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Author(s): Vehniäinen, Eeva-Riikka; Siiskonen, Silja; Raatikainen, Marja; Oikari, Aimo

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SEDIMENTS, SEC 4 • SEDIMENT-ECOLOGY INTERACTIONS • RESEARCH
ARTICLE

**Do laboratory exposures represent field exposures? - Effects of sediments
contaminated by wood industry on yolk-sac fry of rainbow trout (*Oncorhynchus
mykiss*)**

Eeva-Riikka Vehniäinen • Silja Siiskonen • Marja Raatikainen • Aimo O. J. Oikari

E.-R. Vehniäinen (✉) • S. Siiskonen • M. Raatikainen • A. O. J. Oikari

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

e-mail: eeva-riikka.vehniainen@jyu.fi

M. Raatikainen

present address: Finnish Food Safety Authority Evira, Mustialankatu 3, 00790 Helsinki,
Finland

(✉) **Corresponding author:**

Eeva-Riikka Vehniäinen

Phone number: +358400735959

Fax number: +358142602321

e-mail: eeva-riikka.vehniainen@jyu.fi

Abstract

Purpose: Risk assessment of contaminated sediments is routinely based on laboratory exposures. The purpose of this work was to study if sediments contaminated by chemical wood industry cause developmental defects in fish fry, and how well a laboratory exposure correlates with a field exposure.

Materials and methods: Newly hatched yolk sac fry of rainbow trout (*Oncorhynchus mykiss*) were exposed in the laboratory and *in situ*. In the laboratory, the fish were placed in contact with either clean or contaminated sediment in aquaria. In the field, half of the fish were placed in contact with the lake sediment, and the other half were similarly caged 2 m above it, to discern the effects of the sediment from that of the effluent. When approximately 3/4 of the yolk was consumed, the fry were examined for blue sac disease (BSD) symptoms, their length and yolk volume was determined, and *cyp1a* and *cyp1c2* transcript abundances measured with quantitative PCR.

Results and discussion: The sediments did not cause mortality, developmental defects, or up-regulation of *cyp1a* or *cyp1c2* in the laboratory. No severe BSD was detected in the field exposure either, but mortality was higher in embryos caged on the sediment than in those kept 2 m above the bottom and in those exposed in the laboratory. Unlike the laboratory exposure, the field exposure to contaminated sediments reduced the growth of the fry.

Conclusions: Laboratory exposures may underestimate the risk that contaminated sediments pose to developing fish. This should be taken into account in risk assessment.

Keywords Developmental toxicity • Early life stage • Ecological risk assessment • Laboratory - field comparison • Pulp and paper mills • Sediment toxicity

1 Introduction

Contaminated sediments may be stressors to the organisms in aquatic ecosystems (Burton and Johnston 2010). The altered bleaching methods and modern wastewater treatment have reduced the ecotoxicological impacts of the chemical wood industry in many countries, but the contaminated sediments still contain substances that are potentially harmful to wildlife (Ratia et al. 2013). In the past, the wastewaters of pulp and paper industry caused recruitment failures in fish populations that led to the absence of salmonid species in downstream areas (Hakkari 1992, Hakkari and Bagge 1992). The wastewaters of pulp and paper mills are considered to be of relatively low risk to fish nowadays, but salmonid populations are still largely absent. For example in Southern Lake Saimaa, in SE Finland, the fish community structure downstream of the pulp and paper mills still differs remarkably from clean reference, with bleak and roach dominating in the former and vendace in the latter (Karels and Tiitinen 2013). As the lake sediments are natural spawning sites for some salmonids, could the “legacy from the past”, the contaminated sediments, still affect the recruitment of salmonid fishes?

The pulp and paper mills that use softwood as raw material still release resin acids in their wastewater (Ramanen et al. 2010). Some of the resin acids end up in the sediments downstream of the mills, and are converted to retene by microbes (Tavendale et al. 1997, Leppanen and Oikari 1999, Leppanen et al. 2000). Sediment-borne retene may desorb to water and be bioavailable to aquatic organisms (Oikari et al. 2002, Merilainen et al. 2006). In fish embryos, exposure to waterborne retene causes dioxin-like toxicity observed as blue sac disease (BSD) that coincides with aryl hydrocarbon receptor (AhR) activation detectable as up-regulation of *cyp1a*. The BSD symptoms include

hemorrhaging, yolk and pericardial sac edema, spinal and craniofacial deformities, and fin rot. Salmonid fishes are among the most sensitive to dioxin-like developmental toxicity (Elonen et al. 1998, Billiard et al. 1999), and thus teratogenicity of retene-contaminated sediments might be an explanation for the apparent recruitment failures or impairments of salmonids.

The toxicity testing of sediments is almost routinely carried out in the laboratory, because it is cheaper and less laborious, more controllable and convenient, and bears a smaller risk of unforeseen problems (such as vandalism, waterway traffic and extreme weather conditions) than exposures done *in situ*. Nevertheless, a key question remains whether the results obtained in a laboratory setting correctly predict the effects in the field.

The aim of this study was to find out if native retene-contaminated sediments cause BSD, *cypla* and *cyplc2* induction, or growth retardation in early life-stage rainbow trout, and if the effects are similar in the laboratory and *in situ*.

2 Materials and methods

2.1 Study area and sediments

The study area near the Kaukas pulp and paper mills in Lappeenranta, Southern Lake Saimaa, is located in South-Eastern Finland (Fig. 1). The pulping industry started in the area in 1897, followed by papermaking within a few decades. Major environmental improvements took place in 1992, when the mill started to produce elemental chlorine free (ECF) pulp, and began to treat the wastewaters with the aid of activated sludge. Nowadays the Kaukas (UPM) mill produces softwood and birch pulp, paper, sawn timber, plywood, and wood-based chemicals. In 2011, it released around 100 000 m³ of

wastewater per day. In 2011, the average daily emissions to water consisted of 334 kg of nitrogen, 16.5 kg of phosphorus, and 270 kg of adsorbable organic halogens (AOX); and the daily chemical (COD) and biological oxygen demand (BOD) of the effluent was 30 760 kg and 590 kg, respectively (Anon. 2012). The effluent is known to contain chlorophenolics, resin acids, and phytosterols (e.g. β -sitosterol), and substantially smaller amounts of chlorophenolics than before 1992 (Karels et al. 2001).

The sediment samples were taken just after the spring turnover in May 2010. The sediments were collected with an Ekman device (sediment depth ca. 0 – 10 cm; area 225 cm²), and replicate samples were combined. Six replicate samples were taken at locations 1 and 3 km downstream of the pulp and paper mills (see Fig. 1), and ten samples at the reference site 10 km upstream of the mills (Fig. 1). The sediment samples were stored in sealed polycarbonate containers in the dark at 4 °C. The total organic carbon contents of the sediments were 6.5, 8.9 and 15.9 % (dry weight) for the upstream reference, and the 1 km and 3 km sites, respectively.

2.2 Laboratory exposure

Newly hatched (<24 h) rainbow trout (*Oncorhynchus mykiss*) yolk-sac fry were obtained from the fish farm Hanka-Taimen (Hankasalmi, Finland). The water used in the laboratory experiment was filtered and aerated well water of the University of Jyväskylä. The pH, oxygen concentration, and conductivity were measured daily. The light:dark cycle was 16 h:8 h.

The exposures were carried out in 18-l glass aquaria. The fry were placed in the aquaria in 10 cm × 10 cm × 5 cm stainless steel cages (densely perforated plate with Ø 2 mm

holes) that were covered with polyester net. Each cage housed 10 fish, and there were three cages in each aquarium. The water in all aquaria was continuously aerated, and the fish in the cages were examined daily for mortality.

There was 3 kg of wet sediment in each sediment exposure aquarium, and the water volume above the sediment was 12 l. The sediments were let to settle for 24 hours after carefully adding the water, after which the uppermost water was aerated for 72 hours before starting the exposures. Half of the water was changed on day 6 to ensure that there was one liter water day⁻¹ g fish⁻¹. The sediment exposures were terminated on day 11.

The water exposures were carried out in a volume of 6 l. The positive control treatment was 32 µg l⁻¹ retene (nominal concentration), which, based on our earlier experience, causes clear symptoms of BSD but negligible mortality (Vehniäinen, unpublished). The negative control was clean well water, and the solvent control 0.00016 % DMSO (the same concentration as in the retene treatment). Because retene is a very hydrophobic compound ($\log K_{ow} > 6$), half of the exposure solutions were changed daily in the water exposures to keep the retene concentration more stable. The temperature in the water exposures was 1 °C lower than in the sediment exposures, and therefore they were terminated a day later than the sediment exposures (on day 12). In this way similar day-degrees could be reached in all exposures, and the stage of larval development was similar.

Water samples were collected for retene analysis from all aquaria at the beginning, on days 1 and 7, and at the end of the exposure. The water samples were stored in glass bottles in -20 °C until analysis.

2.3 Field exposures

The experiments were carried out in May 2011 in Southern Lake Saimaa. The sites were the same as where the sediment was collected for the laboratory exposures: 1 and 3 km downstream of the Kaukas pulp and paper mills, and a reference site 10 km upstream of the mills (Fig. 1).

The newly hatched rainbow trout fry were of the same stock as those used in the laboratory exposures, but from a different batch that hatched five weeks later. The fry were transferred to the study site for experimental field exposure in similar metal cages that were used in the laboratory exposures. Six cages, each housing 10 fish, were laid in direct contact with the sediment at each site by a diver; these exposures are designated as bottom exposures. Weights kept the cages attached to the sediment. Five to six cages per site were put in bigger enclosures (one in each) made of polyethylene plastic sieves, and these were positioned 2 m above the sediment surface at each site; these exposures are named water exposures.

The water temperature was measured every three to four days, and in attempt to reach similar day-degrees for all sites the exposures were terminated on day 12 for the cages 2 m above the sediment at the 1 and 3 km sites, and on day 14 for the rest of the cages. The oxygen content, pH, conductivity, visibility, solids and Na^+ content were analyzed from water samples taken near the sediment surface and 2 m above the sediment surface at the end of the field experiment.

Water samples for retene analyses were collected at the beginning and at the end of the experiment near the sediment surface and 2 m above the sediment surface. The samples were stored in glass bottles in $-20\text{ }^{\circ}\text{C}$ until analysis.

168

169 2.4 Chemical analyses

170 The retene concentration in the sediment was determined with a gas chromatograph -
171 mass spectrometer (GC-MS) (Ramanen et al. 2010, Ratia et al. 2014). Briefly, the freeze-
172 dried sediment sample (1 g) was Soxhlet-extracted (Büchi B-811, Switzerland) with 180
173 ml of hexane:2-propanol solution (2:1, v/v). The internal standard was [^{10}H]anthracene
174 (purity 98% atom D, IsotecTM, USA). The extract was evaporated to dryness in a vacuum
175 and dissolved in hexane, dried in nitrogen gas flow and dissolved in hexane:chloroform
176 (10:3, v/v). The sample was filtered through a solid-phase extraction (SPE) cartridge (1 g
177 Sep-Pak[®] Vac silica 6CC, Waters Corporation, USA) and eluted with hexane. The
178 samples were analyzed with a GC-MS (Hewlett Packard 6890 MS, Germany; Hewlett
179 Packard 5973 GC, USA, 30 m * 0.25 mm ID HP-5 polysiloxane polymer column, phase
180 thickness 0.25 μm , volume of injection 1 μl) using the temperature programs described in
181 Rämänen et al. (Ramanen et al. 2010).

182 For the retene analysis of the water samples, a 500 ml sample was filtered through a SPE
183 cartridge (Oasis HLB 3CC, Waters Corporation, USA) after the addition of the internal
184 standard [^{10}H]anthracene, and eluted with hexane:dichloromethane (1:1, v/v). The
185 extract was gently dried in nitrogen gas flow, dissolved in 1 ml of hexane and analyzed
186 with GC-MS with the same procedure as used in the sediment analyses.

187

188 2.5 Sampling, BSD scoring and severity index

189 The fry were anesthetized with 100 mg l^{-1} MS-222 (Sigma) and examined under the
190 microscope for the signs of BSD: hemorrhaging, yolk sac and pericardial sac edema, fin

rot, craniofacial deformities and spinal deformities. The length of the fish as well as the width and height of the yolk sac were measured to the nearest 0.5 mm. The fry were sacrificed, the yolk sacs removed, and the remaining part of the body was placed in a microcentrifuge tube and frozen in liquid nitrogen. The samples were preserved in -80 °C until RNA extraction.

BSD severity index was calculated with the formula (1)

$$SI = \frac{\left[\sum_{i=1}^n (PE * E_i) + \sum_{j=1}^n (YE * E_j) + \sum_{k=1}^n (OH * E_k) + \sum_{l=1}^n (CF * E_l) + \sum_{m=1}^n (SD * E_m) + \sum_{o=1}^n (FR * E_o) \right]}{\max \quad SI \quad score} \quad (1)$$

where SI = severity index, PE = pericardial edema, YE = yolk sac edema, OH = hemorrhaging, CF = craniofacial deformities, SD = spinal deformities, and FR= fin rot. E_i , E_j , E_k , E_l , E_m , and E_o equal the number of embryos displaying pericardial edema, yolk sac edema, hemorrhaging, craniofacial deformities, spinal deformities, and fin rot, respectively (Villalobos et al. 2000, Scott and Hodson 2008, Scott et al. 2009).

2.6 Growth measurements

The length of the fry as well as the width and height of the yolk sac were determined under the microscope. The yolk volume was calculated with the formula (2)

$$V_y = \frac{\pi l h^2}{6} \quad (2)$$

where V_y is the yolk volume, l is the major axis, and h is the minor axis (mm) (Kamler 2008).

In the field exposure, the yolk volume and length of the fish were normalized to effective day-degrees to take into account the effect of temperature differences between the sites. The effective day-degrees were calculated with the formula (3)

$$D_{eff}^{\circ} = \tau (t - t_0) \quad (3)$$

where τ is time (days), t is temperature ($^{\circ}\text{C}$), and t_0 is the temperature of biological zero for rainbow trout (the threshold temperature at which ontogeny is theoretically arrested) (Kamler 2002). The t_0 used in the calculations was 1.7°C (Kamler 2002).

2.7 Quantitative PCR

The primers were designed with AmplifX (version 1.5.4 by Nicolas Jullien ; CNRS, Aix-Marseille Université - <http://crn2m.univ-mrs.fr/pub/amplifx-dist>) and checked for specificity with Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The features of the primers are shown in the Electronic Supplementary Material 1. *elongation factor 1 alpha (ef1a)* and *nadh dehydrogenase 1 alpha subcomplex subunit 8 (ndufa8)* were chosen as reference genes because in previous experiments their expression in retene- and control-exposed rainbow trout yolk-sac fry had been the most stable of a set of reference genes tested (Vehniäinen, unpublished).

Total RNA was extracted with Tri reagent (Molecular Research Center, USA) according to the manufacturer's instructions. Three individual fish per cage in three cages per treatment (total $n = 9$ fish per treatment) were used for all sites, except for the 3 km bottom exposure, where all viable fry ($n = 8$) from the remaining cage were taken as samples. The quantity of the RNA was measured, and the purity checked using the 260:280 nm optical density ratio (NanoDrop 1000, Thermo Scientific). The quality of the RNA was monitored with Agilent 2100 BioAnalyzer, using Eukaryote total RNA 6000 nano kit (Agilent Technologies Inc., USA). The samples with RNA integrity number > 7 were included in the analyses. The DNase treatment (DNase I Amp Grade, Invitrogen) was conducted for 1 μg total RNA, after which it was reverse transcribed to cDNA (iScript cDNA Synthesis Kit, Bio-Rad, USA) and diluted 1+9 with nuclease-free water. One 25 μl qPCR reaction consisted of 5 μl of the diluted cDNA, 0.75 μl each of forward and reverse primers (final concentration 300 nM), 6 μl sterile H_2O and 12.5 μl of iQ SYBR Green Supermix (Bio-Rad, USA). The qPCR was run on a CFX96 Real-Time PCR cycler (Bio-Rad, USA). The protocol was 3 min at 95 $^{\circ}\text{C}$; 40 cycles 10 s at 95 $^{\circ}\text{C}$, 10 s at 58 $^{\circ}\text{C}$, and 30 s at 72 $^{\circ}\text{C}$; 10 s at 95 $^{\circ}\text{C}$, and melt curve from 65 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$. No template controls were run for all genes on all plates, and their C_q s were always over 38. Melt curves showed a single peak, confirming formation of only one PCR product. The expression values were calculated with CFX Manager™ Gene Study (Bio-Rad).

2.8 Statistics

The yolk volume and length of fry (both normalized to effective day-degrees), and mortality were not normally distributed, so the statistical testing for the effects of the

treatments was done with Kruskal-Wallis test for these variables. If statistical differences were observed, Dunnett's T3 test was used to find the differences between the treatments. The effect of treatment on ln-transformed *cyp1a* and *cyp1c1* expression values, and on BSD index was tested with one-way ANOVA followed by Tukey's test. Mann-Whitney U tests were performed for mortality, length, and yolk volume, and t-tests for BSD index, and ln-transformed *cyp1a* and *cyp1c2*, to test if the effects of the field exposure and laboratory exposure to the sediment originating from the same site were similar.

3 Results

3.1 Exposure conditions

The conditions in the laboratory exposures revealed that the temperature in the sediment exposures was 1 °C higher than in the water-only exposures (Electronic Supplementary Material 2).

At the start of the field experiment the water temperature over the research area varied from 6.6 to 11.0 °C on the bottom and from 8.6 to 12.8 °C in the water exposure sites, the reference area water being colder than the water in the other areas (Electronic Supplementary Material 3). Nevertheless, during the course of the experiment, the water warmed over the area so that at the end of the experiment it was 12.5 °C at the reference site and 13.0 - 13.2 °C at the other sites, and there were no differences between the bottom and water exposures. The average water temperature was 1.5 °C colder at the reference site than at the contaminated sites. The oxygen saturation was 100 % in the reference area and 90 - 92 % at the other sites (Electronic Supplementary Material 3).

At the 3 km experimental site, the sediment was so soft that one of the cages sank into it completely, causing the death of the fish. In addition, four cages were lost at this site because their holding strings had broken, and due to the poor visibility in the area even the divers could not find them. Thus all the analyses for the 3 km bottom were conducted with viable fry (n=8) from the only remaining cage.

3.2 Retene concentration

The retene concentration in the sediments 1 and 3 km downstream of the mills was (mean $\pm$ SD) 325 ± 73 and $140 \pm 6 \mu\text{g g}^{-1}$ d.w., respectively. Some retene trace was also detected in the reference sediment, but it was below the limit of quantification.

In the laboratory experiments, a small, non-quantifiable retene peak was detected in the water samples taken from the sediment exposure tanks before the start of the experiment.

At the end of the experiment, retene was detected both in the 1 and 3 km sediment tanks, but not in the reference sediment tank. The concentration of retene in the positive control (nominal concentration $32 \mu\text{g l}^{-1}$) was $29.4 \mu\text{g l}^{-1}$ at the time of water change.

In the field experiment, retene was detected in the water samples taken near the bottom at all sites, but its amount was below the limit of quantification ($< 2.5 \mu\text{g l}^{-1}$). No retene was detected in the water samples taken 2 m above the sediment.

3.3 Mortality and BSD severity index

In the laboratory experiments, one fish died on day 1 in the reference sediment exposure.

In the field, only one fish out of the 160 caged 2 m above the sediment died. Mortality in the bottom cages was higher at the reference and 3 km sites (Fig. 2a). Nevertheless, it

must be taken into account that the 3 km site data are derived from one cage, so they must be interpreted with caution.

In the laboratory, the positive control retene caused BSD, and it was the only treatment in which the BSD severity index differed from controls (Fig. 2b). There was no difference in the BSD severity index between the sites in the field exposure (Fig. 2c). Though the BSD severity index seemed to be higher in the bottom exposures at every site in the field, this finding was not statistically significant (Fig. 2c).

3.4 Growth

Retene treatment attenuated growth by 6 % in the laboratory experiment (Fig. 3a). The fry exposed to the sediment 3 km downstream of the mills were 4 % longer than the control fry, but the other treatments had no effect on the length of the fish (Fig. 3a). Accordingly, the mean yolk volume was largest in the positive control treatment, but this was significantly different only from the water only control, reference sediment and 1 km sediment (Fig. 3b). The fry exposed to the reference sediment had the smallest yolk sacs at the end of the experiment (Fig. 3b).

In the field experiments, the length of the fish and yolk volume were normalized to efficient day-degrees to take into account the temperature differences in the treatments. The 1 and 3 km sediments attenuated growth, as the fry caged on the sediment were significantly shorter than those caged 2 m above it, and smaller than those caged on the sediment in the reference area (Fig. 3c). The yolk volumes of the fry from the reference water exposure were smaller than those of the fry from the reference bottom and 3 km bottom exposures (Fig. 3d).

326

327 3.5 *cyp1a* and *cyp1c2* transcript abundance

328 In the laboratory, retene caused a 10-fold up-regulation of *cyp1a* and almost three-fold
329 up-regulation of *cyp1c2* (Figs. 4a and 4c). The *cyp1c2* expression did not differ between
330 the other treatments. The fry from the water-only control and 1 km sediment treatments
331 had the lowest expression values of *cyp1a*; the fish from all other treatments had
332 significantly more *cyp1a* mRNA (Fig. 4a). In the field, there was no effect between the
333 sites in *cyp1a* and *cyp1c2* mRNA levels (Figs. 4b and 4d).

334

335 3.6 Laboratory and field comparison

336 The effect of sediment contact in the laboratory was compared to that in the field (bottom
337 cages) (Table 1). The reference sediment, 10 km upstream from the industrial area, caused
338 more mortality in the field than in the laboratory (mean 44 % and 3 %, respectively). The
339 BSD index values did not differ between the laboratory and field exposures at any site.

340 The fry had more yolk left at the end of the field exposure settings than the laboratory
341 exposures at all sites (Table 1). The length of the fry differed between the laboratory and
342 field exposures: The fish exposed to the 1 and 3 km sediments in the field were shorter than
343 the ones exposed in the laboratory, whereas those exposed to the reference sediment in the
344 field were longer than those exposed in the laboratory (Table 1).

345 For all study sites, the fish from the field exposure possessed more *cyp1a* mRNA than those
346 from the laboratory exposure, but *cyp1c1* transcript abundance was similar in the laboratory-
347 and field-exposed organisms (Table 1).

348

349 **4 Discussion**

Studying the effects of contaminated sediments on fish yolk sac fry in field conditions may be problematic. Monitoring the annual recruitment yield of fishes may include bias due to e.g. migration and field conditions. Caging makes it possible to manipulate the timing, duration and location of the exposure in the field, and thus makes the field exposure more comparable to laboratory exposure than collecting live fry from the area (Oikari 2006).

There have been only a few true comparisons of the effects of contaminated sediments *in situ* and in the laboratory (Conrad et al. 1999, Pereira et al. 2000, Kater et al. 2001, Ringwood and Keppler 2002, Anderson et al. 2004, Phillips et al. 2004, Mann et al. 2010). We are neither aware of any previous laboratory to field comparisons using fish fry, nor of those using sediments contaminated by pulp and paper mills. In this work, the laboratory exposures underestimated the effects of the contaminated sediments on rainbow trout fry. The mortality was higher in the field than in the laboratory. Also the BSD indices appeared higher in the field, but they did not differ statistically between the laboratory and field exposures. The contaminated sediments attenuated the growth of the fry only in the field exposures. Differences in the outcomes between laboratory and *in situ* sediment exposures have also been described previously among invertebrates (Conrad et al. 1999, Pereira et al. 2000, Kater et al. 2001, Ringwood and Keppler 2002, Anderson et al. 2004, Mann et al. 2010) and fishes (Costa et al. 2011a, Costa et al. 2011b, Costa et al. 2012). In most cases, the sediments have been less toxic in the laboratory than in the field (Conrad et al. 1999, Pereira et al. 2000, Kater et al. 2001, Ringwood and Keppler 2002, Anderson et al. 2004, Hose et al. 2006, Mann et al. 2010). Therefore, sediment toxicity tests conducted in the laboratory must be interpreted with caution. As

for practical reasons laboratory testing will remain the choice for the majority of cases, the application of a safety factor could be justified.

The more pronounced toxicity in the field exposures could not be explained by the chemical analyses done in this study. The retene concentrations in water were at a similar, very low level in the laboratory and field exposures at all sites, and they did not correlate with toxicity. The rainbow trout fry could be exposed to retene and other contaminants also by feeding on the sediments, and indeed sediment could be found in the intestines of the fry both in the laboratory and in the field (bottom) exposures. Despite the feeding on the sediments in both settings, toxic effects were seen only in the field setting. As there was no difference between the sites or in the bottom vs. water exposures in *cyp1a* transcript abundance, it can be concluded that AhR activating compounds were presumably not bioavailable from the sediments. The same conclusions were drawn in a recent study in the same area, where brown trout juveniles caged on the sediment did not show elevated EROD activity when compared to the ones caged in water or in a reference area (Oikari et al. 2010). These findings lead to the conclusion that retene was not the compound behind the toxic effects.

In addition to retene, the contaminated sediments in the study area contain resin acids (Ratia et al. 2014). There is no published data on the effects of resin acids on fish embryo development. Nevertheless, one of the most abundant resin acids in the sediments downstream of pulp and paper mills, dehydroabietic acid, affects the energy metabolism of fish and might therefore be capable of interfering with growth (Rissanen et al. 2003, Pandelides et al. 2014).

It has been suggested that especially the contaminated overlying water plays a role in the more pronounced toxicity seen in *in situ* exposures vs. laboratory exposures (Phillips et al. 2004, Hose et al. 2006). In our study, no signs of toxicity were seen in the fry housed in the water cages without contact to the sediment, but there may still be factors in the water that potentiate the toxicity of the sediment at the contaminated sites. Indeed, the water 1 and 3 km downstream of the mills is still affected by the effluent discharge from the pulp and paper mills, as the water analysis shows that it contains more suspended solids and sodium (a tracer for effluent), and has a higher conductivity (also a marker of effluent), than the water at the reference site.

Another factor that may have an effect on the toxicity *in situ* is oxygen. Hypoxia has been shown to delay development and affect the growth of fish embryos and fry (Shang and Wu 2004, Ciuhandu et al. 2005). The O₂ concentrations were similar in the water samples taken from the bottom and 2 m above it at the end of the exposure. Nevertheless, hypoxic conditions on the sediment surface in the contaminated areas during the course of the experiment cannot be ruled out.

It must also be borne in mind that though the sediments in the laboratory assay were from the same sites as the field experiment, there may still be differences in the contaminant composition and concentrations between the laboratory and field settings. For instance, the Ekman device takes the uppermost 10 cm layer of the sediment, whereas in the field the fry are probably exposed to the uppermost 2 cm layer only. Also the exact location of the sediment sampling for the laboratory exposures can never be exactly the same as that of the field exposure. It is known that as the surface area in laboratory sediment assays increases, bioavailability and toxic effects of organic contaminants increase as well

(Fragoso et al. 2006). It can be assumed that the sediment surface area *in situ* is always larger than that in a laboratory setting, and thus one could expect more toxicity in the field. This should be taken into account in the risk assessment, for example by using a safety factor.

There was more *cypla* mRNA present in the fry exposed in the field than in the laboratory. There are two possible explanations for this. The field and laboratory exposures were conducted with different lots of fry, and the level of *cypla* transcripts could have differed between the lots. Another reason could be some substance(s) that were present or more abundant in the field (e.g. constituents of boat fuel). The *cypla* transcript abundance did not differ between the sites or between the bottom and water exposures, and thus if caused by a substance(s) in the field, it had to be something that was present in the water at all sites. The elevated *cypla* transcription coincided with the larger yolk sacs detected in the fry exposed in the field, and one of the symptoms associated with dioxin-like toxicity is the difficulty in absorbing yolk. The larger yolk sacs may be interpreted as developmental delay, which may have an effect on survival in the long run, because it is considered that the longer the fish spend in their most vulnerable stages, the lower the survival success is.

5 Conclusions

The study showed that sediment-bound retene does not cause acute developmental defects in rainbow trout fry. Nevertheless, the fish early life stages developing downstream of chemical wood industry may still be at risk. Sediment assays conducted in

laboratory conditions may underestimate the effects in the field, and this should be taken into account in the risk assessment.

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Figure legends

Fig. 1 The study area at the Southern Lake Saimaa. The black spots mark the field exposure and sediment sampling sites (1 km, 3 km and Reference). The water flow direction is marked by arrows, and the effluent discharge point by a cross. Water from the clean background area is brought to the reference site by a pump station (rate ca 45 m³ sec⁻¹) to dilute the effluents from the downstream mills

Fig. 2 Mortality (a) and BSD severity index (b, c) in rainbow trout yolk sac fry exposed to sediments or water downstream of the pulp and paper mills in the laboratory (a, b) and *in situ* (a, c). The bars represent mean $\pm$ SD. * denotes statistical significance from other treatments ($P < 0.05$). $n = 3$ groups of 10 individuals in laboratory exposures, 5 or 6 groups of 10 individuals in field water exposures, and 6 groups of 10 individuals in field bottom exposures, except the 3 km bottom in which the data are from one group of 10 individuals as other cages were lost or buried into the sediment

Fig. 3 Effect of sediments downstream of the pulp and paper mill on growth and development of rainbow trout yolk sac fry in the laboratory (a, b) and in the field (c, d). The data are presented as mean $\pm$ SD. Groups denoted by the same letter do not differ statistically from each other. $n = 3$ groups of 10 individuals in laboratory exposures, 5 or 6 groups of 10 individuals in field water exposures, and 6 groups of 10 individuals in field bottom exposures, except the 3 km bottom in which the data are from one group of 10 individuals as other cages were lost or buried into the sediment

Fig. 4 Effects of sediments on *cyp1a* (a, b) and *cyp1c2* (c, d) mRNA abundance in rainbow trout yolk sac fry after laboratory (a, c) and field (b, d) exposure. Groups marked with the same letter do not differ statistically from each other. * denotes statistical significance from other groups ($P < 0.05$). $n = 9$ individuals in all exposures except the field 3 km bottom in which the data are from 8 individuals

Figure 1

Figure 2.

Figure 3.

Figure 4.

618

619 Table 1. Effects of exposure to sediment contaminated by wood industry on rainbow trout yolk sac fry in the laboratory and in the
 620 field. Values represent mean $\pm$ SD. Asterisks denote statistically significant difference between laboratory and field exposure: *=
 621 $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$ (t-test for BSD index, *cyp1a* and *cyp1c2* expression; Mann-Whitney U for others).

622 ^a NA = not analyzed because four cages were lost and one buried into the sediment

		1 km	3 km	reference
mortality %	laboratory	0	0	3.3 $\pm$ 5.8 *
	field	13 $\pm$ 16	NA ^a	44.0 $\pm$ 30.5
BSD index	laboratory	0.11 $\pm$ 0.03	NA ^a	0.09 $\pm$ 0.02
	field	0.24 $\pm$ 0.15		0.41 $\pm$ 0.32
length of animal (mm/eff. dd)	laboratory	0.17 $\pm$ 0.01 ***	0.17 $\pm$ 0.01 ***	0.17 $\pm$ 0.01 ***
	field	0.16 $\pm$ 0.01	0.15 $\pm$ 0.01	0.18 $\pm$ 0.01
yolk volume (mm ³ /eff. dd)	laboratory	0.006 $\pm$ 0.007 ***	0.018 $\pm$ 0.011 **	0.005 $\pm$ 0.005 ***
	field	0.046 $\pm$ 0.026	0.053 $\pm$ 0.028	0.060 $\pm$ 0.029
<i>cyp1a</i> expression	laboratory	0.06 $\pm$ 0.02 ***	0.10 $\pm$ 0.01 **	0.09 $\pm$ 0.02 **
	field	0.17 $\pm$ 0.04	0.17 $\pm$ 0.09	0.14 $\pm$ 0.03
<i>cyp1c2</i> expression	laboratory	0.58 $\pm$ 0.24	0.44 $\pm$ 0.14	0.50 $\pm$ 0.17
	field	0.43 $\pm$ 0.14	0.59 $\pm$ 0.29	0.39 $\pm$ 0.18

623

624 Table S1. Features of the primers used in the quantitative PCR.
 625

Gene name	Accession	Primer	Product length	Efficiency
<i>cyp1a</i>	U62796	F: CAGTCCGCCAGGCTCTTATCAAGC R: GCCAAGCTCTTGCCGTCGTTGAT	94	96.9
<i>cyp1c2</i>	NM_001185032.1	F: CAACCACGACCCTTTACAGTGGAA R: CAGATCCTTGTCAAGGGCACCATT	88	92.8
<i>ndufa8</i>	NM_001160582.1	F: TTCAGAGCCTCATCTTGCCTGCT R: CAACATAGGGATTGGAGAGCTGTACG	119	99.5
<i>efla</i>	NM_001124339.1	F: CTTTGTGCCCATCTCTGGTT R: TTACGTTCGACCTTCCATCC	90	98.1

636 Table S2. Water characteristics during the laboratory exposures of rainbow yolk sac fry to sediments contaminated by pulp and paper
 637 mills and clean reference. Values represent mean $\pm$ SD of daily measurements.
 638

	Temperature (°C)	O ₂ concentration mg/l	pH	Conductivity (μ S/cm)	639 640
1 km sediment	12.9 $\pm$ 0.3	11.8 $\pm$ 0.3	7.60 $\pm$ 0.05	213 $\pm$ 15	641
3 km sediment	12.9 $\pm$ 0.3	11.7 $\pm$ 0.3	7.60 $\pm$ 0.07	193 $\pm$ 15	642
reference sediment	12.8 $\pm$ 0.1	11.6 $\pm$ 0.4	7.50 $\pm$ 0.10	210 $\pm$ 15	643 644
negative control	11.9 $\pm$ 0.1	12.1 $\pm$ 0.3	7.80 $\pm$ 0.07	210 $\pm$ 14	645
solvent control	12.0 $\pm$ 0.1	12.0 $\pm$ 0.3	7.80 $\pm$ 0.05	207 $\pm$ 16	
positive control (32 μ g/L retene)	11.8 $\pm$ 0.3	12.1 $\pm$ 0.3	7.80 $\pm$ 0.03	208 $\pm$ 14	

646 Table S3. General water characteristics in Southern Lake Saimaa during the field exposures. Data is from one water sampling taken in
 647 the end of the experiment, except values for temperature represent mean $\pm$ SD of five measurements conducted during the experiment.
 648

Site		Depth from surface (m)	Temperature (°C)	Effective day-degrees	O ₂ concentration (mg/L)	pH	Conductivity (µS/cm)	Visibility (m)	Solids (mg/L)	Sodium (mg/L)
1 km	bottom water	5.5	11.4 $\pm$ 1.3	141.7	9.6	7.3	145	1.8	4.0	25.0
		3.5	12.1 $\pm$ 1.0	127.7	9.5	7.4	146			
3 km	bottom water	5.0	11.5 $\pm$ 1.1	142.8	9.7	7.5	169	1.9	2.8	21.0
		3.0	12.0 $\pm$ 1.1	125.9	9.5	7.5	170			
Reference	bottom water	6.0	9.8 $\pm$ 2.5	121.3	10.8	7.4	71.5	2.3	1.9	7.0
		4.0	10.4 $\pm$ 1.6	128.5	10.9	7.3	69.3			

649