto suspended material and sedimentation. This would suggest that high concentrations of retene could also be found in the sediments contaminated by pulping effluents.

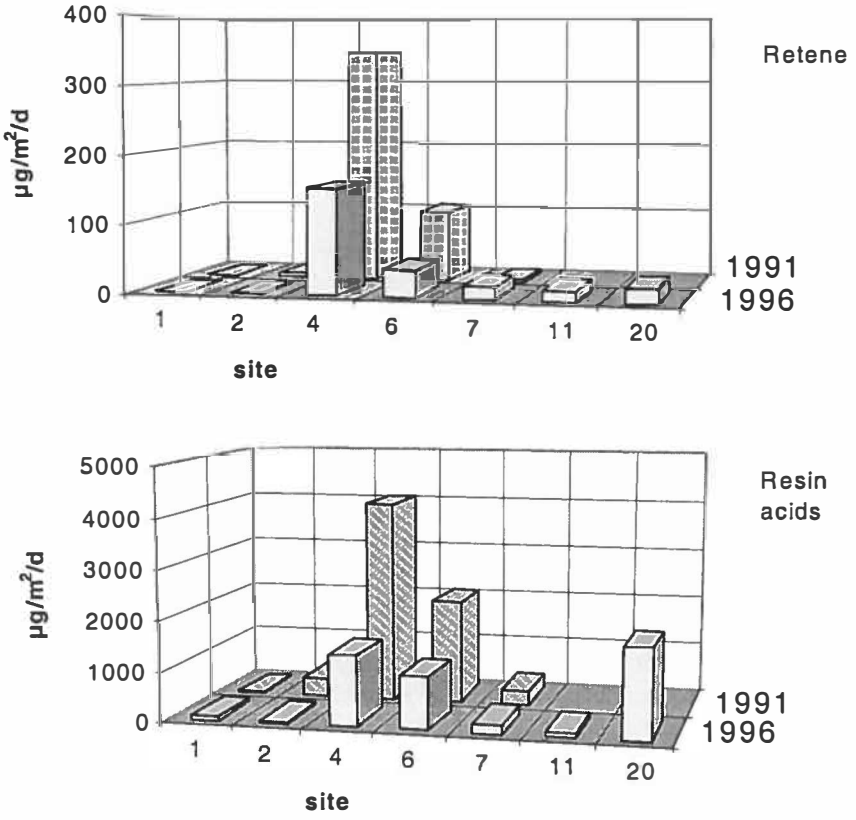

FIGURE 5 The sedimentation rate of resin acids and retene (µg/m^2/day) at Southern Lake Saimaa prior to (1991) and after (1996) the alterations in wastewater treatment at Mill A (sites 4 - 7, Fig. 2). In 1996 the sites 11 and 20 represent adjacent areas of Mills B and C, respectively. Sites 1 and 2 were upstream references.

4.2 Base neutrals and resin acids in transport bottom sediments of a lake polluted by BKME

Since resin acids and retene appeared to partition onto sedimenting suspended material (III) the occurrence of these compounds in sediment profiles of the study area was investigated. The biotransformation of abietic type resin acids in sediment cores was also assessed.

Retene was the most abundant base neutral in sediments at all BKME exposed sites in Southern Lake Saimaa and DHAA the most abundant resin acid (I). Downstream of Mill A (sites 3,4,5 and 7, Fig. 2), both retene and resin acid concentrations in sediments showed reduction with distance from the mill. The highest concentration of retene (1 680 ± 80 µg/g d.w.) was found 1 km downstream of Mill A at a sediment depth of over 30 cm. Fichtelite appeared to be the second most abundant base neutral, its highest concentration being 80 ± 7 µg/g

d.w., implying that retene and fichtelite are the most resistant base neutrals to further biotransformation (Tavendale et al. 1997b; V). At site 3 (see Fig. 2), there was a significant negative correlation between DHAA and retene concentrations, strongly suggesting that DHAA is transformed to retene (Fig. 6, I). The concentrations of THR also correlated negatively with retene concentration but the concentrations of DHA did not. This would indicate that the formation of retene from DHAA may not involve DHA as an intermediate as was also suggested by Tavendale et al. (1997b). Alternatively, the formation of THR from DHA may be rapid process compared to transformation of THR to retene (IV). The higher concentration of DHA in sedimenting particles (III) than in the top five centimeters of sediment also supports the fast biotransformation of DHA in anaerobic sediments. However, these correlations were not detected at the other sites (I). In fact, at other sites retene and resin acid concentrations at different depths of sediment correlated positively with OC content and with each other, indicating that the precursor - metabolite relationships are difficult to interpret from field data in a generalized manner.

The highest concentration of retene in a surface sediment (600 ± 15 µg/g d.w.) was at site 20, which receives effluents from Mill C. The concentration of resin acids at 5 - 10 cm were the highest recorded in the study (1 500 ± 30 µg/g d.w.). The relatively low concentration of retene in deeper sediment layers compared to the concentration of DHAA indicates that little biotransformation had occurred. The lack of further biotransformation may be due to high concentrations of resin acids, which could be inhibitive for biotransforming anaerobic microbes (Sierra-Alvarez & Lettinga 1990). The data provided by Saimaa Water Protection Association Inc. shows that the annual average water temperature 0.5 m above the bottom at site 3 was slightly warmer than at site 20 (I). The temperature was suggested to be an important factor in biotransformation of DHAA (V). However, the difference in temperatures observed was probably insufficient to explain the observed differences in biotransformations activities.

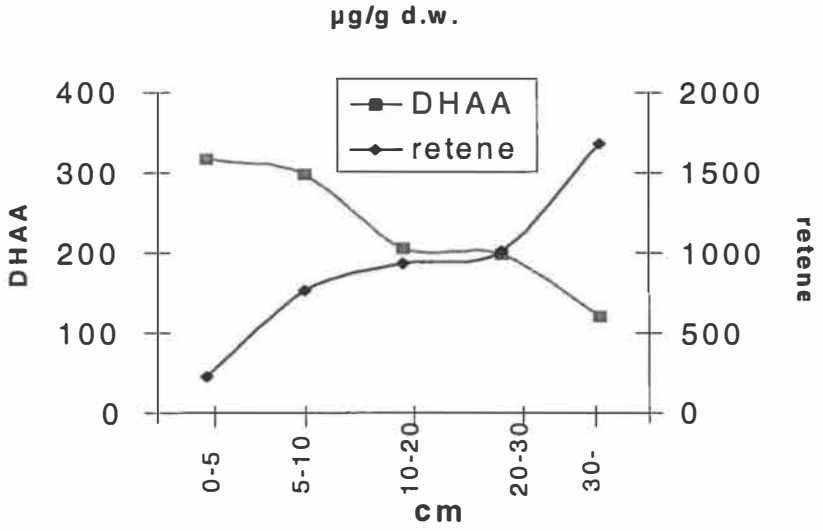

FIGURE 6 Correlation between the concentrations of DHAA and retene in the sediment profile in vicinity of a pulp and paper mill at Southern Lake Saimaa.

The concentrations of resin acids and base neutrals at the sites with complicated and multidirectional water flows, such as sites 11 and 12 downstream of Mill B (Fig. 2), were considerably lower than those observed downstream of Mills A and C. The highest concentration of resin acids was 315 ± 70 μg/g d.w. and of retene 87 ± 7 μg/g d.w.

The retene concentrations at the upstream reference sites 1 and 2 at Southern Lake Saimaa (Fig. 2) were in accordance with what has been reported as background concentrations (<0.1 μg/g d.w.) for brackish (Boulabassi & Saliot 1993) and freshwater areas from forested watersheds (Tan & Hite 1981; Billiard et al. 1999; II). The sediment concentration of resin acids at site 1 was high compared to site 2. This might be due to the pump station displacing water from the main area of Lake Saimaa to the site area. The current transports particulate and dissolved organic material from the lake, and any small molecular substances bound to particulate matrix may be trapped into the sediments and cause elevated concentrations.

4.3 Base neutrals and resin acids in sediments of a lake recovering from exposure to sulphite mill effluents

To find out whether the high concentrations of base neutrals observed in the sediments of Southern Lake Saimaa were exceptional, and to study the stratification of these substances in the sediment against the pollution history of an area, samples were collected from a lake recovering from the exposure to effluents from a sulphite mill.

The concentration of retene in the sediment core approximately dating to the early 1980s (depth of 7 – 11 cm), at site 1, 1.5 km downstream of the pulp mill sewer (Fig. 3), was thus far the highest reported (3300 μg/g d.w., Fig. 7, II). The concentrations of other base neutrals were substantially lower. The concentrations of THR and DHA were rather similar (0.05 – 89 μg/g d.w.), while the concentration of fichtelite was lower (0.3 – 28 μg/g d.w.). The concentrations of resin acids at site 1, in the approximately 20 year old sediment core, were also the highest recorded in the study, with a concentration of 1100 μg/g d.w. The composition of native resin acids was DHAA 91 %, abietic acid 4 %, pimaric acid 3 %, sandaracopimaric acid 0.5 % and isopimaric acid 1.5 %. However, the concentration of a transformed abietic acid, tentatively identified as abietan-18-oic acid by the mass spectrum and the retention data (Tavendale et al. 1995), was approximately three times higher than the concentration of DHAA. This compound has been identified in sediments polluted by pulp and paper mill effluents (Judd et al. 1995). It has been suggested, that abietan-18-oic acid is an intermediate compound in the biotransformation process of abietic acid to fichtelite (Osborne 1991, Fig. 1). In the oxic surface sediment (0-1 cm) the concentration of retene was 62 μg/g d.w. (II).

Further downstream from the pulp mill (site 2, 4.5 km, Fig. 3) the highest concentration of retene was about half of that observed at site 1 (1500 µg/g d.w.) (II). This concentration was in the sediment layer which dated approximately to early 1980s, similar to site 1. The concentrations of other base neutrals varied from 0.14 to 11.9 µg/g d.w. In the oxic surface layer the concentration of retene was low (0.8 µg/g d.w.), but already increasing in the mixed oxic / hypoxic layer (73 µg/g d.w.), supporting the hypothesis, that anaerobic conditions are needed for the formation of retene. The concentration of resin acids was also lower in the uppermost oxic layer of sediment at sites 1 and 2 indicating that the lake is recovering from the pollution.

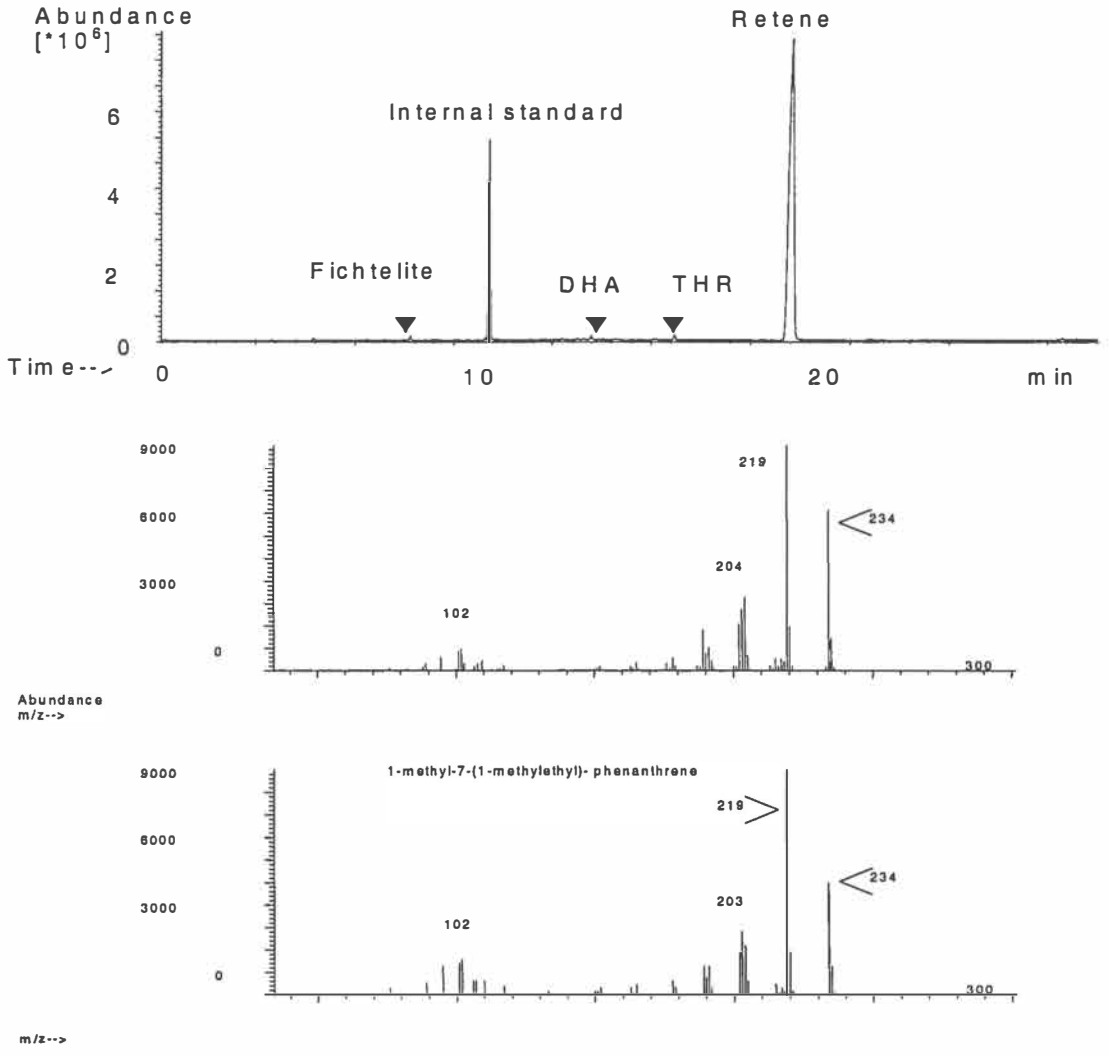

FIGURE 7 The total ion current chromatogram of the base neutral fraction in a sediment core approximately dating to early 1980s at a depth of 7 – 11 cm of site 1, 1.5 km downstream from the pulp mill sewer at Lake Lievestuoreenjärvi (top) and the mass spectrum of the compound identified as retene (middle). For comparison the mass spectrum of the retene standard used is also presented (bottom).

The concentrations of base neutrals and resin acids in sediment layers at site 3 (12.3 km downstream from the mill, Fig. 3) were considerably lower than in sediments at the same distance from a mill at Southern Lake Saimaa (I). This is most likely due to hydrological reasons. The slow movement of the water mass, as indicated by the long hydrological retention time of the Lake Lievestuoreenjärvi (four years), causes the mostly particle bound hydrophobic compounds such as resin acids and base neutrals to settle close to the mill. The concentration of retene ranged from 0.06 μg/g d.w. to 2.2 μg/g d.w. being the most abundant base neutral followed by DHA (0.1 – 0.6 μg/g d.w.), THR (0.05 – 0.5 μg/g d.w.) and fichtelite (0.05 – 0.3 μg/g d.w.). DHAA was the most abundant resin acid with a highest concentration of 39 μg/g d.w.

The concentrations of resin acids and base neutrals in sediments at the reference site of Lake Kuusvesi were in accordance with what has been reported as background concentrations for these compounds (Tan & Heit, 1981; Boulabassi & Saliot 1993; Judd et al. 1995, 1998; Billiard et al. 1999). The concentrations of retene, DHA and fichtelite ranged from 0.01 – 0.13 μg/g d.w., whereas the concentration of THR was somewhat lower. DHAA was the most abundant resin acid with a concentration ranging from 0.3 μg/g d.w. to 1.5 μg/g d.w. Similar concentrations of base neutrals were also observed in sediment cores dating to the pre-pollution age at sites 2 and 3. At site 1 the concentration of retene was higher (3 μg/g d.w.). The concentration of DHAA was higher at all polluted sites in the sediment cores dating to the pre-pollution age (5 – 32 μg/g d.w.), indicating probably a slight mixing of the cores during sampling.

The most notable difference qualitatively in the composition of base neutrals in polluted sediments of Lake Lievestuoreenjärvi (II) and Southern Lake Saimaa (I) was the abundance of fichtelite in the sediments. At Southern Lake Saimaa fichtelite was the second most abundant base neutral after retene, whereas at Lake Lievestuoreenjärvi it was the least abundant. High concentration of abietan-18-oic acid, a suggested precursor of fichtelite was tentatively identified in the sediment cores. This might indicate differences in environmental conditions or microbial activity between Lake Lievestuoreenjärvi and Southern Lake Saimaa.

4.4 The exposure of feral fish to retene

The bioavailability of retene to biota is the key issue when considering its ecotoxicological significance in aquatic environments. To assess the bioavailability to fish we determined its concentration in the bile of roach (*R. rutilus*) and perch (*P. fluviatilis*) caught 1 - 2 km downstream of pulp and paper mill A and in the reference area. It was recently demonstrated that retene and its metabolite(s) are excreted to the bile of rainbow trout, the concentrations being positively corre-

lated with the induction of cytochrome P-450 isozyme CYP1A1 in the liver (Fragoso 1998; Fragoso et al. 1998).

Retene was found in the bile from roach caught 1-2 km downstream of the pulp mill sewer, with an average concentration of 3.1 μg/mL without any difference between the sexes (I). Upstream to the effluent source, no retene in roach bile was measurable. Interestingly, Karels et al. (1998) observed that the EROD activity in roach downstream Mill A was four times higher than in roach caught from the upstream reference site. Retene in roach bile occurred in native form (I), but unidentified metabolites of retene were also likely to be present in hydrolyzed samples. According to previous knowledge on metabolism of PAHs (Krahn et al. 1987), the most probable metabolites would be mono- or dihydroxylated derivatives of retene.

No retene was found in bile from perch caught in the same downstream area as roach nor at the reference site upstream from the mill (I). We suggest, that the most probable reason for this is different feeding habits. Roach graze primarily on benthic invertebrates and ingest some of the sediment too. However, although young perch may feed on benthos, larger specimens prefer prey from the water column (Koli 1990). When compared to roach, another reason for a lack of retene in the bile of perch could be its higher constitutive (uninduced) capacity for phase I biotransformations as indicated by much higher CYP1A activity (Lindström-Seppä & Oikari 1990b), which could make perch a better eliminator of retene. Resin acids and their metabolic conjugates were present at higher concentrations in bile from roach than perch caught downstream from Mill A. Dehydroabietic acid was the most abundant resin acid in the bile followed by abietic acid, reflecting the composition both in sediments and effluents.

Other resin derived neutral compounds (THR, DHA and fichtelite) were not detected in the bile of roach and perch caught downstream of Mill A or from upstream locations serving as reference.

4.5 The validation of the biotransformation of DHAA

Since high concentrations of DHAA and resin derived base neutrals were observed in BKME polluted sediments (I,II), we wanted to study the biotransformation of DHAA by BKME adapted sedimental microbial consortium in the laboratory (IV). The assesment was conducted by incubating the spiked and unspiked sediments under anaerobic conditions at 4 °C and 24 °C and under aerobic conditions at 24 °C for one year. The temperatures were chosen to represent the natural circumstances prevailing most of the year in Nordic lake sediments (4 °C) or to accelerate the biological processes (24 °C) in order to assess the full potential for change.

The methane production under anaerobic conditions revealed a distinct difference in the dynamics of the anaerobic activity at 4 °C and 24 °C. The methane production at 24 °C rose within a week after the start of the incubation

and about half of the total methane production occurred during the first 16 weeks. However, at 4 °C the methane production started after 8 weeks and remained low for the first 30 weeks. After this there was a rapid increase in the methane production rate, actually approaching the early maximum rate at 24 °C (IV). Since the volume produced was higher than the theoretical amount that the degradation of the methanol added could produce, it would appear that sedimentary organic material had also been degraded. At 4 °C the DHAA spiked samples produced only 54 % of the methane that the methanol spiked samples produced, suggesting that the DHAA concentration used inhibited methanogenesis at the low temperature. This was not observed at 24 °C (IV).

In anaerobic incubations at 24 °C with no spike other than methanol, the concentrations of all the resin acids (pimaric, isopimaric, sandaracopimaric, and dehydroabietic acids) and base neutrals (retene, THR, DHA and fichtelite) decreased with respect to the sterile controls (Fig. 8) (IV). This indicates that these hydrophobic compounds, though possibly being more tightly bound to the sediment with aging (Hatzinger & Alexander 1995) than just recently introduced spiked compounds, are degraded or transformed under anaerobic methanogenic conditions (IV). This is also consistent with an earlier report (Tavendale et al. 1997a). Little information is available about the anaerobic biodegradation of retene. However, several research groups have reported anaerobic biodegradation of PAHs with similar structure, especially phenanthrene. Rockne & Strand observed a complete removal of phenanthrene within 14 days by a nitrate-reducing fluidized bed reactor enrichment of bacteria from creosote-contaminated marine sediment. Even faster biodegradation rates were observed with organisms in pure cultures under denitrifying conditions which degraded phenanthrene in 12 - 44 h, the rate depending on the history of contaminant exposure of the microbial community (McNally et al. 1998). However, in sediment incubations the biodegradation rates have been considerably slower. Under sulfate-reducing conditions the total removal of phenanthrene has been observed within 30 - 150 days, the rate again depending on the pollution history of the sediment (Coates et al. 1996, 1997; Zhang & Young 1997). It has been suggested that carboxylation is an initial reaction in the anaerobic metabolism of phenanthrene (Zhang & Young). Aromatic compounds have also been reported to be degraded under methanogenic conditions (Kazumi et al. 1997).

In the anaerobic incubations with a spiked concentration of DHAA at 24 °C the concentration of DHAA also decreased (IV). At the same time the retene concentration in active cultures increased (Fig. 9). The concentrations of other base neutral compounds increased except for fichtelite, which remained practically the same in active and control incubations. This is consistent with earlier reports (Tavendale et al. 1997b). On a molar basis the sum increase of base neutrals was 8.5 micromoles / 50 mL in contrast to 17 micromoles of DHAA removed (IV). This indicates that DHAA was also transformed to other products or mineralized, or retene and other base neutrals were further transformed or degraded during the incubation. Tavendale et al. (1997a; 1997b) observed that after 120 days of anaerobic incubation at 25 °C the base neutrals (except fichtelite) and resin acids in sediments started to degrade.

The concentrations of pimaric type resin acids (pimaric, sandaracopimaric and isopimaric acids) were virtually the same in autoclaved controls and in active DHAA spiked cultures after 52 weeks, indicating the recalcitrant nature of these compounds under anaerobic conditions.

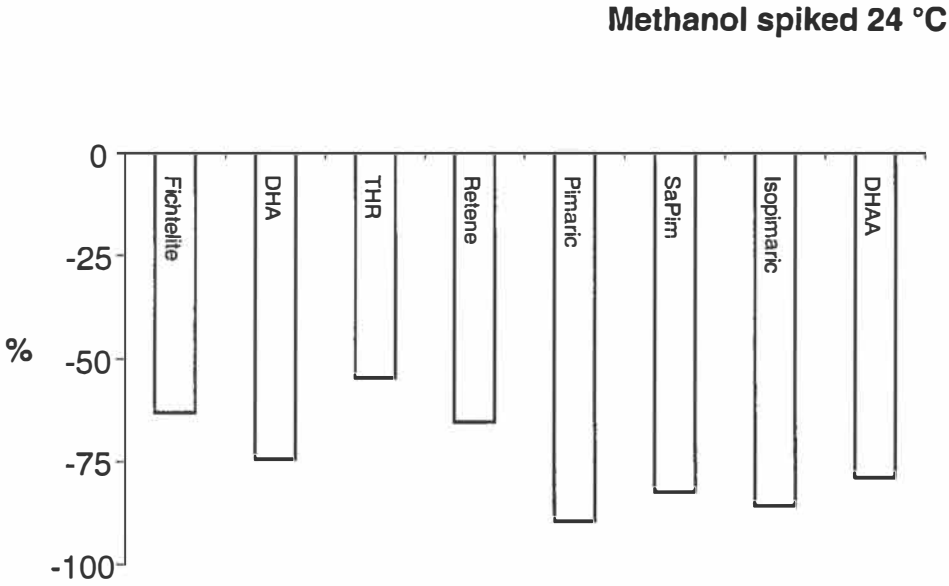

FIGURE 8 Disappearence of resin acids and resin acid-derived compounds with additional carbon source methanol: Percentage net changes under anaerobic conditions at 24 °C are compared to the sterile controls. Abietic acid is not presented, because it´s transformation is principally abiotic. DHA = dehydroabietin, THR = tetrahydroretene, SaPim = sandaracopimaric acid, DHAA = dehydroabietic acid.

Under anaerobic incubation at 24 °C with a high concentration of retene no changes in concentrations of DHAA or retene were observed (IV). Neither were changes observed in the resin acid or base neutrals compositions during anaerobic incubations at 4 °C (IV). This demonstrates the significance of temperature on the rate of biotransformation of DHAA and the formation of retene. This might also support the suggestion that the variations in water temperature from one lake area to another could explain the observed differences in the concentration of retene in BKME polluted sediments at Southern Lake Saimaa (I). On this basis it might be expected that DHAA biotransformation products will remain present far into the future, since the biotransformation of DHAA in anaerobic subsurface sediments of cold Nordic lakes is slow and most of it may only occur during summer as water temperature increases.

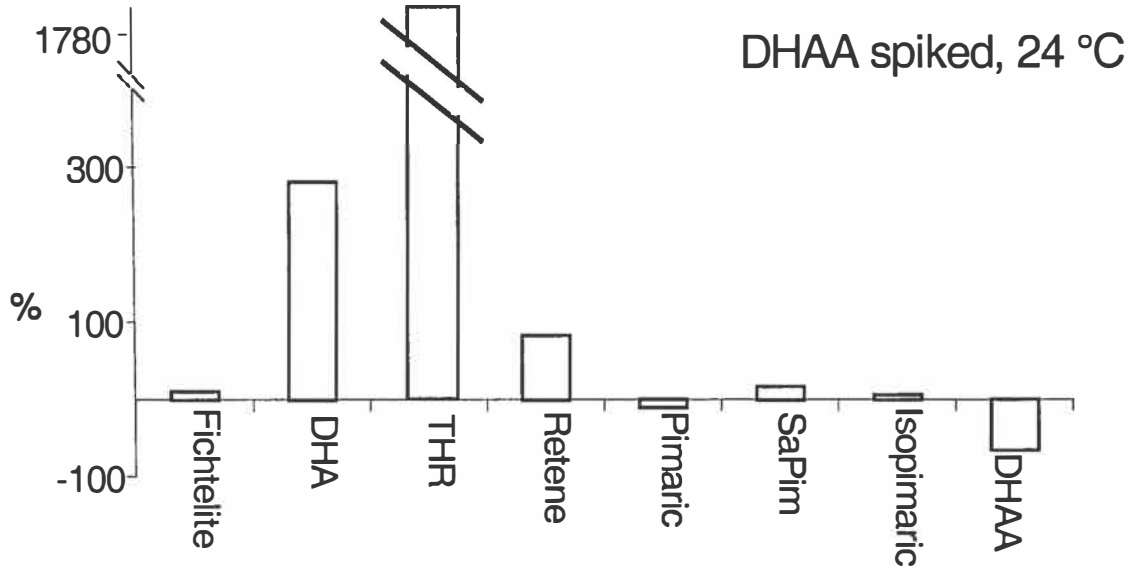

FIGURE 9 Formation of retene and disappearence of DHAA in anaerobic incubations at 24 °C with additional carbon sources methanol and DHAA: Percentage net changes compared to the autoclaved controls. The concentration of DHAA has decreased and the concentration of retene increased in the incubation. On molar basis the increase in retene concentration was 4.5, DHA 1.0 and THR 3 micromoles per 50 mL experimental unit, whereas 17 micromoles of DHAA was removed.

During aerobic incubations spiked with DHAA at 24 °C the levels of all the other compounds of interest decreased by 53 – 94 %, except the concentrations of pimaric type resin acids, which remained rather constant. In the cultures spiked with retene the concentrations of fichtelite, DHA and sandaracopimaric acid showed no significant reduction, whereas the concentrations of DHAA and retene decreased 63 and 93 %, respectively. The more recalcitrant nature of pimaric type acids under aerobic conditions is in accordance with previous reports (Hemingway & Greaves 1973; Wilson et al. 1996). The degradation of retene is consistant with reports of degradation of other PAHs, especially phenanthrene, under aerobic conditions (Shiaris 1989; McNally et al. 1998). Interestingly the concentration of DHA at Southern Lake Saimaa (III) was higher in sedimenting particles than in the few top centimeters in sediment in the same site (I). This might indicate, that the biotransformation of DHA in anaerobic conditions is fast, whereas under aerobic conditions it is more recalcitrant, or it is formed under aerobic conditions.

4.6 Fish bile metabolites as exposure biomarkers to pulp and paper mill effluents

The use of fish bile metabolite analyses as exposure markers in to pulp and paper mill effluents was reviewed in the literature (VI), and the utility of bile analyses was studied at Southern Lake Saimaa by experimentally exposing whitefish to biologically treated effluents from pulp and paper mills using ECF-bleaching (V). The effluent dilution in the lake was determined by sodium (Na^+) analysis of the lake water at the sampling sites. The best chemical markers of exposure were the biliary metabolites of CPs and especially chloroguaiacols, chlorocatecols and 6-monochlorovanillin (Fig. 10) since these correlated positively with sodium concentration. This was somewhat expected, since unlike resin acids, chlorophenolics, especially those with lower chlorine substitution arising from ECF bleaching, are quite water soluble and therefore are unlikely to be impacted by particulate partitioning and sedimentation processes. Before the bleaching process and wastewater treatment changes the amount of CPs in bile of whitefish 3.3 km downstream of mill A (site 4, Fig. 2) was 520 μg/mL (Petänen et al. 1996). Levels of CPs in bile were 99.6 % lower after the changes (V).

Biliary resin acids did not correlate with the effluent dilution at Southern Lake Saimaa. However, the biliary resin acids did correlate positively with the concentration of resin acids in water. This would indicate, that there are resin acids originating from sources other than pulping at Southern Lake Saimaa. The only resin acid detected in bile and in lake water was dehydroabietic acid.

Johnsen et al. (1995) detected elevated concentrations of conjugated fatty acids and cholesterol in bile of rainbow trout exposed to dithionite-bleached untreated pulping effluents in the laboratory. However, the alkali hydrolysis used did not release any additional cholesterol from the bile of whitefish at Southern Lake Saimaa and the conjugated fatty acids did not correlate with the effluent dilution. The concentrations of total biliary resin acids, fatty acids and cholesterol in the bile correlated with each other positively, but these were impossible to connect to pulping activities.

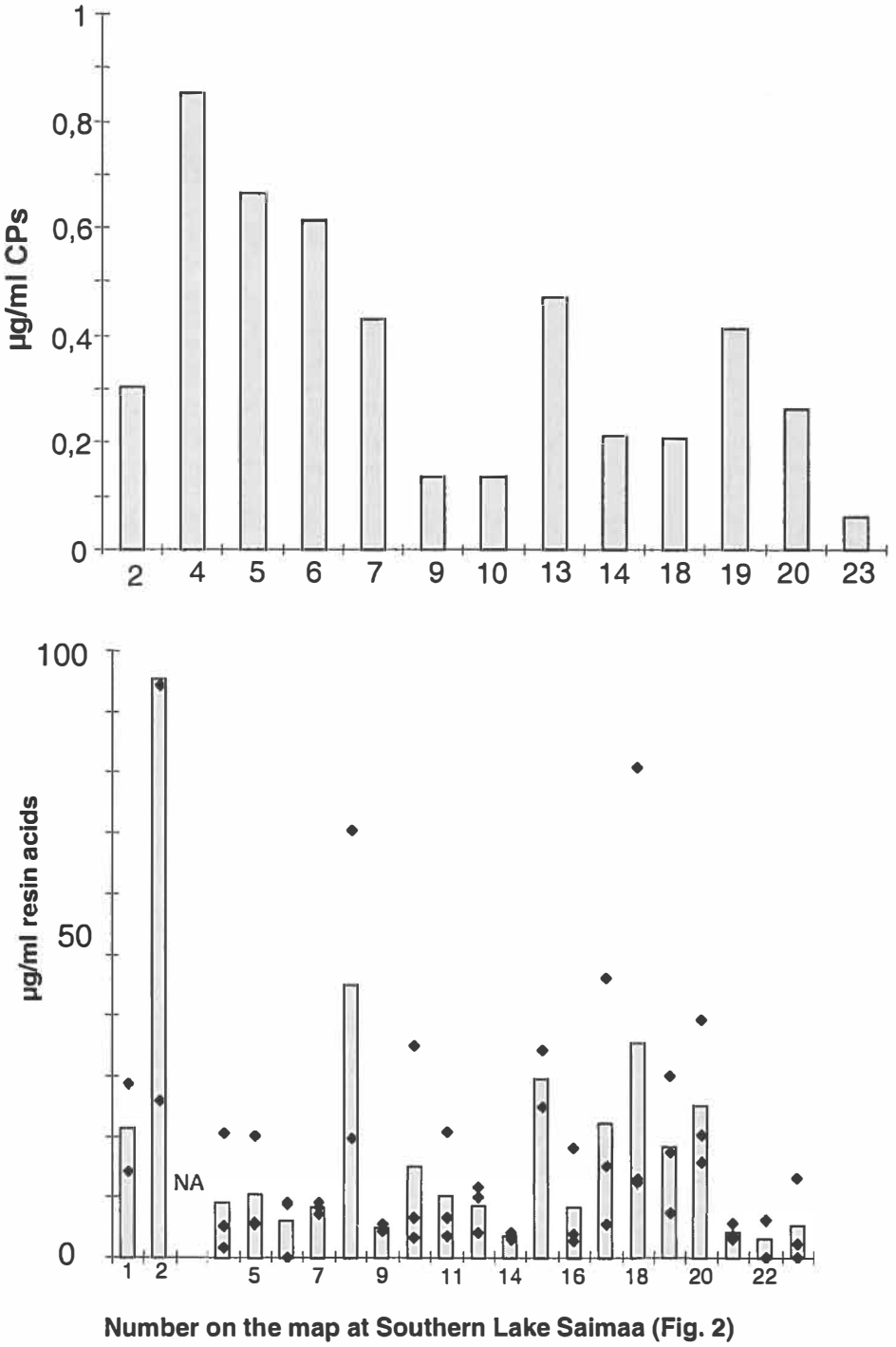

FIGURE 10 The sum concentration of chloroguaiacols, chlorocatecols and chlorovanillins (top) and resin acids (bottom) in the bile of whitefish exposed to the water quality for 30 days at Southern Lake Saimaa. The concentrations of CPs correlated with sodium concentrations used as the effluent tracer, whereas the concentrations of resin acids were impossible to connect to pulping activities.

5 CONCLUSIONS

The transition of pulp manufacturing technology from chlorine bleaching to ECF and TCF bleaching techniques has drawn more attention to wood-derived and other non-chlorinated compounds in treated discharges of pulp mills. Therefore this study has examined the occurrence and fate of resin acids and resin acid-derived compounds in aquatic environments.

High concentrations of these substances were observed in sedimenting particles collected from the BKME exposed area (highest concentration of retene 54 and resin acids 1470 μg/g d.w.) indicating rapid partitioning onto suspended material and sedimentation. As a result high concentrations of retene (up to 3300 μg/g d.w.) and resin acids (total concentration up to 1500 μg/g d.w.) were also found in the sediments contaminated by pulping effluents. The concentrations of other base neutrals (DHA, THR and fichtelite) were considerably lower. The high concentrations of retene suggested, that this, though largely in particle bound form, could be a biologically significant effector, assuming its bioavailability to biota. The bioavailability of retene in natural ecosystem was demonstrated by bile analyses of feral roach.

The biotransformation of DHAA to retene was assessed by the concentration data of the sediment cores. At one site in the vicinity of a pulp and paper mill, a significant negative correlation between DHAA and retene concentrations was detected, indicating biotransformation of the former to the latter. Besides that it was observed, that precursor - metabolite relationships are difficult to interpret from field data in a generalized manner. While validating the biotransformation of DHAA, retene was formed in an anaerobic incubation by sedimental microbial consortium at 24 °C. However, the molar changes indicated, that DHAA was also degraded or transformed to compounds other than retene. At 4 °C no changes were detectable after a year. In aerobic incubations the concentrations of both retene and DHAA decreased.

Bleaching-derived chlorinated compounds (chlorocatechols, chloroguaiacols and 6-chlorovanillin) proved to be the most suitable markers of exposure to biologically treated, ECF-BKME in the bile of experimentally exposed whitefish. On the other hand, biliary resin acids, fatty acids or cholesterol did not reflect the dilution of the effluent determined by the water Na^+ concentration in lake ecosystem.

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YHTEENVETO

Hartsihappojen ja hartsihappoperäisten yhdisteiden ympäristökohtalo kemiallisen puunjalostusteollisuuden likaamissa vesistöissä

Paperi- ja selluteollisuudessa on tapahtunut monia muutoksia 1990-luvulla kaikissa tärkeissä tuottajamaissa. Selluteollisuuden vaikutuspiirissä olevien vesistöjen kannalta keskeisimpiä uudistuksia ovat olleet happea ja klooridioksidia käyttävien valkaisutekniikoiden (ECF- eli alkuaine klooriton ja TCF- eli täysin klooriton valkaisu) sekä biologisten jätevedenpuhdistamoiden käyttöönotot. Biologisissa jätevedenpuhdistamoissa jätevedet puhdistetaan mikrobien avulla. Näiden uudistusten ansiosta monien ympäristön kannalta haitallisten yhdisteiden, kuten kloorifenoleiden pitoisuudet jätevesissä ovat laskeneet jopa 95 – 99%. Tämä on heijastunut myönteisellä tavalla selluteollisuuden alapuolisten vesistöjen elpymisenä. Ekotoksikologisia vaikutuksia voidaan kuitenkin edelleen mitata selluteollisuuden jätevesille altistuneissa eläimissä. Aikaisemmin kloorivalkaisuun liitettyjä vaikutuksia on havaittu eliöissä valkaisematontakin sellua valmistavien tehtaiden alapuolisissa vesissä. Tämä viittaa siihen, että vaikuttavat yhdisteet ovat klooratumattomia, mahdollisesti puuperäisiä yhdisteitä tai näiden muuntumistuotteita.

Tässä työssä tutkittiin puun pihkan pääkomponenttien, hartsihappojen, sekä hartsihappoperäisten neutraaliyhdisteiden, kuten reteenin, dehydroabietiinin, tetrahydroreteenin ja fichteliitin esiintymistä selluteollisuuden jätevesille altistuneissa sedimenteissä. Reteenin on laboratoriokokeissa havaittu olevan toksinen kalan alkio- ja poikasvaiheille. Kuitenkin havaitut pitoisuudet jäte- ja järvivesissä ovat alle vaikutustason. Hartsihapot puolestaan ovat akuutisti toksisimpia yhdisteitä puhdistetuissa selluteollisuuden jätevesissä. Korkeita reteeni- ja hartsihappopitoisuuksia löydettiin Etelä-Saimaan ja Lievestuoreenjärven sedimenteistä. Reteenipitoisuus Lievestuoreenjärven sedimentissä on korkein raportoitu maailmassa (3300 μg/g kuivattua sedimenttiä). Myös järvivedestä kerätyissä partikkeleissa pitoisuudet olivat korkeita.

Avainkysymys tämän jälkeen oli selvittää, onko reteeni eliöiden saatavilla sedimentistä. Saadaksemme vastauksen tähän kysymykseen analysoimme sellutehtaan alapuolisista vesistä ja tehtaan yläpuolella olevalta vertailualueelta pyydystettyjen särkien ja ahventen sappinesteitä. Reteeniä löytyi tehtaan alapuolelta pyydystettyjen särkien sappinesteestä. Sen sijaan ahvenista reteeniä ei löytynyt. Tämän perusteella voimme sanoa, että reteeni on ainakin pohjakalojen saatavilla likaantuneista sedimenteistä.

Reteenin uskotaan olevan dehydroabietiinihapon, yleisimmän likaantuneissa sedimenteissä olevan hartsihapon, biomuuntumistuote. Selvittääksemme tarkemmin reteenin syntyä ja lämpötilan vaikutusta siihen, inkuboimme dehydroabietiinihappoa ja sedimenttiä suljetuissa pulloissa hapellisissa ja hapettomissa oloissa 4 °C ja 24 °C lämpötiloissa. Korkeammassa lämpötilassa re-

teeniä syntyi lisätystä dehydroabietiinihaposta, mutta reteeniä muodostui vähemmän kuin dehydroabietiinihappoa kului. Tästä voitaneen päätellä, että dehydroabietiinihappoa myös hajosi inkuboinneissa tai dehydroabietiinihappo muuttui myös muiksi tuotteiksi kuin reteeniksi. Alhaisemmassa lämpötilassa reteeniä ei yhden vuoden mittaisen kokeen kuluessa syntynyt, mikä viittaa lämpötilan mahdolliseen merkitykseen myös luonnossa. Hapellisissa oloissa sekä reteenin että dehydroabietiinihapon määrä väheni.

Kalan sappianalyyseja on käytetty usein selluteollisuuden jätevesille altistumisen seuraamiseen. Kuitenkaan tutkimuksia, joissa sappianalyyseja olisi käytetty biologisesti puhdistetuille, kloorittomasti valkaistuille (ns. ECF-teknologia) jätevesille altistumisen seuraamiseen, ei ole paljon. Analysoimme selluteollisuuden vaikutusalueella kuukauden ajan sumputettujen planktonsiikojen sappinesteitä nähdäksemme, mikä yhdiste tai mitkä yhdisteet sappinesteessä parhaiten kuvaisivat kalojen altistumista jätevesille. Parhaan tuloksen antoivat valkaisuperäiset klooriyhdisteet alhaisista pitoisuuksista huolimatta. Hartsihapot, rasvahapot ja kolesteroli eivät osoittaneet etäisyys- ja laimennusriippuvuutta eivätkä siksi olleet hyviä altistuksen osoittajia.

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ORIGINAL PAPERS

I

Occurrence of retene and resin acids in sediments and fish bile from a lake receiving pulp and paper mill effluents

Harri Leppänen and Aimo Oikari

Environmental Toxicology and Chemistry 18: 1498 - 1505

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II

Retene and resin acid concentrations in sediment profiles of a lake recovering from exposure to pulp mill effluents

Harri Leppänen and Aimo Oikari

Journal of Paleolimnology (submitted)

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III

Concentration of retene and resin acids in sedimenting particles collected from a bleached kraft mill effluent receiving lake

Harri Leppänen, Jussi Kukkonen and Aimo Oikari

Water Research (in press)

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IV

The biotransformation of dehydroabietic acid under anaerobic and aerobic conditions at different temperatures by sedimental microbial consortium

Harri Leppänen and Aimo Oikari

Environmental Science and Technology (submitted)

The biotransformation of dehydroabietic acid under anaerobic and aerobic conditions at different temperatures by sedimental microbial consortium

Harri J.T. Leppänen* and Aimo O.J. Oikari
University of Jyväskylä, Biological and Environmental Sciences, P.O. Box 35, FIN-40351, Jyväskylä, Finland

* to whom the correspondence should be addressed

ABSTRACT

The biotransformation of dehydroabietic acid (DHAA) under anaerobic conditions at 4 °C and 24 °C, and under aerobic conditions at 24°C, was studied. The experiment was carried out by incubating sediments spiked with methanol, DHAA in methanol or retene in methanol for 52 weeks in serum bottles in dark, and comparing the concentrations of resin acids and base neutral compounds (fichtelite, dehydroabietin, tetrahydroretene and retene) to autoclaved controls. The biotransformation of DHAA to retene was observed under anaerobic incubation of samples with spiked DHAA at 24 °C. In samples with no spike the concentrations of resin acids and base neutrals decreased by 54 to 89 %, respectively. No changes in composition of base neutrals and resin acids in anaerobic incubations spiked with retene were observed at 24 °C, nor in any samples incubated under anaerobic conditions at 4°C. Under aerobic conditions significant decreases were observed in concentrations of base neutrals and resin acids (53 – 94 %), except pimaric type acids. No retene was formed under aerobic conditions.

Keywords – dehydroabietic acid, retene, biotransformation, base neutral compounds, resin acids, anaerobic, aerobic

INTRODUCTION

The hydrophobic nature of resin acids causes them to adsorb to suspended solids during aerobic effluent treatment in pulp and paper mills [1,2] as well as in receiving aquatic environments [1,3]. Because of this, elevated concentrations of resin acids can been found in sediments affected by pulp and paper mill effluents [4-6]. Concentrations up to 1600 μg/g d.w. were recently observed in sediments near mills operated over six to nine decades [6], without proper effluent treatment in the past.

Presently the information about the biodegradation of resin acids is limited. However, aerobic bacterial strains which grow on various resin acids have been isolated and characterized [7-9]. Also some fungi can transform resin acids, but none have been shown to degrade them [10-14]. The anaerobic fate of resin acids is poorly studied. Sierra-Alvarez et al. [15] indicated that resin acids were poorly removed through anaerobic wastewater treatment. In an anaerobic sediment incubation at 25 °C only a 50 % decrease in the total resin acid concentration was observed after 264 days of incubation [16]. Additionally many resin-derived compounds were formed. Sediments in areas receiving pulp and paper mill effluents are usually organically enriched and can be considered as anoxic resin acids sinks. Thus, to determine environmental fate of resin acids, it is important to know their anaerobic biotransformation. Based on the analyses of potential biotransformation products of resin acids in sediments and effluents, possible anaerobic pathways include progressive saturation, decarboxylation

and aromatization [16-19]. The anaerobic degradation pathways proposed for abietic acid are illustrated in Figure 1 [20]. However, also alternative biotransformation pathways have been proposed. Tavendale et al. [16] suggested a pathway in which decarboxylation of dehydroabietic acid to form dehydroabietin competes with an aromatisation – decarboxylation process to form tetrahydroretene. Conditions favoring the decarboxylation over decarboxylation-aromatisation are unclear. They observed that retene is not the only transformation product formed from tetrahydroretene: also a compound tentatively assigned as methyl tetrahydrophenantrene was formed. On the other hand, there were indications that retene is possibly also formed from sources other than DHAA. These sources are at present unknown. It is important to realize that some of the biotransformation products, like retene, are more lipophilic and recalcitrant than the parent compounds [19,21]. Overall, the environmental fates of these recalcitrant biotransformation products are poorly understood.

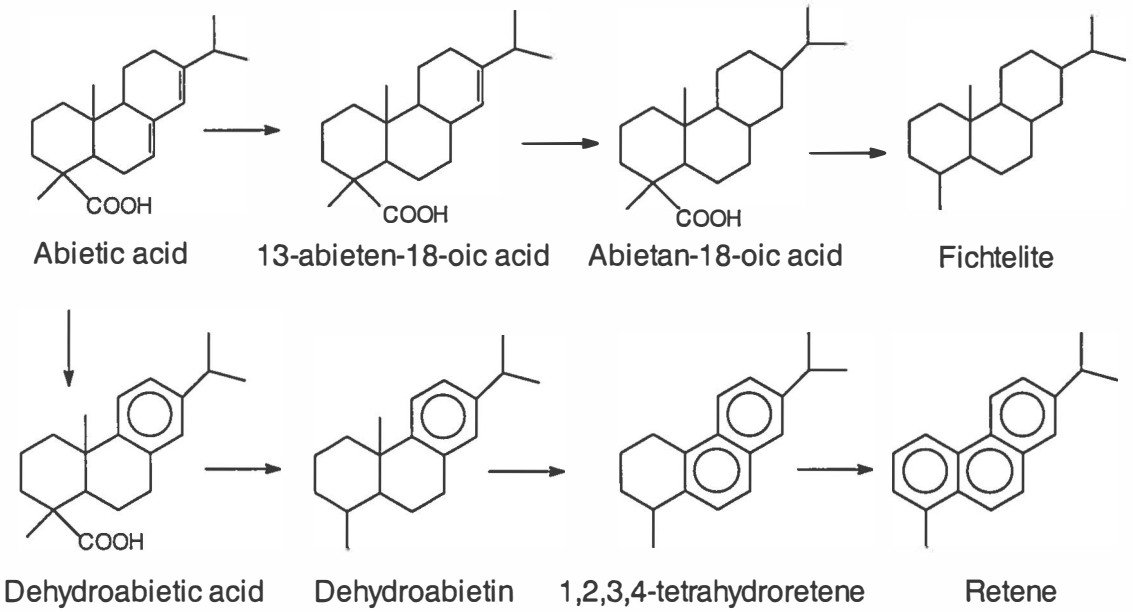

Figure 1. The biotransformation pathways proposed for abietic acid under anaerobic conditions [20].

The aim of this study was to characterize the biotransformation and degradation of resin acids and base neutral compounds (dehydroabietin, tetrahydroretene, retene and fichtelite) by sediment microbes under anaerobic conditions at 4 °C and 24 °C and under aerobic conditions at 24 °C. The temperatures were chosen to represent the natural circumstances prevailing most of the year in Nordic lake sediments (4 °C) or to accelerate the biological processes (24 °C) in order to assess the potential of changes. The assesment was conducted by incubating the spiked and unspiked materials for a year in serum bottles and comparing the concentrations and proportions of these compounds to those in killed control sediments.

MATERIALS AND METHODS

Sediment inoculum. The sediment used in the incubations was collected from an area with a long history of contamination by pulp and paper mill effluents at the Southern Lake Saimaa, SE Finland. High concentrations of resin acids and base neutral compounds were detected at this "site 3" in a recent study [6]. Three subsamples taken with an Ekman type of device were combined from the top 10 – 15 cm of surface sediment. The sample was stored in the dark for two weeks at 4 °C in a sealed glass jar prior the incubations. Because of high concentration of resin acids and base neutral compounds already present in the sample, the contaminated sediment was diluted with a reference sediment collected from an unpolluted site (Lake Palosjärvi in Central Finland, 1: 9, v/v, polluted / unpolluted). The concentrations of the pollutants of interest in the formed inoculum are listed in Table 1. Total organic carbon content of the inoculum was 127 g/kg dry weight.

Table 1. The initial amounts of the pollutants in the incubations / bottle (50 ml). The amounts are averages of 3 analysed serum bottles ± standard deviation.

	Before spiking / methanol spiked [mg / 50 ml]
Pimaric acid	0.39 ± 0.06
Sandaracopimaric acid	0.09 ± 0.01
Isopimaric acid	0.29 ± 0.05
Dehydroabietic acid	3.66 ± 0.32
Abietic acid	0.24 ± 0.03
Fichtelite	0.26 ± 0.02
Dehydroabietin	0.10 ± 0.02
Tetrahydroretene	0.09 ± 0.01
Retene	2.53 ± 0.32

Anaerobic incubations. Strict anaerobic techniques were followed in incubations throughout the study. The sediment inoculum described was added to a methanogenic mineral salts medium, supplemented with vitamins and trace elements (modified from [22]) to form a 10 % (v/v) sediment slurry. The rationale for using a vitamin-suplemented mineral salts medium was to provide

optimum conditions for microbial activity. To anaerobic incubations the following mineral salts were added to 1 liter of degassed distilled water: KCl (1.3 g), KH_2PO_4 (0.2 g), NaCl (1.17 g), NH_4Cl (0.5 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), $MgCl_2 \cdot 6H_2O$ (0.18 g), $NaHCO_3$ (2.5 g), $FeCl_2 \cdot 4H_2O$ (0.37 g), $Na_2S \cdot 9H_2O$ (0.5 g), $MgSO_4 \cdot 7 H_2O$ (3.0 g), $MnSO_4 \cdot H_2O$ (0.5 g), $FeSO_4 \cdot 7H2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.1 g), $ZnSO_4 \cdot 7H2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (0.01 g), H_3BO_4 (0.01 g), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g). Vitamins supplemented were biotin (2.0 mg), folic acid (2.0 mg), thiamine HCl (5.0 mg), riboflavin (5.0 mg), nicotinic acid (5.0 mg), calcium pantothenate (5.0 mg), p-aminobenzoic acid (5.0 mg) and thioctic acid (5.0 mg).

50 mL of slurry was transferred to 50 mL serum bottles (actual volume 59 - 60 ml), CO_2 / N_2 (30 / 70 %) added as the headspace gas, and the bottles sealed with teflon coated butyl rubber stoppers and aluminium cramp sealers. The methanogenic conditions were selected by adding an excess of carbonate [23]. The samples were spiked with DHAA or retene to the final concentrations (5.50 mg of DHAA / 50 mL of slurry and 4.65 mg of retene / 50 mL of slurry, respectively). DHAA and retene were dissolved and added in 15 µL of methanol. To one set of bottles only methanol was added. Sterile controls were prepared in the same way and autoclaved twice (120 °C, 100 kPa, 25 min.). It has been demonstrated that sterilisation of sediment by autoclaving results in an insignificant change in it´s major resin acid and derived neutrals composition [16]. Bottles were incubated statically in dark either at 4 °C or at 24 °C for 52 weeks. Cultures were mixed by shaking once a week when methane was measured. All cultures were carried out in duplicate, including the autoclaved controls.

Aerobic incubations . The sediment inoculum described was added to a mineral salts medium, excluding $Na_2S \cdot 9H_2O$ and $FeCl_2 \cdot 4H_2O$ and supplemented with vitamins and trace elements (modified from [22]) to form a 10 % (v/v) sediment slurry. A volume of 50 mL of slurry was transferred to 100 mL serum bottles and sealed loosely with teflon coated silicone stoppers and aluminum cramp sealers. Headspace was flushed with air once a week by opening the bottle. Sterile controls were prepared in the same way and autoclaved twice, but were not flushed with air. The samples were spiked with DHAA or retene in 15 µL of methanol to the final concentrations. Bottles were incubated in dark at 24 °C for 52 weeks without any mixing of solid material. All cultures were carried out in duplicate, including the autoclaved controls.

Analyses.

Methane. Methane concentration in the headspace of anaerobic incubations was measured weekly with Perkin Elmer Autosystem XL gas chromatograph equipped with flame ionization detector (FID). Column used was PE-Alumina, leghth 30 m, inner diameter 0.53 mm. The sample from a serum bottle was obtained through the stopper with an gas-tight syringe.

The analyses of resin acids and base neutral compounds. The well-settled aqueous phase from the incubation bottle was transferred to an extraction vessel without further centrifugation. The internal standards (heptadecanoic acid and androstane) were added and the pH adjusted to 9.5 with 1 M KOH. Aquous phase was extracted twice with 20 mL of dichloromethane by shaking for 30 minutes in a mechanical horizontal shaker in a separatory funnel. The combined extracts were evaporated to 5 mL in a rotary evaporator and divided into two Kimax tubes. The extracts were further evaporated to dryness under gentle stream of nitrogen and redissolved with hexane and MTBE for base neutrals and resin acid analyses, respectively. 80 μL of N,O-bis(trimethylsilyl)-trifluoroacetamid with 1% was added to the Kimax tube containing the MTBE fraction and the sample was let to silylate in 70 °C for 30 minutes.

The sediment slurry remaining in the incubation bottle was dried with anhydrous sodium sulphate. 40 mL of dichloromethane and the internal standards (heptadecanoic acid and androstane) were added to the bottle and the bottle was resealed with teflon coated butyl stopper. The sample was extracted in a mechanical horizontal shaker overnight. The extract was transferred to a roundbottom flask through a prewashed cotton wool plug and evaporated to 5 mL. The sample was divided into two Kimax tubes and evaporated to dryness under gentle stream of nitrogen. The remains were redissolved with hexane or methyl *tert*-butyl ether (MTBE) for base neutrals and resin acid analyses, respectively. 150 μL of N,O-bis(trimethylsilyl)trifluoroacetamid with 1% trimethylchlorosilane was added to the Kimax tube containing the MTBE fraction and the sample let to silylate in 70 °C for 30 minutes. The amount of the compounds in the aquous phase and in the dried slurry are reported jointly as the total amount of the compounds per bottle (50 mL). The amounts of retene and DHAA in duplicate control cultures varied 5 – 13 % and in active cultures 5 – 20 %. Because of the low concentrations the amounts of other resin acids and base neutrals varied 5 – 30 % in active and control cultures.

All analyses were carried out with a HP 6890 GC equipped with a HP 5973 MS detector. Column used was HP-5 (25 m, 0.2 mm i.d., 0.33 μm phase thickness); analyses were run in scan mode (mass range m/e 35 - 600, scan rate 1 scan/s). Dehydroabietin, tetrahydroretene and fichtelite were quantitated using response factor 1:1 to retene. The changes in the amounts are calculated by comparing the average amount of the compound in the duplicate active incubations to the average amount of the compound in the corresponding control incubations. Comparison of recoveries using the method described with a method described previously [6] are presented in Table 2, indicating good accordance.

Table 2. Comparison of sediment material analysis method described in this paper (shaker) with a method described earlier (Soxhlet, [6]) ± the range of 2 analyses).

	Shaker [µg/g d.w.]	Soxhtlet [µg/g d.w.]
Pimaric acid	55 ± 0.0	61 ± 2.0
Sandaracopimaric acid	9.5 ± 0.5	11 ± 0.0
Isopimaric acid	35 ± 1	37 ± 2.0
Dehydroabietic acid	202 ± 2	231 ± 10
Abietic acid	57 ± 0.5	61 ± 2.0
Fichtelite	24 ± 0.0	34 ± 9.0
Dehydroabietin	8 ± 0.0	9.0 ± 1.0
Tetrahydroretene	5.5 ± 0.0	7.0 ± 2.0
Retene	518 ± 13	468 ± 19

RESULTS AND DISCUSSION

Methane production in anaerobic incubations

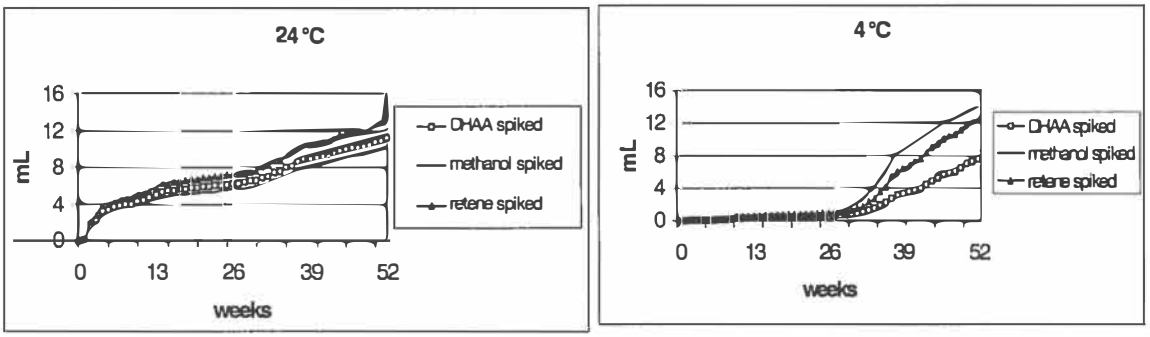

Figure 2. Cumulative methane production in anaerobic incubations at 24 °C and 4 °C. Each symbol presents the mean methane production of two identical incubations. No methane production was observed in the autoclaved controls.

There was a distinct difference in the dynamics of the anaerobic activity, measured as methane production, at two temperatures (Fig. 2). The methane production in incubations at 24 °C started within a week after the start of the incubation. About half of the total methane production occurred during the first 16 weeks. In this period the microbes apparently utilized most of the easily available organic carbon and subseqently the methane production rate decreased. The final volume of methane produced was 10.1 – 13.0 ml. Since the amount of methanol added (15 µl) can theoretically produce 6.9 ml of methane it can be suggested that degradation of sedimentary organic material had additionally occurred. At 4 °C instead, the methane production started after 8 weeks from the sealing of the bottles and remained low for the first 30 weeks. After this there was a rapid increase in the methane production rate actually approaching the

early maximum rate at 24 °C. Eventually the final volume of methane produced was about similar in samples spiked with methanol (14.0 ml) and retene (12.4 ml) than in incubations at 24 °C. At 24 °C the retene spiked samples produced methane the most, whereas the methanol spiked samples produced the least. However, the difference in final volume of methane produced was not substantial between the methanol and retene spiked samples (22%) and the degradation data obtained (Fig. 3 and 4b) implies that the contributory methane was not derived from degradation of retene. On the other hand, during the 52 weeks at 4 °C the DHAA spiked samples produced only 54 % of the methane that the methanol spiked samples produced. The lower methane production rate suggests, that at the low temperature the DHAA concentration used (ca. 110 μg/ml) had inhibited methanogenesis.

Changes in resin acid and base neutral composition in anaerobic incubations

In anaerobic incubations with no spike other than methanol, the concentrations of all the compounds of interest decreased (Fig. 3) in respect to the autoclaved controls. The average decrease in the amount of retene in parallel incubations was 10 micromoles (65 %), dehydroabietin 0.4 micromoles (74 %), tetrahydroretene 0.4 micromoles (54 %), fichtelite micromoles (63 %), DHAA 11 micromoles (79 %), pimaric acid 2.6 micromoles (89 %), sandaracopimaric acid 0.8 micromoles (82 %) and isopimaric acid 1.8 micromoles (86 %) per 50 mL experimental unit. This indicates that the hydrophobic compounds like retene, DHAA and pimaric type acids, though possibly being more tightly bound to the sediment with aging [24] than recently introduced spiked compounds, are degraded or transformed under anaerobic methanogenic conditions. This is also consistent with an earlier report [16]. Little information is available about the anaerobic biodegradation of retene. However, several research groups have reported anaerobic biodegradation of PAHs with similar structure, especially phenanthrene. Rockne & Strand [25] observed a complete removal of phenanthrene by a nitrate-reducing fluidized bed reactor enrichment of bacteria from creosote-contaminated marine sediment within 14 days. Even faster biodegradation rates were observed with organisms in pure cultures under denitrifying conditions which degraded phenanthrene in 12 - 44 h, the rate depending on the history of contaminant exposure of the microbial community [26]. However, in sediment incubations the biodegradation rates have been considerably slower. Under sulfate-reducing conditions the total removal of phenanthrene has been observed within 30 - 150 days, the rate again depending on the pollution history of the sediment [27-29]. It has been suggested that carboxylation is an initial reaction in the anaerobic metabolism of phenanthrene [29]. Aromatic compounds have also been reported to be degraded under methanogenic conditions [30].

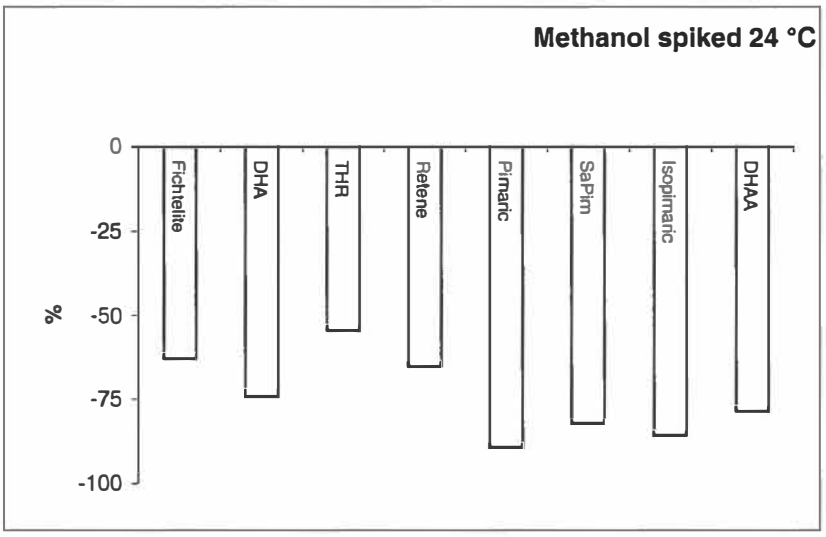

Figure 3. Percentage net changes in anaerobic incubations at 24 °C spiked only with methanol compared to the autoclaved controls. Abietic acid is not presented, because it´s transformation is principally abiotic. DHA = dehydroabietin, THR = tetrahydroretene, SaPim = sandaracopimaric acid, DHAA = dehydroabietic acid. The changes are calculated by comparing the averages of two parallel incubations to averages of two parallel incubated controls.

The most notable change in resin acid and base neutral composition in anaerobic incubation with increased, i.e. spiked, concentration of DHAA (Table 1, Fig. 4a) at 24 °C was the decrease in DHAA content per experimental unit. After 52 weeks the average amount of DHAA in active cultures was 35 % of that in autoclaved controls. At the same time the retene content in active cultures in respect to autoclaved controls increased 45 % (Fig. 4). Given on the molar basis, it means that the amount of DHAA decreased by 17 micromoles per 50 mL experimental unit and the amount of retene increased by 4.5 micromoles indicating no full conversion from the first to the second. There were also increases in concentrations of other base neutral compounds: dehydroabietin by 280% and tetrahydroretene by 1 800% representing, however, only 1.0 and 3.0 micromoles, respectively. The concentration of fichtelite was practically the same in active and control incubations (increase 10%), similar to and consistent with earlier reports [16]. When corresponding molar changes are also included to the mass balance the sum increase of base neutrals is only 8.5 micromoles / 50 ml in contrast to 17 micromoles of DHAA removed.This indicates that DHAA was also transformed to other products or degraded, or retene was further transformed or degraded during the incubation. Tavendale et al. [16,19] observed that after 120 days of incubation under anaerobic conditions at 25 °C the concentrations of base neutrals (except fichtelite) and resin acids in sediments started to decrease, indicating that biodegradation had occurred.

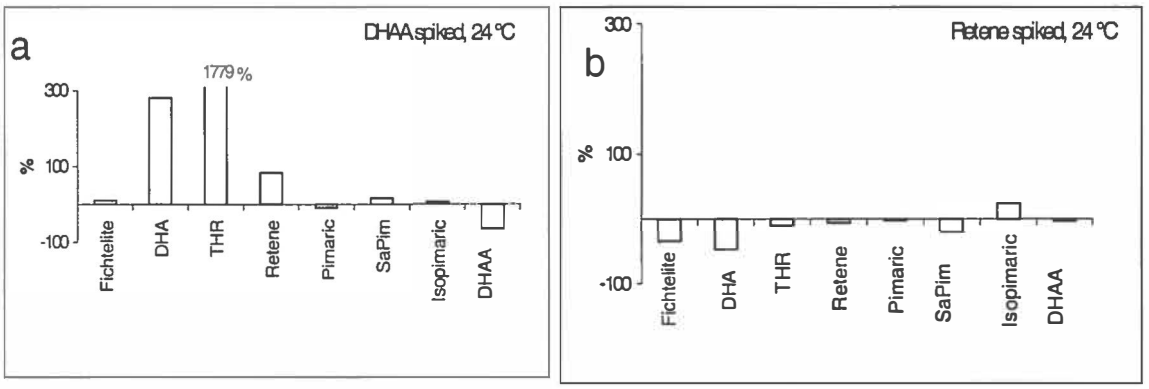

Figure 4. Percentage net changes in anaerobic incubations at 24 °C spiked with DHAA (a) or retene (b) compared to the autoclaved controls. Note that the scaling is different and the THR column in the figure representing the DHAA spiked incubations is cut. For the abbreviations, see Figure 3. Abietic acid is not presented, because it´s transformation is principally abiotic. The changes are calculated by comparing the averages of two parallel incubations to averages of two parallel incubated controls.

The concentrations of pimaric type resin acids (pimaric, sandaracopimaric and isopimaric acids) were virtually the same in autoclaved controls and in active DHAA spiked cultures after 52 weeks, indicating the more recalcitrant nature of pimaric type resin acids compared to abietic type acids [16]. Surprisingly, there was no decrease in the concentration of abietic acid, on the contrary, in all incubations it´s concentration remained constant or slightly increased. Most likely, however, the concentration of abietic acid did not truly increase in the incubations, but instead decreased in the sterile controls. This indicates that under anaerobic conditions the transformation of abietic acid was principally abiotic [16,31,32].

In anaerobic incubation with high concentration of retene (Table 1, Fig. 4b) at 24 °C the only change in composition of base neutrals was the decreases in the amount of fichtelite (34 %). In micromoles the decrease was 3.0. There was also a decrease in the amount of dehydroabietin (46 %), but in micromoles this decrease was only 0.3. However, based on methane production, there was no inhibition of methanogenic activity due to high concentration of retene (Fig.2). Of resin acids the amount of isopimaric acid increased 23 % and the amount of sandaracopimaric acid decreased 20 %. Though, given in micromoles the decrease in the amount of sandaracopimaric acid was only 0.03 and the increase in the amount of isopimaric acid 0.3 micromoles per 50 mL experimental unit.

Importantly however, in anaerobic incubations at 4 °C no changes in concentrations of resin acids or base neutrals were observed in DHAA, retene or methanol spiked samples. This demonstrates the paramount influence of temperature in the rate of biotransformation of DHAA and the formation of retene. This

might also support our suggestion in a recent study, that the varations in water temperature from one lake area to another could explain the observed differences in the concentration of retene in sediments receiving bleached kraft mill effluents [6]. Since the fast production of methane in samples incubated at 4 °C had occurred (Fig. 2), and no changes in composition of base neutrals and resin acids was detected, it can be suggested, that the biotransformation of DHAA under methanogenic conditions occurs mainly after the easily degradable organic matter has been utilized by an anaerobic consortium [16,19]. This also indicates, that the biotransformation of DHAA in anaerobic subsurface sediments of cold Nordic lakes is slow and most of it may occur during summer as water temperature increases. Therefore the presence of DHAA biotransformation products extend their presence far to the future.

Changes in resin acid and base neutral composition in aerobic incubations

Aerobic metabolization was studied at 24 °C only. There was a clear decrease in concentrations of DHAA and retene both in DHAA and in retene spiked experiments when compared to sterile controls. In DHAA spiked samples the decreases per experimental unit were as follows: DHAA 14 micromoles (78 %), retene 15 micromoles (94 %), fichtelite 0.8 micromoles (70 %), dehydroabietin 0.06 micromoles (74 %) and tetrahydroretene 0.09 micromoles (53 %). The concentrations of pimaric type resin acids remained rather constant (± 20 %). Similar results were also obtained from retene spiked incubations: the decrease in the amount of DHAA was 7.6 micromoles (68 %) and that of retene 15 micromoles (93 %). Of the other base neutrals only the amount of tetrahydroretene decreased by 0.5 micromoles (66 %). Fichtelite and dehydroabietin showed no reduction. Again the pimaric type resin acids showed no reduction. As expected no net formation of retene was noted under aerobic conditions [5,19] The more recalcitrant nature of pimaric type acids than abietic type acids under aerobic conditions is in accordance with previous reports [9,33]. The degradation of retene is consistant with reports of degradation of other PAHs, especially phenanthrene, under aerobic conditions [26,34].

The results obtained in aerobic and anaerobic spiking experiments of DHAA and retene with microbial consortium, adapted to sediments contaminated by bleached kraft mill effluents (BKME) are summarized in Table 3.

Acknowledgements – The funding for this study was provided by ESAITOX-project supported by Finnish pulp and paper industry consortium and the Academy of Finland Research Council for the Environment and Natural Resources.

Table 3. Summary of observed changes in concentrations of resin acids and base neutral compounds in respect to autoclaved controls after 52 week incubation at 24 °C. ++ = clear increase in concentration (>60 %); + slight increase in concentration (20 – 40 %) – – = clear decrease in concentration; – decrease in concentration; ± = no clear effect on concentration (± 20 %).

	Anaerobic			Aerobic	
	DHAA Spiked	Retene Spiked	Methanol Spiked	DHAA Spiked	Retene Spiked
Fichtelite	±	±	– –	– –	±
Dehydroabietin	++	±	– –	– –	±
Tetrahydroretene	++	±	–	–	– –
Retene	++	±	– –	– –	– –
Pimaric acid	±	±	– –	±	–
Sandaracopimaric acid	±	–	– –	±	±
Isopimaric acid	±	±	– –	±	–
Dehydroabietic acid	– –	±	– –	– –	– –
Abietic acid[1]	–	–	–	–	–

1) decrease in concentration in both autoclaved controls and active incubations, indicating abiotic transformation.

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V

The use of fish bile metabolite analyses as exposure biomarkers to pulp and paper mill effluents

Harri Leppänen, Sanna Marttinen and Aimo Oikari

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VI

Chemical markers of exposure to pulp and paper mill effluents in aquatic animals - a review

Harri Leppänen and Aimo Oikari

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Chemical markers of exposure to pulp and paper mill effluents in aquatic animals - a review

Harri Leppänen* and Aimo Oikari

University of Jyväskylä, Dept. of Biological and Environmental Sciences
P.O.Box 35, FIN-40354 Jyväskylä, Finland

* to whom the correspondence should be addressed

ABSTRACT

Despite some significant process and waste water treatment changes employed by the pulp and paper industry in 1990ies, residual ecotoxicological effects still linger on in the effluent receiving aquatic environment. To connect these effects with the exposure to pulp and paper mill effluents, exposure biomarkers are needed. Due to historical reasons chlorinated compounds have been the most studied chemicals as exposure markers. However, the employment of low-chlorine and elemental chlorine free bleaching techniques have made the use of such chlorinated markers as chlorophenolics more difficult. Another well-studied group for markers are resin acids. Modern secondary waste water treatment systems have reduced the concentrations of resin acids in the effluents remarkably, making the use of these compounds as markers difficult many times. In all, in light of the literature reviewed, new candidates for markers are thus needed. In this article the exposure markers are divided into chlorinated and non-chlorinated tissue residues and metabolites, always when possible indicating the bleaching sequence used by the pulp mill in question. Biochemical markers are briefly reviewed. By comparing exposure marker results from a study conducted in the field using experimentally exposed fish, with data from a laboratory exposure with the same fish species and effluent dilutions, it seems that laboratory exposures can simulate true pollution sufficiently.

Keywords: Pulp, Paper, Chemical marker, Tissue residues, Metabolites

INTRODUCTION

Pulp and paper industry in all major producing countries in 1990ies is largely different than earlier. The most significant changes in the industry aimed to improve the well-being of aquatic life in waters receiving effluents have been the employment of elemental chlorine-free bleaching techniques and biological wastewater treatment systems. Still residual effects linger on. For example, effects that formerly were believed only to be connected to bleached pulp mill effluents, are also observed in receiving waters of unbleached pulp mills [1]. It is also highly possible that unidentified biotransformation products of substances present in untreated effluents are produced and released during the biological wastewater treatment [2].

Pulp and paper mill wastewaters are complicated mixtures of hundreds of chemicals. In a recent survey [3] 323 different chemicals were identified from pine pulp TCF and ECF wastewater samples including ethers, ethylbenzene, saturated aliphatic and alicyclic hydrocarbons, sulphur compounds, ketons, unsaturated aliphatic and alicyclic hydrocarbons, alcohols, aldehydes, terpenoic alcohols, phenols, amids, phtalates, polyaromatic derivatives, phosphates, nitrates, stilbene derivatives, resin acids, phenantrene derivatives, esters,

aromatic acids, amins and aliphatic carboxylacids. In TCF effluents the aliphatic carboxyl acids (including fatty acids) were most abundant compounds, followed by saturated and unsaturated alicyclic and aliphatic hydrocarbons and resin acids. Aliphatic carboxyl acids, saturated and unsaturated alicyclic and aliphatic hydrocarbons, and phtalates were most abundant in ECF wastewaters. When ECF waste waters were compared to those derived with use of chlorine, given as AOX, up to 90 % decrease was observed [4]. However, chlorophenols, chloroquaiacols and chlorocatechols are still present in effluents in concentrations up to few micrograms per litre [5].

Because of the chemical complexity of pulp and paper mill effluents it is necessary to try to specify general characteristics of compounds of potential ecotoxicological significance. To evaluate environmental hazard caused by an individual compound, although only a part of complex mixture, data about acute and chronic toxicity as well as on the characteristics determining their environmental fate, i.e. broadly including bioaccumulation and persistance of the substance, is needed. Also the concentration of the substance in effluent and in the environment is important.

OECD guidelines for testing of chemicals [6] lists 16 physical and chemical properties necessary to know, when evaluating environmental fate and risk that a substance is posing for the environment. These include UV-VIS absorption spectra, melting point, boiling point, vapour pressure curve, water solubility, adsorption/desorption, K_{ow}, complex formation ability in water, density of liquids and solids, particle size distribution (or fibre length and diameter distribution), hydrolyzation as a function of pH, dissociation constants in water, screening test for thermal stability and stability in air, viscosity of liquids, surface tension of aqueus solutions and fat solubility of solid and liquid substances. In addition the chemical´s effects on biotic systems (growth tests on algae, reproduction test on daphnia, and acute toxicity tests on daphnia and fish), degradation and accumulation (ready and inherent biodegradability, biodegradability in soil, aerobic sewage treatment simulation, and bioaccumulation in fish under various test conditions) and health effects (various acute, subchronic and long term toxicity tests including carcinogenity studies) should be investigated [6]. The K_{ow} -value can succesfully be used to predict the bioconcentration potential of a compound [7,8]. Geyer et al. [9] revealed that the bioconcentration of organochlorines in the adipose tissues of human correlated closely to K_{ow} -values. Könemann [10] also established a positive relationship between toxicity to fish and the K_{ow} - value for a range of environmental pollutants.

Chemicals that are ephemeral in the environment are less likely to have adverse effects than persistant chemicals. Measured or predicted environmental half-lifes of chemicals, or rate constants for removal processes, are used as a measure of persistance. Other things that must be taken into account are the environmental properties of the possible persistent biotransformation products of the compound and the environmental mobility of the substance [11].

Chemical compounds of interest in effluent and aquatic environment

Chlorine bleached BKME

The use of elemental chlorine and other active chlorine containing bleaching agents, such as chlorine dioxide in bleaching of chemical pulp, leads to the formation of complex mixture of chlorinated organic constituents belonging to various compound classes. These are primarily formed as a result of chemical reactions between chlorine or chlorine containing bleaching agent and the lignin remaining in the pulp. The environmental fate of organic halogen compounds discharged from pulp bleaching is poorly understood. Some of the low molecular weight compounds such as chlorophenolics are known to bioaccumulate in aquatic organisms [12] but these compounds represent only a few percent of the AOX discharged [13]. A substantial portion (85 - 95 %) of AOX has a molecular weight smaller than 1, 000 Da with the peak in the molecular weight distribution in the region of 200 - 700 Da [14]. This high mol. wt. AOX has remained only partially characterized ([15] and the literature cited therein). Major part of the low molecular weight fraction has been identified as chlorinated phenolic compounds. It has been suggested that chlorinated phenolics in large part, especially chlorocatecols and -guaiacols are bound to dissolved high molecular weight matter before they enter the recipient water course [16,17]. Other identified groups of chlorocompounds in chlorine bleached effluents include chloroform [18], chlorocymenes and -cymenenes [19] and chlorinated acids [20]. It has been repeatedly documented, that the introduction of ECF-bleaching reduces the degree of chlorination of chlorophenolic compounds detected in effluents, so that mono- and dichlorinated compounds are more dominating [4]. The low-degree of chlorination generally reduces the persistence and toxicity of compounds as well as their potential for bioaccumulation. Overall, in most countries producing pulp and paper, the use of chlorine based technology ceased till early 1990ies but, on the other hand, the AOX and EOCl load still remains in sediments [21].

Elemental chlorine free (ECF) and total chlorine free (TCF) BKME

Despite the substitution of chlorine with chlorine dioxide and oxygen as the bleaching agents, a small amount of chlorinated compounds are formed and released to the aquatic environment in the pulping process. However, probably the most interesting and most studied group of compounds in ECF and TCF effluents are non-chlorinated wood extractives. Wood extractives are nonstructural wood constituents soluble in neutral organic solvents or water. They comprise a large number of compounds with both lipophilic and hydrophilic nature. In wood they derive biochemically via many intermediate steps from glucose, formed originally in photosynthesis. Wood extractives can be grouped to aliphatic compounds, terpenoids and phenolic compounds. Aliphatic wood extractives include n-alkanes, fatty alcohols, fatty acids, fats, waxes and polyestolids (ω-hydroxy acid esters of dicarboxylic acids). Terpenoids are polymerization products of 2-methyl butadiene, and can be grou-

ped to mono-, seskvi-, di- and triterpenes. Resin acids are diterpene derivatives, whereas sterols are triterpenes. Stilbenes, lignans and flavonoids are members of phenolic compounds [22].

The structures of some wood extractives are presented in Fig. 1. Resin acids are very abundant in canal resin of coniferous trees (50 % of canal resin of spruce consist of resin acids [22]). Today, in Nordic mills typical concentrations of resin acids in biologically treated pulp and paper mill effluents are 40 - 150 µg/L [5,23]. Traditionally resin acids were the most acutely toxic compounds of chlorinated BKME to aquatic life [24], but no similar systematic mapping has been conducted with mills of 1990ies. The 96-h LC_{50} values of common resin acids to salmonid fishes are approximately 1 mg/L, but vary to some extent (0.3 - 1.7 mg/L) [25]. The most abundant resin acid, dehydroabietic acid (DHAA) has been shown to produce sublethal effects at concentrations down to about 5 to 20 µg/L [26,27]. Physiological effects of DHAA and other resin acids have been found to include inhibition of a conjugation enzyme UDP-glucuronosyltransferase [27,28], inhibition of bile acid uptake and perturbation of the potassium transport, tested with isolated rainbow trout hepatocytes [29].

Other wood extractives commonly found in biologically treated pulp mill effluents include fatty acids, phytosterols, stilbenes, flavonoids and lignans. Of these, fatty acids are very abundant in BKME (200 - 500 µg/l) whereas the concentration of sterols is lower (50 - 200 µg/l) [5,23]. Stilbenes and flavonoids are present at smaller amounts [30].

Additives used in the paper making

In 1992 the Finnish pulp and paper industry used over 900 different chemicals and additives, which comprised of about 400 effective compounds. These compounds belong to various chemical classes, including chlorinated and non-chlorinated solvents, acids, biocides, slimicides, chelating agents (i.e. EDTA, DTPA), dyes, resins, glues, clarifiers, tensides and dispergents. The total amount used was 4.3×10^9 kg. These chemicals were used in all stages of the paper making process [31]. It was estimated that of the total amount of chemicals used 75% ends up into the final product, 20% into the process waters, 1% into the air and 4% into the solid waste. In 1992 the pulp and paper industry in Finland released about 1500 tons of iron, 21 tons of copper, 7 tons of chromium, 15 tons of nickel, 9 tons of lead and 100 tons of zinc originating from various sources including chemicals, piping, wood and process equipment. However, the concentrations of heavy metals in effluents were typically below the accepted levels for drinking water [31].

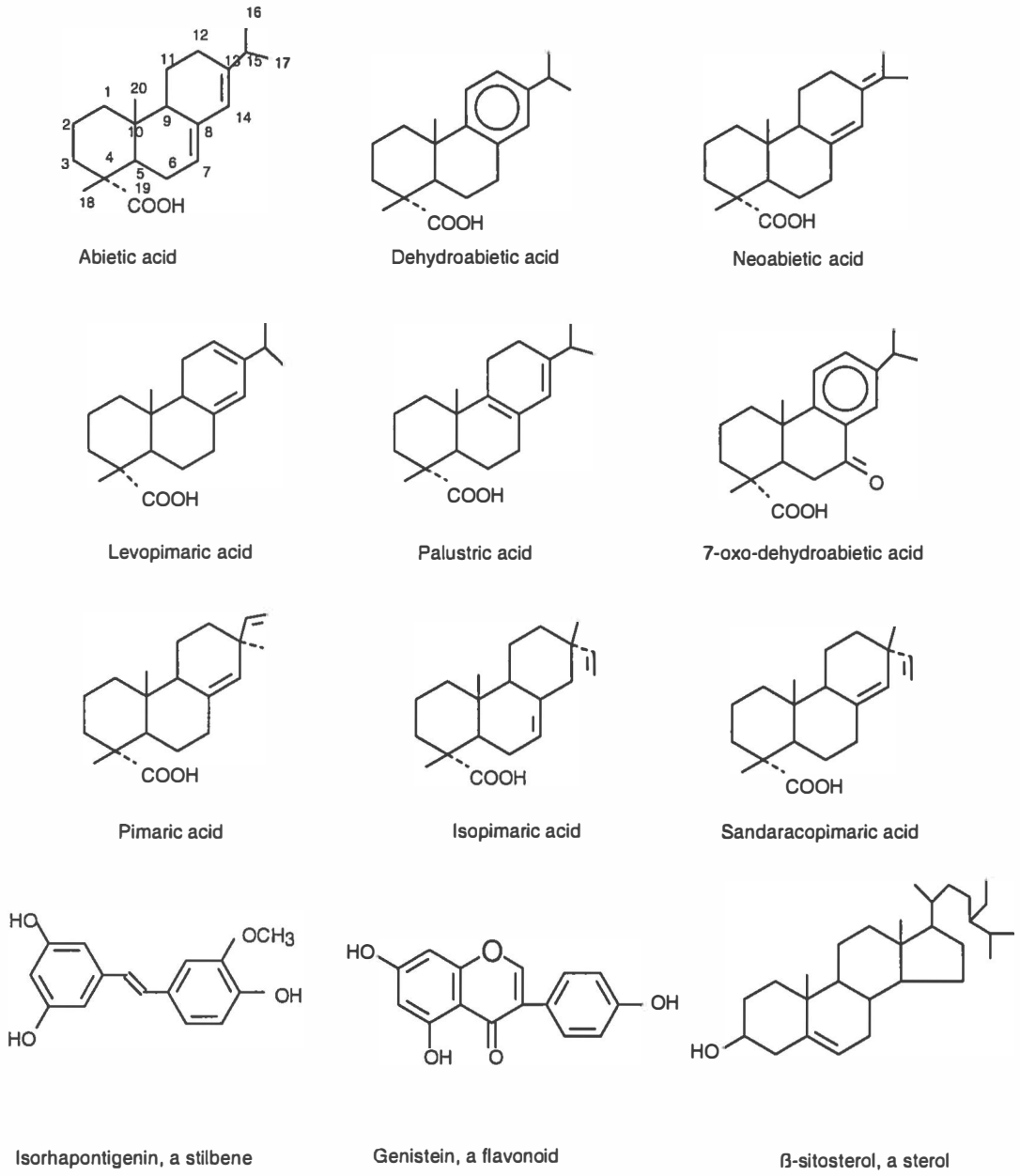

Figure 1. Structures of some resin acids and other wood derived compounds

Seven of the chemicals used in pulp and paper industry are categorized as dangerous for the environment in Finland. These are 1,1,1-trichloroethane, 2-bromo-2-nitropropan-1,3-diol, carbon tetrachloride, glutaraldehyde, chloro-dioxide, hexane and hydrokinone [31].

There are only few studies about the environmental occurrence and fate of the additives used in the pulp and paper industry. Koistinen et al. [32] identified alkylated dibenzothiophenes in pulp mill effluents, biosludge and in defoamer used in a mill. Mikkelson et al. [33] identified the same compounds in the grease of eulachon (*Thaleichthys pacificus*) exposed to effluents of unbleached

pulp mill effluents. It has also been shown, that some defoamers made from oils with high aromatic content may act as a source for precursors of dibenzo-p-dioxins and dibenzofurans, which in bleaching stages with chlorine or chlorine dioxide may become converted to highly toxic polychlorinated congeners [34,35].

TISSUE RESIDUES

The residues of pulping related compounds present in animal tissues have been used as chemical markers of exposure to BKME. The animals used in these studies include fish, crab, mussels and insects. Tissues that has been used to monitor exposure to pulp and paper mill effluents include muscle, liver, intestinal fat tissue, crab hepatopancreas, gut, gills, brain and blood plasma. Component and compounds used as tracers include organically bound chlorine (EOCl or EOX), chlorinated phenolics, chlorinated dioxins and furans, resin acids, resin acid-derived neutrals, fatty acids, chlorinated cymenes and cymenenes, alkyl polychloronaphthalenes, -bibenzyls and -phenantrenes.

Chlorinated markers

Residues of bleaching-derived chlorinated compounds in tissues of biota have frequently been used as markers of exposure to pulp mill effluents. Compounds most frequently measured include polychlorinated phenols, -catechols and -quaiacols. There are 43 mono- and polychlorinated structures possible of these all, including 19 phenols, 15 quaiacols and 9 catechols [36]. The benefits of these compounds are low detection limit, when using gas chromatography equipped with electron capture (EC) detection, relatively high solubility in water of some of these compounds and the specificity to bleaching of compounds like 3,4,5 -trichlorocatechol and 3,4,5 - and 4,5,6-trichloroguaiacols. However, the employment of low-chlorine and chlorine free bleaching techniques have dramatically reduced the amounts of these compounds in pulp mill effluents, making the application of these compounds as markers much more difficult. On the other hand, in ECF-BKME chlorophenols, chloroquaiacols, chlorocatechols and chlorovanillins are still present in effluents in concentrations up to few micrograms per litre [5,23]. There are also some new candidates for markers, like 6-monochlorovanillin [23,37]. The sum parameter EOCl in fish fillets was utilized in Sweden to measure exposure to chlorinated compounds [38,39]. Overall, however, fish in remote areas without industrial sources had substantial background levels of fillet EOCl limiting the usefulness of EOCl in low exposure situations [39,40].

Mäkelä et al. [41] studied, along with biological endpoints, the suitability of the freshwater mussel *Anodonta anatina* for long term field incubations in BKME polluted river by measuring extractable organic chloride (EOCl), ext-

ractable organic halides (EOX) and chlorophenolic concentrations in soft tissues of the animals. At the time of the study, the given pulp mill used chlorine bleaching and the wastewater treatment consisted of mechanical treatment and anaerobic and aerated lagoons. They concluded, that EOCl and EOX in tissues does not seem to be a good indicator of pulp mill effluents. Furthermore, the only chlorinated phenol, found in the digestive gland of the animal was 2,3,4,6-tetrachlorophenol, a component released mainly in wood preservation and combustion rather than pulp bleaching [42]. On the other hand Soimasuo et al. [43] measured significantly higher amount of EOX in gut lipids of whitefish (*Coregonus lavaretus*) caged downstream of a pulp mill using chlorine bleaching than on the reference site. Also the concentration of chlorophenolics in gut lipid was significantly higher in fish caged downstream of the mill. The most abundant chlorophenolics were 3,4,5- and 4,5,6-trichloroguaiacols and tetrachloroguaiacol, all of which are understood to have their origin in chlorine bleaching, although the possibility that tetrachloroguaiacol may be naturally produced has been raised [44]. In a laboratory exposure simulating lake pollution [45] the chlorophenolics´ residues in the fat of whitefish were significantly higher even in the most dilute effluent concentration (1.3 vol%) compared to controls. In contrast, the concentration of EOX accumulated into the intestinal fat tissue was not significantly higher in BKME exposed fish than in control animals. Very high concentrations of EOCl (up to 1200 µg/g) have been reported in lipid of eel (*Anguilla anguilla*) in a recipient of a sulfite mill [46], while lower concentrations were detected in lipid of perch caught from coastal waters near a chlorine bleached pulp mill [38] as well as eels and flounders near a kraft mill [46].

In Finland, Herve and co-workers used the lake mussel (*Anodonta piscinalis*) for monitoring the organochlorine compounds in two areas receiving pulp and paper mill effluents, a lake and a river [47-49]. In the beginning the monitoring program (1984) the mills used elemental chlorine for bleaching and operated without biological wastewater treatment, whereas at the end of the program (1992) chlorine was substituted with chlorine dioxide in bleaching and an activated sludge wastewater treatment system was used. In data processing the chlorinated compounds were divided in two groups: the ones originating from pulp and paper industry (2,4- and 2,6-dichlorophenols, 2,4,5-trichlorophenol, 3,4- dichlorocatechol, 3,4,5-trichlorocatechol, tetrachlorocatechol, 4,5-dichloroguaiacol, 3,4,5- and 4,5,6-trichloroguaiacols, tetrachloroguaiacol and trichloro-2,6-dimethoxyphenol), and those from wood preservation, combustion and pesticide use (2,4,6-trichlorophenol, tetrachlorophenol and pentachlorophenol). In 1984 the concentration of bleaching related chlorinated compounds in mussels incubated for four weeks was as high as 10 µg/g lw (lipid weight). The biological treatment of wastewaters decreased the concentrations of these compounds in mussels significantly, to a level of 1 - 3 µg/g lw. Further, the introduction of ECF bleaching reduced the concentrations in most cases below 1 µg/g lw i.e. by an order of magnitude. On the other hand, at the same time, other organochlorine compounds from wood preservation, combustion and pesticide use did not show this clear trend. Still,

and overall, there appeared to be a fair correlation between the bleaching originated chlorinated compounds in exposed mussels and the dilution based organic load values at the sampling stations. Tetrachloroguaiacol appeared to be one of the dominant bioaccumulating components, as was concluded erlier also by Paasivirta et al. [42] and Suntio et al. [15].

In order to study the bioaccumulation of chlorobleaching originating aromatic chlorohydrocarbons (polychlorocymenes, polychlorocymenenes, alkylpolychloronaphthalenes, alkylpolychlorobibenzyls and alkylpolychlorophenanthrenes) in pulp mill recipients, Rantio et al. [50] used the same mussel species and pike (*Esox lucius*). The concentrations of these compounds in discharges of chlorine bleached pulp are relatively low (0.01 - 7 µg/L), while the bioaccumulated levels of these compounds in mussel lipid (four week incubation) varied from 20 to 450 ng/g lw and the lipid-based bioconcentration factors (calculated from dilution-based concentrations in water) from 1 800 to 1 410 000. The highest bioconcentration factor was for alkylpolychlorophenanthrenes (polychlororetenes). The concentrations in fish lipid varied from below detection limit to 1 610 ng/g lw and the lipid-based bioconcentration factors from 2 800 to 31 000 the last one representing polychlorocymenes.

Carlberg et al. [51] studied extractable organic halide concentrations (EOCl) and persistant extractable organic halide concentrations (EPOCl, recoverable after treatment with concentrated sulphuric acid) in fat of perch (*Perca fluviatilis*), burbot (*Lota lota*), roach (*Rutilus rutilus*) and whitefish (*Coregonus lavaretus*) caught downstream of sulphite pulp mill using chlorine bleaching without a wastewater treatment at Hunselva river outlet in Norway. They discovered that the EOCl concentration in whitefish was four times higher than in burbot and roach, and almost ten times higher than in perch. Also the EPOCl concentration was highest in whitefish, but the differences were smaller. It was also demonstrated that EOCl and EPOCl in fat of fish caught 16 months after the mill was closed down, was of the same magnitude as in animals caught while the mill still was in operation. The authors concluded, that sedimented chlorinated material had acted as a source of these compounds to fish. In a follow-up study conducted three and a half years after the mill´s closure the levels of EOCl and EPOCl had decreased to the same magnitude as in fish caught from a reference area, indicating that the material sedimented no longer acted as a source of chlorinated compounds to fish on the area. All fish species in the recipient had mono- and dichlorocymenes in their fat while the mill was still in operation, but these were all below detection limit 16 months after the mill closed down [51]. Ahokas et al. [52] observed a significant downward gradient in EOX concentration in fillets of carp (*Cyprinos carpio)* downstream to a pulp mill discharging biologically treated effluents. The concentration of EOCl in mountain whitefish (*Proposium williamsoni*) fillets decreased from 13 - 43 mg/L to 1 - 2 mg/L after some process and wastewater treatment improvements conducted at the pulp mill, including the convertion of the bleaching process to chlorine dioxide post-delignification [37].

Paasivirta et al. [36] analysed concentrations of 14 chlorophenolics in benthic evertebrate animals and eight species of fish in a lake 5 km downstream from a mill using conventional chlorine bleaching. They observed, that the bioaccumulation of chlorinated compounds is very specific depending on living and feeding habits of the species: filtering animals in shallow water collect strongest the water-soluble compounds, like 24-dichlorophenol and 3,4-dichloroguaiacol, whereas sediment-herbivorae species accumulated more hydrophobic, material-bound components, like 4,5,6-trichloroguaiacol and 2,6-dimethoxy-3,4,5-trichlorophenol.

Oikari et al. [28] used the chlorophenolics concentration in blood plasma of experimentally exposed rainbow trout to trace the water contamination downstream of pulp mill producing chlorine bleached pulp. The most abundant chlorophenolics in plasma were 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol both in free and hydrolyzable form, with some tetrachloroguaiacol only in hydrolyzable form. However, since 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol were also detected in blood plasma of fish caged upstream from the mill, they were most likely unrelated to the point source of pulp mill effluent investigated. Owens et al. [53] detected 2,4,6-trichlorophenol, pentachlorophenol, tetrachloroguaiacol and tetrachloroveratrole in fillets of mountain whitefish collected downstream from a pulp mill using low chlorine bleaching. However, the concentration were low compared to the total concentration – free plus conjugated - of these compounds in the bile. They also detected 15 chlorophenolic compounds in *Hydropsyche* caddisflies collected 140 km downstream from the mill at a rapidly flowing Canadian river. *Hydropsyche* are filter feeders in the larval stage and the level of chlorophenolics in them seemed to be approximately the same as in suspended sediments collected at the same site.

Oikari et al. [54] studied the bioaccumulation of radiolabelled (^{14}C) 3,4,5-trichloroguaiacol (CG-3) and pentachlorophenol (PCP) into the tissues of lake trout (*Salmo trutta*) in 48 h static exposure. They found out that trout accumulated less CG-3 than PCP. They also observed large differences of accumulated concentrations of the toxicants between tissues: liver contained the highest concentration of CG-3, whereas red blood cells, backbone and swimbladder had the lowest concentrations. Lateral musculature, heart, spleen, head kidney and brain also enriched CG-3 only slightly. The picture of accumulated PCP in different organs of trout was largely similar to CG-3. However, the bile accumulated both toxicants clearly the most: the proportion of GC-3 and PCP in the bile was 92 % and 78 % respectively, of the total body burden. They also concluded, that if the major route of elimination of the contaminants in fish is hepatobiliary-intestinal route, like in the case of CPs, the tissue contamination of fillets can be effectively monitored by means of the concentration of contaminants in the bile.

Fish tainting is a well documented phenomenon in pulp mill recipients, especially when chlorine bleaching was used. The compounds most frequently

connected with this problem are chloroanisoles and -veratroles. However, these compounds are not directly released into the environment by the pulp mills, but are bacterial *O*-methylation products of chlorophenols, chlorocatechols and chloroguaiacols, components of chlorine bleachery effluents [55,56]. The amendments passed to the Pulp and Paper Effluent regulations in Canada in 1992 requires the industry to conduct a fish tainting study if any records of public concerns related to this should arise [57]. Neilson et al. [56] found tetrachloroveratrole in the liver of four-horned sculpin (50 - 400 μg/kg total liver fat), flounder (40 - 70 μg/kg) and eel (50 μg/kg) caught from three stations receiving chlorine bleachery effluents at Baltic Sea. They also found 3,4,5-trichloroveratrole in the liver of these fish, but in lower concentrations. These compounds were also detected in low concentrations in muscle tissue fat from eel (10 - 45 μg/kg total fat). Veratroles were absent in fish from uncontaminated localities. Paasivirta et al. [58] found significantly higher concentrations of chlorophenols, chloroanisoles and chloroveratroles in fillets of pike and burbot caught 5 - 10 km downstream from a pulp mill using chlorine bleaching in a riverine lake system of Äänekoski, Central Finland, and apparently, but not significantly increased levels in fish caught from as far downstream as 60 km from the mill, than the background levels on fish caught from an unpolluted area. They also found these toxicants in fillets of Baltic salmon. However, the highest concentrations were found in fillets of trout exposed for 3 months to 0.5 vol % total kraft mill effluent (chlorine bleached). From the fact that polychlorinated anisoles and veratroles were non-detectable (< 1 ng/L) in lake water they concluded, that the bioconcentration factor of these compounds must be high (> 10 000) or they have been formed of chlorophenols in fish. In fact, Neilson et al. [56] determined with zebrafish (*Brachydanio rerio*) the log BCF of tetrachloroveratrole to be 4.4 (BCF 25 000) and that of 3,4,5-trichloroveratrole 3.5 (BCF 3 200). The measured log P_{ow} values for tetrachloro- and trichloroveratroles are 5.9 and 5.25, respectively.

Also Renberg et al. [59], Watanabe et al. [60] and Veijanen et al. [61] have identified several different polychlorinated anisoles and veratroles in fish. Miyazaki et al. [62] and Watanabe et al. [60] have demonstrated these compounds also in shellfish. However, bleach plant effluents are not the only source of compounds with tainting potential in pulp mills. Craig and Stasiak [63] suggested that the terpenes α-pinene, cumene and thujane were associated with flavour impairment of the grease of eulachon (*Thaleichthys pacificus*) caught from the Kitimat river in Canada, downstream of the discharge of a nonbleaching kraft mill. Another study [64] showed elevated amounts of bad tasting terpenes α-pinene, β-pinene, menthene-2, α-terpinene and three carenes in tainted eulachon grease. Mikkelson et al. [33] noted, that 3,5-dichloroanisole and 2,4,5-trichloroanisole were detectable in all tainted and nondetectable in all nontainted eulachon grease samples. Furthermore, the levels of these polychlorinated anisoles in tainted eulachon grease were similar to levels of polychlorinated anisoles and veratroles in bad tasting fish from a water course receiving pulp mill wastes. Surprisingly, elevated concentrations of these compounds were

also detected in the grease of the fish caught from a reference river as well as in eulachon fish exposed to 20 % Kitimat pulp mill effluent in a tank for 4 to 6 days. The origin of chloroanisoles is not clear, since Kitimit pulp mill does not use bleaching processes. Further, these compounds are not typical microbially methylated chlorobleaching or water chlorination wastes [58,65] and they do not seem to originate from wood preservative chlorophenols or from combustion either [66]. The authors suggest, that 2,4,5-trichloroanisole could originate from the heavy use of the herbicide 2,4,5-T. Other possible source could be the contamination of raw material by the use of chlorophenolic fungicides or lindane as insecticide. Finally, dibenzothiophene, methyldibenzothiophenes and dimethyldibenzothiophenes occurred at significantly higher levels in the grease of unbleached KME-exposed eulachon than in reference fish. The source of these compounds could be an oil-based defoamer used in the Kitimat mill [33].

Polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) have been identified as trace constituents in pulp and paper mill effluents [34]. These compounds reveal high chronic toxicity and have shown strong potential to bioaccumulate. PCDDs/PCDFs are formed in the bleaching stages of pulping, when dibenzo-p-dioxin and dibenzofurans, that contaminate the pulping liquoers, react with chlorine [34]. The major source of precursors was identified as defoamers made from oils with high aromatic content. Polychlorophenol contamination of wood chips has also been identified as a precursor of hexachlorinated dioxins in the mill digesters [67]. The elimination of these precursors from pulping processes, substitution of chlorine with chlorine dioxide in the bleaching, efficient chemical mixing and employment of secondary wastewater treatment have reduced the amounts of these compounds released into the environment [34,35,67].

The concentration of 2,3,7,8-TCDD and 2,3,7,8-TCDF in mountain whitefish (*P. williamsoni*) caught from Wapiti / Smoky river system decreased by 85 % after process and wastewater treatment improvements conducted at the pulp mill, including the convertion of the bleaching process to chlorine dioxide bleaching. The same trend was also shown in burbot (*Lota lota*) livers [37]. No differences could be observed in concentrations of PCDDs and PCDFs in the grease of eulachon caught downstream of a pulp mill producing unbleached pulp and from a reference site [33].

Servos et al. [68] measured several congeners of PCDDs and PCDFs in white sucker (*C. commersoni*) liver samples and fillets from seven pulp and paper mill sites and three reference sites in Canada. The mills included five chlorine bleached-kraft mills, with and without secondary treatment, and two sulphite mechanical mills. They found out, that 2,3,7,8 - TCDD and 2,3,7,8-TCDF were the dominant congeners in liver and fillet samples at all bleached kraft mill sites, the observed TCDF concentrations ranging 4 - 16 times higher than those of TCDD. The same pattern of congeners in exposed fish was also detected by Hodson et al. [69] and Whittle et al. [70]. 2,3,4,7,8 - PeCDF was also consistently detected in samples from bleached kraft mill sites, but it contributed less

than five percent of the measured TCDF concentrations. The concentrations of PCDDs/PCDFs in the liver tissue downstream chlorine bleached kraft mills were remarkably higher than at reference sites even when the data were expressed on a lipid-corrected basis. Downstream of a BKME source the concentration of PCDDs/PCDFs in the liver of a fish could be as much as 45 times higher than in the fillet of the same fish on the wet-weight basis, but the difference was eliminated if the concentrations were corrected for lipid content [68]. The congener concentration, expressed as TEQ (toxic equivalent concentration) correlated with AOX measured in the receiving water at each site, the AOX being inversely related to the dilution of the effluents. TEQs also correlated with levels of chloride and 3,4,5-trichloroguaiacol in water, also tracers of BKME origin. Elevated concentrations of PCDDs and PCDFs in fillets of carp *(C. carpio)* caught downstream of an Australian pulp mill, were also observed by Ahokas et al. [52].

Similar pattern of BKME-related PCDD/PCDF congeners was also observed by Whittle et al. [70] in marine environment, They analysed samples of marine biota (crab muscle and hepatopancreas, prawn and shrimp tissue, and oyster and clam tissue) with several freshwater animals (sucker, whitefish, chub, Dolly Varden, char, burbot, walleye and bullhead) from the recipients of 46 pulp mills using chlorine bleaching. They also noted, that the concentration of tissue lipid and the feeding habits of the species were the most significant variables determining the biological accumulation of these contaminants.

Principal components analysis (PCA) of the concentrations of PCDDs and PCDFs in 469 samples of crab hepatopancreas collected from the recipient of 9 mills in 1987 - 1993 in British Columbia show that both the proportion of the most toxic 2,3,7,8- chlorinated congeners and the overall chlorodioxin and -furan concentrations have decreased since 1987 [71]. It was also noted, that variations in the proportions of tetrachlorofurans from chlorine bleaching and hexachlorodioxins derived from pentachlorophenol have produced striking differences between the PCDD / PCDF profiles of crabs from different mill sites. The mill-related tetrachlorofurans are removed faster from the biota than the hexachlorodioxins [71]. Mussels caged dowstream an Alaskan pulp mill accumulated higher concentrations of dioxins and furans than clams in laboratory exposures and showed better relationship with those chemicals in sediment [72]. It was therefore suggested, that mussels exposed under natural conditions in the water column are useful animal models of exposure and effects.

Non-chlorinated markers

Because of the conversion of the bleaching process from elemental chlorine bleaching to chlorine dioxide and oxygen delignification, there has been an increasing interest in 1990ies to discover the employment of non-chlorinated markers to demonstrate exposure of biota to pulp and paper mill effluents. The most frequently used class of compounds are resin acids. Uptake of dehydroabietic acid (DHAA), the most abundant resin acid in pulp mill ef-

fluents, into fish exposed to DHAA containing effluent was first shown qualitatively by Mahood and Rogers [73]. Shortly afterwards Fox et al. [74] reported twenty-fold bioconcentration of DHAA on whole body basis into rainbow trout exposed for two days to kraft mill effluents containing 0.1 - 0.7 mg DHAA / L, and Oikari et al. [75] studied in more detail the distribution of DHAA and a mixture of resin acids into tissues of trout (*Salmo gairdneri*). In the latter work the fish were exposed to 0.5 to 2.0 mg/L of DHAA or 0.8 to 2.2 mg/L of resin acid mixture for 4 and 2 days, respectively. The highest accumulation of DHAA was observed in the blood plasma ($BCF_{plasma/water}$ = 200), whereas the concentration in the liver was about half of that. The concentration in anterior kidney, posterior kidney and heart was about one third of that in blood plasma. In the experiment with a mixture of resin acids, the highest concentration of total resin acids was in in liver, with the acids occurring in the liver tissue in similar ratios to those found in external water, suggesting that different acids are rather similar in their accumulation characteristics.

In the blood plasma of caged rainbow trout exposed for 10 days to BKME in a lake, the sum bioconcentration factor of all resin acids was 40 - 60. Free resin acid concentrations in the bile, on the other hand, were higher than in the plasma [76]. However, in feral perch (*Perca fluviatilis*) caught from the same area, the concentrations of resin acids were clearly higher in plasma than in the bile [76]. The authors suggested, that this is probably due to differences in mechanisms of detoxification between these two species. In another 15-day field study with experimentally exposed rainbow trout [28], average bioconcentration factor of resin acids to plasma was 570 with rather high variation, probably reflecting the fluctuation of the concentrations of resin acids in water. Also hydrolyzable resin acids, i.e. bound in some unspecific way, were detected in plasma. Overall, the BCFs (bioconcentration factors) calculated from field data are many times approximate, because the water samples collected during the experiment may not be representative of the whole exposure period. The use of uncontrollable field material, like feral fish introduces additional sources of error to the calculations: wild specimen might migrate around in waters where pollutant concentrations differ from the determined values and pollutants can also be taken up by fish via food which may already have undergone bioconcentration.

Johnsen et al. [77] noted, that the resin acid concentrations in liver, gill and muscle of rainbow trout exposed to diluted effluents of dithione-bleached thermomechanical pulp (TMP) for 8 weeks in the laboratory, showed a clear positive concentration dependence. The total concentration of resin acids in water during the experiment varied from 30 to 170 µg/L. In all doses the highest concentration of total (free plus conjugated) resin acids was found in the bile, followed by liver (BCF = 1 800), gills (BCF = 300) and muscle (BCF = 150). The relative distribution and bioconcentration factors of resin acids in tissues of brown trout (*Salmo trutta*) exposed *in situ* in land-based pools upstream and downstream of a mill producing TMP were comparable to those observed in the laboratory study described above [78]. It was also shown, that

resin acids accumulate effectively to gonads of brown trout exposed to TMP effluent in a laboratory for four months (BCF = 2000; [78]). DHAA seemed to accumulate only slightly to gonads of rainbow trout in a four day experiment (BCF = 5; [75]). Brumley et al. [79] studied bioaccumulation of resin acids and resin acid-derived neutral compounds (fichtelite, dehydroabietin, retene and tetrahydroretene) into tissues of rainbow trout exposed to pulp mill treatment system sludge and to an extract of this sludge. The order of bioaccumulation of total resin acids (free plus conjugated) was bile > blood serum > liver > gills > muscle, whereas the more lipophilic resin acid-derived neutral compounds accumulated in order liver > bile > muscle. Resin acid-derived neutrals were not detected in blood serum and gills.

Koistinen et al. [80], besides measuring neutral compounds in primary and secondary clarifier sludges and secondary clarifier effluents, also analysed these as residues in muscle tissue of whitefish (*C. lavaretus*) exposed to diluted primary clarifier effluents. They detected alkylated indanes, alkylated benzenes, alkylated phenyl benzenes, biphenyl and alkylated biphenyls, alkylated diphenyl methane, alkylated fluorenes, naphthalene, phenyl naphthalene and alkylated naphthalenes, C1-alkylated phenantrene, octahydro methano indene and alkylated ester of adipic acid in the tissues of fish. The most abundant of these compounds were alkylated benzenes and alkylated naphthalenes. The alkylated naphthalenes might originate from the oil-based defoamers used at the integrated mill. The same alkyl benzenes and naphthalenes were detected in the contaminated sediment, implying that they may survive the secondary treatment too. Burgraaf et al. [81] detected resin acids and fichtelite in freshwater mussels (*Hyridella menziesi*) incubated at the outlet of a kraft pulp and paper mill wastewater treatment system. The mean mussel dry weight based BCFs for individual resin acids varied from 110 to 330. The BCF for fichtelite was 4 900, an order of magnitude higher than that of the resin acids. The biological half-lives of resin acids were 3 d and for fichtelite 12 d.

METABOLITES

Presence of a xenobiotic chemical in tissues or body fluids of an animal can be considered as a conclusive evidence of its bioavailability from an ambient environment. The biotransformation and metabolism of many chemicals (e.g. PAHs, chlorophenolics and resin acids) in the liver of an animal results in the formation of numerous conjugated and unconjugated metabolites that are excereted, and have been used as biomarkers of exposure to these chemicals [82,83]. Biotransformation is a part of body detoxification of an organism, a mechanism for the elimination of xenobiotics that have become bioavailable [84]. Biotransformation is biphasic. It is run by enzymatic reactions that involve oxidation, reduction and hydrolysis (phase I), and those that consist of conjugations (phase II). During phase II reactions, the foreign chemical or its

product formed by phase I reactions is covalently linked to a normal cellular constituent such as glutathione, glucuronic acid or sulfate. Both phase I and II reactions render the initial xenobiotics more water soluble and permit their excretion from the organism [85]. From the liver, the conjugated xenobiotics are secreted into the bile. Xenobiotic molecules containing hydroxyl-, carboxylic acid or other nucleophilic groups are potential substrates for glycosylation. The major glycoside conjugates formed *in vivo* in fish are glucuronides [86]. It has been verified that resin acids and some chlorophenolics are conjugated with glucuronic acid moiety in the liver of rainbow trout with some minor portions of sulphate conjugates too [82,87]. In other fish species similar reactions can be suggested, too. Importantly, the concentration of a xenobiotic metabolites can be substantially higher in the bile than in surrounding blood plasma [82,87]. Thus, the total gradient of a cholephilic compound between the bile and ambient environment of fish is formed in two steps, by passive uptake from water (or sediment) into the blood, followed by active canalicular transport into primary bile [88-90].

Most frequently used biliary compounds to assess the exposure of organism to pulp mill effluents are chlorophenolics and resin acids.

Chlorinated markers

The levels of conjugated CPs in bile of rainbow trout exposed to chlorine-bleached KME in the laboratory was demonstrated to be positively correlated with the concentration of these toxicants in water [91]. In a follow-up study [82] in early 1980ies, conducted with feral fish species caught from the recipient of a mill producing chlorine bleached pulp and using aerated lagoon wastewater treatment, it was noted, that the concentration of conjugated CPs in roach bile in a lake system decreased continually with increasing distance from the mill. The highest concentration of the total chlorophenolics (conjugated plus free) detected in the bile of roach and perch were 1150 μg/mL and 1550 μg/mL, respectively. The levels of conjugated xenobiotics, tetrachloroguaiacol being most abundant, in roach bile were still significantly higher 15 km downstream from the mill compared to upstream reference values. Unlike roach the results obtained from perch did not reveal such a consistent relationship between the distance of the effluent source and bile CPs concentration. In particular, the levels closest to the mill (1 km) were lower than at the more distant sampling locations, despite the obviously higher concentrations in water. The author suggests, that explanation for this might be the differences of cruising areas of the species, roach probably being more local species than perch or different response to the environmental conditions by the species, including high levels of toxicants which may interfere with the secretory processes of metabolites out of the liver of fish. Interestingly, the same suppression in biliary accumulation of CPs at the exposure site closest to the mill (1 km) was also observed in a study with rainbow trout [28] implying, that the poorest quality of the water, both in terms of direct toxicity as well as other

ambient factors such as low water oxygen concentration, interfered with the metabolism. Further away there was a gradual decrease in bile CPs concentrations, tetrachloroguaiacol and 2,3,4,6-tetrachlorophenol being the most abundant chlorophenolics. By comparing the results of CPs´ analysis from water, bile and blood plasma samples, authors concluded, that bile analyses are a much more sensitive way to trace water contamination by these substances than the analysis of blood plasma. Lindström-Seppä and Oikari [92] and, while ECF technology was in use, Soimasuo et al. [43] observed a distance related decrease in the amount of chloroguaiacols and chlorophenols in the bile of experimentally exposed rainbow trout and whitefish at the same Saimaa study area as mentioned above without any suppression in biliary accumulation of CPs. However, the site closest to the mill was moved further downstream (3 km instead of 1 km). Between 1992 and 1993 the given pulp and paper mill changed over to ECF bleaching and installed an activated sludge plant in place of aerated lagoons. Due to these improvements the CPs concentrations in bile of experimentally exposed whitefish eventually decreased by up to 99% at the sampling station closest downstream (3 km) of the mill [23,93-95].

With the process changes also the quality of chlorophenolics changed from compounds with high degree of chlorination (i.e. tetrachloroguaiacol and -catechol) to compounds with lower degree of chlorination (dichlorinated catechols and -guaiacols and 6-monochlorovanillin) [23,53]. The bleaching related chlorophenolics in bile were the best exposure biomarkers to elemental chlorine free (ECF) bleached, biologically treated pulp mill effluent in a study conducted by exposing juvenile whitefish [23,95]. Other parameters surveyed were biliary resin acids, fatty acids and cholesterol. Water Na^+ concentration was used as a measure of effluent dilution. On the other hand, in two laboratory simulations with biologically treated effluents from a mill producing ECF bleached pulp, no dose-response relationship of BKME dilution and the concentrations of CPs in bile of whitefish could be demonstrated [45,96] (Fig. 2). Owens et al. [53] observed a concentration gradient of biliary chlorophenolics in mountain whitefish as far as 230 km downstream of a mill using low chlorine bleaching in Wapiti and Smoky River system. Similar gradient was not evident for longnose sucker. On the basis of this finding, along with the observation that filterfeeding invertebrates have similar chlorophenolic levels to suspended sediments, the authors suggest that in addition to gill and gut uptake from the water column, the food web uptake might have increased biliary levels of chlorophenolics in mountain whitefish relative to longnose sucker. They also concluded, that fish bile may be the only matrix to provide the sensitivity to monitor exposure to many mill compounds as environmental discharges along process modernizations fall.

Swanson et al. [37] measured EOCl concentrations up to 192 mg/L in the bile of mountain whitefish in a vicinity of a pulp mill producing chlorine bleached pulp. After the mill converted the bleaching process to chlorine dioxide delignification the level of biliary EOCl dropped below detection limit. Owens et al. [53] noted, that the chlorine of 20 specifically analysed chlorophenolic com-

pounds comprised less than 10% of the observed chlorine as EOCl in bile of mountain whitefish downstream a low-chlorine bleachery. While the bile and fillet EOCl values appeared to correspond to exposure in mountain whitefish, the authors concluded that EOCl is not of a high analytical priority in future studies because of the presence of EOCl at the uncontaminated reference site and the lack of the clear response in longnose suckers.

Tavendale et al. [97] observed elevated concentrations CPs in bile of goldfish (*Crassius auratus*) residing in a reservoir receiving a biologically treated bleached kraft mill discharge. The most abundant chlorophenolics were 2,4,6-trichlorophenol, 3,4,5- and 4,5,6-trichloroguaiacols and tetrachloroguaiacol. Attenuated levels of these compounds were identified 2.4 km downstream, while levels 9.1 km downstream were only marginally greater than those identified at the upstream site.

Research for the types and levels of chlorinated phenolics in bleached eucalypt pulp effluent indicate, that 2-chlorosyringaldehyde (2-CSA) is quantitatively the major chlorinated phenol produced by the ECF bleaching, while 2,6-dichlorosyringaldehyde dominates when there is a high proportion of molecular chlorine used [98]. Haritos et al. [99] found that about 99% of metabolised 2-CSA excreted into the bile of sand flathead (*Platycephalus bassensis*) is conjugated with glucuronic acid or sulphate and that the metabolite (2-chloro-4-hydroxy-3,5-dimethoxy-benzylalcohol, 2-CB-OH) indicates 2-CSA exposure in a dose related manner.

Resin acids

In 1976 Statham et al. [100] proposed the use of fish bile as an aid in monitoring certain waterborne chemicals and in 1977 Kaiser et al. [101] reported, that the fish living in receiving waters of a pulp mill in Lake Superior are contaminated by dehydroabietic acid. The unconjugated resin acids were quantitated in bile of experimentally exposed rainbow trout and feral perch living downstream of a pulp mill [76]. The existence of resin acid conjugates in fish bile was demonstrated for the first time by Oikari et al. in 1984 [87]. A distance related decrease in the biliary resin acid concentration of feral perch and roach downstream of a pulp mill was demonstrated in 1986 [82]. In a study with caged rainbow trout in a lake system, elevated concentrations of resin acids up to 11 km downstream of a pulp mill sewer were observed, with the composition of biliary resin acids reflecting well the composition of resin acids in water [28]. The highest concentrations 1 km downstream of the mill were over 3000 μg/ml.

Rather variable results were obtained a decade later, when whitefish were experimentally exposed to biologically treated, ECF bleached kraft mill effluent [23,95]. In these studies the resin acid concentrations in water as well as in fish bile were difficult to connect to pulping activities. Leppänen et al. [23] detec-

ted the highest concentration of resin acids both in water and in fish bile in fish caged upstream of the pulp mill. The source of the resin acids at the upstream site was a dredging conducted in a nearby harbour apparently releasing earlier stores of resin acids from the sediments. Although the resin acid concentration in bile of whitefish correlated with the resin acid concentration in water at all sampling sites, they did not correlate with the water Na^+ concentration, used as an effluent tracer. In all the studies referred, dehydroabietic acid (DHAA) has been predominant, sometimes the only, resin acid present in the bile. Along with this discussion, and before, Owens et al. [53] concluded, that in a river system receiving BKME, biliary fatty and resin acids are not useful markers of exposures in longnose sucker and mountain whitefish, since natural levels appeared to be present near concentrations released from biologically treated discharge. They suggest, that the modified monochloroabietic acids, being direct bleachery products, could be used as effluent tracers when levels are measurable. Tavendale et al. [97] analysed the contents of the bile of goldfish from a hydrolake receiving pulp mill effluents, and found that the predominant resin acids were abietanic, 13-abietanic and secodehydroabietic acids, i.e. compounds that had been established as transformation products of natural resin acids in the biological treatment of the mill´s wastewaters. The level of resin acids in bile of goldfish was comparable to background reference levels at 9.1 km downstream of the mill, but the differences in resin acid compositions indicated that the fish resident at that site might get exposed to pulp mill effluents. Fraikin et al. [102] studied the concentrations of resin and fatty acids in bile and flesh of mountain whitefish and longnose sucker downstream of three pulp and paper mills in Canada. They concluded, that these compounds need to be re-assessed as suitable tracers to modern BKME because of the low levels detected in exposed animals.

Brumley et al. [79] studied the bioconcentration factors of resin acids in the bile, liver, muscle, blood serum and gills of rainbow trout. He subdivided the resin acids to aromatic, dienic, monoenic and saturated acids. The accumulation of all classes of resin acids was highest to the bile compared to other tissues. Furthermore, the log bioconcentration factors of different types of resin acids into the bile were rather similar (5.40 - 5.95, log BCF_{bile} of total resin acids 5.70).

In laboratory studies Soimasuo et al. [45] found a reasonably good correlation between the dilution of biologically treated (aerated lagoons) low chlorine kraft mill effluent and the concentration of biliary resin acids in whitefish. However, no such correlation could be observed with the same dilutions in a study with elemental chlorine free bleached effluents, that were treated with a low-loaded activated sludge process [96]. Johnsen et al. [77] observed a clear relationship between the biliary resin acid level of rainbow trout and the exposure to untreated dithionite-bleached thermomechanical pulping effluent. The bioconcentration factor of resin acids from water to bile were approximate 10^5 in all studies.

Other markers

There are some other pulping derived compounds that have been detected in the bile of fish. Johnsen et al. [77] detected elevated concentrations of conjugated fatty acids and cholesterol in bile of rainbow trout exposed to dithionite-bleached untreated pulping effluents in the laboratory. However, these were not observed by alkali hydrolysis in the bile of whitefish experimentally exposed to discharges from a modern ECF-mill in the field [23]. Nevertheless, there was a positive correlation between the concentrations of total resin acids, fatty acids and cholesterol in the bile, but these were impossible to connect to pulp mill effluents. Tavendale et al. [97] detected several polyaromatic hydrocarbons such as anthracene, phenanthrene and their corresponding metabolites such as phenanthrol in the bile of goldfish from a hydrolake receiving pulp mill discharge. Indicatively, the concentrations of these compounds in the bile decreased with increasing distance from the mill discharge point. They also detected traces of retene, vanillin and acetovanillone. Brumley et al. [79] detected resin acid-derived neutral compounds dehydroabietin and retene in the bile of rainbow trout exposed to contaminated sediment and to an organic extract of that sediment. Leppänen and Oikari [103] quantified the concentration of retene in the bile of feral roach caught downstream from a pulp mill, this being the the first direct evidence on its bioavailability in a natural ecosystem.

BIOCHEMICAL MARKERS

Although other biochemical response markers have variably been connected to BKME, no others have been applied to monitoring like EROD (7-ethoxyresorufin O-deethylase) activity. The cytochrome P450 1A (CYP 1A) system plays a pivotal role in the metabolism of many lipophilic environmental pollutants. Induction of the CYP1A proteins by these xenobiotics, which fit to the cytosolic Ah receptor is one of the major characteristics of this system [104]. Through the action of CYP1A, functional groups are introduced into the substrate molecule, facilitating the further metabolism of xenobiotics by conjugating enzymes such as UDP-GT (uridine-5´-diphospho glucuronosyltransferase) and GST (glutathione S-transferase). The induction can be caused by highly toxic co-planar polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and chlorinated dioxins and furans [1]. The most potent inducer is TCDD due to the size and the planarity of its ring structure [105,106]. Increasing or decreasing the chlorine substitution or changing the pattern of the substitution decreases the potency. Likewise, some PCBs are more potent inducers than others. PAHs that induce MFO activity are multi-ringed, aromatic, and planar, but not chlorinated, so they are less potent than TCDD [1].

The ability of chlorine-bleached pulp mill effluents to induce liver CYP1A-type monooxygenase has been well documented [39,43,107,108]. However, induction in fish is known to be caused by effluents from sulphite, ground-

wood, thermomechanical (TMP), chemi-thermomechanical (C-TMP), unbleached and bleached kraft mills, including mills that use a high degree of ClO_2 substitution or that do not use chlorine at all for bleaching [77,109-114]. Martel et al. [112] proposed that MFO induction was primarily a result of kraft pulping rather than of bleaching. However, a 80 - 90% decrease of EROD activity was noted in caged whitefish after the shift to ECF bleaching and the implementation of the activated sludge effluent treatment in the mill [43,93,95]. Schnell et al. [115] observed 89 - 98% reduction in the MFO induction potency of bleached kraft mill and simulated whole effluent (a volumetric combination of individual waste streams from a bleached kraft mill) after the treatment by bench scale activated sludge, stabilization basin, and aerated stabilization processes. However, MFO inducers survived even the best secondary treatment in Canada [116].

There has been a lot of research to reveal the nature and identity of MFO inducers in pulp and paper mill effluents. Hewitt et al. [116] discovered that ultrafiltration by reverse osmosis to remove high molecular weight compounds eliminates the potency of mill effluents to induct MFO. This implies that either high molecular weight compounds are inducers, which is unlikely since they would not traverse membranes easily, or that the inducers were absorbed or adsorbed by either the reverse osmosis membrane or the high molecular weight compounds. Since resin acids, terpenes, chlorophenolics, aliphatic alkanes, plant sterols and chlorinated dimethyl sulfones survived this process, they could not be considered inducers. Burnison et al. [117] found that MFO inducers consistently eluted with marker compounds having log K_{ow} of about 4.5 - 5.1, a range typical of moderate hydrophobicity. This fraction did not contain typical EROD inducers like PCBs, chlorodioxins and -furans. Peaks shown by fluorometric detection included phenanthrene and substituted phenanthrenes. However, Parrott et al. [118] demonstrated that phenanthrene does not induce EROD in rainbow trout.

Some field studies have demonstrated good correlations between concentrations of chlorodioxins (or total dioxin equivalents) and MFO activity in the field exposed fish [52,68,69,119- 122]. Also the reduction in dioxin emissions from a mill was associated with reductions in MFO activity of whitefish (*C. lavaretus*) in receiving waters [120]. However, Munkittrick et al. [123,124] noted, that during a mill shutdown MFO activity declined rapidly both in free-swimming and caged whitesuckers downstream of the mill. The induction was also lost rapidly after the fish were transfered from BKME to clean water. This behaviour is not typical for persistant inducers, such as TCDD [125,126].

Resin acids appear structurally similar to known inducers, such as PAHs. However, Oikari and Lindström-Seppä [127] demonstrated, that dehydroabietic acid did not induce EROD. Similar results were reported by Ferguson et al. [128] for dehydroabietic acid, resin acid mixture and rosin gum. In contrast retene (7-isopropyl-1-methylphenantrene), primarily derived from the anaerobic microbial aromatization process of dehydroabietic acid, appears to

induce EROD in rainbow trout quite strongly [118,129]. Also other alkyl-substituted phenantrenes induce EROD in fish [118]. Lehtinen et al. [130] observed a slight, statistically non-significant increase in EROD activity of rainbow trout after the exposure to 10 μg/L of phytosterol mixture, mostly ß-sitosterol.

CAN LABORATORY EXPOSURE SIMULATE TRUE POLLUTION?

In order to investigate BKME-related ecotoxicity, Soimasuo and co-workers conducted two field studies with experimentally exposed whitefish along a pollution gradient downstream of a pulp and paper mill, with two parallel laboratory experiments simulating the lake pollution [43,45,95,96]. The effluent dilution at the study area was determined by the water Na^+ concentration and the dilution in the laboratory was set accordingly. The chemical exposure markers used in the first pair of experiments were chlorophenolics and EOX in total tissue lipids, and total chlorophenolics (CPs) in the bile. Between the first pair of studies, the field and the laboratory experiments, the pulp mill converted its bleaching sequence from conventional chlorine bleaching to low-chlorine bleaching (Cl_2 1%), reducing the concentration of CPs in water by up to 92 % [43,45]. This was directly reflected in the concentrations of the chlorinated compounds accumulated in fish. Thus the laboratory experiment was not fully equivalent with the earlier field experiment.

The other set of studies was conducted with effluents from ECF-bleaching both in laboratory and in the field. The markers measured were chlorophenolics and resin acids in the bile and hepatic EROD activity (Fig. 2). On the basis of these studies it can be concluded, that laboratory experiments predict the levels of the chemical markers studied in tissues of whitefish rather sufficiently, eventhough concentrations appear to be somewhat higher in the field studies. This could be a sign of pollution unrelated to present-day pulping activities in the field, or the effect of loading coming from the polluted sediments.

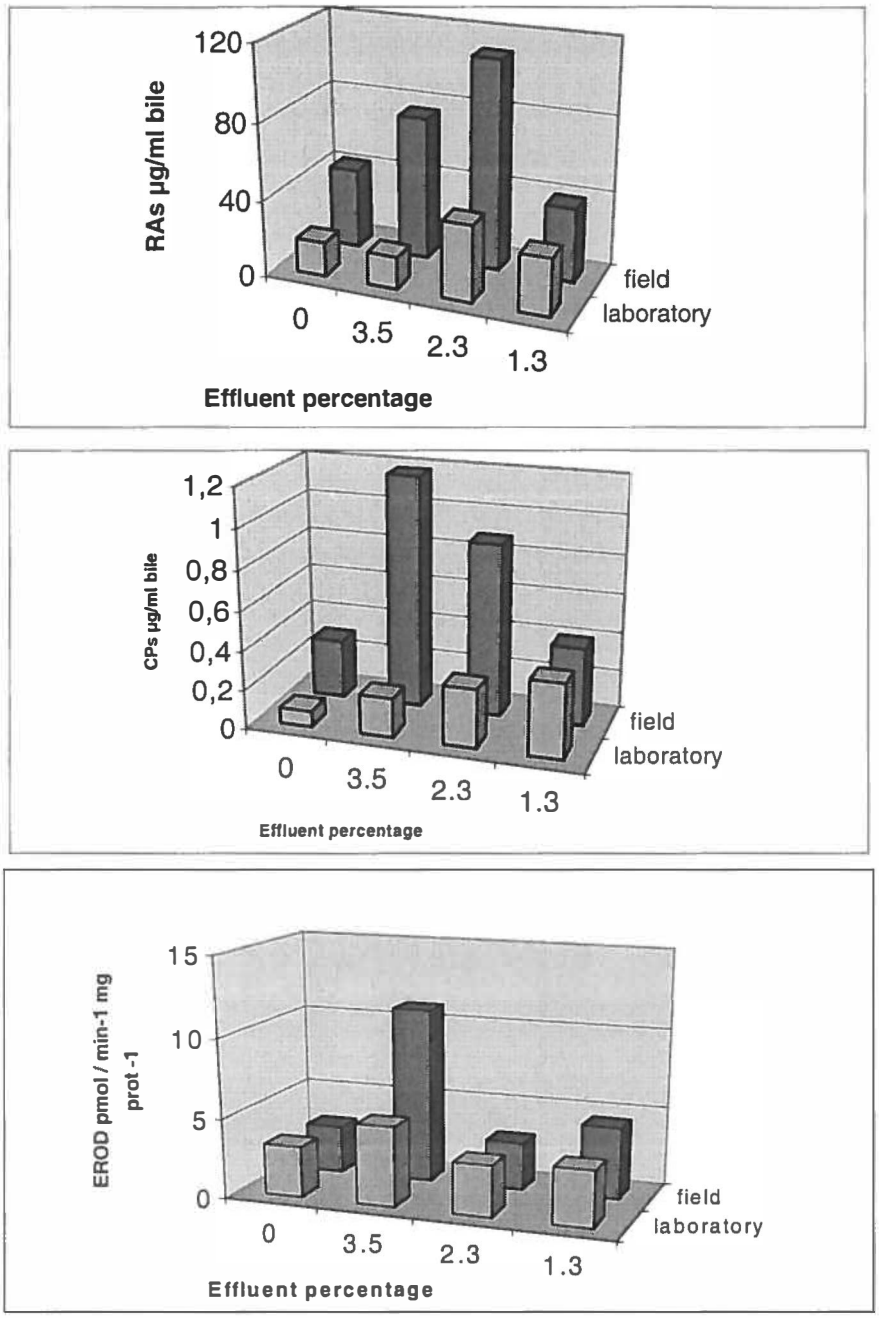

Figure 2. The concentration of resin acids and chlorophenolics in bile of whitefish exposed to effluents from ECF-bleaching in laboratory and in the field and the respective hepatic EROD activity [94,96].

CONCLUSIONS

On the basis of the literature reviewed and due to historical reasons, it is obvious that the chlorinated compounds have been studied the most as chemical markers of exposure to pulp mill effluents in aquatic animals. However, in

1990's in particular, the conversions in the bleaching sequences and other production technologies conducted by the pulp mills along with the improvements in the wastewater treatment have remarkably reduced the amounts of chlorinated compounds released into the aquatic environment, making the current use of chlorinated markers difficult. Resin acids are another well-studies group of compounds for markers, but the wastewater treatment improvements have reduced the amounts of these compounds, too. Resin acids are also released into the environment from sources other than current pulping activities (e.g. dredgings). Fatty acids do not seem to be promising markers, either. The biotransformation products of compounds present in the pulping effluents (chloroveratroles and -anisoles, retene and other resin acid-derived compounds) can be used as markers succesfully at some sites, but it can be difficult to distinguish, whether the animals have been exposed to present effluents or to sediments contaminated earlier. Some results of the literature reviewed are summarized in table 1.

TABLE 1 Summary of the suitability of tissues or body fluids of some species for monitoring exposure markers of pulp mill effluents on the basis of literature reviewed (VI). + = rated suitable in the study, - = rated unsuitable.

Species	Marker	Tissue or fluid	Suitability	Bleaching	Ref.
Freshwater mussel	EOCl, CPs	Soft tissues	-	Cl_2	[41]
Whitefish, mountain whitefish	EOCl, CPs	Filet, bile	+	Cl_2	[37,43,53]
Mountain whitefish	EOCl	Bile	-	Cl_2, ECF	[37, 53]
Lake mussel	CPs, aromatic chlorohydrocarbons[1]	Lipid	+	Cl_2, ECF	[47-50]
Four-horned sculpin, pike, eel, flounder, burbot	Chloroanisoles, -veratroles	Liver, filet	+	Cl_2	[56,58]

(continued)

Carp, crab, White sucker,	PCDDs, PCDFs	Liver, filet	+	Cl_2	[52,68-70]
Rainbow trout	Resin acids, CPs	Blood plasma	-	Cl_2	[28,76]
Roach, rainbow trout, whitefish	CPs	Bile	+	Cl_2, ECF	[23,28,43,82, 92,95]
Whitefish, mountain whitefish, longnose sucker	Resin acids, fatty acids	Bile	-	Cl_2, ECF	[23,53,95,102]
Perch, roach, rainbow trout	Resin acids	Bile	+	Cl_2	[28,82]
Goldfish	Transformed resin acids[2]	Bile	+	ECF	[97]

[1] polychlorocymenes, polychlorocymenenes, alkylpolychloronaphthalenes, alkylpolychlorobibenzyls and alkylpolychlorophenantrenes

[2] abietanic, 13-abietanic and secodehydroabietic acid

There are also some considerations that must be taken into account when choosing the animal species for the monitoring of the pollution. The water-solubility of the chemicals released into the aquatic environment have a strong effect on their immediate environmental fate: the hydrophilic or only moderately hydrophobic compounds are transported as dissolved in water, whereas lipophilic compounds are bound into particles. Therefore the exposure of fish and invertebrates to lipophilic compounds can differ remarkably. It must be also kept in mind, that there is still no technique to address the question of duration of the exposure of the fish to the effluent, because of the rather fast accumulation, metabolization and excretion of most toxicants by fish. Research on the use of evertebrates, native or transposed, to monitor pulp mill effluent exposure is needed.

In conclusion, it seems that new candidates for markers of exposure of aquatic animals to modern pulp mill effluents are needed. One group could be phytosterols present in pulp mill effluents. 6-monochlorovanillin is also suggested as a promising marker of ECF-bleached effluents. Other compounds, that could serve as exposure markers are chlorocymenes, chlorophenantrenes and chlorodibenzothiophenes. Further, new markers representing exposure to sedi-

ments, and thus to the earlier industrial history also, are emerging. Retene, with record surface sediment concentration (600 μg/g d.w. [103]), is a good candidate for further assessment. The addition of an inert chemical marker during mill operations, that could serve as a tracer has also been suggested [131]. However, there are numerous disadvantages in this approach. These include regulatory concerns, costs, developing the necessary data base about the fate of the chemical in diverse aquatic environments, and the toxicological effects on all organims.

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