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Running head: Phototoxicity of retene to coregonid larvae

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PHOTOINDUCED LETHAL AND SUBLETHAL TOXICITY OF RETENE, A RESIN ACID DERIVED PAH, TO COREGONID LARVAE

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1 **Abstract —** A comparative investigation on the acute phototoxicity of retene to
2 vendace (*Coregonus albula*) and whitefish (*Coregonus lavaretus*), both having
3 pelagial larvae in spring, was conducted. In order to test the concept of early warning
4 of sublethal biomarkers in relation to lethality to posthatch stages, we examined the
5 effects of ultraviolet-B (UV-B) and retene on the levels of CYP1A and HSP70 by
6 exposing the animals to elevated levels of these factors for 48 and 72 h, respectively.
7 While UV-B and retene on their own were not lethal, simultaneous retene and UV-B
8 exposure caused very high mortality to both species. **The LC50 (lethal
9 concentration 50%, i.e. the concentration at which half of the larvae died) of
10 retene as a precursor was 41 μg/L for vendace and 15-16 μg/L, depending on UV
11 dose, for whitefish.** Retene evoked substantial induction of CYP1A in larvae of both
12 species and UV-B induced CYP1A in whitefish. In vendace, no effect on HSP70
13 levels by any factor was observed. In whitefish, however, UV-B radiation and water
14 retene alone upregulated HSP70, but no additive response was detected. CYP1A is a
15 biomarker of exposure to retene in both species. HSP70 is an early warning signal of
16 UV-B exposure in whitefish. As a species vendace appears to be more resistant to the
17 phototoxicity of retene than whitefish, as indicated by the higher tolerance.
18
19
20 **Keywords —** Phototoxicity Fish Retene Ultraviolet-B Cytochrome
21 P4501A Heat shock protein 70
22

INTRODUCTION

UV-B radiation (280 – 315 nm) levels have increased due to stratospheric ozone depletion [1]. Enhanced UV-B radiation poses a threat to organisms due to its potential to cause harmful biological effects, directly or indirectly. Of the latter effects of UV-B, photoinduced toxicity of environmental contaminants like polynuclear aromatic hydrocarbons (PAHs) has been documented [2]. Pelagic or semipelagic stages of animals, such as planktonic larvae of coregonid fishes, may potentially be exposed to such a combination in aquatic ecosystems.

Many PAHs can absorb energy from the UV spectrum of sunlight thereby resulting in excited state molecules. The excited state is lost resulting either in the production of active oxygen (photosensitization) or modification of the PAH to new chemical species (photomodification) [2]. Active oxygen is biologically damaging and also the photomodification products are often more toxic than the parent compound.

Retene (7-isopropyl-1-methylphenantrene) is a PAH that is formed from resin compounds during incomplete combustion of resinuous softwood and via action of anaerobic microbes [3-4]. In addition to being formed from coniferous resins, it may originate from algal and bacterial precursors [5]. High concentrations of retene **(up to 1.6 mg/g dry weight)** have been found in pulp mill sludges and sediments downstream of pulp and paper mill discharges [6-9]. As a natural product, it is also

present in sediments not close to pulp mills, although at negligible concentrations **(< 4 ng/g dry weight)** [6, 10].

Retene is not acutely lethal to fish at water-soluble concentrations [11]. On the other hand, its subchronic toxicity is high (10 – 32 μg/l) as evidenced by teratogenic effects, most distinctly the blue sac disease in fish embryos and larvae [12]. The acute toxicity of retene to *Daphnia magna* is enhanced under UV-B radiation [13].

Autumn-spawning vendace and whitefish lay their eggs on lake bottom where they may come in contact with sediment retene, shown to be bioavailable to fish [14]. **Newly hatched larvae may also be exposed to retene in water or particulate material [9].** After hatching the positively phototactic larvae of both species swim near the surface for the first months, thus likely to be exposed to episodes of solar UV-B [15].

The objective of this study was to examine the effects of sole and simultaneous exposures to retene and UV-B on larval vendace and whitefish. **The focus was on UV-B because its levels are rising and because retene has an absorption peak in the UV-B region of the sunlight spectrum [13]. The endpoints were mortality and two inducible proteins, CYP1A and HSP70.** We suggested that these proteins, while serving as early response biomarkers, allow comparison of the two coregonid species at the larval stages.

Cytochrome P4501A (CYP1A), a heme-containing microsomal monooxygenase, catalyzes the first step in the biotransformation of many xenobiotics.

72 **It was chosen as an endpoint because it is known to be induced by many PAHs,**
73 **including retene [12, 16]. The aim was to investigate whether the induction**
74 **differs between species and whether UV-B has an effect on the induction caused**
75 **by retene (e.g. if more potent CYP1A inducers are generated by UV-B).**
76
77 Heat shock protein 70 (HSP70) is a class of stress proteins with a molecular
78 weight of 70 – 72 kDa. Cells express some proteins of this family constitutively (heat
79 shock cognates, HSC's) and some are upregulated by a variety of environmental
80 stressors **that generate denatured proteins**, including UV radiation and bleached
81 kraft pulp mill effluent [17-19]. **HSP70 is believed to have a protective role against**
82 **tissue damage as it binds to denatured proteins and attempts to restore their**
83 **tertiary structure and function [19].**
84
85 **MATERIALS AND METHODS**
86
87 *Organisms and water quality*
88
89 The developing eggs of vendace (Lake Pyhäselkä stock) were obtained from
90 the University of Joensuu and those of whitefish (Rautalampi stock) were obtained
91 from a local hatchery (Laukaa). Once transferred to the University of Jyväskylä in
92 late March, the embryos were further incubated and hatched in flow-through hatchery
93 cones at 6 – 8 ^{o}C. The water was colourless (<5 mg Pt/L), **filtered groundwater with**
94 **pH 6.6 – 7**, having a high penetrability for UV-B (65% in 15 cm). **The water**
95 **hardness (Ca + Mg) was 1.3-1.4 mmol/L and conductivity 26.2 mS/m.** One-day

96 old larvae were kept in the same well-aerated water after hatching and used for the
97 experiments.
98
99 *Exposure system and sampling*
100
101 The vendace and whitefish larvae **(45 and 25 per replicate, respectively)**
102 were carefully transferred to retene (nominal concentrations 3.2; 10, 25, 32 and 100
103 μg/L and 10, 32 and 100 μg/L, respectively) or control treatments (water only or 0.1%
104 dimethylsulfoxide (DMSO), used as carrier) with large-bore pipettes and pre-exposed
105 for 24 hours in Pyrex glass bowls. The depth and volume of pre-aerated water were 5
106 cm and 1liter, respectively. A portion of the water was changed daily. **There were**
107 **three replicates for each treatment.** After a 24-h accumulation period, the larvae
108 were further exposed to retene and, in addition, irradiated with either UV-B or visible
109 light. UV-B was provided in the laboratory using a fluorescent lamp (UVB-313, Q-
110 Panel, Cleveland, OH, USA). Ultraviolet-C (UV-C; under 280 nm), **which in the**
111 **nature is blocked by the atmosphere and does not reach the surface of the earth,**
112 was blocked with a cellulose diacetate filter (Clarifoil, Derby, UK), replaced after
113 each 3-hour UV-B radiation. Control treatments without UV-B received visible light
114 (TLD 36 W/950 daylight, Philips, Eindhoven, Netherlands). The photoperiod was
115 16h:8h (light:dark).
116
117 The larvae received UV-B or visible light 3 h per day (between 24 – 27 hours
118 and 48-51 hours after the beginning) to mimic midday exposure, for two days. UV
119 was quantified using Hamamatsu Photonic Multichannel Spectral analyser (model
120 PMA-11), measuring the wavelength area 280 – 380 nm. The UV-B intensities were

121 measured at the water surface in the beginning and the end of each experiment **at**
122 **various spots to ensure equal doses for all replicates in each treatment.** The daily
123 doses were 2.8 kJ/m^2 for the lower UV-B intensity and 5.4 kJ/m^2 for the higher,
124 calculated as CIE-weighted **(Commission Internationale de l'Eclairage;**
125 **International Commission on Illumination**) J/m^2, i.e., **the action spectrum specific**
126 **for human erythema [20]**. These UV-B doses correspond with slightly subambient
127 (7%) and 80% increase relative to the maximum daily doses in Finland **(approx. 60 –**
128 **70 ^{o}N)** in early May. The vendace were exposed only to the higher and the whitefish
129 to both intensities.
130
131 The larvae were sampled after 3 days (72 hours) from the start by
132 anesthetizing the animals with MS222 (50 mg/L, 2 min) and freezing in liquid
133 nitrogen. The samples were transferred to –80 ^{o}C and preserved there until processed
134 further.
135
136 *Positive controls for Western blotting*
137
138 The positive control used for Western blotting of CYP1A was obtained by
139 injecting four one-year-old juvenile whitefish with 30 μg/g β-naphthoflavone in 95%
140 ethanol : olive oil (1:1). The fish were further kept at 12 ^{o}C in a 40-liter aquarium for
141 4 days, anesthetized with MS-222 (50 mg/L) and sacrificed. The organs were
142 removed and frozen immediately in liquid nitrogen. Once thawed on ice the livers
143 were homogenized with potassium gluconate buffer (70 mmol/L, pH 7.8) and
144 centrifuged for five minutes (3000 x *g*). The supernatant was used for the blotting.
145

146 Heat-shocked vendace and whitefish larvae were used as positive controls for
147 the responsivity and Western blotting of HSP70. Larvae acclimated at 6 ^{o}C were held
148 in 16 ^{o}C for 1 hour, transferred back to 6 ^{o}C and sampled after 3 hours. The animals
149 were frozen in liquid nitrogen and preserved in –80 ^{o}C.
150
151 *Western immunoblotting*
152
153 Larvae frozen in liquid nitrogen (30 – 40 mg, corresponding to 10 larvae of
154 vendace or 5 larvae of whitefish) were homogenized in a glass homogenizer with
155 potassium gluconate buffer (5 mmol/L $MgSO_4$, 5 mmol/L NaH_2PO_4, 40 mmol/L
156 HEPES, 70 mmol/L potassium gluconate, 150 mmol/L sorbitol, pH 7.8) [21]. The
157 homogenates were centrifuged at 1000 *g* for 5min.
158
159 Total protein concentration of the supernatant was determined by modified
160 Lowry method (BioRad DC, Bio Rad Laboratories, Hercules, CA, USA) adapted for
161 96-well plates [22]. Five µl of each diluted sample was pipetted into a microtiter plate,
162 in triplicate. Similarly, 5 µl aliquots of protein standards (bovine serum albumin, 0 –
163 1.5 mg/ml) were pipetted into the 96-well plate. An aliquot of 25 µl of Bio-Rad
164 reagent A was added and 200 µl reagent B pipetted into each well. The plate was put
165 into the microplate reader for mixing, incubated for 15 min and absorbance read at
166 690 nm.
167
168 The CYP1A and HSP70 determination was made by Western blot after
169 sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation
170 (100 and 20 µg protein / lane, respectively), using a Mini-Protean II apparatus [21,

171 23-24]. The positive controls were the liver of β-naphthoflavone injected juvenile
172 whitefish (3.15 μg protein / lane) and heat-shocked vendace and whitefish larvae (20
173 μg protein / lane) for CYP1A and HSP70, respectively. Samples were diluted in a
174 buffer solution (0.125 mol/L Tris-HCl, 2% SDS, 20% glycerol, 0.02% bromophenol
175 blue, 5% 2-mercaptoethanol) and loaded onto gels after heating (4 min, 95 ^{o}C).
176 Proteins were run on a SDS-PAGE gel for 1 h (V = 100 V for 5 min, 200 V for 55
177 min), after which gels were soaked in blotting buffer (25 mmol/L Tris, 192 mmol/L
178 glycine, 20% methanol, pH 8,3). Proteins were transferred to a nitrocellulose
179 membrane (Trans-Blot Transfer Medium, Bio-Rad) using Bio Rad Mini-Protean II
180 apparatus at 100 V for 90 min. The membrane was stained with 0.2 % Ponceau S in 3
181 % trichloroacetic acid for 1 min to confirm protein transfer, rinsed twice with Tris-
182 buffered saline-Tween (TBST; 0.9% NaCl, 10 mmol/L Tris, 0.1% Tween-20, pH 7.4)
183 and incubated in blocking buffer (9% non-fat dry milk in TBST) for 14 h in 4 ^{o}C.
184
185 The blot was probed with 3 μg/ml anti-CYP1A (Mab 1-12-3, kindly provided
186 by Dr. John Stegeman) or 1:5000 anti-HSP70 (MA3-006, Affinity BioReagents,
187 Golden, CO, USA) in blocking buffer for 2 h. These antibodies recognize CYP1A or
188 HSP70, respectively, of many species including fish, amphibians, and mammals
189 (Murphy SP, Fox S, Myers MP , Morimoto RI. Unpublished data. Affinity
190 Bioreagents) [25]. After washing (TBST, 1x15 min, 2x5 min), the blot was probed
191 with secondary antibody, 1:3000 peroxidase labeled anti-mouse IgG (A9044, Sigma-
192 Aldrich Chemie, Steinheim, Germany), in TBST for 1 hour. After washing (TBST,
193 1x15 min, 2x5 min) the immunodetection was performed via enhanced
194 chemiluminescence using Star-Glo chemiluminescent substrate (ICN Biomedicals,

195 Irvine, CA, USA). Hyperfilm ECL® high performance chemiluminescence film
196 (Amersham Pharmacia, Uppsala, Sweden) was used for visualization.
197
198 The exposed films were scanned and, for densitometric analysis, the pictures
199 were analyzed using Scion Image 4.0.2. Different blots were made comparable to
200 each other by calibrating them with positive controls, fixing each positive control at a
201 value of 1.
202
203 *Statistical analysis*
204
205 **Differences in mortality between control and treatment groups were**
206 **tested using two-way analysis of variance (ANOVA) and Tukey's test. LC50**
207 **values were calculated using Probit analysis. Values for CYP1A and HSP70**
208 **induction were log-transformed and the differences between control and**
209 **treatment groups tested using two-way ANOVA and Tukey's test. The level of**
210 **significance of $p < 0.05$ was considered as statistically significant.**
211
212 **RESULTS**
213
214 *Lethality and behavioural effects*
215
216 UV-B or retene alone caused no acute mortality. However, UV-B radiation
217 increased the toxicity of retene significantly. On day three, after two daily 3-h periods
218 of UV-B, most vendace larvae in 100 μg/L retene treatments were dead (Fig. 1). All
219 whitefish larvae in 100 μg/L and nearly all in 32 μg/L retene treatments died by day

220 three, at both UV-B intensities. **As retene in itself did not cause lethality, we call**
221 **the type of toxicity as retene-based lethality potential, but the concentrations are**
222 **expressed as those of the precursor, retene. The LC50 values (72 h) calculated**
223 **from nominal concentrations of the precursor, retene, were 41 μg/L (95%**
224 **confidence limits 30 and 58 μg/L) for vendace, and 16 μg/L (13, 20 μg/L) and 15**
225 **μg/L (13, 17 μg/L) for whitefish exposed to the lower and higher dose of UV-B,**
226 **respectively. Vendace is statistically more tolerant, but the whitefish exposed to**
227 **lower and higher dose of UV-B do not differ statistically from each other.**
228
229 The vendace larvae exposed to 100 μg/L retene and receiving UV-B radiation
230 showed coughing and decreased motility after the first and second UV exposure. The
231 whitefish larvae showed similar symptoms but even more frequently. Symptoms
232 typically lasted for 3 – 4 hours.
233
234 *CYP1A induction*
235
236 Retene caused induction of CYP1A both in the prescence and absence of UV-
237 B radiation in both species. In vendace the CYP1A induction was approximately
238 proportional to the concentration of retene, being greatest with the highest
239 concentration (100 μg/L). In vendace, UV-B radiation alone caused no induction of
240 CYP1A (Fig. 2).
241
242 In whitefish the induction was greatest with 32 μg/L retene, being slightly
243 submaximal (***p*** **> 0.05, Tukey**) with the highest retene concentration applied (100
244 μg/L). The lower UV-B intensity caused induction in non-retene-exposed whitefish

245 larvae. In retene-exposed whitefish (10 μg/L **only, because the higher**
246 **concentrations of retene as precursor were lethal**) the lower UV-B level also
247 seemed to have an additive effect, but this finding was not statistically significant (***p*** **>**
248 **0.05, Tukey**) (Fig. 3).
249
250 When comparing the maximal responses of CYP1A, the inducibility of
251 vendace was slightly higher than that of whitefish (***p*** **> 0.05, Tukey**). The positive
252 control gave a strong dark band in the Western blots even with the low protein amount
253 used (3.15 μg / lane).
254
255 *HSP70 induction*
256
257 In vendace, neither UV-B nor retene had an effect on HSP70 induction that
258 was statistically significant. Also the HSP70 level of the positive control, heat
259 shocked vendace, was similar to control, showing no induction (Fig. 4).
260
261 **In whitefish, the lower intensity of UV-B radiation caused an induction of**
262 **HSP70 in retene non-exposed animals. Also the higher intensity seemed to induce**
263 **HSP70, but this finding was not statistically significant (*p* > 0.05, Tukey). Retene**
264 **upregulated HSP70 slightly in larvae not exposed to UV-B, but only the**
265 **induction by the highest concentration was statistically significant because of**
266 **high variability among individuals. Even though simultaneous exposure to retene**
267 **and UV-B seemed to elevate HSP70 levels, this finding was not statistically**
268 **significant** (Fig. 5). The HSP70 level of the positive control, heat-shocked whitefish,
269 was 2.5 times that of the control fish.

270

271 **DISCUSSION**

272

273 In the clearest oligotropic lakes in Finland, UV-B can penetrate deeper than 1
274 metre and, as larval whitefish and vendace are positively phototactic and swim near
275 the surface for the first weeks, in some years they are likely exposed to episodes of
276 exceptional solar UV-B [15, 26]. Additionally, related to climatic change, up to 90%
277 increase in CIE-weighted UV doses have been predicted in northern latitudes [27].
278 The UV daily doses used in this study, 2.8 kJ/m^2 and 5.4 kJ/m^2, were realistic and
279 correspond with a slightly (7%) subambient or an 80% increase relative to the
280 maximum daily doses in Finland in early May.

281

282 UV-B radiation has been shown to be detrimental to fishes, especially at
283 embryonic and larval stages [28]. In our study, however, UV-B on its own had no
284 effect on the behaviour or mortality of the larvae of vendace and whitefish. This is in
285 accordance with former results showing that vendace and whitefish are relatively
286 resistant to UV-B [29]. As species, therefore, they appear to be well adaptable in
287 respect to scenarios of the future UV-climate. Importantly, however, UV-
288 photoinduced ecotoxicity of xenobiotic chemicals may provide additional risks to
289 adaptable species, as we hypothetized.

290

291 Retene is found at mg/L concentrations in pulp mill sludges and sediments
292 downstream of wastewater discharges [6-9]. Preliminary elutration trials show that
293 sediment retene is dissolved from this matrix (up to 13 μg/L) and is bioavailable to

294 fish [14, 30]. Despite these aspects of its environmental fate, retene is not acutely
295 lethal to fish in water-soluble concentrations [11], as also verified in this study.
296
297 Certain PAHs (e.g. fluoranthene, anthracene, pyrene) have been found to be
298 acutely phototoxic to a number of aquatic organisms including microbes, plants,
299 invertebrates and vertebrates, in the presence of UV radiation [2, 31-34]. It was
300 recently discovered that retene is acutely phototoxic to *Daphnia magna* and to
301 whitefish (*Coregonus lavaretus*) in the presence of UV-B [13, 35] and this study
302 extends those observations with more realistic daily doses of UV-B and with tests of
303 another fish species, vendace. While both coregonids were sensitized due to
304 simultaneous exposure to UV-B and retene, vendace was more tolerant to this **retene-**
305 **based lethality potential** . Importantly, the lower dose of UV-B also evoked similar
306 lethality in whitefish as the higher dose, a UV-B intensity predicted for the coming
307 decades.
308
309 **The retene concentrations we used were below (10 μg/L) and over (32 and**
310 **100 μg/L) the concentrations that are likely to occur in the nature. This was**
311 **needed for a more mechanistic approach of effects. It must be noted that only**
312 **two days of simultaneous exposure to UV-B and 10 μg/L retene (a concentration**
313 **likely to occur in the nature) caused significant mortality (20 – 30%). In addition**
314 **to water as a source, retene can be accumulated by eggs from the sediment, or by**
315 **larvae from particulate material [9, 11]. Furthermore, the applied safety factors**
316 **in risk assessment with the given set of endpoints often range from 10 to 100.**
317

318 **The LC50 values were calculated with nominal concentrations of the**
319 **precursor retene, as the concentrations were measured only in the beginning of**
320 **the experiment, the measured concentrations being similar to nominal**
321 **concentrations (data not shown). In a similar but different experiment the actual**
322 **concentrations were measured also after 24 h, before the first UV-B radiation:**
323 **The concentrations had diminished so that thay were about 80% of nominal**
324 **concentrations (Häkkinen, unpublished results). So the LC50 values are perhaps**
325 **optimistic compared to the real-life situation.**

326

327 As behavioural endpoints, retene caused signs of hypoxia in both species
328 during simultaneous UV-B exposure. Increased ventilation rate and coughing were
329 noted in juvenile sunfish (*Lepomis macrochirus*) and whitefish (*Coregonus lavaretus*)
330 exposed to PAH and UV in combination [31, 35]. The actions behind these symptoms
331 in whitefish larvae seem to be the functional disruption of mucous cells eventually
332 expressed as respiratory stress [35].

333

334 UV-B induces CYP1A1 gene expression in mammalian cells [36]. Irradiation
335 of amino acid tryptophan with UV causes the formation of formylated
336 indolocarbazoles, which are very potent Ah-receptor agonists [37]. Although no
337 CYP1A induction by UV-B could be detected in vendace, a slight induction (10% of
338 the maximum induction detected) was observed in whitefish exposed to the lower
339 UV-B dose (2.8 kJ/m^2). In whitefish larvae exposed to the higher dose (5.4 kJ/m^2),
340 however, no induction by UV-B was detected. Why? We presume that the higher UV-
341 B level may have caused damage to the skin where the CYP1A induction would have
342 taken place. This is in accordance with our earlier observation on UV-B induced skin

343 pathology in larval whitefish exposed to the same high UV-B dose as in the present
344 study [35].
345
346 Subchronic exposure of rainbow trout larvae to retene causes CYP1A
347 induction [12]. In the present study, a three-day retene exposure induced CYP1A in
348 both vendace and whitefish. Induction in vendace was highest with the highest retene
349 concentration (100 µg/L) but this concentration seemed to cause a distinctly
350 submaximal induction in whitefish. Additionally, when comparing the maximal
351 responses of CYP1A in the same larval phase of the two closely related coregonid
352 species, the inducibility of vendace is slightly higher than that of whitefish. Overall,
353 the response of CYP1A in animals exposed to combined UV-B + retene equalled the
354 response to exposure to retene alone, particularly in vendace.
355
356 HSPs are upregulated by a variety of environmental stressors, including
357 elevated temperatures, UV radiation and many xenobiotics [19]. Our results show that
358 heat shock (used as a positive control), retene and UV-B were all inefficient in
359 upregulating HSP70 levels in vendace. One may assume that the treatments we
360 applied were too mild or too short to upregulate HSP70 in larval vendace. The levels
361 of HSP70 in control vendace were slightly lower compared to the levels in control
362 whitefish (data not shown), so it is not likely that the vendace larvae were
363 unresponsive because of previous and ongoing HSP70 induction.
364
365 In contrast to vendace, **the lower dose of UV-B** induced HSP70 in whitefish
366 larvae. The LD50 (14 d) for larval whitefish is 5.1 kJ/m^2 (CIE-weighted) of daily
367 exposure, which corresponds to the total dose of 71 kJ/m^2 (O. Ylönen, personal

368 communication). Supposing the model of cumulative dose for an effect endpoint of
369 UV-B, the total dose in two days (2 x 2.8 kJ/m^2) causing significant induction in
370 HSP70 of whitefish larvae reveals an early signal being 12 times more sensitive in
371 relation to lethality.
372
373 **There was an increasing trend in HSP70 levels in whitefish exposed to**
374 **retene, but the induction varied a lot among individuals and only the highest**
375 **concentration of retene caused an upregulation that was statistically significant.**
376
377 **HSP70 induction is often considered as a protective response and the**
378 **inability to upregulate HSP70 usually correlates with tissue injury [19]. This does**
379 **not directly correlate with our findings where the more tolerant species, vendace,**
380 **was unresponsive and the more sensible species, whitefish, was able to**
381 **upregulate HSP70. Presumably vendace has other protective mechanisms**
382 **making it more tolerant than whitefish.**
383
384 In conclusion, larval vendace and whitefish exposed simultaneously to retene
385 and UV-B revealed different mortality and differ also in the induction of CYP1A and
386 HSP70. Vendace appears to be more resistant to the **retene-based lethality potential**
387 than whitefish, as indicated by the higher tolerance. CYP1A, being elevated even at
388 the lowest sublethal concentration used, was a biomarker of retene exposure in both
389 species. In whitefish, HSP70 acts as an early warning signal of effect due to UV-B
390 exposure. These sublethal responses, however, cannot be used as specific biomarkers
391 of combined retene and UV-B exposures.
392

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398

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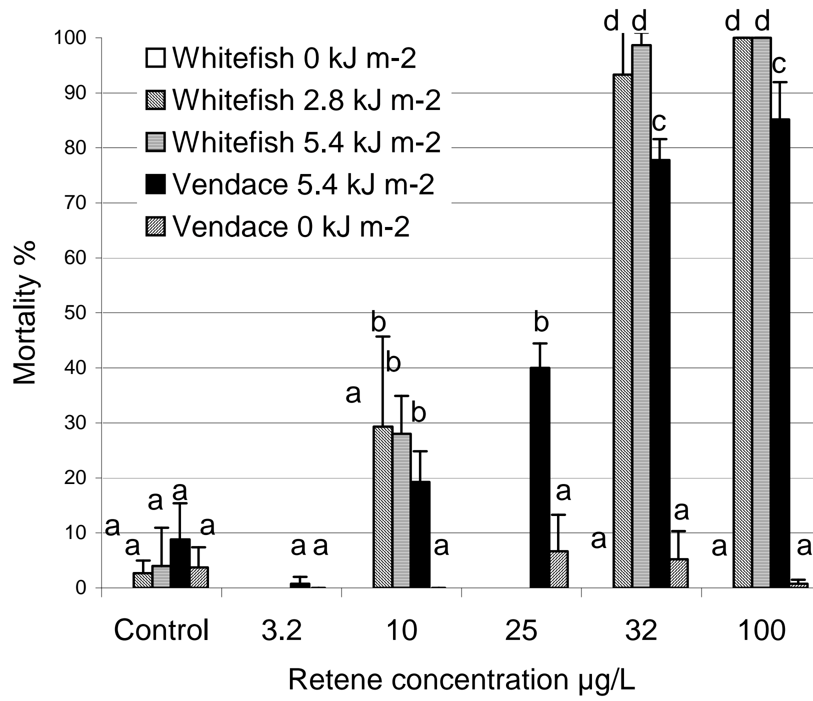
Figure 1. **Retene-based lethality potential of newly hatched vendace and whitefish. Vendace and whitefish larvae were pre-exposed to retene, followed by two 3 h daily irradiations with UV-B (2.8 or 5.4 kJ/m2) or visible light.** Bar denotes standard deviation. Groups denoted by the same letter do not differ significantly from each other ($p \geq 0.05$, Tukey). **UV-B = ultraviolet-B.**

Figure 2. CYP1A induction in larval vendace by retene and UV-B, alone or in combination. **Positive control values were fixed at a value of 1. Bar denotes standard deviation.** Number of determinations 6, each sample pooled of 10 animals. Groups denoted by the same letter do not differ significantly from each other ($p \geq 0.05$, Tukey). **UV-B = ultraviolet-B.**

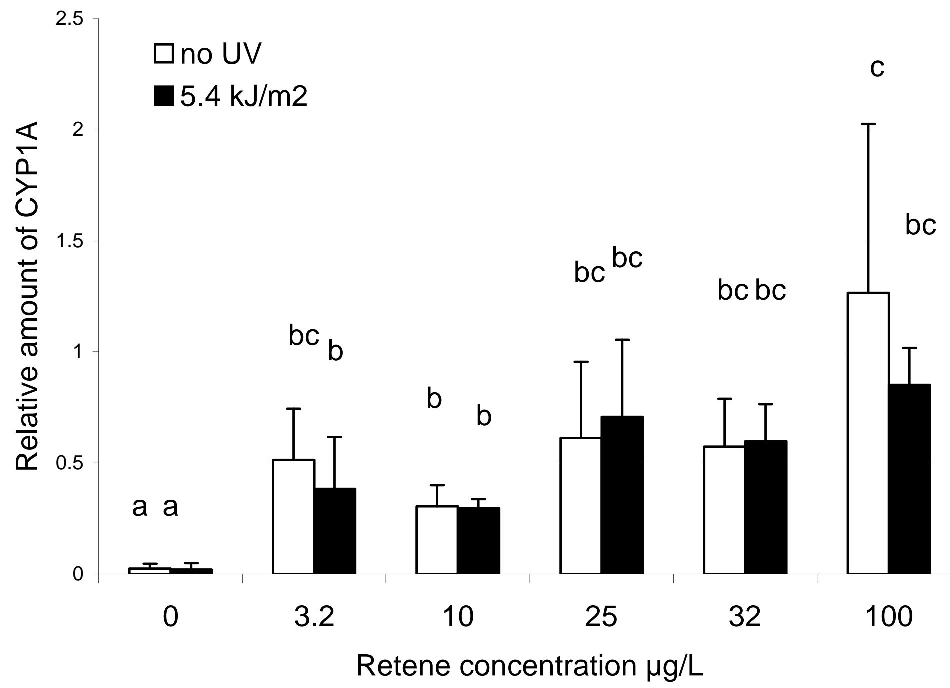
Figure 3. CYP1A induction in larval whitefish by retene and UV-B, alone or in combination. No measurements of CYP1A in animals exposed to 32 µg/L and 100 µg/L retene + UV-B were done, as these treatments were lethal. **Positive control values were fixed at a value of 1. Bar denotes standard deviation.** Number of determinations 6, each sample pooled of 5 animals. Groups denoted by the same letter do not differ significantly from each other ($p \geq 0.05$, Tukey). **UV-B = ultraviolet-B.**

Fig. 4. HSP70 induction in larval vendace by retene and UV-B, alone or in combination. **Bar denotes standard deviation.** For the details, see legend to Fig. 2. **UV-B = ultraviolet-B.**

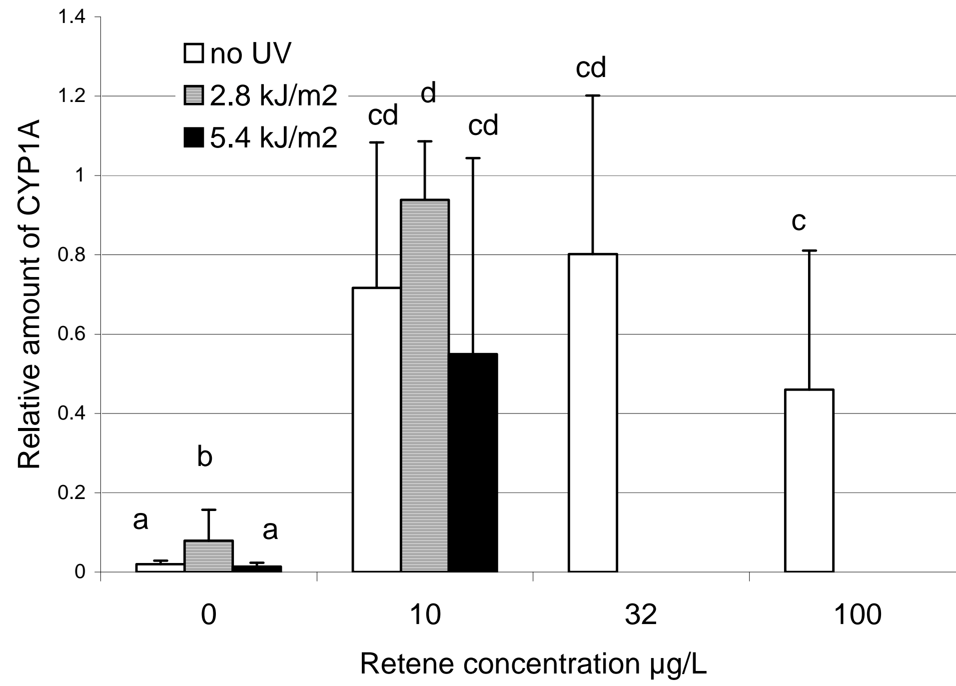
Fig. 5. HSP70 induction in larval whitefish by retene and ultraviolet-B (UV-B), alone or in combination. **Bar denotes standard deviation.** For the details, see legend to Fig. 3. **UV-B = ultraviolet-B.**



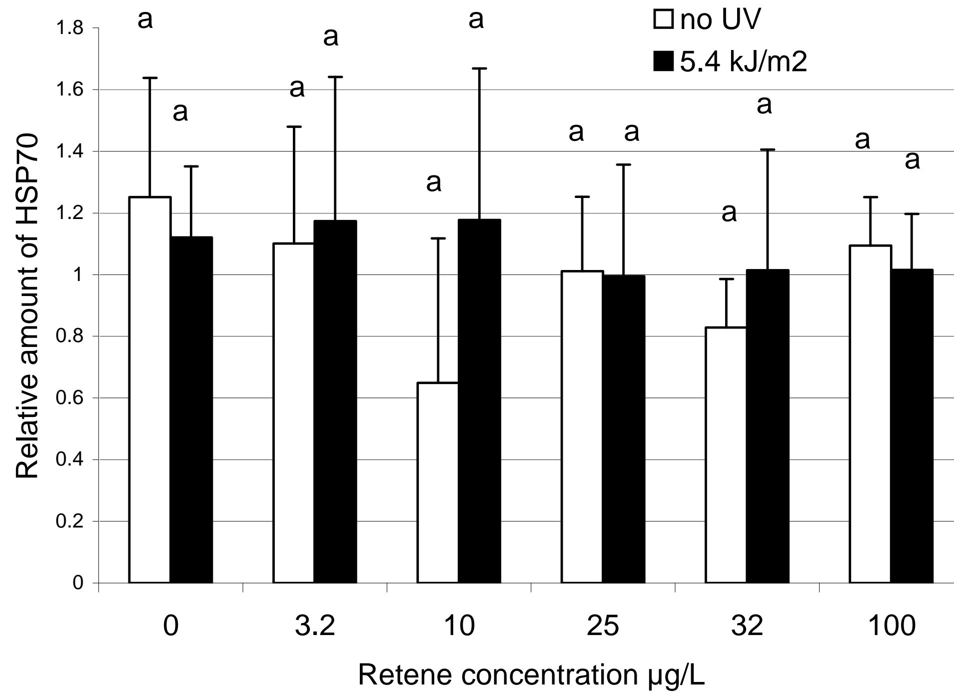
Whitefish 0 kJ m-2
Whitefish 2.8 kJ m-2
Whitefish 5.4 kJ m-2
Vendace 5.4 kJ m-2
Vendace 0 kJ m-2
Mortality %
0
10
20
30
40
50
60
70
80
90
100
Control
3.2
10
25
32
100
Retene concentration µg/L

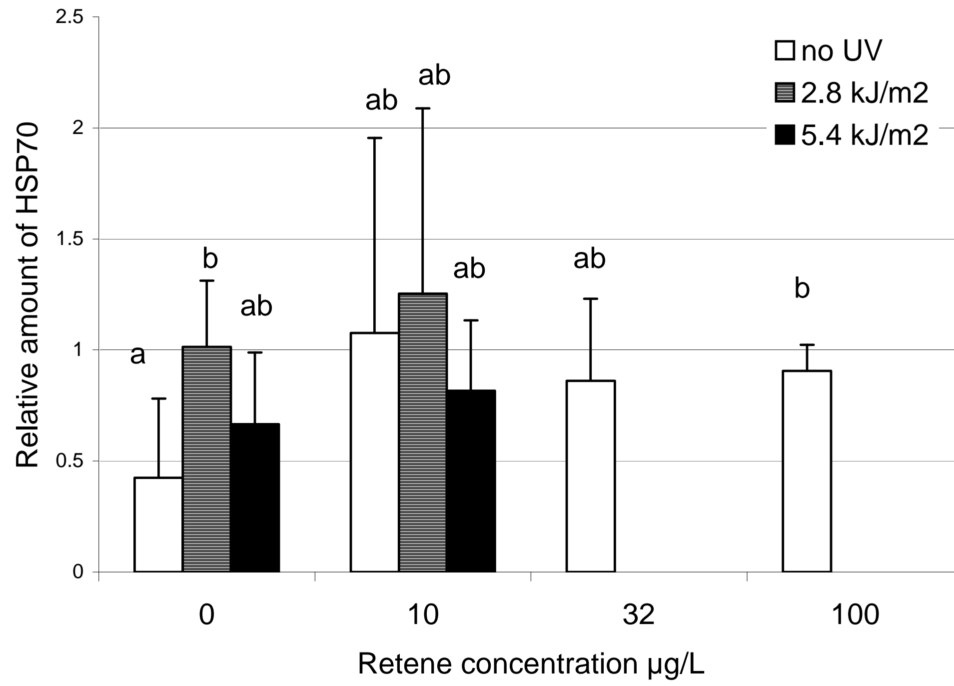


2.5
2
1.5
1
0.5
0
no UV
5.4 kJ/m2
Relative amount of CYP1A
a a
bc b
b b
bc bc
bc bc
c
bc
0
3.2
10
25
32
100
Retene concentration µg/L



1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative amount of CYP1A
no UV
2.8 kJ/m2
5.4 kJ/m2
a
b
a
cd
d
cd
cd
c
0
10
32
100
Retene concentration µg/L


no UV
5.4 kJ/m2
Relative amount of HSP70
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
a
a
a
a
a
a
a
a
0
3.2
10
25
32
100
Retene concentration µg/L


2.5
2
1.5
1
0.5
0
Relative amount of HSP70
no UV
2.8 kJ/m2
5.4 kJ/m2
a
b
ab
ab
ab
ab
ab
b
0
10
32
100
Retene concentration µg/L