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Päivi Meriläinen

Exposure Assessment of Animals to Sediments Contaminated by Pulp and Paper Mills

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Editors
Anssi Lensu
Department of Biological and Environmental Science, University of Jyväskylä
Irene Ylönen, Marja-Leena Tynkkynen
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ABSTRACT

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Yhteenveto: Sellu- ja paperiteollisuuden saastuttamat sedimentit altistavana tekijänä vesieläimille
Diss.

Bioavailability, exposure status and ecotoxicological effects of pulp and paper mill effluent (BKME) -contaminated sediments were studied in the recipient area of a pulp and paper mill and at reference areas in Southern Lake Saimaa, Finland. By dissolution of contaminants into water-sediment elutriate and uptake to feral and laboratory-exposed benthic invertebrates. Additionally, genomic and biotransformation effects of resin acids (RAs) on brown trout were investigated. Differences in biotransformation between fish species was done by measuring RAs in the bile. To compare responses of RA exposed fish to lake conditions, a caging experiment with brown trout was conducted in Southern Lake Saimaa. Finally, exposure status of perch and roach populations was investigated after occurrence of a black liquor spill in June 2003 from a pulp and paper mill in the Southern Lake Saimaa area. Dissolution of RAs and wood sterols (WS) from BKME-sediment into the water phase indicated their potential bioavailability. Uptake and bioaccumulation of RAs, WSs and chlorophenolics in resident populations of invertebrates showed increased body burdens in areas downstream from the mill compared to reference areas, similarly in laboratory-exposed benthic invertebrates to sediments collected from the same areas. The RA concentration in bile of brown trout showed time and concentration dependences. Transcriptomic changes induced by RAs implied high sensitivity to genes implicated in the metabolism of iron and in reactive oxygen species. According to RAs in fish bile, Southern Lake Saimaa had recovered at the latest by three moths, after the black liquor spill. Roach showed the ability to excrete conjugated RAs in higher concentrations than perch. The overall conclusion is that the bioavailability and exposure of aquatic animals to contaminants from BKME sediments indicate that BKME contaminated sediments pose a risk to aquatic environment.

Key words: Bile; bioaccumulation; bioconcentration; biotransformation; dissolution; fish; pulp and paper industry; resin acids; sediment; transcription.

Päivi Meriläinen, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address Päivi Meriläinen
University of Jyväskylä
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä, Finland
E-mail: paivi.merilainen@jyu.fi

Supervisor Professor Aimo Oikari
University of Jyväskylä
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä, Finland

Reviewers Acting professor Matti Leppänen
Laboratory of Aquatic Ecology and Ecotoxicology
Faculty of Biosciences
University of Joensuu
P.O. Box 111
FI-80101 Joensuu, Finland

Dr. George Kruzynski
Institute of Ocean Sciences
P.O. Box 6000
Sidney
B.C. V8L 4B2
Canada

Opponent Professor Peter Hodson
School of Environmental Studies
Queen's University
Kingston
Ontario K7L 3N6
Canada

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on five original articles, which will be referred to by Roman numerals I-V in the text. I am the first author of articles I-IV and have contributed significantly to the planning, data collection and analyses as well as writing, and editorial correspondence of each article, as shown in the table below.

I Meriläinen, P., Lahdelma, I., Oikari, L., Hyötyläinen, T. & Oikari, A. 2006. Dissolution of resin acids, retene and wood sterols from contaminated lake sediments. Chemosphere 65: 840-846.

II Meriläinen, P. S. & Oikari, A. 2007. Uptake of organic xenobiotics by benthic invertebrates from sediment contaminated by pulp and paper industry. Submitted.

III Meriläinen, P. S., Krasnov, A. & Oikari, A. 2007. Time- and concentration-dependent metabolic and genomic responses to exposure to resin acids in brown trout (*Salmo trutta* m. *lacustris*). Environmental Toxicology and Chemistry 26: 1827-1835.

IV Meriläinen, P. S. & Oikari, A. 2007. Exposure assessment of fishes to modern pulp and paper mill effluents. Submitted.

V Oikari, A., Meriläinen, P., Karels, A. & Krasnov, A. 2007. Transcriptomic responses in juvenile brown trout and whitefish exposed subacutely in the field to a modern BKME discharge. Submitted.

Contributors of the original publications

	I	II	III	IV	V
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Writing of article and correspondence	PM, IL, AO	PM, AO	PM, AKr, AO	PM, AO	AO, AKr, PM, AKa,

Päivi Meriläinen (PM), Ilpo Lahdelma (IL), Laura Oikari (LO), Tarja Hyötyläinen (TH), Aimo Oikari (AO), Aleksei Krasnov (AKr), Aarno Karels (AKa).

ABBREVIATIONS

ANOVA	analysis of variance
AOX	adsorbable organic halogen
BCF	bioconcentration factor
BKME	bleached kraft mill effluent
BOD	biological oxygen demand
BSAF	biota sediment accumulation factor
COD	chemical oxygen demand
CP	chlorophenolics
CV	coefficient of variation
CYP 1A	cytochrome P450 1A
DHAA	dehydroabietic acid
dw	dry weight
ECF	elemental chlorine free
EOCl	extractable organochlorine
EROD	7-ethoxyresorufin *O*-deethylase
GC-MS	gas chromatography - mass spectrometry
LOEC	lowest effective concentration
log K_{ow}	logarithmic of octanol water partition coefficient
OC	organic carbon
PAH	polycyclic aromatic hydrocarbon
RA	resin acid
ROS	reactive oxygen species
SD	standard deviation
SQG	sediment quality guidelines
TOC	total organic carbon
WOE	weight of evidence
WS	wood sterol
ww	wet weight
v/v	volume/volume

1 INTRODUCTION

1.1 Sediments as a source of contaminants

During the last fifty years, chemical load from industrial and domestic sources into the environment, together with population size and consumption, has increased significantly. In 2004, the pulp and paper industry was a major industrial sector, accounting to 13 % of industrial production in Finland (Finnish Forest Industries Federation 2006), testifying thereby to the importance of research made on its environmental impacts. Since the 1990s new techniques in bleaching and effluent treatment have improved the quality of bleached kraft mill effluent (BKME) and decreased ecotoxicological effects in the receiving waters of pulp mills (Oikari & Holmbom 1996, Das & Jain 2001). However, concern about past contamination due to sediments as a source of exposure has been expressed (Wenning et al. 2005). Therefore it is important to assess how significant a risk both historical and modern BKME-contaminated sediments pose to the aquatic environment.

Sediments are comprised a heterogeneous mixtures of detritus, organic and inorganic particles, that settle at the bottom of a body of water (Power & Chapman 1992). They serve as a sink for many industrial contaminants (Brownlee et al. 1977, Eder & Weber 1980, Carlberg et al. 1987, Allard et al. 1988, Tavendale et al. 1995, Fattore et al. 1996, Koistinen et al. 1998, Leppänen & Oikari 1999). On the other hand, sediments can act as a source for bioactive chemicals, thus posing a long-term risk to the aquatic environment and even humans (Ingersoll et al. 1997). Sediments located near industrial sites have been receiving these contaminants – e.g. wood extractives and their derivates – for decades and therefore present a source of hydrophobic and persistent contaminants toxic to aquatic species (Eder & Weber 1980, Lee & Peart 1991, Kovacs & Voss 1992, Morales et al. 1992, Tavendale et al. 1995, Kukkonen et al. 1996, Leppänen & Oikari 1999, 2001, Lahdelma & Oikari 2005). Additionally, some organic contaminants are known to biotransform in anaerobic sediments

by aromatization, e.g. resin acids (RAs) biotransform into retene (Tavendale et al. 1997). Due to their relatively high lipophilicity (Peng & Roberts 2000) the contaminants are often sorbed by the organic matrix of particles and tend to remain reversibly associated with sediments (Alexander 1994). Although this initially results in reduced bioavailability, desorption of contaminants back into the water phase increases bioavailability and exposes benthic biota in the long term.

Because the total sediment concentration of contaminants does not serve a predictor of exposure or indicate the bioavailable portion of a contaminant (Bervoets et al. 1994), desorption of organic contaminants has been a recent focus due its contribution to bioavailability (ten Hulscher et al. 2003). According to current knowledge the fraction of a contaminant that desorbs rapidly from the sediment organic matter is bioavailable to sediment organisms (Cornelissen et al. 1998). Therefore, it is essential to study the desorption kinetics of contaminants together with their uptake and effects in the laboratory and in the field.

In nature, desorption of contaminants is accelerated by disturbances along the waterfront, e.g. bioturbation, tides, spring overturn and navigation, or by actions targeted at sediment, such as dredging (Ciarelli et al. 1999, Chapman et al. 2002, Eggleton & Thomas 2004, Landrum et al. 2004). In fact it has been suggested that elutriates mimic the open-water disposal of dredged material and consequently they have been used to determine the emerging toxicity of contaminated sediments. Although whole-sediment exposures seem to be more realistic, laboratory simulations of *in situ* exposures, elutriates or other extracts have been used to demonstrate disturbance situations or evaluate the bioavailable fraction of contaminants (Harkey et al. 1994, Liss & Ahlf 1997). Previous studies have demonstrated the toxicity of elutriates derived from sediments contaminated by PAHs and RAs to *Vibrio fischeri* and *Daphnia magna,* i.e. dissoluted fraction contain bioavailable and toxic elements (Hyötyläinen & Oikari 1999, Lahdelma & Oikari 2005).

BKME derived contaminants, such as RAs and wood sterols (WSs), are harmful to aquatic life because of their toxicity (Kruzynski 1979, Oikari et al. 1983, Bushnell et al. 1985, Mattsoff & Oikari 1987, Hickey & Martin 1995, Kennedy et al. 1996, Peng & Roberts 2000, Rissanen et al. 2003). The effects of BKME-loaded sediments have not been studied as widely as the effluents. Various adverse effects have been documented in biota at downstream sites (Hakkari 1992, Soimasuo et al. 1995, Culp et al. 2003), and the sediment-associated pollutants have been shown to be bioavailable to fish in the habitat (Karels et al. 2001). However, particular by in the field, it is difficult to distinguish the role of current and historical sediments as the origin of exposure when contaminants are continuously released from a nearby source and are present in the water column. Therefore *in situ* bioaccumulation and toxicity analyses in addition to laboratory exposures are essential in determining the role of contaminated sediments in the overall exposure scenario.

1.2 Bioavailability and bioaccumulation of sediment bound compounds – assessment of exposure

The bioavailability of neutral sediment contaminants is determined as potentiality for uptake or absorption by the internal organs of a sediment-dwelling organism, via pore waters or ingested particulates, including prey (Rand 1995), and it is a key factor of sorbed/bound compounds in sediment toxicity. When predicting the partitioning of an organic compound between sediment, pore water and biota, octanol-water partitioning coefficient, K_{ow}, is a good predictor (Landrum et al. 1989, Di Toro et al. 1991). The bioavailability of organic compounds sorbed to sediment particles decreases as log K_{ow} increases, and for compounds with log K_{ow} < 5, the major route for the accumulation is predominantly pore water (Thomann et al. 1992). For more hydrophobic compounds, the involvement of ingested sediment in accumulation increases (Landrum 1989). However, compounds such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans are very hydrophobic (log K_{ow} > 6) but do not bioaccumulate to the expected extent because of the ability of fish to biotransform them to more polar compounds (Opperhuizen & Sijm 1990, Muir & Servos 1996). Additionally, a large molecule size may limit the permeability of cell membranes (Opperhuizen et al. 1985).

In addition to contaminants, physical and chemical properties, the bioavailability of chemicals depends greatly on their interaction with the suspended material in sediment and the adsorption characteristics of the latter, including its particle size distribution (surface area), degree of aging, and organic carbon content and properties (Alexander et al. 1995, Park & Erstfeld 1999, Nikkilä et al. 2001, Lebo et al. 2003). Although hydrophobic compounds are initially sorbed to dissolved organic carbon (DOC), particulate organic carbon (POC) and deposited organic material (Alexander 1994, Kaplin et al. 1997, Kubicki & Apitz 1999, Lebo et al. 2003), benthic organisms can become exposed via the water column lying over the sediment, as chemicals dissolve and penetrate the sediment matrix or pass through micropores (Ball & Roberts 1991, Pignatello & Xing 1995). For ionizable compounds, such as RAs and CPs, the pH of the environment also affects the bioavailability.

Moreover, the characteristics of organisms play a role in bioavailability, and they include the size (surface area to volume ratio), general behavior and movement of a species within the sediments, modes and rates of feeding, ingestion and assimilation (Karickhoff & Morris 1985, Knezovich et al. 1987, Keilty et al. 1988, Landrum 1989, Boese et al. 1990, Leppänen 1995, Mayer et al. 1997, Standley 1997, Baumard et al. 1998, Leppänen & Kukkonen 1998). With respect to bioaccumulation, the most important factor is the lipid content of the organism, which in addition to sediment organic carbon, controls to a large extent the partitioning of organic compounds between sediment, water, and tissue (Lotufo et al. 1998).

Bioaccumulation is a general term describing the net uptake of contaminants from the environment by any or all of the possible routes from any source in the aquatic environment where the contaminants are present (Spacie et al. 1995). Benthic invertebrates are exposed to xenobiotic chemicals through several routes. One is direct contact to sediment, its particles and pore water, and another is through ingestion of contaminated sediment (Landrum 1989, Leppänen & Kukkonen 1998). Bioavailability and organism physiology are the two most important factors influencing the chemical contaminant body burden (Landrum et al. 1996). In sediment risk assessment, bioaccumulation can be used to study cause and effect relationships, e.g. when estimates of tissue concentrations are associated with toxic effects.

1.3 Biotransformation and genomic responses of aquatic animals to BKME contaminants – assessment of effects

For chemicals to elicit an adverse response or to have a toxic effect on an aquatic organism, the chemical must come into contact and react at an appropriate target site(s) on or in an organism at a high enough concentration and for a sufficient length of time. Contact-reaction between the organism and the chemical is called exposure. In the assessment of exposure the most significant factors are the type, duration, and frequency of exposure and the concentration of the chemical of interest (Rand et al. 1995).

Through patterns of elimination, the biotransformation of a toxic chemical is closely related to the body burden of that chemical. For chemicals with rapid biotransformation and elimination, it is commonly difficult to infer exposure from the concentration in animal tissues; instead such inference can be drawn from the concentration in excretory products (Spacie et al. 1995). From a toxicokinetic point of view, RAs are considered to belong to this group of aquatic pollutants, and thus their ecotoxicological risks are due to their continuous emission from industrial processes, in addition to sediment exposure by release/dissolution from BKME-sediments.

The toxicity of RAs is well known: They are acutely toxic to fish in the sub mg/l -range (Oikari et al. 1983, Johnsen et al. 1995), e.g. lowest observed effect concentration (LOEC) for dehydroabietic acid being around 20 µg/l (Oikari et al. 1983). At the cellular level RAs induce energetic imbalance in fish hepatocytes by decreasing cellular ATP content and activating glycolysis (Rissanen et al. 2003). Due to the amphiphilic nature of RAs, they alter the dynamics of the lipid bilayer (Toivola & Isomaa 1991). The most characteristic pathophysiological outcome of relatively high sublethal concentrations (above 0.4 mg/l) of RAs in fish is jaundice (Nikinmaa & Oikari 1982).

Since the 1970s, metabolites in fish bile have been used as a monitoring aid to indicate exposure of fish to certain aquatic pollutants (Statham et al. 1976, Carlberg et al. 1987, Legler et al. 2002). For instance, biliary excretion after

conjugation processes in the liver is the main excretion mechanism for pentachlorophenol, an organic anionic compound (Kobayashi 1979). Black et al. (1995) documented four different elimination routes for pentachlorophenol in rainbow trout, fecal elimination being the predominant one, followed by the urinary route. Although the majority of the conjugates are glucuronides, which are secreted in the bile (Glickman et al. 1977), sulphate conjugates can be eliminated as well (Kobayashi 1979). The biotransformation of RAs, e.g. dehydroabietic acid, has been studied in fish (Kruzynski 1979, Oikari et al. 1984), and 90-98 % of RAs in fish bile are found in the form of metabolic conjugates, indicating exposure and biotransformation (Oikari et al. 1984). Excretion is preceded by conjugation in the hepatocytes and secretion to the bile in the form of glucuronides, within the time frame of a couple of days (Oikari et al. 1984). With regard to the efficiency of the hepatobiliary excretion pathway, RA concentrations can be up to 100 000 times higher in bile than in the water the fish live in (Oikari 1986, Johnsen et al. 1995). In reality, besides RAs, several other xenobiotics as well as physiological ligands may also be metabolized by the same conjugation pathways and transportation routes in the liver and kidney (Black et al. 1995).

Overloading the mechanisms adapted for the regulation of endogenous compounds can cause unbalanced physiological status (Nikinmaa & Oikari 1982). Besides their membrane transport as glucuronides, a major proportion (80-95 %) of free RAs with pKa in the range 5.7-6.4 (Peng & Roberts 2000) is ionized at physiological pH. That the physiological transport system of organic anions is disrupted by exposure to RAs is supported by mechanistic studies performed using renal membrane models (Pritchard et al. 1991). Additionally, RAs cause several effects both in fish erythrocytes and hepatocytes, such as alterations in shape and decrease in intracellular pH (Brushell et al. 1985, Nikinmaa et al. 1999). Furthermore, RAs are known to impair hepatobiliary excretion of bile acids (Toivola & Isomaa 1991) as well as xenobiotics via effects on conjugation with glucuronic acid (Nikinmaa & Oikari 1982, Mattsoff & Oikari 1987).

Although biotransformation of pulp industry-derived contaminants has been widely studied in fish, it is not so in benthic invertebrates (Oikari et al. 1984, Leppänen & Kukkonen 2000, Verrangia Guerrero et al. 2002). Moreover, there is very little information on the possible bioaccumulation of BKME-related bioactive contaminants in benthic fauna. As in to vertebrates, biotransformation of invertebrates includes phase I, in which lipophilic xenobiotics are transformed into primary metabolites, and the phase II synthesis process, which continues by the conjugation of primary metabolites into highly hydrophilic metabolites, targeted at cellular transporters of organic anions and cations (Livingstone 1998).

Although the characteristic detrimental effects of RAs on fish at integrated physiological and cellular levels are known, the gene-level mechanisms of their toxicity remain to be studied. Microarray analyses make it possible to monitor in parallel both the large number of genes implicated in various cellular functions, and the metabolic and regulatory pathways. The salmonid fish

platform has been designed specifically to address responses to environmental stressors, toxicity and pathogens (Koskinen et al. 2004, Krasnov et al. 2007). It was used here for RAs and other BKME-derived contaminants to study more specifically the toxicity mechanism in the fish liver.

1.4 Exposure and risk assessment of contaminated sediments

Internationally, the risk assessment of contaminated sediments has been a steadily evolving area in environmental protection programs. A number of sediment quality guidelines (SQGs), which include sediment quality criteria, sediment quality objectives, and sediment quality standards, are based on ecological or environmental risk assessment (ERA). Environmental risk assessment evaluates two basic elements: exposure and effects (Suter 1990, SETAC 1997, EMEA 2006): 1) exposure is the interaction of stressors with receptors. Measures of exposure can include concentrations of contaminants or physical changes in habitat; 2) the analysis of effects evaluates changes in the nature and magnitude of toxicity and other bio-effects as exposure changes.

The characterization and assessment of exposure, which is the main focus in this thesis, is thus an essential part of the ERA process (USEPA 1992). More specifically, the purpose of characterizing exposure is to predict or measure the spatial and temporal distribution of a contaminant and its co-occurrence or contact with the ecological components of interest. The purpose of characterizing the ecological effects is to identify and quantify the adverse responses elicited by a stressor or stressors and, to the extent possible, to evaluate cause-and-effect relationships (USEPA 1992). Establishing causality can be obtained by linking the contaminants with observed effects by Koch's postulates used widely in biology (USEPA 1992). The negative effect of the toxicant must be regularly associated with exposure to the toxicant and any contributory causal factors. Since evidence of causality can be obtained from observational evidence or experimental data, causality is further strengthened when both types of information are available. Acceptance or rejection of causality can be based on criteria determined according to the so-called Hill's principles applied more widely in epidemiology (e.g. Guzelian et al. 2005): time order, strength, specificity, consistency, coherence, analogy, plausibility, experimental evidence, biological gradient, statistical probability and predictive ability. These criteria should be kept in mind in environmental risk assessment, since the causality outcome, the weight-of-evidence, is affected all of them.

In 1990, Peter Chapman introduced the Sediment Quality Triad, which consisted of sediment chemical analysis, examination of the benthic community structure on site and the *in situ* experimental exposures, and measurements of sediment toxicology (Fig. 1). Correlations of the three components are investigated in an attempt to define the significance of chemical measurements and to provide evidence of the causes of adverse effects. The Triad is suitable

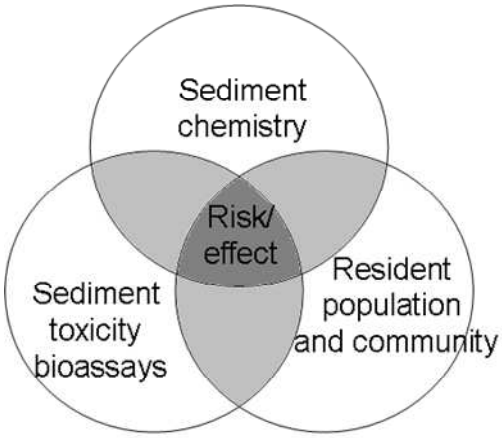

FIGURE 1 Conceptual model of the Sediment Quality Triad, which combines data from chemistry, toxicity and on site population studies. Chemistry and bioassay estimates are based on measurements in laboratory with sediments collected from the field. On site studies include measures of benthic population community structure, and can also include measurements of resident organism pathology and bioaccumulation or metabolism (based on Chapman et al. 1990).

for in-place pollutant control (contaminated sediments), for source control (on-going contamination of sediments), and for disposal applications (dredged material). In order to limit costs, tiered testing is applied in the initial sediment chemistry and sediment toxicity measures. Recently the Triad has been improved by the inclusion of bioaccumulation measurements (Chapman 1995, 2000, Chapman et al. 1997), as is also done in this thesis. Important questions when evaluating contaminated sediments are: are contaminants getting into the system? Are contaminants bioavailable? Is there a measurable response? Are the contaminants causing this response? These key questions are addressed in this thesis.

After assessing sediment characteristics and overall risk, a process called sediment management, a decision making process, considers the actions to be taken with respect to contaminated sediments. Five strategies, which are based upon evaluation of site-specific risks and goals, are possible: no action, which is applicable when sediments do not pose a risk; monitoring natural recovery, when sediments pose some risk, but the risk has been determined as low enough that natural processes may reduce the risk over time in a reasonably safe manner; *in situ* containment, when sediment contaminants are left in place but isolated from target organisms; *in situ* treatment; and dredging or excavation, which may be followed by ex situ treatment, disposal or reuse. The processes of evaluating the risk associated with each option are different and should always be designed site specifically to evaluate and support management goals and options (Apits & Power 2002).

2 OBJECTIVES

The objective of this thesis was to determine whether BKME-contaminated sediments pose a risk to the environment from the exposure point of view. Different environmental compartments were studied from lake sediment to fish populations in order to assess the presence, bioavailability, bioaccumulation, biotransformation and genomic responses, and risks of the compounds studied. More specifically, the following objectives were examined:

1. To study the dissolution of resin acids, retene and wood sterols from sediment into the water phase (I)
2. Investigate the bioavailability and bioaccumulation of resin acids, β-sitosterol and chlorophenolics in BKME-contaminated sediment to benthic invertebrates (II)
3. Assess the sensitivity and the capacity of the fish hepatobiliary system by measuring RAs in the bile of juvenile brown trout exposed subacutely to RAs in laboratory and in field (III, V)
4. Analyze genomic responses in the liver of juvenile brown trout exposed subacutely to resin acids in the laboratory and in the field (III, V)
5. Compare the biotransformation differences between species in hepatobiliary system by measuring resin acids in bile (IV)
6. Monitor the recovery of exposed fish populations in an area contaminated by black liquor spill (IV)
7. Assess the exposing ability and status of sediment derived resin acids to fish (IV)
8. Assess risk of BKME-contaminated sediments (I, II, IV)

3 MATERIALS AND METHODS

3.1 Chemicals

The organic compounds studied were seven RAs, of which three were pimaric-type (pimaric, sandaracopimaric, and isopimaric acid) and four abietic-type (dehydroabietic, abietic, neoabietic, and palustric acid) RAs, three WSs (β-sitosterol, stigmastanol and campesterol), cholesterol, retene (1-methyl-7-isopropyl phenanthrene), and five CPs (2,4,6-trichlorophenol, 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, pentachlorophenol and tetrachloroguaiacol).

3.2 Study areas

The study area was located in the western part of Southern Lake Saimaa, in Southeast Finland (Fig. 2) (I, II, IV, V). The mean depth of water in the area is 14 m and the water surface area 100 km^2. Near the city of Lappeenranta is an integrated bleached kraft pulp and paper mill. In 2003, the mill, which has been in operation since 1897, produced 670 000 t of mechanical pulp and 550 000 t of soft- and hardwood-based kraft pulp. The characteristics of treated effluent in June 2004, as reported by the company to the environmental authorities, were as follows: the effluent flow 105320 m^3/d, conductivity 251 mS/m, pH 8, Na concentration 510 mg/l, COD (chemical oxygen demand) 31 mg O_2/l, and AOX (adsorbable organic halogen) 3.2 mg/l. The total concentration of chlorophenolics, RAs, β-sitosterol, N and P approximated 4.2 mg/l, 47 mg/l, 69 mg/l, 233 mg/l, and 11 mg/l, respectively (Karels et al. 2001). The mill complex also includes a sawmill. To improve the water quality near the mill, a pump station was built in 1936 replacing a ca. 40 m^3/sec flow of water from the clean

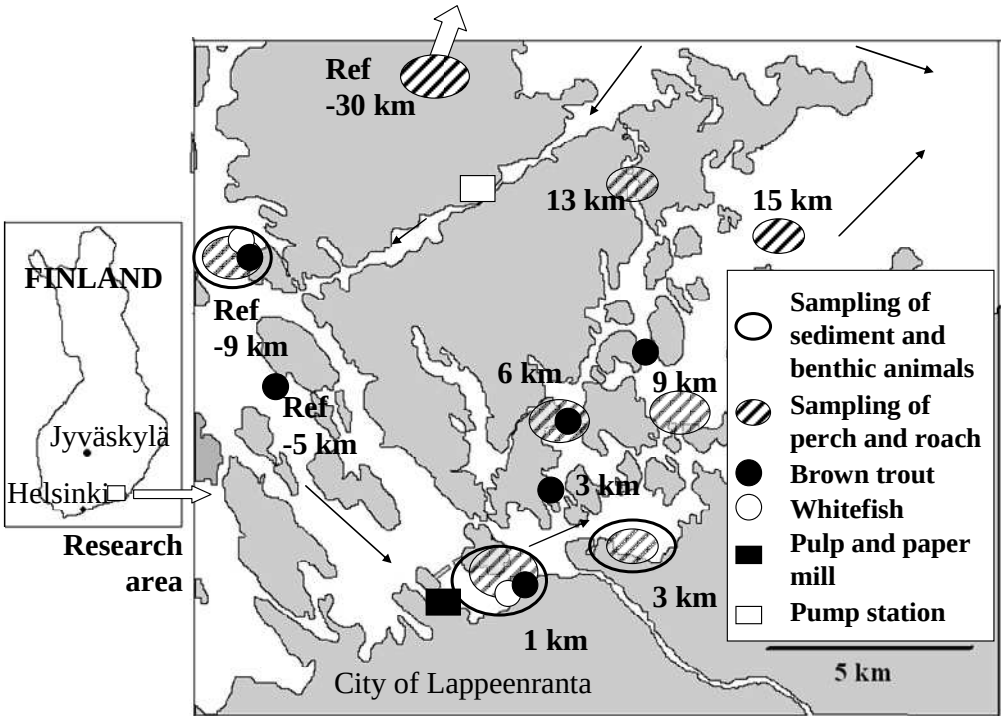

FIGURE 2 The study area in Southern Lake Saimaa, S-E Finland. Upstream areas (Ref -5, -9 km and -30 km) were reference areas. Sediment and benthic fauna sampling (in 2002) was done in areas 1 and 3 km downstream and -9 km upstream from the mill. Sampling areas for perch and roach studies (2003-2004) were located upstream (-9 and -30 km) and downstream (1, 3, 6, 9, 13 and 15 km) of the mill. The experimental exposure areas of brown trout (in 2004) were located in the areas 1, 4, 6 and 9 km downstream and -5 and -9 km upstream from the mill. Experimental exposure areas of whitefish (2004) were 1 km upstream and -9 km downstream from the mill. Black arrows indicate the direction of water flow in the lake. (Original base map by Riku Suutari.)

area of the lake to the watercourse upstream of the mill, causing a net flow from west to northeast in the study area and diluting the effluents (Fig. 2).

The study area was divided into upstream reference areas (Ref -5, -9 and -30 km), considered to be outside the mill's impact zone, and downstream exposure areas, located 1-15 km from the mill. The sediments were collected in the areas Ref -9 km, 1 km and 3 km downstream from the mill (I-II). Additional reference sediment was collected from an unindustrialized area, Lake Palosjärvi, Toivakka, Finland. The benthic invertebrates (Diptera and Oligochaeta) were collected in the same areas as mentioned above (II). Fish biles in 2003-2004 were collected in reference areas -9 and -30 km and in the downstream sampling areas (1-15 km) (IV). The experimental exposure of brown trout (2004) was done in the areas 1, 3, 6 and 9 km upstream and -5 and -9 km upstream from the mill. The whitefish exposure areas (2004) were -9 km upstream and 1 km downstream from the mill (V).

3.3 Accidental release of black liquor in 2003

In June 27th 2003, there was an unscheduled discharge of black liquor from a pulp and paper mill, which exceeded the mill's environmental permit conditions. Altogether 910 tons of black liquor was released into Southern Lake Saimaa, extending at least 15 km the water area to the north-east (Fig. 4) (Korjonen-Kuusipuro et al. 2004, IV). Moreover, the spill caused a large aerobically degrading organic load on the downstream lake system, decreasing the oxygen concentration from 10-11 mg/l to less than 3 mg/l for about two weeks (Korjonen-Kuusipuro et al. 2004). The BKME load of the pulp and paper mill during the spill in 2003 is shown in Table 1. Additionally, there were accidental releases of soft soap from June 16th to June 21st, and June 23rd, which did not exceed the mill's environmental permits.

The spills occurred during a start-up of the mill after major repair operations and a midsummer stoppage just before many Finns were starting their summer holidays in the lake district of Southern Lake Saimaa with its thousands of summer cottages. The Saimaa Water Protection Agency began a hydrological follow-up a few days after the spill occurred. There were large fish kills in the water area that local people use for recreational and minor commercial fishing purposes in addition to the dramatic decrease in lake water quality. These consequences attained lot of attention and debate in the local and national media until late autumn. The attention and activity of the local inhabitants, municipal authorities and local regional environmental authorities led to the consideration by the public prosecutor of bring in charges against the mill for exceeding environmental permit (Korjonen-Kuusipuro et al. 2004). Eventually, however, at the end of 2004, the charges were dropped. According to the law, the polluter is responsible for remedying a contaminated site. The monitoring of perch and roach populations started immediately after the company's request for an investigation into the post-spill situation in Southern Lake Saimaa (IV).

TABLE 1 BKME load of Kaukas pulp and paper mill from 24th of June to 6th of July 2003 (13 days), during the black liquor spill (Korjonen-Kuusipuro et al. 2004).

Parameter	Pulp and paper mill effluent (inflow)	Treated pulp and paper mill effluent (outflow)	Total		Permit limit
	t/13 d	t/13 d	t/13 d	t/d	t/d
Suspended solids	5 80	190	770	59	-
COD_{Cr}	2 900	360	3200	250	75
BOD_7	520	42	560	43	7.5
total N	43	25	46	3,5	0.75
total P	5	0.34	5.3	0.41	0.07

3.4 Sampling of sediments

Sediment samples from Southern Lake Saimaa were taken in 1997 by a Limnos-corer for dissolution of retene (I). For dissolution of RAs and WSs, the sediments were collected with an Ekman sampler from the four above-mentioned areas in 2002. The water depth in the sampling areas averaged 10 m. Five grab samples representing a sediment depth of 0 to max. 10 cm were pooled and sieved to remove large objects. Sediments were stored under a water layer in the dark at 4 ^{o}C (I).

3.5 Sampling of benthic animals

Samples of benthic animals were collected from Southern Lake Saimaa in October 2002 in two areas located 1 km and 3 km downstream from the pulp and paper mill (II). The reference area was 9 km upstream from the mill. Several samples, taken with an Ekman-Birge device were pooled and taken to the laboratory. Sediment was sieved with a 0.50 mm screen with lake water, and the animals identified by their order. The dominant orders were Diptera and Oligochaeta. Animals were kept in water from the same area for 6-8 hours in temperature of 0-4 °C to clean their gut and for sieving and were gently blotted by filter paper and stored at -80 °C until the analyses (II).

3.6 Perch and roach populations

Follow-up of the post-spill situation was conducted with RA analyses of biles from wild fish, perch *(Perca fluviatilis*) and roach (*Rutilus rutilus*) (IV). Bile samples were collected in four periods: the first was one month after the spill, in July 2003 (period I; 24th July - 6th August 2003), the second at three months, in September 2003 (period II; 9th - 22nd September 2003), third at eleven months, in May 2004 (period III; 13th - 24th May 2004), and fourth at 12 months after the spill, in July 2004 (period IV; 3rd - 14th July 2004). Fish were caught in sampling periods I and II with Nordic net serie and in 2004 with a weir. In 2004, the fish were caged at the sites of catch for two days to obtain a higher volume of bile fluids. Biles were frozen in liquid nitrogen and kept at -80 °C until RA-analyses (IV).

3.7 Setup of laboratory exposures

3.7.1 Dissolution experiment (I)

The dissolution potency of RAs and WSs was examined at three different stirring speeds. The experimental design is described in detail in (I). In the first experiment, contaminated field sediment (1 km) and Ref -9 km sediment were mixed (sediment/water ratio; 1 + 4 v/v) with artificial freshwater in Kimax glass tubes (50 ml) to a total liquid volume of 25 ml. The suspensions were mixed by a mechanical horizontal shaker (200 rpm) at 8 ^{o}C. Sampling was performed after 0, 1, 3, 7, 14 days.

Similarly, in the second experiment, dissolution of retene was examined over 23 days from 1 km sediment. Samples were prewashed twice with artificial fresh water to ensure that retene present originally was mainly solid-sediment-associated. The headspace in the 250 ml jars was half of the total volume and artificial freshwater was added (sediment/water ratio; 1 + 4 v/v). The suspensions were mixed with a mechanical horizontal shaker (110 rpm) at 8 ^{o}C. Subsamples from the suspensions (12 ml) were removed in the following time-series: 0, 0.07, 0.23, 0.7, 2.3, 7 and 23 days.

Third dissolution of WSs was investigated in a 43-day experiment. Lake Palosjärvi sediment was used as a spiking matrix for a WS dissolution experiment. A mixture of wood-derived sterols (Ultra sitosterol) containing 79% of β-sitosterol, 16% of stigmastanol and 5% of campesterol was dissolved to diethyl ether and added to wet sediment with mechanical mixing at a temperature of 15 ^{o}C as in (I). After 33 d contact time, sediments were washed twice with artificial freshwater (50:50, v/v). The samples contained 96 µg/g dw (spiked sediment a) and 478 µg/g dw (spiked sediment b) of β-sitosterol, stigmastanol and campesterol in total. Sediments were introduced into an Erlenmeyer flask (250 ml) and the suspensions were mixed with a mechanical horizontal shaker at 100 rpm at 8 ^{o}C. Subsamples (8 ml) were taken from the suspensions immediately and after 2, 10, 16 and 43 days. While largely settled over this period, the suspensions were investigated over an additional 24 h at a faster stirring speed (160 rpm).

3.7.2 Benthic invertebrates (II)

The laboratory culture of *Lumbriculus variegatus* Müller (Oligocheata) was maintained at 20 ± 1 °C in a 16:8 h light:dark cycle in an aerated 20 l glass aquarium containing artificial freshwater (Ca + Mg hardness 1.0 mmol/l) (Nikkilä et al. 2001). Larvae of Dipteran, *Chironomus plumosus*, collected from Lake Mälsäjärvi (Tampere, Finland), were obtained from a local fishing store (Perhokolmio, Jyväskylä, Finland). The animals were acclimated in laboratory conditions for one week before exposure. Water was aerated artificial freshwater as above and sand was used as a substrate. Animals were fed three

times a week with a fish food, but unfed for two days before the beginning of the experiment (II).

L. variegatus and *C. plumosus* were exposed to authentic BKME sediments (II) collected from Southern Lake Saimaa with an Ekman sampler at 1 km (Sed 1 km) and 3 km (Sed 3 km) downstream from the pulp and paper mill (Fig. 2). Reference sediments were from Lake Palosjärvi (Ref Palos) and site 9 km upstream from the mill (Ref -9 km). Since the total organic carbon content of the Ref Palos sediment was 23 %, we adjusted it with fine prewashed quartz sand (Ø 0.05-0.25 mm) to 5.6 %, approximately the same as that in Ref -9 km (TOC 6 %). The exposures were performed in 2 l glass beakers at 15 °C, 16:8 h light:dark cycle. 250 ml of sediment (depth 3 cm) and 1 l of aerated artificial freshwater (pH 6.6) was added to the glass beakers one day before 100 oligochaetes and 70 chironomid larvae were transferred. Three separate replicates were made from each, with an additional blank prepared without the animals, as in (II). Test duration was 14 d, except for one additional group with 1 km sediment which continued for 28 days to check for the possible need of a longer exposure time.

Sediment samples were taken before and after exposure, and the water samples collected at the end. At the end of the exposure, a one to two cm upper layer of sediment was sampled separately from the layer beneath. Animals were collected from the sediment with forceps and placed in clean artificial freshwater for 6 hours to purge their guts. Animals with empty guts were carefully blotted dry, weighed and stored in glass vials at -20 °C.

3.7.3 Laboratory exposures with brown trout (III)

Hatchery-reared juvenile 1-year-old brown trout (*Salmo trutta* m. *lacustris*) were studied in January-February 2005 (III). Before the experiments, the brown trout were acclimatized in continuously changing unchlorinated active carbon filtered municipal water (pH 7.7 ± 0.1, 10.0 ± 0.5 °C) for two weeks. Fish were fed on alternate days with pellet food *ad libitum* with a ratio equal to 0.5 % of fish biomass. Feeding was stopped four days before the experiment, and during the exposure fish were not fed.

Eight RAs in a Polish wood rosin (Hercules Co., Wilmington, USA) containing 95.5 % RAs were used for exposures. A stock solution with 1200 mg/l wood rosin was prepared as in (III). Time series (0.1-192 h) was performed at 10.0 ± 0.2 °C. The initial concentration of the two aquaria with added RAs, performed 12 h before animals were transferred, was 19 µg/l. The average RA concentration in water was 8 µg/l. Fish were transferred gently via water to avoid stressing the animals. In order to prevent any possible aerial contamination by RAs of the control treatment, fish exposed to RAs and their controls were held in separate rooms. When the exposure was initiated, each aquarium (volume 2160 l) contained 24 randomly transferred brown trout. The brown trout were distributed into three identical all-steel tanks of standing preaearated water. Sampling was done with the experimental animals after 0.1, 6, 12, 24, 48, 96, 168 and 192 h, and with controls at 0.1, 96 and 192 h. Fish were netted from a tank, and stunned with a blow to the head. Each fish was

dissected, and its gallbladder together with bile was separated, frozen in liquid nitrogen (LqN), and stored at -80 °C. Water samples were collected before and after the exposure period, and daily to obtain a combined sample to represent the average of the total exposure time.

In concentration-dependence exposure, the preacclimatized fish were randomly distributed into five identical all steel tanks (540 l), six animals in each. Physical conditions were as in (III). Fish in four RA concentrations and one control were kept in water without aeration for ten days. Measured total RA concentrations were initially 1, 7, 18 and 110 µg/l, and averaged 0.6, 4, 14 and 78 µg/l. In order to maintain RA concentrations near target levels, half of the water volume was changed on the fourth day. Liver samples for the microarray analyses were dissected, rinsed with cold 0.7 % NaCl, dried with blotting paper, and frozen immediately in LqN. Liver samples were stored in LqN until analyzed.

3.7.4 Multispecies exposure to resin acids (IV)

Hatchery-reared juvenile 1-year-old brown trout (*Salmo trutta* m. *lacustris*), rainbow trout (S*almo gairdneri*), and whitefish (*Coregonus lavaretus)*, were studied in March 2005. Perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) were caught with a weir from Lake Konnevesi, Konnevesi, Central Finland, an unindustrialized area, and transported to the laboratory. All species were placed in fish tanks (3 °C), and the temperature was adjusted gradually to 10 °C over three days (IV).

Before the experiments, fish were acclimatized in continuously changing unchlorinated active carbon filtered municipal water (pH 7.7 ± 0.1, temperature 10.0 ± 0.5 °C) for two weeks. The fish, including perch and roach, were fed on alternate days with pellet food *ad libitum* with a ration approximately 0.5 % of fish biomass. Feeding was stopped four days before the experiment, and during the exposure fish were not fed.

A stock solution of eight RAs was prepared as described above. The initial concentration in the two aquaria with added RAs, made 12 h before the transfer of animals, was 60 µg/l. At the start, 10 randomly chosen fish per species were placed in two identical all-steel tanks with standing preaearated water (volume 2160 l, 10.0 ± 0.2 °C), 50 fish in total. On the fourth day, half of the water volume was changed.

Samplings were done after seven days. Fish were randomly netted from a tank, without disturbing the other animals therein, and stunned with a blow to the head. Biles were collected as described above (III). Water samples were collected before and after the exposure period, and daily for a combined sample to represent the average of the entire exposure time, and stored as in (III).

3.8 Experimental exposure of fish in the field (V)

One-year-old brown trout (*Salmo trutta* m. *lacustris*) and plankton-feeding coregonid whitefish (*Coregonus lavaretus* s.l.) were used. Brown trout were transferred first to the wet laboratory of the University of Jyväskylä for acclimatization (7 days, 10 °C). Whitefish were transferred directly from the hatchery.

Fish were transferred to Southern Lake Saimaa field sites (Fig. 2) for caging as described elsewhere (Soimasuo et al. 1995, Oikari 2006) (V). Animals were not fed for the last three days before their transfer to the field. Oval 280-liter cages were submerged on the bottom of the lake at a depth of 4-5 m, and held there for 10 d without the animals being fed. Brown trout (two cages per site) and whitefish (one cage per site) were exposed in separate units. Liver and bile samples were collected by the lake shore near each experimental site. Animals were randomly netted form the cage, immobilized, and sampled as described above for their bile and liver.

3.9 Analytical methods

3.9.1 Determination of resin acids, chlorophenolics and wood sterols in water, sediment, and animals (I-V)

Resin acids, WSs and retene in sediment-water elutriates (I) and sediment (II) were analysed by gas chromatography mass spectrometry (GC-MS) using the method modified from Leppänen and Oikari (1999). The method is described in more detail in papers (I, II). Resin acids in water (II, III, IV) was analysed according to Soimasuo et al. (1998a). The method is described in more detail in (II, III, IV).

Free and bound RAs, β-sitosterol and CPs in benthic invertebrates (II) were analysed with GC-MS using the modified method described by Lahdelma and Oikari (2005) and (III), modified from Oikari and Ånäs (1985).

The free and conjugated RAs in the bile of brown trout (III, IV), rainbow trout (IV), whitefish (IV), perch (IV) and roach (IV) were analysed by GC-MS using the modified method described by Oikari and Ånäs (1985), and in (III,IV).

3.9.2 Total organic carbon (I)

Concentrations of total organic carbon (TOC) in freeze-dried sediment samples were determined using an EA 110 Elemental Analyzer equipped with Eager 200 software (CE Instruments, Milan, Italy) (II). Before the measurement, a drop of hydrochloric acid (3 mol/l) was added and the sample was kept at 80 ^{o}C for 12 h to dry and remove inorganic carbon as CO_2.

3.9.3 EROD analyses (V)

Hepatic CYP1A activity of brown trout was measured from the mitochondrial supernatant (S9 fraction) as the deethylase of 7-ethoxyresorufin (Burke et al. 1985, Hodson et al. 1996, V). Samples were held in LqN until analyzed (about eight weeks). The protein concentration of the assay sample was measured by a Bio-Rad DC kit (CA, USA) using bovine serum albumin as standard.

3.9.4 Transcriptome analyses (III, V)

The design of the microarray is described in detail in Krasnov et al. (2005) (III,V). In brief, the livers, frozen and preserved in liquid nitrogen, were homogenized using glass homogenizers, and total RNA was extracted using Tri Reagent (Sigma) or Trizol (Invitrogen). Labeling with cyanine dyes Cy-3 and Cy5-dCTP (Cy5-2'-deoxycytidine 5'-triphosphate) was done using Superscript III (Invitrogen) and oligo(dT) primer; cDNA was purified with Microcon YM30 (Millipore). Each sample was hybridized for two microarrays. For the first slide, test and control cDNA were labeled with Cy5 and Cy3, respectively, and for the second array the dye assignments were reversed. The slides were pretreated with 1% BSA, fraction V, 5 x SSC, 0.1 % SDS (30 min at 50 ^{o}C) and washed with 2 x SCC (3 min) and 0.2 x SCC (3 min) and hybridized overnight in a cocktail containing 1.3 x Denhardt's, 3 x SCC 0.3 % SDS, 0.67 µg/µl polyadenylate and 1.4 µg/µl yeast RBA. Scanning was performed with a ScanArray 5000 and the images processed with a TIGR Spotfinder.

The measurements in spots were filtered by criterion (I-B)/(SI+SI)>1, where I and B are the mean signal and background intensities and SI, BI are the standard deviations. After subtraction of the background, Lowess normalization (Cleveland et al. 1992) was performed separately for each slide, using the dye swap hybridization design, each gene was analyzed in 12 spot replicates. The data from the two slides were consolidated and differential expression was assessed by the difference between the log-expression ratios at the reverse labeling (Student's t-test, $p < 0.01$).

In the laboratory experiment (III), analyses were done from each RA-exposure concentration, and fish exposed to 78 µg/l were divided into two subgroups, four in each, and analyzed separately. In caging experiment (V), analyses were done on fish from cages located at 1 km, 4 km and 9 km from the source of BKME. Brown trout from both unpolluted sites were used as controls.

4 RESULTS

4.1 Sediment characteristics

The RA concentrations in the sediment of Southern Lake Saimaa showed a clear relationship to distance regarding to the location of the sediments from the pulp mill (Table 2). In the reference area (9 km from the mill), the total RA concentration was 12 µg/g dw, in the area 1 km downstream from the mill, it was 3300 µg/g dw, and at 3 km site 2100 µg/g dw. Dehydroabietic acid predominated in all sediments: 40 % for Ref -9 km, 62 % for 1 km and 54 % for 3 km (Table 2). The vertical stratification of the RAs in Southern Lake Saimaa sediment is shown in Table 3.

4.2 Dissolution

The first set of dissolution experiments simulated a ''worst case'' situation. This was done by rapid stirring (200 rpm) for 14 days, until desorption equilibrium (I). Resin acids achieved a steady state in three days, whereas sterols reached equilibrium in seven days (Fig. 3). The highest concentration of dissolved sterols (3.6 mg/l) exceeded that of RAs (2.3 mg /1), although RAs were more abundant in the sediment (Table 2). The most abundant RA was dehydroabietic acid, which accounted for about 50% of the total RA concentration in the elutriate (Fig. 3). By comparison, the desorption of sterols and RAs from the reference matrix was very low, remaining under 0.05 mg/l.

Release of retene was investigated with a somewhat slower stirring speed (140 rpm) than that used for RAs; however, full mixing throughout the sediment and water was achieved. Importantly, the desorption outcome of retene was distinctly slower than that of wood extractives. The concentration of dissolved retene increased throughout the experimental period, peaking (13 µg/l) at the end of the experiment, after 23 days.

TABLE 2 The mean initial concentrations of resin acids (RAs), wood sterols (WSs), chlorophenolics (CPs) and total organic carbon (TOC) in sediments of Southern Lake Saimaa (Ref -9 km, 1 km, 3 km) and Lake Palosjärvi (Ref Palos) used in the 14-d exposure with benthic invertebrates. The RAs and WSs concentrations were analyzed from the 10-15 cm uppermost layer of sediment, and CPs from the uppermost 5 cm of surface sediment, in areas with water depth of 10 m.

Measurement (µg/g dw)	Reference (Ref Palos)	Reference (Ref -9 km)	1 km from the mill (Sed 1 km)	3 km from the mill (Sed 3 km)
Pimaric-type RAs	3.4	3.8	890	310
Pimaric acid	0.8	0.9	360	41
Abietic-type RAs	17	7.8	2400	1800
Dehydroabietic acid	8.2	6.3	2000	1200
Total RAs [1]	21	12	3300	2100
WSs [2]	15	50	1750	250
β-sitosterol[3]	12	20	900	100
CPs [4]	n.a.	< 0.1	500	< 0.1
Retene [5]	n.a.	0	1700	n.a.
TOC (%)	23	6.1	18	20

[1] Dehydroabietic, abietic, pimaric, isopimaric, palustric, sandaracopimaric and neoabietic acid

[2] β-sitosterol, stigmastanol and campesterol (Lahdelma & Oikari, 2006).

[3] Lahdelma & Oikari, 2006

[4] 2,4,6-trichlorophenol, 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, pentachlorophenol and tetrachloroguaiacol (Lahti 2005)

[5] Leppänen & Oikari 1999

n.a. = not analyzed

TABLE 3 Vertical sediment profile of resin acids and wood sterols in sediments of Southern Lake Saimaa (Ref -9 km, 1 km, 3 km) (Lahdelma & Oikari 2005, 2006).

Compound (µg/g dw)	Area	Depth (cm) 0 - 5	5 - 10	10 - 15	15 - 20	20 - 25	25 - 30
Resin acids	Ref -9 km	10	10	3	0.4	0.3	0.5
	1 km	720	630	420	360	430	920
	3 km	470	390	790	940	240	350
Wood sterols	Ref -9 km	50	38	35	17	11	22
	1 km	1760	1550	1340	1080	1390	1530
	3 km	250	300	770	630	260	250
β-sitosterol	Ref -9 km	30	20	20	8	5	10
	1 km	920	800	700	490	600	670
	3 km	110	120	330	280	120	130

[1] dehydroabietic, abietic, pimaric, isopimaric, palustric, sandaracopimaric and neoabietic acid (Lahdelma & Oikari 2005).

[2] cholesterol, cholestanol, campesterol, campestanol, stigmasterol, β-sitosterol, stigmastanol (Lahdelma & Oikari, 2006).

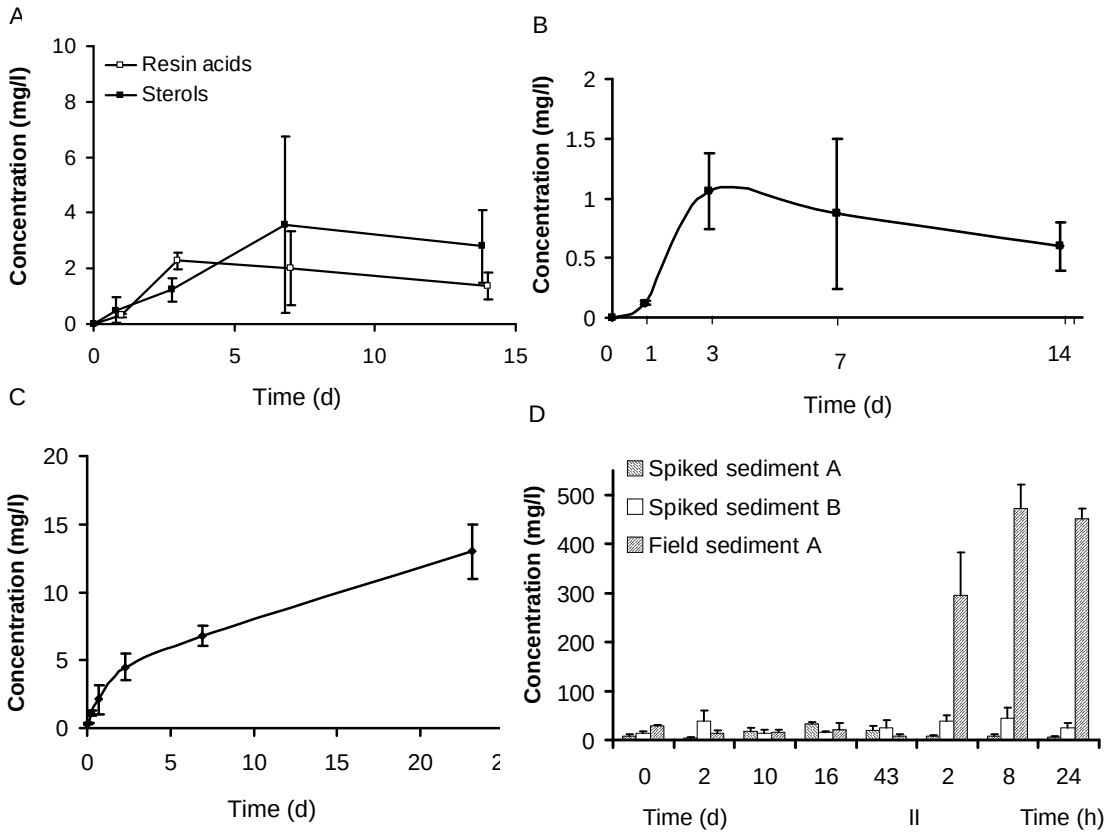

FIGURE 3 A) Dissolved concentrations of resin acids (sum of dehydroabietic, abietic, pimaric, isopimaric, palustric, sandaracopimaric and neoabietic acid) and wood sterols (β-sitosterol stigmastanol and campesterol), and B) dehydroabietic acid from sediment contaminated by pulp and paper industry effluents (1 km) in Lake Saimaa, during the 14-day experiments. C) Dissolved concentration of retene in pulp and paper industry-contaminated sediment from Lake Saimaa (1 km), during the 23-day experiment. D) Dissolved concentrations of wood sterols in field-contaminated sediment from Lake Saimaa (1 km), and in the spiked sediments over 43 days (five columns on the left) and in the subsequent 24-h experiment (three columns on the right). The bar depicts SD of three replicate experiments.

Desorption of extractives was further investigated in the third experiment in spiked and field-contaminated sediments at an even slower stirring speed (100 rpm) for 43 days. During this experiment, only the sediment surface was mixed, i.e. total resuspension did not take place. Sterols dissolved more easily from the spiked sediment matrix than from the naturally contaminated sediment. It can be supposed that the desorption and absorption of sterols followed each other on the sediment surface, as the variation between replicates was high and depended randomly on the time of sampling. After an acceleration of speed to 160 rpm for 24 h, in the total dissolution test after incubation for 43 days, WSs

dissolved more effectively from the field-contaminated sediments than spiked (Fig. 3).

4.3 Uptake of contaminants into benthic invertebrates

Uptake of RAs, β-sitosterol and CPs into resident benthic invertebrates were analysed in samples collected near the pulp mill (II). The highest RA concentration in Diptera was found at 3 km distance from the mill, 7800 µg/g dw, which was over 14 times higher than in the reference area (Fig. 4). For Oligochaeta, the concentration of RAs at 1 km (1700 µg/g dw) was nearly two times higher and in the 3 km area (3300 µg/g dw) nearly four times higher than in the reference area (860 µg/g dw). In contrast to RAs and β-sitosterol, the higher concentration of CPs was at 1 km from the pulp mill, 1000 µg/g dw for Diptera and 700 µg/g dw for Oligochaeta (Fig. 4). As with RAs, the highest concentration of β-sitosterol in Diptera was at 3 km downstream from the mill (3600 µg/g dw) (Fig. 4). For Oligochaeta, β-sitosterol levels were higher in the 1 km area, 700 µg/g dw, than 3 km area, where the concentration was below detection limit. There were no statistically significant differences between the

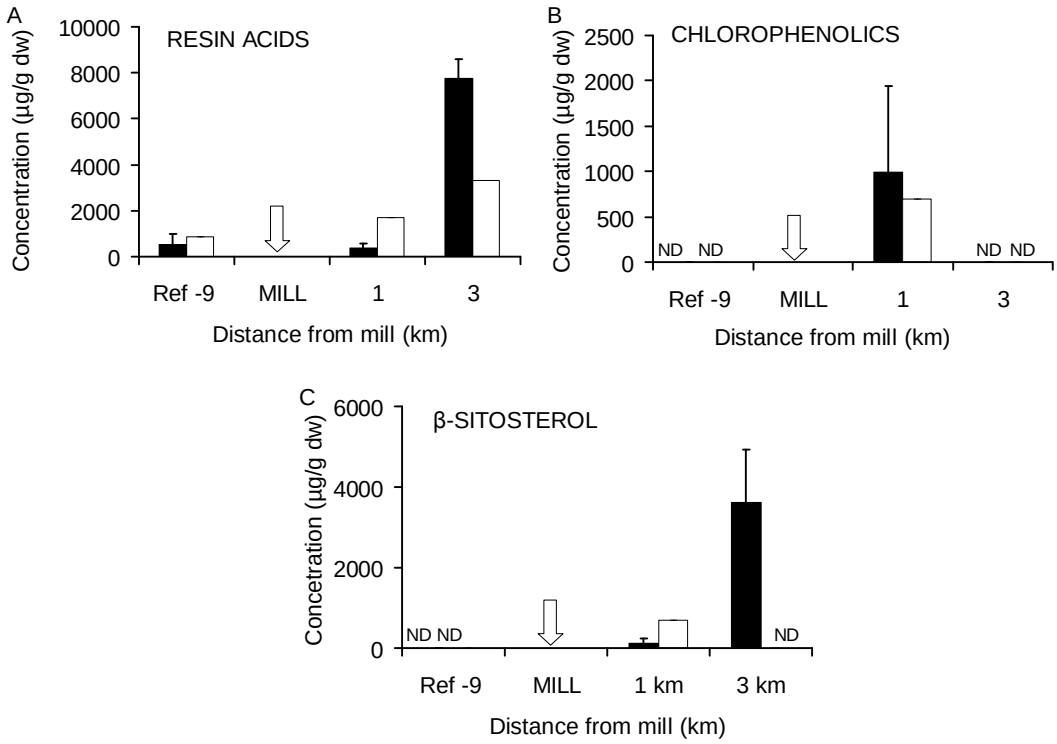

FIGURE 4 Concentration of A) resin acids, B) chlorophenolics and C) β-sitosterol in resident Diptera and Oligochaeta in Southern Lake Saimaa in upstream reference area (-9 km) and downstream exposure areas (1 km and 3 km) from the mill (II). ■ = Diptera, □ = Oligochaeta, ND = not detected.

sampling areas for any of the compounds analysed.

The validation experiment revealed uptake of BKME contaminants in laboratory situations. The behavior of test animals was observed daily during the exposure. According to visual examination, *C. plumosus* caused bioturbation, which made the water layer turbid in all the exposure beakers occupied by both chironomids and oligochaetas. However, the turbidity of the water decreased over the 14-d exposure period, especially in the reference exposures (Fig. 5).

In Sed 1 km, *C. plumosus* and *L. variegatus* did not spend time on the sediment surface. Instead, in all the exposure sediments *L. variegatus* had burrowed into the sediment one day after the experiment started. Mortality was observed in all sediment exposures (Table 4); however, it was not related to the distance from the pulp mill.

In the reference sediment (Ref Palos), the body concentration of RAs in *C. plumosus* was 7 µg/g dw (Fig. 6a), which was very similar to Ref -9 km (5 µg/g dw). In the exposures with contaminated sediment, the concentration of RAs in *C. plumosus* were lower in Sed 1 km (20 µg/g dw) than in Sed 3 km (70 µg/g dw) (Fig. 6).

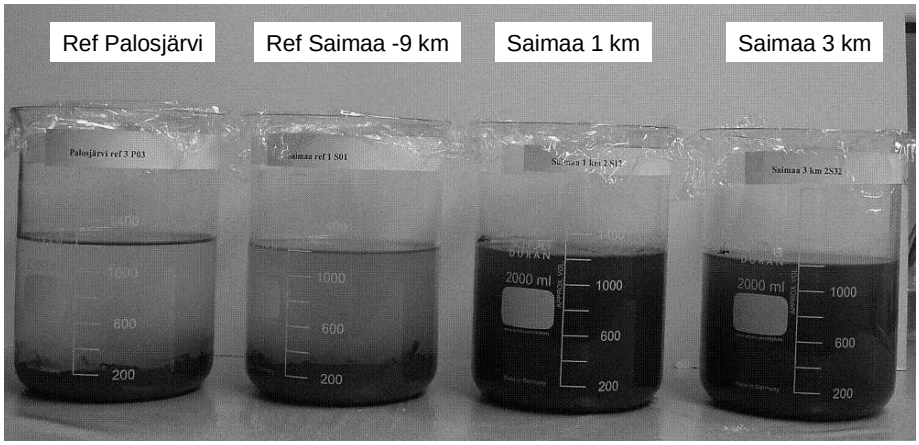

FIGURE 5 Exposure beakers of 14-d sediment exposure with *C. plumosus* and *L. variegatus*. The water clarity increased with distance from the pulp mill; Reference sediments Ref Palosjärvi and Ref Saimaa -9 km were clear compared to sediments 1-3 km downstream from the mill.

TABLE 4 Total mortality percentage (with SD) in BKME-contaminated sediment exposures for 14 d (References Palos and -9 km, exposure sediments 1 and 3 km from the mill) of *C. plumosus* and *L. variegatus*.

Sediment	*C. plumosus*	*L. variegatus*
Ref Palos	25 (6)	27 (15)
Ref -9 km	10 (3)	20 (3)
1 km	19 (3)	20 (2)
3 km	15 (6)	24 (8)

With respect to β-sitosterol in *C. plumosus*, the concentrations in Ref Palos and Ref -9 km were 20 and 10 µg/g dw, respectively. In the exposures with BKME-contaminated sediment, the concentration of β-sitosterol in *C. plumosus* in Sed 1 km (50 µg/g dw) averaged about one sixth of that in Sed 3 km (210 µg/g dw) (Fig. 6b). Additionally, when exposed to Sed 1 km, the concentrations of β-sitosterol remained unchanged from 14 to 28 days.

In *C. plumosus*, the concentration of cholesterol was 40 µg/g dw in Ref Palos, but much higher in Ref -9 km (470 µg/g dw). Similar patterns were seen with β-sitosterol, as the concentration of cholesterol in *C. plumosus* exposed to sediment collected one km from the mill (110 µg/g dw) was one fourth of that in Ref -9 km, and five times lower than in Sed 3 km (570 µg/g dw).

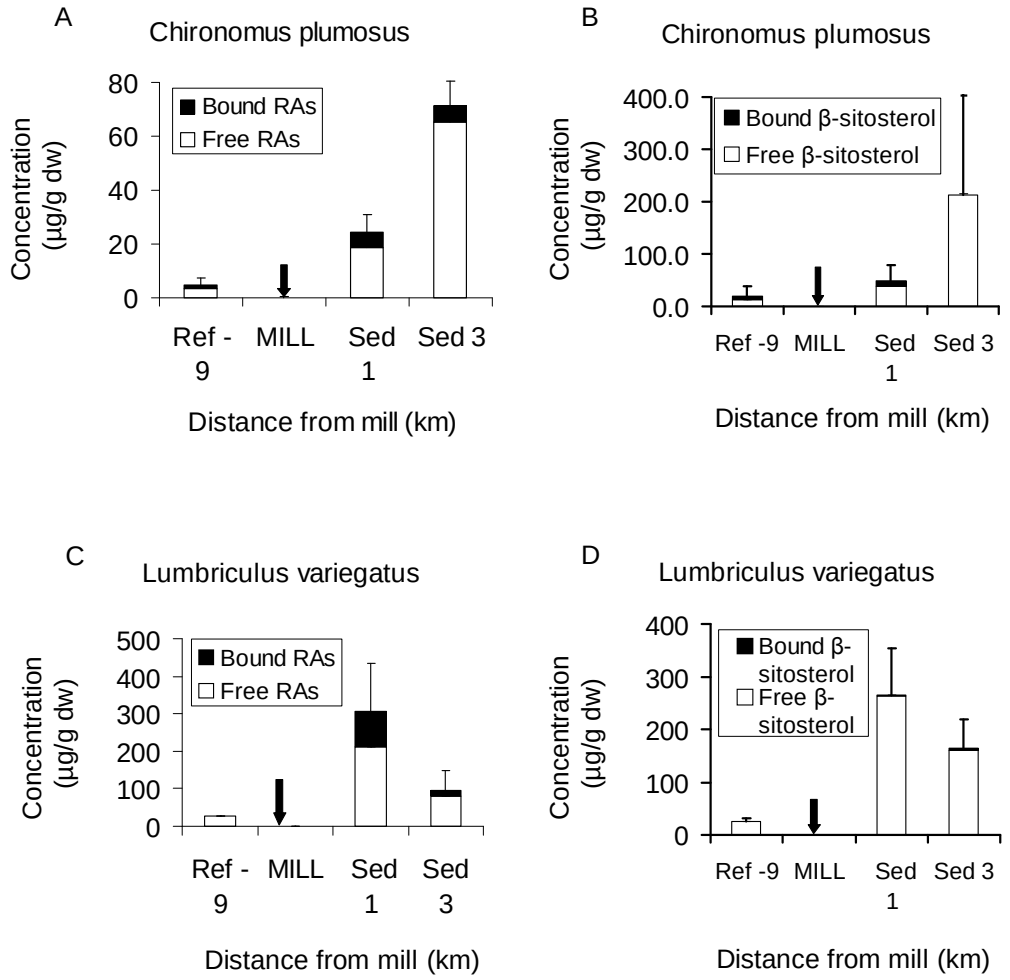

FIGURE 6 Concentrations of free and bound fractions of resin acids (RAs) (dehydroabietic, abietic, pimaric, isopimaric, palustric, sandaracopimaric and neoabietic acid) and β-sitosterol in *C. plumosus* (A, B) and *L. variegatus* (C, D) exposed to sediments collected from Southern Lake Saimaa contaminated by pulp and paper mille effluent. Ref -9 km represents reference exposure (14-d), Sed 1 km and Sed 3 km represent exposures in sediment collected downstream from the mill in 14 d. The bar depicts SD of three replicate experiments.

In *L. variegatus*, the concentrations of RAs in the Ref Palos sediment were 40 µg/g dw, and in Ref -9 km 30 µg/g dw (Fig. 6c). In contrast to *C. plumosus*, *L. variegatus* showed a relationship to distance in their body residues of RAs. In the exposures with BKME-contaminated sediment, the concentration of RAs was 70 % lower at 3 km than 1 km (Fig. 6c).

The concentrations of β-sitosterol in *L. variegatus* exposed to Ref Palos sediment were 30 µg/g dw and to Ref -9 km sediment 20 µg/g dw. In the exposures with BKME-contaminated sediment, β-sitosterol behaved similarly to RAs: the concentration decreased with distance from 270 to 160 µg/g dw (Fig. 6d). There was no difference in the bioaccumulation of β-sitosterol between 14 and 28 d.

In *L. variegatus* the concentrations of cholesterol were higher that in *C. plumosus*, indicating physiological differences between species. In the Sed 1 km the cholesterol level in *L. variegatus* was 9300 µg/g dw, which is over 10 times higher that in Ref -9 km (730 µg/g dw).

4.4 Biotransformation capability of benthic invertebrates

The difference in biotransformation between Diptera and Oligochaeta was investigated by extracting and analyzing the free and conjugated/bound fraction of RAs in animal tissue. In feral populations, conjugation of all the compounds studied was found in Diptera. The hydrolyzed fractions averaged 11, 28 and 63 % for β-sitosterol, RAs and CPs, respectively. In contrast, Oligochaeta showed no contaminants conjugation, i.e. all the compounds were found in the free fraction.

The properties in biotransformation of laboratory-exposed *C. plumosus* and *L. variegatus* were different from those of the resident benthic invertebrates. The results revealed that in *C. plumosus* in the laboratory experiment 13 % of total RAs were in conjugated form, the rest being in free form. In contrast to the feral Oligocheata, in *L. variegatus* in the laboratory experiment 27 % of RAs were in conjugated form (Fig. 6).

4.5 Bioconcentration of resin acids in fish bile

4.5.1 Time- and dose-dependent exposures

In order to find out the time needed to reach a steady concentration of RAs in the bile of brown trout, a time series with a concentration of 8 µg/l RAs in water was conducted (III). The concentration of total RAs in bile at 0.1 h was 5 µg/ml (Fig. 7a), which was already higher than in controls. The sum of seven RAs revealed a steady concentration of RAs in bile in 24 h, stabilizing thereafter (160-180 µg/ml of RA in bile). Pimaric acid was the most abundant RA in bile

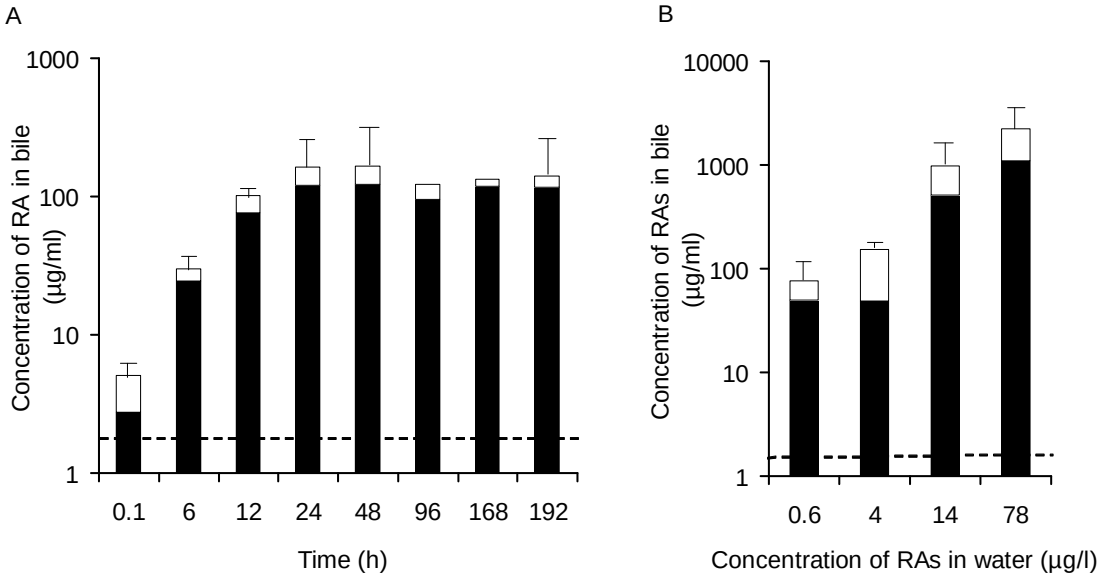

FIGURE 7 Mean concentration of total resin acids (RAs; µg/ml) in bile of brown trout on logarithmic scale: A) time-dependent exposures with water concentration of 8 µg/l of RA up to 192 h and B) in concentration-dependent exposures with 0, 0.6, 4, 14 and 78 µg/l of RA for 10 d. The total column depicts the mean and the bar the standard deviation (SD). Dashed line shows the background concentrations of total RAs in control exposures (no added RAs). A statistically significant difference in total RAs in bile was found between all the exposure concentrations compared to the 0-control (ANOVA, $F=3.50$, $P < 0.05$). □ = abietic-type RAs (dehydroabietic, abietic, neoabietic and palustric acid), ■ = pimaric-type RAs (pimaric, sandaracopimaric and isopimaric acids).

(Fig. 8). Thus, in contrast to the composition of RAs in water, the ratio of pimaric- and abietic-types in bile changed dramatically, showing a dominance pimaric-type. With time, the proportion of pimaric-type acids increased some more from 54 % (0.1 h) to 87 % (162 h) in the time series exposure (Fig. 8).

Numerically, the highest exposure concentration (78 µg/l) resulted in 2250 µg/ml of total RAs in bile (Fig. 7b). Additionally, in the dose experiment the behavior of the pimaric- and abietic-type ratio showed a similar change to that in the time series, but to a lesser extent. The ratio of pimaric- to abietic-type RAs in bile was 66:34 in the 0.6 µg/l, 32:68 in the 4 µg/l, 53:47 in the 14 µg/l, and 49:51 in the 78 µg/l exposures (Fig. 8).

The average concentrations of the ambient RAs were used to calculate the log $BCF_{bile(RA)}$ values (Table 5). The log $BCF_{bile(RA)}$ of total RAs, which reflects the relative excretion capacity of absorbed RAs, varied from 4.46 to 5.11 (Table 5). There was a clear correlation between the concentrations of total RAs in water and bile (Pearson $r = 0.80$, $p < 0.05$).

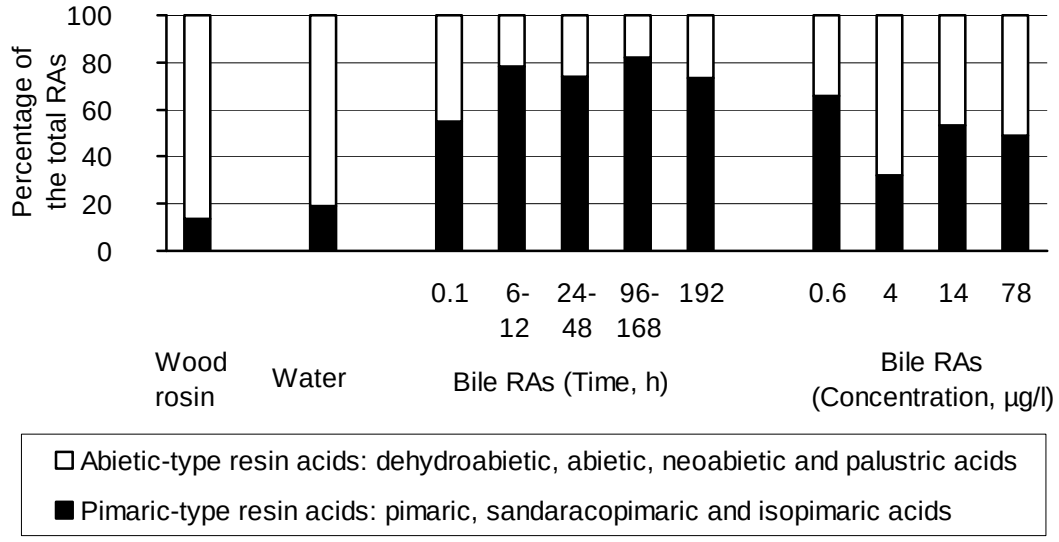

FIGURE 8 Percentage of summed pimaric-type (pimaric, sandaracopimaric, and isopimaric acid) and abietic-type (dehydroabietic, abietic, neoabietic, and palustric acid) resin acids (RA) in the wood rosin stock, in the exposure water at the beginning of the experiments, and in the bile of brown trout when exposed (8 µg/l) for various times and to various concentrations in water (10-d). Values are means of three samples.

TABLE 5 Average bioconcentration factors (log $BCF_{bile(RA)}$)of resin acids (pimaric-type RAs, pimaric acid, abietic-type RAs, dehydroabietic acid and total RAs) in the bile of juvenile brown trout, expressed as log BCF_{bile} of the sum of free and conjugated RAs relative to free RAs in water. There were no statistically significant differences between log BCF_{bile} values between exposure concentrations (ANOVA, F= 0.91, $p > 0.05$).

Resin acid	Concentration of RAs in water (µg/l)			
	0.6	4	14	78
Pimaric-type RAs	5.28	4.13	5.33	4.69
Pimaric acid	5.18	4.11	5.68	4.75
Abietic-type RAs	4.89	5.49	4.66	4.35
Dehydroabietic acid	4.25	4.62	3.45	3.10
Total RAs	5.11	4.56	4.85	4.46

4.5.2 Simultaneous exposure with five species

Simultaneous exposures with five species in an identical concentration of RAs in water revealed different bioconcentration and excretion capacities between species (IV). The lowest RA concentrations were detected in perch bile, 660 ml (Fig. 9). The sum of seven RAs revealed a concentration of 1500 µg/ml in roach bile, 1670 ± SD 380 µg/ml in brown trout bile, and 1650 µg/ml in rainbow trout bile. Concentrations of RAs in whitefish bile, 4330 µg/ml, indicated that whitefish had the highest bioconcentration ability of all five species.

Perch and whitefish were statistically different from each other in their RA concentrations in bile (ANOVA, $p < 0.05$). The perch/roach ratio of bile RAs under uptake from water averaged 0.4.

The average total concentration of the water RAs (23 µg/l) was used to calculate the log $BCF_{bile(RA)}$ values for each species (Table 6). The mean log $BCF_{bile(RA)}$ varied between species from 4.45 to 5.27 (IV). The lowest value was for DHAA, 3.84-4.04, and the highest for pimaric acid, 4.23-5.51 (Table 6).

Since the rainbow trout is a worldwide model species, ratios that can be used as conversion factors in interspecies extrapolation, e.g. in monitoring situations, were estimated (Table 7) (IV). When the overall average values in water over the 7-day experiment (23 µg/l) were used, with the assumption of steady-state of bile RA, the ratio was lowest in perch (0.4), close to one in roach and brown trout (0.9 and 1.0), and highest in whitefish (2.6) (Table 7).

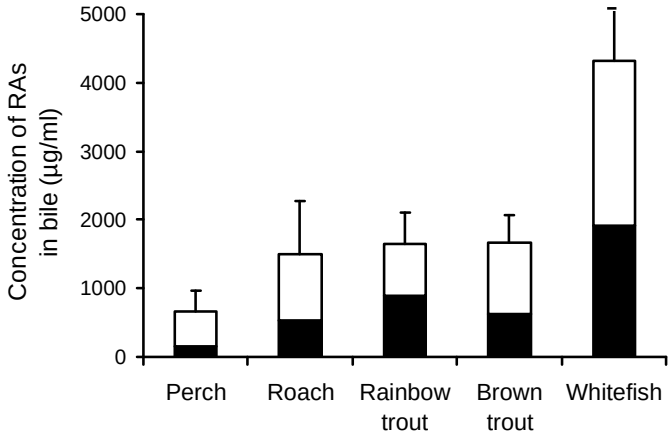

FIGURE 9 Concentration of resin acids (RAs) in the bile of perch, roach, rainbow trout, brown trout, and whitefish exposed to waterborne RAs (average concentration 23 µg/l) in a 7-day laboratory exposure. All the fish exposed to resin acids had statistically different bile RA concentrations compared to the control fish (Student's T-test, $p < 0.05$). Number of analyses and the total number of animals in them were 6/15 for perch, 11/19 for roach, 8/20 for rainbow trout, 7/21 for brown trout and 7/17 for whitefish. □ = abietic-type RAs (dehydroabietic, abietic, neoabietic and palustric acid), ■ = pimaric-type RAs (pimaric, sandaracopimaric and isopimaric acids).

TABLE 6 Average bioconcentration factors of resin acids (log $BCF_{bile(RA)}$) in the bile of perch, roach, rainbow trout, brown trout, and whitefish exposed to waterborne RAs (average concentration 23 µg/l) in a 7-day laboratory exposure. Values are expressed as log BCF_{bile} of the sum of total RAs relative to RAs in water. No statistically significant differences were found between species in log BCF_{bile} values (ANOVA, F=1.48, $p > 0.05$)

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

TABLE 7 Average ratio of RA concentrations in fish bile with rainbow trout as standard (taken as 1.00). Values for perch, roach, brown trout and whitefish compared to rainbow trout were calculated on basis of their bile RA concentration when simultaneously exposed for seven days to RAs (total concentration 23 µg/l in average). In addition, perch/roach ratios from the same exposure are shown (Fig. 9). Coefficients of variation for total RAs are shown in the parentheses.

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

4.5.3 Field experiment with fish

In early summer 2004, juvenile brown trout and whitefish were caged in Southern Lake Saimaa (Fig. 2) (V). In both brown trout and whitefish a clear difference was seen between exposures in upstream (reference) and downstream (1 km) areas in relation to distance from the mill (ANOVA, $p < 0.001$; Tukey's test, $p < 0.001$) (Fig. 10). In brown trout, no statistically significant difference beyond the nearest caging site (1 km) was observed for RAs (Tukey's test, $p > 0.05$), nor did the exposure to BKME change the bile concentrations of β-sitosterol in either species (ANOVA, $p > 0.05$; Tukey's test, $p > 0.05$).

Interestingly, assuming identical environmental concentrations of RAs in the lake water of adjacent cages (1 km), the three-times higher bile concentration in whitefish than in brown trout imply a species difference in the capacity to excrete RAs, as also demonstrated in the simultaneous exposure experiment with brown trout and whitefish in the laboratory (Fig. 7).

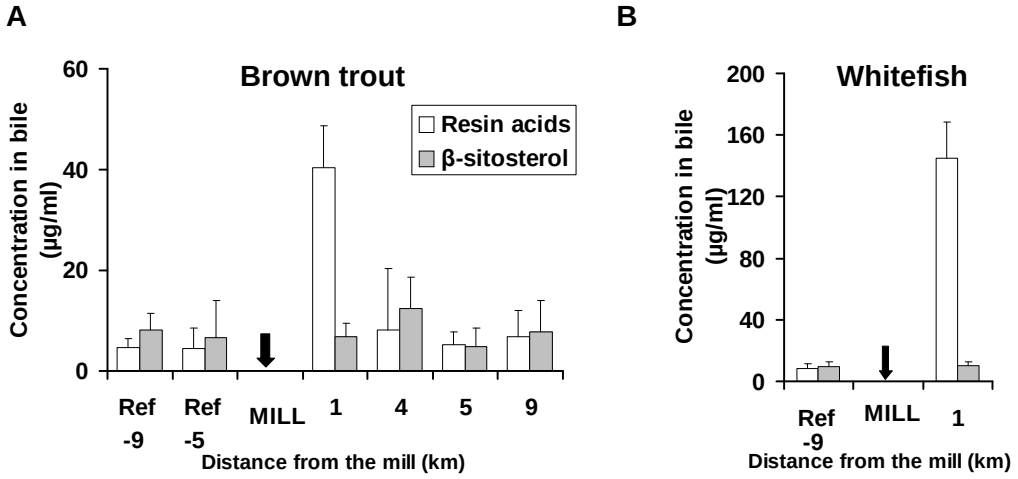

FIGURE 10 Concentrations of total resin acids (conjugated and free) in bile of A) brown trout and B) whitefish exposed by caging for 10 days to bleached pulp and paper mill effluents (BKME) at Southern Lake Saimaa (V). Means with SDs are denoted.

4.6 BKME-induced changes in EROD activity

Hepatic EROD activity was considered as a measure of the effects of chemical exposure in caged fish (V). In Southern Lake Saimaa, when compared to the reference area 9 km upstream, there was no difference in the activity of liver EROD in brown trout in relation to distance from the pulp and paper mill (reference: mean 0.21 with SD 0.47 µmol/mg PMS protein). Even the values at the nearest caging site (1 km) from the mill were similar (1 km: mean 0.12 ± SD 0.16 µmol/mg protein) to the reference values. In one downstream site (9 km) a significant induction was observed in trout EROD (1.39 µmol/mg protein; V). The presence of a local but unknown source of compounds able to induce EROD activity is suggested.

4.7 BKME-induced changes in transcription

Resin acids induced or repressed a number of genes in brown trout and dose-dependent changes were found (III). The highest sensitivity to RAs was observed in the genes implicated in the metabolism of iron and reactive oxygen species (ROS), as evidenced by the decrease in transcripts for iron transporters and haem-containing proteins. The expression of hemoglobins, ferritin heavy chain selenoprotein P and plasma glutathion peroxidase decreased even at the lowest concentration of RA (0.6 µg/l).

The genes involved in carbohydrate metabolism and mitochondrial synthesis of ATP (glyceraldehyde-3-phosphate dehydrogenase, carbonyl reductase and cytochrome oxidase subunit III) were also down-regulated. This could be evidence of depression of the energy metabolism, which coincided with the coordinated down-regulation of ribosomal proteins. Decreased expression was seen in the majority of the fifty-six genes for ribosomal proteins presented on the microarray platform. Dose-dependency was observed in gene expression in relation to RA concentration of exposures.

Up-regulation was observed in genes for apolipoproteins E and A, which are known for the ability to bind lipophilic compounds. Additionally, a number of changes in gene expression indicated recovery and remodelling of hepatic tissues. Resin acids also suppressed the expression of several humoral immune factors, such as the endothelial leukocyte cell adhesion protein, leukocyte cell-derived chemotaxin complement factor H and serine protease-like protein.

The relative alteration in gene expression can be presented by the lowest observed effect concentration (LOEC). The lowest tested concentration of RAs which produces a statistically significant gene-level effect is 0.6 µg/l.

In contrast to the laboratory experiment, the caging experiment in Southern Lake Saimaa (V) showed no up- or down-regulation of the genes that were sensitive to RAs (III). Three groups of genes with highly co-ordinated expression patterns (Pearson $r > 0.7$) were found. One group consisted of genes for mitochondrial proteins involved in oxidative phosphorylation and transport of ATP. Two groups included genes implicated in immune responses, metabolism of nucleotides and protein folding as well as genes with unknown functions. All three groups showed down-regulation in areas receiving BKME and the magnitude of the effect was markedly enhanced with decreasing distance from the source of BKME.

4.8 Monitoring of fish populations after a black liquor spill

In summer 2003, one month after the black liquor spill, the sampling and monitoring of fish populations at the Southern Lake Saimaa began. In the reference areas, the total concentrations of RAs in bile in all sampling periods from July 2003 (one month post-spill) to July 2004 were less than 5 µg/ml in perch (Fig. 11) and 7 µg/ml in roach (Fig. 12). Since there were no statistically significant differences between the periods (Bonferroni's test, $p > 0.05$), two reference areas were combined for statistical analyses.

Downstream from the pulp and paper mill, the total concentration of RAs in perch bile decreased with distance from the mill in all sampling periods. In the sampling area 1 km from the mill, the perch bile averaged 5.5 µg/ml both in July 2003 and in July 2004 (5.7 µg/ml). The situation was similar in the other sampling areas the RA concentrations in perch bile in July 2003 and 2004 were compared. The highest exposure to RAs in perch was 1 km from the mill in May

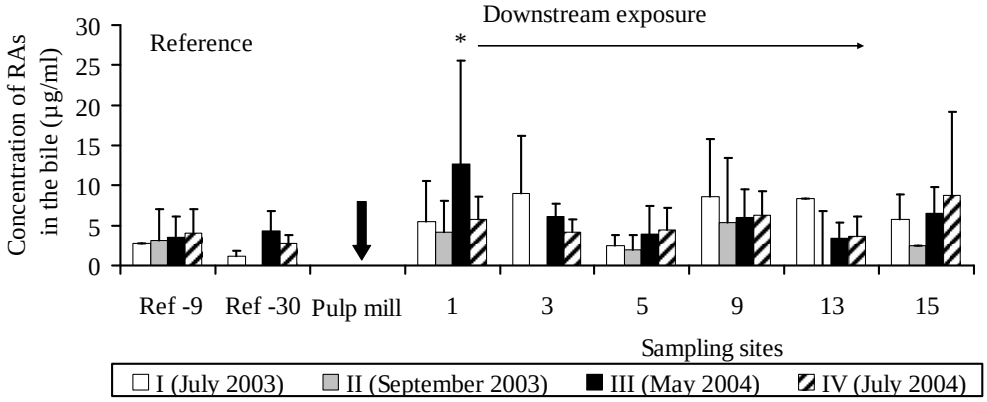

FIGURE 11 Concentrations of total resin acids (conjugated and free) in bile of perch exposed by caging for 10 days to bleached pulp and paper mill effluents (BKME) in Southern Lake Saimaa. Means with SDs are denoted and I-IV denote sampling periods from July 2003 to July 2004. Asterisk denotes statistically significant difference compared to respective reference areas ($p < 0.05$).

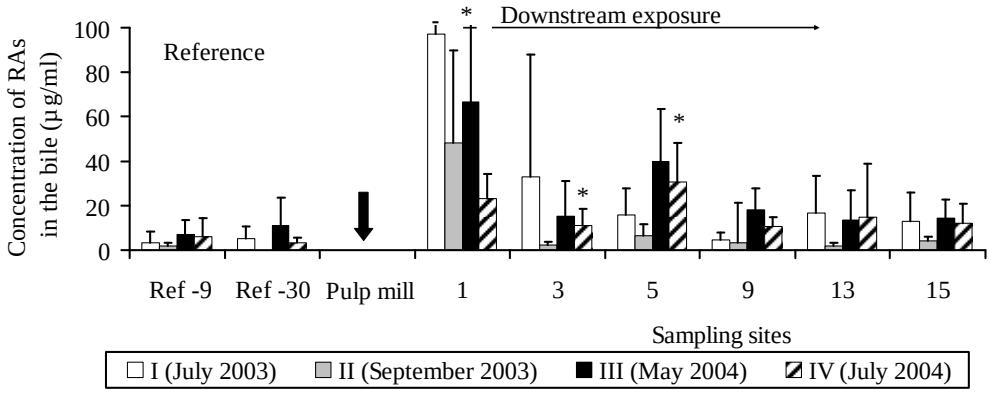

FIGURE 12 Concentrations of total resin acids (conjugated and free) in bile of roach exposed by caging for 10 days to bleached pulp and paper mill effluents (BKME) in Southern Lake Saimaa. Means with SDs are denoted and I-IV denote sampling periods from July 2003 to July 2004. Asterisk denotes statistically significant difference compared to respective reference areas ($p < 0.05$).

2004 (12.7 µg/ml), right after the spring mixing of the water column. At no other post-spill monitoring period was any impact observed on perch populations, in terms of increased exposure to RAs, which could be attributed to the previous black liquor spill (Fig. 11). Bile RA concentrations decreased after July 2003 following the accidental black liquor spill. However, there were no statistical significances between the sampling periods in perch (Bonferroni's

test, $p > 0.05$), although the exposure of perch bile showed a decreasing trend at 3 km downstream from the mill (Fig. 11). Overall, in perch, the total RA concentration in the bile was 94 % lower than in roach 1 km downstream from the mill in September 2003.

In roach, in the areas 1 and 3 km from the mill, the highest concentrations post-spill were observed in July 2003 (1 km: 97 µg/ml; Fig. 12). In the same areas the RA concentrations were 76-66 % less in July 2004 than in July 2003, indicating a significant decrease in the exposure of roach during one year. More precisely, between July 2003 and September 2003, RA concentrations in roach bile decreased in all the sampling areas; however, concentrations increased (38-59 %) in spring 2004, apparently due to the seasonal mixing of the water column at that time. The trend towards decreased exposure was clearest at 1 km from the mill.

Regarding the nearest one km, Southern Lake Saimaa had recovered from the accidental release of black liquor, at the latest, by September 2003. This is indicated by the results showing that the RA concentrations in perch bile downstream from the pulp mill in September 2003 were statistically the same as those in the reference areas and reference period, i.e. July 2004. Although best shown by perch, this conclusion is also supported by the observations on roach. Even though the concentrations were higher in roach than in perch, in both species the concentrations can be assumed 'normal' permit-based exposures, given the presence of the pulp and paper mill industry in the area.

4.9 Exposure role of sediment to fish populations

Perch and roach had distinctly different levels of RAs in their bile after the black liquor spill in Southern Lake Saimaa (Fig. 11 and 12). Because of the continuous low-level exposure of fish populations to BKME derived RAs in the Southern Lake Saimaa area due to the normal operation of the pulp mill, it is difficult to distinguish between the different origins of the exposure (water/sediment). The annual peak concentrations of RAs in fish bile in the spring suggest that BKME-contaminated sediment might, owing to resuspension of its surface layer into the water compartment, serve as an additional source of RAs. Therefore the possible exposure of roach to contaminated sediment was investigated (IV).

Perch and roach have largely different living habitats and ways of foraging. In the great majority of fish in any sampling area downstream of the pulp mill, the bile RA concentrations were much lower in perch than in roach. Two reasons can be proposed for this; species differences in biliary excretion and unidentified sources of RAs in the environment. The first was based on the perch/roach ratios of bile RAs (Table 8) and the second on the results of the laboratory experiment (see 4.5.3, IV). Since perch can be assumed to accumulate RAs only via water, the following hypotheses may be formulated. First, increased uptake from sediment can follow if higher concentrations of RAs exist

TABLE 8 Average perch/roach ratios of RA concentrations in perch and roach bile in the Southern Lake Saimaa from July 2003 to July 2004 (IV). The closest areas (1-5 km), in addition to the spill of black liquor in June 2003, are exposed to the pulp and paper mill effluents (Figure 2). Additionally, control values from water-only exposure (average ambient concentration of RAs 23 µg/l) in the laboratory are shown (Figure 9, IV). The coefficient of variation (%) is shown in parentheses.

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

n.a.= data not available

in the sediment (Table 3). In July 2004 the RA concentration in the water was assumed to be at the normal operational level, and the relative exposure of roach to sediment to be at its highest. Moreover, the reliability of the perch/roach ratio was higher in July 2004 than in June 2003 due to low variation (Table 8), indicating the highest possible sediment uptake of roach. Perch/roach ratios were 0.25 (CV 25 %) in the 1 km area, and 0.38 (CV 25 %) in the 3 km area. For comparison, the uptake from sediment by roach is zero when this species is exposed to water-only RAs (perch-roach ratio 0.44; IV). Areas with higher sediment RA concentrations are the most likely potential source of high RAs in roach bile. Therefore the area one km from the pulp mill was used in comparing the laboratory water-only based ratio to that obtained in the field in July 2004. The results of the comparison show that the lower ratio in the latter, the greater the difference between perch and roach in RA concentrations, indicating that roach may have an additional origin of exposure to RAs, plausibly sediment-derived RAs. However, the difference between water-only and sediment exposure was not great when considering the variation, and therefore more research is needed to verify the assumption of sediment as an exposure source of RAs to roach.

5 DISCUSSION

5.1 Are contaminants released from sediment? – dissolution

High concentrations of RAs, retene, and WSs have been measured in BKME-contaminated sediments in Southern Lake Saimaa (Holmbom et al. 1992, Leppänen & Oikari 1999, Lahdelma & Oikari 2005, 2006) and in other water areas elsewhere in the world (Brownlee et al. 1977, Morales et al. 1992, Judd et al. 1995, Tavendale et al. 1995, Brumley et al. 1998). However, the total concentration of contaminants in a sediment cannot be considered a reliable measure of the potential toxicity of that sediment, since a number of factors limit or enhance the bioavailability of bioactive chemicals. Further, it is impossible to measure the concentrations of all the chemicals in the contaminant cocktail referred to as sediment.

Data from Southern Lake Saimaa clearly show that sediment RA concentration decreases with distance from the pulp and paper mill (Leppänen & Oikari 1999, Lahdelma & Oikari 2005), while the vertical sediment profile indicates that the majority of RAs are located in the uppermost layer (Lahdelma & Oikari 2005). In addition, the highest concentration of retene, an anaerobic transformation product of RAs, has been observed in the deeper anoxic layers of the sediment in Southern Lake Saimaa (Leppänen & Oikari 1999). Due to the bleaching techniques previously used in the pulp and paper industry, the sediments also contain chlorophenolics in amounts, for example, of around 50 µg/g dw at 3 km from the mill (Kukkonen et al. 1996).

Recently, desorption has become in increasingly common subject in organic chemical fate assessment studies in sediments, and dissolution of specific compounds from contaminated sediment provides more information about sediment chemistry and interactions. According to the results presented here (I), dissolution of RAs demonstrated that the compounds present in sediment are likely to desorb into water when appropriate disturbance is applied. The maximum desorption of RAs was faster than that of sterols, possibly due to the higher lipophilicity and stronger binding of sterols to the organic sediment matrix (Kaplin et al. 1997, Kubicki & Apitz 1999, Lebo et al.

2003). The outflux from the sediment matrix is commonly described in terms of the bi- or triphasic desorption kinetics of pollutants (Cornelissen et al. 1997, ten Hulscher et al. 1999). The fast fraction comes into equilibrium rapidly with the aqueous phase, within hours to days, while the following slowly desorbing fraction is related to the diffusion of intraorganic matter and hindered by pore water diffusion via first-order kinetic processes and thus requires months and even years (Johnson & Weber 2001). Additionally, the fast fraction is often connected with the bioavailability of the contaminant (Lamoureux & Brownawell 1999). The results of the present study imply that RAs may belong to the fast fraction since the highest concentration of RAs dissoluted in the first days, and therefore are potentially bioavailable to benthic and above fauna in sediment disturbance situations. Additionally, the water-sediment elutriate has been shown to have toxic potential (Lahdelma & Oikari 2005), which indicates the bioavailability of toxic compounds in the sediment.

The presence and dissolution of retene, an aromatized derivate of RAs (Tavendale et al. 1997), further supports the assumption of that Southern Lake Saimaa sediments are contaminated by alkylated PAHs from BKME. Although the highest concentrations of retene are deposited in the deeper layers in sediments (25-30 cm), significant amounts are present in the top layer and thus available to aquatic and benthic animals. Retene was also dissoluted in much lower concentration than RAs or WSs. The relatively high TOC of the sediment probably contributed to the slowness of the process, which also reflects the lower bioavailability of this compound with increasing TOC to the oligochaete worm (Nikkilä et al. 2001).

Retene is also toxic to fishes (Billiard et al. 1999, Oikari et al. 2002) and is highly resistant to further transformation under anaerobic conditions in sediment, unlike RAs (Leppänen & Oikari 1999, 2001). The concentration of retene obtained in this study (13 µg/l; I) is high enough to cause pathologies, such as edemas in the yolk sac and pericardial cavity (Brinkworth et al. 2003), in developing larvae during the post-hatch phase of the Pacific herring (Oikari et al. 2001). To support the potential bioavailability of retene, it is notable that the compound has been measured in roach bile in Southern Lake Saimaa (Leppänen & Oikari 1999), and its bioavailability from contaminated Lake Saimaa sediment to juvenile rainbow trout has been demonstrated by analyzing the metabolites in the bile of this species (Oikari et al. 2002).

Regarding the stability of WSs in BKME, β-sitosterol and stigmasterol was recently found to undergo transformation into their respective ketones under chlorine dioxide bleaching conditions (van den Heuvel et al. 2006), although sterols have appeared to be recalcitrant in waste water and sludge (Kostamo & Kukkonen 2002). The bacterial degradation of wood sterols (Mahato & Garai 1997) has not been documented in pulp and paper treatment systems, despite the fact that bacterial transformation products can cause endocrine effects in fish (Howell & Denton 1989). The transformation of sterols seems to an environmentally important process as the ketones were observed to occur at higher levels in pulp and paper biosolids than the parent sterols. Additionally, the oxidized sterols did not reveal androgenic or estrogenic potency (van den

Heuvel et al. 2006), effects which have been observed earlier with BKME- and BKME-derived sterols (Mellanen et al. 1996, Tremblay & Van der Kraak 1999, Ellis et al. 2003).

Although RAs and WSs are present in high concentrations in sediments in Southern Lake Saimaa, and the compounds are released into the water phase during both rapid and slow disturbance situations, the question arises as to whether the results are environmentally realistic. Eggleton and Thomas (2004) and Chapman et al. (2002) have reviewed factors affecting the release of contaminants during sediment disturbance events. Dredging allows the mixing of anaerobic layers with biologically more diverse surface sediments, leading to the release of contaminants associated with the sediment particles and pore water. Sediment resuspension has been shown to accelerate the desorption (I), partitioning, bacterial degradation and oxidation of organic contaminants. Various natural disturbances caused by daily tidal currents, wind energies and seasonal flooding can occur. Moreover, storms in coastal and estuarine areas can cause periodical remobilization of surface sediments, possibly leading to the dissolution and release of sorbed contaminants to the more bioavailable water fraction. In fact, mixing speed seems to be a crucial factor in assessing dissolution. This was clearly seen after the 43-day incubation experiment (I), when the increase in the stirring speed of field sediment had a dramatic effect on the dissolution outcome. Heavy mixing, which can be regarded as the equivalent of dredging activities, released considerable amount of contaminants into the water phase. Overall, human activities can increase the desorption outcome of organic contaminants due to the increase in the sediment/water interface, thereby possibly increasing the bioavailability of the compounds in question (I).

In conclusion, modern BKME contains RAs and WSs, which are discharged into the water body during normal operations (permit limits) and during accidental spills (exceed of permit limits). The compounds in the area sorbed to POC in the sediment; hence high amounts of RAs and WS are present in the top sediment layer. In addition CPs and retene are found in the deeper layers of historical sediments. This is clear evidence of the presence of contaminants in the pulp and paper industry-contaminated sediments studied here, and the dissolution of these compounds into water enhances the possible bioavailability of sediment-bound BKME contaminants and thus the environmental risk of these sediments.

5.2 Are sediment-associated contaminants bioavailable? – uptake into benthic invertebrates

Uptake of contaminants present in sediment into benthic fauna can be considered as clear evidence of the bioavailability of RAs and other BKME-related compounds from sediments near pulp and paper industry. Additionally,

it confirms the assumption, that benthic invertebrates are exposed to BKME-related compounds in these sediments. The results presented in (II) show increased uptake of RAs, CPs and β-sitosterol downstream from the pulp mill when compared to upstream reference areas.

Unexpectedly, at three km downstream the concentration of RAs in resident animals was 2-4 times higher than in those closer to the mill. With respect to sediment dynamics, it is noticeable that no annual stratification takes place in Southern Lake Saimaa. Changes in hydrodynamics along the gradient downstream from the industrial source (Seppälä 1986, Lahdelma & Oikari 2005), with changes in the transport type of sediment surface, may have transferred the more contaminated resuspended material further away from the BKME discharge point (Oikari & Holmbom 1996). This will then have exposed the CPs deposited in the sediment during the decades before the year 1992, as evidenced by the CP concentrations in benthic animals in (II). This is due to the heavy loading of CPs in the area near the pulp and paper mill prior to 1992, when the mill employed elemental chlorine free (ECF) bleaching. However, the CP concentrations in the sediment in Southern Lake Saimaa were higher in this study than in a previous one, when concentrations of 50 μg/g dw were measured 3 km from the mill (Kukkonen et al. 1996). Moreover, in a more recent study the sediment surface (5 cm) contained only 0.25 μg/g dw CPs at 5 km distance from the mill (Lahti 2006). Pentachlorophenol, the predominant CP in the sediment, was found to desorb effectively into water from German estuarine sediments (Eder & Weber 1980), which may increase its bioavailability to benthic animals as also in the case of Southern Lake Saimaa.

Moreover, a critical increase in contaminant concentrations, such as RAs, in sediment increases the concentration in the elutriated water (I). Additionally, differences of RA concentration in benthic animals between downstream areas may be explained by differences in feeding behavior due to different habitats, feeding habits and exposure caused by possibly different particle distribution and binding of contaminants.

Surprisingly, tetrachloroguaiacol, an indicator of earlier chlorine bleaching activities, was detected only in traces. It is known to be the most abundant chemical indicator of the pulp and paper industry (Paasivirta et al. 1985). Concentrations of CPs in zoobenthos and fish were recorded in areas contaminated by Finnish pulp and paper mills in the 1980s. In benthic fauna CPs were of the magnitude of 50 ng/kg ww (ppb) at five km from the mill in Äänekoski, Central Finland (Paasivirta et al. 1985). In a Canadian river system downstream from a pulp mill, concentrations of chlorophenolics in *Hydropsyche* caddisflies were similar to those in sediments at the same sites, i.e. around 60 μg/g ww (Owens et al. 1994). These are significantly higher amounts than obtained from Southern Lake Saimaa in 2002. However, the changes in bleaching technology, mean that the historical CP-rich sediments are buried under fresh layers of sediment have much lower CP, and therefore bioaccumulate to a lesser extent in benthic animals. The studies by Oikari et al. (1984) and Leppänen and Oikari (1999) support this assumption, as only low

levels of chlorophenolics were found in fish bile in the Southern Lake Saimaa area.

Diptera larvae had higher concentrations of all compounds than Oligochaeta in the resident benthic community in the areas downstream of the pulp mill. However, in the laboratory exposure to sediments collected from the same areas as the resident animals, *L. variegatus* had significantly higher concentrations of RAs and WSs compared to *C. plumosus*. The difference may be related to the different feeding behavior of the *L. variegatus* (sediment feeder) and *C. plumosus* (deposit feeder), and to the higher lipid content of *L. variegatus* (Penttinen et al. 1996) and incapability to metabolize certain organic contaminants, such as PAHs, compared to Chironomids (Harkey et al. 1994, Penttinen et al. 1996). In previous studies *L. variegatus* accumulated more 2,4,5-trichlorophenol than *Chironomus riparius* (Verrangia Guerrero et al. 2002). The behavior of the animals indicated that *L. variegatus* spend more time burrowing inside the sediment than *C. plumosus*, which mainly inhabit the upper one cm of the sediment along with the sediment-water interphase. Increased lipid content may decrease the overall body burden and protect important sites of toxic action; a factor that needs to be considered in risk assessment (Lasiter & Hallam 1990). Additionally, the heavy bioturbation caused by *C. plumosus* may have increased the bioaccumulation efficiency of adjacent *L. variegatus* individuals (Ciarelli et al. 1999, Landrum et al. 2004).

Although the results of the laboratory test can be extrapolated to the field with a reasonable degree of certainty (Chapman et al. 1998, Ingersoll et al. 2003), the laboratory results in this study underestimated the uptake of xenobiotics when compared to the field. It may be that field conditions are more heterogenous than those in the laboratory and influenced more by external factors, and thus the resident animals may undergo some degree of acclimation when exposed to contaminants for months or even years. Additionally, in the laboratory, only two species were used as opposed to the field experiments which included combinations of many species of the classes Diptera and Oligochaeta. While successful field validations by laboratory test exist (Ferraro & Cole 2002), the extrapolation of results from laboratory-to-field must be done with caution, since the laboratory only provides a snapshot of specific conditions (Chapman et al. 1995).

In conclusion, the contaminants of interest were found in the tissues of resident benthic invertebrate populations adjacent to the pulp mill in Southern Lake Saimaa, and the bioaccumulation was confirmed by the laboratory studies. This can be deemed clear evidence of exposure, and indicates that BKME-contaminated sediments pose a potential risk to benthic invertebrate populations in contaminated areas.

5.2.1 Adverse effects of BKME sediments on animals

Despite the evidence of the bioavailability and uptake of sediment-derived contaminants into benthic animals, bioaccumulation in itself is not an adverse effect. It is only when the contaminants accumulate within the organism to

levels that elicit an adverse response that we can state that the bioaccumulation indicated adverse effects.

The quality of pulp and paper mill effluents has improved with the changes in bleaching techniques and the use of modernized effluent treatment to the extent that CPs are the least abundant of the three groups of contaminant (RAs, WSs, CPs) in benthic animals. However, modern BKME continues to have several negative effects on aquatic animals (Gagnon et al. 1992, Karels et al. 2001, Ellis et al. 2002). Among invertebrates, there are studies on the negative effects of RAs. Fåhraeus-Van Ree and Payne (1999) reported degeneration of the digestive cells in marine mussels, *Mytilus edulis,* when exposed to rosin (nominal concentration 20 mg/l) for 15 days. Additionally, BKME-contaminated sediments containing 1900 mg/kg dw of RAs caused significant reduction of survival and reproduction in amphipod and oligochaeta (Hickey & Martin 1995). Recent interest in endocrine disruption in aquatic invertebrates (Soin & Smagghe 2007, Lafont & Mathineu 2007) is also a relevant issue in connection with BKME-contaminated sediments. Observations of fish endocrine disruption and the decrease of fish populations have been found in areas near pulp and paper mills (Hodson et al. 1992, Tremblay & Van der Kraak 1999, Ellis et al. 2003), including Southern Lake Saimaa (Karels & Niemi 2002). Since, among other WSs, weakly estrogenic β-sitosterol (Mellanen et al. 1996) is present in BKME sediments, it can be proposed that adverse effects in e.g. reproduction may be associated with sterols.

Studies on communities of benthic invertebrates, which are highly sensitive to BKME, have been widely conducted in areas contaminated by the wood processing industry over the last few decades (Scrimgeour 1989, Harris et al. 1992, Vuori 1992, Sibley et al. 1997, 2000, Culp et al. 2003). Culp et al. (2003) conducted a study in a Canadian river, using a mesocosms to investigate the effects of sulfite pulp mill effluent (5 and 10 % vol/vol) on benthic assemblages. Although pulp mill effluent had little effect on benthic invertebrate density and total richness, the structure of the community showed significant change, with an increase in the number of Chironomids and a decrease in the number of Oligochaetes, caddisflies and hydra larvae. Earlier studies conducted before the introduction of modern bleaching and effluent treatment technology have shown that BKME discharge into lakes has resulted in reduced diversity and altered taxonomic composition of invertebrates and fish communities (Scrimgeour 1989). Moreover, settled pulp fibers have formed a blanket over the river bottom downstream of the pulp mill, reducing the areas for invertebrate feeding (Hilton et al. 1980). However, the improvements in BKME-treatment technology have contributed to water quality and decreased the negative effects, also in community structures (Harris et al. 1992, Vuori 1992, Pöykiö et al. 2004). Vuori (1992) found evidence of the recovery of the guild structure of caddisflies in Finnish rapids that earlier were heavily polluted by pulp mills but where water quality had recently improved.

Changes in the community structure of benthic fauna caused by the chemical residues of suspected chemomarkers has hardly been studied, not least in pulp and paper industry recipient areas. When comparing

contaminated downstream with reference upstream areas in Southern Lake Saimaa, it is clear that benthic fauna in downstream areas have a higher concentration of pollutants. Gagnon et al. (1995) observed fish population-level effects in the St. Maurice River in Canada. In addition, population level effects have been observed in downstream areas of pulp and paper mills. Hakkari (1992) reported that fish communities in Finnish lakes polluted by BKME were dominated by perch, roach and ruffe, whereas more sensitive salmoniformes have disappeared. In more recent studies, Sibley et al. (2000) demonstrated a significant relation between benthic community structure to EOCl concentration (5200 mg/kg dw) in sediment adjacent to pulp mill. However, opposite results, showing no significant effects on resident benthic animals, also exists (Felder et al. 1998). In conclusion, the results presented here, combined with previous findings on alterations in community structure and on the presence, bioavailability and bioaccumulation of BKME-derived contaminants may pose a risk to aquatic invertebrates.

5.2.2 Biotransformation capability of benthic invertebrates

The biotransformation of contaminants is an important factor in determining and predicting bioaccumulation. In vertebrates, during metabolic transformation the parent compound or its metabolites from phase I are conjugated in phase II with a molecule of glucuronic acid, glutathione, glucose, sulphate or phosphate (Di Giulio et al. 1995). It is still unclear, how far conjugation predominates with regard to RAs, WSs and chlorophenolics in invertebrates. The concentration of bound RAs in tissues in the freshwater mussel *Hyridella* caged in a BKME outlet pond was around 40 %, and the animals reached a steady state of accumulation in seven days (Burggraaf et al. 1996). However, fishes are known to excrete RAs and CPs in their bile as glucuronide conjugates (RAs and CPs) and sulphate conjugates (CPs) (Oikari et al. 1984, Oikari & Ånäs 1985). Additionally, pentachlorophenol is known to conjugate with sulphate and with β-glycoside, which are the most common pathways for detoxifying the compound in aquatic animals (Verrangia Guerrero et al. 2002).

Little is known about the biotransformation of xenobiotics in aquatic invertebrates (Kukkonen & Oikari 1988, Kamaya et al. 2005) or about the biotransformation of pollutants in benthic Diptera and Oligochaeta in particular. The present results demonstrate the ability of Diptera to metabolize RAs, β-sitosterol and CPs in the field (II). In addition, *C. plumosus* conjugated RAs in the laboratory exposure (14 %), but to a lesser extent than Diptera in the field (28 %). The difference may by due to species-specific differences between field and laboratory animals, and to different exposure and environmental conditions in the field and in the laboratory. With respect to the limited number of samples, however, the conjugation values among this taxa can be regarded as similar.

Oligochaetes did not show biotransformation in the field, but in the laboratory 27 % of RAs of *L. variegatus* was in the hydrolyzable conjugated

fraction. Interspecies differences in biotransformation may be the biggest reason, since the field animals were sorted only to group level. Additionally, laboratory species may differ from wild ones in their biotransformation capabilities. Moreover, the laboratory and wild microbial communities, also active in biotransformation, might have been different. In previous studies *L. variegatus* has been shown to biotransform PAHs, e.g. benzo[a]pyrene (Leppänen & Kukkonen 2000). However, the metabolism of PAHs is highly variable in invertebrates, even within taxonomic groups (polychaetes) (Kane-Driscoll & McElroy 1996), and it has been demonstrated that Oligochaeta do not metabolize or biotransform certain organic contaminants, such as PAHs and phenols, to a great extent (Harkey et al. 1994, Hyötyläinen & Oikari 2004).

The uptake of WSs into wild and laboratory animals may give rise to questions about whether they or their possible microbial modifications can cause adverse effects in benthic invertebrates on the physiological level. In the present study, the cholesterol levels of *L. variegatus* were higher downstream than upstream from the pulp and paper mill. The adverse effects of WSs in fish have been associated with a steroid-like structure, which can bind to hormone receptors and elicit hormonal responses in animals (Cody & Bortone 1997). In fact, studies on pulp mill effluents have reported, for instance, decreased levels of sex hormones and reproduction capacity in fish due to sterol transformation products (Ellis et al. 2003). Since insects cannot synthesize cholesterol, it comes from their diet, and is further derived from ecdysone and other insect hormones (LeBlanc et al. 1999). Among invertebrates, according to an old reference, marine crustacean *Panaeus japonicus* is capable of converting β-sitosterol into cholesterol (Teshima 1971). Cholesterol is the parent molecule for the production of the sexual steroids in mussels, among other groups, and increased levels of cholesterol have been measured in zebra mussels, *Dreissena polymorpha,* when exposed to municipal effluent (Quinn et al. 2004). In conclusion, it is possible that the endocrine system of benthic invertebrates is affected by BKME contaminants, perhaps in relation to WSs.

5.3 Bioconcentration of BKME contaminants in fish bile — useful tool for monitoring exposure to sediment?

5.3.1 Excretory patterns of RAs in fish bile

The total concentrations of RAs and other organic contaminants accumulated in bile have been used as detection and monitoring tools for several species and ambient conditions (Statham et al. 1976, Kobayashi 1979, Oikari et al. 1984, Förlin & Wachtmester 1989, Niimi & Lee 1992, Johnsen et al. 1995, Tavendale et al. 1996, Leppänen & Oikari 1999, Karels et al. 2001). On the basis of the concentrations of RAs in water and in bile, the present bioaccumulation factor values (log $BCF_{bile(RA)}$) for total RAs were 3-5 (III), higher than those of Oikari et

al. (1980). Johnsen et al. (1995) calculated similar log BCF_{bile} values (6.2-6.8) from newsprint mill effluents for brown trout to those found in this thesis. With respect to the rate of hepatobiliary excretion, metabolites in fish bile were detected already at the first sampling time, 0.1 h. It is likely that metabolites appear in the bile within minutes to one hour of exposure, depending on the compound (Förlin & Wachtmester 1989, Brumley et al. 1998). For RAs, it has been speculated that the RA conjugation system would be in dynamic steady-state within a week of exposure (Oikari et al. 1984). However, according to the results presented here, the conjugation system is fully saturated after only 24 h (III).

Considering the sensitivity of the hepatobiliary biotransformation and excretory system in fish, the concentrations of RAs can be up to 110 000 times higher in bile than in the ambient water (Oikari 1986, Johnsen et al. 1995, III). The results from (III) demonstrate that it is possible to measure the exposure status of fish in RA water concentrations comparable to those in receiving waters. With regard to sensitivity, an RA water concentration of 0.6 µg/l revealed a RA bile concentration of 100 µg/ml, around 170 times than that measured in clean control brown trout. This can be precisely measured by modern GC-MS. Thus the method appears to detect RAs in fish bile samples when the water concentrations are within the range of tens of nanograms per liter.

When fish have exposed to a mixture of RAs, the ratio of pimaric- to abietic-type RAs was different between water and bile (III, IV). In water, abietic-type acids were predominant (81 %). However, in the time-dependent exposures, the proportion of pimaric-type acids in the fish bile increased up to 87 %. The same phenomenon was observed in the concentration-dependent exposures as well, though to a lesser extent (III). However, in the multispecies exposure, abietic-type acids were predominant (77 %) in the bile of most fish species (56-77 %), only rainbow trout showing an increase of the proportion of pimaric-type acids up to 54 % (IV). The dominance of pimaric acid in the bile of brown trout may be due to the lower water solubility of some RAs than others, isopimaric acid (water solubility 1.7 mg/l) being the least soluble of these acids (Peng & Roberts 2000). It possesses nonconjugated double bonds and therefore cannot undergo isomerization (Morales et al. 1992). Dehydroabietic acid is the most soluble (water solubility 5.11 mg/l), due to the aromatic ring in its structure (Peng & Roberts 2000). In addition, isopimaric acid has been shown to have higher toxic potency than dehydroabietic acid (Råberg et al. 1992). This may be due to the higher K_{ow} of pimaric acid (log K_{ow} around 5; estimated with Alogps 2.1 by Tetko & Bruneau 2004) compared to dehydroabietic acid (log K_{ow} 1.74; Råberg et al. 1992).

At an ambient water pH 7.7, the acid dissociation constant (pKa) is 5.7 for dehydroabietic acid and 6.4 for isopimaric acid (Liss et al. 1997). In addition to lower water solubility, the fact that a higher proportion of isopimaric acid than dehydroabietic acid occurs in neutral form also promotes faster transmembrane transfer. Thus, at ambient and extracellular pH, e.g. at 7.4, a higher proportion of isopimaric acid (10 %) than DHAA (4 %) remains in fast absorptive acidic

form. Thus the acidic form of an RA is more likely to penetrate into fish and accumulate in hepatocytes, to be then eliminated via the hepatobiliary biotransformation route.

The bile reflects the exposure status of the animal over the past 24-48 h (Oikari et al. 1984). However, the maximum capacity of the hepatobiliary route, as expressed by bile concentrations of RAs, to eliminate RAs remained unknown as no saturation was evident in the highest exposure concentration used in this study i.e. 78 µg/l (III). In the concentration-dependent experiments a clear dose-response was seen. This indicates that in subacute exposure to RAs, despite the competition between bile acids and RAs (Råberg et al. 1992), the hepatobiliary route is a highly effective RA removal path.

Several species differences in RA excretion were observed in this study (IV). Of the five species investigated, whitefish had the highest concentrations of RAs, seven times higher than that of perch, which had the lowest. Moreover, roach, brown trout, and rainbow trout had bile concentrations of RAs two to three times higher than that in perch.

Some species, such as rainbow trout, perch, and brown trout, prefer to feed at advanced age on small fish and invertebrates from nonbenthic sources (Rask 1986, Koli 1998, Horppila et al. 2000). The coregonid whitefish, probably the most pelagic species, feeds on zooplankton (Koli 1998). Roach, a benthic fish, feeds typically on sediment-associated invertebrates (Rask 1989, Horppila et al. 2000), and may thus be exposed to compounds that are reversibly sorbed to sediment particles. In the field, according to Karels et al. (2001), near the same pulp and paper mill studied in the present work, the concentration of RAs in roach bile was 20 % higher than in perch bile. Overall, the differences between perch and roach in bile RA concentrations in the field and laboratory supported the assumption that sediments act as a source of RAs to roach in BKME-contaminated areas (see 5.3.3).

5.3.2 Monitoring of fish populations after a black liquor spill – population-level exposure

The number of unscheduled non-permit spills in the pulp and paper industry may be rising in the world. For example in Canada, they increased from an average of 98 spills per year during 1984-1989 to an average of 354 spills per year during 1990-1995 (Environment Canada 1998), and in Finland from 29 to 59 during 1986-1990 (Ettala & Rossi 1992). In Southern Lake Saimaa, the first of the accidental releases occurring first (June 16-23, 2003) were within the environmental permits of the mill, but those the later releases containing more black liquor (June 27-30, 2003) exceeded them. On the other hand, the pulp and paper industry has performed well in its recent permit-based discharges (IV).

TABLE 9 Average concentrations of total resin acids (µg/ml) in the biles of fishes in the Southern Lake Saimaa from 1983 to 1997. The reference area was located five km upstream from the pulp and paper mill and the exposure area one km downstream from the mill.

Year	Species	Concentration of RAs downstream (1 km)	Concentration of RAs upstream (Reference area)	Reference
1983	Rainbow trout (caged)	3300	4	Oikari & Kunnamo-Ojala 1987
1983	Perch	300	>10	Oikari 1986
1983	Roach	600	>10	Oikari 1986
1997	Perch	260	3	Karels et al. 2001
1997	Roach	320	3	Karels et al. 2001
1997	Whitefish (caged)	140	3	Karels 2000

The area investigated, Southern Lake Saimaa, has been studied widely before (Oikari 1986, Soimasuo et al. 1995, Karels et al. 2001). Therefore the pre-spill exposure of fish to RAs and the effects of the pulp and paper mill on the fish community and populations in the area were already known (Table 9). Because on the basis of the kinetics of hepatobiliary excretion (III), the bile reflects the exposure status of fish over the past 24-48 hours (Oikari et al. 1984, III), the results in this study served as a biomarker of fish exposure to RAs at specific time periods during each sampling period, not just that of a random moment of time as is the case when collecting water samples. Importantly, bile RA measures represent the bioavailable fraction of RAs. Thus, the results presented here can be used to assess the recovery of fish populations in Southern Lake Saimaa area after the black liquor spill.

After the black liquor spill in June 2003, there were fish kills in the areas close to the mill, which were connected to the reduction in the water oxygen level. However, the oxygen levels (3 mg/l) were not necessarily in a lethal range to most fish (lethal level < 2 mg/l; Sprague 1995). It is known, however, that the toxicity of BKME and RAs increases, due to joint effect, when dissolved oxygen levels fall (Kruzynski 1979, Sprague 1995).

Resin acids in modern treated BKME range from 40 to 100 µg/l (Johnsen et al. 1995, Verta et al. 1996, Leppänen et al. 1999), and in black liquor from 240 to 400 mg/l depending on the softwood type (Sjöström 1993). Over the last 15 years, the concentrations of RAs in water 1-8 km downstream from the mill in Southern Lake Saimaa have been low: 0.6-2.1 µg/l in May 1996 (Leppänen et al. 1998), and 0.5-2.4 µg/l in July 2004 (Lahti 2006). According to the RA levels in perch bile, estimates of ambient RA concentrations in the areas downstream from the mill (1-3 km) were in the range of 0.4 to 0.9 µg/l in July 2003. This agrees accords relatively well with the random values in water in pre-spill situation, when BKME emissions were within the permitted level (Leppänen et al. 1999). The BCF_{bile} values were also used to back calculate lake RA concentrations from RA concentrations of indigenous fish bile in Southern Lake

Saimaa (IV). However, since only one water concentration was used in the laboratory exposure, the calculated estimates of water RA concentration must be interpreted with caution. Comparison of the BKME-contaminated areas and reference areas in July 2003 showed that the water RA concentration was three times higher at 1 km distance from the mill. The estimated RA concentrations in water decreased by 30 % from July 2003 to September 2003 (1 km), but returned to the same level in July 2004 as one year previously. The majority of the downstream fish sampling areas followed the same pattern in this way, except the areas 6 and 15 km from the mill, where the estimates increased from July 2003 to July 2004. This may indicate an additional source of RAs in the areas. However, the overall results confirm the assumption that the RA concentration in water was in the same range as permitted pre-spill situation (relative to June 2004) as early as one month after the black liquor spill of June 2003.

In studies performed at other freshwater areas contaminated by pulp and paper mill effluents, a clear distance relationship has been observed when using metabolites of fish bile (Tavendale et al. 1996). RA concentrations of 3000 µg/g bile have been measured in rainbow trout exposed to 10 % pulp mill effluent for 50 days (Lindesjöö et al. 2002). When the results for fish bile RAs in this study are compared to those of previous studies conducted in Southern Lake Saimaa area, it is evident that even in July 2003, one month after the black liquor spill, the bile RAs concentrations in perch and roach were lower than under the normal operating conditions of the factory in winter 1997. For comparison, the bile RA concentrations of perch and roach were 10-30 times higher than those in the reference areas (Karels et al. 2001).

Although the results from the caging experiment (V) showed elevated bile RAs in whitefish and brown trout that directly indicate increased exposure of the caged fish to BKME discharged from the mill, as compared to fish in the upstream area, the overall situation has remained on the same level as in previous studies. In 1997 (Karels 2000), the concentration of RAs in the bile of caged whitefish 1-2 km downstream from the mill were in the same range as in 2004 (V), implying that there had been no increase in the exposure of fish to RAs between those dates.

5.3.3 Can sediment serve as a source for RAs in fish?

The laboratory exposure was conducted after the field monitoring to clarify the reason for the different concentrations of RAs in the biles of perch and roach in the field (IV). Since roach always had a higher RA concentration in the bile and thus higher log $BCF_{bile(RA)}$ values than perch, there is a possibility that a part of exposure status of roach to RAs may be of sediment origin. Perch are assumed to accumulate RAs only via water and food items therein. One possibility to assess this numerically would be through the introduction of perch/roach ratios, from both the field and laboratory experiments. Although roach bioconcentrated over two times more bile RAs than perch in the simultaneous laboratory exposure to RAs (average perch/roach ratio 0.40), expressing a species difference, wild roach in Southern Lake Saimaa, e.g. at 1 km from the

mill (July 2003), had bile RA concentrations 18 times higher than those in perch. Since high concentrations of RAs exist in the Southern Lake Saimaa sediments (see 4.1), it was suggested that part of the RA exposure of roach derived from sediments contaminated by RAs. In June 2004, the perch/roach ratios 1 km from the pulp mill were lower than those in the laboratory exposures, indicating possible additional RA exposure to roach from sediment. In addition, in May 2004 RA concentrations in fish bile showed a slight increase, which may be partially due to exposure to sediment (I) and the upwelling of contaminants from the lake bottom during the annual mixing of the water column. The same phenomenon had been observed earlier in May 1996, when the perch/roach ratio was around 0.30 in the 1 km area, indicating the possible exposure of roach to sediment (Karels & Oikari 2000). However, in winter 1997, the perch/roach ratio was around 0.80 at 1 km from the mill (Karels et al. 2001), indicating that the two species were similarly exposed. The reason for the higher ratio during winter may be a significant stratification of the BKME layer at the bottom of the lake in Southern Lake Saimaa downstream from the mill during that season (Seppälä 1986). This would expose perch more than roach to the effluent layer. Moreover, after the annual mixing of the water column, including the BKME layer, the exposure of roach to sediment increases. In conclusion, the perch/roach ratio may indicate an additional origin of roach RA exposure at 1 km downstream from the mill. However, the suggested perch/roach ratios must be interpreted with caution, and more experiments need to be done on this subject, e.g. exposure to BKME-contaminated sediment only. Overall, the results presented here on the biliary concentration of RAs imply that feral fish populations are exposed to BKME contaminants from water, food and possibly from sediment.

5.4 BKME-induced effects on fish – evidence of a measurable response?

5.4.1 BKME-induced changes in EROD activity

Hepatic EROD activity, indicating mono-oxygenase activity, has been used in several studies to demonstrate exposure to BKME in Southern Lake Saimaa (Lindström-Seppä & Oikari 1990, Oikari & Holmbom 1996, Soimasuo et al. 1998b, Karels et al. 2001) and other BKME-contaminated areas (Hodson et al. 1997). Although in the early 1990s in the 1 km area EROD activity in caged whitefish was around 10 times higher than in reference areas (Petänen et al. 1996), the levels had decreased close to reference levels by 1997 (Karels 2000). In summer 2004, there was no evidence of EROD-inducing compounds bioavailable to brown trout or whitefish downstream from the mill.

Traditionally EROD activity is considered an exposure marker of dioxin-like contaminants (Stegeman & Hahn 1994, Hodson 1996), and is also associated

with PAH-exposure (Fraguso et al. 1999, Oikari et al. 2002, Bauder et al. 2005). Although the underlying mechanism of EROD-induction has not been linked to specific effects, retene-exposed rainbow trout showed CYP1A induction and depletion of tissue antioxidants, indicating oxidative stress (Bauder et al. 2005). This implies that EROD-activity could also be used as a marker of biological effects, not only exposure.

5.4.2 BKME-induced changes in transcription - comparison of laboratory and field experiments

Microarray analysis is a new and increasingly popular technology for exploring transcriptomic responses to external or internal variables that specifically affect gene expression (Neumann & Galvez 2002). The measurements of gene expression levels upon exposure to a chemical can be used both to provide information about the mechanism of action of the toxicant and to form a sort of "genetic signature" for the identification of toxic products (Lettieri 2006). Additionally, ecotoxicogenomics can be seen as linkage between exposure and effects in aquatic risk assessment (Miracle & Ankley 2005), and thus as a strengthening factor in establishing causality. The results presented here demonstrate the difficulty of identifying compound-specific responses in gene expression when comparing results between field and laboratory studies.

To complement existing studies of RA toxicity, multiple gene expression analyses were performed following laboratory exposure of juvenile brown trout to waterborne RAs. This approach made it possible to monitor in parallel several functional groups as well as metabolic and regulatory pathways. Due to their common amphiphilic effect on the cellular membranes (Bushnell et al. 1985), RAs can be considered as a group of contaminants that have similar modes of action at the cellular level. Overall, the results of the present multiple gene expression analyses are in line with existing knowledge on the cellular mechanisms of RA toxicity, which can lead to jaundice, the best known pathological consequence of exposure to RA, caused by liver overload with damaged erythrocytes (Nikinmaa & Oikari 1982, Oikari & Nakari 1982, Oikari et al. 1983, Bushnell et al. 1985, Matsoff & Oikari 1987).

A marked down-regulation of hemoglobins, observed already at the lowest dose of RAs (0.6 µg/l), may explain the need to alleviate this burden. Due to their high sensitivity to different chemical stressors, including RAs, genes encoding hemoglobins can be regarded as a promising early warning group of markers for RAs. The present results indicate that the gene-level LOEC is around 1 µg/l, lower than the previously suggested 20 µg/l, physiological LOEC by enzyme endpoints (Oikari et al. 1983).

In addition to hemoglobins, a large proportion of the genes that responded to the RA exposures, demonstrated high sensitivity to chemical stressors different in their chemical nature (III, V). One third of the genes have shown differential hepatic expression in more than 67% of exposures of brown trout to cadmium and carbon tetrachloride (in total 17 study groups) (Krasnov

et al. 2007) and hence can be considered as candidate markers of generalized chemical stress.

However, owing to bidirectional regulation, caution is advised in using gene-level responses for diagnostic purposes. For example, in two four-day exposures of juvenile brown trout to cadmium and CCl_4 that were carried out under similar maintenance conditions hemoglobins showed opposite responses to toxicity (Krasnov et al. 2007). In fact, temporal fluctuations in physiological and biochemical parameters are common in stress responses. The decrease in hemoglobin expression may be due to depletion of bioavailable iron, which is regulated mainly by transferrin and ferritin (Hentze et al. 2006). The present results RAs included a more profound iron metabolic depression than in related experiments with model chemicals (Krasnov et al. 2007). Exposure to RAs resulted in consistent down-regulation of hemoglobins, other heme-containing proteins and transferrin. Although ferritin showed different responses to different doses, it is uncertain if this is accounted for by the specific effects of RAs or by the subacute (10-d) duration of exposure allowing acclimatization.

As expected, depression of the iron metabolism coincided with negative effects on the genes implicated in energy metabolism and protein biosynthesis. RAs have been reported to cause e.g. disruption of circulatory and plasma ion regulation, resulting in decreased plasma Na^+ and CI^- concentrations and plasma osmolality (Kruzynski 1979, Mattsoff & Oikari 1987), interference with respiration and energy metabolism (Oikari et al. 1988), including reduction in the oxygen carrying capacity of blood and in the aerobic metabolism (via inhibition of succinic dehydrogenase) (Nikinmaa & Oikari 1982), and elevated levels of lactate dehydrogenase and aspartate aminotransferase in heart muscle (Oikari et al. 1983). Down-regulation of cytochrome c oxidase is further evidence of the deficiency of bioavailable iron in the liver of RA-exposed fish (III). Koskinen et al. (2004) reported stimulation of cytochrome oxidase in the liver of rainbow trout exposed to CCl_4 and pyrene, while the same compounds did not affect this gene in brown trout. Coordinated down-regulation of ribosomal proteins was also specific for exposure to RAs. Impairment of the energy balance is one of the best known effects of RA on fish hepatocytes and erythrocytes (Bushnell et al. 1985, Nikinmaa et al. 1999, Rissanen et al. 2003).

With respect to the results obtained from experimental exposure in the field, the microarray analyses revealed gradiental down-regulation in three groups of co-regulated genes. First group included genes to the mitochondrial proteins involved in oxidative phosphorylation and transport of ATP. Two more groups characterized with heterogenous gene composition showed dose-dependent responses to cadmium, carbon tetrachloride, and pyrene in yolk sac fry of rainbow trout (Koskinen et al. 2004). Importantly, their expression decreased at high doses of these contaminants. Therefore the expression patterns observed in the field exposure may be evidence of chemical stress. Laboratory exposures of brown trout to Cd, CCl_4, pyrene and RAs affected hepatic expression of genes for hemoglobins, extracellular and immune proteins, chaperones, regulators of the cell cycle and proteins involved in cellular and

oxidative stress (Krasnov et al. 2007, III). Resin acids had also strong negative impact on the metabolism of the iron and protein biosynthesis mechanism (III). Surprisingly, none of these changes was found in the experimentally exposed brown trout in Southern Lake Saimaa.

To conclude, although the responses obtained from the laboratory and field were dissimilar, the results can be used in developing multiple gene expression analyses towards obtaining a more reliable diagnostic tool in the case of water-related contaminants. In addition, endpoints in transcription can be seen as biomarkers of exposure and effects. However, compound-specific effects should be discussed with caution, since the responses, even of a single species, may depend on various factors, such as dose, duration of exposure, age and the physiological condition of the fish. With respect to future perspectives, one aim would be to conduct a laboratory exposure of BKME in a dose-related manner.

5.5 Exposure assessment of biota to sediments in BKME-contaminated areas

The evidence of relevance in the exposure and risk assessment BKME contaminated sediments with respect to ERA (Suter 1990) and the sediment quality triad (Chapman 1990) obtained by the present and previous studies conducted in Southern Lake Saimaa and similar freshwater areas near pulp and paper mills is summarized below (Fig. 13).

Problem formulation
Modern BKME and black liquor contain RAs and WSs, which are discharged into the water column during normal operations (see Fig. 13 point 1A; permit limits) and during accidental spills (1B; exceed of permit limits). Surface sediments contain high amounts of RAs and WSs (Fig. 13 point 2). Additionally, biotransformation product of RAs, retene, and chlorophenolics in particular are present in the deeper layers of BKME sediments. These facts can be considered as an answer to the question whether the contaminants are accessible to the aquatic system or not.

Assessment of exposure
The contaminants of interest are released, desorbed or dissoluted from the sediment into the water phase when appropriate force is applied (see Fig. 13 point 4). These contaminants of concern are found in the tissues of resident benthic invertebrate populations adjacent to the pulp mill (see Fig. 13 point 5). Bioaccumulation was confirmed in the laboratory studies. Perch (see Fig. 13 point 6) and roach (see Fig. 13 point 7) populations and experimentally exposed brown trout and whitefish (see Fig. 13 point 8) in Southern Lake Saimaa are

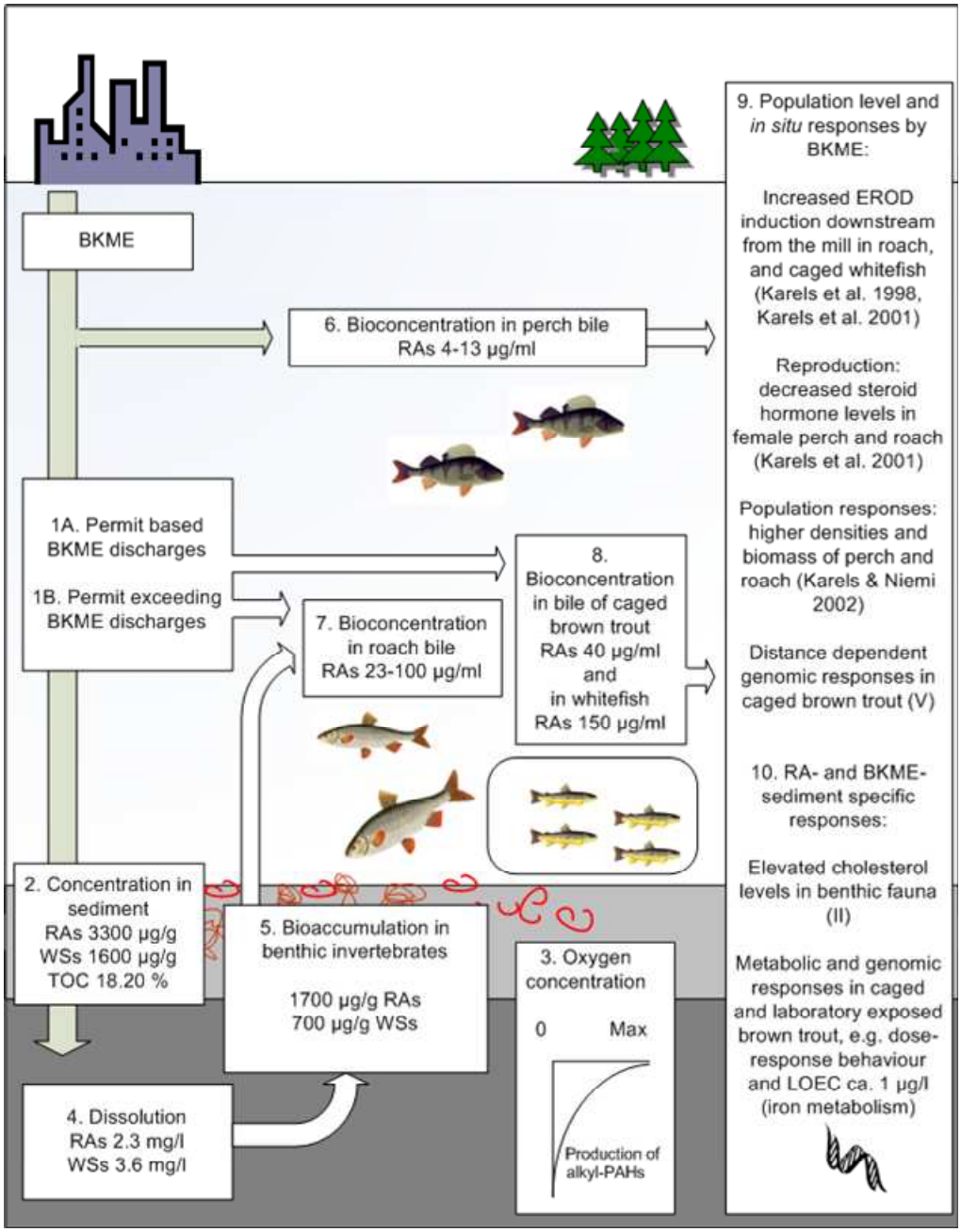

FIGURE 13 Fate and effects of BKME and its contaminants (resin acids, RAs and wood sterols, WSs) in the aquatic environment. 1. Discharge of BKME A) permit-based and B) in excess of permit. 2. Presence in sediment. 3. Oxygen concentration in sediment. 4. Dissolution of compounds from sediment in water phase. 5. Bioaccumulation into benthic invertebrates. 6-8. Bioconcentration in bile of perch, roach and caged brown trout and whitefish. 9. Effects of BKME in Southern Lake Saimaa areas on the population level. 10. Physiological, biotransformation and genomic effects of RAs and BKME-contaminants in field- and laboratory-exposed brown trout. (Images of fishes obtained with permission by Federation of Finnish Fisheries Associations, www.ahven.net.)

exposed to BKME-derived contaminants, as shown by the higher, gradient-dependent, RA concentrations in their bile in areas downstream from the mill when compared to reference areas. Furthermore, time- and concentration-dependent relationships with respect to biliary RAs were demonstrated with brown trout following laboratory exposure to a waterborne mixture of RAs, thus connecting the experimental evidence to the local species in the population survey. Species differences in RA-excretion into bile were also demonstrated in the laboratory exposure, indicating a higher biotransformation rate in roach compared to perch. Although it is difficult to assess the proportion of exposure from sediment due to the continuous discharge of BKME, the perch/roach ratio provided some evidence that the resident roach population is exposed to sediment derived-contaminants. Together these facts demonstrated are considered as evidence of the bioavailability and bioaccumulation of BKME-derived contaminants both in the waterborne laboratory exposures and those present in sediment in the field.

Assessment of effects

In the laboratory experiments, RAs and WS had several negative effects on fish and invertebrates on the genetic, cellular and physiological level (Kruzynski 1979, Nikinmaa & Oikari 1982, Oikari et al. 1983, Mellanen et al. 1996, Lahdelma & Oikari 2005, III). Moreover, the bioaccumulation and bioconcentration can be considered as an adverse effect (II, III, IV). In Southern Lake Saimaa, the fish population showed elevated biomass levels and densities in the industrial areas compared to reference areas. Benthic invertebrates showed elevated levels of cholesterol and changed community structure near the mill, and previous studies have shown effects on the reproductive status of perch. However, EROD induction in fish has decreased during the last decade in the area. Additionally, brown trout showed genomic and biotransformation responses following in laboratory exposure to waterborne BKME-related RAs. However, in the experimental field exposure of brown trout none of the RA-sensitive genes were affected, although other chemical stress-related genes showed gradiental down-regulation close to the source of BKME. Taken together all the results presented here and in previous studies, indicate that there are measurable responses (genomic, biotransformation, community level) in BKME-contaminated areas and that RAs are among the causes of these responses.

Risk assessment

On the basis of the results and other conclusions presented above, the sediments in Southern Lake Saimaa pose a risk to the aquatic environment, exposing benthic animals to harmful contaminants in concentrations that can elicit negative effects on the genomic, metabolic, physiological and population levels.

In addition, causality between the concentration of RAs in sediment and adverse observation in aquatic animals can be shown, when Koch's postulates are applied from the point of view of ERA. Accordingly, the following four

criteria can be posited: 1) the effects of RAs are regularly associated with exposure to the toxicant, e.g. bioconcentration in fish bile and fish with jaundice; 2) the effects are observed when organisms, e.g. brown trout, are exposed to RAs under controlled conditions in the laboratory and 3) the same effects are observed in the field; and 4) indicators of exposure to RAs, i.e. conjugated RAs in fish bile and genomic responses in fish liver are found. The weight of the evidence can be strengthened by applying Hill's criteria, such as specificity and consistency, which are evident in the case of biliary RAs. However, since the genomic responses were dissimilar between the laboratory and field experiments, RA-sensitive genes are not the strongest evidence of effects when considering exposure to BKME-related contaminants.

Although no clear correlations regarding sediments as a source of exposure to fish populations were obtained in this thesis, there are various strands of evidence in the literature (Fig. 13) that BKME-sediments pose a risk to aquatic animals, and thus sediment management strategies may be needed (see 1.4; Apits & Power 2002). In the areas nearest to pulp and paper mills which have significantly elevated concentrations of RAs and other BKME-contaminants in their sediment, the industry could consider creating a reliable monitoring program. Additionally, supplementary risk and effect assessments should be made in the areas adjacent to pulp and paper mills. In the most serious cases of BKME-contaminated sediments, i.e. in hot spots, remedial actions, such as *in situ* containment or dredging, could be applied. As far as the RA concentrations in the sediment are concerned, the target values of possible remedial actions should be set closer to those of the reference areas than the contaminated areas. However, the threshold or target values of BKME-sediments are difficult to set, and the values should be assessed case-specifically.

With regard EU legislation, the EU Water Framework Directive (WFD, 2000/60/EC) addresses to only a very limited extent the issue of sediment quality. However, the WDF requires that water quality and ecological status are addressed within a set time frame (15 years), both of which are closely linked to sediment quality; thus eventually sediment fluxes and management will need to be taken up by the EU (Föstner 2002, Brils 2004). A more detailed list of the international and national conventions and regulations relating to sediments, and in most cases with its quality (e.g. dredged material) are given in the final draft of the SedNet booklet Contaminated Sediments in European River Basins (Salomons & Brils 2004). Although legislation directly concerning BKME-contaminated sediments does not exist in Finland or Europe, in severe cases of contamination the Instructions for dredging and depositing dredged materials (Ministry of the Environment 2004) can be applied.

6 CONCLUSIONS

The aim of this work was to study the exposure of aquatic animals to pulp and paper industry-contaminated sediments, with Southern Lake Saimaa as a model area. Sediment and sedimenting particles contain significant concentrations of contaminants derived from pulp and paper mills, such as RAs, retene and WSs. These contaminants were the focus of this study. The possible bioavailability of wood extractives and retene in downstream effluent sites was established by dissolution experiments, where toxic amounts of contaminants were released in the water phase when sufficient disturbance was applied. It is important to note that sediments may remain fairly undisturbed in natural conditions, and thus the results presented here are mostly applicable to slow stream-like areas and areas subject to erosion characterized by relatively heavy turbulence and mechanical disturbances such as dredging.

Total contaminant concentrations in sediments are poor predictors of their levels in sediment-dwelling invertebrates, since multiple physical, chemical and biological factors affect on the uptake and bioavailability of the contaminants to animals. Therefore the approach used in this study, measuring the uptake of contaminants into the wild and in the laboratory, provided environmentally relevant information on the level of exposure of benthic fauna to BKME-derived sediments. The ability of Diptera to biotransform contaminants was demonstrated with the field material. Additionally, *C. plumosus* as well as *L. variegatus*, were able to bind, possibly conjugate, RAs and β-sitosterol in laboratory exposure. Nevertheless, more assessment research is needed regarding the biotransformation properties of benthic invertebrates. Although the experiment revealed that laboratory exposures can underestimate the exposure situation in the field, both data showed the bioavailability of BKME-derived contaminants in sediments and their uptake into benthic invertebrates. Given the changes in benthic community levels recorded in similar areas adjacent to pulp and paper mills, there is a reasonable possibility that BKME-contaminated sediments pose a risk to benthic invertebrates in such areas.

By analyzing RAs in fish bile it is possible to obtain quantitative sensitive biomarkers of exposure. The conjugation and excretory status of the brown trout, the main model species used, was in steady state within one day from the onset of exposure. Resin acids in the bile showed a clear concentration-dependent behavior in waterborne exposure in the bile of brown trout. This result is of major interest in evaluating the environmental effects and monitoring remedial actions taken in areas contaminated, e.g., by pulp and paper effluents. In addition, the species differences that were investigated allow interspecies extrapolation of bioconcentrated RAs, which can then be applied in risk assessment of BKME-contaminated areas. Moreover, differences in the biliary excretion of RAs between perch and roach, combined with their different exposure status in Southern Lake Saimaa, suggest the possibility that sediments serve as an additional source of exposure to roach.

Multiple gene expression at subacute exposures to RAs together with metabolic bile analyses outlined the possible targets of RA toxicity. The genomic responses observed are in line with the previously known physiological and cellular effects of RAs in fish. Combination of present data with that of other studies on the effects of RAs as aquatic contaminants substantially enhanced the possibilities to search for RA-specific responses and the development of novel diagnostic markers for this group of contaminants. Moreover, a new LOEC-value, applicable in risk assessment, was determined for RAs (1 µg/l). However, the genomic responses found here must be viewed with caution since there are uncertainties in the new technology, as found when applied from laboratory to *in situ* conditions.

In conclusion, BKME-contaminated sediment may act as a source of bioactive chemicals in the aquatic environment in Southern Lake Saimaa. Because benthic invertebrates are an important food source for fish, and fish bile showed elevated levels of RAs *in situ* and in the laboratory, more research needs to be done on this subject, e.g. laboratory exposures of perch and roach with BKME-sediment. Further, the genomic study suggested that possible early-warning signs should be studied in more detail in the future. The bioaccumulation and bioconcentration of contaminants from water and sediment in animals was evident, and the levels found in the present study in exposed biota can elicit negative effects on the genomic, biotransformation, physiological and population levels.

To conclude, the overall goal of this thesis was to consider whether contaminated sediments constitute a long-term ecotoxicological risk and the results showed elevated levels of contaminants in benthic animals, implying the presence of such a risk.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Sellu- ja paperiteollisuuden saastuttamat sedimentit altistavana tekijänä vesieläimille

Sellu- ja paperiteollisuuden jätevesien sisältämien vierasaineiden haittavaikutuksia kaloihin ja muihin eliöihin on tutkittu jo vuosikymmeniä, mutta niiden saastuttamien sedimenttien tutkiminen on noussut merkittävään asemaan vasta viime aikoina. Tässä työssä tutkittiin sellu- ja paperiteollisuuden saastuttamien sedimenttien osuutta altistavana lähteenä vesieläimille Etelä-Saimaan alueella, jossa vertailtiin erityisesti alueen sellu- ja paperitehtaan alapuolisen vesistön tilannetta puhtaisiin vertailualueisiin. Tutkimuksessa mitattiin haitta-aineiden (hartsihapot, reteeni ja puusterolit) biosaatavuutta vesiuuttojen avulla saastuneesta sedimentistä veteen ja näiden haitta-aineiden siirtymistä sedimentistä paikallisiin pohjaeläimiin (kaksisiipisten toukat ja harvasukamadot) sekä laboratoriossa altistettuihin surviaissääsken toukkiin (*Chironomus plumosus*) ja harvasukamatoihin (*Lumbriculus variegatus*). Lisäksi tutkittiin hartsihappojen geeni- ja aineenvaihduntatason vasteita järvitaimenella (*Salmo trutta* m. *lacustris*) altistamalla kaloja laboratoriossa hartsihapposeokselle. Sapen hartsihappopitoisuuksia määrittämällä vertailtiin myös viiden eri kalalajin eroja sappiaineenvaihdunnan suhteen. Lisäksi järvitaimenia ja planktonsiikoja altistettiin sellutehtaan lähialueilla, jotta laboratoriokokeista saatuja geeni- ja metaboliatason vasteita voitiin vertailla luonnonoloihin. Lopuksi tutkittiin kesäkuussa 2003 Etelä-Saimaalla tapahtuneen mustalipeäpäästön vaikutuksia alueen kalapopulaatioiden altistumiseen hartsihapoille tarkkailemalla alueen ahventen (*Perca fluviantis*) ja särkien (*Rutilus rutilus*) sapen hartsihappopitoisuuksia. Hartsihappojen ja puusterolien dissoluutio saavutti tasapainon viikossa osoittaen yhdisteiden mahdollisen biosaatavuuden saastuneesta sedimentistä. Hartsihappojen, puusterolien ja kloorifenolien siirtyminen sedimentistä pohjaeläimiin havaittiin sellutehtaan alapuolisella alueella kenttäaineistossa sekä osoitettiin laboratoriokokein, jolloin havaittiin kaksisiipisten toukissa tehtaan alapuolisella alueella kymmenkertaisia hartsihappopitoisuuksia verrattuna kontrollialueeseen. Lisäksi osoitettiin surviaissääsken toukkien ja harvasukamatojen kyky biotransformoida hartsihappoja, eli muuntaa yhdisteitä aineenvaihdunnan kautta vesiliukoisempaan muotoon. Altistettaessa järvitaimenia hartsihapoille havaittiin, että kala erittää hartsihappokonjugaatteja sappeen sekä aika- että pitoisuusriippuvaisesti saavuttaen tasapainon 24 tunnissa. Logaritminen biokonsentroitumiskerroin oli järvitaimenelle 4,46 – 5,11. Laboratoriossa altistetuissa järvitaimenissa havaittiin geenitason vasteita mm. raudan aineenvaihdunnan ja happiradikaalien torjunnan tai tuotannon geeneissä jo erittäin matalassa hartsihappopitoisuudessa (0,6 µg/l). Tutkitun tehtaan jätevesien leviämisalueen lähellä altistetuissa järvitaimenissa ei kuitenkaan havaittu samoja geenitason vasteita kuin laboratoriokokeissa. Ahven- ja särkipopulaatioiden sappianalyysien perusteella eteläisen Saimaan alue toipui heinäkuussa 2003 tapahtuneesta mustalipeäpäästöstä viimeistään syyskuuhun 2003 mennessä. Särjen kyky erittää konjugoituneita hartsihappoja sappeen oli suurempi kuin ahvenella, mutta toi-

saalta pohjan lähellä elävä ja siellä ruokaileva särki on kosketuksissa sedimenttiin enemmän kuin ahven. Näin ollen särjet altistuivat myös sedimentistä lähtöisin oleville vierasaineille. Tutkimuksen tulokset osoittavat, että sellu- ja paperiteollisuuden saastuttamissa sedimenteissä on haitallisia vierasaineita, jotka ovat biosaatavia eläimille. Eliöt myös altistuvat näille vierasaineille, joten sedimentit muodostavat mahdollisen riskin alueen eläimille.

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