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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*)

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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, *cyp1a* and *cyp1c2* 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 m^3 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 cm^2), 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

122 holes) that were covered with polyester net. Each cage housed 10 fish, and there were
123 three cages in each aquarium. The water in all aquaria was continuously aerated, and the
124 fish in the cages were examined daily for mortality.
125 There was 3 kg of wet sediment in each sediment exposure aquarium, and the water
126 volume above the sediment was 12 l. The sediments were let to settle for 24 hours after
127 carefully adding the water, after which the uppermost water was aerated for 72 hours
128 before starting the exposures. Half of the water was changed on day 6 to ensure that there
129 was one liter water day^{-1} g $fish^{-1}$. The sediment exposures were terminated on day 11.
130 The water exposures were carried out in a volume of 6 l. The positive control treatment
131 was 32 μg l^{-1} retene (nominal concentration), which, based on our earlier experience,
132 causes clear symptoms of BSD but negligible mortality (Vehniäinen, unpublished). The
133 negative control was clean well water, and the solvent control 0.00016 % DMSO (the
134 same concentration as in the retene treatment). Because retene is a very hydrophobic
135 compound ($\log K_{ow} > 6$), half of the exposure solutions were changed daily in the water
136 exposures to keep the retene concentration more stable. The temperature in the water
137 exposures was 1 ºC lower than in the sediment exposures, and therefore they were
138 terminated a day later than the sediment exposures (on day 12). In this way similar day-
139 degrees could be reached in all exposures, and the stage of larval development was
140 similar.
141 Water samples were collected for retene analysis from all aquaria at the beginning, on
142 days 1 and 7, and at the end of the exposure. The water samples were stored in glass
143 bottles in -20 °C until analysis.
144

145 2.3 Field exposures

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

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

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

165 Water samples for retene analyses were collected at the beginning and at the end of the
166 experiment near the sediment surface and 2 m above the sediment surface. The samples
167 were stored in glass bottles in -20 °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 [10^2H]anthracene
174 (purity 98% atom D, Isotec™, 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 [10^2H]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 ºC 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)

$$D^{\circ}_{eff} = \tau\,(t - t_0) \qquad (3)$$

where τ is time (days), t is temperature (°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 °C; 40 cycles 10 s at 95 °C, 10 s at 58 °C, and 30 s at 72 °C; 10 s at 95 °C, and melt curve from 65 °C to 95 °C. No template controls were run for all genes on all plates, and their C_qs 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

257 treatments was done with Kruskal-Wallis test for these variables. If statistical differences
258 were observed, Dunnett's T3 test was used to find the differences between the treatments.
259 The effect of treatment on ln-transformed *cyp1a* and *cyp1c1* expression values, and on
260 BSD index was tested with one-way ANOVA followed by Tukey's test.
261 Mann-Whitney U tests were performed for mortality, length, and yolk volume, and t-tests
262 for BSD index, and ln-transformed *cyp1a* and *cyp1c2*, to test if the effects of the field
263 exposure and laboratory exposure to the sediment originating from the same site were
264 similar.
265

266 **3 Results**

267 3.1 Exposure conditions

268 The conditions in the laboratory exposures revealed that the temperature in the sediment
269 exposures was 1 ºC higher than in the water-only exposures (Electronic Supplementary
270 Material 2).
271 At the start of the field experiment the water temperature over the research area varied
272 from 6.6 to 11.0 ºC on the bottom and from 8.6 to 12.8 ºC in the water exposure sites, the
273 reference area water being colder than the water in the other areas (Electronic
274 Supplementary Material 3). Nevertheless, during the course of the experiment, the water
275 warmed over the area so that at the end of the experiment it was 12.5 ºC at the reference
276 site and 13.0 - 13.2 ºC at the other sites, and there were no differences between the
277 bottom and water exposures. The average water temperature was 1.5 °C colder at the
278 reference site than at the contaminated sites. The oxygen saturation was 100 % in the
279 reference area and 90 - 92 % at the other sites (Electronic Supplementary Material 3).

280 At the 3 km experimental site, the sediment was so soft that one of the cages sank into it
281 completely, causing the death of the fish. In addition, four cages were lost at this site
282 because their holding strings had broken, and due to the poor visibility in the area even
283 the divers could not find them. Thus all the analyses for the 3 km bottom were conducted
284 with viable fry (n=8) from the only remaining cage.
285
286 3.2 Retene concentration
287 The retene concentration in the sediments 1 and 3 km downstream of the mills was (mean
288 ± SD) 325 ± 73 and 140 ± 6 μg g^{-1} d.w., respectively. Some retene trace was also
289 detected in the reference sediment, but it was below the limit of quantification.
290 In the laboratory experiments, a small, non-quantifiable retene peak was detected in the
291 water samples taken from the sediment exposure tanks before the start of the experiment.
292 At the end of the experiment, retene was detected both in the 1 and 3 km sediment tanks,
293 but not in the reference sediment tank. The concentration of retene in the positive control
294 (nominal concentration 32 μg l^{-1}) was 29.4 μg l^{-1} at the time of water change.
295 In the field experiment, retene was detected in the water samples taken near the bottom at
296 all sites, but its amount was below the limit of quantification (< 2.5 μg l^{-1}). No retene was
297 detected in the water samples taken 2 m above the sediment.
298
299 3.3 Mortality and BSD severity index
300 In the laboratory experiments, one fish died on day 1 in the reference sediment exposure.
301 In the field, only one fish out of the 160 caged 2 m above the sediment died. Mortality in
302 the bottom cages was higher at the reference and 3 km sites (Fig. 2a). Nevertheless, it

303 must be taken into account that the 3 km site data are derived from one cage, so they
304 must be interpreted with caution.
305 In the laboratory, the positive control retene caused BSD, and it was the only treatment in
306 which the BSD severity index differed from controls (Fig. 2b). There was no difference
307 in the BSD severity index between the sites in the field exposure (Fig. 2c). Though the
308 BSD severity index seemed to be higher in the bottom exposures at every site in the field,
309 this finding was not statistically significant (Fig. 2c).
310
311 3.4 Growth
312 Retene treatment attenuated growth by 6 % in the laboratory experiment (Fig. 3a). The
313 fry exposed to the sediment 3 km downstream of the mills were 4 % longer than the
314 control fry, but the other treatments had no effect on the length of the fish (Fig. 3a).
315 Accordingly, the mean yolk volume was largest in the positive control treatment, but this
316 was significantly different only from the water only control, reference sediment and 1 km
317 sediment (Fig. 3b). The fry exposed to the reference sediment had the smallest yolk sacs
318 at the end of the experiment (Fig. 3b).
319 In the field experiments, the length of the fish and yolk volume were normalized to
320 efficient day-degrees to take into account the temperature differences in the treatments.
321 The 1 and 3 km sediments attenuated growth, as the fry caged on the sediment were
322 significantly shorter than those caged 2 m above it, and smaller than those caged on the
323 sediment in the reference area (Fig. 3c). The yolk volumes of the fry from the reference
324 water exposure were smaller than those of the fry from the reference bottom and 3 km
325 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).

395 It has been suggested that especially the contaminated overlying water plays a role in the
396 more pronounced toxicity seen in *in situ* exposures vs. laboratory exposures (Phillips et al.
397 2004, Hose et al. 2006). In our study, no signs of toxicity were seen in the fry housed in
398 the water cages without contact to the sediment, but there may still be factors in the water
399 that potentiate the toxicity of the sediment at the contaminated sites. Indeed, the water 1
400 and 3 km downstream of the mills is still affected by the effluent discharge from the pulp
401 and paper mills, as the water analysis shows that it contains more suspended solids and
402 sodium (a tracer for effluent), and has a higher conductivity (also a marker of effluent),
403 than the water at the reference site.
404 Another factor that may have an effect on the toxicity *in situ* is oxygen. Hypoxia has been
405 shown to delay development and affect the growth of fish embryos and fry (Shang and
406 Wu 2004, Ciuhandu et al. 2005).The O_2 concentrations were similar in the water samples
407 taken from the bottom and 2 m above it at the end of the exposure. Nevertheless, hypoxic
408 conditions on the sediment surface in the contaminated areas during the course of the
409 experiment cannot be ruled out.
410 It must also be borne in mind that though the sediments in the laboratory assay were from
411 the same sites as the field experiment, there may still be differences in the contaminant
412 composition and concentrations between the laboratory and field settings. For instance,
413 the Ekman device takes the uppermost 10 cm layer of the sediment, whereas in the field
414 the fry are probably exposed to the uppermost 2 cm layer only. Also the exact location of
415 the sediment sampling for the laboratory exposures can never be exactly the same as that
416 of the field exposure. It is known that as the surface area in laboratory sediment assays
417 increases, bioavailability and toxic effects of organic contaminants increase as well

418 (Fragoso et al. 2006). It can be assumed that the sediment surface area *in situ* is always
419 larger than that in a laboratory setting, and thus one could expect more toxicity in the
420 field. This should be taken into account in the risk assessment, for example by using a
421 safety factor.
422 There was more *cyp1a* mRNA present in the fry exposed in the field than in the
423 laboratory. There are two possible explanations for this. The field and laboratory
424 exposures were conducted with different lots of fry, and the level of *cyp1a* transcripts
425 could have differed between the lots. Another reason could be some substance(s) that
426 were present or more abundant in the field (e.g. constituents of boat fuel). The *cyp1a*
427 transcript abundance did not differ between the sites or between the bottom and water
428 exposures, and thus if caused by a substance(s) in the field, it had to be something that
429 was present in the water at all sites. The elevated *cyp1a* transcription coincided with the
430 larger yolk sacs detected in the fry exposed in the field, and one of the symptoms
431 associated with dioxin-like toxicity is the difficulty in absorbing yolk. The larger yolk
432 sacs may be interpreted as developmental delay, which may have an effect on survival in
433 the long run, because it is considered that the longer the fish spend in their most
434 vulnerable stages, the lower the survival success is.
435

436 **5 Conclusions**

437 The study showed that sediment-bound retene does not cause acute developmental
438 defects in rainbow trout fry. Nevertheless, the fish early life stages developing
439 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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References

Anderson B, Hunt J, Phillips B, Nicely P, Tjeerdema R, Martin M (2004) A comparison of in situ and laboratory toxicity tests with the estuarine amphipod *Eohaustorius estuarius*. Arch Environ Contam Toxicol 46:52-60

Anonymous (2012) Environmental performance 2011 UPM Kaukas

Billiard S, Querbach K, Hodson P (1999) Toxicity of retene to early life stages of two freshwater fish species. Environmental Toxicology and Chemistry 18:2070-2077

Burton GA, Johnston EL (2010) Assessing contaminated sediments in the context of multiple stressors. Environmental Toxicology and Chemistry 29:2625-2643

Ciuhandu C, Stevens E, Wright P (2005) The effect of oxygen on the growth of *Oncorhynchus mykiss* embryos with and without a chorion. J Fish Biol 67:1544-1551

Conrad A, Fleming R, Crane M (1999) Laboratory and field response of *Chironomus riparius* to a pyrethroid insecticide. Water Res 33:1603-1610

Costa PM, Chicano-Galvez E, Caeiro S, Lobo J, Martins M, Ferreira AM, Caetano M, Vale C, Alhama-Carmona J, Lopez-Barea J, Angel DelValls T, Costa MH (2012) Hepatic proteome changes in *Solea senegalensis* exposed to contaminated estuarine sediments: a laboratory and in situ survey. Ecotoxicology 21:1194-1207

466 Costa PM, Caeiro S, Lobo J, Martins M, Ferreira AM, Caetano M, Vale C, DelValls TA,
467 Costa MH (2011a) Estuarine ecological risk based on hepatic histopathological indices
468 from laboratory and in situ tested fish. Mar Pollut Bull 62:55-65

469 Costa PM, Miguel C, Caeiro S, Lobo J, Martins M, Ferreira AM, Caetano M, Vale C,
470 DelValls TA, Costa MH (2011b) Transcriptomic analyses in a benthic fish exposed to
471 contaminated estuarine sediments through laboratory and in situ bioassays.
472 Ecotoxicology 20:1749-1764

473 Elonen G, Spehar R, Holcombe G, Johnson R, Fernandez J, Erickson R, Tietge J, Cook P
474 (1998) Comparative toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin to seven freshwater
475 fish species during early life-stage development. Environmental Toxicology and
476 Chemistry 17:472-483

477 Fragoso NM, Hodson PV, Zambon S (2006) Evaluation of an exposure assay to measure
478 uptake of sediment PAH by fish. Environ Monit Assess 116:481-511

479 Hakkari L (1992) Effects of pulp and paper mill effluents on fish populations in Finland.
480 Finnish Fisheries Research 13:93-106

481 Hakkari L, Bagge P (1992) Reproductive success of *Coregonus* species in areas loaded
482 by effluents from paper-mills. Hydrobiologia 243:405-412

483 Hose GC, Murray BR, Park ML, Kelaher BP, Figueira WF (2006) A meta-analysis
484 comparing the toxicity of sediments in the laboratory and in situ. Environmental
485 Toxicology and Chemistry 25:1148-1152

486 Kamler E (2002) Ontogeny of yolk-feeding fish: an ecological perspective. Rev Fish Biol
487 Fish 12:79-103

488 Kamler E (2008) Resource allocation in yolk-feeding fish. Rev Fish Biol Fish 18:143-200

489 Karels A, Markkula E, Oikari A (2001) Reproductive, biochemical, physiological, and
490 population responses in perch (Perca fluviatilis L.) and roach (Rutilus rutilus L.)
491 downstream of two elemental chlorine-free pulp and paper mills. Environmental
492 Toxicology and Chemistry 20:1517-1527

493 Karels A, Tiitinen V (2013)
 Etelä-Saimaan ja Vuoksen kalataloudellinen
494 tarkkailu vuonna 2012
 :1-48

495 Kater B, Postma J, Dubbeldam M, Prins J (2001) Comparison of laboratory and in situ
496 sediment bioassays using *Corophium volutator*. Environmental Toxicology and
497 Chemistry 20:1291-1295

498 Leppanen H, Kukkonen J, Oikari A (2000) Concentration of retene and resin acids in
499 sedimenting particles collected from a bleached kraft mill effluent receiving lake. Water
500 Res 34:1604-1610

501 Leppanen H, Oikari A (1999) The occurrence and bioavailability of retene and resin acids
502 in sediments of a lake receiving BKME (bleached kraft mill effluent). Water Science and
503 Technology 40:131-138

504 Mann RM, Hyne RV, Simandjuntak DL, Simpson SL (2010) A rapid amphipod
505 reproduction test for sediment quality assessment: *in situ* bioassays do not replicate
506 laboratory bioassays. Environmental Toxicology and Chemistry 29:2566-2574

507 Merilainen P, Lahdelma I, Oikari L, Hyotylainen T, Oikari A (2006) Dissolution of resin
508 acids, retene and wood sterols from contaminated lake sediments. Chemosphere 65:840-
509 846

510 Oikari A, Fragoso N, Leppanen H, Chan T, Hodson PV (2002) Bioavailability to juvenile
511 rainbow trout (Oncorynchus mykiss) of retene and other mixed-function oxygenase-
512 active compounds from sediments. Environ Toxicol Chem 21:121-128

513 Oikari A, Lahti M, Merilainen P, Afanasyev S, Krasnov A (2010) Do historical
514 sediments of pulp and paper industry contribute to the exposure of fish caged in receiving
515 waters? Journal of Environmental Monitoring 12:1045-1054

516 Oikari A (2006) Caging techniques for field exposures of fish to chemical contaminants.
517 Aquatic Toxicology 78:370-381

518 Pandelides Z, Guchardi J, Holdway D (2014) Dehydroabietic acid (DHAA) alters
519 metabolic enzyme activity and the effects of 17β-estradiol in rainbow trout
520 (Oncorhynchus mykiss). Ecotoxicol Environ Saf 101:168-176

521 Pereira A, Soares A, Goncalves F, Ribeiro R (2000) Water-column, sediment, and in situ
522 chronic bioassays with cladocerans. Ecotoxicol Environ Saf 47:27-38

523 Phillips BM, Anderson BS, Hunt JW, Nicely PA, Kosaka RA, Tjeerdema RS, de
524 Vlaming V, Richard N (2004) In situ water and sediment toxicity in an agricultural
525 watershed. Environmental Toxicology and Chemistry 23:435-442

526 Ramanen H, Lassila H, Lensu A, Lahti M, Oikari A (2010) Dissolution and spatial
527 distribution of resin acids and retene in sediments contaminated by pulp and paper
528 industry. Journal of Soils and Sediments 10:349-358

529 Ratia H, Ramanen H, Lensu A, Oikari A (2013) Betulinol and wood sterols in sediments
530 contaminated by pulp and paper mill effluents: dissolution and spatial distribution.
531 Environmental Science and Pollution Research 20:4562-4573

532 Ratia HM, Vehniainen E, Rusanen AT, Oikari AOJ (2014) Recovery of historically
533 contaminated watercourse polluted by the chemical wood industry: EROD activity in fish
534 as biomarker. Soil Sed Contam 23:211-225

535 Ringwood A, Keppler C (2002) Comparative in situ and laboratory sediment bioassays
536 with juvenile *Mercenaria mercenaria*. Environmental Toxicology and Chemistry
537 21:1651-1657

538 Rissanen E, Krumschnabel G, Nikinmaa M (2003) Dehydroabietic acid, a major
539 component of wood industry effluents, interferes with cellular energetics in rainbow trout
540 hepatocytes. Aquatic Toxicology 62:45-53

541 Scott JA, Ross M, Lemire BC, Hodson PV (2009) Embryotoxicity of retene in co-
542 treatment with 2-aminoanthracene, a cytochrome P450 1A inhibitor, in rainbow trout
543 (*Oncorhynchus mykiss*). Environ Toxicol Chem 28:1304-1310

544 Scott JA, Hodson PV (2008) Evidence for multiple mechanisms of toxicity in larval
545 rainbow trout (*Oncorhynchus mykiss*) co-treated with retene and alpha-naphthoflavone.
546 Aquat Toxicol 88:200-206

547 Shang E, Wu R (2004) Aquatic hypoxia is a teratogen and affects fish embryonic
548 development. Environ Sci Technol 38:4763-4767

549 Tavendale M, McFarlane P, Mackie K, Wilkins A, Langdon A (1997) The fate of resin
550 acids .2. The fate of resin acids and resin acid derived neutral compounds in anaerobic
551 sediments. Chemosphere 35:2153-2166

552 Villalobos S, Papoulias D, Meadows J, Blankenship A, Pastva S, Kannan K, Hinton D,
553 Tillitt D, Giesy J (2000) Toxic responses of medaka, d-rR strain, to polychlorinated
554 naphthalene mixtures after embryonic exposure by in ovo nanoinjection: A partial life-
555 cycle assessment. Environmental Toxicology and Chemistry 19:432-440

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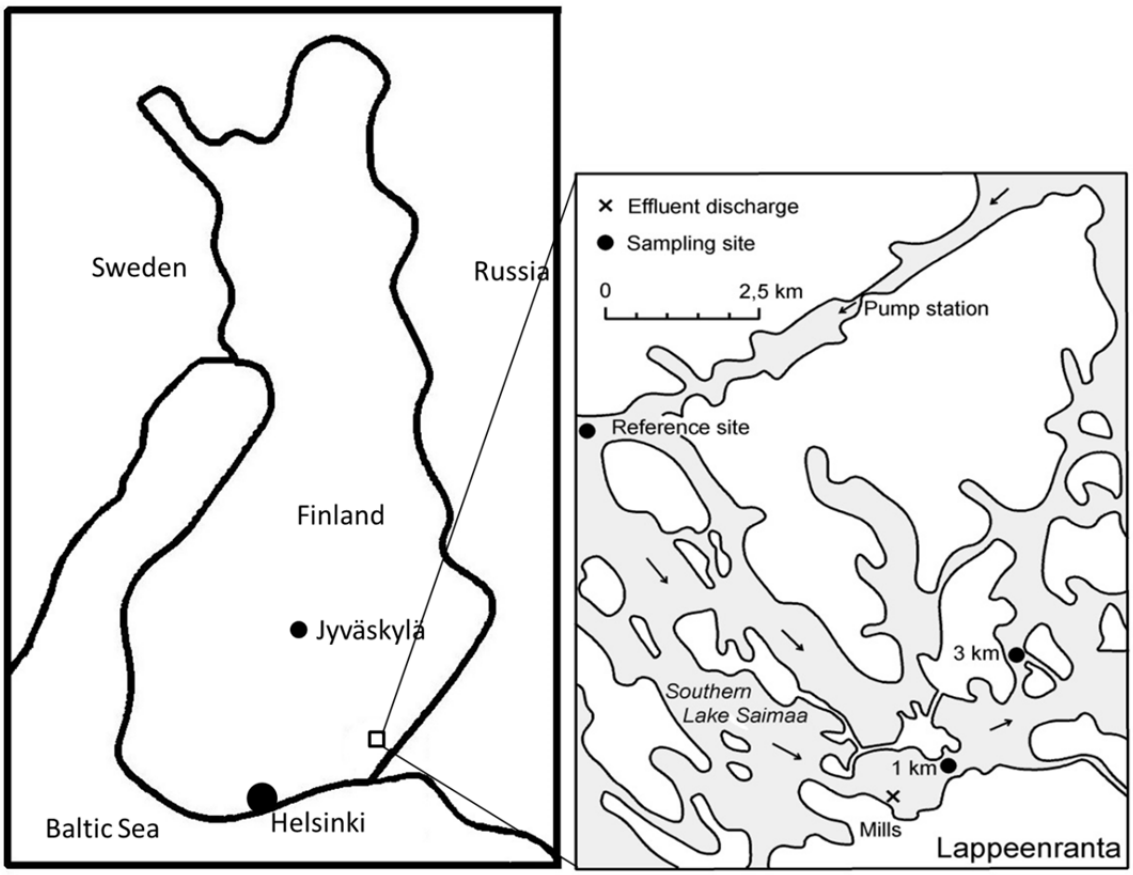
Figure legends

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 m^3 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 ± 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 ± 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



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585 Figure 1

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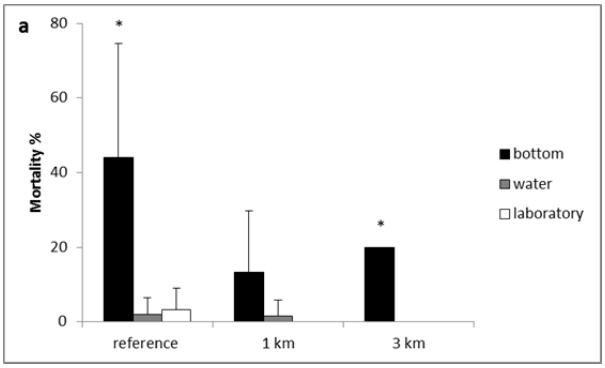

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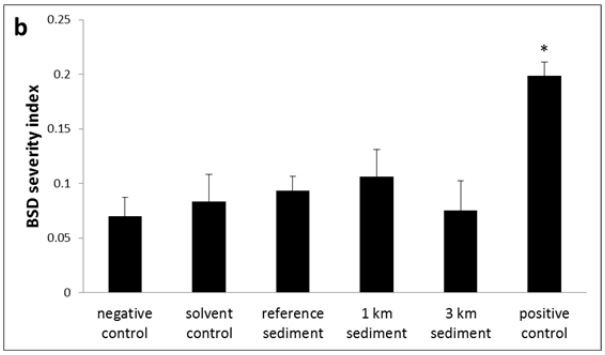

b
BSD severity index
0.25
0.2
0.15
0.1
0.05
0
*
negative control
solvent control
reference sediment
1 km sediment
3 km sediment
positive control

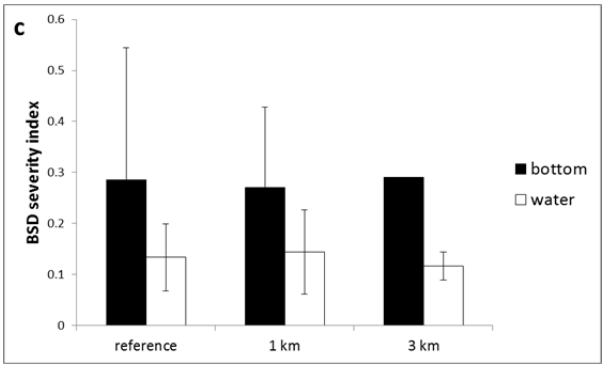
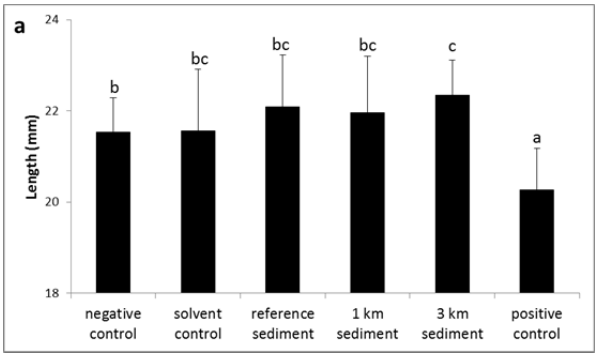
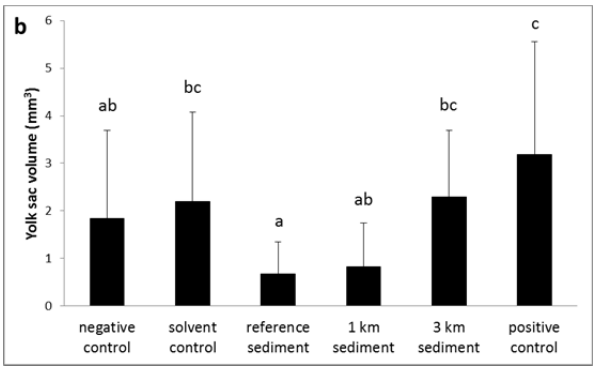

c
BSD severity index
0.6
0.5
0.4
0.3
0.2
0.1
0
bottom
water
reference
1 km
3 km

Figure 2.


a
Length (mm)
24
22
20
18
b
bc
bc
bc
c
a
negative control
solvent control
reference sediment
1 km sediment
3 km sediment
positive control


b
Yolk sac volume (mm³)
6
5
4
3
2
1
0
ab
bc
a
ab
bc
c
negative control
solvent control
reference sediment
1 km sediment
3 km sediment
positive control

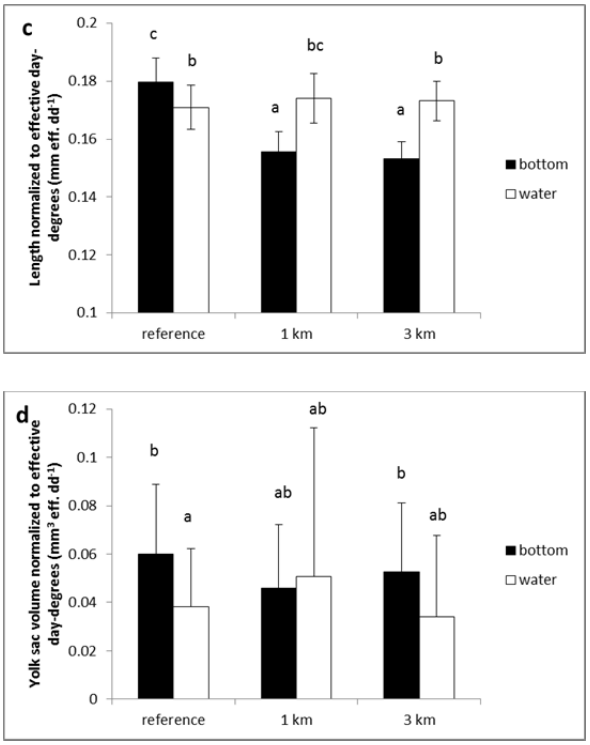
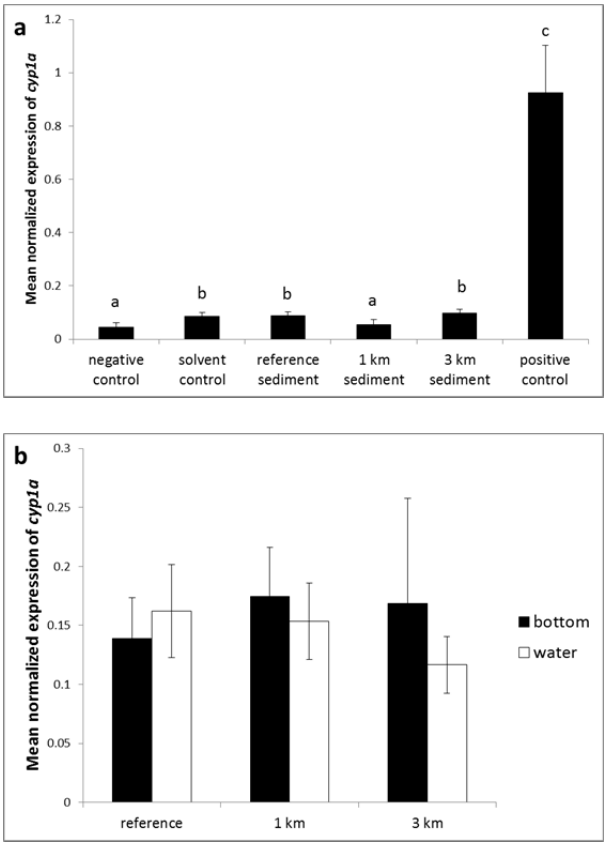
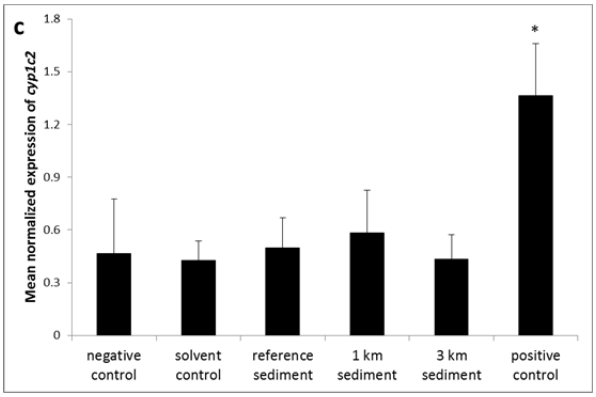

c
Length normalized to effective day-degrees (mm eff. dd-1)
0.2
0.18
0.16
0.14
0.12
0.1
c
b
a
bc
a
b
bottom
water
reference
1 km
3 km
d
Yolk sac volume normalized to effective day-degrees (mm3 eff. dd-1)
0.12
0.1
0.08
0.06
0.04
0.02
0
b
a
ab
ab
b
ab
bottom
water
reference
1 km
3 km

Figure 3.


a
Mean normalized expression of cyp1a
1.2
1
0.8
0.6
0.4
0.2
0
a
b
b
a
b
c
negative control
solvent control
reference sediment
1 km sediment
3 km sediment
positive control
b
Mean normalized expression of cyp1a
0.3
0.25
0.2
0.15
0.1
0.05
0
bottom
water
reference
1 km
3 km

610



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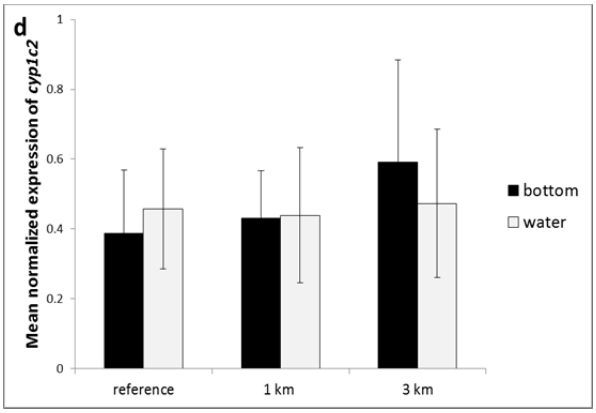

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615 Figure 4.
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619 Table 1. Effects of exposure to sediment contaminated by wood industry on rainbow trout yolk sac fry in the laboratory and in the
620 field. Values represent mean ± SD. Asterisks 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 ± 5.8 *
	field	13 ± 16	NA[a]	44.0 ± 30.5
BSD index	laboratory	0.11 ± 0.03		0.09 ± 0.02
	field	0.24 ± 0.15	NA[a]	0.41 ± 0.32
length of animal (mm/eff. dd)	laboratory	0.17 ± 0.01 ***	0.17 ± 0.01 ***	0.17 ± 0.01 ***
	field	0.16 ± 0.01	0.15 ± 0.01	0.18 ±0.01
yolk volume (mm^3/eff. dd)	laboratory	0.006 ± 0.007 ***	0.018 ± 0.011 **	0.005 ± 0.005 ***
	field	0.046 ± 0.026	0.053 ± 0.028	0.060 ± 0.029
cyp1a expression	laboratory	0.06 ± 0.02 ***	0.10 ± 0.01 **	0.09 ± 0.02 **
	field	0.17 ±0.04	0.17 ± 0.09	0.14 ± 0.03
cyp1c2 expression	laboratory	0.58 ± 0.24	0.44 ± 0.14	0.50 ± 0.17
	field	0.43 ± 0.14	0.59 ± 0.29	0.39 ± 0.18

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624 Table S1. Features of the primers used in the quantitative PCR.
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Gene name	Accession	Primer	Product length	Efficiency
cyp1a	U62796	F: CAGTCCGCCAGGCTCTTATCAAGC R: GCCAAGCTCTTGCCGTCGTTGAT	94	96.9
cyp1c2	NM_001185032.1	F: CAACCACGACCCTTTACAGTGGAA R: CAGATCCTTGTCAAGGGCACCATT	88	92.8
ndufa8	NM_001160582.1	F: TTCAGAGCCTCATCTTGCCTGCT R: CAACATAGGGATTGGAGAGCTGTACG	119	99.5
ef1a	NM_001124339.1	F: CTTTGTGCCCATCTCTGGTT R: TTACGTTCGACCTTCCATCC	90	98.1

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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 ± SD of daily measurements.
638

	Temperature (°C)	O_2 concentration mg/l	pH	Conductivity (µS/cm)
1 km sediment	12.9 ± 0.3	11.8 ± 0.3	7.60 ± 0.05	213 ± 15
3 km sediment	12.9 ± 0.3	11.7 ± 0.3	7.60 ± 0.07	193 ± 15
reference sediment	12.8 ± 0.1	11.6 ± 0.4	7.50 ± 0.10	210 ± 15
negative control	11.9 ± 0.1	12.1 ± 0.3	7.80 ± 0.07	210 ± 14
solvent control	12.0 ± 0.1	12.0 ± 0.3	7.80 ± 0.05	207 ± 16
positive control (32 µg/L retene)	11.8 ± 0.3	12.1 ± 0.3	7.80 ± 0.03	208 ± 14

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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 ± SD of five measurements conducted during the experiment.
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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	5.5	11.4 ± 1.3	141.7	9.6	7.3	145	1.8	4.0	25.0
	water	3.5	12.1 ± 1.0	127.7	9.5	7.4	146			25.0
3 km	bottom	5.0	11.5 ± 1.1	142.8	9.7	7.5	169	1.9	2.8	21.0
	water	3.0	12.0 ± 1.1	125.9	9.5	7.5	170			21.0
Reference	bottom	6.0	9.8 ± 2.5	121.3	10.8	7.4	71.5	2.3	1.9	7.0
	water	4.0	10.4 ± 1.6	128.5	10.9	7.3	69.3			6.5

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