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Heli Ratia

Ecotoxicological Status of a Watercourse Recovering from Heavy Loading by Pulp and Paper Industry

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ABSTRACT

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The Äänekoski watercourse in Central Finland has been loaded over 100 years by chemical wood industry. It was heavily polluted until the recovery started in 1985 due to the mill modernization and improved wastewater treatment. However, large amounts of harmful compounds may still be discharged due to high effluent volumes. In addition, resuspension of contaminated sediments may release harmful compounds to the water. To evaluate the current ecotoxicological risks, the concentrations of the natural wood extractives (resin acids, betulinol and wood sterols) and retene (a derivative of resin acids) in the sediments were measured, and their dissolution potency was investigated. Also persistent organochlorines (polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls) were analyzed from the sediment. As a novel approach, to evaluate the risks of contaminated sediments, spatial distribution of the most common wood extractives were calculated to assess the maximal loads due to physical disturbances, such as extensive floods. Also biological indicators were used to evaluate the recovery. Hepatic EROD activity, a biomarker of exposure to the dioxin-like compounds, was measured from wild perch and roach, and from juvenile rainbow trout exposed to the contaminated sediment in the laboratory, and no increased EROD induction was observed. To assess the current ecotoxicological status with invertebrates, species composition and gill abnormalities of caddis larvae were analyzed in 1999–2008, and also these results revealed the recovery. Although no effects on animals were observed, based on the sediment dissolution experiments and on the literature, concentrations of resin acids and betulinol may be high enough to cause toxic effects on animals in the event of severe sediment erosion. Instead, retene, wood sterols and the studied organochlorines do not pose a risk at the study area.

Keywords: Caddis larvae; dissolution; EROD activity; gill damage; pulp and paper industry; sediment; wood extractive.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

In article I, I am the second author, as Lassila, and contributed to the sediment analytics and participated in writing. In article II, I am the first author equal with Rämänen, and contributed significantly to the sediment sampling, laboratory work and finalized the paper. I am the first author of articles III-V, and contributed significantly to the planning, data collection, analyses and writing. I have been the corresponding author in all articles (I-V).

I Rämänen H., Lassila H., Lensu A., Lahti M. & Oikari A. 2010. Dissolution and spatial distribution of resin acids and retene in sediments contaminated by pulp and paper industry. *Journal of Soils and Sediments* 10: 349–358.

II Ratia H., Rämänen H., Lensu A. & Oikari A. 2012. Betulinol and wood sterols in sediments contaminated by pulp and paper mill effluents: dissolution and spatial distribution. *Environmental Science and Pollution Research.* DOI 10.1007/s11356-012-1381-3.

III Ratia H. & Oikari A. 2012. Vertical distribution of AhR-activating compounds in sediments contaminated by modern pulp and paper industry. Submitted manuscript.

IV Ratia H., Vehniäinen E.-R., Rusanen A. & Oikari A 2012. Recovery of historically contaminated watercourse polluted by chemical wood industry — EROD activity in fish as biomarker. Submitted manuscript.

V Ratia H., Vuori K.-M. & Oikari A. 2012. Caddis larvae (Trichoptera, Hydropsychidae) indicate delaying recovery of a watercourse polluted by pulp and paper industry. *Ecological Indicators* 15: 217–226.

ABBREVIATIONS

AhR	aryl hydrocarbon receptor
AOX	adsorbable organic halides
BOD	biological oxygen demand
COD	chemical oxygen demand
CYP1A	cytochrome P450 1A
DHAA	dehydroabietic acid
dw	dry weight
ECF	elemental chlorine free
EROD	ethoxyresorufin-*O*-deethylase
GC-MS	gas chromatograph – mass spectrometer
HA	hydropsychidae index
IPA	isopimaric acid
i.p.	intraperitoneal
log K_{ow}	logarithm of octanol water partition coefficient
LOI	loss on ignition
MFO	mixed-function oxygenase
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
RA	resin acid
TCF	total chlorine free
TOC	total organic carbon
WS	wood sterol
ww	wet weight

1 INTRODUCTION

1.1 Aquatic pollution by pulp and paper industry

The pulp and paper industry is one of the most serious polluters of aquatic resources in the world. In boreal latitudes, excluding Russia and Norway, the chemical wood industry has been the most notable industrial activity disturbing aquatic ecosystems over a hundred years. Therefore, the impacts on aquatic organisms caused by pulp and paper effluents have been widely studied and reviewed, especially in Northern Europe and North America (Södergren et al. 1988, Owens 1991, Ali & Sreekrishnan 2001, Hewitt et al. 2008). Nowadays pulp and paper production has shifted more to Asia and South America, but it still has a remarkable role in the northern latitudes also. In Scandinavia and North America, environmental research on aquatic impacts traditionally focused on water quality parameters such as oxygen depletion and nutrient load until the 1980s, when concern shifted to chemical toxicity (Owens 1991).

Pulp and paper mill effluents contain more than 250 identified chemicals, many of which are hazardous to aquatic animals (Suntio et al. 1988, Ali & Sreekrishnan 2001). The loading of bioactive compounds has remarkably decreased since the 1970s and 1980s due to modern technology with efficient wastewater treatment and altered bleaching methods (Dahlman et al. 1991, Owens et al. 1994, Solomon 1996, Strömberg et al. 1996, Hewitt et al. 2008). Although the purification efficiency of the current effluents has been improved, some amounts are still released and the loading is continuous (Chapman et al. 1991, Strömberg et al. 1996, Kaplin et al. 1997, Koistinen et al. 1998, Leppänen et al. 1998, Makris & Banerjee 2002, Pokhrel & Viraraghavan 2004). For instace, effects on reproduction and other adverse impacts on fish have still been observed downstream from the modern elemental chlorine free (ECF) pulp mill with tertiary effluent treatment (Chiang et al. 2011). In addition, worldwide, pulp and paper production has been continuously increasing (Forström et al. 2006) and due to high effluent volumes large amounts of bioactive compounds may still be discharged.

Besides chemicals previously added and formed during the pulp and paper making processes, such as polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), there are also natural wood extractives, nutrient loads, and suspended solids, which may cause harmful effects on biota (Owens 1991, Ali & Sreekrishnan 2001, Hewitt et al. 2008). Elevated levels of natural wood extractives and their derivatives, for instance retene (7-isopropyl-1-methylphenantrene), have been commonly found in the sediment downstream from pulp and paper mills (Tavendale et al. 1997, Koistinen et al. 1998, Leppänen & Oikari 2001, Lahdelma & Oikari 2005, 2006, Meriläinen et al. 2006). Furthermore, historically contaminated sediment may constitute a risk if they act as sources of the contaminants (Sibley et al. 1997, Meriläinen et al. 2006, Hollert et al. 2007, Westrich et al. 2007). Especially in the river-like watercourses, as in case of this study, the harmful compounds originally discharged from the pulp and paper industry may be released to the water column due to sediment resuspension (Meriläinen et al. 2006).

More abundant floods may further increase the risk of sediment remobilization (Hollert et al. 2007, IPCC 2007, Westrich et al. 2007), as the annual precipitation has been predicted to rise 10–40 % in the boreal latitudes within this century (IPCC 2007). The uppermost sediment is the most sensitive layer to the erosion (Förstner et al. 2004) and the presence of endocrine disrupting chemicals, such as betulinol (Mellanen et al. 1996, Christianson-Heiska et al. 2004, 2008) and wood sterols (WSs) (Tremblay & van der Kraak 1998, 1999) raises a concern about safety to aquatic animals. It is known that during periods of flooding, sediments polluted by organic hydrophobic contaminants can function as a secondary source of contamination (Stachel et al. 2004, Hilscherova et al. 2007). For instance in the River Elbe, a massive flood in 2002, caused resuspension of fine suspended material (Baborowski et al. 2004, Stachel et al. 2004) where the hydrophobic compounds tend to be sorbed (Servos et al. 1989a, 1989b, Fletcher & McKay 1993, Lebo et al. 2003). Therefore, being reversible process, it can be assumed that historically adsorbed contaminants can be released and expose aquatic biota, thus causing harmful effects on ecosystem.

1.2 Ecotoxicological effects on biota

During elemental chlorine bleaching and inefficient wastewater treatment, large amounts of chlorinated compounds, such as chlorophenolics, chlorinated lignin, PCDDs and PCDFs were discharged to the waters (Södergren et al. 1988, Dahlman et al. 1991, Solomon 1996, Strömberg et al. 1996). Adsorbable organic halids (AOX) has been a commonly used parameter of chlorinated organic material. The majority of AOX consists of chlorinated lignin which is a large molecular weight compound and therefore considered to be fairly non-toxic (Kringstad & Lindström 1984), although, Pessala et al. (2004) suggested that the

amount of lignin has more significant role than the molecular size regarding the toxicity of softwood effluents. However, probably the most discussed compounds in the pulp and paper mill effluents are persistent chlorinated organic compounds and natural wood extractives, as they have been commonly present in the effluents and many of them may cause harmful effects on biota (Owens 1991, Billiard et al. 1999, Ali & Sreekrishnan 2001, Vehniäinen et al. 2003, Christianson-Heiska et al. 2004, Meriläinen et al. 2006, Hewitt et al. 2008, Orrego et al. 2010).

Before modern bleaching and advanced wastewater treatment techniques, various effects on fish populations were observed, such as low biomass and changes in species composition (Södergren et al. 1988, Södergren 1991, Hakkari & Bagge 1992). Reproductive impacts have been also widely documented (Andersson et al. 1988, Södergren et al. 1988, Södergren 1991, Sandström 1996, Solomon 1996, Karels et al. 1998, van den Heuvel 2006). One example of strong effluent toxicity in the 1980s is the laboratory experiment, in which mature female fish were exposed to 0.5 % (v/v) effluent. This led to the decreased egg numbers and effects were seen also on the offspring, such as symptoms of blue sac disease, morphological changes and mortality (Vuorinen & Vuorinen 1985).

Improved wastewater treatment techniques in 1980s and transition to elemental chlorine free (ECF) and total chlorine free (TCF) bleaching in the 1990s remarkably decreased the loadings to the watercourses in Finland (Table 1) and worldwide (Dahlman et al. 1991, Owens et al. 1994, Solomon 1996, Strömberg et al. 1996, Hewitt et al. 2008) despite continuous increase in pulp and paper production. For instance, in Finland, biological oxygen demand (BOD) in the pulp and paper mills effluents has decreased about 95 % since 1980, and also the loading of nutrients has remarkably decreased (Table 1) (Metla 2012). These improvements possibly have affected biota due to decreased eutrophication and increased oxygen concentrations (Granberg et al. 1987, Palomäki & Salo 2008). In addition, loading of AOX has decreased about 90 % after ECF bleaching has started in the early 1990s (SYKE 2012).

TABLE 1 Mean annual production of sulphite pulp, softwood and hardwood sulphate pulp, unbleached pulp, and paper and board (millions tonns) in Finland in 1970–2010. Discharged water quality parameters (tons per year): biological oxygen demand (BOD_7), total nitrogen (N), total phosphorus (P) (Metla 2012) and adsorbable organic halides (AOX) (SYKE 2012).

Year	Sulphite pulp	Sulphate softwood	Sulphate hardwood	Sulphate unbleached	Paper & Board	BOD_7	Total N	Total P	AOX
1970	1460	1440	–	1290	4260	–	–	–	–
1975	1090	770	530	970	3990	–	–	–	–
1980	810	1650	1060	1090	5920	262 770	3910	570	–
1985	450	1830	1550	860	7450	199 410	4330	750	–
1990	290	2240	1960	670	8970	88 850	4110	650	8660
1995	–	2930	2170	680	10 940	31 140	3180	320	1660
2000	–	3500	2900	710	13 510	17930	2820	210	1100
2005	–	3470	2780	530	12 390	11550	2490	170	1010
2010	–	3970	2300	460	11 760	9180	2440	150	1040

Although remarkable reduction in toxicity of the effluents was observed after shifting to more efficient wastewater treatment and ECF bleaching, it did not totally remove endocrine disruptive effects on fish, which have still been documented in 2000s and more recently in 2008. It has been suggested that these endocrine disruptive effects may be caused by wood extractives in the effluents (van den Heuvel 2006, Hewitt et al. 2008, Chiang et al. 2011).

1.3 Bioactive compounds in the effluents

1.3.1 Resin acids

Resin acids (RAs), together with fatty acids, are the dominant wood extractives in softwood pulping effluents (Kaplin et al. 1997). These acids are weak hydrophobic acids (log K_{ow} 6.52; Mellanen et al. 1996) and toxic to fish (Hutchins 1979, Ali & Sreekrishnan 2001) and invertebrates (Hickey & Martin 1995). Two types of RAs exist based on their chemical structure: abietic and pimaric (Sjöström 1993). Dehydroabietic acid (DHAA) and abietic acid are the most abundant abietic type of RAs (Leach & Thakore 1976, Kaplin et al. 1997, Kostamo & Kukkonen 2003). DHAA is less toxic, but more water soluble compared to pimaric type of RAs (Peng & Roberts 2000).

The toxicity of RAs was observed already in the early 1970s. For instance Rogers (1973) found that the mortality of salmonids was related to the concentration of RAs in pulp mill effluents. An acutely lethal concentration was about 2 mg l^{-1} (Rogers 1973). In the case of individual RAs, lethal concentrations for trout are 1.1 mg l^{-1} DHAA and 0.4 mg l^{-1} isopimaric acid (IPA) (Leach & Thakore 1976), i.e. below their water solubilities (DHAA 5.1 mg l^{-1}, IPA 1.7 mg l^{-1}; Peng & Roberts 2000).

RAs cause increased and altered use of body energy reserves (Oikari et al. 1983, Rissanen et al. 2003), decreased plasma volume and impaired liver function (Oikari et al. 1983), as well as cellular effects, such as disturbed iron metabolism and decreased expression of genes encoding for enzymes degrading reactive oxygen species (Meriläinen et al. 2007). DHAA may cause reproductive disturbances at a concentration of 50 µg l^{-1} (Christianson-Heiska et al. 2008), and it has been suggested to cause indirect anti-estrogenic effects on fish (Orrego et al. 2010). However, the lowest effective concentration of DHAA on trout (*Salmo gairdneri*) was long thought to be about 20 µg l^{-1} (Oikari et al. 1983), but genomic responses have recently been measured at levels below 1 µg l^{-1} (Meriläinen et al. 2007). RAs are toxic to invertebrates also, for example to amphipods, clams and oligochaetes, affecting their survival and reproduction (Hickey & Martin 1995).

Although the latest technology efficiently removes RAs from the effluent (94–96 % purification) (Dahlman et al. 1991, Makris & Banerjee 2002, Kostamo & Kukkonen 2003, Pokhrel &Viraraghavan 2004), their loading still is continuous (Kaplin et al. 1997, Koistinen et al. 1998, Leppänen et al. 1998, Makris &

Banerjee 2002, Pokhrel & Viraraghavan 2004). In addition, sediment contaminated by the pulp and paper industry may act as sources of RAs (Sibley et al. 1997, Meriläinen et al. 2006), and thus the risk of the exposure may be continuous.

1.3.2 Retene

Under anaerobic conditions DHAA may transform to retene, alkylated polycyclic aromatic hydrocarbon (PAH) (Tavendale et al. 1997, Leppänen & Oikari 1999b). Retene is a hydrophobic compound (log K_{ow} 6.4; Basu et al. 2001) and common xenobiotic found in the sediment downstream from pulp and paper industry (Koistinen et al. 1998, Leppänen & Oikari 1999a, 1999b, 1999c, 2001, Meriläinen et al. 2006). The highest reported concentration of retene (3300 µg g^{-1}, dw) has been measured from the buried sediment of Lake Lievestuoreenjärvi in Central Finland (Leppänen & Oikari 2001). However, in sediment contaminated by the pulp and paper industry, concentrations may commonly range roughly between 500–1000 µg g^{-1}, dw (Lahdelma & Oikari 2005). This contrasts dramatically with natural background under 0.1 µg g^{-1}, dw (Billiard et al. 1999).

Retene can be bioavailable to animals in the sediment (Leppänen & Oikari 1999a, Nikkilä et al. 2001) and it is toxic to the early life stages of fish causing reduced growth, yolk sac edema and mortality (Billiard et al. 1999, Vehniäinen et al. 2003). Deleterious malformations in fish embryos are formed at low nominal concentrations (10–32 µg l^{-1}) (Billiard et al. 1999, Brinkworth et al. 2003). In addition, the toxicity of retene increases under UV radiation (Vehniäinen et al. 2003). Therefore, if retene would happen to be remobilized through dissolution, as in cases of floods or dredging, it can cause harmful effects on aquatic biota.

1.3.3 Betulinol

Betulinol (betulin) is the main extractive in the birches, i.e. *Betula* spp., as even 54–82 % of dried birch bark extract is betulinol (Abyshev et al. 2007) whereas it is missing totally from the softwoods (Vikström et al. 2005, Abyshev et al. 2007). Betulinol has been studied for its medical aspects, as it is known to inhibit bacterial growth (Salina et al. 2010). In addition, betulinic acid, a derivative of betulinol, has anti-cancer, anti-malarial and anti-inflammatory effects (Moghaddam et al. 2012).

Betulinol may have estrogenic effects on fish (Mellanen et al. 1996, Christianson-Heiska et al. 2004, 2008). Endocrine-disrupting effects have been observed at a concentration of just 5 µg l^{-1} (Christianson-Heiska et al. 2004, 2008), which is the concentration measured from the paper mill effluents (Kaplin et al. 1997). Betulinol may also weaken the disease defense mechanism of fish (Christianson-Heiska et al. 2004). Overall, the environmental fate and risks of betulinol are still poorly known.

1.3.4 Wood sterols

The β–sitosterol, stigmastanol and campesterol are the most abundant sterols in soft wood and hard wood species which are used for paper manufacturing in Finland and Sweden (Sjöström 1993). Therefore, those are also detected from the pulp and paper mill effluents (Kostamo & Kukkonen 2003). Due to their strong hydrophobicity (log K_{ow} 9.65 for β–sitosterol; Kinney et al. 2006) they tend to sorb to the sediment (Sjöström 1993), and thus become bioaccessible by the benthic animals (Meriläinen & Oikari 2008a), and bottom feeding fish.

WSs may cause harmful effects on biota (Owens 1991, Christianson-Heiska et al. 2004, Meriläinen et al. 2006). They may cause hormonal disruption in fish, for example β-sitosterol and stigmasterol are able to bind to the estrogen receptors and affect the reproductive functions in rainbow trout (Tremblay & van der Kraak 1998, 1999). Not only estrogenic effects are noted but also the masculinization of female fish have been detected in pulp and paper mill contaminated waters (Howell et al. 1980, Ellis et al. 2003), which probably could be explained by microbial degradation and transformation of phytosterols into bioactive androgens (Jenkins et al. 2003). However, high concentrations of β-sitosterol were needed to cause severe consequences on maturing trout (*Salmo trutta lacustris*). The observed effects have been egg mortality, smaller egg size, lower mean weight of the yolk sac larvae and higher prevalence of deformed or otherwise diseased larvae (Lehtinen et al. 1999). Faster hatching and increased hatchability in rainbow trout, medaka (*Oryzias latipes*) and flagfish (*Jordanella floridae*) embryos are also documented (Orrego et al. 2011).

1.3.5 Organochlorines — PCDDs, PCDFs and PCBs

PCDDs and PCDFs have been by-products of pulp bleaching (Rappe et al. 1987, 1989, 1990, Owens 1991) whereas polychlorinated biphenyls (PCBs) were used in manufacturing of carbonless copy paper from 1950s to 1970s (Carr et al. 1977). PCDDs, PCDFs as well as PCBs are included in the twelve initial persistent organic pollutants under the Stockholm Convention (Stockholm Convention 2012).

PCDDs and PCDFs are hydrophobic compounds (log Kow 6.64–8.60; Servos et al. 1989a) which are able to bioaccumulate in the organisms (Rappe et al. 1987, Södergren et al. 1988, Servos et al. 1989a, 1989b, Fletcher & McKay 1993, Koistinen et al. 1993). The most toxic dioxin, 2,3,7,8-TCDD, causes severe effects on fish, for instance symptoms of yolk sac disease (Henry et al. 1997), reduced embryonic brain development (Hill et al. 2003), and morphological abnormalities (Spitsbergen et al. 1988). In the coastal area of Sweden, concentrations of 2,3,7,8-TCDD and 2,3,7,8-TCDF in perch (*Perca fluviatilis*) decreased with increasing distance from the pulp mill discharge of Norrsundet. In that facility, the use of elemental chlorine for pulp bleaching was reduced ca. 40 % between 1983–1985 which reflected in the less accumulated concentrations in fish (Södergren et al. 1988). In relation to PCBs, coplanar PCBs may cause oxidative stress and immune responses in fish (Duffy et al. 2002).

The pulp and paper industry around the world has used TCF or ECF bleaching methods since the early 1990s which have decreased but not totally eliminated the formation of the PCDDs and PCDFs (Strömberg et al. 1996). The use of PCBs was banned in many countries already in the 1970s and 1980s, and the production was ceased entirely in 1993 (AMAP 2000). Although the concentrations of these compounds have decreased in the effluents (Oikari & Holmbom 1996, Solomon 1996) and in the sediment since the 1970s and 1980s (Rappe et al. 1987, Huestis et al. 1997, Sanctorum et al. 2011), hotspots still exist in many polluted watercourses and in the coastal areas (Breivik et al. 2004, Verta et al. 2007, Sundqvist et al. 2009, Heimann et al. 2011). Due to the persistence and toxic characteristics these chemicals are widely studied and still monitored in aquatic environment (Owens 1991, Ali & Sreekrishnan 2001, Verta et al. 2007, Sundqvist et al. 2009).

1.4 History of the Äänekoski watercourse in Central Finland

1.4.1 Chemical wood industry in Äänekoski

This study is focused on the downstream waterways of the City of Äänekoski, Central Finland, where the pulp and paper industries have existed for over 100 years (Tuuri et al. 1996, Meriläinen et al. 2001). The chemical wood industry in Äänekoski began around 1890s when the board mill was established there, followed by paper production in 1906 and newsprint production in 1917. In the 1960s, manufacturing of coated printing papers and wallpapers began, and a new board mill was initiated. In 1987, a second paper machine was started. The paper mill was closed in 2011 but the board mill is still producing fully coated bleached paperboards, at an annual capacity of 240 000 tons. Loading of the paper and board mill effluent to the water has been chemical oxygen demand (COD) 9.4 kg t^{-1}, AOX 0.07 kg t^{-1}, total N 0.09 kg t^{-1} and total P 0.01 kg t^{-1} in 2008. The paper mill and board mills have their own wastewater treatment plant using chemical and mechanical methods (Metsä Board 2012).

Pulp production was started in 1938, producing sulfite cellulose (Tuuri et al. 1996, Meriläinen et al. 2001). Chlorine-based bleaching began in the 1950s (Reunala et al. 1998), and in 1961 a second pulp mill was built, producing sulfate cellulose (Granberg et al. 1987). In the early 1960s, the sulfite and sulfate cellulose production were about 45 000 and 87 000 tons per year, respectively (Rämänen 2008). The first mechanical wastewater treatment plants were built in the 1970s (Kukkonen & Välttilä 1988). Since the early 1970s, the production volumes were about 50 000 and 120 000 tons of sulfite and sulfate cellulose per year, respectively. Both birch and spruce were used. The proportion of birch cellulose was about 70 % in the 1970s and decreased to 30 % in the 1980s (Rämänen 2008). The sulfite process was discontinued in 1984, and one year later another old pulp mill was also closed (Granberg et al. 1987).

In 1985, a new pulp mill, using the sulfate method and activated sludge wastewater treatment, was opened. Before that only mechanical wastewater treatment was used (Tuuri et al. 1996). In 1993, an ECF bleaching process with oxygen delignification was introduced, which led to decreasing discharge of process waters. In 1999, pulp washing and oxygen delignification were improved, which resulted in a 30 % decrease of COD by wastewaters (Anonymous 2006a). Since 2009, mainly kraft pulp from birch has been produced, and wastewaters are treated by an activated sludge plant. In 2011, the production volume was about 503 000 tons of pulp, and the discharge included about 14 650 000 m^3 a^{-1} of process waters. In 2011, the load to Lake Kuhnamo was about 11.5 kg COD, 0.10 kg AOX and total phosphorus 17 kg per ton produced pulp (Metsä Fibre 2012).

1.4.2 Changes in water quality

Since the first chemical pulping unit was introduced in 1938 (Tuuri et al. 1996, Meriläinen et al. 2001), the Äänekoski watercourse was progressively loaded and finally heavily polluted for several decades due to untreated wastewaters (Auer 1971, Granberg et al. 1987, Kaplin et al. 1997). Impaired lake water quality was detected already in the 1950s (Palomäki et al. 2006), and the wastewaters were discharged without treatment until the 1970s when the mechanical treatment was started (Kukkonen & Välttilä 1988).

In the basin of Lake Kuhnamo (see map on p. 26), into which effluents are discharged, anoxic conditions were monitored between 1975 and 1993. Over the decades, Lake Kuhnamo was not monitored as regularly as Lake Vatianjärvi 15 km downstream, where monitoring was started already in the 1960s. In the 1960s and 1970s, anoxic conditions were commonly measured in the hypolimnion of Lake Vatianjärvi. Oxygen depletion was detected also in the epilimnion. However, oxygen concentrations improved both in the epilimnion and the hypolimnion following the introduction of the new pulp mill in April 1985. The effects of more efficient wastewater treatment were notable in the water column already in the same year, as in August 1985 COD had decreased and oxygen concentrations increased especially in the epilimnion compared to the concentrations in previous years (Fig. 1; HERTTA 2012). Granberg et al. (1987) reported the decrease of BOD of the effluent to one tenth within one year after modernization of the mills. Similar observations were made also in Southern Lake Saimaa, South-East Finland, where reductions of COD and BOD were 76 % and 98 %, respectively, after the activated sludge treatment was initiated and ECF bleaching was introduced in 1992 (Oikari & Holmbom 1996). In Lake Vatianjärvi, increased oxygen concentration was also reflected in the decreased concentration of dissolved phosphorous (HERTTA 2012), as in aerobic conditions metal oxides, for instance aluminium and iron oxides, of the upper sediment layer will efficiently adsorb phosphate (Liu et al. 2009). Furthermore, the oxygen concentration also increased in the Kuusaankoski rapids, where oxygen air saturation values have been higher than 77 % since June 1985 (Fig. 1; HERTTA 2012).

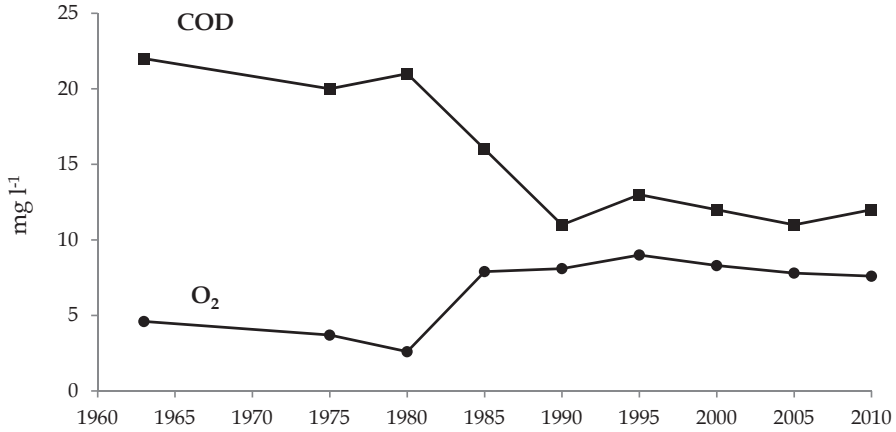

FIGURE 1 Chemical oxygen demand (COD) and dissolved oxygen concentration (O_2) measured from water column in one meter depth of Lake Vatianjärvi, 15 km downstream from the pulp and paper mills in Äänekoski. Samples were taken in late July or in August in 1963-2008 (HERTTA 2012).

Monitoring of harmful compounds released from the pulp and paper industry to the Äänekoski watercourse has been limited, as in the routine water analyses chlorinated organic compounds or other bioactive substances are not measured. However, Paasivirta et al. (1988) measured concentrations of chlorinated compounds in receiving lake before and after the introduction of the new kraft pulp mill, and detected significant decrease in concentrations of chloroform and chlorophenols. After mill modernization and transition to ECF bleaching, reduction of the total organic carbon (TOC) and AOX in the outflow of activated sludge treatment plant were more than 80 % and 60 %, respectively (Anonymous 2006b). In comparison, a notable reduction in AOX values also resulted in Southern Lake Saimaa, where the transition from elemental chlorine to chlorine dioxide bleaching and finally to full ECF method occurred after 1992, together with introduction of activated sludge treatment, which led to a 62 % decrease in AOX emission (Oikari & Holmbom 1996). In some Canadian mills, the amount of total AOX have been only 0.1 kg per ton produced pulp, which indicates a reduction of over 90 % in AOX discharge due to modern bleaching and effluent treatment technologies (Shimp & Owens 1993).

1.4.3 Effects on fish populations

Wood-derived bioactive compounds in the effluents caused heavy oxygen depletion and declined species diversity of fish biota in the Äänekoski watercourses (Granberg et al. 1987, Hynynen et al. 2004). Water quality also affected the local fisheries as the fish were inedible (Granberg et al. 1987). In autumn 1982, massive fish death was observed in the lakes downstream from the Äänekoski mills, and several thousand fish were lost probably due to exceptionally dry summer and autumn, which lead to the decreased rate of flow and thus to higher exposure to the mill discharge (Anonymous 1982).

In the early 1980s, pike (*Esox lucius*) was the most abundant fish species (41 %) in Lake Kuhnamo, followed by bream (*Abramis brama*, 25 %) and whitefish (*Coregonus lavaretus*, 17 %). Pikeperch (*Stizostedion lucioperca*) and especially brown trout (*Salmo trutta*) were nearly absent and the size of whitefish and brown trout had decreased. Previously, for instance the average weight of brown trout had been over three kilograms, whereas in 1981 it was only about one kilogram. In addition, the colour of bream skin was brownish yellow, its liver was black, and an unsavoury taste was observed in fish caught from Lake Kuhnamo, the recipient water basin for the effluents of the Äänekoski mills (Anonymous 1981). After mill renewal, the fish community started to recover. In spring 1986, pike continued to be the most dominant species in Lake Kuhnamo but juvenile vendace (*Coregonus albula*) and whitefish were already present in Lake Vatianjärvi, 15 km downstream (Granberg et al. 1987).

The exposure of fish to the readily metabolized chemicals can be monitored by measuring metabolites in bile (Oikari et al. 1984, Meriläinen et al. 2007). In 1992, elevated concentrations of chlorophenols and RAs were measured in the bile of pike, especially in Lake Vatianjärvi. In addition, PCBs were analyzed from their muscles. In 1992, PCB concentrations in the muscle tissues of pike were about 50 % in Lake Vatianjärvi compared to the concentrations in 1989 (Anonymous 1992).

Nowadays, a much more diverse fish community is present in the Äänekoski watercourse (Anonymous 2006b, Palomäki & Salo 2008, Palomäki et al. 2009). Since 2000, no notable changes have occurred in fish fauna; for instance pike, bream and pikeperch are abundant, and also brown trout and vendace are present. Restocked salmonids are present in all rapids, and brown trout is breeding naturally in the Äänekoski watercourse (Anonymous 2006b, Palomäki & Salo 2008). In 2007, perch (*Perca fluviatilis*) was the most abundant species (38 %) in Lake Kuhnamo, and pikeperch, which was nearly absent in the early 1980s (Anonymous 1981), is now quite common (14 %) (Palomäki & Salo 2008). However, even in 2003, RAs, chlorophenols and PCBs were detected in some fish, but in such low concentrations that no restriction on consuming them needed to be ordered (Anonymous 2006b).

1.4.4 Effects on invertebrates

Impacts were detected not only in the fish community (Granberg et al. 1987), but also in invertebrates, for instance in caddis flies (Trichoptera, Hydropsychidae) (Mäkelä 1984), which are common benthic macroinvertebrates in running waters (Wallace et al. 1990, Williams and Feltmate 1992). Until the beginning of the 1980s, *Hydropsyche* species were absent in the Kuusaankoski rapids, 15 km downstream from the Äänekoski pulp and paper mills (Mäkelä 1984). The reason for that was probably the low oxygen level (HERTTA 2012) as it should be higher than 50 % air saturation value for some *Hydropsyche* species, whereas for example *H. pellucidula* requires an oxygen level higher than 70 % (Becker 1987). In North America in the 1970s,

populations of caddis flies were shown to decline if exposed to the primary treated pulp mill effluent (Hutchins 1979). Also Warren & Seim (1975) reported reduced abundance of *Hydropsyche* species in pulp and paper mill affected areas in United States, and they suggested that a lowered oxygen level was primarily responsible for the reduction. Thus, probably due to increased oxygen concentration, caddis larvae re-colonized the Kuusaankoski rapids in 1991, six years after the remodeling of the mills, when six *Hydropsyche* species were found. However, high amounts of morphological abnormalities, such as deformities in anal papillae (the ionoregulatory organ), were detected (Vuori 1992).

In many invertebrate studies, biomass has increased in the areas affected by pulp and paper industry, which is likely to result from increased organic nutrient levels (Culp et al. 2000, 2003). However, Södergren et al. (1988) pointed out that this may lead to flawed interpretation, as hydrophobic toxic compounds may still be abundant in the sediment. Therefore, Södergren et al. (1988) caution that deformities and disturbed reproduction may occur in invertebrates despite they exhibit unchanged or increased biomass. In the Äänekoski watercourse, the decrease in the biomass of the sediment burrowing invertebrates, as well as increased species richness, was already noted in Lake Vatianjärvi within one year after the mill renewal (Granberg et al. 1987). Furthermore, increased oxygen concentrations downstream were measured (HERTTA 2012). Although, the loading of the harmful compounds have decreased nowadays, it should be taken into account that the contaminated sediment may still react as source of the chemicals (Meriläinen et al. 2006). Especially in a river-like watercourse, sediment resuspension may pose a risk.

The effects of the current pulp and paper industry on the Äänekoski region can be seen even nowadays in the benthos of Lake Kuhnamo, the closest lake to the mills, where the diversity is still low and a Diptera (*Chaoborus flavicans*), is the dominant benthic invertebrate. Further downstream, in Lake Vatianjärvi, *Chironomus* species were dominant in the 1980s, but nowadays the biomass of Chironomidae has decreased. Still, species that tolerate low oxygen concentrations, for instance *Chaoborus* sp. and Oligochaeta *Tubifex tubifex*, are the most abundant species in Lake Vatianjärvi. On the other hand, a relatively high abundance of some mesotrophic species (e.g. Chironomidae *Sergentia* sp.) has already been noted (Palomäki et al. 2009). To return to Södergren et al. (1988) remark, chironomids may have been commonly present in the polluted sites near the pulp and paper mills, but the incidence of mentum deformities in *Chironomus* species larvae could have been high (Hämäläinen et al. 2000).

Some effects of or associations with the chlorinated compounds on the benthic invertebrates have been reported (Sibley et al. 1997, Pöykiö et al. 2004). Sibley et al. (1997) reported a significant correlation between extractable organochlorines in the sediment with the mortality of different groups of macroinvertebrates, including mayflies (*Hexagenia* spp.) and water fleas (*Daphnia magna*). Pöykiö et al. (2004) observed the recovery of the benthic fauna in the pulp and paper mill contaminated area after the reduction in the

discharge of organochlorine compounds. In the Äänekoski watercourse, only a few studies with specific organochlorines have been made. Concentrations of chlorophenols were detected in the experimentally exposed lake mussels (*Anodonta piscinalis*). Concentrations of chlorophenols decreased with increasing distance from the mills, and a decreasing trend was also shown between 1984–1998, as the concentrations of chlorophenols started to decrease after mill renewals in 1985 (Herve et al. 2001).

1.5 Ecotoxicological bioindicators

1.5.1 Bioindicators in environmental monitoring

Use of biological indicators is recommended in the process of ecological risk assessment (WHO 1993, Adams et al. 2001). Bioindicator has various types of meaning however. It can indicate community structure (consisting of a species and number of individuals), population structure (density, age structure and sex ratio) or indicator species which have the requirements for the specific environmental conditions, for instance with dissolved oxygen or nutrient level. It also can mean the biological response which reflects an exposure to the specific type of pollutants, for instance inhibited or increased enzymatic activity as a biomarker after chemical exposure. Altogether, bioindicators reflect the behavioral, biochemical or physiological responses of an organisms or populations to altered environmental conditions or to the quantity of environmental pollutants (Hellawell 1986, WHO 1993, Rand 1995).

In this study, hepatic Ethoxyresorufin-*O*-deethylase (EROD) activity of fish, and species composition and morphological structure in caddis flies were chosen as ecotoxicological bioindicators, as they are known to be responsive indicators for contamination caused by pulp and paper industry (Camargo 1991, Vuori 1992, Vuori & Parkko 1996, Karels et al. 1998, van der Oost et al. 2003, Oikari et al. 2010).

1.5.2 EROD activity of fish

EROD activity is a sensitive ecotoxicological biomarker of exposure to many traditional xenobiotics, including discharge from the chemical wood industry (Andersson et al. 1988, Goksoyr & Förlin 1992, Oikari & Holmbom 1996, Karels et al. 1998, van der Oost et al. 2003, van den Heuvel 2006, Wartman et al. 2009). Chemicals which activate the aryl hydrocarbon receptor (AhR) and induce the cytochrome P450 1A (CYP1A) system can be detected as increased hepatic EROD activity. Such chemicals are typically dioxin-like planar compounds, for instance many PCDDs and PCDFs (van der Oost et al. 2003). Furthermore, some of the PCBs (Schmitz et al. 1995, van der Oost et al. 2003) and PAHs, for instance retene (Fragoso et al. 1998, Billiard et al. 1999, van den Heuvel 2006)

and benzo(*a*)pyrene (Oikari & Jimenez 1992), are known EROD inducers. Thus, AhR agonists exist among both halogenated and nonhalogenated chemicals.

Increased EROD activity has been commonly measured from fish exposed to pulp and paper mill effluents (Förlin et al. 1995, Karels et al.1998, van der Oost et al. 2003, Oikari et al. 2010). Of course, not all bioactive chemicals in pulp and paper mill effluents are EROD inducers. For instance, no correlation between EROD activity and chlorophenolics or resin acids in water has been observed (Soimasuo et al. 1998). Instead, retene may induce EROD activity at a concentration of 10 $\mu g\ l^{-1}$ in water. Therefore, if retene is present in the effluent, or released from the sediment, elevated EROD activity can occur in exposed fish. On the other hand, if the exposure to the retene is only temporal, EROD activity decreases within few days (Fragoso et al. 1998).

The EROD activity varies naturally between species, and between sexes and seasons due to the reproduction cycle (Karels et al. 1998, Blanchard et al. 1999, Karels & Oikari 2000, Ruus et al. 2006). For instance, in clean water areas, the activity in perch liver can be 30 times that in roach (Karels & Oikari 2000), and EROD activity is commonly higher in males than females (Karels et al. 1998, Blanchard et al. 1999, Karels & Oikari 2000, Ruus et al. 2006). It would seem that if EROD activity is used as a biomarker for the exposure, sampling should be avoided around the spawning season when the EROD activity is naturally fluctuating (Blanchard et al. 1999, Karels & Oikari 2000). Instead, the optimal sampling time is after the spawning period, i.e. for spring-spawning species in late summer or early autumn, when the difference in EROD activity between the sexes is absent or minimum (Karels et al. 1998, Blanchard et al. 1999). Thus EROD activity can be used as a warning biomarker of dioxin-like exposure if seasonal variations as well as differences between the species and sexes are taken into account.

1.5.3 Gill damage in caddis larvae

Worldwide, the order Trichoptera is the second largest group of aquatic insects including ca. 11 500 species. It is classified into two suborders, the first one Annulipalpia containing eight families, one of those being Hydropsychidae. In the Hydropsychidae family, 158 species are included (Thorp & Covich 2001). The second suborder, Integripalpia, includes 25 families, which are mainly tube makers (Grimaldi & Engel 2005).

Caddis larvae are suitable for biological monitoring because of their widespread abundance in diverse temperatures and water current velocities (Thorp & Covich 2001, Wiggins 2004), as well as due to variable tolerances of the species in relation to organic pollution (Thorp & Covich 2001). In addition, they are relatively stable organisms, and their life cycle is quite long, typically one year in Northern Europe (Andersen & Klubnes 1983). Thus the caddis larvae will be chronically exposed to the contaminants and other environmental stressors over their annual cycle (Slack 1936, Williams & Feltmate 1992), potentially indicating long term water quality. Caddis larvae of the Hydropsychidae family are net spinning filter feeders, feeding on particles

carried in suspension (Wiggins 2004), and thus may become exposed to the hydrophobic compounds sorbed to the filtered food particles.

Caddis larvae have a closed tracheal system with branched external gill tufts, and oxygen absorption also occurs largely through the cuticula (Korboot 1964). *Hydropsyche* species have 19 gill tufts; nine pairs in each segment and one gill tuft in the middle of the thoracic segment. *Hydropsyche siltalai* (Döhler) is an exception, as a gill pair is absent from the seventh segment, the total number of gill tufts thus being 17 (Philipson & Moorhouse 1973). All *Hydropsyche* species also have four anal papillae in the caudal part of the body, which function as ion exchange organs (Wigglesworth 1974).

Hydropsyche species have been used in biological monitoring by evaluating the incidence of external damage in the gill tufts and anal papillae as biomarkers of altered water quality (Vuori & Parkko 1996, Leslie et al. 1999, Vuori & Kukkonen 2002). Incidence of anal papillae and tracheal gill darkening has been shown to increase with increased exposure to metals (Vuori 1994, Leslie et al. 1999, Vuori & Kukkonen 2002). Such abnormalities have also been associated with pollution by crude oil (Simpson 1980) and organochlorine compounds (Camargo 1991, Vuori & Parkko 1996). Gill and anal papillae abnormalities have also been discovered downstream from pulp and paper industry (Vuori 1992).

The gill damage or deformities in the anal papillae are shown to develop rapidly (within 72 h) of exposure to the metals in laboratory conditions. In addition, damaged gill or anal papillae do not recover during instar development (Vuori 1994), as caddis flies have five larval instars before pupating (Andersen & Klubnes 1983). Caddis larvae are thus potential bioindicators of running waters, although more laboratory assays with pure compounds or their mixtures, as well as the effects of environmental factors such as pH or oxygen level, should be conducted to amplify the know-how of the development of pathologies. In fact, very few laboratory experiments have been done, and therefore the tolerance against specific organic chemicals and environmental conditions is still poorly known. Therefore, if caddis larvae were used as bioindicators, the quality of contaminated sites could be either under- or overestimated without additional toxicity tests in laboratory with specific compounds and many *Hydropsyche* species.

In field monitoring, the sampling method and timing of sampling should be the same especially if species composition and Hydropsychidae index (HA), are used as bioindicators. Kick net sampling is a commonly used method in macroinvertebrate studies, but as in the case of HA, there does not yet exist any harmonized sampling methodology. Kick net sampling could be difficult and dangerous in deep and fast flowing water whereas colonization bricks can be placed there as well. Caddis larvae colonize the bricks very well, and even hundreds of larvae can be picked up from one colonization brick set-up (Ratia, personal observation). The third sampling method is collecting the stones from the bottom and picking the larvae from the stones by tweezers. However, by using this method, the sampling depth is shallow (< 50 cm) and some species

are probably absent. For instance, the last instar of *H. pellucidula* favors high current velocities, higher than 0.5 m s^{-1} (Vuori & Parkko 1996) and also inhabits undersides of the stones, whereas *H. siltalai* favors a moss cover and the upper sides of the stones during summertime (Hildrew & Edington 1979).

2 OBJECTIVES

The aim of this study was to evaluate the recovery of the pulp and paper mill contaminated water course from an ecotoxicological perspective in the Äänekoski region of Central Finland. The hypothesis was that high loads of contaminants in the effluents have impacted on the aquatic organisms downstream and historically contaminated sediments may still pose harm due to sediment resuspension. The objectives were:

- Risk identification. Pulp and paper mill contaminated sediment was expected to have elevated concentrations of bioactive compounds, such as wood extractives or persistent organochlorines. Therefore, vertical distribution of resin acids (I), retene (I), betulinol (II) and wood sterols (II) as well as PCDDs, PCDFs and PCBs (III) were analyzed from the sediment.
- To evaluate the worst case scenario in case of resuspension of bioactive compounds from the surface sediment. Despite the efficient wastewater treatment, large amounts of wood extractives may be discharged due to high effluent volumes. Therefore, according to the spatial distribution, the amounts of the most abundant wood extractives were calculated from the surface sediment (I, II). In addition, the dissolution potency was investigated to assess the maximal load due to the physical disturbances, such as bioturbation or extensive floods (I, II). Sediment investigation was complemented with the laboratory bioassays describing both the capacity (III) and simulating the impact (IV) of the sediment to cause ecotoxicity.
- To assess the ecotoxicological status of fish and invertebrate populations in the pulp and paper mill contaminated watercourse. Sediment surveys and laboratory bioassays were complemented with the ecotoxicological field studies. Activity of biotransformation system of fish was measured from wild roach and perch caught downstream from the pulp and paper mill effluents discharge (IV). Caddis larvae were collected in 1999–2008 downstream from Äänekoski mills for evaluation of the species composition and analyses of the gill abnormalities (V).

3 MATERIALS AND METHODS

3.1 Sampling sites

The watercourse of Äänekoski is a river-like system, composed of several narrow lakes and retention time in lakes is only three days. Wastewaters from the mills are discharged to Lake Kuhnamo (I, II), which collects inflows from two sources: the humic Saarijärvi–Naarajärvi source (colour 125 mg Pt l^{-1}) and from the oligotrophic Viitasaari–Keitele source (20 mg Pt l^{-1}). The combined discharge rate in the Äänekoski watercourse after the snow melt is approximately 140 m^3 s^{-1} (max. 230 m^3 s^{-1}, SYKE 2013), and it decreases to 50 m^3 s^{-1} during the summer months. From September to April discharge varies from 60 m^3 s^{-1} to 85 m^3 s^{-1} (SYKE 2011). From Lake Kuhnamo, the watercourse continues through the Kapeenkoski rapids to Lake Vatianjärvi (I–IV), followed by the Kuusaankoski rapids (V) and Lake Saraavesi where the water discharges through the Kuhankoski rapids (V) to Lake Leppävesi (I–III), and finally through the Haapakoski rapids (V) to Northern Lake Päijänne (Fig. 2). The Siikakoski and Simunankoski rapids (V) and Lake Konnevesi (IV) are located in the Rautalampi watercourse, which contains mainly oligotrophic water (Table 2). The Rautalampi watercourse runs to Lake Saraavesi in the Äänekoski watercourse (Fig. 2). Lake Palosjärvi (IV) is also oligotrophic, and is located in Toivakka, Central Finland. Characteristics of the lakes are presented in Table 2.

Lake Saimaa is the largest lake in Finland, and the water area of its southern part is 609 km^2. Southern Lake Saimaa (III, IV) is a widely studied watercourse loaded by pulp and paper industry (Karels et al. 1998, Soimasuo et al. 1998, Leppänen & Oikari 1999a, 1999b, Karels & Oikari 2000, Lahdelma & Oikari 2005, Meriläinen et al. 2006, Oikari et al. 2010). It has quite a similar industrial contamination history to the Äänekoski watercourse, e.g. in relation to bleaching processes and wastewater treatment. The pump station displaces water from the clean area, and dilutes effluents from the pulp mill downstream (Fig. 3). In contrast to Lake Vatianjärvi, Southern Lake Saimaa is oligotrophic (Table 2) and the theoretical retention time is longer, about 60 days.

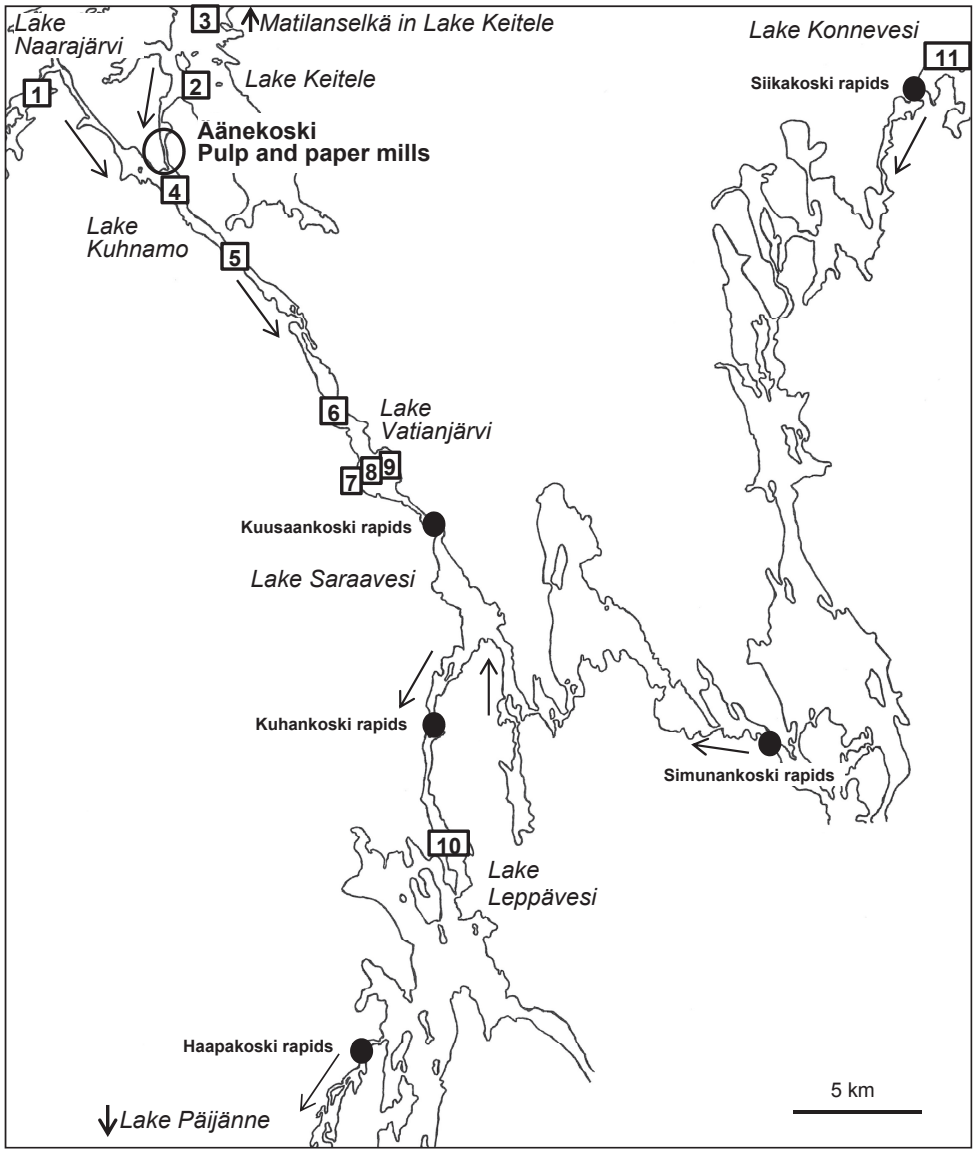

FIGURE 2 The research area. Sampling sites in the lakes are numbered from 1 to 11 and the rapids are marked by black spots. The industrial area in Äänekoski City is marked by a circle and the water flow directions by arrows.

TABLE 2 Characteristics of the research lakes (Palomäki et al. 2006, HERTTA 2012, Järviwiki 2013, SYKE 2013).

Lake	Area (km^2)	Mean depth (m)	Maximum depth (m)	Flow rate ($m^3 s^{-1}$)	Colour (mg Pt l^{-1})	Secchi depth (m)	pH	Oxygen (mg l^{-1})	P (µg l^{-1})	N (µg l^{-1})	COD (mg l^{-1})	Conductivity (mS m^{-1})	Data measured
Kuhnamo	3.7	5	30	50–230	50	1.4	7.1	6.6	21	400	10	9.3	16.8.2010**
Vatianjärvi	6.3	4	27	50–230	50	1.6	6.9	7.6	20	300	10	8.3	16.8.2010**
Leppävesi	36	11	43	100-380	40	2.4	7.2	7.7	12	350	8.6	6.7	16.8.2010**
Southern Saimaa	609	8	67	40	60	0.4–1*	7.3	8.0	22	440	10	15.0	6.7.2006
Keitele (ref.)	88	–	45	40–120	20	2.8	7.0	8.4	5	310	6.6	4.0	2.9.2010**
Naarajärvi (ref.)	2.1	5	32	–	160	0.6	6.5	8.7	24	760	22	3.3	3.7.2012
Palosjärvi (ref.)	2.6	–	30	–	15	4.0	7.4	8.1	5	250	4.1	3.1	17.8.2006
Konnevesi (ref.)	189	11	57	40–150	25	3.0	7.1	8.3	6	300	7.2	4.6	17.8.2010**
Rautniemi (ref.)	609	8	67	40	30	2.2	7.2	8.0	9	330	6.0	6.6	7.8.2003

* Oikari A. personal communication
** Flow rates are annual range of 2012 (SYKE 2013)

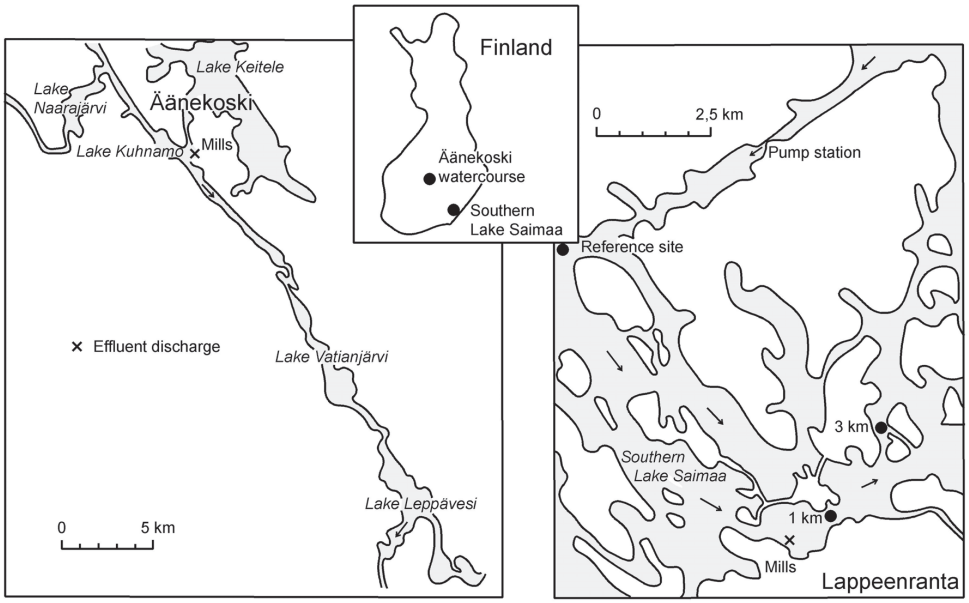

FIGURE 3 Äänekoski watercourse on the left, and Southern Lake Saimaa on the right. The inset map shows the locations of the study sites within Finland. Sediment sampling sites in Southern Lake Saimaa (III, IV) are marked by black dots, and effluent discharge points by cross. The water flow directions are marked by arrows.

3.2 Sampling methods

3.2.1 Sediment sampling

Sediments for the wood extractive (I, II) and retene (I) analyses were collected in late April and early May in 2007 from the series of lake basins comprising the Äänekoski watercourse. The site nearest to the source was Lake Kuhnamo in Äänekoski, and site locations continued for 33 km from the mills. From Lake Kuhnamo, sediment samples were taken one km (Fig. 2, site 4) and 3.5 km (Fig. 2, site 5) downstream from the effluent source. Lake Vatianjärvi was sampled at 12 km (Fig. 2, site 6) and 15 km (Fig. 2, sites 7–9) downstream from the mills. The farthest site, Northern Lake Leppävesi (Fig. 2, site 10), was located 33 km downstream from Äänekoski. Two reference sites were selected: the humic Lake Naarajärvi (Fig. 2, site 1) and the oligotrophic Lake Keitele (Fig. 2, site 2), 8.5 km and 5 km upstream from Äänekoski, respectively. Samples were taken by Kajak-corer and in the laboratory, sediment samples were allowed to resettle in the dark at 4 °C for 24 h before they were sectioned for the uppermost 20 cm: 0–2, 2–5, 5–10, 10–15, and 15–20 cm. Two to three subsamples were combined (I, II).

For the dissolution experiments (I, II), reference sediment samples (layer ca. 0–10 cm) were collected from the upstream sites in Lake Keitele (Fig. 2, site 2) and Lake Naarajärvi (Fig. 2, site 1). The downstream sites were Lake Kuhnamo (Fig. 2, site 5), Lake Vatianjärvi (Fig. 2, site 7), and Lake Leppävesi (Fig. 2, site 10).

For the PCDD, PCDF and PCB analyses and for the sediment extract bioassay (III) sediments were collected in October 2011 from Lake Vatianjärvi (Fig. 2, site 7) and Lake Leppävesi (Fig. 2, site 10). Matilanselkä in Lake Keitele (Fig. 2, site 3), 16 km upstream from the mills, was used as a reference sediment. Also sediment from Rautniemi, located about 10 km upstream from the pulp and paper mills in Southern Lake Saimaa (sampled in 2011), was used as a reference. Downstream sites of Southern Lake Saimaa were sampled in 2010, the site one km downstream from the mill served as a positive control of an authentic contamination (Fig. 3) (III).

In the sediment with water bioassay (IV), Southern Lake Saimaa sites one and three km downstream from the mills were the positive controls (Fig. 3). Lake Vatianjärvi (Fig. 2, site 7) and Lake Leppävesi (Fig. 2, site 10) sediment samples were the same as those sampled and analyzed in I and II.

3.2.2 Sampling of fish and caddis larvae

In the field study, hepatic EROD activity was analyzed from wild roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) (IV), caught from Lake Vatianjärvi (Fig. 2, site 7). Reference sites were Lake Konnevesi (Fig. 2, site 11) and Lake Palosjärvi.

The main study site of the caddis larvae (V) was the Kuusaankoski rapids, about 15 km downstream from the wood industries of Äänekoski. Other rapids in the same watercourse were the Kuhankoski and Haapakoski rapids, approximately 30 km and 50 km downstream from the mills, respectively. The reference sites were the Siikakoski and Simunankoski rapids (Fig. 2).

3.3 Sediment characteristics

3.3.1 Total organic carbon and loss on ignition

The concentration of total organic carbon (TOC) was determined from the sites downstream from the Äänekoski mills. Samples were freeze-dried, homogenized, acidified by HCl and dried at 80 °C. TOC was analyzed in an accredited laboratory using nitrogen-carbon-analyzer (I, II).

The content of organic matter can be determined by analyzing loss on ignition (LOI) (550 °C, 2 h). For the sediments used in the wood extractive and retene analyses (I, II), LOI was determined as two replicates from the freeze-dried samples (SFS 1990) (I, II). Dry weights and LOI were analyzed from the sediment samples used for the PCDD, PCDF and PCB analyses (III), and from Rautniemi and Southern Lake Saimaa sediment samples as well (III, IV).

3.3.2 Sediment age determination

To determine the timing of deposited compounds and the samples of bioassays (III), the sediment age of each two centimeter layer was determined. Dating of the sediment was based on the activity level of ^{137}Cs, as the peak of it can be seen after the Chernobyl fallout in 1986 (Saski et al. 1997). Gamma-radiation associated with ^{137}Cs decay was measured with an Ortec GMX series high-purity germanium coaxial photon detector (Sanada et al. 2002). Each sample was measured for 24 hours to accumulate the necessary statistics from ^{137}Cs activity. Measurements were done in the Department of Physics in the University of Jyväskylä, by professor Ari Jokinen and Mr. Mikko Koikkalainen.

3.4 Bioactive compounds in the sediment

3.4.1 Wood extractives and retene

Vertical distribution of RAs, retene (I), betulinol and WSs (II) were analyzed from the sectioned uppermost 20 cm of the sediment samples (0–2, 2–5, 5–10, 10–15, and 15–20 cm). Analyses were done according to Lahdelma & Oikari (2005), Leppänen & Oikari (1999a) and Tavendale et al. (1995), with minor modifications. The internal standards were heptadecanoic acid for RAs, anthracene-d^{10} for retene and 17α-methyltestosterone for WSs. Freeze-dried sediment samples were Soxhlet-extracted with hexane:2-propanol solution (2:1, v/v). The extracts were evaporated to a small volume, diluted with hexane and extracted with 0.1 M K_2CO_3. RAs were separated into the K_2CO_3 fraction (I) and retene (I), WSs and betulinol (II) into the hexane fraction.

For retene (I), WS and betulinol (II) analyses, the hexane fractions were combined, concentrated and further evaporated under a nitrogen gas stream to dryness. The sample was then diluted with hexane:chloroform (4:1, v/v) and absorbed in the deactivated and conditioned silica gel column. The retene was eluted with hexane, while WSs and betulinol were eluted with chloroform. Both fractions were concentrated and evaporated by nitrogen until dry (I, II). The hexane fraction, including retene, was diluted with hexane and analyzed with gas chromatograph - mass spectrometer (GC-MS) (I). The chloroform fraction, including WSs and betulinol, was diluted with pyridine and combined with RA samples before silylation (II).

For RA analysis (I), the pH of the K_2CO_3 fraction was adjusted to nine with H_2SO_4. The sample was extracted with dichloromethane, concentrated and further evaporated with nitrogen stream to dryness. The RAs were diluted with pyridine, combined with WS and betulinol fraction, and silylated with *N,O-bis*(trimethylsilyl)-trifluoroasetamid + 1 % trimethylchlorosilane. The RAs, WSs and betulinol were analyzed by GC-MS (I, II).

3.4.2 Organochlorines — PCDDs, PCDFs and PCBs

For PCDD, PCDF and PCB analyses (III) vertical sediment samples were sliced into the two cm layers. For screening the presence of these compounds concentrations of PCDDs, PCDFs and PCBs were first analyzed from the 14–16 cm sediment layer of Lake Vatianjärvi, which is a layer formed approximately 15 years ago (Saski et al. 1997). Based on the results, only three sediment depths (2–4, 8–10 and 14–16) from each sampling site were chosen for further analyses and for the fish bioassays (III).

Sediment samples were extracted with a mixture of petrol ether, acetone, hexane and diethyl ether (9:5.5:2.5:1, v/v) by Dionex Ace 200 extractor. PCB-30 and ^{13}C labeled PCDD/PCDF were used as the internal standards. After extraction, acetone and dimethyl ether were evaporated, and the extracts concentrated to a small volume and washed with sulfuric acid. Afterwards activated Cu powder was added to remove the sulfur. PCBs were analyzed by the Institute for Environmental Research by GC (Agilent 6890) with an electrochemical detector by using an accredited method based on ISO (2002), SFS (2008) and Paasivirta et al. (1986). For PCDD/PCDF analyses extracts were purified by alkaline Al_2O_3 column and washed by 2 % dichloromethane in hexane and eluted by dichloromethane:hexane solution (1:1, v/v) and analyzed by GC-MS (III).

3.5 Dissolution of wood extractives and retene

To simulate the worst-case disturbances in the surface sediment, the dissolution potencies of the wood extractives and retene were determined from the 0–10 cm sediment layer (I, II). Sediment samples were collected from the Äänekoski watercourse as described in chapter 3.2.1. Dissolution of sediment-bound RAs (I), retene (I), betulinol (II) and WSs (II) were studied by mixing wet sediment with artificial freshwater (1:4, v/v) with two agitation schemes. After agitation, elutriates were centrifuged and analyzed. The RAs (I) were analyzed according to Meriläinen & Oikari (2008a). Retene (I), betulinol and WSs (II) were analyzed by GC-MS with the same method as used for the sediment samples.

3.6 Spatial distribution of wood extractives and retene

The concentrations of the most common wood extractives: DHAA, IPA, betulinol and β-sitosterol, from two uppermost sediment layers (0–2 and 2–5 cm) were used for spatial interpolation and, further, to assess the distribution of the total amounts (kg ha^{-1}) in the Kuhnamo and Vatianjärvi basins (I, II). The program used was ArcGIS (GIS, Geographic Information System) applying the Inverse Distance Weighting (IDW; Shepard 1968) interpolation method.

3.7 Fish bioassays and population surveys

3.7.1 Sediment extract bioassay

Two types of fish bioassays were carried out in the laboratory. In the first type of bioassay, the potency of pulp and paper mill contaminated sediment to change liver CYP1A detoxification system was studied by injecting sediment extract intraperitoneally (i.p.) into the juvenile rainbow trout (III). Sediment extracts were prepared according to Oikari et al. (2002), except retene and other labile organic compounds were not removed. Thus the whole range of AhR-activating compounds was included in the exposure. Hepatic EROD activity was used as a biomarker of exposure to the dioxin-like ligands.

Three sediment layers from Lake Vatianjärvi (Fig. 2, site 7), Lake Leppävesi (Fig. 2, site 10) and Lake Keitele (Fig. 2, reference site 3) were extracted: 2–4 cm, 8–10 cm and 14–16 cm. Sediment of Southern Lake Saimaa (Fig. 2, site 1 km) and retene (50 µg g^{-1} fish) were used as the positive controls. In the sediment samples from Lake Vatianjärvi and Lake Leppävesi the proportion of organic matter is about half of that in Southern Lake Saimaa sediment. Therefore, double amount of sediment (50 g, dw) from Lake Vatianjärvi and Lake Leppävesi was extracted compared to the sediment of Southern Lake Saimaa (25 g, dw). Lake Keitele sediment was very wet and thus the amount of dried sediment remained lower and extracted amount was 20 g (dw) (III).

Juvenile rainbow trout (*Oncorhynchus mykiss*) were purchased from a local hatchery (Hanka Taimen Inc.) and acclimatized to the laboratory conditions for ten days (III) at 11–13 °C in pH 7.3–7.5. Trout were fed with pellet fish food, except one day before the exposures. During the exposures fish were not fed.

Three dose levels of Southern Lake Saimaa sediment extracts were injected: 10, 50 and 100 %, so that 100 % extract included the total content derived from 25 g dry sediment. All extracts were diluted in DMSO:sunflower oil (1:9, v/v). Part of the experiments was repeated with the 8–10 cm sediment extracts of Lake Vatianjärvi and Lake Keitele (reference). Rautniemi sediment from Southern Lake Saimaa was included as another reference, and also positive controls were included (III).

Fish were anesthetized and sediment extracts were injected i.p. at a volume of 5.0 µl g^{-1} fish. After 72 h exposure fish were killed, and livers were removed and stored in liquid nitrogen (III).

3.7.2 Sediment with water bioassay

In the second type of bioassay, contaminated sediment was laid in the bottom of the aquarium before water was added to the aquarium. Thus exposure of fish occurred either by contact with the sediment or by dissolution of the contaminants to the water (IV). Juvenile rainbow trout were from the same source and acclimatized at the same conditions as in the first type of

experiment, except acclimatized period was seven days in (IV). Fish were exposed to the uppermost (ca. 0–10 cm) sediments, which were collected from Lake Vatianjärvi (Fig. 2, site 7) and Southern Lake Saimaa (Fig. 3, sites 1 km and 3 km) in the spring of 2007 and 2010, respectively. Reference sediment was collected from Rautniemi, 10 km upstream from the mills in Southern Lake Saimaa (Fig. 2). The water with DMSO was a blank control and retene in the water (100 $\mu g\ l^{-1}$) was the positive control (IV). Hepatic EROD activity was used as a biomarker of exposure.

The experimental setup was done exploiting the study by Fragoso et al. (2006). Three replicates per treatment and four fish per aquarium were used. After 48 h exposure the fish were killed, their livers were dissected and stored in liquid nitrogen for the EROD analyses (IV).

3.7.3 Field survey of fish populations

EROD activity was measured from roach and perch caught from Lake Vatianjärvi (Fig. 2, site 7) and from two reference lakes, Lake Konnevesi (Fig. 2, site 11) and Lake Palosjärvi, in August and early September in 2009. Fish were caught from the littoral zone. Fish were killed, their sex was determined, and the total length of each was measured. The livers were dissected and immediately stored in liquid nitrogen for the EROD analyses (IV).

3.7.4 Assay of EROD activity

Hepatic EROD activity from postmitochondrial supernatant (S9 fraction) was analyzed according to Vehniäinen et al. (2012). All EROD and protein measurements were made in triplicate or in duplicate. EROD activity was quantified by measuring fluorescence and the total protein in the S9 fraction by modified Lowry method (III, IV).

3.8 Caddis larvae as bioindicators

3.8.1 Sampling of caddis larvae

Caddis larvae of Hydropsychidae family were sampled from the water depth of 20–80 cm. Temperature, oxygen air saturation value, conductivity, pH and current velocity were measured in the field. Caddis larvae were collected during May, June and July in 1999, 2003, 2007 and 2008 from the Kuusaankoski rapids (Fig. 2). In 1999 and 2003, samples were also taken from the Kuhankoski and Haapakoski rapids (Fig. 2). The Siikakoski and Simunankoski rapids (Fig. 2) were the reference sites in 2007 and 2008. Also in 2000, material was obtained from Siikakoski. During transportation, no mortality was observed (V).

3.8.2 Hydropsychidae index

Hydropsychidae index (HA) is based on the collated field and laboratory studies on the relations between water quality and species tolerance towards pollution. HA is focused especially on species tolerance towards eutrophication (Vuori 2002) and now the index was refined by adding scoring criteria also for tolerances towards organic pollution. The Hydropsychidae index value can vary from one to five, so that a higher HA value represents a better water quality and ecological status for the family (Vuori 2002). HA index is described in detail in (V).

3.8.3 Gill abnormalities

Every gill tuft was analyzed from each animal by stereo microscope. The frequency of morphological abnormalities of the gill tufts was calculated as an average in a population. The gill tuft was defined to be healthy if it was totally transparent (Fig. 4a–b) or if there were minor brown spots on less than 50 % of tufts, or if it had only sporadic dark spots. Slightly damaged gill tufts (Fig. 4c) included those with obvious brown spots in more than 50 % of the branches. Seriously damaged gill (Fig. 4d) was totally dark or darkened from the distal or basal parts, often including shortening of the gill. Scar-like darkening (Fig. 4e) was not classified to be abnormal, as it was probably caused by the bites of predators or other caddis larvae (V).

For inspection of the internal gill structures, gill tissues were embedded to the epon. Cross sections of the gills were obtained by cutting 0.5 µm slices, and staining with toluidine blue. Darkening of the gill membrane and the structures inside the gills were inspected with the microscope (V).

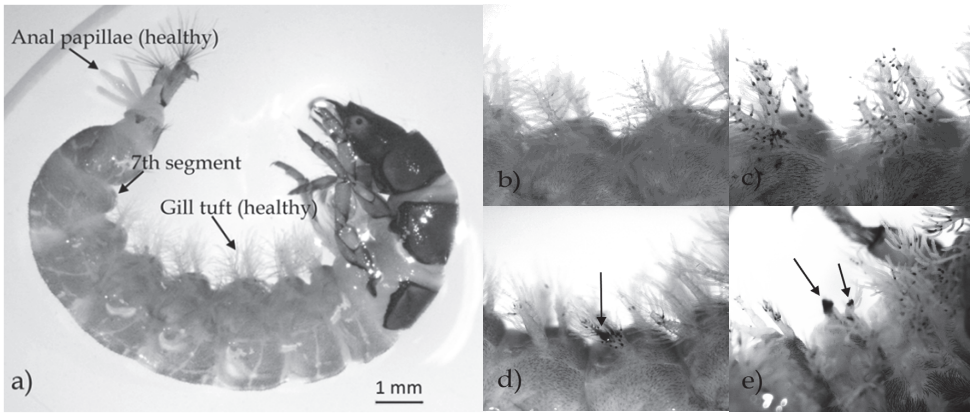

FIGURE 4 *Hydropsyche siltalai*, which has no gills in the 7th segment, unlike other Hydropsychidae species. Gill tufts are the transparent branches on the ventral side, and anal papillae in the caudal part of the body (a). Gills of hydropsychid larvae were classified as either healthy (a, b), slightly damaged (c), or seriously damaged (d). Scar-like darkening (e) was not classified as gill damage.

3.9 Statistics

The normality was analyzed by the Shapiro–Wilk's W-test ($3 \leq n \leq 50$) or by Kolmogorov–Smirnov test with the Lilliefors significance correction ($n \geq 50$), and homogeneity of variances by Levene's test (I–V). In the sediment studies (I, II) the relationship between the studied chemicals and LOI and TOC were determined by Spearman's correlation test due to non-normality of some measurements. Correlations between the concentrations in elutriates and in the sediments were determined by Pearson's correlation test if the data was normally distributed (II).

In the field research (IV), roach and perch were analyzed as the different groups when comparing differences between Lake Vatianjärvi and the reference lakes. As there were no statistical differences between males and females, sexes were combined in the statistical analyses. Also reference lakes were combined. The differences between Lake Vatianjärvi and the reference lakes were analyzed by using independent samples t-test (IV).

In the laboratory bioassays where fish were exposed to the contaminated sediment *via* water (IV), the significance of the differences between the treatments were analyzed by using Welch ANOVA and Dunnett's T3 post hoc test because of the heteroscedasticity of the variances. Correlation between the EROD activity and retene concentrations in the sediments (III, IV) as well as correlation between the treatments in the sediment extract i.p. injections (III) were analyzed by Spearman's rho correlation test.

In the caddis larvae field research (V) the data were not normally distributed and the significance of the differences in the average number of gill abnormalities was analyzed by the Kruskal–Wallis test. The data were analyzed statistically as an average gill damage proportion per larva, so that slight and serious damage were analyzed separately. Pair-wise comparisons between the sampling sites and sampling time were analyzed by using the Mann–Whitney U-test.

In all studies, a significance level of 0.05 was used as indicative of difference between means. Statistical analyses were done using SPSS software (SPSS Inc., Chicago, IL USA).

4 RESULTS

4.1 Sediment analyses

4.1.1 Sediment characteristics

Sediment characteristics, especially content of organic matter, may remarkably affect to the fate of hydrophobic compounds in the aquatic environment. Water content and LOI varied significantly between the analyzed sediment sites. Sediment samples were mostly tightly packed clay except reference sediment from Lake Keitele (Table 3). The highest TOC and LOI were in the sediment of Lake Kuhnamo. TOC and LOI were about 50 % lower in Lake Vatianjärvi, and further decreased downstream in Lake Leppävesi (Table 3) (I, II).

Statistically significant positive dependence was detected between TOC and LOI ($p < 0.001$), TOC and RAs ($p = 0.009$), and TOC and retene ($p = 0.003$) (I). Betulinol and LOI showed a positive dependence in all lakes but the only significant correlation was in Lake Kuhnamo ($p = 0.045$). The dependence between WSs and LOI was significant only in Lake Kuhnamo sediment ($p = 0.038$) and in Lake Naarajärvi reference ($p = 0.037$). No significant correlation between betulinol or WSs and TOC were observed in the lakes downstream from the mills (II).

TABLE 3 Sediment characteristics analyzed from the uppermost 10 cm layer: dry weight (dw), loss on ignition (LOI), and total organic carbon (TOC) (I, II).

Sampling site (km)	dw (%)	LOI (%)	TOC (%)	Description
– 8.5 (ref.)	21.9	8.7	3.7	Fine particles, tightly packed, brown color.
–5 (ref.)	11.6	14.2	4.7	Grainy particles, loosely packed, 0–4 cm carmine color, deeper brownish grey.
3.5	15.9	16.9	9.0	Fine particles, 0–2 cm reddish brown, deeper dark brown.
15	24.4	8.6	4.2	Fine particles, 0–2 cm orange brown, deeper brown.
33	32.6	3.3	1.2	Fine particles, 0–2 cm orange brown, deeper brown.

The sediment age was analyzed from three lakes: Lake Keitele, Lake Vatianjärvi and Lake Leppävesi (Fig. 2). The peak of the ^{137}Cs isotope in the Lake Keitele reference was found from the depth of 4–6 cm, indicating that the Chernobyl accident in 1986 was below that. In Lake Vatianjärvi, ^{137}Cs started to increase at the depth of 16–20 cm. However, more accurate dating cannot be done as the maximum peak was not found within the analyzed vertical profile. In Lake Leppävesi, the sedimentation rate was probably a little slower than in Lake Vatianjärvi (III).

4.1.2 Wood extractives and retene in the sediment

For the risk identification of bioactive compounds, concentrations of wood extractives and retene were measured from the pulp and paper mill contaminated sediments. Considering the uppermost 10 cm of sediment, analyses showed that concentrations of wood extractives and retene decrease linearly downstream from the source (Fig. 5) (I, II).

The total concentration of RAs was highest (246 μg g^{-1}) in the deepest layer (15–20 cm) at the closest site, i.e. one km downstream from the pulp and paper mill point source in Lake Kuhnamo. The highest concentration at the sites 12 and 15 km in Lake Vatianjärvi were found at the sediment depth of 10–15 cm (Fig. 6a). Dehydroabietic acid (DHAA) was the most abundant RA, covering over 60 % of the total RAs. Isopimaric acid (IPA) was the most abundant pimaric type RA, being 11 % of total RAs (I).

Concentrations of retene were lower and declined more rapidly than RAs, being 51 μg g^{-1} (layer 10–15 cm) at site one km, 5–18 μg g^{-1} at site 3.5 km, and less than 2.5 μg g^{-1} further downstream (Fig. 6b). The Spearman's correlation analysis indicated a dependence between the concentrations of RAs and retene, revealing a significant positive dependence in the sediment samples from Lake Kuhnamo ($p < 0.05$) and Lake Vatianjärvi ($p < 0.01$) (I).

Furthermore, in case of betulinol, the highest concentration (1666 μg g^{-1}, dw) was measured from Lake Kuhnamo, one km downstream from the mills (Fig. 5 and 7a). The maximum concentration was at the depth of 5–10 cm whereas in Lake Vatianjärvi and in Lake Leppävesi the maximum concentrations were at the depths of 10–15 and 15–20 cm, respectively. In the reference sites, the betulinol concentration in the sediment was lower in Lake Naarajärvi (<5 μg g^{-1}) than in Lake Keitele, where the concentrations varied between 4.4–44 μg g^{-1} (Fig. 7a). Betulinol and LOI showed a positive dependence in every sample but the only significant correlation was in Lake Kuhnamo sediment ($p = 0.045$) (II).

Similarly, the total concentration of WSs was highest (2886 μg g^{-1}) at the closest site (1 km) downstream from the mills (Fig. 5 and 9b), the maximum concentration was found in the layer 2–5 cm (Fig. 7b). The most abundant WSs were β-sitosterol and stigmastanol, accounting for 36 % and 32 % of the total concentration. The dependence between WSs and LOI was significant only in Lake Kuhnamo sediment ($p = 0.038$) and in one of the two references, Lake Naarajärvi ($p = 0.037$). The significant positive correlation between betulinol

and WS concentrations in the sediments was found in Lake Kuhnamo (p < 0.001) and in Lake Vatianjärvi sediment samples (p = 0.004). Further downstream, in Lake Leppävesi, no dependence was shown, nor was there any dependence shown in the reference sites (II). No significant correlation between sediment TOC and wood extractives were observed in the lakes downstream from the mills (II).

Overall, the concentrations of wood extractives and retene deceased with increasing distance from the source (Fig. 5). Instead, gradients varied vertically between the sampling sites and compounds (Fig. 6 and 7).

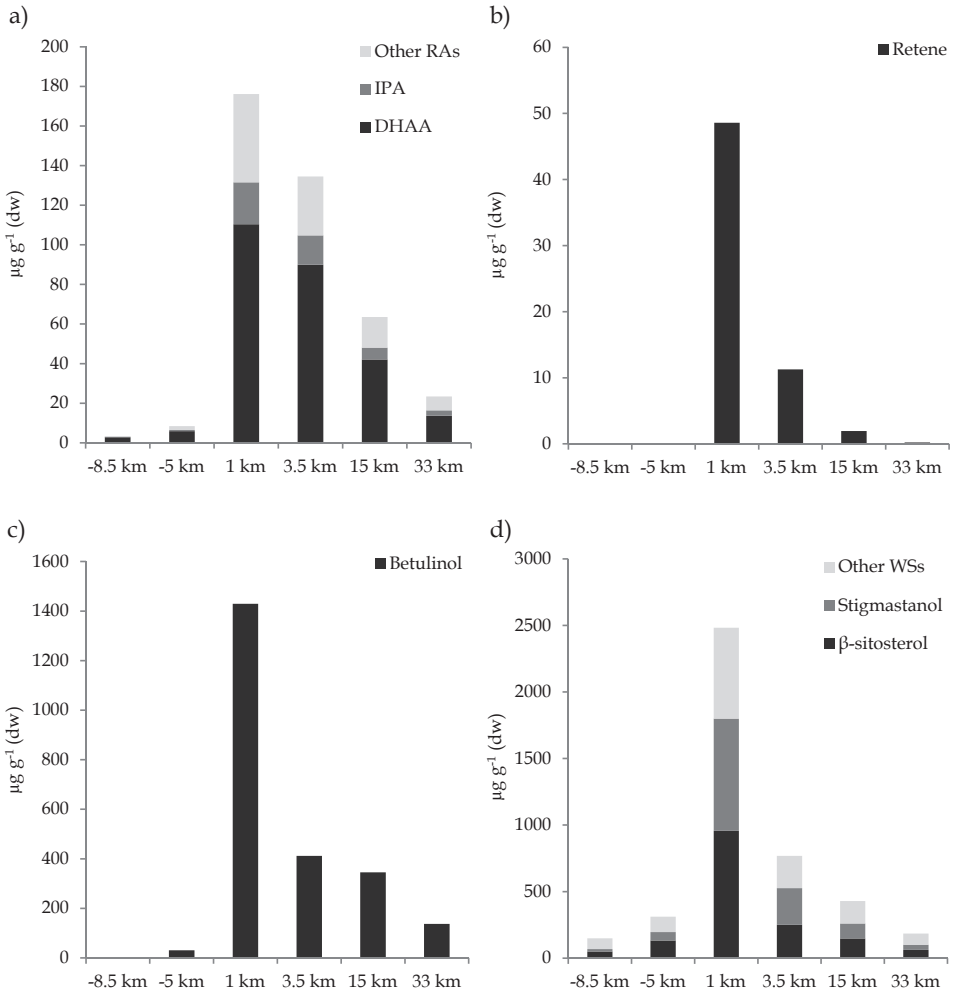

FIGURE 5 Concentrations of a) dehydroabietic acid (DHAA), isopimaricacid (IPA) and other resin acids (RAs), b) retene, c) betulinol, and d) β-sitosterol, stigmastanol and other wood sterols (WSs) in the sediments of the Äänekoski watercourse (0-10 cm) from one to 33 km downstream from the pulp and paper industry, and from two reference sites, 5 km and 8.5 km upstream (I, II).

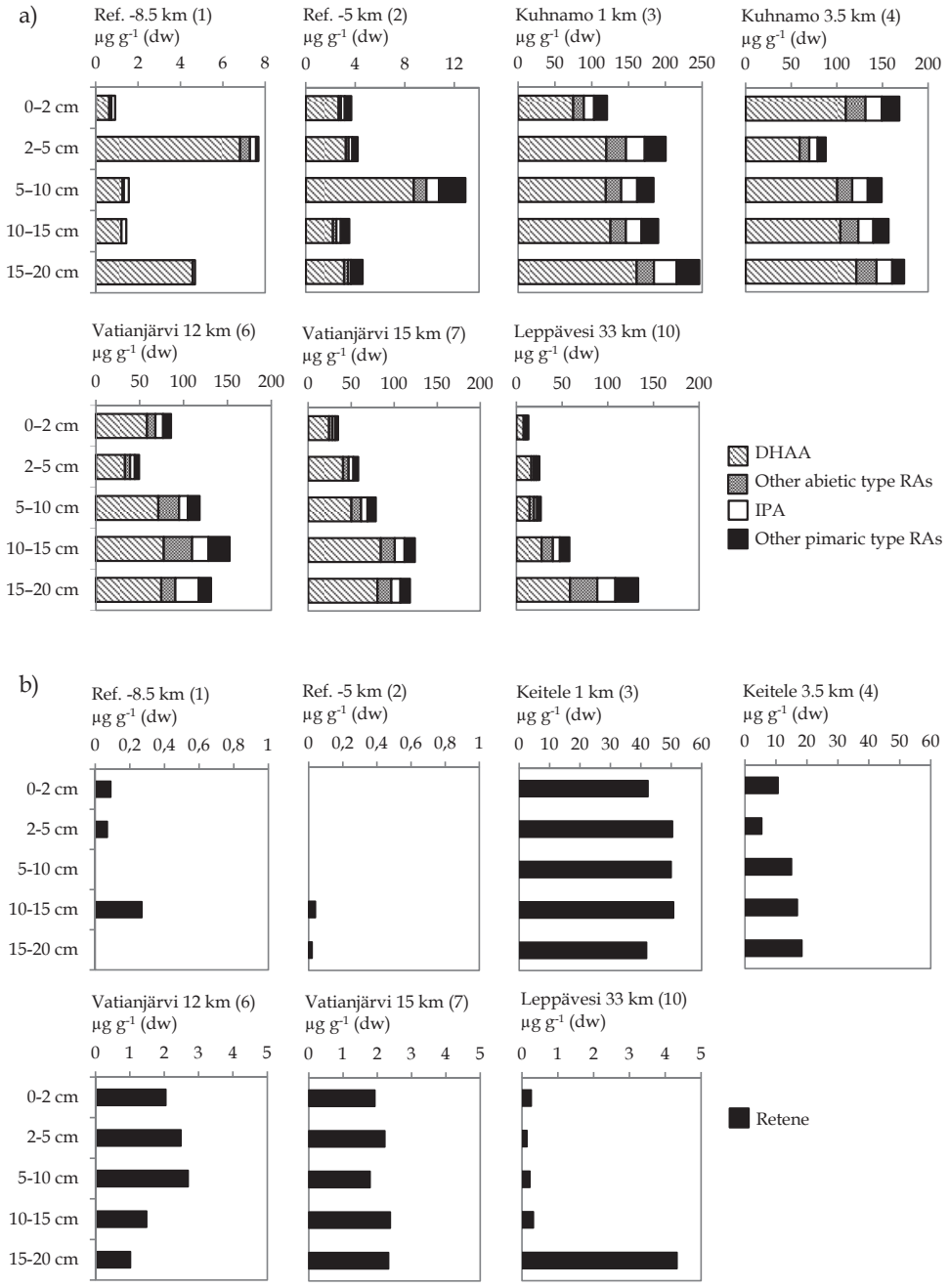

FIGURE 6 Vertical variation of concentrations of a) resin acids (RAs) and b) retene in the sediment samples downstream from the pulp and paper industry in the Äänekoski watercourse, Central Finland. The most abundant abietic and pimaric type RAs, dehydroabietic acid (DHAA) and isopimaric acid (IPA), are presented separately from other RAs. Sampling sites are separated according to their distance from the mills (site numbers in brackets; see Fig. 2 for the exact locations).

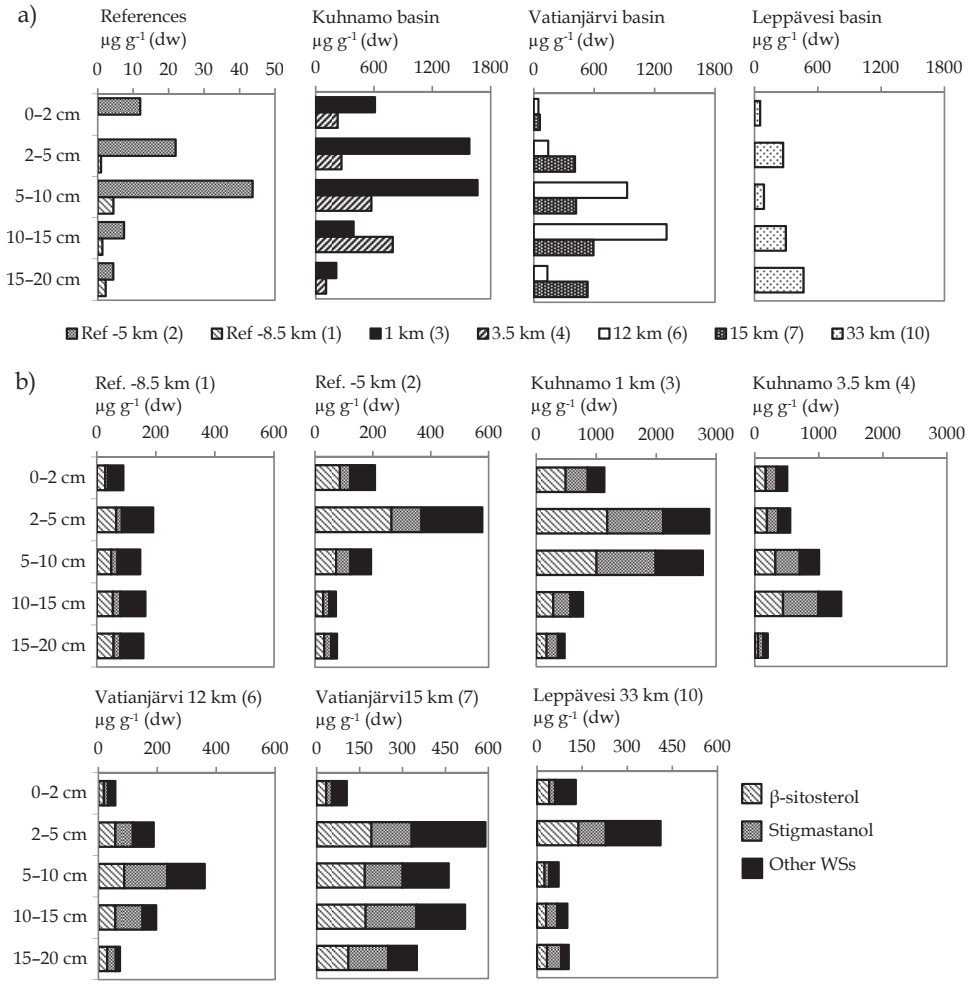

FIGURE 7 Vertical variation of concentrations of a) betulinol and b) wood sterols (WSs) in the sediment samples downstream from the pulp and paper industry in the Äänekoski watercourse, Central Finland. β-sitosterol and stigmastanol are represented separately from other WSs. Sampling sites are separated according to their distance from the mills (site numbers in brackets; see Fig. 2 for the exact locations).

4.1.3 Spatial distribution of wood extractives

Spatial distribution of the most common wood extractives in the uppermost sediment was calculated to evaluate the risk of contaminated sediment in case of physical disturbance causing resuspension. Distributions of DHAA (Fig. 8a–b) and IPA (Fig. 8c–d), betulinol (Fig. 9a–b) and β-sitosterol (Fig. 9c–d) were assessed by interpolating the data from sediment layers of 0–2 and 2–5 cm in Lake Kuhnamo and Lake Vatianjärvi. These were expected to be the layers most sensitive to erosion. Almost without exception, wood extractives were more abundant in the deeper layer compared to the upper one (Fig. 8 and 9) (I, II).

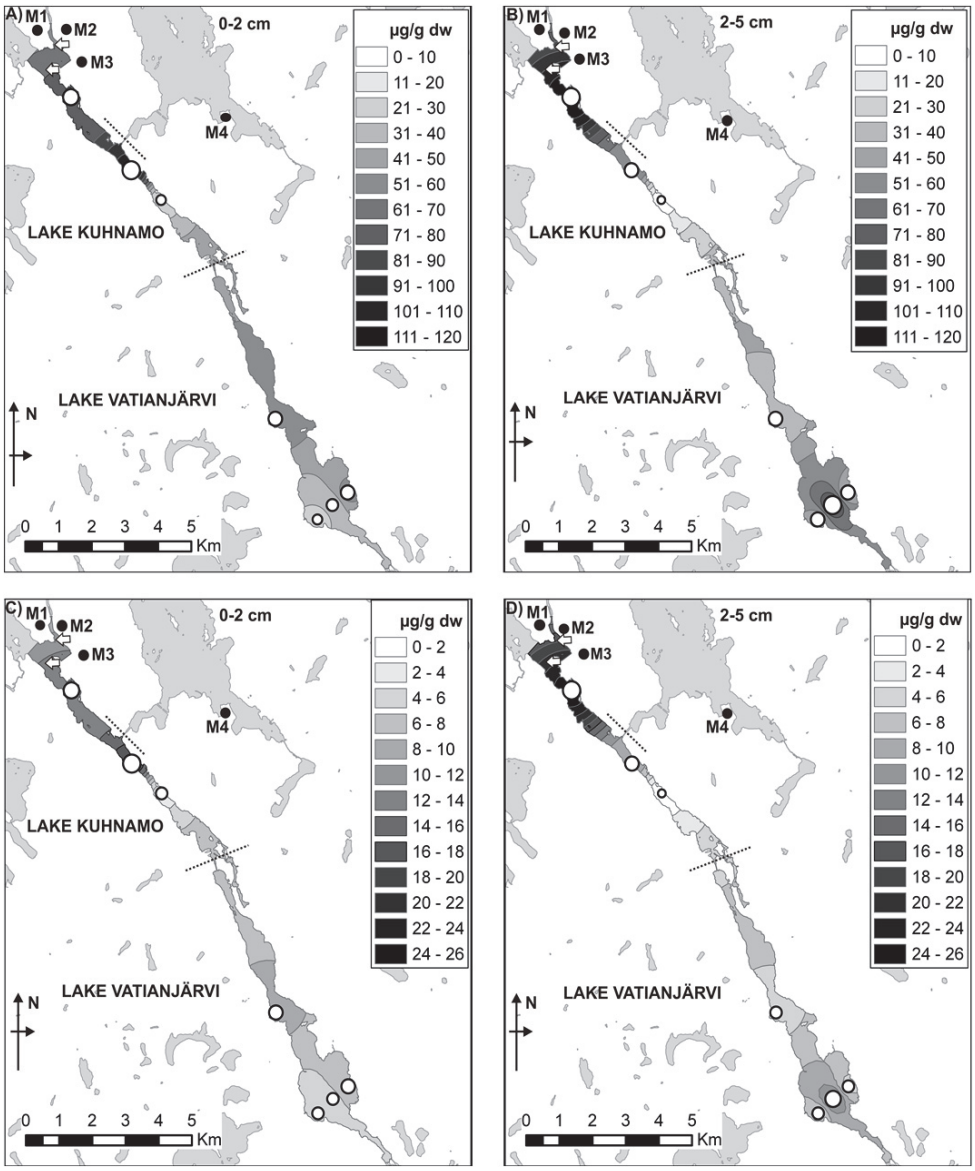

FIGURE 8 Interpolated spatial distributions of the most common resin acids in the sediment samples of Lake Kuhnamo and Lake Vatianjärvi: dehydroabietic acid (DHAA) in the sediment layer a) 0–2 cm and b) 2–5 cm, and isopimaric acid (IPA) in the sediment layer c) 0–2 cm and d) 2–5 cm. Production facilities have been marked in the picture as follows: M1 = paper mill, M2 = board mill, M3 = pulp mill, M4 = plywood mill. Bolded white arrows show outflows from the wastewater treatment plants. The lakes are separated by a dash line, and the white circles (size depends on concentration) represent the sampling sites (Base map © National Land Survey of Finland 892/MML/09) (I).

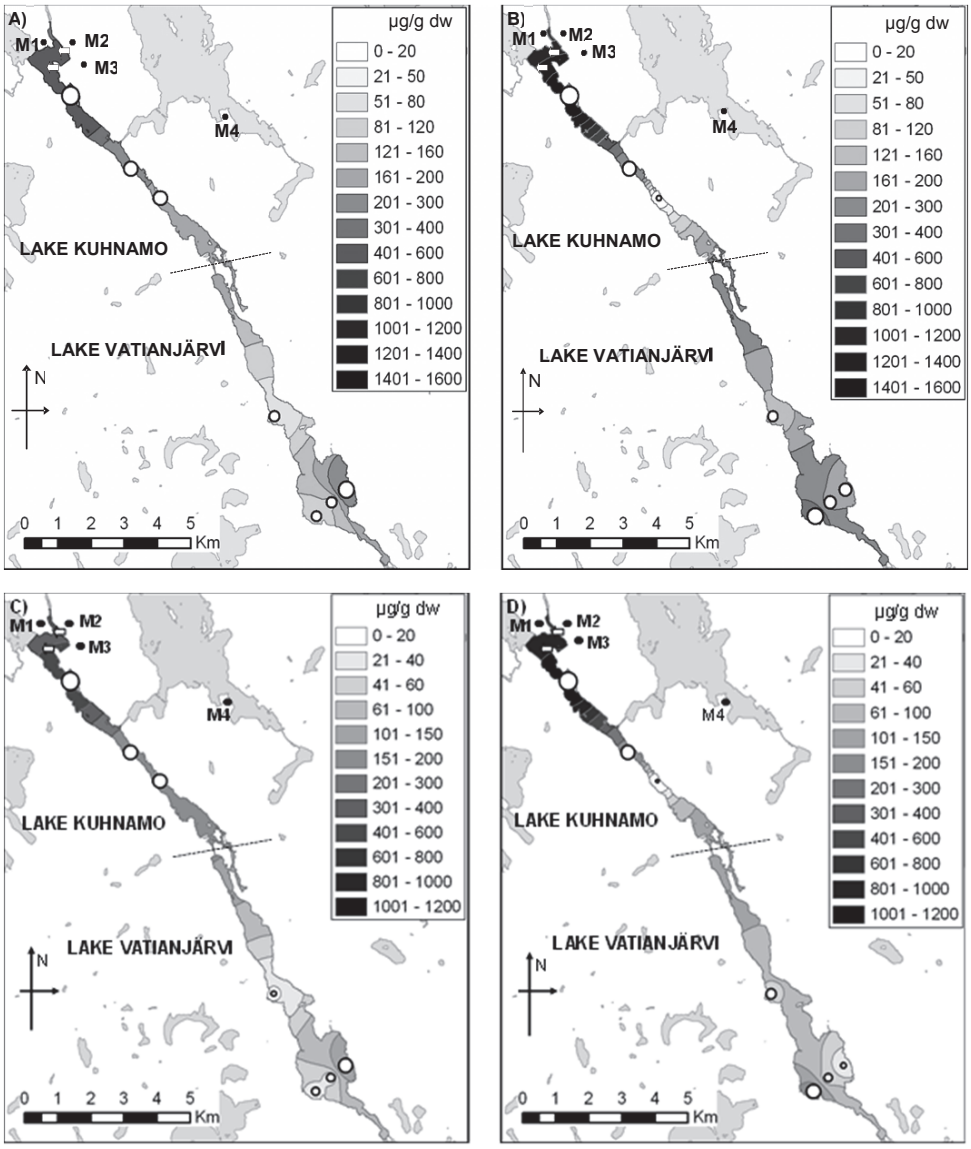

FIGURE 9 Interpolated spatial distributions of the betulinol in the sediment layer a) 0–2 cm and b) 2–5 cm, and β-sitosterol in the sediment layer c) 0–2 cm and d) 2–5 cm in the sediment samples of Lake Kuhnamo and Lake Vatianjärvi. Production facilities have been marked in the picture as follows: M1 = paper mill, M2 = board mill, M3 = pulp mill, M4 = plywood mill. Bolded white arrows show outflows from the wastewater treatment plants. The lakes are separated by a dash line, and the white circles (size depends on concentration) represent the sampling sites (Base map © National Land Survey of Finland 892/MML/09) (I).

The estimated total amounts of DHAA were highest in Lake Kuhnamo being 279 and 437 kg ha^{-1} in the 0–2 and 2–5 cm sediment layers, respectively. In Lake Vatianjärvi, the amounts were 208 and 335 kg ha^{-1}, respectively. The estimations of IPA were similar: in Lake Kuhnamo the amounts were 48 and 85 kg ha^{-1} in the sediment layers of 0–2 cm and 2–5 cm, respectively. In Lake Vatianjärvi the amounts were 32 and 50 kg ha^{-1}, respectively (I).

For betulinol, the highest estimated amount was in the Kuhnamo basin accounting for 3151 and 4726 kg ha^{-1} at the sediment depths 0–2 cm and 2–5 cm, respectively. In the Vatianjärvi basin the estimated amounts were 1455 kg ha^{-1} at a depth of 2–5 cm and half of that in the uppermost two centimeters (II).

Also for the most abundant WS, β-sitosterol, the highest estimated amount, 3571 kg ha^{-1}, was in the Kuhnamo basin at the sediment depth of 2–5 cm, and half of that (1294 kg ha^{-1}) in the uppermost two centimeters. A similar trend was observed in the Vatianjärvi basin, where the estimated amount of β-sitosterol was 311 kg ha^{-1} in the uppermost two centimeters, and 563 kg ha^{-1} at a depth of 2–5 cm (II).

In conclusion, despite the efficient wastewater treatment, large amounts of bioactive RAs and WSs are widely present in the uppermost sediments.

4.1.4 PCDDs, PCDFs and PCBs in the sediment

No PCDDs or PCDFs were found above the limits of detection from the sediment samples of the Äänekoski watercourse from the depths of 0–2, 8–10 or 14–16 cm. Analytically, depending on the compound, the limit of detection varied from 0.01 to 0.1 ng g^{-1}, dw. Trace concentrations of PCB congeners 101, 153, 138 and 180 were found only from the sediment of Lake Vatianjärvi. Concentrations were just above the limit of detection (0.002 ng g^{-1}, dw) (III).

4.2 Dissolution of wood extractives and retene

The dissolution potency was investigated to assess maximal loadings of wood extractives and retene in case of heavy disturbances, such as extensive floods. In the dissolution experiments, some differences were detected between the two agitation times and temperatures. Concentrations of the most wood extractives and retene increased with a longer agitation time (Table 4) (I, II). Desorption of DHAA was the highest of all RAs, being related to its concentration in the sediment samples. Desorption of retene to elutriates was negligible, being less than 0.5 µg l^{-1}, mirroring its low concentrations in the sediments (Table 4) (I).

For betulinol, the significant dependence was observed between the sediment and the elutriate concentrations, both after one hour and 46 h of agitation. During the longer agitation more betulinol was desorbed from the sediment, compared to one hour of agitation, without any exception. The dissolution was highest from the Lake Vatianjärvi sediment followed by Lake Keitele. From Lake Leppävesi and from the reference sediment samples no

dissolution of betulinol was observed above the limit of detection, i.e. 0.12 µg g^{-1}, dw (Table 4) (II).

In the case of WSs, approximately double the amounts of WSs were in the sediment of Lake Kuhnamo and Lake Vatianjärvi compared to the sediment of Lake Leppävesi and to two reference sediments. The dissolution of WSs reflected the concentrations in the sediment in sites downstream from the mills. However, the reference sites provided quite unexpected results. The dissolution of WSs from the Lake Keitele reference sediment was lower compared to the other sediment samples, including the Lake Naarajärvi reference site, although the concentration in the sediment was relatively high (Table 4). The low WS concentration in elutriate from the Lake Keitele could probably be explained by different sediment characteristics, as the sediment was coarse and loosely packed contrary to the other sites. Instead, the reason for the efficient dissolution of WSs from the Lake Naarajärvi reference sediment remains unknown. Similarly with other wood extractives, the dissolution of WSs increased with longer agitation time in the most cases (Table 4). For WSs, positive but not significant correlations were observed between the sediment and elutriate concentrations (II).

TABLE 4 Average concentrations of total resin acids (RAs), dehydroabietic acid (DHAA), retene, betulinol, total wood sterols (WSs) and β-sitosterol of the sediment samples (0–10 cm) in the Äänekoski watercourse (µg g^{-1}, dw), and in elutriates (µg l^{-1}) with two mixing times and temperatures: one hour at 20 °C and 46 hours at 10 °C (I, II). Sampling sites in Fig. 2.

		Distance from the mills				
Compound		–8.5 km	–5 km	3.5 km	15 km	33 km
RAs	Sed.	2.9	8.4	140	62	23
	1 h	2.6	3.8	33	20	5.9
	46 h	2.7	3.0	77	28	8.0
DHAA	Sed.	2.5	5.8	92	41	13
	1 h	2.4	3.8	24	15	4.2
	46 h	2.6	3.0	38	20	6.0
Retene	Sed.	<0.1	<0.1	12	1.9	0.2
	1 h	<0.1	<0.1	0.5	0.2	<0.1
	46 h	<0.1	<0.1	0.5	<0.1	<0.1
Betulinol	Sed.	2.5	31	412	346	137
	1 h	<0.12	<0.12	63	73	22
	46 h	<0.12	<0.12	156	165	31
WSs	Sed.	149	311	768	429	185
	1 h	89	2.7	145	113	76
	46 h	237	8.2	273	106	52
β-sitosterol	Sed.	50	133	250	148	62
	1 h	24	0.7	46	33	20
	46 h	72	2.5	83	30	13

In summary, the dissolution of wood extractives and retene reflected the concentrations in the sediment with some exceptions. However, it must be noted that the dissolution potency cannot be directly compared to the concentrations measured from the dry sediment as elutriates were made from the wet sediment and dry weight of the sediment samples varied considerably.

4.3 AhR-activating compounds in the sediment — EROD activity in fish as biomarker

4.3.1 Sediment extract bioassay

The aim was to analyze the potency of sediment samples collected downstream from the pulp and paper industry to evoke activity of CYP1A system. Regarding to the validity of the assay (III), an increasing trend was observed with increasing retene concentration both in the exposure to pure retene and to sediment extract from Southern Lake Saimaa. The correlation between retene concentration and EROD activity was statistically significant (p = 0.026). Theoretically, the maximum estimated retene doses in 10, 50 and 100 % Southern Lake Saimaa extracts were 7, 14 and 28 µg g^{-1} fish wet weight, respectively. Instead, EROD induction in fish i.p. injected by the extracts from the Äänekoski watercourse sediment were at the same level as in Lake Keitele reference sediment in all depths (2–4, 8–10 and 14–16 cm) (Fig. 10).

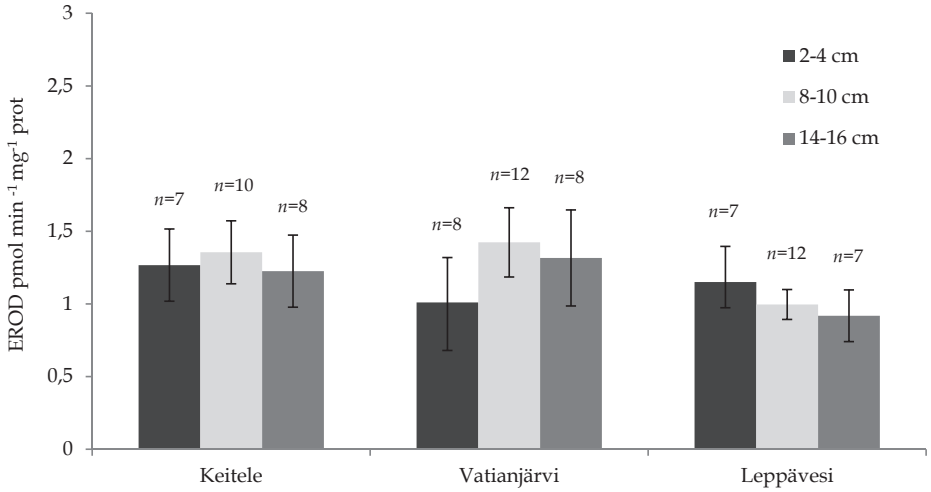

FIGURE 10 Hepatic EROD activity (± S.E.) in juvenile rainbow trout, injected i.p. by the sediment extract isolated from three depths and diluted in DMSO:sunflower oil (1:9, v/v). EROD activity is weighted by loss on ignition. The number of fish is marked by *n*.

No statistical difference was observed between the first and the second serie of bioassays, in which the injection of sediment extracts (8–10 cm) of Äänekoski watercourse sediment was repeated, and therefore the results were combined. All the results were weighted by LOI (III). Thus, to conclude, no AhR-activating compounds were present in the studied sediment depths from the Äänekoski watercourse.

4.3.2 Sediment with water bioassay

To evaluate the impact of the pulp and paper mill contaminated sediment to cause ecotoxicity, juvenile rainbow trout were exposed to the sediment in the laboratory assay (IV). The exposure of fish simulated the natural conditions where bioturbation by juvenile fish may lead to the chemical exposure either by direct contact with the sediment or by dissolution of the contaminants to the water.

In juvenile rainbow trout exposed to the contaminated sediment of Southern Lake Saimaa, both one and three km distance from the pulp and paper mills, the average EROD activity was 2.5 times that found in the Rautniemi reference sediment. The differences between the treatments were statistically significant (p = 0.045) but in the multiple comparisons statistically significant differences were not found because of the small number of analytical samples in one group (n = 3). In exposure to the surface sediment (0–10 cm) of Lake Vatianjärvi, collected in 2007, EROD activity was at the same level as the Rautniemi reference sediment and with water only treament (Fig. 11) (IV).

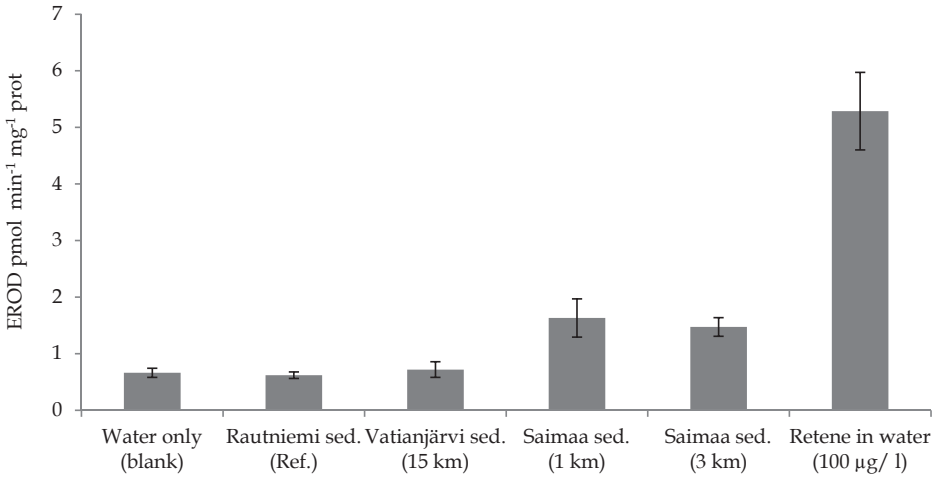

FIGURE 11 Hepatic EROD activity (± S.E.) in juvenile rainbow trout. Fish were exposed to the sediment samples collected from Lake Vatianjärvi 15 km, and from Southern Lake Saimaa 1 and 3 km downstream from the pulp and paper mills. Sediment of the Rautniemi from upstream in Southern Lake Saimaa was a reference, and water with DMSO as a blank control. Retene in water (100 µg l^{-1}) was used as a positive control (IV).

A positive association was observed between the EROD activity and retene concentrations in the sediment samples (p = 0.005, n = 12). Compared to the Southern Lake Saimaa sediment, remarkably lower retene and resin acid concentrations were measured from Lake Vatianjärvi sediment. The concentration of retene in the sediment of Lake Vatianjärvi (5.1 ug g^{-1}, dw) was only 2–5 % of the concentrations at one and three kilometer sites in Southern Lake Saimaa (94 and 224 ug g^{-1}, dw, respectively). Southern Lake Saimaa (1 km) was the only treatment from which retene was observed in the water sample (0.2 µg l^{-1}), the limit of detection being 0.1 µg l^{-1} (IV).

As EROD activity in rainbow trout exposed to the sediment of Lake Vatianjärvi did not differ from the exposure to the reference sediment, it can be concluded that fish were not exposed to the retene or any other AhR-activating compounds (IV). Instead, retene and perhaps some other AhR-activating compounds also are present in the sediment of Southern Lake Saimaa, as increased EROD activity was observed in fish both in this bioassay (IV) as well as in the sediment extract bioassay where the fish were dosed i.p. (III).

4.3.3 EROD activity in wild fish populations

As hepatic EROD activity has not been monitored earlier from the populations of feral fish in Äänekoski watercourse, it was measured in the course of this study from roach and perch sampled from Lake Vatianjärvi. When compared to the populations in the reference lakes, no higher EROD activity was detected in perch and roach caught from Lake Vatianjärvi. In roach, the average EROD activity was slightly lower in Lake Vatianjärvi. In perch, instead, the average EROD activity was about 80 % lower in Lake Vatianjärvi compared to the two reference lakes, and the difference was statistically significant (p < 0.001) (Fig. 12) (IV).

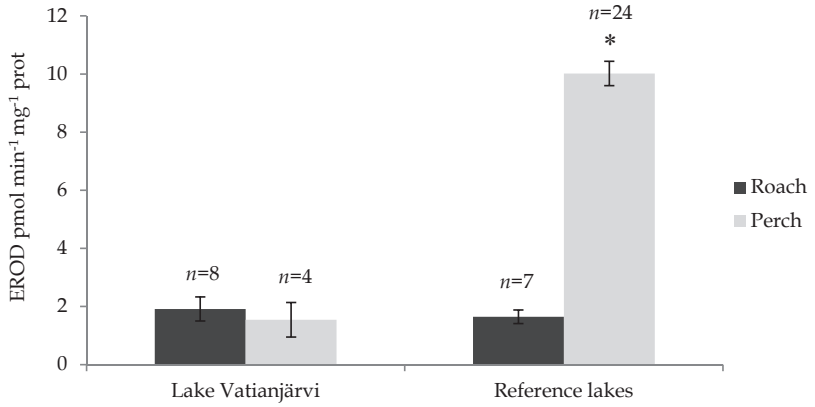

FIGURE 12 Hepatic EROD activity (± S.E.) in roach and perch caught from Lake Vatianjärvi, 15 km downstream pulp and paper industry, in 2009. Reference lakes, Lake Palosjärvi and Lake Konnevesi, are combined. Statistical difference (p < 0.05) from reference sites is marked by an asterisk (*) and the number of fish by n (IV).

4.4 Hydropsychidae index and gill damage in caddis larvae

The aim was to assess the ecotoxicological status of an invertebrate group in the Äänekoski watercourse from 1999 to 2008 by using species composition and gill abnormalities of caddis larvae as bioindicators. During this time, the species composition changed. This was reflected in HA values in the Kuusaankoski rapids, 15 km downstream from the mills. HA increased from 3.4 to 4.0 in 1999–2007 due to the increasing dominance of *Hydropsyche siltalai* which has become the most abundant species since 2007 (Fig. 13). *H. siltalai* was also dominant at the Siikakoski and Simunankoski reference sites in 2007–2008, and *Hydopsyche pellucidula* and *Cheumatopsyche lepida* were also present (V).

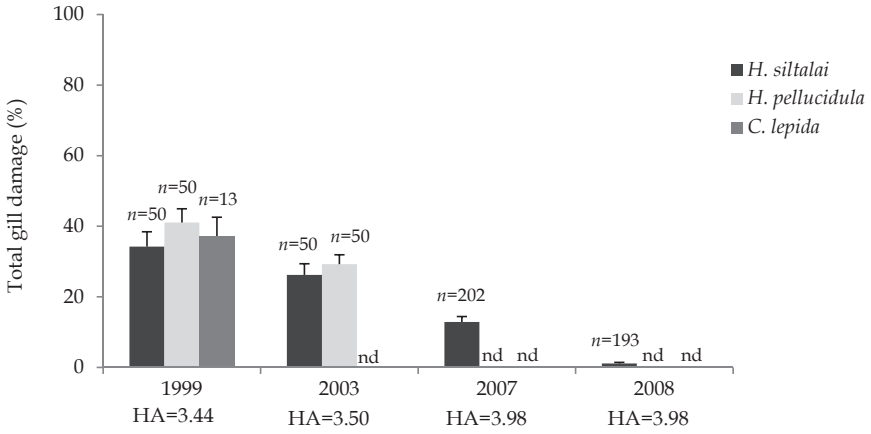

FIGURE 13 Average (± S.E.) proportions of *Hydropsyche siltalai*, *Hydropsyche pellucidula* and *Cheumatopsyche lepida* individuals with damaged gills (slight + serious damage) in the Kuusaankoski rapids from 1999 to 2008. Absence of species is denoted by nd (not detected), and *n* is the number of individuals. HA is Hydsopsychidae index varying from one to five, so that a higher value indicates changed water quality based on lesser organic pollution (V).

The incidence of gill damage of the *H. siltalai* population significantly decreased ($p < 0.001$) in the Kuusaankoski rapids from 1999 to 2007. Further downstream in the Kuhankoski and Haapakoski rapids, *H. pellucidula* was the only species found in 1999 and 2003 and the incidence of its gill damage significantly decreased with increasing distance from the mills (Fig. 14). In 1999, an average of 23 % of *C. lepida* gills were slightly damaged, and 14 % were seriously damaged in the Kuusaankoski rapids. *C. lepida* was absent there in 2003 and 2007, and in 2008 only two individuals were found and no gill damage was observed. Further downstream, *C. lepida* was absent (V).

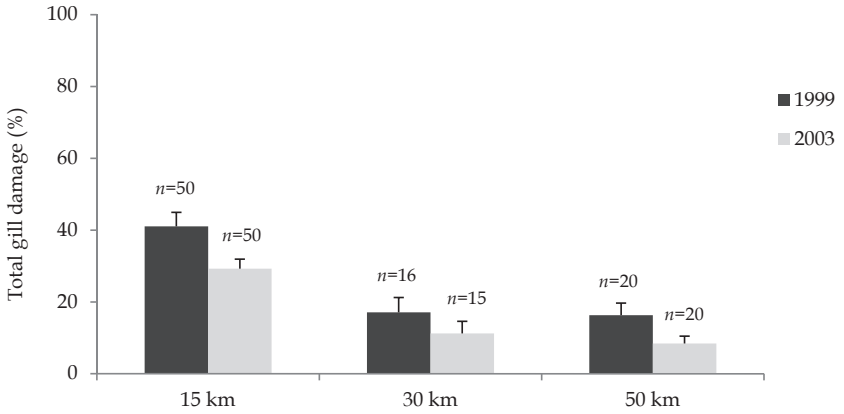

FIGURE 14 Average (± S.E.) proportions of *H. pellucidula* with damaged gills (slight + serious) in the years 1999 and 2003 in the rapids downstream from pulp and paper mills in Äänekoski: Kuusaankoski (15 km), Kuhankoski (30 km) and Haapakoski (50 km), *n* is the number of individuals (V).

Unexpectedly, at the reference sites relatively high numbers of abnormal gills were observed in larvae collected in 2007 in the Siikakoski rapids where 13 % of the gills of *H. siltalai* were slightly or seriously damaged. Only three individuals of *H. pellucidula* were found and the proportion of abnormal gills was 9 %. However, in 2008, less than 1 % of the gills of *H. siltalai* were found to be abnormal, whereas in *H. pellucidula* the proportion of gill damage was 20 %. Gill abnormalities were also detected in the Simunankoski rapids, but only in *H. pellucidula*. There, in 2007 and 2008, 52 % and 27 % of the gills of *H. pellucidula* were damaged, respectively. However, it must be pointed out that in both reference sites most of the abnormal gills (80–95 %) were classified as only slightly damaged (V).

When comparing the histological cross-sections of the healthy gills between the species, it was noticed that *H. siltalai* had a round shape and a smooth membrane (Fig. 15a–b), whereas gills of *H. pellucidula* were more irregular and the outer membrane was coarser (Fig. 15e–f). Inspection of the gill structure revealed evidence of the tissue alterations. From the cross-section of gill tissue it could be seen that healthy and damaged gill tissues had dissimilar structures. In the healthy gills bronchioles were visible, unlike in the damaged gills (Fig. 15c–d and g–h) (V).

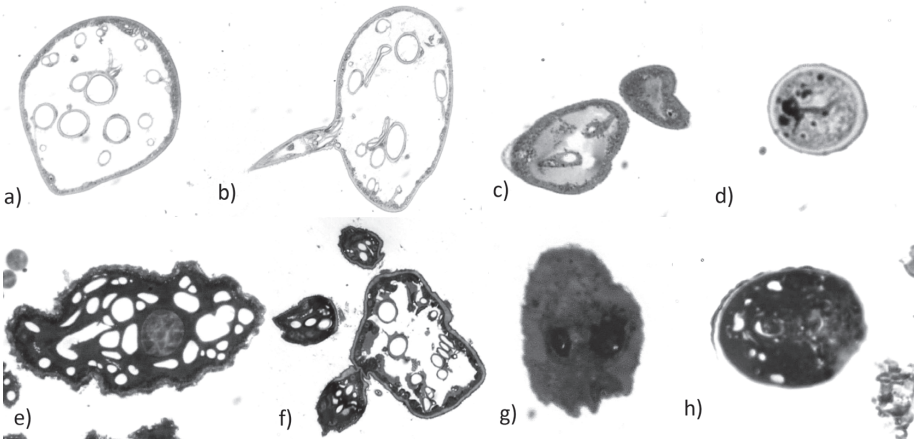

FIGURE 15 Cross sections of healthy (a–b), and damaged gills (c–d) of *Hydropsyche siltalai*, and cross sections of healthy (e–f), and damaged gills (g–h) of *Hydropsyche pellucidula* (V).

In conclusion, the species composition index (HA) as well as gill abnormalities of caddis larvae, as ecotoxicological bioindicators, reflected the impacts of pulp and paper mill discharge. The results indicated a 20-year recovery period of the Äänakoski watercourse.

5 DISCUSSION

5.1 Pulp and paper mill contaminated sediment — sink or source?

There are only a few case areas toxicologically documented well over several decades following major technical transitions in the chemical wood industry. A few examples include Southern Lake Saimaa in Finland, Norrsundet in Sweden and Jackfish Bay in Canada. The monitoring of ecosystem recovery is meaningful, as it provides knowledge about a long term impacts to the populations, success of water management and policy, and possible needs for further technological investments.

The Äänekoski watercourse was heavily impacted until the mid-1980s. Due to technological improvements it started to recover. However, this study has shown that some bioindicators reflected decreased but detectable impacts still over 20 years later. Nevertheless, by the end of the current studies, no significant effects on fish (III, IV) or on invertebrates (V) were observed. However, substantial concentrations of the wood extractives were measured from the sediment, some of them in such high amounts that they have potential to cause harm to the aquatic animals in cases of exceptionally strong disturbances of the sediment, for instance during heavy flood or dredging (I, II). Indeed, besides the direct exposure to the bioactive compounds discharged from the mills, the sediment resuspension is another important exposure route of the pollutants to the aquatic animals. Importantly, while the hydraulic retention time in the lakes downstream from the Äänekoski pulp and paper mills is only three days (Palomäki et al. 2006), the particles with the sorbed hydrophobic compounds may be rapidly transported far downstream in the river-like watercourse.

Annual runoff in the higher latitudes is predicted to increase by 10–40 % by the end of this century. This change may result in an increasing risk of floods (IPCC 2007), which may affect the flow rate and enhance sediment erosion. Critical shear stress is one important factor when assessing the potential of sediment erosion but simple correlation between the sediment characteristics

and the critical bed shear for erosion is difficult to demonstrate. For instance, in the River Elbe the critical shear stress is very low in the uppermost five cm of the sediment, i.e. this layer is highly sensitive to erosion (Förstner et al. 2004). In addition, freshwater species of sediment burrowing invertebrates usually inhabit in the uppermost 5 cm of the sediment (Särkkä 1987, Doig & Liber 2010) and if they accumulate chemicals from the sediment, it may further influence the bottom feeding fish. For these reasons, vertical concentrations of wood sterols and retene were measured, and the total amounts of betulinol and the most common RAs and WSs were estimated from the uppermost five cm of sediment. In this study, the RAs and betulinol were identified as the extractives of concern. While the surface area of Lake Kuhnamo is 3.7 km^2 and Lake Vatianjärvi 6.3 km^2, in case of severe erosion, massive amounts of bioactive wood extractives could be released from the uppermost five cm sediment to the water phase and downstream.

Not only erosion, but also the bioavailability of xenobiotics to the benthic animals and fish is important when assessing the potential risks of the contaminated sediments, as mentioned above. Dissolution potency can be used as an approximate predictor of bioavailability (Lebo et al. 2003, Meriläinen et al. 2006). As expected, the highest concentrations of wood extractives and retene in the dissolution experiments were found in Lake Kuhnamo elutriates. Concentrations in elutriates were site-dependent, and corresponded to the concentrations in the sediment (I, II). In the dissolution experiments, the abundance of organic carbon seemed to decrease desorption from the sediment samples, with some site-related differences. Overall, the OC concentration was associated with the LOI, which remained similar in vertical sediment layers (I, II). The OC and grain size have been shown to be the most crucial factors concerning bioavailability of hydrophobic organic xenobiotics and their toxicity in the sediments (Delle Site 2001, Lebo et al. 2003).

Typically, the rate of desorption decreases over time (Chen et al. 1999, Meriläinen et al. 2006). In this study, the temperature and duration of each experiments varied and kinetic sampling was not done, and therefore the rate of desorption cannot be estimated. However, the differences in desorption of the wood extractives and retene may partly be due to the duration of agitation and the prevailing temperatures probably has an effect also (I, II). The longer agitation at a lower temperature probably simulated more natural case of flooding which occur every spring in Northern countries due to melting snow. Turnover in spring and autumn in boreal lakes may also cause some sediment resuspension and dissolution of the contaminants. Also hydrophobicity of the compounds may affect the dissolution potency. For instance, Meriläinen et al. (2006) have shown the dissolution of WSs being slower than of RAs; for WSs it took seven days to reach the highest state of desorption, whereas RAs reached equilibrium in three days. This probably reflects the higher hydrophobicity of WSs (e.g. log K_{ow} 9.65 for β-sitosterol; Kinney et al. 2006) compared to the RAs (log K_{ow} 6.52 for DHAA; Mellanen et al. 1996), and therefore a stronger binding of WSs to the sediment. Also betulinol is more hydrophobic (log K_{ow} 8.18,

ChemSpider 2012) than RAs. In the following sections, the current status and potential risks of these bioactive compounds and persistent organochlorines in the sediment of Äänekoski watercourse are discussed in more detail.

5.2 Risk identification — bioactive compounds in the sediment

5.2.1 Resin acids

In the Äänekoski watercourse, abietic type RAs were considerably more abundant than the pimaric type RAs (I), confirming earlier observations of the sediment (Judd et al. 1996, Leppänen & Oikari 1999a) and in elutriates (Meriläinen et al. 2006) related to the pulp and paper industry. The most abundant RAs were DHAA and IPA. The overall amounts in a lake-wide area were also estimated. The estimated amount of DHAA, based on the interpolated spatial distribution, was approximately three times higher than amount of IPA (I). However, IPA is probably also a cause for concern because its toxic potency is higher than that of DHAA (Peng & Roberts 2000). Indeed, some evidence of dissolution of RAs has been noted in the field experiments, for instance during spring turnover elevated concentrations of RAs have been detected in fish bile in Southern Lake Saimaa (Karels & Oikari 2000, 2008b). This indicates that sediments may annually and naturally release contaminants to the water. On the other hand, the effluents are also still sources of RAs, as Oikari et al. (2010) measured higher RA concentrations from the bile of trout which were caged near the lake surface of Southern Lake Saimaa, compared to the concentrations in the bile of trout held just above the sediment.

Regarding sediment erosion, the maximum RA concentration in the sediments of the Äänekoski watercourse, as measured one kilometer from the point of discharge, was 246 μg g^{-1} (dw), yielding up to 77 μg l^{-1} in the dissolution experiments (I). This dissolved concentration is high enough to be toxic to brown trout (*Salmo trutta*) (Meriläinen et al. 2007) and rainbow trout (*Salmo gairdneri*) (Oikari et al. 1983) as well as to water fleas (*Daphnia magna*), depending on the composition of the RA mixture (Peng & Roberts 2000). Background levels of RAs in uncontaminated sediments in Finnish lakes vary from 0.3 to 20 μg g^{-1} (dw) (Lahdelma & Oikari 2005).

5.2.2 Retene

Retene's ability to cause deleterious malformations in fish embryos at low concentration levels (10–32 μg l^{-1}) is well-known (Billiard et al. 1999, Brinkworth et al. 2003). In this study, concentrations in the Äänekoski sediments were remarkably lower (< 51 μg g^{-1}, dw) than those measured from other Finnish lakes loaded by chemical wood industry. For instance, retene concentrations of 1600 μg g^{-1} (dw) have been measured from Southern Lake Saimaa and 1070 μg g^{-1} (dw) from Central Lake Päijänne (Lahdelma & Oikari 2005). Furthermore,

the dissolution of retene from the sediment of Äänekoski watercourse was negligible (< 0.53 µg l^{-1}) mirroring its low concentrations in the sediment (I). Even in much higher retene concentrations in the sediment (500 µg g^{-1}, dw), dissolution has been low (Meriläinen et al. 2006), which apparently can be explained by high hydrophobicity of retene (log K_{ow} 6.4; Basu et al. 2001). Therefore, the low water solubility of retene (17 µg l^{-1}; Kiparissis et al. 2003) may be partly responsible for its low concentrations in elutriates. Although the high hydrophobicity decreases the dissolution, it may result in adsorption to the depositing particles, which in a river-like watercourse may transport retene molecules far from the source as was noted in this study (I) and in the previous studies as well (Leppänen & Oikari 1999a, Leppänen & Oikari 1999c).

The low concentrations of retene in the sediments of the Äänekoski watercourse probably can be explained by the relatively low abundance of RAs, retene's precursors (Tavendale et al. 1997, Leppänen & Oikari 1999b), as a significant positive correlation between RAs and retene concentrations were found in the sediment of Lake Kuhnamo (I). Probably aerobic conditions in the sediment may also be affected, while anoxic conditions are needed for retene formation (Tavendale et al. 1997, Leppänen & Oikari 1999b). In fact, in the 1960s and 1970s, during the summer stratification, anoxic conditions were commonly measured in the hypolimnion of Lake Vatianjärvi and even in early 1990s in Lake Kuhnamo (HERTTA 2012). Such conditions should have been suitable for retene transformation from RAs (Tavendale et al. 1997, Leppänen & Oikari 1999b). On the other hand, the sediment samples analyzed in this study were deposited after that, as the sedimentation rate was about three cm in Lake Kuhnamo (Saski et al. 1997) and one cm per year in Lake Vatianjärvi (III, Saski et al. 1997), and the oxygen concentrations increased relatively quickly (HERTTA 2012) due to the introduction of the new pulp mill and efficient wastewater treatment in 1985. Therefore, unsuitable conditions for retene formation may explain the low concentrations in the analyzed sediment layers. Overall, nowadays the concentrations of retene in the sediments are low and dissolution from the sediments does not evoke a risk for aquatic animals in the Äänekoski watercourse (I).

5.2.3 Betulinol

Surprisingly high amounts of betulinol were measured from the sediment of Äänekoski watercourse (II). Betulinol was generally more abundant in the sediment samples than any other individual wood extractive (I, II). Like most of the wood extractives, betulinol is a hydrophobic compound (log K_{ow} 8.18; ChemSpider 2012) which tends to sorb to organic particles (Kostamo et al. 2004). Still the dissolved concentrations in the sediment elutriates were considered to be high enough to cause harmful effects on fish (II). Potentially, betulinol is an endocrine disrupting chemical (Mellanen et al. 1996, Christianson-Heiska et al. 2004, 2008), being effective down to a concentration of only 5 µg l^{-1} and it also may weaken the disease defense mechanisms of fish (Christianson-Heiska et al. 2004). In this study, much higher concentrations, up

to 165 μg l^{-1}, were measured from elutriates. Even the sediment 33 km downstream from the pulp and paper mill may affect fish as betulinol concentrations of 22–31 μg l^{-1} were measured from elutriate made of sediment from Lake Leppävesi (II). Therefore, further ecotoxicological studies of betulinol are highly recommended.

On the other hand, the concentrations of betulinol in the sediment of Äänekoski watercourse were lower than those measured from Southern Lake Saimaa. In this study, betulinol concentrations in the uppermost 10 cm of the sediment samples varied from 610 to 1670 μg g^{-1} (dw) one kilometer downstream from the mills (II) whereas in Southern Lake Saimaa Lahdelma & Oikari (2006) documented betulinol at 3810 μg g^{-1} (dw) from the sediment depth of 20–25 cm, and reported that the surface sediment concentration was about half of that. Also resin acids and retene have been measured at higher concentrations in Southern Lake Saimaa (Leppänen & Oikari 1999a, 1999c, Lahdelma & Oikari 2005) than those in the Äänekoski watercourse (I), probably due to the different hydromorphology of the lakes. Unlike Southern Lake Saimaa, the Äänekoski watercourse is more river-like and therefore the contaminants in the effluents are spread in larger area and further downstream.

Interestingly, in the sediment samples taken from Lake Keitele, the reference site 5 km upstream from the main point source, a similar pattern of betulinol profile was observed as in the Kuhnamo basin, just below the effluent discharge point. However, the concentrations in the Lake Keitele sediments were only about 2 % of that in Lake Kuhnamo (II). Betulinol concentrations in Lake Keitele were approximately the same as measured from the other reference sites in Finland, around 25–30 μg g^{-1} (dw) (Lahdelma & Oikari 2006). On the other hand, a much lower concentration was measured from our other reference site, Lake Naarajärvi. The variation in our reference sites may have arisen from the plywood factory, which releases its overflow waters of the birch reservoir into nearby Lake Keitele, although since 2006 a new pumping system has decreased the overflow volume by 75 % (Anonymous 2006b). In addition, it must be noted that the dissolution potency cannot be directly compared to the concentrations measured from the dry sediment, because elutriates were made from the wet sediment and dry weight of the sediments varied substantially. The differences between the two reference sediment sites may also be explained by the differences between the catchment areas and the sediment characteristics. It is further possible that the reference levels varied considerably for instance because of log floating, which has been practiced in almost every large watercourse in Finland.

5.2.4 Wood sterols

Like betulinol, WSs are also bioactive compounds causing hormonal disruptions (Mellanen et al. 1996, Tremblay & van der Kraak 1998, 1999). However, the effective concentration is much higher compared to betulinol (Lehtinen et al. 1999, Christianson-Heiska et al. 2004, Orrego et al. 2011).

Interestingly, the maximum concentrations of WSs were measured from the surface sediment both in this study in the Äänekoski watercourse (2890 μg g^{-1}, dw) (II) and in the previous study in Southern Lake Saimaa (1760 μg g^{-1}, dw) (Lahdelma & Oikari 2006). In this study, the total WS concentrations were higher in the uppermost 10 cm of sediment (II) than detected in earlier studies from other Finnish lakes loaded by pulp and paper mill effluents (Lahdelma & Oikari 2006). In Central Lake Päijänne, which has also been contaminated by the chemical wood industry for over a hundred years, the maximum WS concentration (1970 μg g^{-1}, dw) was measured from the sediment layer which was deposited during the 1970s when untreated effluents were discharged. The natural background levels of WSs in the previous studies have been around 10–50 μg g^{-1} (dw) (Lahdelma & Oikari 2006). These levels are lower than in our study (80–200 μg g^{-1}, dw) (II). Surprisingly, in the Lake Keitele reference site, the total WS concentration was 580 μg g^{-1} (dw) from the sediment depth of 2–5 cm. In every other sediment layer the concentrations were about 200 μg g^{-1} (dw) or less, as was also in our other reference site, Lake Naarajärvi (II). In relation to the exceptionally high concentration of WSs in Lake Keitele, it could be explained by plywood factory as discussed above in case of betulinol.

Some variation in the dissolution potency of WSs between the sampling sites was observed. The dissolution of WSs from the Lake Keitele reference sediment was lower compared to the other sediment samples, including the Lake Naarajärvi reference site, probably because of different sediment characteristics, as the sediment of Lake Keitele was coarse and loosely packed contrary to the other sediments (II). Lake Keitele is part of the oligotrophic watercourse whereas Lake Naarajärvi is part of the humic watercourse where peat mining, agriculture and forestry are the largest types of land use in the catchment area. It is also possible that in Lake Keitele the origin of the WSs is mainly from the decaying plant material which would explain the high measured concentrations in the sediment when the strong solvents were used in the extraction (II), whereas WSs from this kind of origin probably do not dissolve as easily into the water elutriate. Instead, in the impacted sites, most of the WSs were discharged from the mills and were originally in a dissolved form which may also explain the higher dissolution into elutriates. However, this does not explain an efficient dissolution of WSs from the Lake Naarajärvi reference sediment, as the dissolution of WSs in proportion to the amounts in the wet sediment were higher therein than in the areas downstream from the mills (II). The difference may be explained by the lower TOC in Lake Naarajärvi (3.7 %) compared to Lake Kuhnamo (9.0 %) and Lake Vatianjärvi (4.2 %), as desorption of organic contaminants increase with decreasing OC content (Park & Erstfeld 1999, Lebo et al. 2003). On the other hand, in Lake Leppävesi the dissolution of WSs in proportion to the amounts of WSs in the agitated wet sediment were about the same as in Lake Kuhnamo and Lake Vatianjärvi, although the TOC content was only 1.2 % (II).

The β-sitosterol and the stigmastanol are, along with the campesterol, the most abundant sterols in trees (Sjöström 1993, Kostamo et al. 2004). The β-

sitosterol has been documented as the dominant sterol in the pulp mill effluents (Strömberg 1996). It was the most abundant of WSs in the analyzed sediments in this study as well (II). Stigmastanol was another common WS in the Äänekoski watercourse (II), possibly due to deoxidation of the β-sitosterol (Chan et al. 1998).

While β-sitosterol may cause estrogenic effects, such as induction of vitellogenin production on fish (Mellanen et al. 1996, Tremblay & van der Kraak 1998, 1999), also the masculinization of female fish has been detected in pulp and paper mill contaminated waters (Howell et al. 1980, Ellis et al. 2003). Jenkins et al. (2003) suggested that microbial degradation of pine phytosterols may transform sterols to progesterone and further into androstenedione and other bioactive androgens, which would explain the masculinization. In addition, the reduction of plasma cholesterol and testosterone concentrations has been observed (Tremblay & van der Kraak 1998, 1999). However, moderately high added concentrations of β-sitosterol (10–20 mg l^{-1}) are needed to cause severe consequences on maturing lake trout, such as egg mortality, smaller egg size and lower mean weight of the yolk-sac stage larvae (Lehtinen et al. 1999). A lower addition to water (5 mg l^{-1}) caused early hatching and increased hatchability in rainbow trout, medaka and flagfish embryos (Orrego et al. 2011). In our dissolution experiments, an effective concentration of WSs were not exceeded, as the maximum concentration total WSs was only 0.3 mg l^{-1}, and 0.08 mg l^{-1} for β-sitosterol (II). In conclusion, WSs are capable of creating various disruptive effects on aquatic biota but in the Äänekoski watercourse WSs should not cause harm to biota.

5.2.5 Organochlorines

PCDDs and PCDFs, chlorinated phenolics and chlorinated lignin, making up a part of AOX, had been common compounds in the pulp and paper mill effluents especially before the introduction of biological effluent treatment and replacement of the use of molecular chlorine with the chlorine dioxide (Dahlman et al. 1991, Owens 1991, Solomon 1996, Strömberg et al. 1996, Ali & Sreekrishnan 2001). In the Äänekoski watercourse, monitoring of chlorinated organic compounds or specific bioactive substances released from the pulp and paper industry has been limited. Only a few studies with specific organochlorines have been done (Paasivirta et al. 1988, Anonymous 1992, Herve et al. 2001). Paasivirta et al. (1988) measured concentrations of chlorophenolic compounds in the Äänekoski watercourse before and after the introduction of the new kraft mill in 1985, and detected significant decreases in concentrations after mill modernization. From the 1960s to 1984, the annual discharge of chlorophenolics was about 6.5 tons whereas after mill renewal it decreased to 1.2–1.8 tons in 1986–1990 (Paasivirta 1991). This was reflected on exposure of aquatic animals as well. Concentrations of chlorophenolics were measured from the caged lake mussel (*Anodonta piscinalis*) in 1984–1998 and it was observed that concentrations decreased with increasing distance from the mills, and a

decreasing time trend was also shown, as concentrations started to decrease after mill renewals in 1985 (Herve et al. 2001).

Due to the new activated sludge treatment plant, there were reductions not only of the chlorophenolics, but also of AOX and TOC, which showed reductions of more than 60 % and 80 %, respectively (Paasivirta et al. 1988). Thus, also the discharge of PCDDs and PCDFs, as small parts of AOX, and as hydrophobic compounds, likely decreased even though elemental chlorine bleaching with ECF was still in use in the late 1980s and early 1990s. In the late 1980s, a few years after the mill renewal, the concentrations of PCDDs in Lake Vatianjärvi sediment were 1.4 ng g^{-1} (dw), but 2,3,7,8-TeCDD, the most toxic dioxin, was not observed (Koistinen et al. 1990). Paasivirta (1991) also did not find 2,3,7,8-TeCDD or 2,3,7,8-TeCDF above the analytical limit (0.02 ng g^{-1}, dw). However, Paasivirta (1991) documented several toxic hexa-, hepta- and octachloridated dibenzo-*p*-dioxins and dibenzofurans at concentrations 0.02–0.23 ng g^{-1} (dw) in the sediment of the Äänekoski watercourse in 1988. In the current study no PCDDs or PCDFs were found above the limit of detection (0.01–0.1 ng g^{-1}, dw). Based on the ^{137}Cs peak revealing the sedimentation rate of approximately one centimeter per year in Lake Vatianjärvi (III, Saski et al. 1997), the deepest sediment layer (14–16 cm) used in this study described the concentrations from the years 1995–1997. The ECF bleaching process with oxygen delignification was fully introduced in 1993 (Anonymous 2006a), thus the absence of PCDDs and PCDFs was expected.

PCBs were another group of organochlorines analyzed in this study (III). Although the production of PCBs ceased in many countries already in the 1970s and worldwide in 1993 (AMAP 2000), elevated concentrations are still measured from aquatic environments in the 21st century (Verta et al. 2007, Sundqvist et al. 2009, Heimann et al. 2011). The use of PCBs in the paper production was clearly detectable in the sediment of Lake Jyväsjärvi, another lake in Central Finland loaded by the paper industry. PCB concentrations started to increase in the sediment of Lake Jyväsjärvi in the 1960s reaching the maximum concentration (0.6 mg kg^{-1}, dw) in the early 1970s. After 1979 concentrations were at the background level again (Meriläinen et al. 2003). The cause and effect relationship was observed also in Norrsundet, Sweden, where the PCB concentrations in the sediment and in perch tissues decreased in function of distance to the pulp mill in the 1980s (Södergren et al. 1988). In the Äänakoski watercourse, PCBs were first measured from sediment of Lake Kuhnamo in 1988, when the concentration was about 380 ng g^{-1} (dw) (Paasivirta et al. 1990). One year later PCBs were measured from pike caught from Lake Vatianjärvi. The highest measured concentration of PCBs in the muscle of pike was approximately 160 ng g^{-1} (ww), and concentrations decreased to about 50 % of that until year 1992 (Anonymous 1992). In 2003, PCBs found in fish in the Äänekoski watercourse were still detected but concentrations were so low that no restriction on consuming them as human food was needed to be ordered (Anonymous 2006b). In this study, the only found PCB congeners in the sediments were PCB 101, 105, 138 and 180, and concentrations were near or

below the background level (10–100 ng g^{-1}, dw) (Paasivirta et al. 1990, Särkkä et al. 1993). It can thus be concluded that this group of chemicals are not likely to pose a risk to biota in the Äänekoski watercourse. Similarly, PCDDs and PCDFs are buried so deep in the sediment that they should not be able to cause hazards.

5.3 Recovery of the Äänekoski watercourse

5.3.1 Absence of dioxin-like loading — experimental and field evidence

Regarding to known inducers of CYP1A activity in the pulp and paper mill effluents, hepatic EROD activity in fish was used as a biomarker of exposure to the dioxin-like compounds (III, IV). Such compounds studied in this work were retene, and some PCDDs, PCDFs and PCBs.

Fish bioassays (III, IV) confirmed the results of the sediment investigations: AhR-activating compounds were not present in the sediment samples downstream from the Äänekoski pulp and paper mills at such levels which could induce EROD activity (III, IV). Retene concentrations in the sediment (I, IV) and in elutriates (I) were low compared to the other sites contaminated by the chemical wood industry (Leppänen & Oikari 2001, Lahdelma & Oikari 2005). The PCBs found were at background level (III), and of them only PCB105 is known to induce EROD activity (Schmitz et al. 1995). PCDDs and PCDFs were not found above the limit of detection (0.01–0.1 ng g^{-1}, dw) from the sediment (III) and apparently the effects on biota are negligible.

EROD activity in rainbow trout, which were exposed i.p. to the sediment extracts of Lake Vatianjärvi and Lake Leppävesi, were at the same level with the reference treatments (III). Similar results were also obtained from the laboratory bioassays, where juvenile rainbow trout were exposed to the sediment in water system (IV). In sediment with water bioassays, Southern Lake Saimaa sediment was used as a positive control (III, IV), and the results indicated EROD-potential as in a similar experimental setup (Oikari et al. 2002). The sediment of Southern Lake Saimaa is known to be highly contaminated by retene (Leppänen & Oikari 1999a, 1999b, Oikari et al. 2002, Lahdelma & Oikari 2005, Meriläinen et al. 2006) and retene may induce EROD activity already at a nominal concentration of 10 μg l^{-1} in water (Fragoso et al. 1998). The amounts of retene in the Southern Lake Saimaa sediment extract dosed in our study were 7, 14 and 28 μg g^{-1} fish (III), i.e. lower than what Oikari et al. (2002) used in their study (50 and 150 μg g^{-1} fish). In both studies, hepatic EROD activity of juvenile rainbow trout increased with increasing dose. Oikari et al. (2002) did another exposure with the sediment where the retene was removed by sulfuric acid oxidation. They found that EROD activity did not decrease to any great extent with this treatment, which demonstrated the presence of some other Ah-receptor mediating compounds in the sediment of Southern Lake Saimaa, possibly PCDDs and PCDFs originating from the pulp and paper industry.

Instead, in a field research in 2004 in Southern Lake Saimaa, increased EROD activity was not observed in experimentally exposed juvenile brown trout which were in contact to the sediment or kept apart from it at a depth of three meters of water (Oikari et al. 2010). These observations indicate that in the field conditions in Southern Lake Saimaa EROD activating compounds were not released from the sediment or from the mills in concentrations which could significantly induce this biomarker. However, in our laboratory experiments, sediment of Southern Lake Saimaa was functioning well as a positive control (III, IV). Thus, the validity of bioassays are an evidence of the absence of AhR-activating compounds in the sediment of the Äänekoski watercourse, and the double dosing of Lake Vatianjärvi and Lake Leppävesi sediment extracts without increased EROD activity in the sediment extract exposure (III) confirmed this.

In a field study, roach and perch were chosen for a bioassay (IV), as the difference in their feeding behaviour may affect the exposure pattern to chemicals. Adult perch is piscivorous and prey in the pelagic zone, whereas roach is more common in the littoral zone and is omnivorous, feeding on zooplankton, detritus, plants and benthic invertebrates (Horppila et al. 2000). Therefore, in contaminated areas it is probable that roach will become more exposed to the hydrophobic compounds present in the uppermost sediment and benthos burrowed whereas perch may merely reflect the exposure in water phase. However, in this study, EROD activity in wild fish caught from Lake Vatianjärvi was at the same level or lower than in the reference sites (IV).

Compared to previous studies made in the middle 1970s and the early 1980s, the recovery of the Äänekoski watercourse is obvious. In 1975, the reduction of mixed-function oxygenase (MFO) capacity was observed in pike (*Esox lucius*) caught from Lake Vatianjärvi (Ahokas et al. 1976). Ahokas et al. (1976) hypothesized a disruption in MFO system to be caused by liver damage due to the heavy pollution of Lake Vatianjärvi, as similar hepatotoxic effects were observed as in exposure to carbon tetrachloride, a known hepatotoxic chemical. Later Oikari & Jimenez (1992) demonstrated this by pre-dosing sunfish with carbon tetrachloride and allyl formate, another hepatotoxic chemical, and exposing fish 24 h later to the β-naphtoflavone or benzo[*a*]pyrene, both known as EROD inducers. They observed that both hepatotoxins caused abolished EROD induction and dose related reduction in the EROD activity. However, the reason for the liver damage in pike in Lake Vatianjärvi remained unclear.

In the early 1980s, the bleached kraft mill effluents in Äänekoski were still highly toxic to fishes, including long-term lethality (Vuorinen & Vuorinen 1985, Lindström-Seppä et al. 1989). In 1980, brown trout (*Salmo trutta*) were exposed to pulp mill effluent for three months before spawning time, and 2 % effluent revealed total mortality within 15 days (Vuorinen & Vuorinen 1985). In 1981, long-term exposures to the 0.1–0.5 % (v/v) effluents were carried out with vendace (*Coregonus albula*), revealing approximate LC_{50} in the highest concentration in 120 days (Lindström-Seppä et al. 1989). Sublethal effects, such

as effects on reproduction and teratogenecity were observed in brown trout exposed to 0.5 % (v/v) effluent concentration, for instance, smaller egg size, egg number and lower fertilization rate compared to the control. In addition, abnormality and disturbances in the blood circulation, both symptoms of blue sac disease, were observed in sac fry from the females exposed to 0.5 % (v/v) effluent (Vuorinen & Vuorinen 1985). This can be considered as an evidence of the presence of EROD-activating compounds in the effluent, as the symptoms of blue sac disease have never been observed without the activation of CYP1A system. Moreover, induction of benzo[*a*]pyrene hydroxylase, considered equivalent to EROD as parameter, was observed in vendace by 0.2 % (v/v) discharge of Äänekoski mill effluent (Lindström-Seppä et al. 1989). In the present study, the concentration of the effluent was estimated to range 0.2–0.8 % (v/v) in 2009, and no evidence of the exposure to the AhR-activating compounds was observed in the Äänekoski watercourse anymore (III, IV).

5.3.2 Caddis larvae — and evidence of ecosystem recovery

The impacts of pulp and paper effluents have been observed not only in fish but also in aquatic invertebrates. Some invertebrate groups tolerate pulp and paper mill contamination very well, for instance Oligochaeta, especially *Tubifex tubifex* (Särkkä 1987, Palomäki et al. 2009), but also many *Chironomus* species and Diptera (*Chaoborus flavicans*) (Palomäki et al. 2009). However, teratogenic effects may exist, such as mentum deformities in *Chironomus* species (Hämäläinen et al. 2000). Sibley et al. (1997) also assessed both the acute and chronic effects of bleached kraft pulp mill effluent on different macroinvertebrate groups which were exposed to the pore water, elutriate or to the sediment collected from Jackfish Bay, Canada, contaminated by chemical wood industry. In the acute 48 h assay, sediment pore water caused high mortality in Amphipod (*Hyalella azteca*), water flea (*Daphnia magna*), Diptera (*Chironomus riparius*), mayfly (*Hexagenia* spp.), and Oligochaeta (*T. tubifex*). Elutriates were slightly less toxic than pore water to Amphipod, and approximately 50 % less toxic to water fleas and mayflies. In the ten-day subacute assay the growth decline was noted in *Chironomus tentans* and *H. azteca*. Both in the acute and chronic tests, the mortality of invertebrates correlated significantly with the concentration of extractable organochlorines in the sediment. Effects on invertebrates have been observed in the Äänekoski watercourse as well. Mäkelä (1984) reported the absence of caddis files in the early 1980s and ten years later serious morphological abnormalities was detected in recolonized *Hydropsyche* larvae (Vuori 1992). In this study (V) some amounts of gill abnormalities were still observed more than 20 years after the mill renewal. However, either this study or experimental setup by Sibley et al. (1997) did not determine if the toxicity was caused by some specific chemical.

Although cause of the morphological abnormalities in caddis larvae remained unclear in this study, some evidence of association between abnormalities and chlorinated compounds has been noted. Petersen & Petersen (1984) observed that one percent pulp mill effluent, using chlorine bleaching,

can cause abnormalities to the food capturing nets of caddis larvae (*Hydropsyche angustipennis*). Also gill and anal papillae abnormalities have been found in caddis larvae collected downstream from PCDD contaminated sites (Simpson 1980, Camargo 1991, Vuori & Parkko 1996, Vuori & Kukkonen 2002). Camargo (1991) has demonstrated that chlorine will cause both anal papillae and tracheal gill damage in caddis flies. He collected caddis larvae (*H. pellucidula*) downstream from a hydroelectric power plant, which was known to release chlorinated discharge to a river. It was found that 60 % of sampled larvae had altered tracheal gills and 30 % had darkened anal papillae. Furthermore, Camargo (1991) exposed *H. pellucidula* to the chlorinated tap water (1.73 mg residual chlorine, 1.12 mg hypochlorous acid) which led to the damaged gills in 100 % of larvae and 75 % damaged anal papillae of larvae.

At the Äänekoski watercourse, the concentrations of chlorinated compounds have been measured as AOX. AOX concentrations in water samples taken in 1994-2007 from the Kapeenkoski rapids, 10 km downstream from the Äänekoski pulp and paper mills, have been the same or higher (45–100 $\mu g\ l^{-1}$) (Palomäki et al. 2009) compared to the Kymijoki River (45–60 $\mu g\ l^{-1}$), where extensive gill darkening in *C. lepida* and *H. pellucidula* (Vuori & Parkko 1996), as well as in mayflies (*Potamanthus luteus*) (Vuori 1999), has been observed. However, it must be considered that the group of compounds measured as AOX is very heterogeneous and can vary between the sites. Therefore, without bioassays in the laboratory with specific chemicals, it cannot be known which chemical or combination of chemicals may have the potential to cause gill damage. As in the Äänekoski watercourse, no changes in AOX concentrations in water has occurred during this study (1999–2008), we presumed that the decreased incidence of gill damage in hydropsychid populations (V) could reflect the degree of exposure to the more specific bioactive organic contaminants released from the pulp and paper mills. It was first suggested that retene could be one potential chemical to cause gill darkening but the concentration of retene in the sediments were low and the dissolution of retene was negligible (I). Therefore, the clear decrease of morphological damage in caddis over time in the Kuusankoski rapids raises a question: What led to the ecotoxicological recovery of the study site? The chemical water quality indicators, such as nutrients, oxygen, pH etc. have not noticeably changed in the Äänekoski watercourse over the years 1999–2008 (HERTTA 2012). While exposure to metals is known to cause gill damage to the caddis larvae (Vuori 1994, Leslie et al. 1999, Vuori & Kukkonen 2002), metal concentrations in the Kuusaankoski rapids are so low (HERTTA 2012) that their potential to cause gill damage is negligible.

If considering filter-feeding caddis larvae or other filter-feeding invertebrates, it must be noted that fine organic particles with sorbed hydrophobic compounds could end up in the food capturing nets, and thus exposure and possible accumulation would be continuous although ambient concentrations might be low. Also water moss, *Fontinalis antipyretica*, which caddis larvae consume, absorbs PAHs and chlorinated organic compounds

from the water (Chovanec et al. 1994). It is thus probable that caddis larvae will become exposed to the hydrophobic contaminants *via* food. Hence, as gill abnormalities in caddis larvae of the Kuusaankoski rapids decreased to the same level as at the reference sites in 2008 (V), it is probable that the load of harmful organic compounds had decreased to such levels which did not cause anymore morphological abnormalities to caddis flies in the Äänekoski watercourse.

6 CONCLUSIONS

In the Äänekoski watercourse, the chemical contamination by the pulp and paper industry was demonstrated in the sediment as far downstream as 30 km from the source. Dissolution experiments revealed that wood extractives and retene can be desorbed from the sediment to water, predicting their bioavailability. Dissolution was positively related to the concentrations in the sediment as well as inversely on the content of organic carbon. Interpolated spatial distribution was a novel approach to estimate the amounts of the most abundant wood extractives in the sediment of lake-wide area. This kind of estimation can be used to assess the risks of contaminated sediments in case of severe disturbances, for instance extreme floods, which may lead to resuspension of the surface sediment. As a result, retene and wood sterols were not assessed to cause harm. Instead, concentrations of the resin acids and betulinol were unexpectedly high both in the sediment and in elutriates, and the toxic effects on aquatic biota may occur in the event of severe sediment erosion. Therefore, toxicity studies especially with betulinol are recommended with benthic animals and fish.

However, the bioindicators used in this study indicated the recovery of the Äänekoski watercourse. Dioxin-like exposure, acting *via* hepatic Ah-receptor, was not observed in fish, as increased EROD activity was not detected in any experiment: sediment extract bioassay, sediment with water bioassay or in wild perch and roach populations caught from downstream lake. These observations were consistent with the results of the sediment analyses, in which AhR-activating compounds (retene, PCDDs, PCDFs and some PCBs) were below the detection limit or found only at background concentrations. Also caddis flies indicated the recovery. In the beginning of the 21st century, the effects of pulp and paper industry discharge were still obvious in caddis larvae, as the proportion of larvae with damaged gills were higher near the mills compared to the sites in further downstream. However, the evidence of the recovery was observed in the Kuusaankoski rapids, 15 km downstream the mills, where the gill damage of *H. siltalai*, the most sensitive *Hydropsyche* species

in Finland, decreased to the reference level during the period of field monitoring in 1999–2008.

The results of this study support the conclusions of the changed aquatic and ecological conditions in the Äänekoski watercourse, although remarkably high amounts of certain natural wood extractives are still present in the surface sediment. The load of harmful organic compounds from the mills, as well as dissolution from the sediments, appeared to have decreased to such levels that do not cause harm to aquatic biota today. However, this study showed that even though the discharge of the harmful compounds have significantly decreased and traditional water quality parameters were rapidly reversed by enhanced technology, the overall recovery of the watercourse took considerably longer, more than 20 years.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Metsäteollisuuden voimakkaasta kuormituksesta palautuvan vesistön ekotoksikologinen tila

Boreaalisella vyöhykkeellä Venäjää lukuun ottamatta kemiallinen metsäteollisuus on ollut voimakkain paikallisia paineita vesistöille aiheuttava teollisuudenala. Vaikka vesistökuormitus on vähentynyt ja muuttanut muotoaan etenkin viimeisten kahdenkymmenen vuoden aikana, kuormitus on edelleen jatkuvaa. Tämän tutkimuksen kohteena oli Keski-Suomessa sijaitsevan Äänekosken kaupungin alapuolinen vesistö, joka on ollut metsäteollisuuden kuormituksen alaisena yli sadan vuoden ajan. Vesistön tila oli selvästi muuttunut jo 1950-luvulla, ja orgaaninen kuormitus jatkui voimakkaana 1980-luvun puoliväliin saakka, kunnes siirryttiin uuteen keitto- ja valkaisutekniikkaan sekä otettiin käyttöön jätevesien aktiivilietepuhdistus. Tehdasuudistusten myötä ympäristökuormitus alkoi niin bioaktiivisten puuperäisten haitta-aineiden kuin muunkin kuormituksen osalta vähetä. Saastuneet sedimentit säilyvät kuitenkin muistona voimakkaasta kuormituksesta ja työn hypoteesina oli, että jokimaisessa vesistössä sedimentin sisältävät haitta-aineet voivat vapautua takaisin veteen ja aiheuttaa eliöihin bioakkumuloiduttuaan viivästyneitäkin haittavaikutuksia. Ilmastonmuutoksen myötä tulvien on ennustettu lisääntyvän leveysasteillamme 10 – 40 %, mikä voi osaltaan voimistaa sedimentin eroosiohuippuja. Etenkin jokimaisessa vesistössä vapautuneet haitta-aineet voivat kulkeutua virtauksen mukana kauaskin päästölähteestä.

Tutkimuksessa arvioitiin Äänekosken vesistön sedimentistä mitattujen haitta-ainesisältöjen aiheuttamia riskejä. Sedimentistä analysoitiin syvyysprofiileittain puun luonnollisia uuteaineita: hartsihappoja, betulinolia ja puusteroleja, sekä hartsihappojen muuntumistuotteena syntyvää PAH-yhdistettä, reteeniä. Lisäksi laboratoriokokeissa tutkittiin kyseisten yhdisteiden vapautumista sedimentistä veteen. Kuhnamosta ja Vatianjärvestä, joihin metsäteollisuuden vaikutukset kohdistuvat voimakkaimmin, arvioitiin myös yleisimpien yksittäisten uuteaineiden (dehydroabietiinihapon, isopimaarihapon, betulinolin ja β-sitosterolin) kokonaismassat järvipinta-alaa kohden. Myös polykloorattuja dibentso-*p*-dioksiineja ja dibentsofuraaneja sekä polykloorattuja bifenyylejä (PCB) analysoitiin sedimentistä, koska metsäteollisuus on ollut merkittävä päästölähde näille erittäin pysyville ja haitallisille orgaanisille yhdisteille.

Ekotoksikologisina vasteina mitattiin populaatiotason vasteita kaloista ja selkärangattomista. Metsäteollisuuden alapuolelta pyydystetyistä särjistä ja ahvenista mitattiin maksan EROD-aktiivisuutta, mikä on herkkä ja yleisesti käytetty indikaattori altistuttaessa dioksiininkaltaisille yhdisteille, joihin lukeutuvat klooridioksiinien ja -furaanien lisäksi myös reteeni ja tietyt PCB-yhdisteet. Laboratoriokokeissa nuoria kirjolohia altistettiin sedimenttien haitta-ainesisällölle injektoimalla eri syvyyskerroksista valmistettuja sedimenttiuutteita suoraan kaloihin, jolloin saavutettiin noin vuoden 1995 kuormitustaso syvimmässä sedimenttikerroksessa. Toisessa koeasetelmassa matkittiin luonnollista altistumis-

tapaa asettamalla sedimenttiä akvaarion pohjalle, jolloin hienojakoiset sedimenttipartikkelit sekoittuivat veteen kalojen pöyhiessä pohjaa, mikä puolestaan lisäsi mahdollista altistumista haitta-aineille. Lisäksi seurattiin lähes kymmenen vuoden ajan vesiperhosten lajikoostumusta sekä kidusvaurioita Äänekosken alapuolisessa vesistössä.

Tulokset osoittivat, että jokimaisessa Äänekosken reitissä sedimentin uuteaine- ja reteenipitoisuudet olivat pienempiä verrattuna niin ikään metsäteollisuuden kuormittamaan Etelä-Saimaseen, joissa läpivirtaus on selkeästi Äänekosken reittiä hitaampaa. Tutkimuksessa selvisi myös, että sedimentin eroosio voi vapauttaa haitallisia puun uuteaineita ja hartsihappojen muuntumistuotteena syntyvää reteeniä takaisin vesistöön. Äänekosken alapuolisessa vesistössä hartsihappoja voi vapautua haitallisia määriä veteen, mutta reteenin ei todettu aiheuttavan riskiä. Reteenin pieni pitoisuus Äänekosken reitillä oli mielenkiinen havainto, sillä hapettomat olosuhteet tutkimusvesistössä 1980-luvulla ja Kuhnamossa ajoittain jopa 1990-luvun alkupuolella ovat olleet otollisia reteenin muodostumiselle sedimentissä, eivätkä reteenipitoisuudet kohonneet suuriksi edes syvemmissä sedimenttikerroksissa. Koivusta peräisin olevaa betulinolia sen sijaan oli runsaasti myös pintasedimentissä ja sitä saattaa vapautua siinä määrin veteen, että haittavaikutukset kaloissa ovat mahdollisia. Myös puusteroleja oli sedimentissä runsaasti, mutta haitallisia pitoisuuksia ei oletettavasti vapaudu vesistöön. Polykloorattuja dibentso-*p*-dioksiineja ja dibentsofuraaneja ei Äänekosken sedimentistä löytynyt sellaiselta syvyydeltä, että ne voisivat olla biosaatavia eliöille. PCB-yhdisteitä havaittiin vain muutamia ja nekin olivat taustapitoisuuksien tasolla.

Ekotoksikologiset vasteet osoittivat Äänekosken vesistön palautuneen siinä määrin, että kalat eivät enää altistu dioksiinin kaltaisille aineille, sillä kohonnutta EROD-aktiivisuutta ei havaittu laboratoriokokeissa eikä luonnosta pyydetyissä kaloissa. Vesiperhosten kidusvauriot palautuivat referenssitasolle vasta vuonna 2008, yli 20 vuotta tehdasuudistusten jälkeen. Vesiperhosissa havaitut vauriot ovat todennäköisesti aiheutuneet metsäteollisuuden kuormituksesta, sillä vauriot vähenivät lineaarisesti sekä ajan että etäisyyden suhteen, mutta tämän tutkimuksen perusteella tarkkaa syytä vaurioille ei kuitenkaan voitu esittää.

Yhteenvetona voidaan sanoa, että Äänekosken vesistön sedimentit voivat tiettyjen yhdisteiden osalta olla edelleen riski vesieliöstölle, mikäli sedimentin pintakerros pääsee sekoittumaan veteen esimerkiksi suurtulvien aiheuttamien voimakkaiden virtausten myötä. Vesistön tila on kuitenkin palautunut reilun 20 vuoden aikana sellaiseksi, että se ei tämän tutkimuksen perusteella aiheuta enää haittaa kaloille eikä virtavesien herkille selkärangattomille.

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ORIGINAL PAPERS

I

DISSOLUTION AND SPATIAL DISTRIBUTION OF RESIN ACIDS AND RETENE IN SEDIMENTS CONTAMINATED BY PULP AND PAPER INDUSTRY

by

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SEDIMENTS, SEC 1 • SEDIMENT QUALITY AND IMPACT ASSESSMENT • RESEARCH ARTICLE

Dissolution and spatial distribution of resin acids and retene in sediments contaminated by pulp and paper industry

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Abstract
Purpose Wood extractives in sediments originating from the wood industry may interfere with benthic biota in aquatic environments. The research area was the Äänekoski watercourse in Central Finland, which has been affected by the chemical wood industry for over a century. The goal was to determine the dissolution potency of resin acids (RAs) and their derivative, retene, in the sediment, and their current vertical and spatial stratification to assess the load due to potential erosion.
Materials and methods Sediments were collected from two upstream reference sites and three lake-like basins, located as far as 33 km downstream from the mills. The dissolution potency was studied by two different agitation times and temperatures from sediment-water (1+4 v/v) elutriates. The vertical distribution of extractives was determined from the uppermost 20 cm of sediment. Using spatial interpolation, the distribution of extractives was estimated from two upper sediment layers (0–2 and 2–5 cm) downstream from the source. According to the interpolation, the total amount of dehydroabietic (DHAA) and isopimaric acids (IPA) were calculated as kg/ha in the whole sediment area.
Results and discussion The total concentration of RAs in the surface sediment reached up to 168 μg/g dw, and they were found to desorb to water up to 77 μg/l. The concentrations of retene were low both in the sediment (<51 μg/g dw) and elutriate (<0.53 μg/l). Spatial interpolation showed that the highest calculated amounts of the most abundant RAs were in Kuhnamo basin, in the sediment layer 2–5 cm; the estimated amount of DHAA and IPA were approximately 440 and 85 kg/ha, respectively.
Conclusions Disturbances may change the exposure situation, causing desorption of sediment-associated compounds in levels that may be harmful to aquatic animals. The amount of desorption varies depending on the concentration of contaminants in sediment, the nature of disturbance, and the sediment organic carbon content. Low retene concentrations can be explained by oxic conditions and low abundance of RAs in the sediments.

Keywords Dissolution · Pulp and paper mill effluents · Resin acid (RA) · Retene · Wood extractives

1 Introduction

Climate change is becoming a greater concern when considering the increasing frequency of extreme weather conditions such as drought and floods. Indeed, the risk of coastal floods is predicted to increase due the global rise in sea level. Floods may have detrimental effects on health and even cause numerous deaths, for instance, during the years 1992 to 2001 flooding was the most frequent natural disaster globally affecting thousands of people (McMichael et al. 2006). There is a probability that climate change will cause flooding to be more harmful than has been previously observed (IPCC 2007). In Finland, the most severe flood 'valapato' occurred in the late 19th century, during the era of Russian occupation (Anonymous 2003). The flood water covered over 1,400 km^2 and caused considerable financial loss. This kind of 'very severe' flood usually occurs averagely once every 250 years, and 'severe' flood once a century. Springtime, as the snow melts, river floods are

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more common and may raise the water level above the normal (Ollila et al. 2000). When the water level in rivers and lakes rises, the water flow and sediment erosion increases. This causes silt formation and increases desorption of sediment-bound compounds, thus making them bioavailable to aquatic animals, sometimes with severe consequences (Eggleton and Thomas 2004). Therefore, floods may cause long-term damage to aquatic environments. However, no watercourse-wide assessment has been carried out thus far.

In addition to wood derivatives, wastewaters discharged from the pulp and paper industry contain numerous bioactive and toxic compounds, modified in the pulping process (Sjöström 1993), which subsequently end up in sediments (Judd et al. 1995, 1996; Lahdelma and Oikari 2005; Leppänen and Oikari 1999a; van den Heuvel et al. 2006). Resin acids (RAs) are among the most abundant wood extractives. They can be divided according to their structural differences into abietic type RAs: dehydroabietic (DHAA; Fig. 1), levopimaric, palustric, abietic, and neoabietic acid, and pimaric type RAs: pimaric, sandarakopimaric, and isopimaric acid (IPA; see Fig. 1) (Sjöström 1993). It has been observed that abietic type RAs are somewhat less toxic and more water soluble than the pimaric type RAs (Peng and Roberts 2000). RAs are sublethally toxic to fish and daphnia at concentrations of over 20 μg/l (Oikari et al. 1983; Peng and Roberts 2000), and cellular responses can be seen already at a concentration level of 0.6 μg/l (Meriläinen et al. 2007). In sediment downstream from chemical wood industry in Finland, the total RA concentration may be as high as 3300 μg/g dw (Meriläinen and Oikari 2008b) when natural background levels are usually under 5 μg/g dw (Leppänen and Oikari 1999a, 2001). In anaerobic sediments, retene (7-isopropyl-1-methylphenantrene; see Fig. 1) can be biotransformed by microbes from RAs, especially from DHAA (Tavendale et al. 1997). Retene may be detected in downstream sediment in levels of up to 3300 μg/g dw (Leppänen and Oikari 2001), whereas, typical background concentrations are under 0.1 μg/g dw (Lahdelma and Oikari 2005; Leppänen and Oikari 1999a). Retene seems to be highly toxic to fish in their early developmental stages; for instance, causing blue sac disease (BSD) in rainbow trout (*Oncorhynchus mykiss*) embryos at a concentration level of 10 μg/l (Brinkworth et al. 2003). RAs and retene remobilization through dissolution was recently identified as an ecotoxicological risk to benthic and epibenthic biota (Meriläinen et al. 2006).

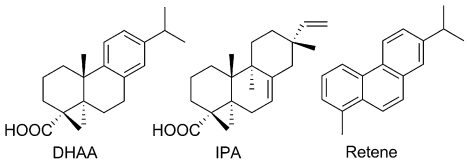

Fig. 1 Molecular structures of the most abundant abietic and pimaric type RAs, dehydroabietic acid (DHAA) and isopimaric acid (IPA), and RAs' derivative, retene (7-isopropyl-1-methylphenantrene)

The sediments studied in the present research are from the Äänekoski watercourse in Central Finland (Fig. 2), where mechanical and chemical pulp, as well as paper and board production, has loaded the watercourse since 1899 (Anonymous 2006; Auer 1971). The wastewaters from the mills were discharged into the watercourse without purification until the 1970s, when the first mechanical wastewater treatment plants (WWTPs) were built. However, the organic material continued to be discharged into the waterway to a significant degree until 1985, when the new pulp mill and active sludge process were introduced. Currently, the Äänekoski mill's capacity to produce elemental chlorine-free (ECF) pulp is around 500,000 tonnes per year, with most of it going to the paper and board mills (both produced about 160,000 t/a) in the same area.

In this study, the main goal was to determine the dissolution potency of RAs and their derivative, retene, in silted sediments in the worst-case situation caused by severe sediment erosion. The worst-case scenario of unpredicted flooding was projected according to the spatial distribution of the most abundant RAs (DHAA and IPA, kg/area unit of sediment) in the uppermost sediment layers (0–2 cm and 2–5 cm). A further goal of this work was to assess the recovery of the watercourse since its highest organic and toxic loading 20 to 30 years ago. This is the first time that the spatial distribution of individual wood extractives has been estimated on the basis of a whole lake area.

2 Materials and methods

2.1 Sampling of sediments

The watercourse downstream from the Äänekoski mills is composed of several narrow lakes with an average flow of approximately 80 m^3/s. The watercourse collects water from two directions; more humic water comes from the Saarijärvi source, and oligotrophic water from the Viitasaari source. Therefore, two sediment reference sites were selected; the first located 8.5 km upstream from Äänekoski, in Lake Naarajärvi (Ref. –8.5 km), and the second 5 km upstream, in Lake Keitele (Ref. –5 km) (see Fig. 2). Wastewater from the two WWTPs in Äänekoski mills discharge to Lake Kuhnamo, with an area of about 3.5 km^2. There were three sampling sites: 1, 3.5, and 4.5 km downstream from the mills, with water depths of 19, 20, and 16 m respectively. From Lake Kuhnamo, water flows to Lake Vatianjärvi, which has an area of 5.5 km^2. There,

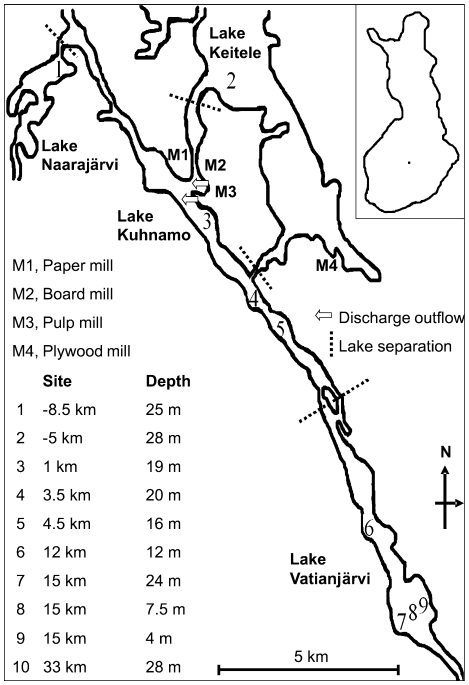

Fig. 2 The research area. Scale is about 1:175 000

four sampling sites were chosen; one 12 km downstream from the mills (water depth 12 m) and three approximately 15 km downstream, with water depths 24, 7, and 4.5 m respectively. The farthest sampling site, the Northern Lake Leppävesi, with a water depth of 28 m, is located 33 km downstream from the source. In its entirety, this basin is larger than the other two, having an area of 36 km^2.

The sediment samples were taken in late April and early May of 2007. Sediments intended for use in dissolution experiments were collected with an Ekman device (area 225 cm^2, sediment depth 0–10 cm). In the reference sites, ten and eleven samples were taken and combined from Ref. –8.5 km and Ref. –5 km, respectively. Downstream from the mills, six samples from 3.5 and 33 km, and seventeen from the 15 km site were taken and combined similarly. The samples were stored at 4°C in darkness.

The sediment samples used in the vertical distribution analysis of RAs and retene were taken using a Kajak-corer, with a diameter of 50 mm and column length of 30–50 cm. Three subsamples were combined, apart from two from Ref. –5 km and one core from the 4.5 km site, which was due to the hard bottom in the latter site. After the sampling stage, the sediments were left to settle in the dark at 4°C for 24 h before they were sectioned for the uppermost 20 cm (0–2, 2–5, 5–10, 10–15, and 15–20 cm). The combined subsamples were frozen at –18°C before freeze-drying, and were thereafter stored in an exicator until analysis.

2.2 Sediment analyses

Loss on ignition (LOI, 550°C/2 h) was determined as two replicates from the freeze-dried samples taken with the Kajak-corer (SFS 1990). The concentration of total organic carbon (TOC) was determined from the combined Ekman-samples and Kajak-samples from the sediment layer 0–2 cm from all of the downstream sites, and from the layer 2–5 cm at one site in each lake basin. In addition, TOC was analysed from all five layers of the 15 km site, as well as from the deepest layer (15–20 cm) of the 33 km site. For TOC, pre-treated (freeze-dried, homogenized, acidified by HCl and dried at 80°C) sediments were analysed in an accredited laboratory using an NC-analysator (Thermo Finnigan, Delta Plus Advantage Flash 1112 Series EA, Bremen, Germany).

The concentrations of RAs and retene were analysed in accordance with the methods used by Lahdelma and Oikari (2005), Leppänen and Oikari (1999a), and Tavendale et al. (1995), with some modifications. In short, the freeze-dried sediments were Soxhlet-extracted (Büchi B–811, Switzerland) by 180 ml of hexane:2-propanol solution (2:1, v/v; Rathburn Chemicals HPLC-Grade, UK). The internal standards were heptadecanoic acid (~99%, Fluka, Switzerland) for RAs, and anthracene-*d*10 (98% atom D, Isotec™, USA) for retene. Compound identification was performed with Polish wood rosin (Hercules, Wilmington DE, USA) and retene standard (98%, ICN Biomedicals, Cosa Mesa, USA). After seventy cycles, the extracts were rinsed with hexane and evaporated to a small volume in a vacuum. The concentrated samples were diluted with 50 ml of hexane and extracted three times with 40 ml of K_2CO_3 (>99%, Merck, Germany) in a separation funnel. The hexane fraction containing retene was vacuum-evaporated to a small volume, and further evaporated under a nitrogen gas stream to dryness (Leppänen and Oikari 1999a). The sample was diluted with 500 µl of hexane:chloroform (4:1, v/v) and absorbed in the 5% deactivated and conditioned silica gel (0.062–0.200 mm, Baker Analyzed®, The Netherlands) column. The retene was then eluted with 12 ml of hexane and evaporated by nitrogen until dry. The fraction was diluted with 300 µl of hexane and analysed with a gas chromatograph/mass spectrometer (GC-MS, Hewlett Packard 6890 MS, Germany; Hewlett Packard 5973 GC, USA, 30 m * 0.25 mm ID HP-5 polysiloxane polymer column, phase thickness 0.25 µm, volume of injection 0.1 µl). Helium (Aga, USA) was used as a carrier gas. The following GC-MS temperature programme for retene was used: start at 120°C (held for 1 min), raising 5°C/min up to 250°C (5 min), and raising 5°C/min up to 290°C

(5 min) (Lahdelma and Oikari 2005). The retene concentrations were corrected with a recovery factor.

In order to separate the RAs, the fractions from K_2CO_3 extractions were combined and the pH-value adjusted to 9 with 5 M H_2SO_4. The solution (120 ml) was then extracted in a horizontal shaker for 16 h with 50 ml of dichloromethane. After extraction, the solvent was vacuum-evaporated to a volume of approximately 5 ml and further evaporated with nitrogen stream to dryness. The RAs were diluted with 125 µl pyridine (purity >99%, J.T. Baker, The Netherlands) and silylated with 175 µl *N,O-bis* (trimethylsilyl)-trifluoroasetamid (BSTFA) + 1% trimethylchlorosilane (TMCS) (GC-quality, Fluka, Switzerland) at 70°C for 30 min. The extracts were analysed by GC-MS according to the temperature programme; starting at 120°C and raising 8°C/min up to 190°C, raising 2°C/min up to 240, raising 10°C/min up to 280°C (8 min), and raising 10°C/min up to 290°C (20 min) (Leppänen and Oikari 1999a).

2.3 Dissolution of sediment-bound wood extractives

Artificial freshwater (AFW) was prepared in distilled water and contained 11.8 g/l calcium chloride, 4.9 g/l magnesium sulphate, 2.6 g/l sodium bicarbonate, and 0.2 g/l potassium chloride; its pH was adjusted to 7±0.1. In the first dissolution experiment, wet sediment was mixed with AFW (1+4, v/v), and shaken horizontally (180 rpm) for one hour at 20°C. In the second experiment, agitation was conducted in the dark at 10°C for 46 h. The sediment water mixtures were centrifuged for 15 min at 2000 rpm.

The RAs were analysed from elutriates using the same method as that employed by Meriläinen and Oikari (2008a), within four days, while stored in the dark, at 4°C. The compounds in acidified (H_2SO_4; pH~2.5) elutriates (200 ml) were extracted three times with 25 ml of hexane: acetone (3:1, v/v) by using a horizontal shaker for 5 min (180 rpm). Solvent fractions were combined, centrifuged for 5 min at 2,000 rpm, and rinsed with hexane. The fraction was concentrated in a vacuum and evaporated to dryness with a stream of nitrogen. The samples were diluted with 50 µl of pyridine, silylated with 150 µl BSTFA + 1% TMCS, and analysed with the same method as the sediment samples. The retene was analysed after five days of storage at 4°C in dark: it was extracted once from 200 ml of elutriate with 50 ml of hexane by using a horizontal shaker for 4 hours (150 rpm). The extract was diluted with 250 µl of hexane, concentrated, and analysed as described above.

2.4 Statistics and spatial interpolation

The relationship between the sediment concentrations of wood-derived chemicals, LOI and TOC in sediments were determined by Spearman's correlation test, using SPSS software (Statistical Product Service Solutions, Chicago, IL USA). Spearman's correlation test was chosen because of some groups of measurements were not normally distributed.

The extractive concentrations of two sediment layers (0–2 and 2–5 cm) from ten sampling sites were used for spatial interpolation and, further, to assess the distribution of the total RAs, DHAA and IPA in the Kuhnamo and Vatianjärvi basins. The programme used was ArcGIS (GIS, Geographic Information System) and the Inverse Distance Weighting (IDW; Shepard 1968) interpolation method was applied due to the small number of sampling sites. The weighting exponent used in the IDW interpolation was two. In compliance with the interpolation, we calculated the amounts of DHAA and IPA (kg/ha) that would be released from these sediments in a worst-case scenario of unpredictable erosion in association with flooding.

3 Results

3.1 Sediment characteristics

The sediment in site Ref. –8.5 km was fine-grained and overall brown: in comparison, the Ref. –5 km sediment grain size was notably larger and the colour was mostly grey for the first uppermost 15 cm. In the surface sediments (0–10 cm), the LOI and TOC contents in Ref. –8.5 km were twice as much as in Ref. –5 km (Table 1). Sediment sampled 3.5 km downstream from the mills was mostly dark brown, lightly packed, and smelled strongly of hydrogen sulphide. Furthermore, the LOI and TOC contents were highest (30% and 17.6% per dw) in this site. Further downstream, the sediment colour was lighter brown and it was more tightly packed than the sediments taken from closer to the mills. The LOI and TOC decreased by approximately 70% (LOI ~8.5% dw and TOC ~4.2% dw) at the 15 km and 33 km sites, both of which averaged 3% per dw.

3.2 Spatial distribution of wood extractives in the sediments

All of the sediment concentrations were determined as dry weights. DHAA was the most abundant RA in all of the sediments, accounting for 63.4% (±SD 8.1%) of the total RA concentration (Fig. 3). The high abundance of DHAA resulted in the proportions of abietic type RAs being 77.1% (±SD 4.8%). IPA was the most abundant of the pimaric type RAs, (11±2.3% of total RAs). Vertical analyses revealed that the total concentration of RAs was highest (246 µg/g dw) in the deepest layer (i.e. 15–20 cm) at the closest site (1 km) downstream from the mills. The highest concentration in the 12 and 15 km sites were found at a sediment depth of 10–15 cm, indicating a period before the process

Table 1 Dry weight (dw), loss on ignition (LOI) and total organic carbon (TOC) in the sediments, sampled by Kajak corer and Ekman device 0–20 cm and 0–10 cm, respectively. Samples marked by line were not analysed. Sampling sites are demonstrated in Fig. 2

	Sampler type	Sediment depth (cm)	Ref. –8.5km	Ref. –5km	1km	3.5km	12km	15km	33km
dw (%)	Kajak	0–2	17.4	9.6	7.9	14.1	27.6	20.9	54.9
		2–5	21.5	10.8	7.8	15.9	27.6	23.9	54.7
		5–10	23.8	12.8	13.9	16.6	31.3	26.1	56.5
		10–15	24.3	13.7	9.5	17.0	30.5	25.8	62.9
		15–20	25.4	11.6	9.6	17.2	25.2	23.3	84.5
	Ekman	0–10	21.7	11.5	–	15.8	–	24.3	55.7
LOI (%)	Kajak	0–2	8.0	16.7	30.0	17.0	8.1	8.6	2.6
		2–5	9.0	15.8	29.5	16.8	10.7	8.6	3.1
		5–10	8.8	12.3	22.7	17.0	9.3	8.6	3.6
		10–15	8.9	10.7	26.8	16.6	7.8	8.8	3.8
		15–20	8.4	14.6	28.4	17.2	9.6	8.1	3.2
	Ekman	0–10	8.7	14.3	–	16.9	–	8.6	3.2
TOC (%)	Kajak	0–2	-	-	17.6	9.6	4.2	4.4	2.6
		2–5	-	-	-	8.2	-	4.3	2.5
		5–10	-	-	-	-	-	4.4	-
		10–15	-	-	-	-	-	4.4	-
		15–20	-	-	-	-	-	3.9	3.9
	Ekman	0–10	3.7	4.7	-	9.0	-	4.2	1.2

modification in the mills that occurred in the mid-1980s, when discharge was still untreated.

Retene was less abundant than RAs, and its highest concentration (51 μg/g dw) was detected in the 1 km site, at a depth of 10–15 cm (Table 2). Retene concentrations decreased to the range of 5–18 μg/g dw at a distance of 3.5 km, and even more with increasing distance from the mill. The Spearman's correlation indicated a dependence

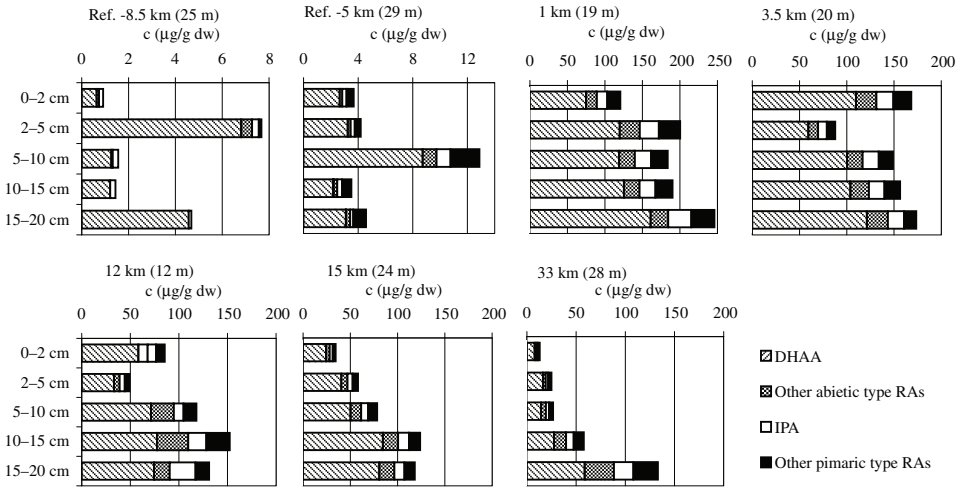

Fig. 3 Concentrations (μg/g dw) of resin acids (RAs) and retene in the sediments. Dehydroabietic acid (DHAA) and isopimaric acid (IPA) are presented separately from other abietic and pimaric type RAs, respectively. Sampling sites are separated according to their distance from the mills, and the water depths of the sites are shown in brackets (see Fig. 2 for site locations)

Table 2 Concentrations (μg/g dw) of retene in the sediments. Sampling sites are separated according to their distance from the source of discharge. Sampling sites are demonstrated in Fig. 2

Depth (cm)	Concentration (μg/g dw)					
	Ref. –8.5km	Ref. –5km	1km	3.5km	15km	33km
0–2	<0.10	0	42.4	10.7	1.9	0.26
2–5	<0.10	0	50.4	5.4	2.2	0.14
5–10	0	0	50.0	15.0	1.8	0.22
10–15	0.27	<0.10	50.8	16.9	2.4	0.32
15–20	0	<0.10	41.9	18.4	2.3	4.3

between concentrations of RAs and retene, revealing a significant positive dependence in the sediment of Lake Kuhnamo ($r=0.846$, $p<0.001$, $n=13$). Statistically significant positive dependence was also detected between TOC and LOI ($r=0.913$, $p<0.001$, $n=20$), TOC and RAs ($r=0.565$, $p=0.009$, $n=20$), and TOC and retene ($r=0.622$, $p=0.003$, $n=20$).

Spatial distributions of DHAA and IPA were assessed by interpolating the data from lakes Kuhnamo and Vatianjärvi, from sediment layers 0–2 and 2–5 cm (Fig. 4). As the DHAA and IPA were the most abundant RAs, spatial distribution showed similar results. The estimated total amounts of DHAA bound to the sediment were approximately twice as high in the 2–5 cm layer (437 kg/ha in Kuhnamo basin and 335 kg/ha in Vatianjärvi basin) as in the upper 0–2 cm layer (279 kg/ha in Kuhnamo basin and 208 kg/ha in Vatianjärvi basin). The estimations of IPAs were similar: in Lake Kuhnamo 48 kg/ha and 85 kg/ha and in Lake Vatianjärvi 32 kg/ha and 50 kg/ha in the sediment layers of 0–2 cm and 2–5 cm, respectively.

3.3 Dissolution of wood extractives

Some differences in the RAs and retene dissolution potency between the two agitation times and temperatures were detected, and most of the concentrations increased with a longer agitation time (Table 3). The highest RA concentration (76.5 μg/l) was found in the Lake Kuhnamo elutriate, via the longer agitation period (46 h), which was approximately twice as much as that revealed with the shorter agitation time (1 h). The desorption of DHAAs was the highest of all RAs; similar to its concentration in sediments. Desorption of retene to elutriates was practically non-existent (<0.5 μg/l), mirroring its low concentrations in the sediments.

4 Discussion

The main goal of this study was to determine the dissolution potency of sediment-bound xenobiotics when severe mechanical force causes erosion, especially in the case of unpredictable flooding. It is well-known that climate change cause more severe drought in arid areas and, conversely, increase precipitation and runoff in areas of high latitude. Indeed, precipitation levels have already significantly increased from the beginning of the 20th century to 2005 in Northern Europe, North and South America and in Central Asia. Furthermore, annual runoff in higher latitudes is predicted to increase by 10–40% (10–20% in Central Finland) by the end of this century; resulting in increasing flood risk (IPCC 2007). This increase may affect the flow rate and enhance sediment erosion, especially in river-like watercourses such as examined in this study. Usually, natural events such as yearly floods and daily tidal currents only cause minor sediment movement. In contrast, anthropogenic events such as dredging may cause more severe erosion (Eggleton and Thomas 2004), which, with more vigorous mixing, makes increasing resuspension of sediment-bound contaminants possible (Meriläinen et al. 2006).

In the present study, sediment RA concentrations (246 μg/g dw or less), 1 km from the point of discharge, were not as high as recorded in earlier studies with the same distance (Lahdelma and Oikari 2005; Leppänen and Oikari 1999a). On the other hand, the impact of the wood industry was detectable further downstream (over 30 km) in the watercourse compared with similar riverine habitats (Judd et al. 1996; van den Heuvel et al. 2006). As expected, the concentrations of RAs in the dissolution experiments were site-dependent, and corresponded to the conditions in the sediment yielding concentrations (up to 77 μg/l) potentially toxic to aquatic animals (Meriläinen et al. 2007; Oikari et al. 1983; Peng and Roberts 2000). DHAA is more water-soluble (5.11 mg/l) than IPA (1.70 mg/l) (Peng and Roberts 2000). In elutriates, the highest concentration of IPA (4.4 μg/l) was detected at a distance of 3.5 km, and DHAA was found at concentration up to 38 μg/l, which can be harmful for fish (Meriläinen et al. 2007; Oikari et al. 1983). The exposure scenario near the sediment surface can be cyclic, as Chen et al. (1999) showed that adsorption and desorption of xenobiotics from sediments is in balance after the maximum adsorption capacity is reached. Adsorption can be either reversible or irreversible. The reversible

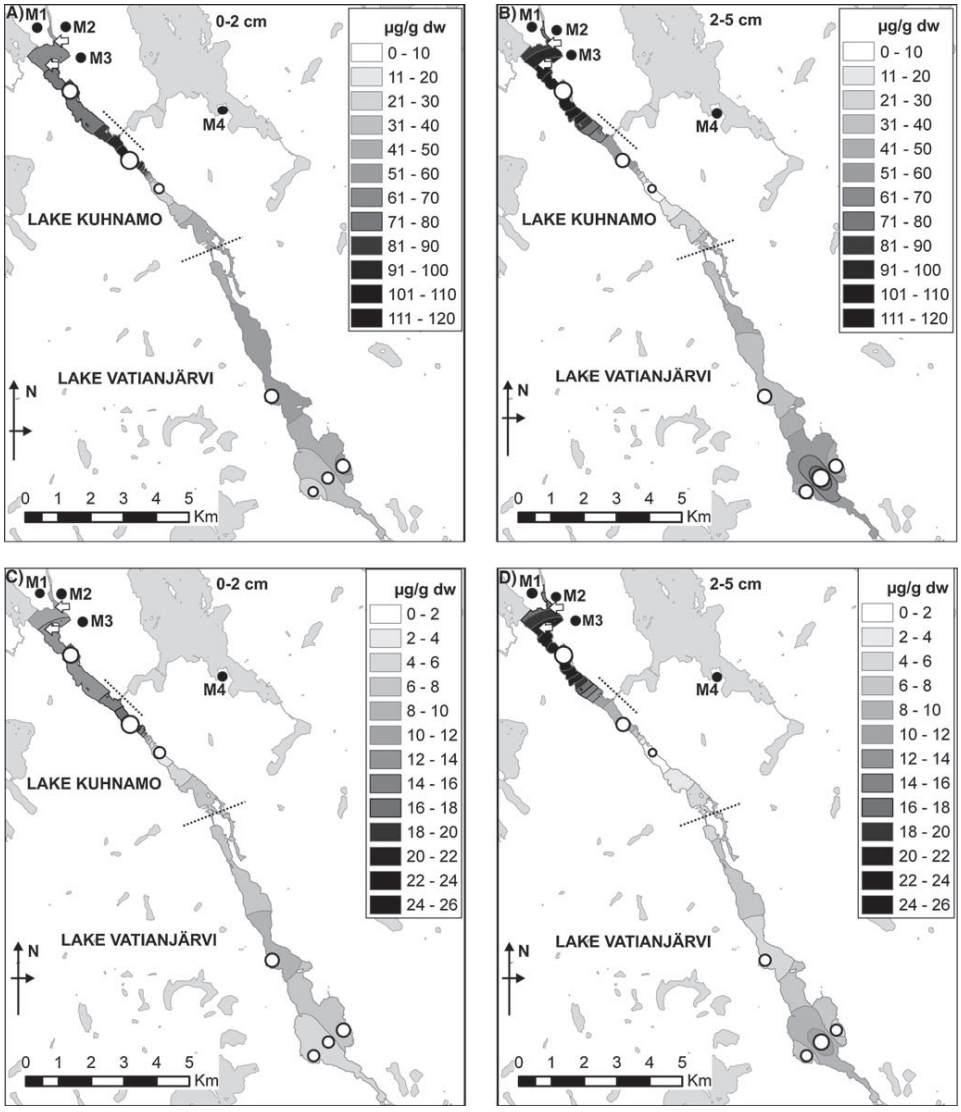

Fig. 4 Interpolated spatial distributions of DHAA, **a**) sediment layer 0–2 cm and **b**) 2–5 cm, and IPA, **c**) 0–2 cm and **d**) 2–5 cm, in the sediments of lakes Kuhnamo and Vatianjärvi. Production facilities have been marked in the picture as follows: M1 = paper mill, M2 = board mill, M3 = pulp mill, M4 = plywood mill. Bolded arrows show outflows from the wastewater treatment plants. The lakes are separated by a dash line, and the white circles (size depends on concentration) represent the sampling sites (Base map © National Land Survey of Finland 892/MML/09)

Table 3 Total average concentrations of resin acids (RAs), dehydroabietic acid (DHAA), isopimaric acid (IPA) and retene in the sediments (sed. µg/g dw; 0–10 cm) and elutriates (µg/l) with two mixing times and temperatures in darkness: 1 hour at 20°C and 46 hours at 10°C. Sampling sites are demonstrated in Fig. 2

Site	Total RAs			DHAA			IPA			Retene		
	Sed. µg/g dw	1h µg/l	46h µg/l	Sed. µg/g dw	1h µg/l	46h µg/l	Sed. µg/g dw	1h µg/l	46h µg/l	Sed. µg/g dw	1h µg/l	46h µg/l
–8.5 km	2.9	2.6	2.7	2.5	2.4	2.6	0.23	0.17	0.14	<0.10	0	0
–5 km	8.4	3.8	3.0	5.8	3.8	3.0	0.67	0	0	0	0	0
3.5 km	138.5	32.7	76.5	92.4	23.5	37.9	15.3	2.5	4.4	11.5	0.53	0.49
15 km	62.4	20.3	27.5	41.3	14.7	19.8	5.9	1.5	2.0	1.9	0.16	0
33 km	22.8	5.9	8.0	13.3	4.2	6.0	2.7	0.43	0.40	0.21	0	0

adsorption is influenced by competitive sorption, which may enhance desorption, depending on a compound (Chen et al. 2000). Typically, the rate of desorption decreases over time (Chen et al. 1999), and Meriläinen et al. (2006) have demonstrated that with more intense agitation, the rate of desorption increases until it reaches the steady state. In this study, however, we cannot assess the rate of desorption, because the temperatures and durations of each experiment were different and kinetic sampling was not done. Overall, we assume that the differences between the experiments may partly be due to agitation time, simulating the length of the flooding; however, temperature differences also probably had an effect. It is also possible that the extractives have started to decompose in oxic conditions during longer periods of agitation (Leppänen and Oikari 1999b).

In the case of sediment erosion, we assume that the RA concentrations in sediment were high enough to cause toxic effects in aquatic animals. However, this hypothesis needs to be confirmed by field measurements. This could be done with benthic fish, as their RA exposure can be monitored by measuring metabolites in bile (Meriläinen et al. 2007). The Southern Lake Saimaa, in Finland, is another lake with sediment contaminated by effluents discharged from a pulp mill; there, more elevated concentrations of RAs have been detected in fish bile during spring turnover. This possibly indicates that sediment may desorb contaminants in nature (Karels and Oikari 2000; Meriläinen and Oikari 2008a). Elevated levels of RA concentrations have also been detected in the bottom dwellers (Meriläinen and Oikari 2008b) and the bile of eel (van den Heuvel et al. 2006). It has also been observed that, in nature, there are differences in whole body concentrations of RAs between species of benthic fauna (Meriläinen and Oikari 2008b). In addition, bottom feeding roach (*Rutilus rutilus*) have been shown to have higher concentrations of RAs in their bile than, for example, open-water perch (*Perca fluviatilis*) (Karels and Oikari 2000; Leppänen and Oikari 1999a; Meriläinen and Oikari 2008a). However, in the water-only experiment, there were no differences between species, leading into conclusion that exposure partly originates from sediment (Meriläinen and Oikari 2008a).

In contrast to the levels of RAs, retene was present at very low concentrations in the sediments and elutriates. Desorption of retene was shown to be considerably slower than that of RAs (Meriläinen et al. 2006), probably reflecting its higher hydrophobicity. Therefore, the low water solubility of retene (17 µg/l) (Kiparissis et al. 2003) may be partly responsible for its low elutriate concentration. On the other hand, the low retene concentrations in sediments can be explained by the low abundance of RAs, retene's precursors, and probably oxic conditions in the sediment while anoxic conditions are needed for retene formation (Leppänen and Oikari 1999b; Tavendale et al. 1997). At the Äänekoski watercourse the oxic conditions in the sediments have probably been recovered since the mid-1980s. Such recovery has been previously demonstrated in cases in which the pulp and paper industry has ceased (Hynynen et al. 2004; Leppänen and Oikari 2001) or the technique improved (Lahdelma and Oikari 2005; Leppänen and Oikari 1999a; Matinvesi 1996; Meriläinen et al. 2001). However, retene has been detected to be possibly bioavailable with semipermeable membrane device in the sediment of Lake Vatianjärvi, despite its low abundance (Lassila et al. to be published). Retene's ability to cause deleterious malformations in fish embryos at low (10–32 µg/l) concentration levels is well-known (Billiard et al. 1999; Brinkworth et al. 2003; Oikari et al. 2001), but we do not consider it to be a risk factor at the concentration levels observed in this study.

Dissolution potency may be used as an indicator of bioavailability, which is critical when assessing sediment toxicity (Lebo et al. 2003; Meriläinen et al. 2006). The most crucial factor concerning xenobiotics bioavailability and toxicity in sediment is organic material, more specifically organic carbon (OC) (Lebo et al. 2003; Park and Ertsfeld 1999; Ristola et al. 1996), and grain size (Lebo et al. 2003; Park and Erstfeld 1999). In our dissolution experiments, the abundance of OC seems to decrease desorption from sediments, with some site-related differences. In the present study, the content values of LOI in both deeper and upper sediment layers remained similar, and OC concentration was depending on the LOI content in sediment. However, our

observation does not exclude the possibility of the microbial decaying of OC in deeper sediment that has been detected in previous studies (Håkanson and Jansson 1983; Leppänen and Oikari 1999b).

In this study, abietic type RAs were considerably more abundant than the pimaric type RAs, confirming the earlier observations in sediments (Judd et al. 1996; Leppänen and Oikari 1999a) and elutriate (Meriläinen et al. 2006) related by the pulp and paper industry. After we detected the possibility of dissolution, we estimated the overall dissolution potential in a lake-wide area. Interpolated spatial distribution showed the estimated total abundance of the most abundant of both RA types; namely DHAA and IPA. The estimated total amounts of xenobiotics were approximately three times higher with DHAA than IPA. However, IPA is probably more of a concern due to its toxic potency being higher than that of DHAA (Peng and Roberts 2000). Indeed, we propose that, in the event of severe flooding with surface sediment erosion, this kind of estimation can be used when assessing the risks of contaminated sediments. In addition, this novel approach may be particularly useful when it is necessary to assess the exposure of organisms such as perch, which prefer surface waters and are not in direct contact with the sediment.

5 Conclusions

The industrial-era vertical distribution of process-derived extractives in sediments implies that, in the present study, the net recovery of sediments and benthic habitats is due to the major technological improvements during the 1980s. This study confirms that the effect of pulp and paper production can be demonstrated as far as 33 km, and probably also further, downstream from the source. Dissolution experiments revealed that RAs and retene can be desorbed from sediments to water, predicting their bioavailability for epibenthic biota. We suggest that desorption of wood extractives depends directly on their concentration in the sediment as well as on the OC content of sediments. We conclude that if the worst-case scenario came to pass, considerable amounts of RAs would be desorbed, possibly with very severe consequences for aquatic animals, in downstream habitats. We recommend that long-term desorption experiments, especially with various temperatures and dynamics, be carried out in order to provide more comparable results. Moreover, we particularly recommend that further bioavailability experiments with elutriates be carried out.

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II

BETULINOL AND WOOD STEROLS IN SEDIMENTS CONTAMINATED BY PULP AND PAPER MILL EFFLUENTS: DISSOLUTION AND SPATIAL DISTRIBUTION

by

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RESEARCH ARTICLE

Betulinol and wood sterols in sediments contaminated by pulp and paper mill effluents: dissolution and spatial distribution

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Abstract The goal was to determine dissolution potency of betulinol and wood sterols (WSs) from pulp and paper mill-contaminated sediments and the current stratification for assessment the load due to potential erosion in the river-like watercourse. Both compounds are wood extractives, which may be toxic to benthos and fish. This research continues a study in which other wood extractives, resin acids and their derivative, retene, were analysed. Sediments were collected from 1, 3.5, 12, 15, and 33 km downstream from the pulp and paper mills, and from 2 upstream reference sites. The dissolution potency into sediment–water elutriates (1+4 *v*/*v*) was studied by two agitation times and temperatures. The vertical amounts of extractives were determined from the uppermost 20 cm of sediment. The amounts of extractives potentially released were estimated from the sediment layers 0–2 and 2–5 cm by using spatial interpolation. According to the interpolation, the total amount of betulinol and β-sitosterol was calculated as kg/ha in the whole sediment area. Significant concentrations of betulinol (1,666 μg/g, dw) and WSs (2,886 μg/g, dw) were measured from the sediments. According spatial interpolation, the highest calculated amount of betulinol (4,726 kg/ha) and that of the most abundant WS, β-sitosterol (3,571 kg/ha), were in the lake where the effluents were discharged. In the dissolution experiment, the highest concentration of betulinol in sediment (0–10 cm) and elutriate was 412 μg/g (dw) and 165 μg/l, respectively. For WSs, concentrations were 768 μg/g (dw) in sediment and 273 μg/l in elutriate. In a worst-case scenario, betulinol may be desorbed to water in concentrations which are hazardous to aquatic animals. Instead WSs are not a risk in this study area. The amount of desorption varied depending on the concentration of contaminants in sediment, the nature of disturbance, and the sediment organic carbon content.

Keywords Betulinol · β-sitosterol · Dissolution · Pulp and paper mill effluents · Wood extractives · Wood sterol (WS)

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Introduction

Pulp and paper mill effluents contain more than 250 identified chemicals (Suntio et al. 1988; Ali and Sreekrishnan 2001). Besides chemicals used and formed during the pulp and paper making processes, also natural wood extractives, such as resin acids and wood sterols (WSs), have been commonly found from the effluents (Hutchins 1979; Kaplin et al. 1997) and from the sediments downstream from pulp and paper mills (Rogers 1978; Koistinen et al. 1998; Leppänen and Oikari 2001; Lahdelma and Oikari 2005; Lahdelma and Oikari 2006; Meriläinen et al. 2006). The composition and content of extractives differ in hardwood and softwood species. For instance, betulinol (also called betulin) is the main extractive in birches, i.e. *Betula* spp.; even 54–82 % of dried birch bark extract is betulinol (Abyshev et al. 2007) whereas it is missing totally from softwoods (Vikström et al. 2005; Abyshev et al. 2007). On the other hand, resin acids are much more abundant in softwood, for instance pine contains about 12 times more resin acids than birch (Vikström et al. 2005). The most abundant, and therefore common, plant sterols in trees are β-sitosterol, stigmastanol, campesterol, and stigmasterol (Chan et al. 1998; Ayebo et al. 2006). Cholesterol and cholestanol are plentiful both in plants and animals (Sjöström 1993), whereas coprostanol is considered a biomarker of human and other mammalian feces (Chan et al. 1998; Ayebo et al. 2006).

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Wood extractives may cause harmful effects on biota (Owens 1991; Ali and Sreekrishnan 2001; Christianson-Heiska et al. 2004; Meriläinen et al. 2006). WSs may cause hormonal disruptions in fish, for example β-sitosterol and stigmasterol are able to bind to the estrogen receptor and affect reproductive functions in juvenile rainbow trout (Tremblay and van der Kraak 1998; 1999). Like many wood extractives, also WSs are highly hydrophobic (Sjöström 1993) and tend to adsorb to the sediment, being bioaccessible to the benthic animals (Meriläinen and Oikari 2008), and thus pose a risk to bottom feeding fish. Also resin acids are toxic to fish, causing for instance impaired liver function (Oikari et al. 1983) and genomic effects (Meriläinen et al. 2007). Resin acids are toxic to invertebrates also, affecting the survival and reproduction (Hickey and Martin 1995). Retene, an anaerobic biotransformation product of resin acids, can be bioavailable to animals inhabiting sediments (Leppänen and Oikari 1999; Nikkilä et al. 2001) and cause reduced growth, yolk sac edema and mortality on early life stages of fish (Billiard et al. 1999; Vehniäinen et al. 2003). Betulinol is mostly studied for its medical aspects, as it is known to inhibit bacterial growth (Salina et al. 2010). In addition, betulinic acid, one of its derivatives, has anticancer, anti-malarial, and anti-inflammatory effects (Moghaddam et al. 2012). Betulinic acid, as well as dihydrobetulinic acid, inhibit HIV activity (Fujioka et al. 1994). In aquatic environment betulinol may have estrogenic-disrupting properties on fish (Mellanen et al. 1996; Christianson-Heiska et al. 2004; 2008). However, the environmental fate and risks of betulinol are still poorly known.

Nowadays, compared to early 1980s for instance, the pulp and paper mill effluents can be efficiently treated (Makris and Banerjee 2002). However, some amounts of extractives are still released and the loading is continuous (Koistinen et al. 1998; Makris and Banerjee 2002; Pokhrel and Viraraghavan 2004). In addition, historically contaminated sediments may constitute a risk if they act as a source of the contaminants (Meriläinen et al. 2006; Hollert et al. 2007; Westrich et al. 2007). The dissolution property is a critical in the risk assessment, as it predicts bioavailability potential of the contaminants from the sediment (Lebo et al. 2003; Meriläinen et al. 2006; Rämänen et al. 2010). One crucial factor affecting the sediment capacity to absorb and desorb organic compounds is the content of organic material (Park and Erstfeld 1999). On the other hand, increasing dissolution potency may occur due to the physical disturbances. Especially in the river-like watercourses, harmful compounds may be released to the water column due to sediment erosion (Meriläinen et al. 2006; Rämänen et al. 2010) and abundant floods may further increase the risk of sediment erosion (Hollert et al. 2007; IPCC 2007; Westrich et al. 2007). For instance, in 2002 extreme flooding in the River Elbe caused a massive increase in the amount of suspended particulate matter in the water column, advancing transportation of the lipophilic contaminants as well (Stachel et al. 2004). In Nordic countries, flooding occurs every spring along with snow melting, perhaps with increasing tendency as the annual precipitation has been predicted to raise 10–40 % in boreal latitudes within this century (IPCC 2007). In addition, the vertical turnover of water twice a year breaks the temperature stratification in boreal lakes and this is probably churning the uppermost sediment layer. Therefore, it is important to assess the possible impacts of the uppermost sediment but also the deeper layers should be taken into account in case of severe erosion.

In this study, the main goal was to determine the dissolution potency of some endocrine disrupting wood extractives, betulinol and WSs, in the pulp and paper mill-contaminated sediments in a boreal watercourse. Sediment slurry mimicking the case of sediment erosion caused by flooding was made to estimate the worst-case situation. The spatial distributions of individual wood extractives were estimated from the two sediment layers (0–2 and 2–5 cm). Despite the efficient wastewater treatment, large amounts of wood extractives may be discharged due to high effluent volumes. Therefore, according to the spatial distribution, the amounts of betulinol and β-sitosterol were calculated as the amount in the sediment area (kg/ha). To a similar extent, betulinol has not been previously determined from sediments downstream pulp and paper mills. This is a further study to the resin acid and retene investigation in the same study area in Äänekoski watercourse in Central Finland (Rämänen et al. 2010).

Materials and methods

Study areas

The research area was the Äänekoski watercourse, in Central Finland (Fig. 1), where wood industry began in 1886 when the board mill was established, followed by paper and saw industry shortly thereafter. In 1938, the first chemical pulp mill was introduced (Tuuri et al. 1996). In 1961, another pulp mill was built, producing sulfate cellulose. In the early 1960s, the sulfite and sulfate cellulose produced were about 45,000 and 87,400 tons per year. Since the early 1970s, the amounts were 50,000 and 12,000 tons sulfite and sulfate cellulose per year, respectively. Both birch and spruce were used. The proportion of birch cellulose was about 70 % in the 1970s and decreased to 30 % in the 1980s (Rämänen 2008). The sulfite process was discontinued in 1984, and 1 year later another old pulping mill was closed as well (Granberg et al. 1987). In 1985, a new pulp mill using the sulfate method, and activated sludge wastewater treatment was initiated. Before that only mechanical wastewater treatment was used (Tuuri et al. 1996). In 1993, an ECF (elemental chlorine free) bleaching process with oxygen

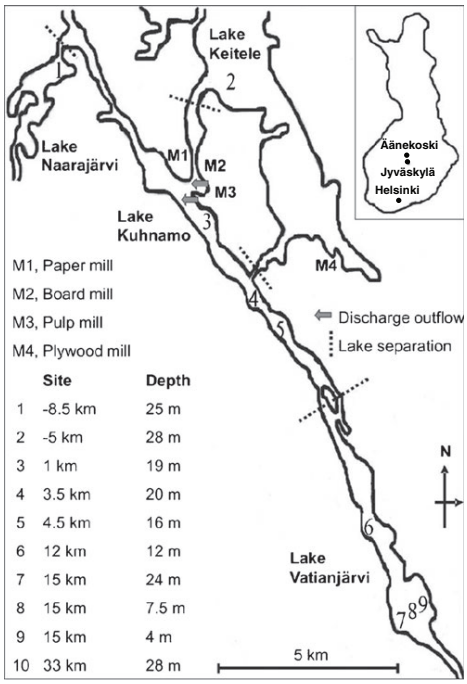

Fig. 1 The research area, the Äänekoski watercourse in Central Finland. Lake Leppävesi (site 10, not shown in the map) is located about 15 km further downstream from Lake Vatianjärvi. Lakes are separated by *dash line* and discharge outflow of mills (*M*) by *grey bolded arrows*

delignification was introduced which led to decreasing discharge of process waters. In 1999, pulp washing and oxygen delignification were improved, which resulted in a 30 % decrease of chemical oxygen demand (COD) by wastewaters (Anonymous 2006a).

During the sediment sampling in 2007, birch and softwood were used equally in pulp production. In 2007, the discharge from the pulp mill included process waters (14,826,000 m^3/a) purified in an activated sludge treatment plant (Anonymous 2007). Paper and board mills had their own wastewater treatment plant using chemical and mechanical methods. In 2006, the wastewater discharge from the paper mill was 820,000 m^3 (Anonymous 2011a). In addition, the plywood factory is located next to Lake Keitele, one of the reference lakes in this study. Wood has been stored in the waters, for instance in year 2003, 8,000 and 300 m^3 birch and conifer logs were stored, respectively. The factory has released overflow waters from the conifer reservoirs into the lake, for instance in 2004 the estimated amount of overflow was 70,000 m^3. In addition, the birch storage reservoir (10,000 m^3) is released to Lake Keitele once a year during the mill shutdown in summer. However, since 2006, a new pumping system has decreased the overflow volume by 75 %, being 20,000 m^3/a in maximum (Anonymous 2006b).

The watercourse downstream from Äänekoski mills is composed of several narrow lakes. The discharge rate in the lakes downstream from pulp and paper mills is approximately 140 m^3/s during the spring floods in May, and decreases to 50 m^3/s during the summer months. From September to April, the discharge varies from 60 to 85 m^3/s (Anonymous 2011b). The watercourse collects inflows from two directions; humic water from the Saarijärvi source and oligotrophic water from the Viitasaari source. The two reference sites located in Saarijärvi and Viitasaari sources 8.5 and 5 km upstream from the pulp and paper mills in Äänekoski, respectively (Fig. 1). Wastewaters from the Äänekoski mills and from the municipal wastewater treatment plant of the city of Äänekoski are discharged to Lake Kuhnamo, with an area of about 3.7 km^2. In Lake Kuhnamo, sediment samples were taken 1, 3.5, and 4.5 km downstream from the mills. From Lake Vatianjärvi (an area of 6.3 km^2), samples were taken 12 and 15 km downstream from the mills, and further downstream from Northern Lake Leppävesi (area 36 km^2), approximately 33 km from Äänekoski (Fig. 1).

Sediment sampling

The sediment samples were taken in late April and early May in 2007. Sediments for the dissolution experiments were collected with an Ekman device (area 225 cm^2, sediment depth ca. 0–10 cm) and replicates were combined. Six samples from Lake Kuhnamo (3.5 km) and Lake Leppävesi (33 km), and 17 from Lake Vatianjärvi (15 km) were taken. From the reference sites, 10 samples were taken from Lake Naarajärvi (Ref. −8.5 km) and 11 samples from Lake Keitele (Ref. −5 km). The samples were stored in darkness at 4 °C.

The sediment samples for the vertical distribution analysis of betulinol and WSs were taken by Kajak-corer (diameter 50 mm, column length 30–50 cm). In each site, three subsamples were combined, except two from Ref. −5 km, and from the 4.5-km site, only one subsample was taken, due to the hard bottom. The sediments were left to settle for 24 h in the dark at 4 °C and thereafter sectioned for the uppermost 20 cm (0–2, 2–5, 5–10, 10–15, and 15–20 cm).

Sediment analyses

The concentrations of betulinol and WSs were analysed as described by Lahdelma and Oikari (2006) and Leppänen and Oikari (1999), with some modifications. In short, the freeze-dried sediments were Soxhlet-extracted (Büchi B–811, Switzerland) by 180 ml of hexane/2-propanol solution

(2:1, *v/v*, Rathburn Chemicals HPLC-Grade, UK). The internal standard was 17α-methyltestosterone (17α-methyl-4-androsten-17β-ol-3-one, Sigma®, USA). Identification was performed according to the mass spectra comparisons with library spectra and with Ultra Sitosterol (UPM-Kymmene Corp., Kaukas mill). After 70 cycles, the extracts were rinsed with hexane and evaporated to a small volume in a vacuum. The concentrated samples were diluted with 50 ml of hexane and extracted three times with 40 ml of K_2CO_3 (>99 %, Merck, Germany) in a separation funnel. The hexane fraction containing betulinol and WSs was vacuum-evaporated to a small volume, then further evaporated under a nitrogen gas stream to dryness (Leppänen and Oikari 1999). The sample was diluted with 500 μl of hexane/chloroform (4:1, *v/v*) and absorbed in the 5 % deactivated and conditioned silica gel (0.062–0.200 mm, Baker Analyzed®, The Netherlands) with column recovery of 77–83 %. The column was first eluted with 12 ml hexane and then with 15 ml of chloroform (Merck, Germany) and evaporated by nitrogen gas until dry. The fraction was diluted with 125 μl of pyridine (purity >99 %, J.T. Baker, The Netherlands) and silylated with 175 μl*N,O-bis* (trimethylsilyl)-trifluoroasetamid (BSTFA)+1 % trimethylchlorosilane (TMCS; GC-quality, Fluka, Switzerland) at 70 °C for 30 min. A gas chromatograph/mass spectrometer (Hewlett Packard 6890 MS, Germany; Hewlett Packard 5973 GC, USA, 30 m HP-5 polysiloksane polymer column, ID 0.25 μm, volume of injection 0.1 μl) was used for analysis temperature program starting from 100 °C (kept stable for 1.5 min), raising 6 °C/min up to 180 °C (15 min) and further raising 4 °C/min up to 290 °C (13 min). The carrier gas used was helium (Aga, USA; Lahdelma and Oikari 2006). Loss of ignition (LOI) and the concentration of total organic carbon (TOC) were analysed according to Rämänen et al. (2010).

Dissolution of sediment-bound wood extractives

Artificial freshwater (11.8 g/l of calcium chloride, 4.9 g/l of magnesium sulphate, 2.6 g/l of sodium bicarbonate, and 0.2 g/l of potassium chloride) was made with distilled water (pH 7) and used for elutriation of sediment-bound compounds using two protocols. Wet sediment (150–188 g) and artificial fresh water (1+4, *v/v*) were agitated horizontally (180 rpm) for 1 h at 20 °C and for 46 h at 10 °C in dark. Thereafter, sediment–water mixtures were centrifuged for 15 min at 200 rpm/min, and further 5 min 2,000 rpm/min. Within 4 days of storing in the dark at 4 °C, elutriates were analysed as described in Rämänen et al. (2010) based on the same method as used for the sediment samples.

Statistics and spatial interpolation

The relationship between the sediment concentrations of betulinol and WSs, and proportion of LOI and TOC in sediments were determined by Spearman's correlation test due to non-normality of some measurements (data from Lake Keitele and Lake Leppävesi). Correlations between elutriate and sediment concentrations were determined by Pearson's correlation test as the data were normally distributed. Statistical analyses were done by SPSS software (SPSS Inc., Chicago, IL USA).

The extractive concentrations of two sediment layers (0–2 and 2–5 cm) from ten sampling sites in the Kuhnamo and Vatianjärvi basins were used for spatial interpolation and to assess the distribution of betulinol and β-sitosterol by using the same program and method as described in Rämänen et al. (2010). The sedimentation rate is approximately 3 cm per year in Lake Kuhnamo and 1 cm in Lake Vatianjärvi (Saski et al. 1997), and thus the uppermost 0–2 and 2–5 cm describe the very recent distribution. The amounts of betulinol and β-sitosterol (kg/ha) that would be released from these sediments in a worst-case scenario of unpredictable erosion in association with flooding were interpolated.

The total amount calculations for betulinol and β-sitosterol were done by multiplying the mean values of the interpolated amounts for both substances (ug/g, dw) by the density of the sediment (kg/m^3) by the volume of the studied sediment layers (m^3), and then converted into kg/ha values by dividing the obtained result with total area of the lake (ha). These calculations therefore assume (somewhat unrealistically) that the sediment layer of the whole lake would have behaved the same way as a sink for the studied substances as the sediment at the sampling locations, and that the sedimentation would occur the same way regardless of water depth and water currents (of which information was not available for the whole lakes). In addition, it was assumed that the interpolation would give the correct kind of values on average, as it should, if the number of sampling points is high enough. Therefore, based on these assumptions, the results of total amount of betulinol and β-sitosterol within certain sediment depth should be addressed with caution.

Results

Spatial distribution of wood extractives in the sediments

The highest concentration of betulinol (1,666 μg/g, dw) was measured from Lake Kuhnamo 1 km downstream from the mills from the depth of 5–10 cm (Fig. 2); whereas in Lake Vatianjärvi and in Lake Leppävesi, the maximum concentrations were at the depth of 10–15 and 15–20 cm, respectively. In the reference sites, the betulinol concentration in the sediment was lower in Lake Naarajärvi (Ref. −8.5 km; <5 μg/g, dw) than in Lake Keitele (Ref. −5 km), where the concentrations varied between 4.4–44 μg/g, dw (Fig. 2). Limit of detection (LOD) for betulinol in the sediment was 0.12 ug/g, dw.

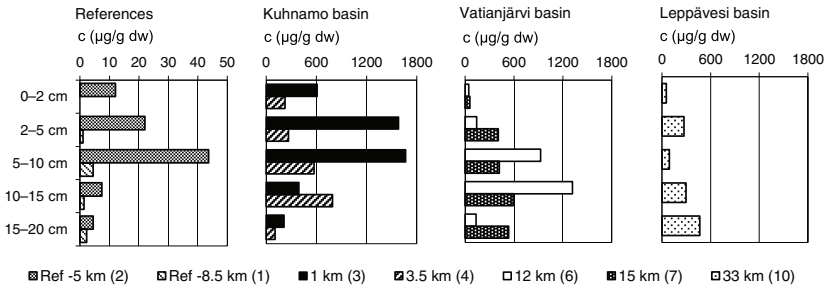

Fig. 2 Concentrations (µg/g, dw) of betulinol in the sediments downstream from pulp and paper industry in the Äänekoski watercourse, Central Finland. The scale is different in the reference sites than in the other sampling sites. Site numbers are in *brackets* (see Fig. 1 for the exact locations)

The most abundant WSs in the sediments were β-sitosterol and stigmastanol, accounting for 36 % (±SD 8.3 %) and 32 % (±SD 8.3 %) of the total WS concentration. Vertical analyses revealed that the total concentration of WSs was highest (2886 µg/g, dw) in the layer 2–5 cm at the closest site (1 km) downstream from the mills (Fig. 3). LOD for β-sitosterol, the most abundant WS, was 0.11 ug/g, dw.

The dependence between betulinol and WS concentrations in the sediments was determined according to Spearman's correlation. It showed significant positive dependence in Lake Kuhnamo (r=0.995, p<0.001, n=13) and in Lake Vatianjärvi sediments (r=0.614, p=0.004, n=20). Further downstream, in Lake Leppävesi, no dependence was shown nor in the reference sites. Betulinol and LOI showed a positive dependence in all the lakes but the only significant correlation was in Lake Kuhnamo (r=0.564, p=0.045, n=13). The dependence between LOI and WSs was significant only in Lake Kuhnamo sediment (r=0.580, p=0.038, n=13) and in the reference, Lake Naarajärvi (r=0.900, p=0.037, n=5). No significant correlation between TOC and wood extractives were observed in the lakes downstream from the mills. From the reference sites, TOC was analysed only from the Ekman sample, and thus, it was impossible to make the statistical analysis.

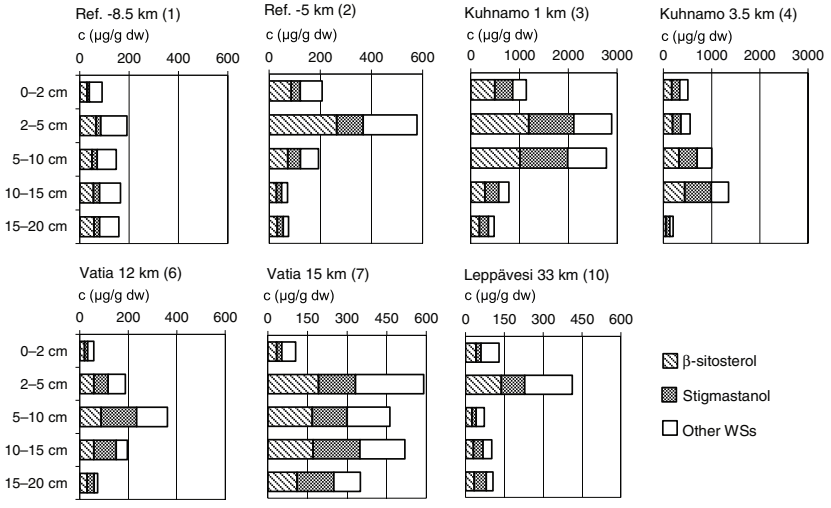

Fig. 3 Concentrations of WS (µg/g, dw) in the sediments downstream from pulp and paper industry in the Äänekoski watercourse, Central Finland. β-sitosterol and stigmastanol are represented separately from other WS. The scale is different in 1 and 3.5 km sites. Sampling sites are separated according to their distance from the mills (site numbers in *brackets*; see Fig. 1 for the exact locations)

Spatial distributions of betulinol (Fig. 4a–b) and β-sitosterol (Fig. 4c–d) were assessed by interpolating the data from Lake Kuhnamo and Lake Vatianjärvi sediment layers 0–2 and 2–5 cm, which were expected to be the layers most sensitive to erosion. Almost without exception, betulinol and β-sitosterol were more abundant in the deeper layer compared to the upper one. The highest estimated amount of betulinol in the Kuhnamo basin was 3,151 and 4,726 kg/ha at the sediment depths 0–2 and 2–5 cm, respectively. In the Vatianjärvi basin, the estimated amounts of betulinol were 1,455 kg/ha at a depth of 2–5 cm, i.e. more than twice that in the uppermost 2 cm (619 kg/ha). Also for β-sitosterol, the highest estimated amounts were 3,571 kg/ha in the Kuhnamo basin at the sediment depth of 2–5 cm, and only half of that (1,294 kg/ha) in the uppermost 2 cm. A similar trend was observed in the Vatianjärvi basin, where the estimated amount of β-sitosterol was 311 kg/ha in the uppermost 2 cm, and 563 kg/ha at a depth of 2–5 cm.

Dissolution of betulinol and wood sterols

Some differences in the dissolution potency of betulinol and WSs between the two agitation times and temperatures were detected, and most of the concentrations increased with a longer agitation time (Table 1). In the contaminated sediments, during the longer agitation (46 h), more betulinol desorbed from the sediments, compared to 1 h agitation, without any exception. It must be noted that the dissolution potency cannot be directly compared to the concentrations measured from the dry sediment as the elutriates were made from the wet sediment and dry weight of the sediments varied a lot (Table 2). Therefore, the amounts of betulinol, WSs, and β-sitosterol per agitated sediment were calculated and correlations between the dissolved concentrations and the sediment concentrations were analysed for the both sediment dry weight and wet weight concentrations.

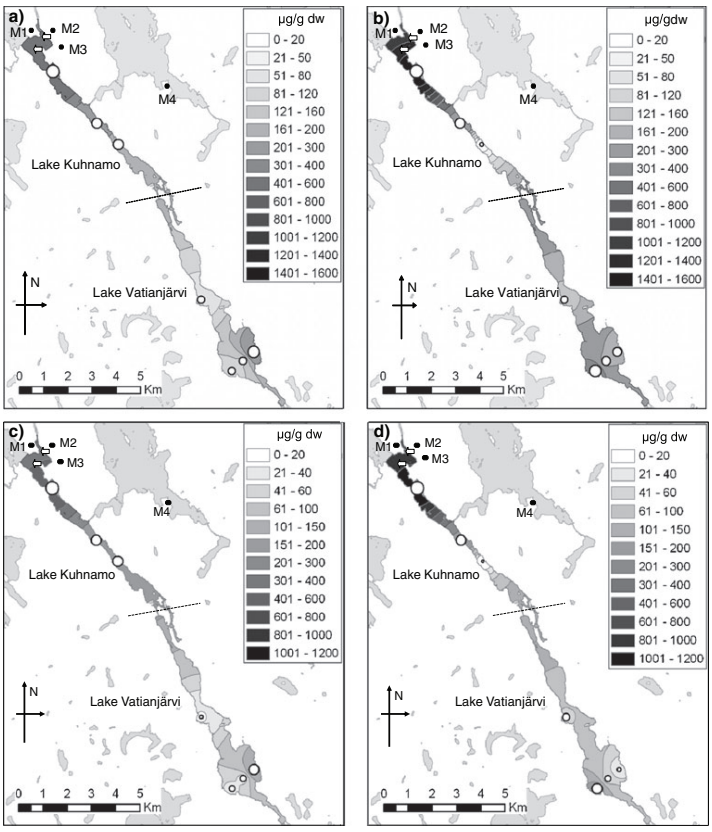

Fig. 4 Spatial distributions of betulinol **a** 0–2 cm, **b** 2–5 cm and β-sitosterol, **c** 0–2 cm, and **d** 2–5 cm in the sediments of Lake Kuhnamo and Lake Vatianjärvi. Production facilities are marked as: *M1*=paper mill, *M2*=board mill, *M3*=pulp mill, and *M4*=plywood mill. *Bold white arrows* show outflows from the WWTPs. The lakes are separated by a *dash line*, and the *white circles* (size depends on concentration) represent the sampling sites (Base map © National Land Survey of Finland)

Table 1 Average concentrations of betulinol, total wood sterols and β-sitosterol at a sediment depth of 0–10 cm taken by Ekman sampler, and in the sediment–water elutriates produced with 1+4 *v*/*v*, with two agitation times and temperatures in darkness: 1 h at 20 °C and 46 h at 10 °C; sampling sites are represented in Fig. 1

Sampling site	Betulinol			Wood sterols			β-sitosterol		
km	Sed. (μg/g dw (μg/g dw)	1 h (μg/l)	46 h (μg/l)	Sed. (μg/g dw)	1 h (μg/l)	46 h (μg/l)	Sed. (μg/g dw)	1 h (μg/l)	46 h (μg/l)
−8.5 (ref.)	2.5	<0.12	<0.12	149	89	237	50	24	72
−5 (ref.)	31	<0.12	<0.12	311	2.7	8.2	133	0.7	2.5
3.5	412	63	156	768	145	273	250	46	83
15	346	73	165	429	113	106	148	33	30
33	137	22	31	185	76	52	62	20	13

The amounts (μg) of betulinol in agitated wet sediments were about 1–1.5 % in Lake Naarajärvi (ref. −8.5 km) and 5–10 % in Lake Keitele (ref. −5 km) compared to the amounts in the sediments downstream from the mills. For betulinol, a positive and significant dependence was observed between contaminated sediment (dw) and elutriate concentrations both after one hour (r=0.970, p=0.006, n=5) and 46 h agitation (r=0.978, p=0.004, n=5). The significant correlation was observed also between betulinol concentrations in the elutriates and in the wet sediment both after 1 h (r=0.970, p=0.006, n=5) and 46 h agitation (r=0.932, p=0.021, n=5). In the reference sites, no dissolution of betulinol occurred.

In the case of WSs, about the same amounts were present in the agitated reference sediments and in Lake Leppävesi sediment 33 km downstream from the mills. Instead, compared to the reference and Lake Leppävesi sediments, about double the amount of WSs was in Lake Kuhnamo and Lake Vatianjärvi sediments. The degree of desorption increased with longer agitation time in the reference lakes and in Lake Kuhnamo (3.5 km). Instead, further downstream desorption slightly decreased with longer agitation. β-sitosterol, the most common WS, also desorbed more with the longer agitation, except slightly less in Lake Vatianjärvi and Lake Leppävesi. A positive but not significant correlation was observed between sediment (dw and ww) and elutriate concentrations.

Discussion

The recovery of the Äänekoski watercourse practically started in 1985 after mill renewal. Before that, wood-derived organic and bioactive compounds in the effluents caused heavy oxygen depletion, impaired water quality and various negative effects in fish, for instance the decline of species diversity (Granberg et al. 1987; Hynynen et al. 2004), the decreased size of whitefish and brown trout, and bad taste in fish (Anonymous 1981). Nowadays, more diverse fish species are present, and since 2000 notable changes have not occurred in fish fauna, for instance restocked salmonids are present in all rapids, and brown trout is breeding naturally in the Äänekoski watercourse (Anonymous 2006c; Palomäki and Salo 2008). More sensitive biomarkers, such as EROD activity in fish liver, have recently (2009) measured from perch and roach caught from the Lake Vatianjärvi, results indicating that the exposure to the dioxin-like compounds did not occur in the study site (Ratia et al. unpublished).

Bioindicators and animal diversity indicate the recovery of the watercourse but there are still potential hazardous compounds in the uppermost sediment. Previously Rämänen et al. (2010) demonstrated elevated resin acid concentrations even 30 km downstream from the pulp and paper industry, and in their dissolution experiments concentrations of resin acids desorbed from the sediment at concentrations which may cause some biological effects in

Table 2 Sediment characteristics analysed from the uppermost 10 cm layer along the Äänekoski watercourse, Central Finland: dry weight (dw), loss on ignition (LOI, for dw), and total organic carbon (TOC, for dw)

Sampling site km	dw (%)	LOI (%)	TOC (%)	Description
−8.5 (ref.)	21.9	8.7	3.7	Fine particles, tightly packed, brown color.
−5 (ref.)	11.6	14.2	4.7	Grainy particles, loosely packed, 0–4 cm carmine color, deeper brownish grey.
3.5	15.9	16.9	9.0	Fine particles, 0–2 cm reddish brown color, deeper dark brown.
15	24.4	8.6	4.2	Fine particles, 0–2 cm orange brown color, deeper brown.
33	32.6	3.3	1.2	Fine particles, 0–2 cm orange brown color, deeper brown.

aquatic animals (Peng and Roberts 2000; Meriläinen et al. 2007). Instead retene, PAH compound transformed from the resin acids, was not assessed to cause a risk (Rämänen et al. 2010). This research was a further study to the research by Rämänen et al. (2010), and information about the environmental fate and risks of the other bioactive wood extractives, betulinol and WSs, were find out.

Concentrations and estimated amounts of betulinol and WSs in the sediment

Activated sludge treatment can efficiently remove wood extractives from the wastewaters. Over 98 % of triterpenyl alcohols (mainly betulinol), and over 95 % of WSs can be removed (Kostamo et al. 2004). The remaining concentrations of triterpenyl alcohols and WSs in activated sludge treated wastewaters vary considerably. Mattson et al. (2001) measured ca. 100 μg/l WSs from Finnish ECF kraft mill effluent, whereas Kostamo et al. (2004) found WSs of 2.3 −11 μg/l in water phase but higher concentrations (17 −1294 μg/l) in the particles. In treated and released effluent, 97 % of triterpenyl alcohols and 79 % of sterols were bound in the particle phase (Kostamo et al. 2004). Although wood extractives can nowadays be efficiently removed, large amounts may be discharged due to high effluent volumes. Indeed, in this study, betulinol and WSs were widespread and measured from the sediment over a distance of 30 km downstream from the mills. There have been similar results with other wood extractives measured from the sediments on the same watercourse (Rämänen et al. 2010) as mentioned before. Compared to the average concentrations of total resin acids (140 μg/g, dw), and retene (12 μg/g, dw) in the 0–10 cm sediment of Lake Kuhnamo in the Äänekoski watercourse (Rämänen et al. 2010), multiple times higher concentrations of betulinol (410 μg/g, dw) and WSs (770 μg/g, dw) were measured. The high abundance of plant related WSs (88 %) compared to animal related WSs (cholesterol and cholestanol) indicates the main source being pulp and paper industry and, in part, allochthonous plant material as well.

This is the first research in which the vertical distribution of betulinol has been analysed from the sediment. Betulinol was generally more abundant in the sediments than any individual sterol. Both betulinol (Mellanen et al. 1996; Christianson-Heiska et al. 2004; 2008) and WSs (Mellanen et al. 1996; Tremblay and van der Kraak 1998; 1999) are bioactive compounds, causing hormonal disruptions, the effective concentration of betulinol being much lower than that of WSs (Lehtinen et al. 1999; Christianson-Heiska et al. 2004; Orrego et al. 2011). Like most of the wood extractives, betulinol is a hydrophobic compound (log K_{ow} 8.18) which adsorbs to the particles (Kostamo et al. 2004). In this study, betulinol concentrations in the uppermost 10 cm of sediment varied from 610 to 1,670 μg/g (dw) when sampled 1 km downstream from the mills. Compared to Southern Lake Saimaa, another lake in Finland with a similar history in pulp and paper production and techniques as in Äänekoski, the concentrations in our study were lower (Lahdelma and Oikari 2006). Lahdelma and Oikari (2006) documented betulinol at 3,810 μg/g (dw) from deep sediment (20–25 cm) whereas in the surface sediment concentration was about half of that. Resin acids and retene have also been measured at higher concentrations in Southern Lake Saimaa (Leppänen and Oikari 1999; Lahdelma and Oikari 2005) than in the Äänekoski watercourse (Rämänen et al. 2010), probably due to the different hydromorphology of the lakes, as unlike Lake Saimaa, the Äänekoski watercourse is river-like and therefore the contaminants in the effluents are spread in larger area and further downstream.

Interestingly, in the sediment of Lake Keitele, the reference site 5 km upstream from the main point source, a similar pattern of betulinol profile was observed as in the Kuhnamo basin just below the effluent discharge point. However, the concentrations in the Keitele sediment were only about 2 % of that in Lake Kuhnamo. Betulinol concentrations in Lake Keitele were approximately the same as measured from the other reference sites in Finland being around 25–30 μg/g (dw) (Lahdelma and Oikari 2006). On the other hand, a much lower concentration was measured from our other reference site, Lake Naarajärvi. The variation in our references may have arisen from the plywood factory in Lake Keitele but it may also be explained by the differences between the two catchment areas and the sediment characteristics. It is also possible that background levels varied considerably for instance because of log floating which has previously been done in Finland more or less in every watercourse.

The total WS concentrations were higher in the uppermost 10 cm of sediment than detected in earlier studies from the other Finnish lakes loaded by pulp and paper effluents (Lahdelma and Oikari 2006). β-sitosterol was the most abundant of WSs, confirming earlier observations in sediments related by the chemo-mechanical wood industry (Lahdelma and Oikari 2006). Stigmastanol was another common WS in the Äänekoski watercourse sediments, possibly due to deoxidation of β-sitosterol (Chan et al. 1998). Interestingly, the maximum concentrations of WSs have been measured from the surface sediment both in our study (2,890 μg/g, dw) and in Southern Lake Saimaa (1,760 μg/g, dw; Lahdelma and Oikari 2006). In Lake Päijänne, which has also been contaminated by chemical wood industry over hundred years, the maximum WS concentrations (1,970 μg/g, dw) were measured from the sediment layer which was deposited during the 1970s when untreated effluents were discharged (Lahdelma and Oikari 2006). The background level of WSs in previous studies has been around 10–50 μg/g, dw (Lahdelma and Oikari 2006),

these levels are lower than in our study (80 to 200 μg/g, dw). Surprisingly, in one reference site in our study, Lake Keitele, the total WS concentration was 580 μg/g (dw) in the sediment depth of 2–5 cm. In every other sediment layer, the concentrations were about 200 μg/g (dw) or less as was also in our other reference site, Lake Naarajärvi. Approximately 60 % of the WSs in Lake Keitele and 50 % in Lake Naarajärvi sediment were β-sitosterol and stigmastanol which are, along with campesterol, the most abundant sterols in trees (Sjöström 1993; Kostamo et al. 2004). In relation to the exceptionally high concentration of WSs in Lake Keitele, it could be explained by plywood factory which releases overflow waters of their birch reservoir to Lake Keitele, although since 2006 a new pumping system has decreased the overflow volume by 75 % (Anonymous 2006b). The sedimentation rate in Lake Keitele is about half a centimeter per year (Maatela et al. 1990) and thus the maximum peak at the 2–5 cm depth is deposited before the introduction of the new pumping system in 2006, whereas WSs in the uppermost 2 cm are deposited during the implementation of the new system.

The uppermost sediment is the most sensitive layer to erosion and the presence of endocrine disrupting chemicals raises a concern about safety to aquatic animals. It is known that during periods of flooding, sediments polluted by organic hydrophobic contaminants may function as a secondary source of contamination (Hilscherova et al. 2007). For instance in the River Elbe, a massive flood occurred in 2002, causing re-suspension and transportation of fine suspended material (Baborowski et al. 2004) where the hydrophobic compounds tend to adsorb (Lebo et al. 2003). However, not only erosion but also bioavailability of xenobiotics to the benthic animals is crucial when assessing the potential risks of the contaminated sediment (Meriläinen and Oikari 2008). For these reasons, we also measured vertical concentrations of betulinol and WSs and estimated the total amounts of betulinol and the most common WS, β-sitosterol, in uppermost sediment. Förstner et al. (2004) reported that the critical shear stress is very low in the uppermost 5 cm of sediment in the River Elbe, i.e. this layer is highly sensitive to erosion. The same sediment depths (0–2 and 2–5 cm) were used in our study when the total amounts of the most common wood extractives were estimated. On the other hand, sediment characteristics may vary considerably and Förstner et al. (2004) mentioned that simple correlation between the sediment characteristics and the critical bed shear for erosion is difficult to demonstrate. In the present study, the spatial interpolations of betulinol and β-sitosterol in Lake Kuhnamo and Lake Vatianjärvi showed that large amounts of these extractives have been deposited in the sediment. Almost without exception, both compounds were more abundant in the deeper (2–5 cm) sediment layer. For betulinol, the estimated amounts in the Kuhnamo basin were 3,151 and 4,726 kg/ha at sediment depths of 0–2 and 2–5 cm, respectively. In the Vatianjärvi basin, 15 km from the source, the estimated amounts of betulinol were only 20–30 % of that being 619 kg/ha in sediment at a depth of 0–2 cm and 1,455 kg/ha at a depth of 2–5 cm. The estimated amounts of β-sitosterol were 3,571 kg/ha in the Kuhnamo basin at the sediment depth of 2–5 cm, i.e. more than twice than in the uppermost 2 cm. Like betulinol, the amounts of β-sitosterol were also lower in Lake Vatianjärvi, being only 15–25 % of that in Lake Kuhnamo. Similarly the amount of β-sitosterol in the uppermost 2 cm was only about half of that in the deeper 2–5 cm sediment layer. In conclusion, while the surface area of Lake Kuhnamo is 3.675 km^2 and Lake Vatianjärvi 6.275 km^2, in the case of severe erosion, massive amounts of bioactive wood extractives could be released from the uppermost 5 cm sediment.

Dissolution of betulinol and WSs

Dissolution potency may be used as a predictor of the bioavailability of the contaminants from the sediment (Lebo et al. 2003; Meriläinen et al. 2006). High content of organic carbon (OC) and small particle size decrease the bioavailability of organic contaminants (Park and Erstfeld 1999; Lebo et al. 2003). As detected in earlier study (Leppänen and Oikari 1999), OC concentration is dependent on the LOI content in the sediment. In our dissolution experiments, the content values of LOI in both deeper and upper sediment layers remained similar and the abundance of OC decreased desorption of contaminants from sediments, with some site-related differences (Rämänen et al. 2010).

Betulinol and WSs were present in the sediments in the Äänekoski watercourse, and the dissolution potency of betulinol and WSs was about the same. However, some variation in the dissolution potency of WSs between the sampling sites was observed. The dissolution of WSs from the Lake Keitele reference sediment was lower compared to the other sediments, including Lake Naarajärvi reference, probably because of different sediment characteristic, as the sediment of Lake Keitele was coarse and loosely packed contrary to the other sediments. Lake Keitele is located in the oligotrophic watercourse whereas Lake Naarajärvi is located in the humic watercourse where the peat mining, agriculture, and forestry are the largest types of land use in the catchment area. It is also possible that in Lake Keitele, the origin of the WSs is mainly from the decaying plant material which would explain the high measured concentrations in the sediment when the strong solvents were used in the extraction, whereas WSs from this kind of origin probably do not dissolve so easily into the elutriates. Instead, in the impacted sites, most of the WSs are discharged from the mills and are originally in dissolved form which may explain higher dissolution into the elutriates. However, this does not explain an efficient dissolution of WSs from Lake Naarajärvi reference sediment, as the

dissolution of WSs in proportion to the amounts in the wet sediment was higher in Lake Naarajärvi than in the areas downstream the mills. Probably this may be explained by the lower TOC in Lake Naarajärvi (3.7 %) compared to Lake Kuhnamo (9.0 %) and Lake Vatianjärvi (4.2 %), as desorption of organic contaminants increase with decreasing organic carbon content (Park and Erstfeld 1999; Lebo et al. 2003). However, in Lake Leppävesi, the dissolution of WSs in proportion to the WSs amount in the agitated wet sediment was about the same than in Lake Kuhnamo and Lake Vatianjärvi, although the TOC content was only 1.2 %. On the other hand, it must be realized that elutriates in our experiment does not necessarily describe exactly the same bioavailability potency as freely dissolved concentration of the contaminants, and therefore the dissolution potency can be used only as a rough indicator of bioavailability.

Compared to the resin acids (RAs), the dissolution of WSs were shown to be slower; for WSs it took 7 days to reach the highest state of desorption, whereas RAs reached equilibrium in 3 days (Meriläinen et al. 2006). This probably reflects the higher hydrophobicity of WSs (log K_{ow} 9.65 for β-sitosterol) compared to the RAs (log K_{ow} 6.52 for dehydroabietic acid), and therefore stronger binding of WSs to sediment. Also betulinol is more hydrophobic (log K_{ow} 8.18) than RAs. Typically, the rate of desorption decreases over time (Chen et al. 1999; Meriläinen et al. 2006). In this study, the temperatures and durations of each experiment varied and kinetic sampling was not done, and therefore the rate of desorption cannot be estimated. However, we assume that the differences in desorption of betulinol and WSs may partly be due to agitation time but different temperatures probably had an effect also. The longer agitation at a lower temperature probably simulates more natural case of flooding which occur every spring in Northern countries due to melting snow. Turnover in spring and autumn in boreal latitudes may also cause some sediment erosion and dissolution of the contaminants. For example, elevated concentrations of resin acids have been detected in fish bile during spring turnover in Southern Lake Saimaa (Karels and Oikari 2000; Meriläinen and Oikari 2008).

As expected, the highest concentrations of betulinol and WSs in the dissolution experiments were found in Lake Kuhnamo elutriates. Concentrations were site-dependent, and corresponded to the conditions in the sediment. Surprisingly high amounts of betulinol were measured from the sediment, and dissolved concentrations can be considered high enough to cause harmful effects on fish. Betulinol is an endocrine disrupting chemical (Mellanen et al. 1996; Christianson-Heiska et al. 2004; 2008) but does not include vitellogenin production as for instance β-sitosterol and β-sitostanol (Mellanen et al. 1996). Betulinol may have endocrine-disrupting potency already at a concentration of 5 μg/l and it also may weaken the disease defense mechanism of fish (Christianson-Heiska et al. 2004). In our study, multiple times higher concentrations were measured from the elutriates and, based on these results, even the sediment 33 km downstream from the pulp and paper mill effluent discharge may affect the fish as 22–31 μg/l betulinol was measured from the elutriate. Based on these results, more ecotoxicological studies of betulinol are highly recommended.

β-sitosterol may cause estrogenic effects, such as induction of vitellogenin production on fish (Mellanen et al. 1996; Tremblay and van der Kraak 1998; 1999). Not only estrogenic effects but also the masculinization of female fish has been detected in pulp and paper mill contaminated waters. Jenkins et al. (2003) suggested that microbial degradation of pine phytosterols may transform sterols to the progesterone and further changes into androstenedione and other bioactive androgens which could explain the masculinization. In addition, reduction of plasma cholesterol and testosterone levels has been observed (Tremblay and van der Kraak 1998; 1999). However, moderately high dissolved concentrations of β-sitosterol (10–20 mg/l) are needed to cause severe consequences on maturing lake trout, such as egg mortality, smaller egg size, lower mean weight of the yolk sac stage larvae, and higher prevalence of deformed or otherwise diseased larvae (Lehtinen et al. 1999). Lower concentrations (5 mg/l) caused early hatching and increased hatchability in rainbow trout, Japanese medaka, and American flagfish embryos (Orrego et al. 2011). In our dissolution experiment, effective concentration of WSs were not exceeded, as the maximum concentration total WSs was only 0.3 and 0.08 mg/l for β-sitosterol. In conclusion, WSs are capable of creating various disruptive effects on aquatic biota but in our study site WSs should not cause harm to animals.

Conclusions

In summary, this study supports previous results that wood extractives of pulp and paper production can be transported over 30 km and most likely further downstream from the point of discharge. In spatial interpolation high amounts of betulinol and β-sitosterol were estimated as being distributed in the uppermost sediment in lakes downstream from chemical wood industry. In addition, dissolution experiments revealed that betulinol and WSs can be desorbed from sediments to water and we suggest that desorption of wood extractives depends on their concentration in the sediment. In the case of severe sediment erosion, considerable amounts of betulinol and WSs could be desorbed. Betulinol may be dissolved at concentrations which may be high enough to cause toxic effects in aquatic animals. We recommend that long-term desorption experiments, especially with various temperatures and dynamics, be carried out in order to provide more comparable results. In addition,

toxicity tests with betulinol are recommended with benthic animals and fish.

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III

VERTICAL DISTRIBUTION OF AHR-ACTIVATING COMPOUNDS IN SEDIMENTS CONTAMINATED BY MODERN PULP AND PAPER INDUSTRY

by

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VERTICAL DISTRIBUTION OF AHR-ACTIVATING COMPOUNDS IN SEDIMENTS CONTAMINATED BY MODERN PULP AND PAPER INDUSTRY

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ABSTRACT

Ethoxyresorufin-*O*-deethylase (EROD) activity is a sensitive biomarker of exposure to the chemicals which activate the aryl hydrocarbon receptor (AhR) and induce the cytochrome P450 system, such as many polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). Pulp bleaching was one of the main sources of PCDDs and PCDFs before the use of elemental chlorine ceased in early 1990s. PCBs were used in paper manufacturing before being banned in 1970s. In this study, the contamination history and recovery of a watercourse heavily loaded by the chemical wood industry was studied by analyzing PCDDs, PCDFs and PCBs from vertical sediment samples and by measuring hepatic EROD activity from fish intraperitoneally dosed by the sediment extracts. No PCDDs or PCDFs were found above the chromatographic limit of detection from the study area and only small amounts of PCB congeners 101, 138 153, and 180 were present. No increased EROD activity was observed in fish indicating absence of any AhR-activating compounds in the surface sediment, to about 15 cm depth, representing about the last 20 years. It can be concluded that nowadays organochlorines and other AhR-ligands do not harm the previously heavily polluted watercourse.

Keywords: Ethoxyresorufin-*O*-deethylase; polychlorinated dibenzo-*p*-dioxin; polychlorinated dibenzofurans; polychlorinated biphenyl; pulp and paper industry; sediment.

1 INTRODUCTION

The chemical wood industry was long a remarkable source of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) as by-products of pulp bleaching (Owens 1991, Fletcher & McKay 1993), similar to polychlorinated biphenyls (PCBs) which were used in manufacturing of carbonless copy paper from the 1950s to the 1970s (Carr et al. 1977). PCDDs, PCDFs and PCBs are included on the original list of persistent organic pollutants under the Stockholm Convention (Anonymous 2012a), and due to their persistence and toxic characteristics these chemicals are still widely studied (Owens 1991, Fletcher & McKay 1993, Sundqvist et al. 2009). While the worldwide pulp and paper industry has mostly used total chlorine free (TCF) or elemental chlorine free (ECF) bleaching methods since the early 1990s, this has largely decreased but not totally eliminated the formation of the PCDDs and PCDFs (Chapman et al. 1991, Strömberg et al. 1996). Although the deposition of PCDDs and PCDFs to the sediments has decreased since 1980s (Huestis et al. 1997), elevated concentrations in the surface sediment still exist in polluted watercourses and coastal areas (Sundqvist et al. 2009), and benthic animals may remarkably affect the fate of the chemicals accumulated in the sediments due to bioturbation, migration and trophic transfer (Reynoldson 1987). Regarding to PCBs, many countries banned their use already in the 1970s. However, elevated concentrations of PCBs also are still measured from both terrestrial and aquatic environments (Anonymous 2001).

Hepatic ethoxyresorufin-*O*-deethylase (EROD) is an integrated endpoint to all ligands of aryl hydrocarbon receptor (AhR) activating compounds, which are bioavailable to animals. EROD activity is used as a sensitive biomarker of exposure to the PCDDs and PCDFs and other dioxin-like planar compounds (van der Oost et al. 2003) and its increased activity has been detected from fish exposed to pulp and paper mill discharges (Karels et al. 2000, van der Oost et al. 2003). Furthermore, some of the PCBs (van der Oost et al. 2003) and polyaromatic hydrocarbons, for instance retene (Fragoso et al. 1998), are known EROD inducers. The transition to ECF and TCF technologies decreased EROD activity in fish caught downstream from pulp and paper industry (Karels et al. 1999, 2000).

In this study PCDDs, PCDFs and PCBs were analyzed from sediments contaminated by pulp and paper mills from the depths which have deposited after the use of elemental chlorine in bleaching ceased in 1993. The primary aim of the study was to assess the presence of these compounds in the surface sediment layers, i.e. those which could nowadays be exposure sources to fish or benthic invertebrates either by bioturbation or by other disturbances. Burrowing freshwater invertebrates are most abundant in the uppermost five to ten cm of sediment, for instance chironomids commonly stay in the uppermost two cm (Nogaro et al. 2008), whereas oligochaetes may burrow down to 10 cm (Särkkä 1987). Also other disturbances, like heavy floods or runoffs may

remarkably increase the water flow and hence the sediment remobilization and bioaccessibility of the chemicals present there (Stachel et al. 2004). Accordingly, the aim in this study was to include all the AhR ligands present in the given sediment by direct administration of them to the fish body, followed by EROD activity measurement as a response. The null hypothesis was that nowadays the uppermost sediment layers of studied watercourse should not contain AhR-activating compounds.

2 MATERIALS AND METHODS

2.1 Study sites

The study area is located in a river-like watercourse, downstream from the pulp and paper industry in Äänekoski City, in Central Finland. In 1899, a board mill was established, followed by paper and saw industries shortly thereafter. The first chemical pulp mill was introduced in 1938, producing sulfite cellulose and chlorine-based bleaching was started in the 1950s. The kraft mill started in 1961 and a new board mill in 1966. Untreated wastewaters of the mills were discharged to the watercourse until the 1970s when the mechanical treatment began. However, the watercourse did not start to recover before 1985, when both old pulp mills were closed and a new kraft mill with activated wastewater treatment was initiated. In 1993, ECF bleaching process using chloride dioxide, and oxygen delignification was introduced. Finally, in 1999, pulp washing and oxygen delignification were further improved resulting in 30 % decrease of chemical oxygen demand by wastewaters. The paper and board mills had their own wastewater treatment plant using chemical and mechanical methods. In 2011, the production capacity of the he board mill and ECF bleached pulp was 240 000 and 520 000 tons per year, respectively. Paper production was ceased at the end of 2011.

For comparison, Southern Lake Saimaa in South-East Finland was used as a potential positive reference as previous studies on fishes conducted there have shown induced EROD activity (Karels et al. 1999, Oikari et al. 2002). Moreover, pulp and paper mills discharging to Southern Lake Saimaa, have quite a similar history as the mills in Äänekoski (Lahdelma & Oikari 2005).

2.2 Sediment sampling

Sediment samples were taken from Lake Vatianjärvi and Lake Leppävesi, 15 and 33 km downstream from pulp and paper mills in Äänekoski, respectively. The watercourse collects its inflows from two sources: relatively humic water from the Saarijärvi-Naarajärvi route and oligotrophic water from the Viitasaari-

Keitele route. The reference site is located in oligotrophic Lake Keitele, 16 km upstream from the mills.

Samples were taken by Limnos device in late October 2011 during the turnover which occurs annually in autumn and spring in most boreal lakes. Therefore, the water temperature (7 °C) and oxygen concentration (11–13 mg l^{-1}) were similar in the water surface and near the sediment in every sampling site. Sediment profiles were sectioned to 2 cm slices in the field and stored in cooled boxes during the sampling. For the bioassay, three to five replicates were combined to the glass jars and stored in the dark at 4 °C until extracted and used in the bioassay, within two months. For chemical analysis of chlorinated compounds, one sample (approximately 150 g) was placed to the separate 1.5 dl glass jars and kept in the dark at 4 °C before being freeze dried for analyzes of PCDDs, PCDFs and PCBs.

Surface sediment (0–10 cm) taken by an Ekman device from Southern Lake Saimaa was the positive control. Southern Lake Saimaa sediment is known to induce hepatic EROD activity in rainbow trout (Oikari et al. 2002). Rautniemi sediment from Southern Lake Saimaa was the pristine reference, and was also taken by an Ekman device in 2011.

2.3 Sediment characteristics and deposition rate

Loss on ignition (LOI) was determined from the dry sediments (SFS 1990) (550 °C, 2h) and the results were normalized with LOI, considered as sufficient estimate of the organic carbon concentration. The normalization with the LOI was crucial because AhR-ligands as hydrophobic xenobiotics associate with the organic part of the sediments (Delle Site 2001).

In order to estimate the age of the analyzed and assayed layers of lake sediments were, their vertical aging from each two centimeter layers were determined. About three grams freeze-dried, homogenized sediment was used from each sediment layer. Dating was based on the activity level of ^{137}Cs which ones source is the Chernobyl fallout of 1986 (Sanada et al. 2002). Gamma-radiation associated to ^{137}Cs decays was measured with an Ortec GMX series high-purity germanium coaxial photon detector. The sample and the detector head located inside a low-background shield consisting of 10 cm thick lead walls and additional layers of 3.5 mm of Sn and Cu to eliminate natural background. Each sample was measured for 24 hours to accumulate necessary statistics even from weak ^{137}Cs activities. Measurements were done in the Department of Physics in the University of Jyväskylä.

2.4 EROD-induction bioassay with rainbow trout

Juvenile rainbow trout (*Oncorhynchus mykiss*) (3.0–5.3 g) were transferred from Hanka Taimen Inc. (Hankasalmi, Finland) to the wet laboratory of the University of Jyväskylä, and acclimatized for ten days at 13 ºC in continuously flowing artesian well water (pH 7.3) in 500 liter tanks with a photoperiod of 16:8 light:dark. Fish were fed once a day with Royal Hercules fish food (c.a. 0.5 % of wet fish weight). One day before the start of the experiments and during the exposures fish were not fed. Maintenance and bioassays were performed in accordance with the valid laws on animal testing and were licensed (ESLH-2007-06053/Ym-23) by the Finnish authority.

Rainbow trout was bioassayed with aid of intraperitoneal (i.p.) injection as described in Oikari et al. (2002). For i.p. dosing, sediment extractions were done by hexane from 20–50 g dry sediment according to Oikari et al. (2002). However, retene and other labile organic compounds were not removed by sulfuric acid treatment. Sediment layers from specific depths were combined from three to five replicates. In the sediments of Lake Vatianjärvi and Lake Leppävesi the proportion of organic matter was about half of that in sample of Southern Lake Saimaa sediment. Therefore, double amount of sediment (50 g) of Lake Vatianjärvi and Lake Leppävesi was extracted compared to the sediment of Southern Lake Saimaa (25 g). Lake Keitele sediment was very wet and thus the amount of dried sediment remained lower and extracted amount was 20 g. All the results were weighted by LOI.

Two series of experiments were done. In the first series, the extracts were isolated from three sediment depths: 2–4 cm, 8–10 cm and 14–16 cm from Lake Vatianjärvi, Lake Leppävesi, and Lake Keitele (reference). Retene with i.p. doses 0, 0.1, 10, 50 and 100 µg g^{-1} fish, used as a positive control, revealed good dose dependency in juvenile rainbow trout (Fig. 1). To assure dose dependency, three dose levels from Southern Lake Saimaa sediment extracts were involved: 10 %, 50 % and 100 % extract. Theoretically, in terms of retene, as its concentration in Southern Lake Saimaa sediment sample was 224 µg g^{-1} (dw), the doses corresponded for 7, 14 and 28 µg retene per g fish, respectively. DMSO in sunflower oil (1:9, v/v) was a blank control.

In the second series, treatment was repeated with sediment extracts from the 8–10 cm layers of Lake Vatianjärvi, Lake Leppävesi and Lake Keitele (reference). Additionally, Rautniemi reference sediment from Southern Lake Saimaa was studied. A blank control and positive controls (retene 50 µg g^{-1} fish, and 100 % extract of Southern Lake Saimaa sediment) were included. For further assurance of potency to induce CYP1A, another dosing after 24 h was conducted with these lake sediment extracts. Fish of this second series were from the same origin as in the first one, and the maintenance was carried out in similar conditions.

For dosing, fish were anesthetized with MS222 (75 mg l^{-1}) and the extract of sediment was injected intraperitoneally at a volume of 5.0 µl g^{-1} fish. After

injection, fish were immediately released to the aerated water where they recovered from the narcosis within 30 seconds, and then released to the 50 liter aquaria. Eight juvenile rainbow trout per aquarium were dosed. The same photoperiod, water temperature and pH were maintained as during the acclimatization. After 24 h about half of water was replaced by pre-aerated water.

After 72 h, fish were anesthetized with MS222 (75 mg l^{-1}) and killed by transection of the spinal cord. Livers were immediately removed and stored in liquid nitrogen. EROD activity from hepatic postmitochondrial supernatant (S9 fraction) was analyzed according to Vehniäinen et al. (2012). All EROD and protein measurements were made in triplicate, or in duplicate if the liver was too small for three analyses. EROD activity was quantified by measuring fluorescence by Fluoroscan Ascent fluorometer (Thermo Labsystems) at 30 s intervals, the total scan time being 3 min 30 s, at emission and excitation wavelengths of 584 and 540, respectively. Total protein in S9 fraction was analyzed by modified Lowry method using Bio-Rad DC Protein Assay (CA, USA), with bovine serum albumin as standard. Absorbance was measured by Labsystems iEMS Reader MF spectrophotometer.

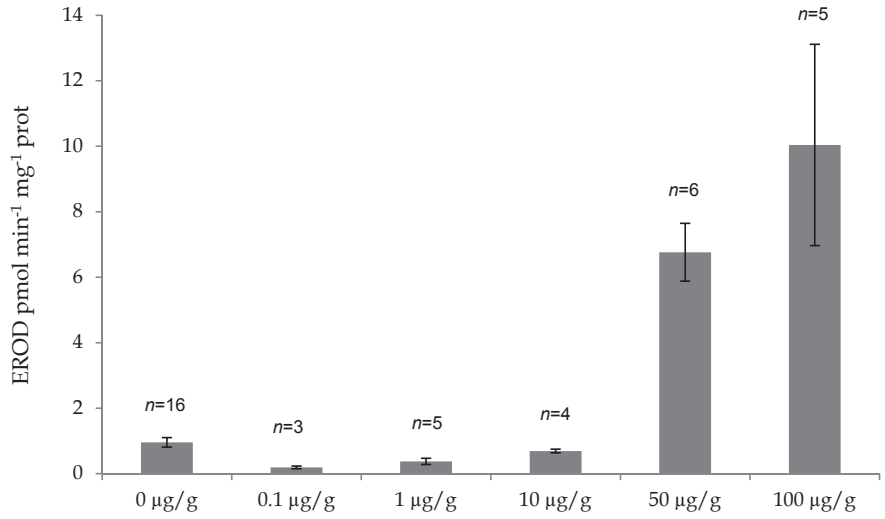

FIGURE 1 Hepatic EROD activity (± S.E.) in juvenile rainbow, injected i.p. by retene diluted in DMSO:sunflower oil (1:9, v/v). The number of fish is marked by *n*.

2.5 Analysis of PCBs, PCDDs and PCDFs

PCDDs, PCDFs and PCBs were analyzed from 5 g freeze dried sediment, which were homogenized and extracted in a Dionex Ase 200 extractor (Anonymous

2012b) with a mixture of petrol ether, acetone, hexane and diethyl ether (9:5.5:2.5:1, v/v). Extraction was done under 1500 psi pressure at 100 °C for 20 min. After extraction, acetone and dimethyl ether were evaporated in a nitrogen stream, and the rest concentrated by rotary evaporator to about 5 ml volume. The extracts were then washed twice with 5 ml sulfuric acid, and activated Cu powder was added to remove the sulfur. PCB analyzes and extraction of PCDDs and PCDFs were done in the accredited laboratory, the Institute for Environmental Research, Jyväskylä, Finland.

PCBs (Table 1) were measured by GC-MS (Agilent 6890) with an electrochemical detector (ED) by using the method (O-20A) based on standards: ISO 10382:2002 (E) and SFS-EN 15308-2008 and those used by Paasivirta et al. (1986). PCB-30 (Dr. Ehrenstorfer, Germany) and ^{13}C labeled PCDD/PCDF (Wellington Laboratories, Canada) were the internal standards for PCBs and PCDD/PCDFs, respectively. For measurements of PCDDs and PCDFs, the extracts were purified by alkaline Al_2O_3 column (Acros Organics) and washed by 2 % dichloromethane in hexane (10 ml). PCDDs and PCDFs were eluted by 10 ml of dichloromethane-hexane solution 1:1 (v/v), which was gently evaporated to dryness with a nitrogen stream, and diluted in toluene. Seven PCDD congeners and ten PCDF congeners (Table 1) were analyzed by GC-MS using the following temperature program: start at 100 °C (held for 50 s), rising 20 °C min^{-1} to 220 °C (held for 8 min), rising 4 °C min^{-1} to 290 °C (held for 1 min) and rising 20 °C min^{-1} to 310 °C (held for 50 s). Concentrations were analyzed first from the 14–16 cm sediment layer of Lake Vatianjärvi and, based on these results, three sediment depths from each sampling site were chosen for further analyses and to the bioassays. PCDDs and PCDFs were not analyzed from the Southern Lake Saimaa sediment.

TABLE 1 Analyzed PCB and PCDD/PCDF congeners with the analytical limits of detection (LOD).

PCBs	LOD (µg g^{-1}, dw)	PCDDs	LOD (ng g^{-1}, dw)	PCDFs	LOD (n g^{-1}, dw)
8	<0.002	2,3,7,8-TeCDD	<0.01	2,3,7,8-TeCDF	<0.02
18	<0.002	1,2,3,7,8-PeCDD	<0.01	1,2,3,7,8-PeCDF	<0.02
28	<0.002	1,2,3,4,7,8-HxCDD	<0.03	2,3,4,7,8-PeCDF	<0.02
52	<0.002	1,2,3,6,7,8-HxCDD	<0.03	1,2,3,4,7,8-HxCDF	<0.03
101	<0.002	1,2,3,7,8,9-HxCDD	<0.03	1,2,3,6,7,8-HxCDF	<0.03
118	<0.002	1,2,3,4,6,7,8-HpCDD	<0.03	2,3,4,6,7,8-HxCDF	<0.03
153	<0.002	OcCDD	<0.1	1,2,3,7,8,9-HxCDF	<0.03
105	<0.002			1,2,3,4,6,7,8-HpCDF	<0.03
138	<0.002			1,2,3,4,7,8,9-HpCDF	<0.03
128	<0.002			OcCDF	<0.1
156	<0.002				
180	<0.002				

2.6 Statistics

Due to heteroscedasticity of the variances the significance of the differences between the retene concentrations and EROD activity was analyzed by using Welch ANOVA and Dunnett T3 post hoc test in both positive controls: retene treatments and Southern Lake Saimaa sediment extracts. Injected retene concentrations via the sediment extract from Southern Lake Saimaa were calculated from the measured concentration in dry weight, and correlation between the sediment retene concentrations and EROD activity was analyzed by Pearson's correlation test. A significance level of 0.05 was taken as indicative of difference between means. Statistical tests were done by using SPSS software (SPSS Inc., USA).

3 RESULTS

3.1 Sediment characteristics and deposition rate

LOI was 9 % and 6 % in Lake Vatianjärvi and Lake Leppävesi, respectively (Supplementary data). In the reference site, Lake Keitele, LOI higher, being 17 % per dw on average. In Southern Lake Saimaa, one kilometer downstream from the mills, LOI was approximately 26 % and in the Rautniemi reference it was 15 %, measured from the 0–10 cm sediment.

In Lake Keitele reference, the regarding sediment aging, the ^{137}Cs peak was at a depth of 4–6 cm, indicating that Chernobyl accident in 1986 was below that. ^{137}Cs activity in the sediment of Lake Päijänne, nearby the study site, was about 30 % of the maximum activity in year 1986, whereas the maximum activity was measured about two years later (Saxén & Outola 2009). Hence, in Lake Keitele, the year 1986 could be located from the depth of 6–8 cm (Fig. 2a). In Lake Vatianjärvi, ^{137}Cs started to increase in the deepest sample in the depth of 18–20 cm (Fig. 2b). Exact dating cannot thus be done as the maximum peak was not found within the analyzed vertical profile. In Lake Leppävesi, sedimentation rate was probably the same or a little slower than in Lake Vatianjärvi as an elevated ^{137}Cs activity was found from the depth of 16–18 cm, although the overall trend is not as clear as in the other lakes (Fig. 2b).

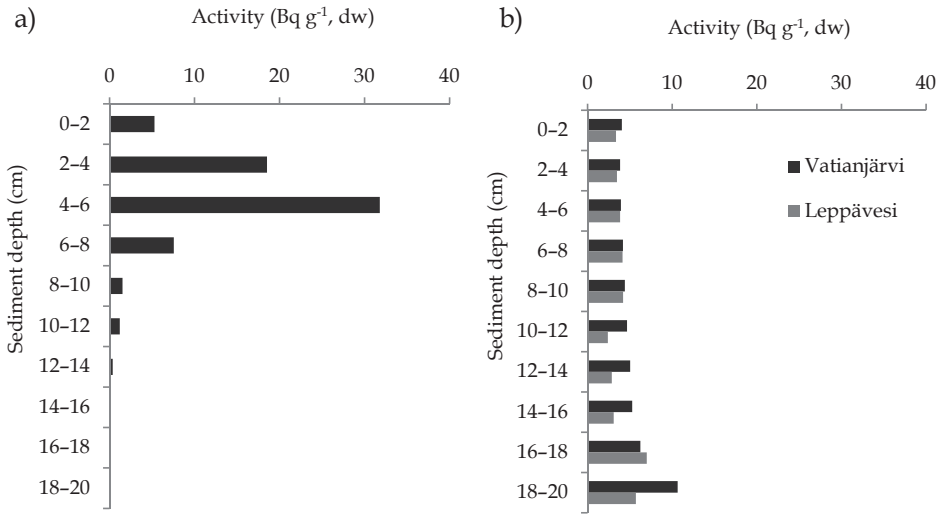

FIGURE 2 Deposition of ^{137}Cs in the sediments of Lake Keitele reference (a), Lake Vatianjärvi and Lake Leppävesi (b).

3.2 Presence of compounds inducing EROD activity

In the positive control, Southern Lake Saimaa, the EROD activity increased with increasing dose (Fig. 3), similar to retene (Fig. 1). A significant positive dependence was observed between the retene concentrations in the sediment and the EROD activity (p = 0.019). Also, the significant difference (p = 0.048) was observed between the Southern Lake Saimaa tretments (10, 50 and 100 % extract), but in the multiple comparisons statistically significant differences were not found because of large variances caused by relatively small number of fish. Compared to the blank, the most considerable difference was observed in the 100 % dose (p = 0.069) (Fig. 3).

EROD induction in fish injected by the extracts of the Äänekoski watercourse sediments were at the same level as in the Keitele reference (Fig. 4). As the values were similar and no statistical differences were observed, both in the first and in the second series of bioassays, data of these two was combined. The null hypothesis was accepted because no statistically significant differences were shown and no increase of liver EROD activity were evident following a doubling the dosing by an equal amount after 24 h from the first dosing of Lake Keitele (reference) or Lake Vatianjärvi sediment extracts.

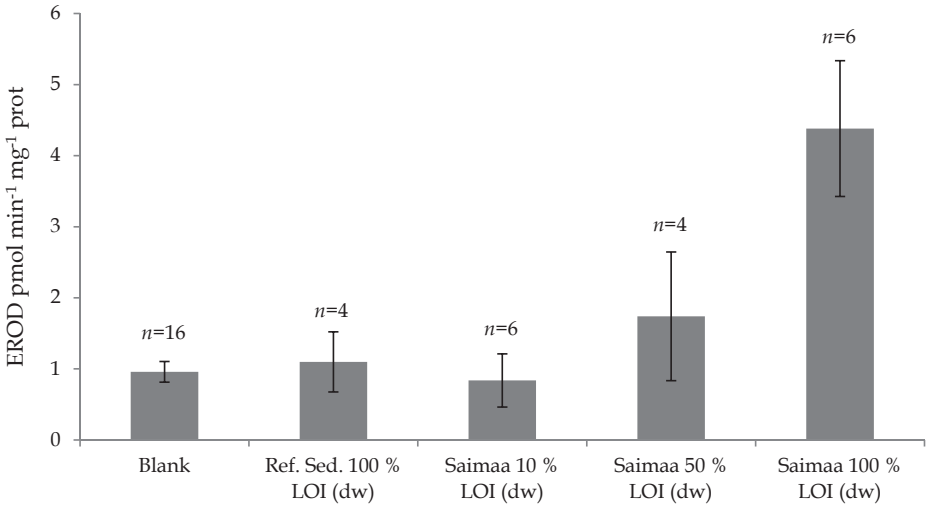

FIGURE 3 Hepatic EROD activity (± S.E.) in rainbow trout, injected i.p. by 10 %, 50 % and 100 % doses of Southern Lake Saimaa sediment extract diluted in DMSO:sunflower oil (1:9, v/v). DMSO in sunflower oil was the blank and Rautniemi sediment as the reference sediment. For 100 %, 25 g dry sediment was extracted. EROD activity is weighted to the relative amount of sediment organic matter, defined by loss on ignition (LOI). The number of fish is marked by *n*.

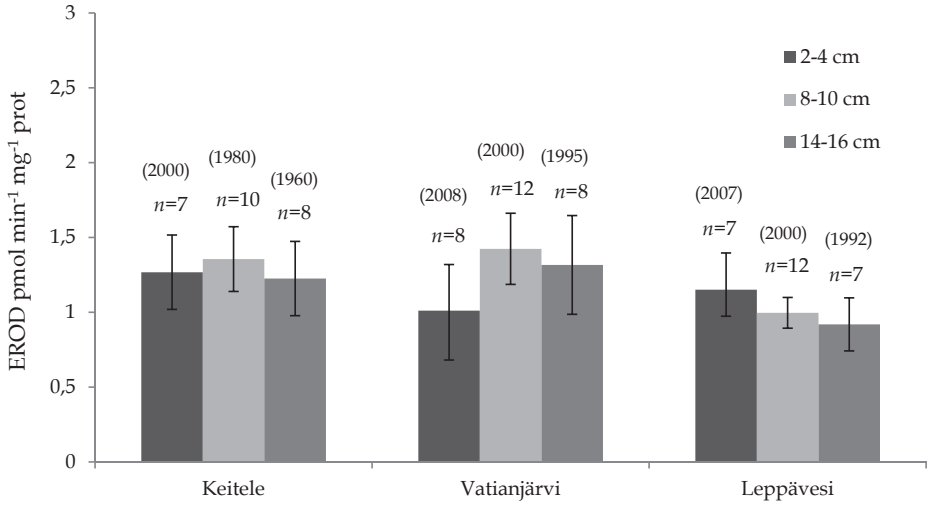

FIGURE 4 Hepatic EROD activity (± S.E.) in juvenile rainbow trout, injected i.p. by the sediment extract isolated from three depths and diluted in DMSO:sunflower oil (1:9, v/v). EROD activity is weighted by loss on ignition. Approximate year of sediment age is given in parenthesis (of. Fig. 2), for Lake Vatianjärvi and for Lake Leppävesi only as a rough estimate. The number of fish is marked by *n*.

3.3 AhR-activating compounds in the sediment

In the sediment samples from the Äänekoski watercourse at the depths of 0–2, 8–10 and 14–16 cm, PCDDs or PCDFs were not measurable above the limits of detection: TeCDD and PeCDDs < 0.01 ng g^{-1}, TeCDF and PeCDFs < 0.02 ng g^{-1}, HxCDDs, HxCDFs, HpCDD and HpCDFs < 0.03 ng g^{-1}, and OCDD and OCDF < 0.1 ng g^{-1}, (dw). Trace concentrations of PCBs were found only from the sediment of Lake Vatianjärvi (Table 2).

TABLE 2 PCB congeners (mg kg^{-1}, dw) in the sediment of Lake Vatianjärvi, 15 km downstream from the pulp and paper mills in Äänekoski City, Central Finland. Samples were taken in October 2011. Limit of detection was 0.002 µg g^{-1} (dw)

PCB congener	2–4 cm	8–10 cm	14–16 cm
101	<0.002	<0.002	0.002
138	0.003	0.003	0.005
153	0.004	0.005	0.006
180	0.004	0.004	0.005

4 DISCUSSION

Monitoring of harmful organic compounds released from pulp and paper industry in the Äänekoski watercourse has been limited. However, the mill renewal and improved wastewater treatment begun in 1985 started the recovery of the watercourse towards preindustrial era. Paasivirta et al. (1988) measured concentrations of chlorinated compounds in lakes receiving effluents before and after the introduction of the new kraft mill, and detected significant decreases in concentrations of chlorophenolics. Reductions of total organic carbon (TOC) and adsorbable organic halides (AOX) in the new activated treatment plant were more than 80 % and 60 %, respectively (Paasivirta et al. 1988). Thus, also the discharge of PCDDs and PCDFs, as parts of AOX, has likely decreased even though elemental chlorine bleaching was still in use until 1993. Despite the modern technology, elevated AOX concentrations, account for mostly biodegradable mono- and dichlorinated compounds, are still measured from water in the Äänekoski watercourse (Anonymous 2006). However, based on this study, chlorinated dioxins and furans should not be a problem anymore. The deepest sediment layer (14–16 cm) studied from two downstream lakes describes the potential impacts in years 1992–1995, based on the ^{137}Cs deposition and observations by Saski et al. (1997). In Lake Leppävesi, the

sedimentation rate probably was slightly slower than in Lake Vatianjärvi. In comparison, Maatela et al. (1990) estimated that the sedimentation rate in Lake Leppävesi might be only half centimeter per year in the uppermost sediment depth of 10 cm, which is slightly less than our study shows.

Ecotoxicologically, for one or two decades before the environmental and technical remodeling of the pulp and paper industry in Äänekoski took place in 1985, the watercourse can be suggested to have been very heavily impacted. In 1975, lower activity of mixed function oxygenase (MFO) system was observed in pike (*Esox lucius*) caught from Lake Vatianjärvi when compared to the reference lake (Ahokas et al. 1976). Ahokas et al. (1976) hypothesized a disruption in MFO system to be caused by chronic liver damage due to the heavy pollution of Lake Vatianjärvi. Related hepatotoxic effects were found in exposure of fish to known hepatotoxic chemicals (Oikari & Jimenez 1992). However, the chemical factors causing the apparent liver damage in Lake Vatianjärvi pike remained unclear (Ahokas et al. 1976). It was therefore unfortunate that the sediment cores assayed in this research did not extend to a depth to reach the age of the early 1970s.

In 1980, the emissions of bleached kraft mill effluent in Äänekoski were still highly toxic to fish. Vuorinen & Vuorinen (1985) carried out long-term experiments with maturing brown trout (*Salmo trutta*), and low concentration (0.5 %) of mill effluent caused serious effects on reproduction. In addition, abnormality of yolk and disturbances in the blood circulation, both signs of blue sac disease (Brinkworth et al. 2003), was observed in sac fry from the females exposed to 0.2 % effluent (Vuorinen & Vuorinen 1985). The latter consequences can be considered as evidence of the presence of EROD-activating compounds in the effluent, as the symptoms of blue sac disease have never been observed without the activation of CYP1A system. In late 1980s, the concentrations of PCDDs in Lake Vatianjärvi surface sediment were 1.4 ng g^{-1} dw, but 2,3,7,8-TeCDD, the most toxic dioxin, was not observed, neither were any PCDFs found (Koistinen et al. 1990).

Today, in the uppermost 16 cm layer of sediment, no effective concentrations of AhR-activating compounds exist in the Äänekoski watercourse. This is supposed by i.p. injection bioassays of Lake Vatianjärvi and Lake Leppävesi sediment extracts and further by analyses of PCDDs, PCDFs and PCBs from the sediment, which all were either below the analytical detection limit or at such level which could not induce EROD activity. Also retene concentrations are low in the sediments of Äänekoski watercourse (Rämänen et al. 2010). For reasons related to quality assurance, we used Southern Lake Saimaa sediment as a positive control, showing equal EROD-potential as in the similar experiment with similar sediment one decade earlier (Oikari et al. 2002). The validity of bioassay is an evidence of the absence of AhR-activating compounds in the sediment of the Äänekoski watercourse, and the double dosing of extracts without increased EROD activity confirmed this. In contrast to the Äänekoski watercourse, sediment of Southern Lake Saimaa is known to be highly contaminated by retene and some other AhR-activating

compounds, with equal persistency as PCDDs and PCDFs (Oikari et al. 2002). The result of this study is further supported by laboratory bioassays where juvenile rainbow trout were exposed to the water with sediment of Lake Vatianjärvi. Also EROD activity in wild fish caught from Lake Vatianjärvi in 2010 was at the same level or perhaps even lower than in two reference lakes (Ratia et al., unpublished).

Regarding to PCBs, another chemical group with some AhR ligands analyzed in this study, similar recovery of the Äänekoski watercourse is obvious. In the late 1980s and early 1990s, some PCBs were measured in the muscles of pike, caught downstream from the Äänekoski mills. The highest concentration of PCBs was approximately 160 ng g^{-1} in 1989 and decreased by about 50 % until year 1992 (Anonymous 1992). However, still in 2003, low concentrations of PCBs were detected in fish (Anonymous 2006). In the present study, small amounts (2–6 ng g^{-1}, dw) of PCB congeners 101, 105, 138 and 180 were measured from the sediment but of these, only PCB105 is known to act as a ligand of AhR and an inducer of CYP1A1 (Schmitz et al. 1995). Concentrations of found PCBs in this study were near or below the documented background levels (10–100 ng g^{-1}, dw) (Paasivirta et al. 1990, Särkkä et al. 1993). For comparison in the late 1980s, the amount of PCBs measured from sediment in Lake Kuhnamo, which is the closest lake of the Äänekoski mills was about 380 ng g^{-1} (dw) (Paasivirta et al. 1990). As both the bioassays with fish and analyses of the sediment support the recovery of Äänekoski watercourse, it can be concluded that the surface sediment in the previously heavily polluted watercourse is nowadays a suitable habitat for the sediment-burrowing invertebrates and it does not pose a risk to fish either. Coincidentally, in the Kuusaankoski rapids, just below Lake Vatianjärvi, morphological damage in caddis larvae were still observed at the beginning of millennium, but decreased to the reference level in 2008 (Ratia et al. 2012). There are thus several evidences that the recovery of the watercourse took over a period of 20 years.

5 CONCLUSIONS

Fish bioassays revealed no increased EROD activity when juvenile rainbow trout were exposed to the sediment extracts from the Äänekoski watercourse. PCDDs and PCDFs were not present above the analytical limits of detection in the uppermost 0–16 cm sediment. Some PCBs were found from Lake Vatianjärvi sediment but concentrations were at background level and should not cause harmful effects to animals. It can be concluded that nowadays the pulp and paper industry in Äänekoski City, or the historically contaminated sediments in the watercourse downstream, do not release CYP1A inducing compounds. Ecotoxicological risks related to dioxin-like pollution are improbable today.

Acknowledgements

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Supplementary data

Loss on ignition (LOI) (% of dw) in the sediments of Äänekoski watercourse, Central Finland.

Sediment depth	Lake Keitele (ref.)	Lake Vatianjärvi	Lake Leppävesi
0–2 cm	20	9.3	6.8
2–4 cm	18	9.4	6.6
4–6 cm	14	8.7	6.4
6–8 cm	14	9.3	6.1
8–10 cm	15	9.2	5.9
10–12 cm	16	8.7	5.8
12–14 cm	16	8.5	5.6
14–16 cm	16	8.7	5.5
16–18 cm	17	8.3	4.7
18–20 cm	19	7.8	5.8

IV

RECOVERY OF HISTORICALLY CONTAMINATED WATERCOURSE POLLUTED BY CHEMICAL WOOD INDUSTRY – EROD ACTIVITY IN FISH AS BIOMARKER

by

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RECOVERY OF HISTORICALLY CONTAMINATED WATERCOURSE POLLUTED BY CHEMICAL WOOD INDUSTRY – EROD ACTIVITY IN FISH AS BIOMARKER

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ABSTRACT

Despite outstanding process alterations over decades, pulp and paper mill contaminated sediments and continuing exposure by the effluents may still have effects on biota. In this study, ecotoxicological impacts in the boreal watercourse were analyzed by measuring ethoxyresorufin-*O*-deethylase (EROD) induction from wild fish populations and from experimentally exposed fish. In order to assess the role of sediment-borne chemicals, juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed in the laboratory to the surface sediments of Lake Vatianjärvi and Southern Lake Saimaa, both watercourses impacted by chemical wood industry for approximately a century. Hepatic EROD activity was measured also from roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) caught from Lake Vatianjärvi. Increased EROD activity was not observed in wild fish caught from Lake Vatianjärvi nor in rainbow trout exposed to the sediment of Lake Vatianjärvi, but it was observed in rainbow trout exposed to the sediment of Southern Lake Saimaa, probably explained by critically high concentrations of retene. The results of both our laboratory and the field study indicate the absence of EROD inducing compounds of the previously heavily contaminated Lake Vatianjärvi.

Keywords: Ethoxyresorufin-*O*-deethylase; pulp and paper industry; resin acids; retene; sediment.

1 INTRODUCTION

The chemical wood industry in Fennoscandia has a history of over a hundred years, and pulp and paper industry is one of the most serious polluters in the world. The impacts in aquatic organisms caused by pulp and paper effluents have been studied and reviewed especially in Northern Europe and North America (Ali & Sreekrishnan 2001, Hewitt et al. 2008). Nowadays pulp and paper productions have shifted more to Asia and South America, but the chemical wood industry still has a remarkable role also in the northern latitudes. Modern technology with efficient wastewater treatment and bleaching methods has significantly decreased the environmental impacts (Makris & Banerjee 2002). However, heavily contaminated sediments have remained and may still cause risks, especially in the case of persistent and toxic pollutants, such as polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (Yunker et al. 2002, Hemming et al. 2003).

Today PCDDs and PCDFs are still bioavailable in the sediments although dramatic reduction in concentrations of chlorinated compounds in the effluents (Oikari & Holmbom 1996, Solomon 1996) and in the sediments (Yunker et al. 2002) has occurred since the late 1980s and early 1990s. Also retene (7-isopropyl-1-methylphenantrene) causes similar toxic effects on fish as the dioxins (Guiney et al. 1997, Billiard et al. 1999, Brinkworth et al. 2003). Retene is a transformation product of resin acids (Tavendale et al. 1997, Leppänen & Oikari 1999a), which are continuously discharged from chemical wood industry (Koistinen et al. 1998, Makris & Banerjee 2002, Wartman et al. 2009). Although activated wastewater treatment efficiently remove most of the resin acids and other wood extractives (Koistinen et al. 1998, Makris & Banerjee 2002), pulp and paper production is continuously increasing worldwide (Forström et al. 2006) which may increase released volume of harmful compounds. Indeed, elevated concentrations of retene and resin acids have been still measured from the uppermost sediment layer (Leppänen & Oikari 1999a, Rämänen et al. 2010), wherefrom it may be bioavailable to aquatic animals (Leppänen and Oikari 1999b). Though concentrations of bioactive compounds may be decreasing compared to the 1980s and 1990s, and further sedimentation will bury heavily contaminated sediment deeper, the characteristics of depositing particles as well as local hydrology and morphology of the waterway is able to affect the fate of the pollutants. For instance, in the riverlike watercourses, as in case of this study, sediment erosion may still release bioactive compounds (Meriläinen et al. 2006, Rämänen et al. 2010) and affect the biota.

Hepatic ethoxyresorufin-*O*-deethylase (EROD) activity is a commonly used biomarker of exposure to the pulp and paper mill effluents (Solomon 1996, van der Oost et al. 2003, Wartman et al. 2009, Oikari et al. 2010). It is one of the most sensitive methods to indicate an exposure to the xenobiotics which are able to activate the aryl hydrocarbon receptor (AhR) and induce the cytochrome P450 (CYP1A) detoxification system (van der Oost et al. 2003). Of course, not all

chemicals in pulp and paper mill effluents induce EROD activity. For instance, the correlation between EROD activity and chlorophenolics or resin acids in the sediment or their metabolites in fish bile has not been observed (Soimasuo et al. 1998). Instead, PCDDs, PCDFs and some of the polychlorinated biphenyls (PCBs) induce EROD activity (van der Oost et al. 2003). Also polycyclic aromatic hydrocarbons (PAHs), such as retene, can act as EROD inducers (Fragoso et al. 1998, Billiard et al. 1999). Thus, AhR agonists exist among both halogenated and nonhalogenated chemicals.

The aim of this study was to analyze today's ecotoxicological impacts in the boreal watercourse in Central Finland, which has been loaded by chemical wood industry over one hundred years. The recovery has been underway since pulp mill renewal and improved wastewater treatment in 1985. However, ecotoxicological effects on biota were still detected more than two decades later. For instance, morphological damage in benthic invertebrates remained elevated until the year 2008 (Ratia et al. 2012). Therefore, to assess the present status of the watercourse, two approaches were taken: sampling of wild roach and perch downstream from the pulp and paper mill effluent discharge, and laboratory assays with juvenile rainbow trout exposed to the uppermost sediment. EROD activity was chosen as a traditional biomarker of exposure to the AhR-activating compounds present in the pulp and paper mill contaminated sediments, or in the effluents.

2 MATERIAL AND METHODS

2.1 Study site

The study site is located in the Äänekoski watercourse, Central Finland. The wood industry began in Äänekoski city in 1886 when a board mill was established, followed by paper and saw industry shortly thereafter. In 1938, the first pulp mill was introduced, using sulfite method (Tuuri et al. 1996). Chlorine-based bleaching began in the 1950s (Reunala et al. 1998), and impaired lake water quality was detected already during the same decade (Palomäki et al. 2006). In 1961, a second pulp mill was built, producing sulfate cellulose. The recovery of the watercourse began in 1985 when remarkable alterations were implemented; the old pulp mills were closed and a new kraft mill was introduced (Granberg et al. 1987). At the same time, wastewater treatment by activated sludge was initiated. In 1993, elemental chlorine free (ECF) bleaching with oxygen delignification was introduced, and in 1999 pulp washing and oxygen delignification were amended resulting in a 30 % decrease of chemical oxygen demand (COD) by wastewaters. Nowadays the pulp mill in Äänekoski is producing mainly hardwood pulp, about 500 tons per year. The volume of the discharged wastewaters is about 15 100 000 m^3 per year. During this study,

in 2009, the mills produced paper and board also, treating the wastewaters chemically and mechanically. In 2011, the paper production finished but board continues to be manufactured.

The Äänekoski watercourse is composed of several narrow lakes. The average flow rate is approximately 100 m^3 s^{-1}, and 225 m^3 s^{-1} at maximum. Wastewaters from the pulp and paper mills are discharging to Lake Kuhnamo (theoretical dilution 0.2–1 %, v/v) which has theoretical retention time only three days. From Lake Kuhnamo water flows to Lake Vatianjärvi, 15 km downstream (Table 1, Fig. 1). Lake Vatianjärvi was chosen as the primary study site, as the effects of pulp and paper industry has been documented there though it is moderately far from the source (Ahokas et al. 1976, Rämänen et al. 2010, Ratia et al.). Lake Vatianjärvi is eutrophic, its mean depth is four meters and maximum depth about 27 m. Reference sites were in Lake Palosjärvi and Lake Konnevesi, both oligotrophic and located in Central Finland (Fig. 1). Mean depths of Lake Palosjärvi and Lake Konnevesi are 10 and 12.5 m, and maximum 48 and 56 m, respectively. Southern Lake Saimaa in Southeast Finland was selected as a comparison lake, because of its quite similar pulp and paper mill contamination history, and it is also a very widely studied watercourse (Karels et al. 1998, Soimasuo et al. 1998, Leppänen & Oikari 1999a, Leppänen and Oikari 1999b, Karels & Oikari 2000, Lahdelma & Oikari 2005, Meriläinen et al. 2006, Oikari et al. 2010). The pulp industry started there in l896, and in 1992 elemental chlorine bleaching was replaced by chlorine dioxide, and activated sludge wastewater treatment was initiated. In 1995, chlorine dioxide was replaced by oxygen delignification. In contrast to Lake Vatianjärvi, Southern Lake Saimaa is oligotrophic and the theoretical retention time there is 60 days. Overall, Lake Saimaa is the largest lake in Finland, and the water area of its southern part, Southern Lake Saimaa, is 609 km^2. Exact locations, hydromorphological characteristics and water quality parameters of the sampling sites are presented in Table 1.

TABLE 1 Coordinated and characteristics of the sediment and fish sampling sites.

Lake	Coordinates	Flow rate (m^3 s^{-1})	Colour (mg Pt l^{-1})	pH	Oxygen (mg l^{-1})	P (µg l^{-1})	N (µg l^{-1})	COD (mg l^{-1})	Conductivity (mS m^{-1})
Vatianjärvi	6930100-3443360	50–230	50	6.9	7.6	20.0	460	10.0	7.0
Saimaa (1 km)	6775068-3567511	4	50	7.3	8.7	21.0	390	8.2	13.1
Saimaa (3 km)	6776850-3568646	4	50	7.3	8.7	21.0	390	8.2	13.1
Saimaa (ref.)	6780873-3562111	40	30	7.2	8.0	8.0	330	6.0	6.6
Palosjärvi (ref.)	6882320-3455909	–	15	7.4	8.1	5.0	250	4.1	7.4
Konnevesi (ref.)	6946630-3469797	40–125	15	7.7	8.2	8.0	350	6.4	4.6

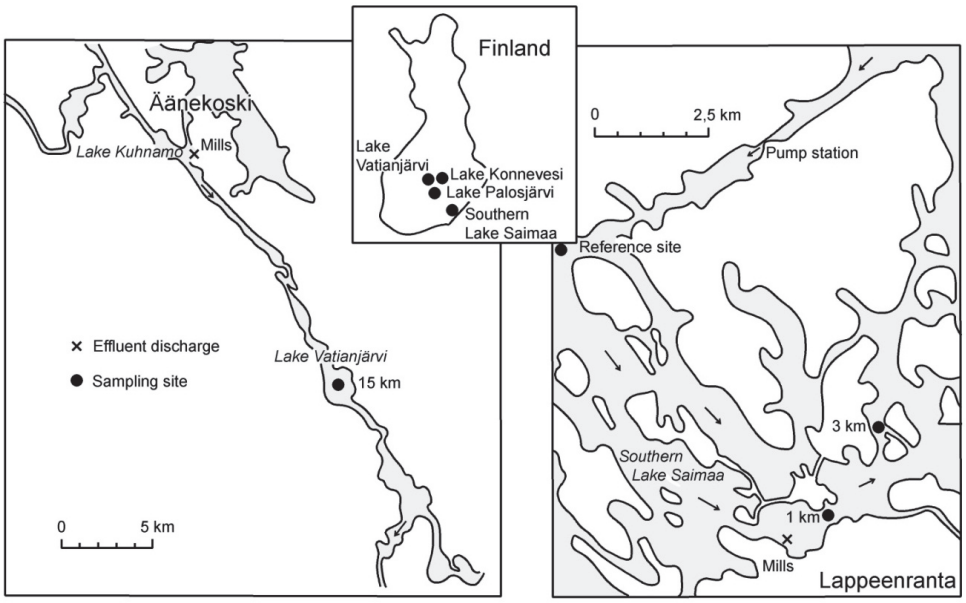

FIGURE 1 The study sites: Lake Vatianjärvi,(Lake Konnevesi and Lake Palosjärvi on the left and Southern Lake Saimaa on the right. The inset map shows the location of the study sites within Finland. Sediment sampling sites are marked by black dots and effluent discharge points by black crosses. In Southern Lake Saimaa the pump station displaces water from the clean area, and dilutes effluents from the pulp mill downstream (theoretical dilution ca. 3.5 % vol.). Approximate water flow directions are marked by arrows.

2.2 Sediment sampling

Sediments for the chemical analysis and for the laboratory bioassays were collected by an Ekman device from Lake Vatianjärvi and Southern Lake Saimaa (Fig. 1) in spring 2007 and 2010, respectively. The Lake Vatianjärvi sediment was from the deep basin 15 km downstream from the mills. In Southern Lake Saimaa, sediment samples were collected 1 and 3 km downstream from the pulp and paper mills. Reference sediment was collected from a site 10 km upstream. Sediment samples were stored in sealed polycarbonate containers in 4 °C in the dark.

2.3 Field study

Roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) were chosen to the field investigation as the difference in the feeding behaviour between roach and perch may affect the exposure pattern to chemicals. Adult perch is piscivorous

and prey in the pelagic zone, whereas at young stages they feed on zooplankton and benthic invertebrates (Hjelm et al. 2000, Horppila et al. 2000). On the other hand, roach is more common than perch in the littoral zone, and is omnivorous, feeding on zooplankton, detritus, plants and benthic invertebrates (Horppila et al. 2000). In contaminated areas it is thus probable that roach, compared to adult perch, will become more exposed to the hydrophobic compounds present in the uppermost sediment and benthos burrowed in it which may reflect also to the EROD activity. This type of information is important for understanding the recovery time and process in continuously loaded and heavily contaminated waterways.

Fish were caught from Lake Vatianjärvi and from two reference lakes, Lake Konnevesi and Lake Palosjärvi (Fig 1), in August and early September 2009. Fish were caught with a hook and worm in or with weir trap (12 mm mesh) in the littoral zone, about 500 m from the sediment sampling site. Fish caught with hook were immediately placed in recovery cages (55×55×55 cm) at approximately 1-2 m water depth. When the weir was used, fish were kept there to avoid additional stress caused by handling. Sampling of roach and perch was done in field within 24 h of capture in Lake Vatianjärvi and Lake Konnevesi, and within three days in Lake Palosjärvi. Fish were killed by a blow to the head, sex was determined, and the total length was measured. The livers were dissected and immediately stored in liquid nitrogen. EROD analyses were done within four months.

2.4 Laboratory exposure experiments

Juvenile rainbow trout (*Oncorhynchus mykiss*) (0.8–1.6 g) were transported in late May 2010 from the Hanka-Taimen Oy hatchery (in Central Finland), and acclimatized for seven days in the laboratory at 11 °C and pH 7.5 in aerated artesian well water. Temperature and pH was approximately the same as in the hatchery. The light cycle was 16:8 light:dark. The fish were fed once a day with Royal Hercules fish food (ca. 0.5 % of wet weight), except one day before the experiments.

Fish were exposed for 48 h to the uppermost (ca. 0–10 cm) sediments of Lake Vatianjärvi and Southern Lake Saimaa. Reference treatments were the Rautniemi sediment and the water with 5µg l^{-1} DMSO (Sigma-Aldrich). The positive control was retene in water (100 µg l^{-1}), DMSO used as a carrier solvent. Experimental setup was done exploiting the study by Fragoso et al. (2006). Around 2 cm layers (400 g) of homogenous sediment were added on the bottom of glass aquaria (dimension 19×19×32 cm, volume 10.8 l, water depth ca. 20 cm). Eight liters water was allowed to flow to each aquarium, trying to avoid mixing of the sediment. Water for the treatments was taken from the same source in which the fish were acclimatized (aerated artesian well water). Aquaria were covered with black plastic to minimize disturbing the fish. A glass lid was placed on the top of each aquarium and aeration pipe (a Pasteur pipet of glass)

was taped to the uppermost part of water column. Sediments were permitted to settle for one day and water aeration was started one to two hours before starting the experiment. Fish were transferred from acclimatization container to assay aquaria keeping them in water all the time. Three replicates per treatment and four fish per aquarium were used. The biomass of fish was less than one gram per one liter water. The light cycle, water temperature and pH were the same as during the acclimatization. The water was not changed and fish were not fed during the experiment. After 48 h exposure the fish were killed by a blow to the head, and total weight and length were measured. The livers were dissected and immediately stored in liquid nitrogen. EROD analyses were done within one month.

2.5 Chemical analyses

For sediments used in the laboratory bioassays, retene concentrations were analyzed. As resin acids are precursors of retene, also they were analyzed according to Tavendale et al. (1995) with some modifications described in Rämänen et al. (2010). Freeze-dried sediment samples were Soxhlet-extracted with hexane:2-propanol solution (2:1, v/v, Rathburn Chemicals, HPLC-Grade, UK). The internal standards were heptadecanoic acid (~99 %, Fluka, Switzerland) for RAs and anthracene-*d*10 (98 %, atom D, Isotec™, USA) for retene. The extracts were evaporated to a small volume, diluted with hexane and extracted with 0.1 M K_2CO_3 (>99 %, Merck, Germany). RAs were separated into the K_2CO_3 fraction and retene into the hexane fraction. For retene analysis, the hexane fractions were combined, concentrated and further evaporated under a nitrogen gas stream to dryness. The sample was then diluted with hexane:chloroform (4:1, v/v) and absorbed in the deactivated and conditioned silica gel column (0.062–0.200 mm, Baker Analyzca®, The Netherlands). The retene was eluted with hexane and evaporated by nitrogen until dry, diluted with small volume of hexane and analyzed with GC-MS. For RA analysis, the pH of the K_2CO_3 fraction was adjusted to nine with H_2SO_4. The sample was extracted with dichloromethane (Rathburn Chemicals, HPLC-Grade, UK), concentrated and further evaporated with nitrogen stream to dryness. The RAs were diluted with pyridine (>99 %, J.T. Baker, The Netherlands) and silylated with *N,O-bis*(trimethylsilyl)-trifluoroasetamid + 1 % trimethylchlorosilane (GC-quality, Fluka, Switzerland).

Serving as the positive control, retene concentrations were measured from experimental waters also. For that, 500 ml sample was filtered by solid-phase extraction (SPE) cartridge (Oasis HLB 3CC) and anthracene-*d*10 was added as the positive control. The SPE cartridge was washed with dichloromethane, 2-propanol (Rathburn Chemicals, HPLC-Grade, UK) and MilliQ water (ultrapure, conductivity 0.056 μS cm^{-1}, resistivity 18.2 MΩ-cm), 3 ml each, 6 mbar, 2.5 ml min^{-1}. The sample was then filtered (5 ml min^{-1}), washed with 3 ml MilliQ water (2.5 ml min^{-1}), and dried for 20 min in vacuum. After that the sample was eluted

four times with one milliliter of hexane:dichloromethane (1:1, v/v). At each step the sample was allowed to react for one minute and then eluted by 6 mbar 2.5 ml min^{-1}. The extract was gently dried in nitrogen gas flow, dissolved to 1 ml of hexane.

Retene and resin acids were measured with a gas chromatograph/mass spectrometer (GC-MS, Hewlett Packard 6890 MS, Germany; Hewlett Packard 5973 GC, USA, 30 m * 0.25 mm ID HP-5 polysiloxane polymer column, phase thickness 0.25 µm, volume of injection 1 µl) by using the temperature programs described in Rämänen et al. (2010).

2.6 EROD analyses

CYP1A activity was analyzed by measuring the 7-ethoxyresorufin *O*-deethylase (EROD) activity from hepatic post-mitochondrial supernatant (S9 fraction) according to Vehniäinen et al. (2012). In shortly, the livers were weighed and homogenized on ice in HEPES buffer solution (pH 7.5). The homogenates were centrifuged at 10,000×*g* for 20 min in +4 °C, and the supernatant containing S9 fraction was used for analyses. 20 µl of S9 fraction with 140 µl reaction buffer (100 mM HEPES sodium salt, pH 7.8) and 20 µl 2.5 µM ethoxyresorufin were pipetted to the microwell plate. After 5–8 minutes incubation 20 µl of 5 mM NADPH was added. EROD activity was quantified immediately by measuring the fluorescence of resorufin with a Fluoroscan Ascent fluorometer (Thermo Labsystems, Finland) at 30 s intervals, total scan time being 3 min 30 s, at emission and excitation wavelengths of 584 and 540 nm, respectively.

All EROD and protein measurements were made in triplicate. EROD activity was measured separately in each roach and perch, i.e. livers were not pooled, but the livers of four juvenile rainbow trout per aquarium were pooled for one analysis. Livers of rainbow trout injected intraperitoneally with β-naphtoflavone (100 µg g^{-1} wet weight) were used as a positive control for field populations.

Total protein in S9 fraction was analyzed by modified Lowry method using Bio-Rad DC Protein Assay (CA, USA). Bovine serum album was used as standard. Absorbance was measured with a Labsystems iEMS Reader MF photometer (Thermo Labsystems, Finland).

2.6 Statistical analyses

In the field research, species were analyzed as different groups when comparing differences between the lakes. As there were no statistical differences between sexes, those were combined in statistical analyses. Also reference lakes were combined.

The normality was analyzed using the Shapiro-Wilk's W test and homogeneity of variances by Levene's test. Due to heteroscedasticity of the variances the significance of the differences between the treatments in the laboratory experiment was analyzed by using Welch ANOVA and Dunnett T3 post hoc test. Correlation between the EROD activity and retene concentrations in the sediments was analyzed by Spearman's rho correlation test. In a field research the differences between Lake Vatianjärvi and the reference lakes were analyzed by using independent samples *t*-test. A significance level of 0.05 was used as indicative of difference between means. Statistical tests were done by using SPSS software (SPSS Inc., USA).

3 RESULTS

3.1 Chemical concentrations

Compared to Southern Lake Saimaa sediment, remarkably lower retene and resin acid concentrations were measured from Lake Vatianjärvi sediment. Concentrations of total resin acids in Lake Vatianjärvi were 11–14 % of those in Southern Lake Saimaa (Table 2). For retene, the difference was even higher being only 2–5 % of the concentrations in Southern Lake Saimaa (Table 2).

In the laboratory experiments retene was measured also from the water samples. In the retene 100 µg l^{-1} treatment, concentration was 9.2 µg l^{-1} at the end of the 48 h exposure. In the sediment exposures, Southern Lake Saimaa 1 km was the only treatment wherefrom retene was observed from the water sample (0.2 µg l^{-1}). The detection limit of retene was 0.1 µg l^{-1}.

TABLE 2 Concentrations (µg g^{-1}, d.w.) of retene and resin acid in the sediments (0–10 cm) of Lake Vatianjärvi (2007), Southern Lake Saimaa and the Rautniemi reference (2010).

Site	Retene	Sandaracopimaric acid	Pimaric acid	Isopimaric acid	Abietic acid	Dehydroabietic acid	Resin acids Total
Rautniemi (Ref)	0	0	0	0.1	0.2	1.1	1.4
Vatianjärvi 15 km	5.1	0.5	1.8	3.0	3.7	9.6	19
Saimaa 1 km	224	5.5	13	32	27	57	135
Saimaa 3 km	94	5.6	27	33	51	54	170

3.2 EROD activity in field populations

When compared to reference animals, no increased EROD activity was detected in perch and roach caught from the Lake Vatianjärvi in late summer 2009 (Table 3). In roach, the EROD activity was lower in Lake Vatianjärvi compared to the reference lakes but the difference was not statistically significant (p = 0.711, t-test). In perch, the average EROD activity was 73–84 % lower in Lake Vatianjärvi compared to the reference lakes, the difference being statistically significant (p < 0.001, t-test). In comparison between the species, the EROD activity was significantly higher in perch than in roach in the reference sites (p < 0.001, t-test), but no difference between the species was observed in Lake Vatianjärvi. For the positive control (β-naphtoflavone) EROD activity was 19.2 ± 14.2 (SD) pmol min^{-1} mg^{-1} protein, which proved that the EROD method worked appropriately.

TABLE 3 Number (n) of analyzed roach and perch in late August and early September 2009 from Lake Vatianjärvi and the combined reference lakes: Lake Konnevesi and Lake Palosjärvi. Fish length, weight and hepatic EROD activity are presented as mean ± SD.

Species	Sampling site	n	Length (cm)	Weight (g)	EROD (pmol min^{-1} mg^{-1} protein)
Roach	Vatianjärvi	9	184 ± 34	53 ± 31	1.9 ± 1.2
	Reference lakes	7	220 ± 37	147 ± 23	1.6 ± 0.6
Perch	Vatianjärvi	4	130 ± 14	24 ± 9	1.5 ± 1.2
	Reference lakes	24	147± 18	29 ± 15	10.0 ± 2.0

3.3 EROD activity in laboratory experiments

Significant differences were observed between the treatments (p = 0.045, df = 5, Welch ANOVA) but in the multiple comparisons statistically significant differences were not found probably because of small number of samples (n = 3). The most considerable difference (p = 0.170) was observed between retene 100 µg l^{-1} treatment compared to the Lake Vatianjärvi sediment, Lake Rautniemi reference sediment and water only treatment. In rainbow trout exposed to the contaminated sediment of Southern Lake Saimaa (both 1 km and 3 km), the average EROD activity was 2.5 times that in the Lake Vatianjärvi and Rautniemi reference sediment. In exposure to the surface sediment (0–10 cm) of Lake Vatianjärvi, collected in 2007, EROD activity was at the same level with Rautniemi reference sediment and with water only treament (Fig. 2). A significant correlation was observed between the EROD activity and retene concentration in the sediments (p = 0.005, Spearman's rho).

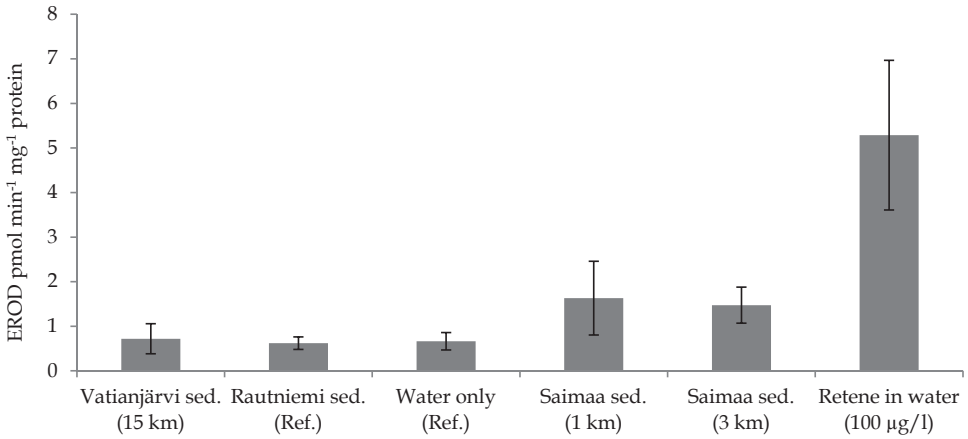

FIGURE 2 Liver EROD activity (± S.D.) in juvenile rainbow trout in laboratory bioassays. Fish were exposed to the sediments collected from Southern Lake Saimaa at 1 and 3 km, and from Lake Vatianjärvi 15 km downstream the pulp and paper mills. Southern Lake Saimaa reference sediment (10 km upstream), and water with DMSO were the reference treatments. Retene in water (nominal 100 µg l^{-1}, 48 h) was used as positive control.

4 DISCUSSION

4.1 Sediment as possible source of AhR-activating compounds

Dissolution potency can be used as an approximate predictor of bioavailability (Lebo et al. 2003, Meriläinen et al. 2006). Retene is tightly adsorbed to natural sediments and its dissolution is low in the studied sediments of Lake Vatianjärvi (Rämänen et al. 2010) and Southern Lake Saimaa (Meriläinen et al. 2006). Fragoso et al. (1998) have shown that EROD activity can be induced already with a retene concentration of 10 µg l^{-1} in water. Therefore, if retene is continuously present in the effluent, released from the sediment, or bioavailable in the sediment, elevated EROD activity can remain in exposed fish. If exposure to retene is only temporal, EROD activity decreases within four days (Fragoso et al. 1998). In this study as well as previous studies, concentrations of AhR-activating compounds, such as retene, differ between Lake Vatianjärvi and Southern Lake Saimaa (Lahdelma & Oikari 2005, Rämänen et al. 2010). Therefore, differences in retene concentrations in the sediments may explain the differences in fish liver EROD activity in our laboratory experiments. In Southern Lake Saimaa, the retene concentrations in the uppermost sediments (0–10 cm) were 224 and 94 µg g^{-1} (d.w.) at one and three kilometers downstream from the mill, respectively, whereas in Lake Vatianjärvi it was only 5 µg g^{-1}. Also concentrations of resin acids, which may transform to retene under anoxic

conditions (Tavendale et al. 1997), were much higher in the sediment of Southern Lake Saimaa than in Lake Vatianjärvi. These lakes share a similar pollution history, and nowadays in both areas pulp mills are using the same pulping-bleaching and wastewater treatment methods. However, the pulp mill in Äänekoski discharging upstream to Lake Vatianjärvi has produced mainly birch kraft pulp since 2009 and concentrations of soft wood originating retene are thus probably further decreasing. Therefore, it is unlikely that retene or other AhR-activating compounds could have any effects on fish in Lake Vatianjärvi anymore.

4.2 EROD activity in wild roach and perch

Populations of both roach and perch have revealed increased CYP1A activity downstream from pulp and paper industry, including the Southern Lake Saimaa (Lindström-Seppä & Oikari 1990). Improvements in the technology and effluent treatment in early 1990s led to recovery of populations there in this respect (Karels & Oikari 2000). In autumn 1995, three years after the wastewater treatment in aerated lagoons was replaced by activated sludge treatment and the ECF bleaching was started in the pulp mill in Southern Lake Saimaa, increased EROD activity was still observed in bottom feeding roach but not in perch (Karels et al. 1998). This indicates that EROD-activating compounds were probably still bioavailable in the uppermost sediment layer, whereas in 1997 no difference in EROD activity of roach and perch was measured anymore (Karels & Oikari 2000).

The EROD activity varies not only between the species, but also between sexes and seasons due to the reproduction cycle (Karels et al. 1998, Blanchard et al. 1999, Karels & Oikari 2000, Ruus et al. 2006). For instance, in the clean areas, the activity in perch liver can be over 30 times that in roach (Karels & Oikari 2000), and EROD activity is commonly higher in males than females (Karels et al. 1998, Blanchard et al. 1999, Karels & Oikari 2000, Ruus et al. 2006). If EROD activity is used as a biomarker for the exposure, sampling should be avoided during the spawning period when the EROD activity is naturally fluctuating (Blanchard *et al.* 1999, Karels & Oikari 2000). Instead, optimal sampling time is after the spawning period, i.e. for spring spawning species in late summer or early autumn, when the difference in EROD activity between the sexes is absent or at minimum (Karels et al. 1998, Blanchard et al. 1999). Therefore, in our study, sampling was done at an optimal time and no difference between sexes existed in the reference lakes. This justified the combination of sexes also for Lake Vatianjärvi, as only unbalanced proportions of males and females for both species were caught. In our study, a statistically significant difference was not observed either between roach and perch in Lake Vatianjärvi. Thus it may be concluded that concentrations of AhR-activating compounds in the water and in the upper sediment are negligible in that area.

The EROD activity in roach caught from the reference lakes was surprisingly significantly higher compared to roach from Lake Vatianjärvi. The reason for this remained unsolved. The catch of fish from Lake Vatianjärvi remained regrettably low, and thus the results of the field study are mainly indicative. On the other hand, the Äänekoski watercourse is loaded not only by chemical wood industry but also by municipal wastewaters. Therefore, theoretically, there might be some EROD inhibiting compounds present in the water column. For instance, many pharmaceuticals are known to inhibit EROD activity (Laville et al. 2004). Also remarkably high concentrations of hepatotoxins may cause reduction of CYP1A function and thus inhibition of the EROD activity (Oikari & Jimenez 1992). However, such situation is very unlikely as the historical contaminants of pulp industry, such as PCDDs and PCDFs have not been found from the uppermost sediment from the Äänekoski watercourse (Ratia, unpublished). Larger number of fish would have been desirable for more reliable conclusions.

4.3 EROD activity in experimentally exposed rainbow trout

A significant correlation was observed between the EROD activity in fish and retene concentration in the sediments. The concentration of retene in the sediment of Lake Vatianjärvi was negligible and retene was not detected from the exposure water. Thus the EROD activity in rainbow trout exposed to the sediment of Lake Vatianjärvi was expectedly at the same level as in the Rautniemi reference. Instead, retene concentration in the sediment of Southern Lake Saimaa was high enough to induce EROD activity in the laboratory bioassay. However, in the field research implemented in Southern Lake Saimaa in 2004, no difference in liver EROD activity was observed in juvenile brown trout which were exposed for ten days in cages with either close contact with the sediment or kept apart from it at the three meters water depth (Oikari et al. 2010). It is thus possible that in the laboratory assays access of fish with sediment caused more mixing of particulates and dissolution of retene or other AhR-activating compounds, whereas in the field similar mixing and dissolution from the sediment did not necessarily occur. In the laboratory assay, the only treatments where retene was observed in water were Southern Lake Saimaa 1 km and, of course, retene benchmark (100 µg l^{-1}). In the retene treatment, the measured concentration was 10 % of the nominal concentration, most likely due to accumulation of retene in the fish and adsorption to the walls of the aquaria (Scott et al. 2009, Räsänen et al. 2012). Possibly small amounts of retene have been dissolved also in the other treatments but concentrations remained below the detection limit.

4.4 Recovery of Äänekoski watercourse

In this research, increased EROD activity was not observed either in roach or perch caught from Lake Vatianjärvi, or in juvenile rainbow trout exposed to the surface sediment of Lake Vatianjärvi. Compared to the times of chlorine bleaching and inefficient wastewater treatment, the recovery of Äänekoski watercourse is obvious. An early study in 1975 indicated hepatotoxic effects in pike (*Esox lucius*) seen as the reduction of CYP1A-system in Lake Vatianjärvi (Ahokas et al. 1976). In Lake Vatianjärvi, benzpyrene hydroxylase activity (comparable to EROD) in pike was 83 % lower compared to the reference lake. Later Oikari & Jimenez (1992) demonstrated that hepatotoxic compounds are able to abolish EROD induction in dose related manner. However, the factors apparently causing hepatotoxicity remained obscure and unresolved. Also in the current study in 2010, EROD activity was significantly lower in perch caught from Lake Vatianjärvi, compared to the reference lakes, whereas in roach no difference was observed. It is possible that EROD activity in perch is naturally lower due to genetically different stocks. On the other hand, theoretically, some unknown EROD inhibitive xenobiotics could be present as discussed above. However, is there any evidence, direct or coincidental, on historical induction of liver CYP1A system in Lake Vatianjärvi? Actually, the potency to induce hepatic benzo[*a*]pyrene hydroxylase in 1981 by Äänekoski mill effluent (dilution 0.2 %) was demonstrated in vendace (*Coregonus albula*) (Lindrtöm-Seppä et al. 1989). Even more importantly, Vuorinen & Vuorinen (1985) demonstrated a blue sac disease-like syndrome in embryos of trout in 1982 caused by bleached kraft mill effluent of Äänekoski mills. Because this pathology coincides with CYP1A induction, it is probable that the assumed 1 % effluent dilution to the Äänekoski watercourse (Saski et al. 1997) has caused CYP1A induction in fish before 1985. However, no watercourse-wide assay on EROD or related endpoints, similar to Southern Lake Saimaa, exists from Lake Vatianjärvi. For the historical view, the potency of vertical sediment profiles to induce EROD activity in fish would be interesting to investigate in Äänekoski watercourse. In addition, more comprehensive analysis would be recommended, such as analysis of the contaminants from fish tissues or metabolites from the bile.

5 CONCLUSIONS

In summary, the results of our study, both in the laboratory and in the field, indicate the recovery of the previously heavily contaminated Lake Vatianjärvi. It may be concluded that the pulp and paper industry in Äänekoski City nowadays, or the historically contaminated sediments in the watercourse downstream, do not release EROD inducing compounds.

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CADDIS LARVAE (TRICHOPTERA, HYDROPSYCHIDAE) INDICATE DELAYING RECOVERY OF A WATERCOURSE POLLUTED BY PULP AND PAPER INDUSTRY

by

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Caddis larvae (Trichoptera, Hydropsychidae) indicate delaying recovery of a watercourse polluted by pulp and paper industry

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ABSTRACT

We studied ecotoxicological responses of aquatic insects to evaluate the recovery of a watercourse with a history of over 100 years as a recipient of pulp and paper mill effluents. The recovery of the water quality began in 1985 due to the improved technology, but ecotoxicological effects on biota have still been evident two decades later, as indicated by morphological abnormalities in tracheal gills of caddis larvae. We hypothesized that these abnormalities are related to organic contaminants, such as resin acids, alkylated PAHs and chlorinated organic compounds, released historically from the mills, and more recently via resuspension from the sediment. Samples were collected between 1999 and 2008, and the species composition and morphological abnormalities in the gill tufts were analysed. We tested a Hydropsychidae index (HA) based on species sensitivity scoring as an indicator of improved overall water quality, especially oxygen concentrations. Gill abnormalities were used as morphological biomarkers indicating potential ecotoxicity. The increased HA values revealed improved oxygen levels and associated recovery of benthic macroinvertebrate community structure. However, the incidence of gill abnormalities remained elevated until the year 2008 indicating slow recovery, as 20-year impacts on population health and ecotoxicity was detected in the watercourse polluted by pulp and paper industry.

1. Introduction

In the boreal latitudes, excluding Russia, the chemical wood industry is the most prominent industrial activity disturbing aquatic ecosystems. In Finland many watercourses receiving effluents from pulp and paper mills were heavily polluted before application of more efficient waste water treatment techniques (Kaplin et al., 1997; Meriläinen et al., 2001; Vuori, 2004). The bleached-kraft mill effluents contain many harmful compounds, such as wood sterols (Koistinen et al., 1998; Lahdelma and Oikari, 2006), resin acids (Tavendale et al., 1995; Koistinen et al., 1998; Leppänen and Oikari, 2001; Lahdelma and Oikari, 2005; Meriläinen et al., 2006), polycyclic aromatic hydrocarbons (PAHs) and the alkyl derivates of all of these (Tavendale et al., 1995; Koistinen et al., 1998). These compounds eventually end up in sediments, and are bioavailable to benthos (Meriläinen and Oikari, 2008a) and bottom feeding fish (Meriläinen and Oikari, 2008b).

Harmful substances can nowadays be effectively removed from the effluents, but some amounts are still released (Koistinen et al., 1998; Leppänen et al., 1998; Makris and Banerjee, 2002). Furthermore, historically contaminated sediments may constitute a risk if they act as a source of the contaminants (Sibley et al., 1997). Especially in river-like watercourses, as described in this case study, harmful compounds originally discharged from the pulp and paper industry may be released to the water column due to re-suspension (Meriläinen et al., 2006; Rämänen et al., 2010). Also more abundant floods in future climate may increase the risk of sediment erosion and pollutant release, as annual precipitation has been predicted to increase some 10–40% in the boreal latitudes within this century (IPCC, 2007).

In this study we evaluated the recovery of the historically contaminated watercourse from an ecotoxicological perspective. The study focuses on the downstream watercourse of the Äänekoski City, in Central Finland, where the pulp and paper industry has existed for over a 100 years (Meriläinen et al., 2001). Impaired water quality was detected as early as the 1950s, and the watercourse was heavily loaded for several decades due to untreated waste waters. Wood-derived bioactive compounds in the effluent, in addition to the heavy nutrient load and compounds causing oxygen depletion resulted in a decline of species diversity in area (Granberg et al., 1987; Hynynen et al., 2004). Impaired water quality also affected the local economy as fish were inedible. Negative impacts were detected not only in the fishes (Granberg et al., 1987), but also in invertebrates, for instance in the caddisflies (Trichoptera, Hydropsychidae) (Mäkelä, 1984; Vuori, 1992), the

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common benthic macroinvertebrates of running waters (Wallace et al., 1990; Williams and Feltmate, 1992).

In autumn 1982, massive fish death was observed in the lakes downstream of Äänekoski mills due to exceptionally high exposure to mill effluents. Therefore, news of a new pulp mill in the early 1980s evoked strong public criticism locally. Nevertheless, the recovery of Äänekoski watercourse started due to the replacement of the old pulp mills with a new one and establishment of an activated sludge wastewater treatment plant in 1985 (Botnia, 2010). A decrease in loading was observed shortly thereafter and anoxic conditions were no longer detected in the epilimnion of the recipient lakes (unpublished, Finnish Environment Institute, HERTTA database). Probably due to improved oxygen concentrations, re-colonization of caddisflies was observed in 1991, six years after the refitting of the mills. At the time six *Hydropsyche* species were found, but a high frequency of morphological abnormalities was detected (Vuori, 1992). Incidence of these morphological abnormalities, including structural deformities and darkening of anal papillae (ion regulatory organs), and tracheal gills have been shown to increase with increased exposure to metals (Vuori, 1994; Leslie et al., 1999; Vuori and Kukkonen, 2002). Such abnormalities have been also associated with pollution by crude oil (Simpson, 1980) and organochlorine compounds (Camargo, 1991; Vuori and Parkko, 1996). Therefore, *Hydropsyche* species have been instrumental in biological monitoring of impaired water quality (Vuori and Parkko, 1996; Leslie et al., 1999; Vuori and Kukkonen, 2002).

We hypothesized that the incidence of morphological abnormalities in hydropsychid populations can reflect the degree of exposure to organic contaminants released from pulp and paper mills. We surmised that modernization of the mills and improved water quality since 1985 could be correlated to both species composition and incidence of abnormalities of caddis larvae. In this study we used the same methods as described by Vuori (1994) and Vuori and Kukkonen (2002), but analysed also the more detailed structures of the gill tissues implying pathological alterations. In previous studies gill damage was analysed by examining only the external structure of the gills (Vuori, 1994; Vuori and Parkko, 1996; Vuori and Kukkonen, 2002). To our knowledge this is the first study in which internal damage of the tracheal gill tissue of caddis larvae has been investigated. In addition, we used, and further developed, the Hydropsychidae index (HA) (Vuori, 2002), based on the hydropsychid species composition and sensitivity to eutrophication and organic pollution, in order to assess the recovery of the watercourse.

2. Materials and methods

2.1. The study area

The City of Äänekoski is located in Central Finland (Fig. 1). Board mill was introduced there in 1899 and paper mill shortly thereafter. Sulphite pulp mill was initiated in 1938 (Meriläinen et al., 2001) and chlorine-based bleaching began in the 1950s (Reunala et al., 1998). In 1961, a second pulp mill was built, producing sulphate cellulose in about twice the amounts of that of sulphite cellulose. The sulphite process was discontinued in 1984, and one year later also another old pulping mill was closed (Granberg et al., 1987). In 1985, Metsä-Botnia opened a new pulp mill replacing the sulphite process with a sulphate one. In the same year water treatment by activated sludge was initiated. In 1993, an elemental chlorine free (ECF) bleaching process was introduced. Nowadays pulp mill of Metsä-Botnia is producing mainly birch kraft pulp (Botnia, 2010). Paper and board mills of M-Real have own waste water treatment plant using chemical and mechanical methods.

The study sites for this work were rapids located 15, 30 and 50 km downstream from the Äänekoski City (Fig. 1). In addition, two reference sites were used: the Simunankoski and Siikakoski rapids. Both reference sites are at high physicochemical and ecological status based on the European Union's Water Framework Directive (Finnish Environment Institute, 2010). Both reference sites and impact sites belong to the same river type (large rivers in mineral soil regions), and have nowadays similar pH and oxygen levels, whereas nutrient concentrations and colour values are elevated in the impact sites.

2.2. Collection of larval material

Species richness and morphological abnormalities in the gill tufts of *Hydropsyche* larvae were investigated between 1999 and 2008 in the Kuusaankoski rapids (Fig. 1). Caddis larvae were collected during May, June and July in 1999, 2003, and 2007–2008. In 2007, after the spring flooding the water level was still too high for working in late May, and only few caddis larvae were found ($n = 18$). Sampling was therefore repeated in late June. In 1999 and 2003 samples were also taken from the Kuhankoski and Haapakoski rapids. The Siikakoski and Simunankoski rapids were used as reference sites in 2007 and 2008. Material was also obtained from Siikakoski rapids in 2000.

For invertebrates, a combination of several sampling methods was used: kick net sampling, colonization substrates (bricks), and picking up the larvae from the stone surfaces. Different methods were used in order to effectively gather representative number of larvae. Larvae collected in 1999, 2000 and 2003 were preserved in a 70% ethanol solution immediately after collection in the field. In 2007 and 2008, however, animals were transported to the laboratory in a cooled box in half-litre plastic containers filled with river water. While the sampling lasted from one to two hours, transport from sampling sites to the laboratory took a maximum of three hours. No larvae mortalities occurred during transportation. In the laboratory, aeration was immediately added to the containers and larvae were kept at 10 °C, until stored (within 24 h) in a 70% ethanol solution.

Water temperature, conductivity and pH were measured at each sampling site (Table 1). In 2007–2008, also flow velocity and sampling depth were measured from four to five measuring points in each rapid. Roughly, the conductivity was higher and pH was lower in the Äänekoski watercourse compared to the reference sites. The average flow velocity did not differ significantly between the sampling sites. However, in 2007, the water level was low in all rapids, and thus the flow velocity was generally lower than in 2008, being 0.3–0.4 m/s and 0.4–0.6 m/s, respectively. The sampling depth was about one and a half metres in 1999 and 2003, when colonization bricks were used as the sampling method. In 2007–2008 the sampling depth varied from 20 to 80 cm and was carried out either by use of a kick net or by collecting larvae from stones by hand. The bottom habitat was similar at each sampling site being fine grain sand, stones and boulders.

2.3. Species analysis and determination of morphological damage

Caddis larvae were determined at the species level (Edington and Hildrew, 1981; Bongard, 1990), and instars were determined from about ten per cent of collected larvae. Morphological damage to the gills was inspected using a stereomicroscope. *Hydropsyche siltalai* (Fig. 2a) has 17 gill tufts and other species in the Hydropsychidae family have 19. The frequency of morphological damage to the gill tufts was calculated as an average in a population. There is no standardized procedure for classifying gill tufts as abnormal. Vuori and Kukkonen (2002) ranked only totally darkened or shortened gill tufts as abnormal. Their approach, relying on severe

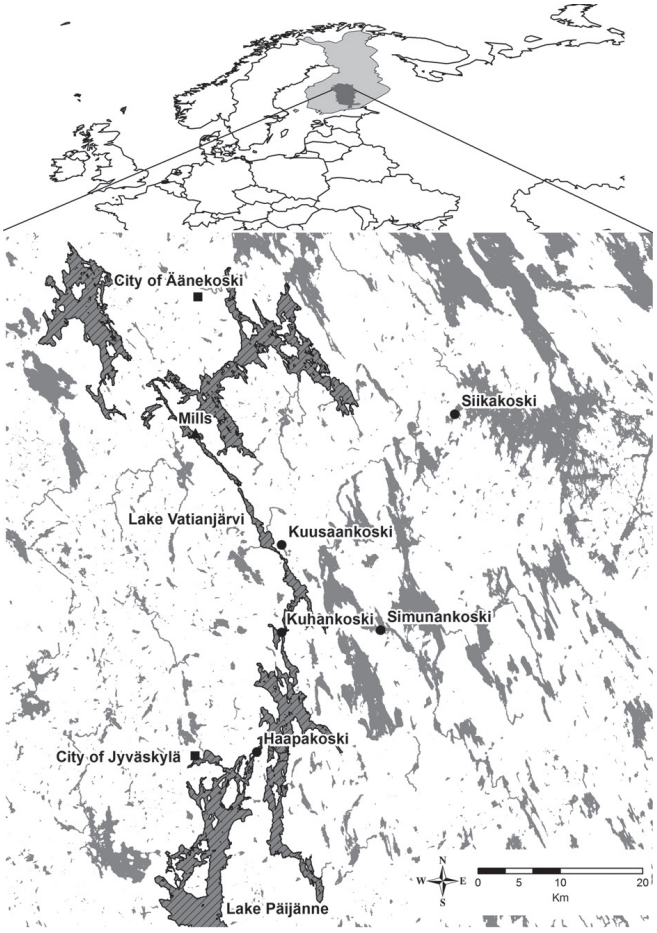

Fig. 1. Location of the study area in Central Finland. Sampling sites are marked with black circles.

Table 1
Sampling date and water quality parameters measured from the sampling sites during the collection of caddis larvae. The distance from the mills are marked after each sampling site, rapids Siikakoski and Simunankoski are reference sites (Ref). Missing data are marked with hyphen.

Sampling site	Date	T (°C)	Oxygen (%)	Conductivity (mS/m)	pH
Kuusaankoski (15 km)	18 May 1999	7.8	97	5.6	6.8
	16 June 2003	14.3	90	6.4	6.9
	28 June 2007	17.5	93	8.9	7.1
	9 June 2008	15.9	99	–	7.1
Kuhankoski (30 km)	18 May 1999	7.8	99	5.2	6.9
	16 June 2003	–	–	–	–
Haapakoski (50 km)	21 June 1999	18.9	101	5.7	7.1
	16 June 2003	14.0	88	6.7	7.0
Simunankoski (Ref)	1 June 2007	14.3	–	3.6	7.3
	11 June 2008	14.5	98	–	7.3
Siikakoski (Ref)	16 June 2000	10.4	–	–	–
	4 July 2007	21.5	100	3.5	–
	10 June 2008	12.4	105	3.7	7.3

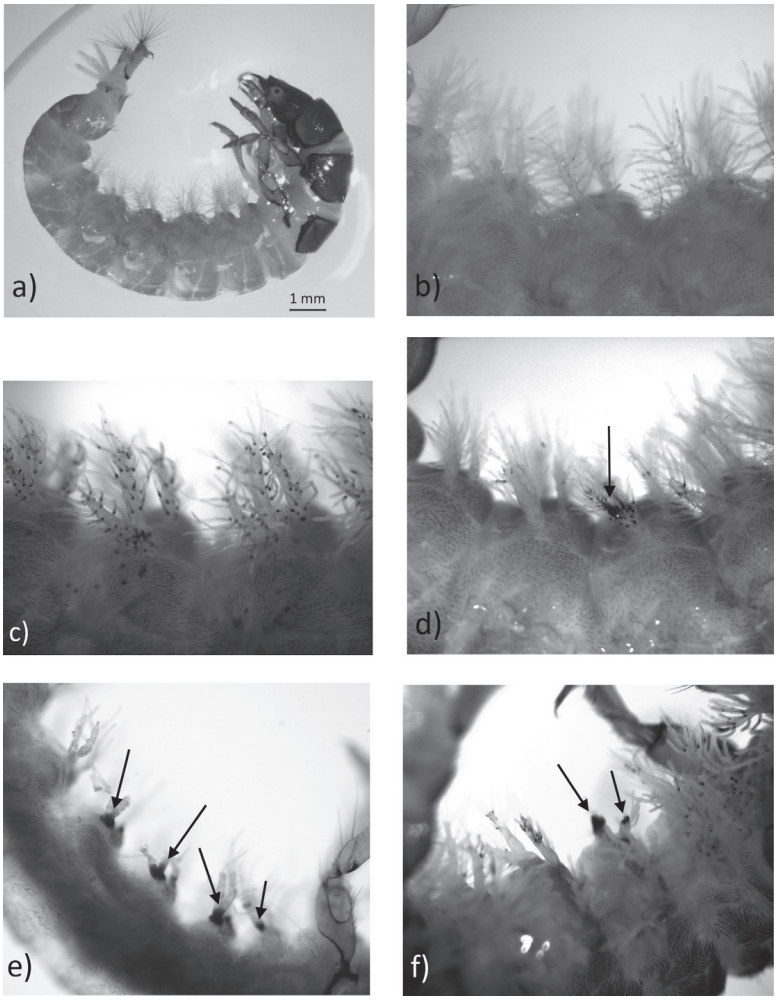

Fig. 2. *Hydropsyche siltalai* (a); gill tufts are the transparent branches on the ventral side. Gills of hydropsychid larvae were classified as either healthy (a and b), slightly damaged (c), or seriously damaged (d and e). Scar-like darkening (f) was not classified as gill damage.

discolouration or malformation of stalk and branches of gills, is a robust one. As reported by Leslie et al. (1999), the abnormalities also include variation in the number of darkened tips of gill branches. In the present study, we observed that gill branch discolouration also occurs in the basal parts of branches, ranging from sporadic occurrence of small dark spots to total coverage of all gill branches with such spots. Here we apply a combination of the above approaches. The gill tuft was defined to be healthy (Fig. 2a and b) if it was totally transparent or if there were minor brown spots on less than 50% of gill tufts, or if it had only sporadic dark spots. Slightly damaged gill tufts (Fig. 2c) included those with obvious brown spots in more than 50% of branches. Seriously damaged gill tufts (Fig. 2d and e) were totally dark, or darkened from the distal or basal parts, often including shortening of the gill. Scar-like darkening (Fig. 2f) was not classified as gill damage, as it is probably caused by bitings of predators or competitors.

The species and instars were analysed from the larvae stored in 70% ethanol. For microscopical sections and inspection of the gill structures, caddis larvae were stored in a buffered glutaraldehyde:formaldehyde fixative (2:5, v/v). The fixative was prepared by dissolving ultrasonically 16 g $NaH_2PO_4{\cdot}H_2O$ to 86 ml nanopure water, and pH was adjusted to 7.4 by adding NaOH. By adding 10 ml of 37% formaldehyde and 4 ml of 25% glutaraldehyde, the final volume was 100 ml.

Table 2
Finnish species of Hydropsychidae and Arctopsychidae families. Optimum habitat description and indicator values based on tolerance towards the pollution are presented. Two different scoring criteria are given, according to the species sensitivity to eutrophication (HA_e) caused by nutrient loading, and oxygen depletion (HA_o) caused by organic pollution. A higher indicator value denotes lower tolerance (modified from Vuori, 2002).

Species	Habitat	HA_e	HA_o
Hydropsychidae			
Cheumatopsyche lepida	Stony bottoms, lake outlets	2	3
Ceratopsyche nevae	Stony and gravelly bottoms, lake outlets	2	3
Ceratopsyche silfvenii	Moss-grown stony bottoms	5	4
Hydropsyche siltalai	Moss-grown stony bottoms, fast current	4	4
H. pellucidula	Stony and gravelly bottoms, lake outlets	2	3
H. contubernalis	Stony bottoms, lake outlet	3	1
H. angustipennis	Stony and gravelly bottoms, lake outlets	1	2
H. saxonica	Stony bottoms	3	4
H. bulgaromanorum	Sandy and gravelly bottoms, slow current	4	3
Arctopsychidae			
Arctopsyche ladogensis	Deep, moss-grown stony bottoms, fast current	5	4

To observe the histological structure of gills, the gill tissues were first rinsed three times in 0.1 M phosphate buffer of pH 7.4 at 4 °C for 10 min. Post-fixation was done in 1% osmium tetroxide (1 h), prepared by diluting equal volumes of 2% osmium tetroxide and 0.2 M phosphate buffer (pH 7.4). The preserved gills were washed again three times with 0.1 M phosphate buffer (pH 7.4) at 4 °C for 10 min. Samples were then dehydrated through the graded ethanol and stained in uranyl acetate: three times five minutes in 70% ethanol, three times five minutes in 96% ethanol, 30 min in 2% uranyl acetate diluted in pure ethanol (99%), and three times five minutes in pure ethanol. Ethanol was first substituted for propylenoxide, three times seven minutes at room temperature, and then propylenoxide was substituted for propylenoxide-epon LX-112 mixture (1:1) for one hour. Samples were then left in 100% epon overnight at room temperature. During the next day, samples were embedded in the final epon solution and polymerized at 60 °C for 48 h. Cross sections of the gills were obtained by cutting the epon blocks to 0.5 μm slides with microtome, and stained with toluidine blue (Fluka Chemica). Darkening of the gill membrane and the structures inside the gills were inspected with a stereo microscope.

2.4. Hydropsychidae index

Vuori (2002) collated field and laboratory studies on the relations between water quality and distribution, abundance and physiological performance of ten hydropsychid species present in Finland and scored them based on their tolerance towards pollution. This Hydropsychidae index (HA) focused especially on species tolerance towards eutrophication. We refined the index by adding scoring criteria also for tolerances towards organic pollution. The index has a formula:

$$HA = \frac{\sum_{i=1}^{n}(HA_i \times A_i)}{\sum_{i=1}^{n} A_i},$$

where HA_i is an indicator value either for sensitivity to eutrophication (HA_e) or oxygen depletion (HA_o) caused by organic pollution (Table 2). A_i is number of the individuals of the species. The Hydropsychidae index value varies from one (poor status) to five (high status) (Vuori, 2002). HA is suitable for use in typical habitats of Hydropsychidae, i.e. fast flowing rapids inhabited by at least two species of the Hydropsychidae family. It must be taken into consideration that indicator values (HA_i) are subjective, and largely based on empirical observations of species distributions, although experimental evidence is available for some species. In addition, sampling methods of caddis larvae should be the same, and at least semi-quantitative. The favourable habitat and indicator values of Hydropsychidae and Arctopsychidae species present in Finland are presented in the Table 2 (modified from Vuori, 2002).

2.5. Statistics

The normality of the data collected in this study was analysed using the Shapiro–Wilk's W test ($n < 50$) or Kolmogorov–Smirnov test with the Lilliefors significance correction ($n \geq 50$). As the data were not normally distributed, the significance of the differences in the average number of gill abnormalities were analysed by the Kruskal–Wallis test. The data were analysed statistically as an average gill damage proportion per larva, so that slight and serious damage were analysed separately. Pair wise comparisons between sampling sites and sampling time were analysed by use of the Mann–Whitney U test. A significance level of 0.05 was used. Statistical tests were done by using SPSS software (Statistical Product Service Solutions, Chicago, USA).

3. Results

3.1. Oxygen concentrations

Before 1985, oxygen depletion was observed in the Kuusaankoski rapids and further downstream. Since 1985, after mill renewal, oxygen levels increased in a whole watercourse downstream from the mills. Reference sites, the Siikakoski and Simunankoski rapids, have not suffered from oxygen depletion (Fig. 3) (unpublished data, HERTTA database of the Finnish Environment Institute).

3.2. Species composition and HA index

In 1999 and 2003, *H. siltalai* and *Hydropsyche pellucidula* were equally present ($n = 50$) in the samples collected from the Kuusaankoski rapids, and also *Cheumatopsyche lepida* was

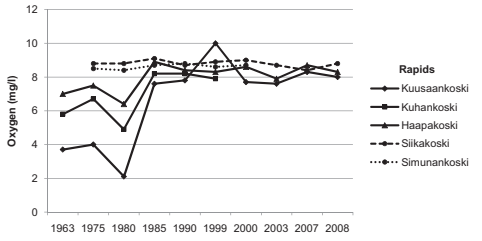

Fig. 3. Oxygen concentration since 1963 in Äänekoski watercourse in Finland: rapids Kuusaankoski, Kuhankoski and Haapakoski, 15, 30 and 50 km downstream from the pulp and paper mills, respectively. rapids Siikakoski and Simunankoski are reference sites.

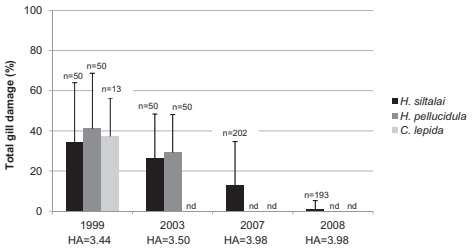

Fig. 4. Average (±S.D.) proportions of *Hydropsyche siltalai*, *Hydropsyche pellucidula* and *Cheumatopsyche lepida* individuals with abnormal gills in the Kuusaankoski rapids from 1999 to 2008. Absence of species ($n \leq 2$) is marked with nd (not detected), and n is number of individuals. HA is Hydsopsychidae index varying from one to five, so that a higher value indicates better water quality based on organic pollution.

commonly found (n = 13–16). HA values in 1999 and 2003 were 3.44 and 3.50, respectively. In 2007 and 2008, HA increased to 3.98 due the dominance of *H. siltalai*. In 2007 only two individuals of both *H. pellucidula* and *C. lepida* were found. In 2008, four individuals of *Ceratopsyche nevae* were found along with *H. siltalai*, but *H. pellucidula* and *C. lepida* were absent. Thus, the composition of the species was changed in the Kuusaankoski rapids, which is also reflected by HA values, which increased from 3.44 in 1999 to 3.98 in 2007 and 2008 (Fig. 4).

Further downstream in the Kuhankoski and Haapakoski rapids, *H. pellucidula* was the only species found in 1999 and 2003. At the Siikakoski and Simunankoski reference sites in 2007–2008, *H. siltalai* was dominant, but *H. pellucidula* and *C. lepida* were also present. However, in 2007 only three larvae of *H. pellucidula* were found from the Siikakoski rapids. In 2008, *H. pellucidula* was more common at both reference sites. In the Simunankoski and Siikakoski rapids, HA values were 3.5 and 3.9 in 2007, and 3.8 and 3.7 in 2008, respectively.

3.3. Cell structure and incidence of abnormal gills

When comparing the histological cross-sections of the healthy gills between the species, we noticed a difference in the shape and the structure of the outer membrane of *H. siltalai* and *H. pellucidula*. *H. siltalai* has a round shape and a smooth membrane (Fig. 5a and b), whereas gills of *H. pellucidula* are more irregular and the outer membrane is coarser (Fig. 5e and f). Inspection of the gill structure revealed evidence of the tissue alterations. From cross-section of gill tissue it was seen that the bronchioles were without characteristic structures found in the control individuals, and were thereby determined to be damaged (Figs. 2d and 2e, and 5c, 5d, 5g and 5h).

3.3.1. Morphological abnormalities in H. siltalai

Incidence of slight and serious gill damage of *H. siltalai*, the most abundant species, had significantly decreased in the Kuusaankoski rapids from year 1999 to 2007 and 2008 ($p < 0.001$, $df = 3$) (Fig. 4). During the 1999–2008, the proportion of slightly damaged gills decreased from 22% to 0.1%, and that of the seriously damaged ones from 12% to 1.0%. In 2008, the proportion of abnormal gills of *H. siltalai* had decreased to same level as the reference sites where the total incidence of gill damage was 1.6% and 0.8%, for the Simunankoski and Siikakoski rapids, respectively. However, inconsistent results were obtained for gill abnormalities in the Siikakoski rapids, where slight and serious gill damage in 2007 amounted to 12% and 1.3%, respectively. This is significantly higher ($p < 0.001$, $df = 1$) than the corresponding values in 2008, when proportions of both slight and serous gill damage were less than 1%. In 2000 there were no abnormal larvae (n = 50).

3.3.2. Morphological damages in H. pellucidula

In 1999, in the Kuusaankoski rapids, 22% of *H. pellucidula* gills were slightly damaged while 15% were seriously damaged; the total number of abnormal gills thus being nearly 40%. In 2003, the incidence of slight gill damage was high (24%), but the proportion of serious damage was less than 5%. In 2007, only two larvae were found, and in 2008 *H. pellucidula* was absent. However, in 1999 and 2003 *H. pellucidula* was common, and for these years, the incidence of gill damage decreased with downstream distance from the mill site (Fig. 6). In 1999, significant decrease in slight ($p = 0.005$, $df = 1$) and serious ($p = 0.047$, $df = 1$) gill damage were observed in the Kuhankoski rapids compared to the proportion of gill damage in the Kuusaankoski rapids. In 2003, significant decrease in slight gill damage was observed between Kuusaankoski and Kuhankoski rapids ($p = 0.010$, $df = 1$) whereas proportion of serious gill damage was significantly lower only in the Haapakoski rapids ($p = 0.025$, $df = 1$) compared to the Kuusaankoski rapids.

Unexpected results were found in the reference sites, as relatively high number of abnormal gills was found from the caddis larvae collected. In 2007, in the Simunankoski rapids, 50% and 2.5%

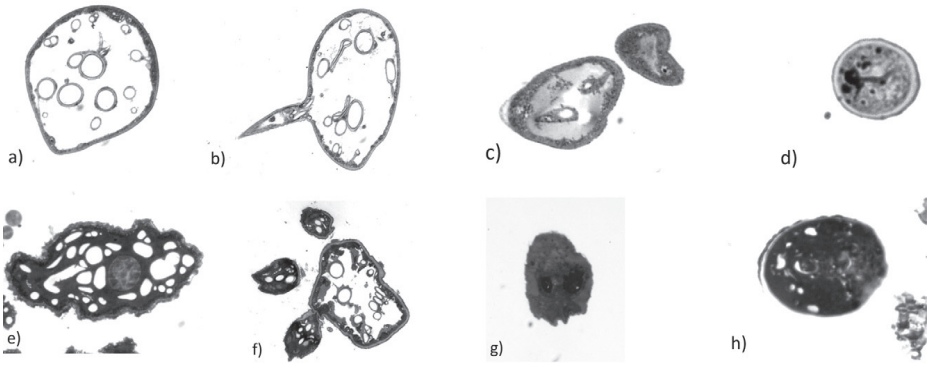

Fig. 5. Cross sections of healthy (a and b), and damaged gills (c and d) of *Hydropsyche siltalai*, and cross sections of healthy (e and f), and damaged gills (g and h) of *Hydropsyche pellucidula*.

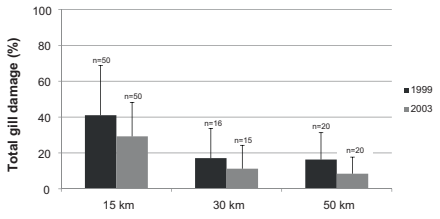

Fig. 6. Average (±S.D.) proportions of *H. pellucidula* individuals with abnormal gills in years 1999 and 2003 in the rapids downstream from pulp and paper mills in Äänekoski, Central Finland: Kuusaankoski (15 km), Kuhankoski (30 km) and Haapakoski (50 km). *n* is number of individuals.

of the gill tufts of *H. pellucidula* ($n = 15$) were observed to be slightly or seriously darkened, respectively. In the same year only three larvae of *H. pellucidula* were found in the Siikakoski reference site, where the proportions of slightly or seriously damaged gills were 7% and 2%, respectively. Gill abnormalities were also observed in *H. pellucidula* at both reference sites in 2008. At the Simunankoski site, 18 larvae were found, and proportions of slight and serious gill damage were 24% and 3%, respectively. In the Siikakoski rapids, 28 larvae were collected, and 19% of the gills were slightly damaged, whereas only 1% was seriously damaged. However, as samples were also collected from the Siikakoski rapids in 2000, no abnormality was observed in *H. pellucidula* ($n = 30$). Even though a relatively high number of abnormal gills were found in 2007–2008, most (80–95%) of the abnormal gills from reference sites exhibited only slight damage (Fig. 2c).

3.3.3. Morphological damage in C. lepida

In 1999, an average of 23% of *C. lepida* gills ($n = 13$) exhibited slight damage, and 14% had serious damage in the Kuusaankoski rapids. *C. lepida* was absent there in 2003 and 2007. In 2008, only two individuals were found and no gill damage was observed. Further downstream, in the Kuhankoski and Haapakoski rapids, *C. lepida* was absent.

At the Simunankoski reference site, *C. lepida* was abundant ($n = 42$) in 2007. On average, 2% of their gills were slightly damaged, and 3% seriously damaged. In 2008, only seven larvae were found. Their gills exhibited 4% and 2% slight and serious damage, respectively. At the other reference site, rapid Siikakoski, *C. lepida* was absent in 2000, and in 2007 very few abnormal gills were found from the 12 individuals collected. No slight damage was found, and only 0.4% (i.e. one gill tuft) was seriously damaged ($n = 12$). In 2008, only 1% and 2% slightly or seriously damaged gills were found, respectively ($n = 15$).

4. Discussion

4.1. Species composition

The increasing values of the HA index since 1999 indicate a recovery of the hydropsychid species guild structure. It is likely that this is related to the increased oxygen concentrations, and changed water quality in the recipient water bodies. The positive influence of improved waste water purification on species richness was detected already in the decade following the early 1980s (Vuori, 1992). In 1981, when the organic load from the pulp mills was still very high, hydropsychids and most other macroinvertebrates were absent from the Kuusaankoski rapids, 15 km downstream from the mills. Further downstream, only one Hydropsychidae species (*Hydropsyche contubernalis*) was found (Mäkelä, 1984; Vuori, 1992). Engels et al. (1996) reported that *H. contubernalis* was the first *Hydropsyche* species to return to the Lower River Rhine after extinction due to the heavy pollution in 1960s and early 1970s. They found a correlation between the oxygen content and re-colonization of caddisflies. *H. contubernalis* is able to survive in water with oxygen level as low as 50% ASV (air saturation value), whereas for example *H. pellucidula* requires oxygen level higher than 70% ASV (Becker, 1987). Hence, one plausible reason for the absence of hydropsychids in the Äänekoski watercourse before the mill renewal can be a low oxygen level. In the 1960s and 1970s, during the summer stratification, anoxic conditions were commonly measured in the hypolimnion of Lake Vatianjärvi, just above the Kuusaankoski rapids. Oxygen depletion was detected also in the epilimnion discharging directly into the Kuusaankoski rapids. Even at the beginning of 1980s, the oxygen concentration in summer dropped to less than 2.5 mg/l (26% ASV) in Kuusaankoski rapids (unpublished data, HERTTA database of the Finnish Environment Institute).

The aerobic conditions improved relatively quickly following the introduction of a new pulp mill in April 1985, aided by more efficient waste water treatment. Granberg et al. (1987) reported the decrease of biological oxygen demand (BOD) to one tenth within one year. Improved conditions were measured also in the Kuusaankoski rapids, and since mid-June 1985 oxygen levels have been 77% ASV or higher (unpublished data, HERTTA database of the Finnish Environment Institute). In the beginning of 1990s, caddisflies re-colonized the area, and six Hydropsychidae species were detected in the Kuusaankoski rapids: *H. siltalai*, *H. pellucidula*, *Hydropsyche saxonica*, *Hydropsyche angustipennis*, *C. lepida* and *C. nevae* (Vuori, 1992). Oxygen level is a critical factor in the feeding behaviour and survival of other lotic macroinvertebrates. For instance, Lowell and Culp (1999) demonstrated reduced grazing intensity by 80%, and a 60–90% lower survival rate in mayflies (*Baetis tricaudatus*, Dodds) exposed to pulp mill and sewage effluents under a relatively low level of dissolved oxygen (5 mg/l). The field experiment carried out by Stuijfzand et al. (1999) support this assumption. They found that the mortality of hydropsychids was very high (95%) in River Meuse as compared to River Rhine (25%), and related this difference to the lower oxygen, and higher metal and pesticide levels in the former. Stuijfzand et al. (1999) concluded that toxicity of metals and pesticides together with low oxygen levels possibly caused the death of the larvae. The combination of toxicity and low oxygen concentrations could have been the case also in the Äänekoski watercourse in early 1980s.

In 1991, Vuori (1992) found the same species as we did from the Kuusaankoski rapids, and also *H. saxonica* and *H. angustipennis* were present. Since late 1990s, sensitive species have become more common, as demonstrated by the increase in value of the HA index in this study. At the Kuusaankoski study site, the number of *H. pellucidula* larvae varied over the period of study due to different samplers, sampling efforts and timing. This impedes interpretation of results, and for further studies sampling methods should be standardized to assure proper HA index values. The most reliable results include years 1999 and 2003, when *H. pellucidula* and *H. siltalai* were the most common species in the Kuusaankoski rapids. In 2007–2008, *H. siltalai* was the most dominant. *H. siltalai* appears to avoid impaired water quality, whereas *H. pellucidula* tolerates it quite well (Vuori, 2002). *H. siltalai* also favours lower temperature compared to *H. pellucidula* (Roux et al., 1992). Both species usually inhabit similar habitats (Badcock, 1974; Bagge, 1991), and favour high stream velocity (Bagge, 1991; Roux et al., 1992). *H. pellucidula* overwinters as its last instar in Northern Europe (Andersen and Klubnes, 1983), Britain (Hildrew and Edington, 1979), and Ireland (Sangpradub et al., 1999). It is thus possible that in 2007 and 2008 *H. pellucidula* had already emerged in Kuusaankoski rapids, as it metamorphoses in mid-June on average (Andersen and Klubnes, 1983), and in 2007 samples were taken in late June (28.6), when

H. pellucidula was in the fifth, i.e. in the last instar. *H. siltalai*, on the other hand, overwinter mainly as the third or fourth instar, and flies later in summer (Hildrew and Edington, 1979; Andersen and Klubnes, 1983; Sangpradub et al., 1999). In 2008, however, sampling was done in early June which does not explain the absence of *H. pellucidula* and *C. lepida* in the Kuusaankoski rapids. Another possible reason for low abundance of *H. pellucidula* in 2007–2008 in the Kuusaankoski rapids can be microhabitat selection. Vuori and Parkko (1996) observed that the final instar of *H. pellucidula* favours higher current velocities (>0.5 m/s) compared to the earlier instars. In addition, Hildrew and Edington (1979) reported that *H. pellucidula* inhabit the underside of stones, whereas *H. siltalai* favours the moss covered upper side of stones during the summer. The substratum of the Kuusaankoski rapids mainly consists of gravel, stones and boulders, and many of the stones are too large to collect by manual sampling. Thus, it is plausible that, due to this, *H. pellucidula* have been present, but were more difficult to detect. In future studies, utilizing HA index standardization of sampling procedures should be assured. Furthermore, although our study species are widely distributed in Europe and Russia, scoring criteria should take into account species habitat requirements specific to each ecoregion.

4.2. Morphological abnormalities

The incidence of gill abnormalities decreased significantly in 1999 and 2003 with increasing distance from the mills (Fig. 6). This implies an association between the industry as a source, and gill pathologies as consequence of pollution causing ecotoxicity, indicating delayed recovery since 1980s. In addition, at Kuusaankoski rapids, the incidence of gill damage significantly decreased during the study period (Fig. 4). Contrary to expectations, a relatively high proportion of abnormal gills in *H. pellucidula* were found at the reference sites, although only very few ranked as serious. One possible reason could be natural gill darkening and structural change of the gills during the transformation from larvae to pupa, as the high proportion of gill damage was found from the last instar *H. pellucidula*, pupating already in mid-June (Andersen and Klubnes, 1983), which was the average sampling time in this study. In addition, in 2007, some abnormalities were found also from the last instar *H. siltalai* from the Siikakoski rapids, and in that year sampling was done there on the beginning of July, which was later than other years. Therefore, natural gill darkening and structural change of the gills during the transformation from larvae to pupa should be investigated in laboratory conditions. Another reason for gill damage in the reference sites could be scar-like darkening, as in dense populations, such as at the reference outlet habitats, hydropsychids are known to fight over net spinning sites and bite each other (Vuori, 1994). The biting of gills may also result in the appearance of shortened and blackened gill branches. Upon inspection, such scar-like darkening (Fig. 2f) was under consideration and not ranked as damage, but such appearances may be difficult to distinguish and may have been included in the results.

Also natural diseases and parasites may have had an effect on gill darkening. Leslie et al. (1999) observed at a reference site that the last instar larvae of *H. pellucidula* with gill damages suffered also from an extensive fungal infection. Such phenomenon was not observed of reference larvae in the present study; hence the reason for occasional gill damage at the reference sites remains unclear. Another possibility is that the prolonged transportation and storage prior to preservation may have induced the gill damage. In 2007 and 2008, larvae were transported and stored in laboratory prior to the preservation in 70% ethanol. All other larvae were preserved immediately after sampling on site. Even if transportation took only couple of hours and larvae were kept in aerated containers at 10 °C, it is possible that prolonged treatment stress may have induced gill darkening at both reference sites and species. Impact of such treatment stress should be experimentally examined in future studies.

The decrease of morphological damage in caddisflies over the time at the Kuusankoski rapids raises a question: what caused the ecotoxicological recovery of the study site? Firstly, the chemical water quality indicators, such as nutrients, oxygen, pH, etc. have not remarkably changed in the Kuusaankoski rapids over the years 1999–2008. Secondly, metal concentrations at the Kuusaankoski rapids were low (unpublished data, HERTTA database of the Finnish Environment Institute), thus their potential to cause gill damage is negligible. Thirdly, it is possible that the decreased incidence of morphological abnormalities is more probably related to decrease or delayed loading of bioactive organic compounds, whose origin is the pulp and paper industry. Harmful substances, not commonly monitored in routine water analysis, include resin acids, retene (7-isopropyl-1-methylphenantrene), chlorinated compounds, and wood sterols. Elevated levels of these compounds have been commonly found from the sediments downstream from pulp and paper mills (Tavendale et al., 1995; Koistinen et al., 1998; Leppänen and Oikari, 2001; Lahdelma and Oikari, 2005, 2006; Meriläinen et al., 2006). Although, the purification efficiency of the current effluents has been improved due to the modern technology (Makris and Banerjee, 2002), resin acids and wood sterols, among other bioactive compounds, are still potentially released to the water (Koistinen et al., 1998; Leppänen et al., 1998; Makris and Banerjee, 2002). Despite their moderately hydrophobic characteristics, they end up in the sediment only temporarily.

The amounts of resin acids and retene in Lake Vatianjärvi were investigated in 2007, when the sediment profiles were analysed along the Äänekoski watercourse downstream of the pulp and paper mills in Äänekoski (Rämänen et al., 2010). In the top layer (0–2 cm), the sediment of Lake Vatianjärvi contained 35 μg/g (d.w.) resin acids and 1.9 μg/g (d.w.) retene. In the deeper and more historically contaminated sediment, the concentrations were about 120 μg/g (d.w.) and 2.3 μg/g (d.w.), respectively. Just below the effluent discharge, the uppermost and the most erodable sediment contained much higher concentrations of resin acids (170 μg/g d.w.) and retene (42.4 μg/g d.w.) (Rämänen et al., 2010). In the studied watercourse, the hydraulic retention time in the lakes downstream pulp and paper mills is only three days (Palomäki et al., 2006). Therefore, besides the direct exposure to the diluted bioactive compounds discharging from the mills, the sediment resuspension is probably another important exposure route of the pollutants to the caddisflies. Probably the load due to the mixture of harmful organic compounds from the mills, as well as dissolution from the sediments, has currently decreased to levels, which do not cause morphological abnormalities to caddisflies. Thus far, the deformities possibly caused by resin acids or alkylated PAHs have not been experimentally studied with caddis larvae. However, gill abnormalities have been found from natural waters polluted by crude oil, containing significant amounts of alkylated and unalkylated PAHs (Simpson, 1980). Retene is a arylhydrocarbon-receptor (AhR) activating alkylated PAH, and therefore, to assure the possible roles of harmful chemicals locally present downstream the pulp and paper industry, laboratory experiments with caddis larvae should be conducted.

Chlorodioxins, and probably also some other chlorinated organic compounds, are another type of pollutants, which have been associated with an increase in gill damage in caddisflies (Simpson, 1980; Camargo, 1991; Vuori and Parkko, 1996; Vuori and Kukkonen, 2002). The Kymijoki River, in southern Finland, is heavily polluted by chlorinated dibenzo-p-dioxins and dibenzofurans discharged from the old manufacture of Ky-5 wood preservative, and up to 27% of gill damage in *C. lepida* and 32% in *H. pellucidula* were observed in that area (Vuori and Parkko, 1996). Vuori and

Kukkonen (2002) found in the Kymijoki River significant correlation between the gill damage in *H. pellucidula* and organochlorine concentrations in aquatic mosses, which are favourable habitats to hydropsychid larvae (Muotka, 1990). Darkened gills were also found from mayflies (*Potamanthus luteus*) collected from the Kymijoki River (Vuori, 1999). Chlorinated compounds, such as chlorinated phenols, chlorinated lignin, chlorinated dioxins and chlorinated furans, usually measured as AOX (adsorbable organic halides), have been common compounds also in the pulp and paper mill effluents (Owens, 1991; Ali and Sreekrishnan, 2001), especially before the introduction of biological effluent treatment, and replacing the use of molecular chlorine with the chlorine dioxide (Owens, 1991). Although, the substitution of elemental chlorine with chlorine dioxide decreased the formation of chlorinated dioxins and furans, some loading by organochlorines is still continuous (Ali and Sreekrishnan, 2001). At the Äänekoski pulp mill the elemental-chlorine-free bleaching method was introduced in 1993 (Botnia, 2010). However, in 1994–2007 the average concentrations of chlorinated organic compounds (AOX) in water samples, 10 km downstream of the mills, have been more than two times higher compared to the Kymijoki River (Palomäki et al., 2009), where extensive gill darkening in *C. lepida* and *H. pellucidula* was observed (Vuori and Parkko, 1996). It must be considered, that the group of compounds measured as AOX is very heterogeneous, and in the Äänekoski watercourse composition of AOX is not known, unlike in the Kymijoki River. However, in the Äänekoski watercourse, few studies with specific organochlorines have been done. Concentrations of chlorophenols, which are typically released from the bleaching process, were measured from the lake mussels (*Anodonta piscinalis*) in 1984–1998. Concentrations decreased with increasing distance from the mills, and concentrations started to decrease after mill renewals in 1985 (Herve et al., 2001). In another study, the concentrations of polychlorinated biphenyls (PCB) were measured from the pike downstream from the Äänekoski mills. The concentration was found to be the highest (157 ng/g f.w.) in pikes caught from Lake Vatianjärvi (Anonymous, 1992), just above the study site of Kuusaankoski rapids. As various chlorinated organic compounds have been measured from the study area, it is possible that some specific compounds, such as chlorinated dioxins, chlorinated furans, or PCB have been causing larval gill damage in our study site. Therefore, to fully understand the cause and effect relationships between candidate groups of chemicals and their potency for pathologies, laboratory experiments with specific chlorinated organic compounds should be carried out.

5. Conclusions

Our study using the gill abnormalities of caddis larvae as an ecotoxicological biomarker indicates a 20-year recovery period of water bodies heavily polluted by the pulp and paper mills. Also our species composition index (HA) reflects the recovery, although variable sampling methods limit interpretation of the results. We conclude that nowadays neither the current discharge from the pulp and paper mills or resuspension of contaminants from the sediments have ecotoxicological impacts on caddisflies at the study area, assuming that the load from the mills or the erosion of the sediments does not increase.

The main question was: which factors enabled the recovery? Indicators based on different caddisfly species and their water quality requirements suggest that the primary factor was an increased oxygen concentration. However, after the start of re-colonization, gill abnormalities still prevailed in caddisflies. High prevalence of abnormalities might have arisen due to toxic compounds. The causes of these abnormalities can be toxicologically bioactive chemicals in recent discharges and/or from past contaminated sediments. We suggest that in the river-like watercourse discharge of the effluents or dissolution of harmful compounds due to sediment erosion may transport the pollutants far from their source, and cause the pathologies. However, without specific laboratory assays, with the single chemicals or representative mixtures, it cannot be concluded that some particular compound originating from the pulp and paper industry has been the specific cause of previous morphological damages over throughout the 1990s. Finally, this study supports the use of morphological damage of caddisflies as a generic biological indicator of water and bottom habitat quality. However, further experimental studies are needed to define the effects of natural environmental factors and treatment stress on the alteration of gill structure. Additional laboratory experiments are also needed to specify indicator values used in HA index.

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