This is an electronic reprint of the original article. This reprint may differ from the original in pagination and typographic detail.

Author(s): Vehniäinen, Eeva-Riikka; Häkkinen, Jani; Oikari, Aimo

Title: Photoinduced lethal and sublethal toxicity of retene, a polycyclic aromatic hydrocarbon derived from resin acid, to coregonid larvae

Year: 2003

Version:

Please cite the original version:

All material supplied via JYX is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.
Running head: Phototoxicity of retene to coregonid larvae

Eeva Vehniäinen
University of Jyväskylä
Department of Biological and Environmental Sciences
P.O.Box 35
40014 Jyväskylä
Finland.
Tel: +358-14-260 4192
Fax: +358-14-260 2321
E-mail: eevehnia@cc.jyu.fi

Total number of words: 5 235
PHOTOINDUCED LETHAL AND SUBLETHAL TOXICITY OF RETENE, A RESIN ACID DERIVED PAH, TO COREGONID LARVAE

EEVA-RIIKKA VEHNÄINEN*, JANI HÄKKINEN AND AIMO OIKARI

Department of Biological and Environmental Sciences, University of Jyväskylä,

P.O.Box 35, 40014 Jyväskylän yliopisto, Finland
*To whom the correspondence should be addressed

eevhnia@cc.jyu.fi
Abstract — A comparative investigation on the acute phototoxicity of retene to vendace (*Coregonus albula*) and whitefish (*Coregonus lavaretus*), both having pelagial larvae in spring, was conducted. In order to test the concept of early warning of sublethal biomarkers in relation to lethality to posthatch stages, we examined the effects of ultraviolet-B (UV-B) and retene on the levels of CYP1A and HSP70 by exposing the animals to elevated levels of these factors for 48 and 72 h, respectively. While UV-B and retene on their own were not lethal, simultaneous retene and UV-B exposure caused very high mortality to both species. The LC50 (lethal concentration 50%, i.e. the concentration at which half of the larvae died) of retene as a precursor was 41 μg/L for vendace and 15-16 μg/L, depending on UV dose, for whitefish. Retene evoked substantial induction of CYP1A in larvae of both species and UV-B induced CYP1A in whitefish. In vendace, no effect on HSP70 levels by any factor was observed. In whitefish, however, UV-B radiation and water retene alone upregulated HSP70, but no additive response was detected. CYP1A is a biomarker of exposure to retene in both species. HSP70 is an early warning signal of UV-B exposure in whitefish. As a species vendace appears to be more resistant to the phototoxicity of retene than whitefish, as indicated by the higher tolerance.

Keywords — Phototoxicity Fish Retene Ultraviolet-B Cytochrome P4501A Heat shock protein 70
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.
It was chosen as an endpoint because it is known to be induced by many PAHs, including retene [12, 16]. The aim was to investigate whether the induction differs between species and whether UV-B has an effect on the induction caused by retene (e.g. if more potent CYP1A inducers are generated by UV-B).

Heat shock protein 70 (HSP70) is a class of stress proteins with a molecular weight of 70 – 72 kDa. Cells express some proteins of this family constitutively (heat shock cognates, HSC's) and some are upregulated by a variety of environmental stressors that generate denatured proteins, including UV radiation and bleached kraft pulp mill effluent [17-19]. HSP70 is believed to have a protective role against tissue damage as it binds to denatured proteins and attempts to restore their tertiary structure and function [19].

MATERIALS AND METHODS

Organisms and water quality

The developing eggs of vendace (Lake Pyhäselkä stock) were obtained from the University of Joensuu and those of whitefish (Rautalampi stock) were obtained from a local hatchery (Laukaa). Once transferred to the University of Jyväskylä in late March, the embryos were further incubated and hatched in flow-through hatchery cones at 6 – 8 °C. The water was colourless (<5 mg Pt/L), filtered groundwater with pH 6.6 – 7, having a high penetrability for UV-B (65% in 15 cm). The water hardness (Ca + Mg) was 1.3-1.4 mmol/L and conductivity 26.2 mS/m. One-day
old larvae were kept in the same well-aerated water after hatching and used for the experiments.

Exposure system and sampling

The vendace and whitefish larvae *(45 and 25 per replicate, respectively)* were carefully transferred to retene (nominal concentrations 3.2; 10, 25, 32 and 100 $\mu$g/L and 10, 32 and 100 $\mu$g/L, respectively) or control treatments (water only or 0.1% dimethylsulfoxide (DMSO), used as carrier) with large-bore pipettes and pre-exposed for 24 hours in Pyrex glass bowls. The depth and volume of pre-aerated water were 5 cm and 1 liter, respectively. A portion of the water was changed daily. There were three replicates for each treatment. After a 24-h accumulation period, the larvae were further exposed to retene and, in addition, irradiated with either UV-B or visible light. UV-B was provided in the laboratory using a fluorescent lamp (UVB-313, Q-Panel, Cleveland, OH, USA). Ultraviolet-C (UV-C; under 280 nm), which in the nature is blocked by the atmosphere and does not reach the surface of the earth, was blocked with a cellulose diacetate filter (Clarifoil, Derby, UK), replaced after each 3-hour UV-B radiation. Control treatments without UV-B received visible light (TLD 36 W/950 daylight, Philips, Eindhoven, Netherlands). The photoperiod was 16h:8h (light:dark).

The larvae received UV-B or visible light 3 h per day (between 24 – 27 hours and 48-51 hours after the beginning) to mimic midday exposure, for two days. UV was quantified using Hamamatsu Photonic Multichannel Spectral analyser (model PMA-11), measuring the wavelength area 280 – 380 nm. The UV-B intensities were
measured at the water surface in the beginning and the end of each experiment at various spots to ensure equal doses for all replicates in each treatment. The daily doses were 2.8 kJ/m² for the lower UV-B intensity and 5.4 kJ/m² for the higher, calculated as CIE-weighted (Commission Internationale de l'Eclairage; International Commission on Illumination) J/m², i.e., the action spectrum specific for human erythema [20]. These UV-B doses correspond with slightly subambient (7%) and 80% increase relative to the maximum daily doses in Finland (approx. 60 – 70 °N ) in early May. The vendace were exposed only to the higher and the whitefish to both intensities.

The larvae were sampled after 3 days (72 hours) from the start by anesthetizing the animals with MS222 (50 mg/L, 2 min) and freezing in liquid nitrogen. The samples were transferred to –80 °C and preserved there until processed further.

Positive controls for Western blotting

The positive control used for Western blotting of CYP1A was obtained by injecting four one-year-old juvenile whitefish with 30 μg/g β-naphthoflavone in 95% ethanol : olive oil (1:1). The fish were further kept at 12 °C in a 40-liter aquarium for 4 days, anesthetized with MS-222 (50 mg/L) and sacrificed. The organs were removed and frozen immediately in liquid nitrogen. Once thawed on ice the livers were homogenized with potassium gluconate buffer (70 mmol/L, pH 7.8) and centrifuged for five minutes (3000 x g). The supernatant was used for the blotting.
Heat-shocked vendace and whitefish larvae were used as positive controls for the responsivity and Western blotting of HSP70. Larvae acclimated at 6 °C were held in 16 °C for 1 hour, transferred back to 6 °C and sampled after 3 hours. The animals were frozen in liquid nitrogen and preserved in –80 °C.

**Western immunoblotting**

Larvae frozen in liquid nitrogen (30 – 40 mg, corresponding to 10 larvae of vendace or 5 larvae of whitefish) were homogenized in a glass homogenizer with potassium gluconate buffer (5 mmol/L MgSO₄, 5 mmol/L NaH₂PO₄, 40 mmol/L HEPES, 70 mmol/L potassium gluconate, 150 mmol/L sorbitol, pH 7.8) [21]. The homogenates were centrifuged at 1000 g for 5 min.

Total protein concentration of the supernatant was determined by modified Lowry method (BioRad DC, Bio Rad Laboratories, Hercules, CA, USA) adapted for 96-well plates [22]. Five μl of each diluted sample was pipetted into a microtiter plate, in triplicate. Similarly, 5 μl aliquots of protein standards (bovine serum albumin, 0 – 1.5 mg/ml) were pipetted into the 96-well plate. An aliquot of 25 μl of Bio-Rad reagent A was added and 200 μl reagent B pipetted into each well. The plate was put into the microplate reader for mixing, incubated for 15 min and absorbance read at 690 nm.

The CYP1A and HSP70 determination was made by Western blot after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation (100 and 20 μg protein / lane, respectively), using a Mini-Protean II apparatus [21,
The positive controls were the liver of β-naphthoflavone injected juvenile whitefish (3.15 μg protein / lane) and heat-shocked vendace and whitefish larvae (20 μg protein / lane) for CYP1A and HSP70, respectively. Samples were diluted in a buffer solution (0.125 mol/L Tris-HCl, 2% SDS, 20% glycerol, 0.02% bromophenol blue, 5% 2-mercaptoethanol) and loaded onto gels after heating (4 min, 95 °C). Proteins were run on a SDS-PAGE gel for 1 h ($V = 100$ V for 5 min, 200 V for 55 min), after which gels were soaked in blotting buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, pH 8.3). Proteins were transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad) using Bio Rad Mini-Protean II apparatus at 100 V for 90 min. The membrane was stained with 0.2 % Ponceau S in 3 % trichloroacetic acid for 1 min to confirm protein transfer, rinsed twice with Tris-buffered saline-Tween (TBST; 0.9% NaCl, 10 mmol/L Tris, 0.1% Tween-20, pH 7.4) and incubated in blocking buffer (9% non-fat dry milk in TBST) for 14 h in 4 °C. The blot was probed with 3 μg/ml anti-CYP1A (Mab 1-12-3, kindly provided by Dr. John Stegeman) or 1:5000 anti-HSP70 (MA3-006, Affinity BioReagents, Golden, CO, USA) in blocking buffer for 2 h. These antibodies recognize CYP1A or HSP70, respectively, of many species including fish, amphibians, and mammals (Murphy SP, Fox S, Myers MP, Morimoto RI. Unpublished data. Affinity Bioreagents) [25]. After washing (TBST, 1x15 min, 2x5 min), the blot was probed with secondary antibody, 1:3000 peroxidase labeled anti-mouse IgG (A9044, Sigma-Aldrich Chemie, Steinheim, Germany), in TBST for 1 hour. After washing (TBST, 1x15 min, 2x5 min) the immunodetection was performed via enhanced chemiluminescence using Star-Glo chemiluminescent substrate (ICN Biomedicals,
Irvine, CA, USA). Hyperfilm ECL® high performance chemiluminescence film (Amersham Pharmacia, Uppsala, Sweden) was used for visualization.

The exposed films were scanned and, for densitometric analysis, the pictures were analyzed using Scion Image 4.0.2. Different blots were made comparable to each other by calibrating them with positive controls, fixing each positive control at a value of 1.

Statistical analysis

Differences in mortality between control and treatment groups were tested using two-way analysis of variance (ANOVA) and Tukey's test. LC50 values were calculated using Probit analysis. Values for CYP1A and HSP70 induction were log-transformed and the differences between control and treatment groups tested using two-way ANOVA and Tukey's test. The level of significance of $p < 0.05$ was considered as statistically significant.

RESULTS

Lethality and behavioural effects

UV-B or retene alone caused no acute mortality. However, UV-B radiation increased the toxicity of retene significantly. On day three, after two daily 3-h periods of UV-B, most vendace larvae in 100 μg/L retene treatments were dead (Fig. 1). All whitefish larvae in 100 μg/L and nearly all in 32 μg/L retene treatments died by day
As retene in itself did not cause lethality, we call the type of toxicity as retene-based lethality potential, but the concentrations are expressed as those of the precursor, retene. The LC50 values (72 h) calculated from nominal concentrations of the precursor, retene, were 41 µg/L (95% confidence limits 30 and 58 µg/L) for vendace, and 16 µg/L (13, 20 µg/L) and 15 µg/L (13, 17 µg/L) for whitefish exposed to the lower and higher dose of UV-B, respectively. Vendace is statistically more tolerant, but the whitefish exposed to lower and higher dose of UV-B do not differ statistically from each other.

The vendace larvae exposed to 100 µg/L retene and receiving UV-B radiation showed coughing and decreased motility after the first and second UV exposure. The whitefish larvae showed similar symptoms but even more frequently. Symptoms typically lasted for 3 – 4 hours.

**CYP1A induction**

Retene caused induction of CYP1A both in the presence and absence of UV-B radiation in both species. In vendace the CYP1A induction was approximately proportional to the concentration of retene, being greatest with the highest concentration (100 µg/L). In vendace, UV-B radiation alone caused no induction of CYP1A (Fig. 2).

In whitefish the induction was greatest with 32 µg/L retene, being slightly submaximal ($p > 0.05$, Tukey) with the highest retene concentration applied (100 µg/L). The lower UV-B intensity caused induction in non-retene-exposed whitefish
larvae. In retene-exposed whitefish (10 $\mu$g/L only, because the higher concentrations of retene as precursor were lethal) the lower UV-B level also seemed to have an additive effect, but this finding was not statistically significant ($p > 0.05$, Tukey) (Fig. 3).

When comparing the maximal responses of CYP1A, the inducibility of vendace was slightly higher than that of whitefish ($p > 0.05$, Tukey). The positive control gave a strong dark band in the Western blots even with the low protein amount used (3.15 $\mu$g / lane).

**HSP70 induction**

In vendace, neither UV-B nor retene had an effect on HSP70 induction that was statistically significant. Also the HSP70 level of the positive control, heat shocked vendace, was similar to control, showing no induction (Fig. 4).

**In whitefish, the lower intensity of UV-B radiation caused an induction of HSP70 in retene non-exposed animals.** Also the higher intensity seemed to induce HSP70, but this finding was not statistically significant ($p > 0.05$, Tukey). Retene upregulated HSP70 slightly in larvae not exposed to UV-B, but only the induction by the highest concentration was statistically significant because of high variability among individuals. Even though simultaneous exposure to retene and UV-B seemed to elevate HSP70 levels, this finding was not statistically significant (Fig. 5). The HSP70 level of the positive control, heat-shocked whitefish, was 2.5 times that of the control fish.
DISCUSSION

In the clearest oligotrophic lakes in Finland, UV-B can penetrate deeper than 1 metre and, as larval whitefish and vendace are positively phototactic and swim near the surface for the first weeks, in some years they are likely exposed to episodes of exceptional solar UV-B [15, 26]. Additionally, related to climatic change, up to 90% increase in CIE-weighted UV doses have been predicted in northern latitudes [27].

The UV daily doses used in this study, 2.8 kJ/m² and 5.4 kJ/m², were realistic and correspond with a slightly (7%) subambient or an 80% increase relative to the maximum daily doses in Finland in early May.

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

Retene is found at mg/L concentrations in pulp mill sludges and sediments downstream of wastewater discharges [6-9]. Preliminary elutration trials show that sediment retene is dissolved from this matrix (up to 13 µg/L) and is bioavailable to
fish [14, 30]. Despite these aspects of its environmental fate, retene is not acutely lethal to fish in water-soluble concentrations [11], as also verified in this study.

Certain PAHs (e.g. fluoranthene, anthracene, pyrene) have been found to be acutely phototoxic to a number of aquatic organisms including microbes, plants, invertebrates and vertebrates, in the presence of UV radiation [2, 31-34]. It was recently discovered that retene is acutely phototoxic to *Daphnia magna* and to whitefish (*Coregonus lavaretus*) in the presence of UV-B [13, 35] and this study extends those observations with more realistic daily doses of UV-B and with tests of another fish species, vendace. While both coregonids were sensitized due to simultaneous exposure to UV-B and retene, vendace was more tolerant to this retene-based lethality potential. Importantly, the lower dose of UV-B also evoked similar lethality in whitefish as the higher dose, a UV-B intensity predicted for the coming decades.

The retene concentrations we used were below (10 μg/L) and over (32 and 100 μg/L) the concentrations that are likely to occur in the nature. This was needed for a more mechanistic approach of effects. It must be noted that only two days of simultaneous exposure to UV-B and 10 μg/L retene (a concentration likely to occur in the nature) caused significant mortality (20 – 30%). In addition to water as a source, retene can be accumulated by eggs from the sediment, or by larvae from particulate material [9, 11]. Furthermore, the applied safety factors in risk assessment with the given set of endpoints often range from 10 to 100.
The LC50 values were calculated with nominal concentrations of the precursor retene, as the concentrations were measured only in the beginning of the experiment, the measured concentrations being similar to nominal concentrations (data not shown). In a similar but different experiment the actual concentrations were measured also after 24 h, before the first UV-B radiation: The concentrations had diminished so that they were about 80% of nominal concentrations (Häkkinen, unpublished results). So the LC50 values are perhaps optimistic compared to the real-life situation.

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

UV-B induces CYP1A1 gene expression in mammalian cells [36]. Irradiation of amino acid tryptophan with UV causes the formation of formylated indolocarbazoles, which are very potent Ah-receptor agonists [37]. Although no CYP1A induction by UV-B could be detected in vendace, a slight induction (10% of the maximum induction detected) was observed in whitefish exposed to the lower UV-B dose (2.8 kJ/m²). In whitefish larvae exposed to the higher dose (5.4 kJ/m²), however, no induction by UV-B was detected. Why? We presume that the higher UV-B level may have caused damage to the skin where the CYP1A induction would have taken place. This is in accordance with our earlier observation on UV-B induced skin
pathology in larval whitefish exposed to the same high UV-B dose as in the present study [35].

Subchronic exposure of rainbow trout larvae to retene causes CYP1A induction [12]. In the present study, a three-day retene exposure induced CYP1A in both vendace and whitefish. Induction in vendace was highest with the highest retene concentration (100 μg/L) but this concentration seemed to cause a distinctly submaximal induction in whitefish. Additionally, when comparing the maximal responses of CYP1A in the same larval phase of the two closely related coregonid species, the inducibility of vendace is slightly higher than that of whitefish. Overall, the response of CYP1A in animals exposed to combined UV-B + retene equalled the response to exposure to retene alone, particularly in vendace.

HSPs are upregulated by a variety of environmental stressors, including elevated temperatures, UV radiation and many xenobiotics [19]. Our results show that heat shock (used as a positive control), retene and UV-B were all inefficient in upregulating HSP70 levels in vendace. One may assume that the treatments we applied were too mild or too short to upregulate HSP70 in larval vendace. The levels of HSP70 in control vendace were slightly lower compared to the levels in control whitefish (data not shown), so it is not likely that the vendace larvae were unresponsive because of previous and ongoing HSP70 induction.

In contrast to vendace, the lower dose of UV-B induced HSP70 in whitefish larvae. The LD50 (14 d) for larval whitefish is 5.1 kJ/m² (CIE-weighted) of daily exposure, which corresponds to the total dose of 71 kJ/m² (O. Ylönen, personal
communication). Supposing the model of cumulative dose for an effect endpoint of UV-B, the total dose in two days (2 x 2.8 kJ/m²) causing significant induction in HSP70 of whitefish larvae reveals an early signal being 12 times more sensitive in relation to lethality.

There was an increasing trend in HSP70 levels in whitefish exposed to retene, but the induction varied a lot among individuals and only the highest concentration of retene caused an upregulation that was statistically significant.

HSP70 induction is often considered as a protective response and the inability to upregulate HSP70 usually correlates with tissue injury [19]. This does not directly correlate with our findings where the more tolerant species, vendace, was unresponsive and the more sensible species, whitefish, was able to upregulate HSP70. Presumably vendace has other protective mechanisms making it more tolerant than whitefish.

In conclusion, larval vendace and whitefish exposed simultaneously to retene and UV-B revealed different mortality and differ also in the induction of CYP1A and HSP70. Vendace appears to be more resistant to the retene-based lethality potential than whitefish, as indicated by the higher tolerance. CYP1A, being elevated even at the lowest sublethal concentration used, was a biomarker of retene exposure in both species. In whitefish, HSP70 acts as an early warning signal of effect due to UV-B exposure. These sublethal responses, however, cannot be used as specific biomarkers of combined retene and UV-B exposures.
Acknowledgement — Antibody against scup CYP1A was kindly provided by Dr. John Stegeman, Woods Hole Oceanographic Institution, MA, USA. The authors would like to thank Mervi Koistinen for excellent technical assistance. This research was supported by the Academy of Finland (Figare/Solar-project and project-Repro to A.O.).

REFERENCES


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 $\mu$g/L and 100 $\mu$g/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.**
Control 3.2 10 25 32 100
Retene concentration µg/L
Mortality %

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
Relative amount of CYP1A

- Retene concentration µg/L

- No UV
- 5.4 kJ/m²

- Bars with letters indicate significant differences:
  - a vs. b vs. c
  - bc vs. bc vs. bc
Relative amount of CYP1A

Retene concentration µg/L

- no UV
- 2.8 kJ/m²
- 5.4 kJ/m²

Significant differences indicated by letters:
- a
- b
- c
- d
Relative amount of HSP70

Retene concentration µg/L

- **no UV**
- **5.4 kJ/m²**
Retene concentration µg/L

Relative amount of HSP70

- no UV
- 2.8 kJ/m²
- 5.4 kJ/m²

a, b, ab