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4   **Do laboratory exposures represent field exposures? - Effects of sediments**

5   **contaminated by wood industry on yolk-sac fry of rainbow trout (*Oncorhynchus***

6   ***mykiss*)**

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24

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.

53 **1 Introduction**

54 Contaminated sediments may be stressors to the organisms in aquatic ecosystems (Burton

55 and Johnston 2010). The altered bleaching methods and modern wastewater treatment

56 have reduced the ecotoxicological impacts of the chemical wood industry in many

57 countries, but the contaminated sediments still contain substances that are potentially

58 harmful to wildlife (Ratia et al. 2013). In the past, the wastewaters of pulp and paper

59 industry caused recruitment failures in fish populations that led to the absence of

60 salmonid species in downstream areas (Hakkari 1992, Hakkari and Bagge 1992). The

61 wastewaters of pulp and paper mills are considered to be of relatively low risk to fish

62 nowadays, but salmonid populations are still largely absent. For example in Southern

63 Lake Saimaa, in SE Finland, the fish community structure downstream of the pulp and

64 paper mills still differs remarkably from clean reference, with bleak and roach

65 dominating in the former and vendace in the latter (Karels and Tiitinen 2013). As the lake

66 sediments are natural spawning sites for some salmonids, could the "legacy from the

67 past", the contaminated sediments, still affect the recruitment of salmonid fishes?

68 The pulp and paper mills that use softwood as raw material still release resin acids in

69 their wastewater (Ramanen et al. 2010). Some of the resin acids end up in the sediments

70 downstream of the mills, and are converted to retene by microbes (Tavendale et al. 1997,

71 Leppanen and Oikari 1999, Leppanen et al. 2000). Sediment-borne retene may desorb to

72 water and be bioavailable to aquatic organisms (Oikari et al. 2002, Merilainen et al.

73 2006). In fish embryos, exposure to waterborne retene causes dioxin-like toxicity

74 observed as blue sac disease (BSD) that coincides with aryl hydrocarbon receptor (AhR)

75 activation detectable as up-regulation of *cyp1a*. The BSD symptoms include

76 hemorrhaging, yolk and pericardial sac edema, spinal and craniofacial deformities, and

77 fin rot. Salmonid fishes are among the most sensitive to dioxin-like developmental

78 toxicity (Elonen et al. 1998, Billiard et al. 1999), and thus teratogenicity of retene-

79 contaminated sediments might be an explanation for the apparent recruitment failures or

80 impairments of salmonids.

81 The toxicity testing of sediments is almost routinely carried out in the laboratory, because

82 it is cheaper and less laborious, more controllable and convenient, and bears a smaller

83 risk of unforeseen problems (such as vandalism, waterway traffic and extreme weather

84 conditions) than exposures done *in situ*. Nevertheless, a key question remains whether the

85 results obtained in a laboratory setting correctly predict the effects in the field.

86 The aim of this study was to find out if native retene-contaminated sediments cause BSD,

87 *cyp1a* and *cyp1c2* induction, or growth retardation in early life-stage rainbow trout, and if

88 the effects are similar in the laboratory and *in situ*.

89

90 **2 Materials and methods**

91 2.1 Study area and sediments

92 The study area near the Kaukas pulp and paper mills in Lappeenranta, Southern Lake

93 Saimaa, is located in South-Eastern Finland (Fig. 1). The pulping industry started in the

94 area in 1897, followed by papermaking within a few decades. Major environmental

95 improvements took place in 1992, when the mill started to produce elemental chlorine

96 free (ECF) pulp, and began to treat the wastewaters with the aid of activated sludge.

97 Nowadays the Kaukas (UPM) mill produces softwood and birch pulp, paper, sawn timber,

98 plywood, and wood-based chemicals. In 2011, it released around $100\,000$ m^3 of

99   wastewater per day. In 2011, the average daily emissions to water consisted of 334 kg of

100   nitrogen, 16.5 kg of phosphorus, and 270 kg of adsorbable organic halogens (AOX); and

101   the daily chemical (COD) and biological oxygen demand (BOD) of the effluent was

102   30 760 kg and 590 kg, respectively (Anon. 2012). The effluent is known to contain

103   chlorophenolics, resin acids, and phytosterols (e.g. β-sitosterol), and substantially smaller

104   amounts of chlorophenolics than before 1992 (Karels et al. 2001).

105   The sediment samples were taken just after the spring turnover in May 2010. The

106   sediments were collected with an Ekman device (sediment depth ca. $0 - 10$ cm; area 225

107   cm^2), and replicate samples were combined. Six replicate samples were taken at locations

108   1 and 3 km downstream of the pulp and paper mills (see Fig. 1), and ten samples at the

109   reference site 10 km upstream of the mills (Fig. 1). The sediment samples were stored in

110   sealed polycarbonate containers in the dark at 4 °C. The total organic carbon contents of

111   the sediments were 6.5, 8.9 and 15.9 % (dry weight) for the upstream reference, and the 1

112   km and 3 km sites, respectively.

113

114   2.2 Laboratory exposure

115   Newly hatched (<24 h) rainbow trout (*Oncorhynchus mykiss*) yolk-sac fry were obtained

116   from the fish farm Hanka-Taimen (Hankasalmi, Finland). The water used in the

117   laboratory experiment was filtered and aerated well water of the University of Jyväskylä.

118   The pH, oxygen concentration, and conductivity were measured daily. The light:dark

119   cycle was 16 h:8 h.

120   The exposures were carried out in 18-l glass aquaria. The fry were placed in the aquaria

121   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^{2}H]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^{2}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

191   rot, craniofacial deformities and spinal deformities. The length of the fish as well as the

192   width and height of the yolk sac were measured to the nearest 0.5 mm. The fry were

193   sacrificed, the yolk sacs removed, and the remaining part of the body was placed in a

194   microcentrifuge tube and frozen in liquid nitrogen. The samples were preserved in -80 ºC

195   until RNA extraction.

196   BSD severity index was calculated with the formula (1)

197

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

199
200   where SI = severity index, PE = pericardial edema, YE = yolk sac edema, OH =

201   hemorrhaging, CF = craniofacial deformities, SD = spinal deformities, and FR= fin rot. E_i,

202   E_j, E_k, E_l, E_m, and E_o equal the number of embryos displaying pericardial edema, yolk sac

203   edema, hemorrhaging, craniofacial deformities, spinal deformities, and fin rot,

204   respectively (Villalobos et al. 2000, Scott and Hodson 2008, Scott et al. 2009).

205

206   2.6 Growth measurements

207   The length of the fry as well as the width and height of the yolk sac were determined

208   under the microscope. The yolk volume was calculated with the formula (2)

209

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

211

212 where V_y is the yolk volume, *l* is the major axis, and *h* is the minor axis (mm)

213 (Kamler 2008).

214

215 In the field exposure, the yolk volume and length of the fish were normalized to effective

216 day-degrees to take into account the effect of temperature differences between the sites.

217 The effective day-degrees were calculated with the formula (3)

218

219 $$D_{eff}^{\circ} = \tau\,(\mathrm{t} - \mathrm{t}_0) \qquad\qquad\qquad (3)$$

220

221 where τ is time (days), t is temperature (°C), and t_0 is the temperature of biological zero

222 for rainbow trout (the threshold temperature at which ontogeny is theoretically arrested)

223 (Kamler 2002). The t_0 used in the calculations was 1.7 °C (Kamler 2002).

224

225 2.7 Quantitative PCR

226 The primers were designed with AmplifX (version 1.5.4 by Nicolas Jullien ; CNRS, Aix-

227 Marseille Université - http://crn2m.univ-mrs.fr/pub/amplifx-dist) and checked for

228 specificity with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The

229 features of the primers are shown in the Electronic Supplementary Material 1. *elongation*

230 *factor 1 alpha* (*ef1a*) and *nadh dehydrogenase 1 alpha subcomplex subunit 8* (*ndufa8*)

231 were chosen as reference genes because in previous experiments their expression in

232 retene- and control-exposed rainbow trout yolk-sac fry had been the most stable of a set

233 of reference genes tested (Vehniäinen, unpublished).

234   Total RNA was extracted with Tri reagent (Molecular Research Center, USA) according

235   to the manufacturer's instructions. Three individual fish per cage in three cages per

236   treatment (total n = 9 fish per treatment) were used for all sites, except for the 3 km

237   bottom exposure, where all viable fry (n = 8) from the remaining cage were taken as

238   samples. The quantity of the RNA was measured, and the purity checked using the

239   260:280 nm optical density ratio (NanoDrop 1000, Thermo Scientific). The quality of the

240   RNA was monitored with Agilent 2100 BioAnalyzer, using Eukaryote total RNA 6000

241   nano kit (Agilent Technologies Inc., USA). The samples with RNA integrity number > 7

242   were included in the analyses. The DNase treatment (DNase I Amp Grade, Invitrogen)

243   was conducted for 1 µg total RNA, after which it was reverse transcribed to cDNA

244   (iScript cDNA Synthesis Kit, Bio-Rad, USA) and diluted 1+9 with nuclease-free water.

245   One 25 µl qPCR reaction consisted of 5 µl of the diluted cDNA, 0.75 µl each of forward

246   and reverse primers (final concentration 300 nM), 6 µl sterile H_2O and 12.5 µl of iQ

247   SYBR Green Supermix (Bio-Rad, USA). The qPCR was run on a CFX96 Real-Time

248   PCR cycler (Bio-Rad, USA). The protocol was 3 min at 95 °C; 40 cycles 10 s at 95 °C,

249   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

250   template controls were run for all genes on all plates, and their C_qs were always over 38.

251   Melt curves showed a single peak, confirming formation of only one PCR product. The

252   expression values were calculated with CFX Manager™ Gene Study (Bio-Rad).

253

254   2.8 Statistics

255   The yolk volume and length of fry (both normalized to effective day-degrees), and

256   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**

350   Studying the effects of contaminated sediments on fish yolk sac fry in field conditions

351   may be problematic. Monitoring the annual recruitment yield of fishes may include bias

352   due to e.g. migration and field conditions. Caging makes it possible to manipulate the

353   timing, duration and location of the exposure in the field, and thus makes the field

354   exposure more comparable to laboratory exposure than collecting live fry from the area

355   (Oikari 2006).

356   There have been only a few true comparisons of the effects of contaminated sediments *in*

357   *situ* and in the laboratory (Conrad et al. 1999, Pereira et al. 2000, Kater et al. 2001,

358   Ringwood and Keppler 2002, Anderson et al. 2004, Phillips et al. 2004, Mann et al.

359   2010). We are neither aware of any previous laboratory to field comparisons using fish

360   fry, nor of those using sediments contaminated by pulp and paper mills. In this work, the

361   laboratory exposures underestimated the effects of the contaminated sediments on

362   rainbow trout fry. The mortality was higher in the field than in the laboratory. Also the

363   BSD indices appeared higher in the field, but they did not differ statistically between the

364   laboratory and field exposures. The contaminated sediments attenuated the growth of the

365   fry only in the field exposures. Differences in the outcomes between laboratory and *in*

366   *situ* sediment exposures have also been described previously among invertebrates

367   (Conrad et al. 1999, Pereira et al. 2000, Kater et al. 2001, Ringwood and Keppler 2002,

368   Anderson et al. 2004, Mann et al. 2010) and fishes (Costa et al. 2011a, Costa et al. 2011b,

369   Costa et al. 2012). In most cases, the sediments have been less toxic in the laboratory

370   than in the field (Conrad et al. 1999, Pereira et al. 2000, Kater et al. 2001, Ringwood and

371   Keppler 2002, Anderson et al. 2004, Hose et al. 2006, Mann et al. 2010). Therefore,

372   sediment toxicity tests conducted in the laboratory must be interpreted with caution. As

373   for practical reasons laboratory testing will remain the choice for the majority of cases,

374   the application of a safety factor could be justified.

375   The more pronounced toxicity in the field exposures could not be explained by the

376   chemical analyses done in this study. The retene concentrations in water were at a similar,

377   very low level in the laboratory and field exposures at all sites, and they did not correlate

378   with toxicity. The rainbow trout fry could be exposed to retene and other contaminants

379   also by feeding on the sediments, and indeed sediment could be found in the intestines of

380   the fry both in the laboratory and in the field (bottom) exposures. Despite the feeding on

381   the sediments in both settings, toxic effects were seen only in the field setting. As there

382   was no difference between the sites or in the bottom vs. water exposures in *cyp1a*

383   transcript abundance, it can be concluded that AhR activating compounds were

384   presumably not bioavailable from the sediments. The same conclusions were drawn in a

385   recent study in the same area, where brown trout juveniles caged on the sediment did not

386   show elevated EROD activity when compared to the ones caged in water or in a reference

387   area (Oikari et al. 2010). These findings lead to the conclusion that retene was not the

388   compound behind the toxic effects.

389   In addition to retene, the contaminated sediments in the study area contain resin acids

390   (Ratia et al. 2014). There is no published data on the effects of resin acids on fish embryo

391   development. Nevertheless, one of the most abundant resin acids in the sediments

392   downstream of pulp and paper mills, dehydroabietic acid, affects the energy metabolism

393   of fish and might therefore be capable of interfering with growth (Rissanen et al. 2003,

394   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

440   laboratory conditions may underestimate the effects in the field, and this should be taken

441   into account in the risk assessment.

442

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448

449   **References**

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

453   Anonymous (2012) Environmental performance 2011 UPM Kaukas

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

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

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

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

462   Costa PM, Chicano-Galvez E, Caeiro S, Lobo J, Martins M, Ferreira AM, Caetano M,
463   Vale C, Alhama-Carmona J, Lopez-Barea J, Angel DelValls T, Costa MH (2012) Hepatic
464   proteome changes in *Solea senegalensis* exposed to contaminated estuarine sediments: a
465   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)   \<br /\>   Etelä-Saimaan ja Vuoksen kalataloudellinen
494 tarkkailu vuonna 2012 \<br /\> :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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558   **Figure legends**

559

560   **Fig. 1** The study area at the Southern Lake Saimaa. The black spots mark the field exposure and

561   sediment sampling sites (1 km, 3 km and Reference). The water flow direction is marked by

562   arrows, and the effluent discharge point by a cross. Water from the clean background area is

563   brought to the reference site by a pump station (rate ca 45 $m^3 sec^{-1}$) to dilute the effluents from the

564   downstream mills

565   **Fig. 2** Mortality (a) and BSD severity index (b, c) in rainbow trout yolk sac fry exposed to

566   sediments or water downstream of the pulp and paper mills in the laboratory (a, b) and *in situ* (a,

567   c). The bars represent mean ± SD. * denotes statistical significance from other treatments (P <

568   0.05). n = 3 groups of 10 individuals in laboratory exposures, 5 or 6 groups of 10 individuals in

569   field water exposures, and 6 groups of 10 individuals in field bottom exposures, except the 3 km

570   bottom in which the data are from one group of 10 individuals as other cages were lost or buried

571   into the sediment

572   **Fig. 3** Effect of sediments downstream of the pulp and paper mill on growth and development of

573   rainbow trout yolk sac fry in the laboratory (a, b) and in the field (c, d). The data are presented as

574   mean ± SD. Groups denoted by the same letter do not differ statistically from each other. n = 3

575   groups of 10 individuals in laboratory exposures, 5 or 6 groups of 10 individuals in field water

576   exposures, and 6 groups of 10 individuals in field bottom exposures, except the 3 km bottom in

577   which the data are from one group of 10 individuals as other cages were lost or buried into the

578   sediment

579   **Fig. 4** Effects of sediments on *cyp1a* (a, b) and *cyp1c2* (c, d) mRNA abundance in rainbow trout

580   yolk sac fry after laboratory (a, c) and field (b, d) exposure. Groups marked with the same letter

581   do not differ statistically from each other. * denotes statistical significance from other groups (P <

582   0.05). n = 9 individuals in all exposures except the field 3 km bottom in which the data are from 8
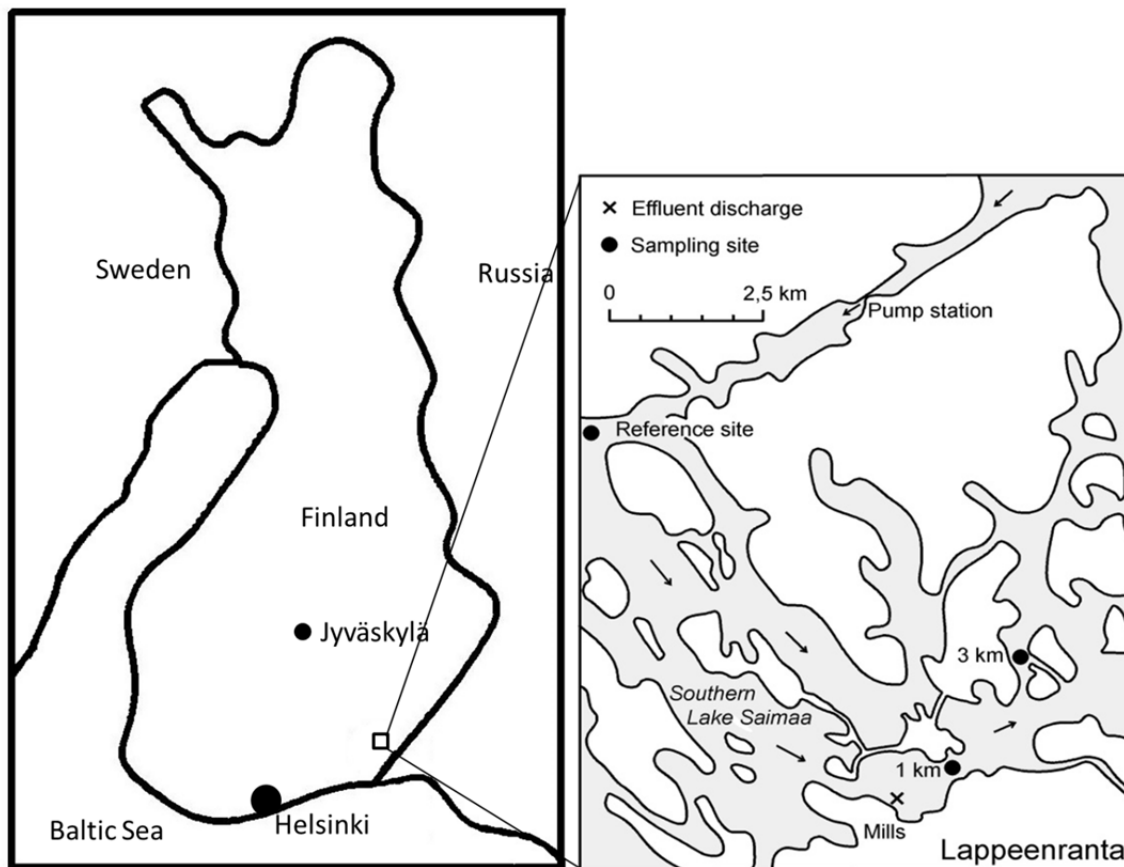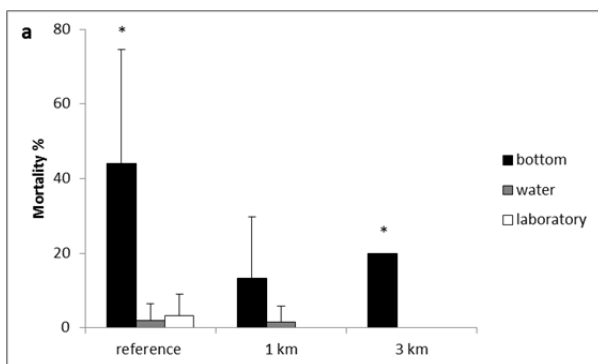
583   individuals
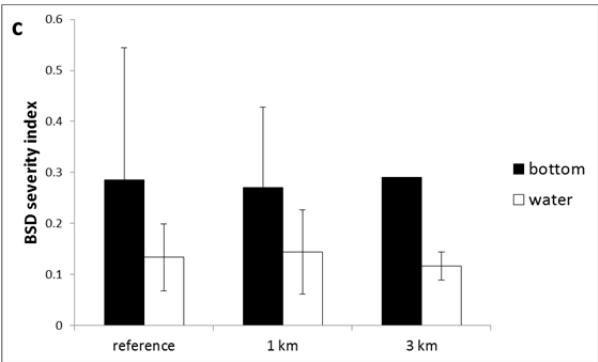
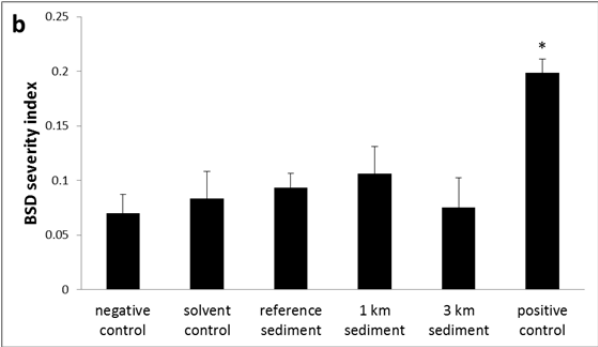584

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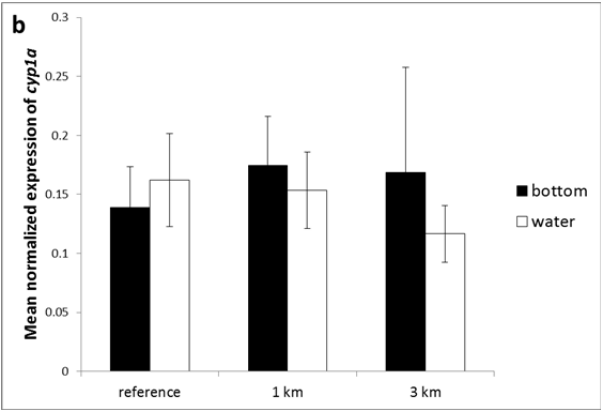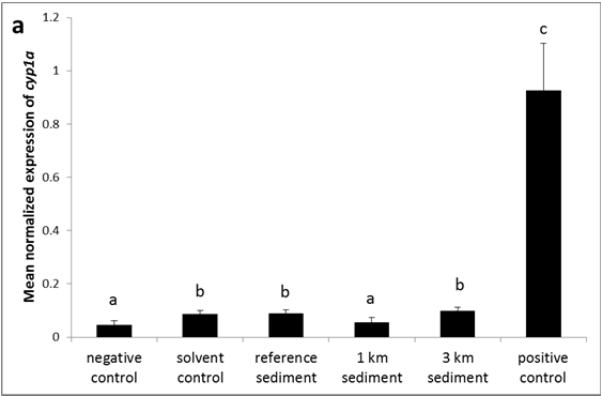605

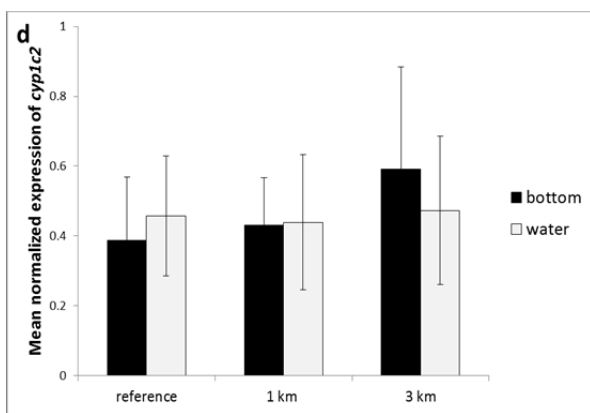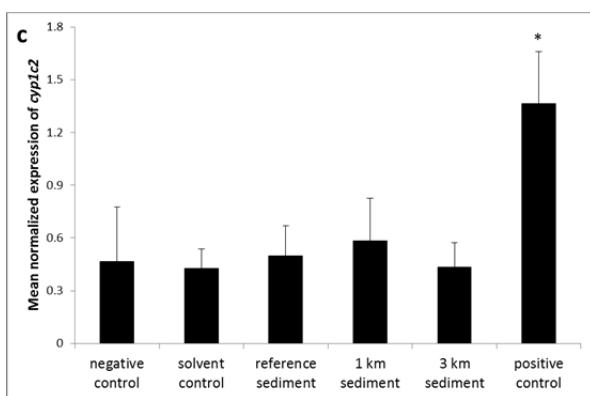606
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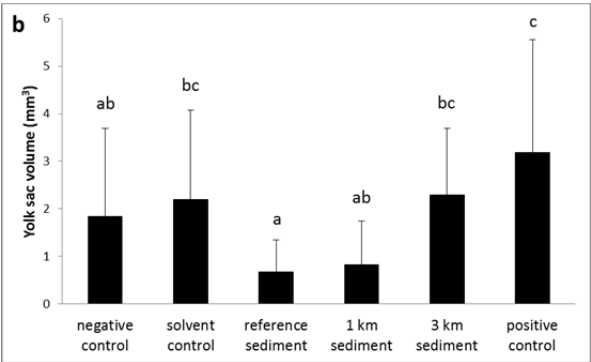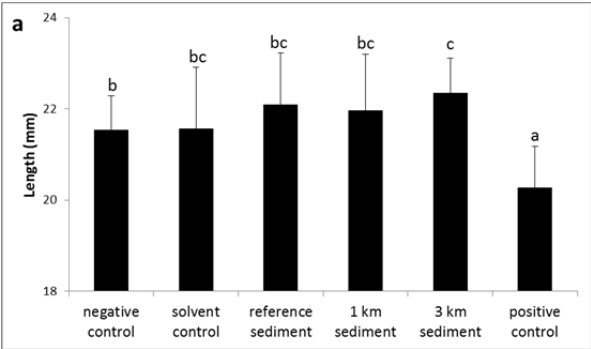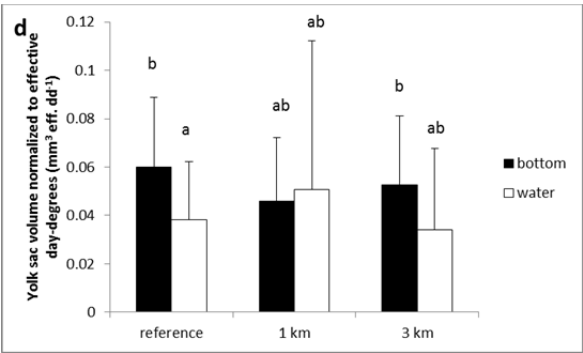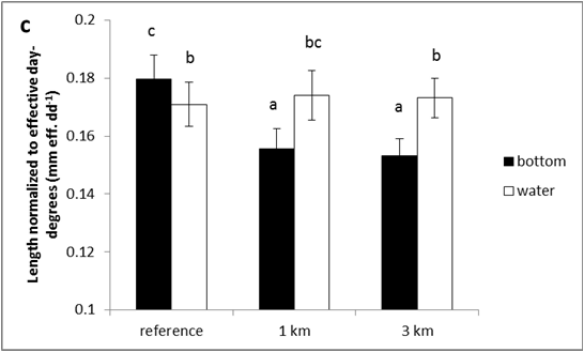
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619 Table 1. Effects of exposure to sediment contaminated by wood industry on rainbow trout yolk sac fry in the laboratory and in the
620 field. Values represent mean ± SD. Asterisks denote statistically significant difference between laboratory and 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.
625

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

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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