JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE 125

Jani Häkkinen

Comparative Sensitivity of Boreal Fishes to UV-B and UV-Induced Phototoxicity of Retene

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Ambiotica-rakennuksen salissa (YAA303)
marraskuun 28. päivänä 2003 kello 12.

Academic dissertation to be publicly discussed, by permission of
the the Faculty of Mathematics and Science of the University of Jyväskylä,
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UNIVERSITY OF JYVÄSKYLÄ

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Publishing Unit, University Library of Jyväskylä

URN:ISBN 9513916472
ISBN 951-39-1647-2 (PDF)

ISBN 951-39-1509-3 (nid.)
ISSN 1456-9701

Jyväskylä University Printing House, Jyväskylä 2003

ABSTRACT

Häkkinen, Jani
Comparative sensitivity of boreal fishes to UV-B and UV-induced phototoxicity of retene
Jyväskylä: University of Jyväskylä, 2003, 58 p.
(Jyväskylä Studies in Biological and Environmental Science,
ISSN 1456-9701; 125)
ISBN 951-39-1647-2
Yhteenveto: Kalojen varhaisvaiheiden herkkyys UV-B säteilylle ja reteenin UV-valoindusoituvalle toksisuudelle

Related to ozone depletion, 20-50 % increases in CIE-weighted UV-B doses have been predicted in northern latitudes in coming decades. In order to investigate whether increased UV-B radiation is a risk factor for the most sensitive larval stages of freshwater fishes (vendace, whitefish and pike) a series of experiments were conducted under lamps in the laboratory and under the sun in the field. Besides direct effects of UV-B, phototoxicity of certain environmental xenobiotics may also follow. The possibility of phototoxicity of retene, a resin acid derived PAH, was investigated. UV-B conditions considered to be realistic in the future were strictly followed. UV-B alone, even at high dose rates, had only minor effects on coregonid larvae, indicated a minor sunburn reaction of the skin, reversible growth response and increased pigmentation. However, pike larvae were highly sensitive to UV-B under laboratory conditions even in current doses, indicated as severe neurobehavioral disorder. Monitoring of animals with the behavioral syndrome revealed substantial late mortality. Interestingly, field experiments with pike suggest that only minor increases in ambient UV-B coming on the earth's surface may cause sublethal effects to larval fish. However, the frequency of behavioral disorders was considerably lower in the field than under laboratory conditions. Newly hatched larvae of whitefish, vendace and pike differed substantially in their reactions to UV-photoinduced toxicity of retene. By itself, retene - when tested up to 100 µg/l - had no short-term toxic effects to the larvae of boreal fishes. In coregonids, however, the combined exposure of retene+UV resulted in dramatic lethality at low concentrations of retene in acute (72 h) exposures. On the other hand, retene had no phototoxic effects to the larval pike. Overall, it can be concluded that for post-hatch larvae of whitefish and vendace the synergism of UV-B and retene is a key factor of potential ecotoxicological risk to be taken into consideration in lake areas chemically contaminated by sources of retene.

Key words: Boreal fishes; larvae; phototoxicity; retene; UV-B.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by Roman numerals I-VI.

I Häkkinen, J., Vehniäinen, E., Ylönen, O., Heikkilä, J., Soimasuo, M., Kaurola, J., Oikari, A. & Karjalainen, J. 2002. The effects of increasing UV-B radiation on pigmentation, growth and survival of coregonid embryos and larvae. Environmental Biology of Fishes 64: 451-459.

II Häkkinen, J., Vehniäinen, E. & Oikari, A. 2003. Histopathological responses of newly hatched larvae of whitefish (Coregonus lavaretus s.l.) to UV-B induced toxicity of retene. Aquatic Toxicology 63: 159-171.

III Vehniäinen, E., Häkkinen, J. & Oikari, A.O.J. 2003. Photoinduced lethal and sublethal toxicity of retene, a resin acid derived PAH, to coregonid larvae. Environmental Toxicology and Chemistry 22: 66-71.

IV Häkkinen, J., Korhonen, H., Oikari, A. & Karjalainen, J. 2003. Melanin concentrations in vendace and whitefish larvae in five Finnish lakes with different optical properties. Boreal Environmental Research 8: 193-201.

V Häkkinen J, Vehniäinen, E. & Oikari, A. Sensitivity of northern pike larvae to UV-B and UV-photoinduced toxicity of retene. Submitted manuscript (Aquatic Toxicology).

VI Häkkinen, J. & Oikari, A. A field methodology to study effects of UV radiation on fish larvae. Submitted manuscript (Water Research).

In addition, some unpublished data are presented.

ABBREVIATIONS

A_{500}	Absorbance of 500 nanometers
CDOM	colored dissolved organic matter
CIE	(Commission Internationale de l'Eclairage; International Commission on Illumination) i.e., the action spectrum specific for human erythema
CYP1A	cytochrome P4501A
dw	dry weight
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
DOC	dissolved organic carbon
HE	haematoxylin eosin
HSP70	heat shock protein 70
LC50	lethal concentration causing 50 % lethality
MAA	mycosporine like aminoacids
MS-GC	mass spectrometry-gas chromatography
PAH	polycyclic aromatic hydrocarbon
PAR	photosynthetically active radiation
PAS	periodic acid schiff
PCB	polychlorinated biphenyl
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
SDS-PAGE	sodiumdodecyl sulphate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
UV	ultraviolet radiation
UV-A	ultraviolet-A radiation (315-400 nm)
UV-B	ultraviolet-B radiation (280-315 nm)
UV-C	ultraviolet-C radiation (200-280 nm)

1 INTRODUCTION

Climatic factors affecting UV-B exposure

Sunlight consists of ultraviolet radiation, visible light and infrared light. UV radiation is divided into three types according to wavebands: Ultraviolet C (UV-C, 200-280 nm), ultraviolet B (UV-B, 280-315 nm) and ultraviolet-A (UV-A, 315-400 nm). The biologically most harmful UV-C is completely absorbed in the upper atmosphere. However, UV-B is only partially attenuated by the stratospheric ozone layer, and UV-A reaches the earth surface almost completely (Madronich et al. 1995). Fortunately UV-A is even 10^4 times less biologically active than UV-B (deGruijl et al. 1993). However, the importance of UV-A in melanoma induction, especially in humans, should not be forgotten (Setlow et al. 1993). Long-term data series on solar UV-B radiation incident at the earth's surface indicate that, over the past 10-20 years, ultraviolet-B levels have increased due to anthropogenically caused stratospheric ozone depletion (Stolarski et al. 1992, Kerr & McElroy 1993, Madronich et al. 1995, Taalas et al. 2000). Related to climatic change, even 20-50 % increases in CIE-weighted doses have been predicted in northern latitudes in coming decades (Taalas et al. 1996, 2000, 2002).

Several factors, besides stratospheric ozone depletion, affect the intensity of UV-B radiation reaching the biosphere including altitude, surface albedo, cloudiness, aerosols and snow cover, as well as solar zenith affected by latitude, season and time of the day (Madronich 1993, Taalas et al. 1996, Madronich et al. 1998). Altitude affects UV-B irradiance considerably (over 10 % increase per 1000 m) (Blumthaler et al. 1997). Small particles suspended in air, i.e. aerosols, can reduce the irradiances of UV-B coming to the earth's surface (Madronich et al. 1998). Clouds usually reduce the irradiance, but it is well known that clouds may also enhance UV irradiances due to reflection of solar radiation at clouds (Mims & Frederick 1994). Highest UV-B radiation can be measured during midsummer and midday in equatorial regions with high solar elevation. The UV-B doses in equatorial regions are several fold higher than in Finland (Taalas 2002). However, the species living under the sun of the equatorial region are well and differently adapted to high UV-B doses (Häder et al. 1998). The

possible problems arise in polar region and mid-latitudes if ongoing ozone depletion exposes the nature to higher UV-B dose levels than organisms are adapted.

UV in natural waters

In northern boreal latitudes (approx. 60-70 °N) lakes and rivers are covered most of the year by ice, which almost totally eliminates the UV-B radiation from the water (Belzile et al. 2001, Schubert et al. 2001). In May, after the ice is shed, the waters in many boreal lakes appear clearer than one or two weeks thereafter, after the onset of spring primary production. This allows deeper penetration of UV-B to the water column (Huovinen et al. 2000), at just the time of spawning and/or hatching of boreal fishes. Furthermore, the ozone depletion is situated in springtime (Taalas et al. 2002). For all of these reasons, it can be hypothesized that this timeframe is the most important when evaluating the effects of UV-radiation to boreal freshwater ecosystems, including the important species making up the largest value of fisheries therein.

Natural levels of UV-B radiation are known to penetrate to biologically significant depths in many bodies of water (Smith & Baker 1979, Smith et al. 1992, Kirk 1994, Morris et al. 1995). The water depth required to remove 90-99 % of the solar radiation at 310 nm depends on the optical quality of water. In the clear oceans, UV-B can penetrate down to 20 metres (Smith & Baker 1979, Kirk 1994). In lakes, attenuation depths for ultraviolet radiation may range from a few centimeters in waters having high concentrations of dissolved organic carbon (DOC) to over 10 m or more in some of the lowest DOC lakes (Kirk 1994, Morris et al. 1995, Williamson et al. 1996, Huovinen et al. 2000). In the clearest lakes in Finland, UV-B radiation is known to penetrate deeper than 1 metre (Huovinen et al. 2000). Increases in UV-B radiation due to ozone depletion alter the spectral balance of UV-A, UV-B and photosynthetically active radiation (PAR; > 400 nm) and increase the exposure of aquatic ecosystems to UV radiation (Smith et al. 1992). Further, changes in UV-B may influence other environmental factors like acidification, precipitation and temperature changes. All these in combination, decrease the concentrations of dissolved organic carbon (DOC) and colored dissolved organic matter (CDOM), mainly controlling the penetration of the solar radiation in marine and freshwaters (Kirk 1994, Schindler et al. 1996, Williamson et al. 1996, Yan et al. 1996).

Impacts of UV-B to aquatic organisms

UV-B radiation, even at current levels, is known to be harmful to organisms and reduce the productivity of ecosystems, including aquatic environments (Häder et al. 1998). UV-B can directly or through oxidative stress cause DNA and macromolecular damage, leading to sublethal or lethal responses (Ahmed & Setlow 1993, Jurkiewicz & Buettner 1994, Renzing et al. 1996, Charron et al. 2000). It has been suggested that UV-B radiation is one possible stress factor explaining worldwide extinctions of amphibian species (Blaustein et al. 1997,

1998). Previous studies with numerous fish species have shown that UV-B radiation can be detrimental to fish, especially at the embryo and larval stages. The most severe effects of UV-B radiation impair larval development and decrease offspring recruitment (Hunter et al. 1979, Williamson et al. 1997, Beland et al. 1999, Flamarique & Harrower 1999, Battini et al. 2000, Browman et al. 2000). Besides direct lethality, several fish species are susceptible to UV-B-induced sunburn (Berghahn et al. 1993, Little & Fabacher 1994, Blazer et al. 1997), reduced growth rate, axial malformations and eye or brain damage (Hunter et al. 1979, Dethlefsen et al. 1996, 2000). Studies with adult fish have shown that UV radiation is an immunosuppressive agent to roach a benthic fish (Salo et al. 1998, 2000a, 2000b, Jokinen et al. 2001).

Protective mechanisms against UV-B

Fish have physiological, morphological and behavioral mechanisms of photoprotection, including screening substances like mycosporine-like amino acids (MAA´s) (Shick & Dunlap 2002) and melanin (Ahmed & Setlow 1993, Lowe & Goodman-Lowe 1996, Speekmann et al. 2000), inducible photorepairing, and photoindependent excision repair (Shima & Setlow 1984, Applegate & Ley 1988, Ahmed & Setlow 1993). Further in some species capability of visually avoiding high UV irradiances has been suggested (Speekmann et al. 2000, Kelly & Bothwell 2002, Ylönen et al. 2003).

Besides protective mechanisms, another important, but usually forgotten, factor that possibly limits the exposure of organism to UV-B radiation is the phenology of species. Merilä et al. (2000) postulated that the cumulative UV-B exposure of aquatic embryos at high latitudes might even exceed that at lower latitudes, having an impact because of lower ambient temperatures and hence slower embryonic development. However, Cummins (2003) showed that this theory was too simplified, and in common frog (*Rana temporaria*) embryos in populations at high-latitude undergo more rapid ontogenetic development than those from lower latitudes, at a given temperature. Further, due to possible global warming the ice cover will be shed earlier, leading to exposure in more sensitive developmental stages. On the other hand, there are also indications that some species have a tendency towards earlier breeding (Terhivuo 1988), and in that case leading to lower UV exposure earlier in the spring.

Combined effects of UV-B with xenobiotics

UV-B can also have harmful interactions with other environmental factors; the enhanced toxicity of environmental contaminants or even synergism with pathogens (Ankley et al. 1994, Kiesecker & Blaustein 1995). Many polycyclic aromatic hydrocarbons (PAHs), being relatively non-toxic as such, however, reveal enhanced toxicity because of UV-induced structural changes of the chemical (photomodification) or through photosensitization, caused by activation of chemicals already bioaccumulated in tissues (Ankley et al. 1994, Mallakin et al. 1999, Little et al. 2000). Photoinduced toxicity of individual PAH-

compounds (e.g. fluoranthene, anthracene, pyrene) has been documented in plants (Huang et al. 1995, Mallakin et al. 1999), zooplankton (Wernesson & Dave 1997, Nikkilä et al. 1999), crustaceans (Boese et al. 1997), amphibians (Hatch & Burton 1998, Monson et al. 1999) and fishes (Oris & Giesy 1985, McCloskey & Oris, 1991, 1993, Little et al. 2000).

Retene (7-isopropyl-1-methylphenanthrene) is a PAH-compound formed from resin compounds either during incomplete combustion of resinuous softwood or via action of anaerobic microbes (Ramdahl 1983, Tavendale et al. 1997). Retene is also formed thermally during forest fires (after burning, soil may contain over 4 µg retene/g d.w.) (Gabos et al. 2001) and municipal incinerators and oil refineries have small amounts of retene in their effluents (Besombes et al. 2001). In natural waters, retene is mainly formed anaerobically from resin acids, oleoresinous constituents of coniferous trees. It has been found in sedimenting particles (highest observed concentration 54 µg/g d.w.) contaminated by treated pulp and paper mill effluents as well as in the sediment surface up to 500-1600 µg/g in lake areas contaminated by the industry (Leppänen & Oikari 1999, Leppänen et al. 2000). Dissolution from contaminated sediments also occurs (Oikari et al. 2001). As a natural product, it can be found in sediments, though at lesser concentrations (Bouloubassi et al. 1997, Judd et al. 1998).

Although retene is hydrophobic by its nature, it is bioavailable to fish, presumably via desorption from sediments (Oikari et al. 2002a). In laboratory exposures, retene appears to accumulate in liver and muscles of fish (Brumley et al. 1997). Chronic exposures have shown that retene is teratogenic to fish embryos at low water concentrations (~ 32 µg/l or below) and induces the mixed function oxygenase (MFO) system in fishes (Fragoso et al. 1998, Billiard et al. 1999, 2000). Further, in a previous study, retene was phototoxic to *Daphnia magna* at concentrations revealing no toxicity without UV-B (Huovinen et al. 2001).

Ecologically relevant species

Autumn-spawning vendace and whitefish lay their eggs on lake bottom where they may be exposed to sedimental retene (Oikari et al. 2002a). Once hatched in April-May, the positively phototactic larvae of both species swim near the surface for the first month (Shkorbatov 1966, Viljanen et al. 1995), thus likely to be exposed to episodes of solar UV-B. On the other hand, northern pike spawn in April-May in shallow littoral waters (water depth under 1 metre) and their larvae use these as nurseries for several weeks after hatching (Turner & Mackay 1985, Urho et al. 1989). Thus, during early developmental stages pike larvae can be exposed to episodically high UV-B dose rates at present and probably even more in coming decades.

2 OBJECTIVES

The purpose of this thesis was to evaluate whether or not increased UV-B radiation will be an environmental risk factor for freshwater fishes at current doses or at those predicted in coming decades. Further, for the first time, the risk of UV-B combined with a potentially hazardous PAH-compound for northern fish was evaluated by simulating future scenarios on enhanced UV-B radiation.

For these goals, more specifically, the following tasks were made:

1) To study whether or not UV-B is biologically harmful for larval fish either in subchronic or acute exposures (I, II, III, V, VI).
2) Investigate adaptive mechanisms against effects of UV radiation (I, IV).
3) Evaluate effects of phototoxicity of PAHs, using retene as a model compound (II, III, V).
4) Solve certain mechanistic associations in UV-induced PAH-phototoxicity with histological and biochemical methods (II, III, V).
5) Compare the sensitivity of boreal fish species to UV-B and UV-induced phototoxicity (I, II, III, V, VI).
6) By using fish models, make an initial risk assessment by comparing suggested and observed UV-effects in boreal lake environment.

3 MATERIALS AND METHODS

The materials and methods are described in more detail in the original articles (I-VI).

3.1 Test animals

Three species were studied: vendace (*Coregonus albula* L.), whitefish (*Coregonus lavaretus* s.l.) and Northern pike (*Esox lucius* L.) - each in larval stages (I-VI). Experiments were started with newly hatched larvae, and extended for several days in the presence of yolk in young (II, III, V, VI) or until yolk absorption up to two weeks (I).

3.2 Experimental UV-B exposures

Q-Panel UVB-313 lamps were used to produce the enhancement of UV-B radiation in the laboratory (I, II, III, V) and in field experiments (VI). The main principle was to use relevant UV-B doses currently existing in nature and those predicted to come in the near future. Harmful shorter wavelengths not existing in nature were blocked with a cellulose diacetate filter (Clarifoil), which was replaced after each 3-hour exposure (I, II, III, V). In a two-week experiment (I), in every treatment including control, the UV-A-lamp (Q-Panel UVA-340) and visible light (Philips TLD 36 W/950 daylight) was present. In the control treatment, however, UV-B radiation was blocked with a Mylar-D filter (DuPont). In a series of short-time UV-B experiments with and without retene, the controls were exposed only to visible light (Philips TLD 36 W/950 daylight) (II, III, V).

UV-B dose rates in experiments were measured either with a Hamamatsu Photonic Multichannel Spectral analyser (Model PMA-11), integrating the

wavelength area from 280-380 nm (II, III, V) or with a Macam spectroradiometer (SR 9910), equipped with a long light guide and a planar cosine collector 30 mm in diameter (IV, VI). The UV-B doses were calculated as J/m^2 (CIE-weighted), i.e. the action spectrum used in experiments was specific for human erythema (McKinlay & Diffey 1987). This includes the biologically most harmful shorter wavelengths that the earth receives and can thus be used as a model for biological effects to all organisms. Experimental daily doses were related either to average (I, V) or maximum (II, III) daily doses (CIE-weighted) measured by the Finnish Meteorological Institute at the beginning of May - between 2-8 day, 1998-2000 - in Jokioinen (62°82'N, 23°50'E). The comparisons are presented in Table 1.

3.3 Subchronic exposures

Because long-term condition can be considered as a proper estimate for cumulative sensitivity of animals to UV-B, a two-week laboratory experiment was conducted (I). Newly hatched embryos of whitefish and vendace were collected to the rearing aquaria and randomly divided into four different treatments. Before exposing fish to various conditions the developmental stage of each group of larvae was determined, and total length (mm) as well as wet and dry weights of 50 individuals were measured as starting values for growth determinations. Newly hatched coregonid embryos were exposed in the laboratory in flow-through aquaria to three CIE weighted UV-B dose levels (Table 1) or control light for 3 h between 1200 and 1500 h to simulate midday exposure each day. All treatments and controls were replicated four times, with 100 vendace and 100 whitefish embryos in each aquarium. Larvae were fed four times a day *ad libitum* with *Artemia* nauplii. Water temperature and other rearing conditions were kept constant. Photoperiod was set at 18 h light and 6 h dark. Dead embryos and larvae were removed and counted daily. First sampling for growth determination was made after a one-week of exposure. At the end of the experiment sampling was repeated, and samples were also taken for the isolation of the melanin pigment (I).

TABLE 1 Weighted and unweighted UV-B (280-315 nm) doses used in laboratory exposures, and their comparison to average and maximum daily doses emitted by the sun in May in Southern Finland.

Species	Paper	Exp. Days	CIE-weighted UV-B daily dose (kJ/m^2)	Unweighted daily UV-B dose (kJ/m^2)	Unweighted cumulative UV-B dose (kJ/m^2)	Compared to mean daily UV-B (CIE) dose in May (1.66 kJ/m^2)	Compared to max daily UV-B (CIE) dose on May (3.0 kJ/m^2)
Both coregonids	I	14	1.4	4.2	59	Subambient	Subambient
Both coregonids	I	14	1.8	6.1	85	9 % higher	Subambient
Both coregonids	I	14	2.2	8.2	114	34 % higher	Subambient
Vendace	III	2	2.8	13.0	26	68 % higher	Ambient
Vendace	III	2	5.4	25.0	50	> 200 % higher	80 % higher
Whitefish	II, III	2	2.8	13.0	26	68 % higher	Ambient
Whitefish	II, III	2	5.4	25.0	50	> 200 % higher	80 % higher
Pike	V	2	1.0	3.2	6.4	40 % lower than ambient	Subambient
Pike	V	2	1.8	5.8	11.6	8 % higher	Subambient
Pike	V	2	2.7	9.0	18	63 % higher	Ambient

3.4 Acute (72 h) phototoxicity experiments

In order to study both UV-B mediated phototoxicity of retene and the effects of high, periodically existing UV-B dose rates on boreal fishes at the same time, a series of acute experiments was conducted. The basic concept of the three semistatic phototoxicity experiments with vendace, whitefish and northern pike was the same (II, III, V). First, newly hatched larvae were pre-exposed in aerated water to several retene concentrations (vendace and whitefish exposed to 3.2-100 µg retene/l and pike 3-82 µg retene/l) or control conditions (water and DMSO, used as a carrier,) in Pyrex glass bowls (volume one litre). After a 24-h accumulation period, larvae were further exposed to retene or to controls (test solutions were renewed daily before UV-B irradiation by carefully replacing 60 % of each solutions) and, in addition, irradiated once a day (twice) with either UV-B (CIE-weighted) or visible light for 3 h (between 1200 and 1500 h) to simulate midday exposure. Daily doses were compared either with average or maximum daily doses in early May in Finnish nature (Table 1). All treatments and controls were replicated at least three times, each comprised of 25-40 newly hatched larvae (II, III, V). Photoperiod (no UV light) in the experiment room was adjusted so that it simulated natural daily rhythm. Behavioral responses were monitored four times a day, also during irradiation. Dead larvae were removed and counted daily. Larvae were sampled after 3 d (72 h) 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 ^{o}C and preserved there until processed further.

3.5 Field sampling for melanin determinations

Melanin concentration was analyzed from vendace and whitefish larvae, sampled in May from five lakes with different UV attenuation depth, water color and transparency (IV). During spring 2001 (Konnevesi, Paasivesi, Puruvesi) and 2002 (SW Pyhäjärvi and Pyhäselkä), larval vendace (*Coregonus albula*) and whitefish (*Coregonus lavaretus*) were sampled by Bongo nets from five different lakes in Southern and Central Finland. The lake basins included a range from oligotrophic to mesotrophic (IV).

The spectral irradiance measurements of air and underwater UV-B and UV-A irradiance were conducted between late July-August in 2001 or 2002, depending on the sampling year of the study lakes. The field measurements were carried out around solar noon under clear sky (no cloud cover). Irradiance spectra were measured with a Macam spectroradiometer (SR 9910), equipped with long light guide and a planar cosine collector 30 mm in diameter. Irradiance from 290 to 800 nm was measured in steps of 1 nm at 8-10 water depths. The attenuation depth, i.e. the depth where the UV irradiance (ranging

from 310 to 390 nm, at 10 nm steps) was reduced to 1 % of the irradiance just beneath the surface was calculated. The data for optical quality of the lakes (water color and transparency) were gathered from the databases of the Finnish Regional Environmental Centres, except the data of Lake Pyhäselkä, this being based on the analyses of the Karelian Institute, University of Joensuu (Anna-Liisa Holopainen, unpublished data).

3.6 On-field experimentation

Field UV exposures with pike were conducted in May 2002 in Lake Palosjärvi, Finland (62°03' N, 26°21' E), which is an optically very clear water basin (DOC 4.8 mg/l). Newly hatched larvae (< 48 h) of northern pike were used as test animals (Lake Päijänne stock)(VI).

An experimental dock for on-field exposures to natural light was constructed on an underwater stone 25 m off the shoreline. On the southern side of the dock, the additional UV-source and a rack holding experimental cuvettes were installed (VI). Larval pike were transferred gently to quartz and glass cuvettes that were placed at selected depths (VI) to vary the UV-exposure regime. The quartz cuvette transmitted 93 % of UV-B range, but the glass only ca. 30 %. All treatments were replicated four times, each comprised of 30 newly hatched larvae. Fish were exposed to natural solar UV radiation at two depths (5 and 15 cm, respectively) or in addition to enhanced UV-B level for 3 h on two consecutive days (unweighted UV-B intensity 1.0 W/m^2; CIE-weighted UV-B dose at lake surface between 1100 and 1400 hours was 2.0 $kJ/m^2/3$ h), produced with a Q-panel UV-B 313 lamp. Surveys of the survival of animals and neurobehavioral disorders were made every day, and larvae were sampled for further analyses after 48 h.

Irradiance spectra were measured several times during the day with a Macam spectroradiometer (SR 9910; calibrated with SR903 calibration source and software), equipped with a long light guide and a planar cosine collector 30 mm in diameter. The planar cosine collector was adjusted to depth with the help of a special rack leaned against the dock and having a long adjustable arm that had a centimeter scale in every side. Also underwater UV-B measurements were conducted (VI).

3.7 Biological parameters

3.7.1 Determination of melanin

Melanin concentrations in whole body of whitefish and vendace were examined from larvae that were exposed to different UV-B levels for two weeks (I) as well

as from larvae collected from lakes with different optical properties (IV). Total melanin (eumelanin and pheomelanin) of both species was analyzed spectrophotometrically by a method developed for mammalian hair (Ozeki et al. 1995, 1996). Larvae kept frozen at –20 °C were thawed for extraction. Before homogenisation heads were removed, to yield samples without the melanin eyes contained. For one analysis 2-3 larvae were combined and homogenized in distilled water with an Ultra-Turrax device for three min (20 mg wet tissue/ml). Samples were placed in 10 ml capped test tubes (glass), to which Soluene-350 was added (1.8 ml). After sonication for 5 min, tubes were vortexed and placed in a boiling water bath, first for 30 min, and once cooled and revortexed, for an additional 15 min. Samples were analysed for absorbances at 500 nm (A_{500}). Although melanin does not have a distinct absorption maximum at 500 nm, shorter wavelengths revealed much larger background due to proteins (Ozeki et al. 1995). The A_{500} value was converted to total melanin by referring it to the A_{500} value of solubilized sepia melanin standard (I, IV).

3.7.2 UV-B absorbing substances

Absorptive pigments other than melanin were analyzed from larvae of coregonids with methanol extraction. Pooled samples, 4-5 larvae in each were homogenized, and extracted in 1 ml of methanol (90 % aqueous methanol) at room temperature (Fabacher & Little 1995, Hofer & Mokri 2000). After 2 h of extraction samples were centrifuged for 3 min at 11000 *g*. Absorbance (200-800 nm) of supernatant was scanned with a Beckman DU-640 spectrophotometer.

3.7.3 Bioenergetics *in vivo*

In order to determine possible metabolic cost caused by UV-B radiation, mass-specific oxygen consumption was measured. Daily (24 h) oxygen consumption was measured at 12 °C for the larvae that were irradiated with UV-B (daily dose 1.81 kJ/m^2) for 14 d with appropriate controls. One experiment was carried out with vendace and two experiments with whitefish. An intermittent-flow respirometer (Forstner 1983), with polarographic oxygen sensor (YSI 5750) and three parallel acrylic chambers was used. The oxygen consumption in each chamber was recorded for 15 minutes each hour, for 24 hours consecutively, and the average rate over the whole period extrapolated to an hourly value. Total lengths and wet weights of animals were measured after the experiment. Three levels of metabolic activity were determined: 1) maximum rate, the average of the three highest hourly values during the 24 h experiment, 2) minimum rate (“near” the standard metabolic rate), the average of the three lowest values during the experiment, 3) routine rate, the average of the all the hourly values, excluding three maximum and three minimum values (Forstner 1983).

3.7.4 Histopathology

Sublethal UV-B induced phototoxicity of retene was evaluated by histological endpoints (II). Only fish exposed to the high UV-B level (5.4 kJ/m^2) were used. Randomly sampled larvae (10/treatment) were analyzed for histology, but in the treatment 32 µg retene/l plus UV-B, all nonmoribund specimens (four out of five) were examined. Larvae were fixed for 24 h in 10 % buffered formalin. Once fixed, the samples were dehydrated through a graded series of ethanol solutions up to 100 %, followed by xylene prior to embedding in paraffin. Animals were sectioned longitudinally along the vertical axis using a Leica microtome at 5 µm. Haematoxylin and eosin (HE) stained sections were prepared from each tissue block and examined at x400 and x650 magnifications using an Olympus IX70 stereomicroscope. Histological abnormalities detected in liver and skin were recorded. Lesions and tissue alterations were defined using several slides from each fish. The postcranial dorsal sector of longitudinal sections in larvae was selected for examination of dorsal skin lesions. The severity of skin lesions was classified to four groups depending on the percentage of the dorsal skin that had lesions: 0) healthy skin, 1) minor lesion (≤ 1 %), 2) pronounced (1-5 %) and 3) severe skin lesion (≥5 %). In addition, the number of mucous cells was counted from skin sections. Selected sections were stained, besides HE, also with Periodic Acid Schiff (PAS)-Alcian blue-Mayer´s haematoxylin (Mowry 1968).

3.7.5 CYP1A and HSP70 determinations

Two inducible biomarker proteins, CYP1A and HSP70, were measured as whole body analysis (more details in III, V). Briefly, specimens frozen in liquid nitrogen were homogenized with potassium gluconate buffer (pH 7.8) centrifuged, and the supernatant used for analysis. Total protein concentrations of the supernatants were analysed by the Lowry method (BioRad DC) adapted for 96-well plates (Lowry et al. 1951).

Proteins (100 and 20 µg protein/lane for CYP1A and HSP70, respectively) were run on a SDS-PAGE gel (Laemmli 1970, Towbin et al. 1979, Clegg et al. 1998). The positive control for CYP1A was the liver of β-naphthoflavone-injected juvenile whitefish (3.15 µg protein/lane). HSP70 from bovine brain (Sigma H9976, 32.5 µg protein/lane) was used as a positive control for HSP70. Proteins were transferred to a nitrocellulose membrane, which was then blocked with 9 % non-fat dry milk and probed with 3 µg/ml anti-CYP1A (Mab 1-12-3, kindly provided by Dr. John Stegeman) or 1:3000 anti-HSP70 (MA3-006, Affinity BioReagents, Inc.). After washing, the blot was probed with the secondary antibody, 1:3000 peroxidase labelled anti-mouse IgG (A9044, Sigma). Immunodetection was performed via enhanced chemiluminescence, and Scion Image 4.0.2. software was used for quantification of immunoreactive bands. Different blots were made comparable to each other by calibrating them with positive controls, fixing each positive control at a value of 1.

3.7.6 Superoxide dismutase (SOD) analysis

Superoxide dismutase activity of pike larvae was measured on a whole body basis analysis (V, VI). Three to four fish per sample were homogenized with 0.3 M phosphate buffer, and centrifuged at 600 *g* for 10 min at 4 °C. SOD activity was measured by the method of Ukeda et al. (1999) (SOD-assay kit; Dodindo Molecular Technologies Inc.). The incubation was at room temperature and absorbances were measured after 30 min incubation time.

3.8 Determination of retene concentration and its photoproducts

Retene concentrations in water were analysed with an MS-GC-system (V). After addition of the internal standard (anthracene), the sample was extracted in a Soxhlet-apparatus with hexane. The solvent was evaporated first with Rotavapor and finally with a nitrogen gas stream. Then the residue was dissolved in 1 ml hexane and analysed with an MS-GC-system (HP 6890 GC equipped with a HP 5973 MS detector). Retene concentrations were analysed right before UV-B exposure started and also from waters collected during first 24 h. Further, in order to determine possible photomodification products, water containing retene (100 µg/l) was irradiated (3 h, dose 2.7 kJ/m^2) and analyzed right after (V).

4 RESULTS AND DISCUSSION

In boreal latitudes in Fennoscandia, the highest relative increase in UV-B radiation takes place in spring, the time preceding the most intensive reproductive phase of several fishes. As previously mentioned, related to climatic change, even 20-50 % increases in CIE-weighted UV-B doses have been predicted in northern latitudes in coming decades (Taalas et al. 2002). At the first time most UV sensitive developmental stages of fish were investigated in boreal freshwater conditions.

Besides UV alone, in accidental or intentional situations newly hatched larvae of fishes may be exposed to chemical contaminants, for instance to polycyclic aromatic hydrocarbons (PAHs). Several PAHs reveal potential photoinduced toxicity. The purpose of this thesis was to examine, with laboratory and field experiments if UV-B radiation will negatively affect larvae of boreal fishes at current dose and irradiance levels or at those predicted to come. In this connection, therefore, phototoxic effects of PAHs were studied, retene acting as a model compound. Research was conducted with ecologically relevant fish species having also high recreational and commercial value in inland waters.

4.1 UV-radiation in Finnish lakes

The water depth that is required to remove 90-99 % of the solar radiation at 310 nm depends on the optical quality of water, and in humic freshwaters mainly on the concentrations of dissolved organic carbon (DOC) (Kirk 1994, Morris et al. 1995, Bukaveckas & Robbins-Forbes 2000). In the clearest ocean waters UV-B attenuation depths are estimated to be tens of meters (Smith & Baker 1979, Morris et al. 1995) and in many lakes in North America greater than 4 m (Williamson et al. 1996), in Finland in one of the clearest lake basins Lake Puruvesi, the corresponding value for 310 nm is only 0.65 m (Table 2). In clear, low DOC waters particulate material especially phytoplankton is important factor attenuating UV (Kirk 1994).

Many Finnish lakes are considerably humic and Huovinen et al. (2000, 2003) showed a strong negative correlation between UV attenuation depth and DOC. Similarly, there was a clear negative correlation between UV attenuation depth and water color (IV); however, transparency did not correlate either with UV attenuation or water color. In the present research, the measured 1 % UV-B (310 nm) penetration ranged from 11 cm in a very humic lake to 65 cm in the clearest study lake (Table 2, IV), supporting the results of Huovinen et al. (2000, 2003). Similarly, the 1 % penetration depth in the UV-A waveband (380 nm) ranged from 0.3 to 2.3 m (Table 2), being slightly higher than values calculated by Huovinen et al. (2000, 2003).

TABLE 2 The optical properties of the five study lakes (IV). Measured UV attenuation depths (m) for different wavelengths where the UV irradiance is 1 % of the value measured just beneath the surface. The other optical values are from the databases of the Finnish Regional Environmental Centre and Karelian Institute, University of Joensuu.

Lake	Location	Att. depth of UV-B (310 nm)	Att. depth of UV-A (380 nm)	Color mg Pt/l	Transparency (m)	Tro phic status
Puruvesi	61°47'N-62°03'N, 29°17'E-29°48'E	0.65	2.29	5	7.5	oligo
Konnevesi	62°38'N-26°21'E	0.22	0.75	20	3.5	oligo
SW Pyhäjärvi	60°54'N-61°06'N, 22°09'E-22°22'E	0.28	0.92	25	1.8	meso
Paasivesi	62°06'N-62°13'N, 29°17'E-29°31'E	0.18	0.54	40	3.9	meso
Pyhäselkä	62°22'-62°38'N, 29°32'-29°55'E	0.11	0.28	68	2.1	meso

It is evident that increased levels of UV radiation enter to waters because of ozone depletion. Increases in UV-B radiation due to ozone depletion alter the spectral balance of UV-B, UV-A and photosynthetically active radiation (PAR; > 400 nm) and increase the exposure of aquatic ecosystems to harmful shorter wavelengths (Smith et al. 1992). UV-B may interact with acidification, precipitation and temperature changes all of which, in combination, decrease the concentrations of DOC (Kirk 1994, Schindler et al. 1996, Williamson et al. 1996, Yan et al. 1996). It can be concluded that biologically significant amounts of UV penetrate the water column to depths reaching many organisms, in Finnish lakes, particularly in oligotrophic ones.

4.2 Sensitivity of fish larvae to UV-B

Regarding the potential risk of UV-B radiation, many studies have demonstrated threat to aquatic organisms, including numerous fish species. Endpoints include direct lethality of fish embryos, impairment of larval development and decreased offspring recruitment (Hunter et al. 1979, 1981, Beland et al. 1999, Gutiérrez-Rodríguez & Williamson 1999, Battini et al. 2000, Browman et al. 2000, Dethlefsen et al. 2001).

A direct comparison between the results of this thesis and earlier reports is not always possible, because of differences in UV-B experimentations (lamps, filters and UV dosimetry varies). In this work, the CIE weighting function (McKinlay & Diffey 1987) was used, since it helps to harmonize the spectra of UV-lamps and the sun, making them comparable to each other. However, as different weighting functions are not directly comparable, also unweighted doses are presented (Table 1) to improve comparison to other UV studies presenting full spectra. It is biologically most important to realize, when comparing reported spectral doses, that the biological effectiveness of two unweighted UV-B doses are not necessarily equal, since lower wavelengths are much more damaging than higher ones.

4.2.1 Lethal endpoints of UV-radiation

Northern pike

The literature survey reveals, that in light of current knowledge, the observed neurobehavioral syndrome in larval pike is the most sensitive response to UV-B measured in fishes: existing in submaximal UV-B daily doses (Table 1) and leading eventually to death of the larvae (V). In larval pike, the two highest CIE-weighted UV-B daily doses (1.8 and 2.7 kJ/m^2) increased acute mortality only by 10-20 % after a 48 h exposure. Compared to mortality, however, the neurobehavioral disorder was a much more significant response. Under laboratory conditions, the frequency of larvae having symptoms was over 80 % (Fig. 1) even at the lowest dose level (CIE 1.0 kJ/m^2/day; unweighted daily dose 3.2 kJ/m^2). In the studies of Salo et al. (1998, 2000a, b) and Steeger et al. (1999), significant negative results were demonstrated at relatively low UV-B dose levels, however, at higher doses than used in these pike experiments. Steeger et al. (1999) demonstrated lowered vitality of plaice embryos (*Pleuronectes platessa*) at a daily dose of 4.86 kJ/m2. A single dose of UV-B (4.3-5 kJ/m^2 unweighted) was sufficient to cause immunosuppression to adult roach (*Rutilus rutilus*). However, lamps used by Salo et al. (1998, 2000a, b) were unfiltered including biologically more harmful shorter wavelengths. Further, both the intensity and the severity of the observed response in pike was more dramatic than earlier studies, since monitoring of animals with the neurobehavioral syndrome resulted in near complete late mortality (V). This

was confirmed by further studies in 2003 (Vehniäinen et al. unpublished). In relation to sublethality in pike larvae, UV-B radiation downregulated whole body HSP70 concentration, and a connection between the status of HSP70 and neurological disorders was suggested (details in section 4.7.1).

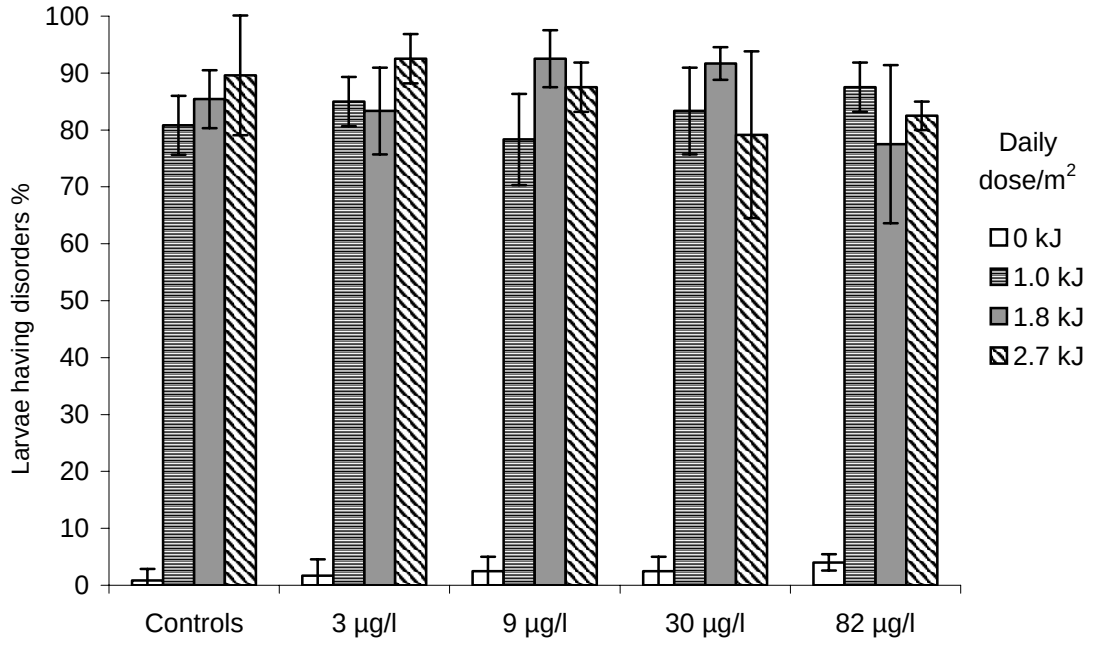

FIGURE 1 The frequency of pike larvae (n = three replicates, 40 larvae in each, however in controls both DMSO and control treatment are combined) suffering from the neurobehavioural disorders in retene and UV-B treatments at the end of 72-hour experiment (V).

Towards environmental realism by experimenting in the field

An absolutely natural spectrum of combined UV- and visible light is impossible to create under standard laboratory conditions. Therefore transfer of experimentation on the field is a necessary step to increase ecological realism of environmental research. In the present work, larval pike were exposed under natural light spectra in the field, in order to assess the impact of current solar UV-B or artificially enhanced UV-B doses on the frequency of lethal disorders (VI). Previously, field experiments with fishes have demonstrated that UV-B can have detrimental effects even at current irradiance level (VI, Kaweewat & Hofer 1997, Williamson et al. 1997, Battini et al. 2000). Nevertheless, there is a considerable lack of *in situ* specific information on the effects of UV-B radiation on fish studied in the field. However, the UV-B effects on a few commercial species such as salmonids (Little & Fabacher 1994, Blazer et al. 1997, Kaweewat & Hofer 1997, Noceda et al. 1997) and plaice *Pleuronectes platessa* (Dethlefsen et al. 1996, Freitag et al. 1998, Steeger et al. 1999) and in particular, cod *Gadus morhua* have been intensively examined both in the laboratory and in field

experiments (Beland et al. 1999, Kouwenberg et al. 1999, Browman et al. 2000, 2003).

In the present study, the environmental dose realism and comparability with laboratory experiments was the main purpose. Therefore UV-B radiation on the surface as well as underwater radiation was directly measured. These measurements revealed that under half of the surface UV-B irradiance might penetrate to a depth of 5 cm (VI). The enhanced UV-B dose rate (CIE weighted 0.9 kJ/m^2/3 h) inside the underwater cylinder in a field experiment (VI) corresponded well with lowest laboratory dose rate (V). As an indication of difference, field experiments revealed that only ca. 20 % of larvae developed neurobehavioral symptoms inside quartz cylinders (while kept at 5 cm depth) in the enhanced UV-B treatment. As UV-A and visible light are needed for photorepairing DNA damage (Applegate & Ley 1988, Ahmed & Setlow 1993) the reason for the observed frequency difference in larvae with neurobehavioral symptom between laboratory and on-field experiment might be lack of UV-A and visible light under laboratory conditions (V).

Vendace and whitefish

Usually early life stages of fishes are sensitive to UV-B radiation and only a few UV-B tolerant fish species exist when exposed at larvae (McFadzen et al. 2000) or juvenile stages (Little & Fabacher 1994, Blazer et al. 1997). Importantly, in coregonids no significant mortality due to UV-B was observed. In fact larvae of vendace and whitefish were highly resistant to both acute (II, III) and subchronic (I) UV-B exposures, even when doses applied were higher than currently exist in nature (Table 1; Fig. 2). UV-B radiation doses up to 2.24 kJ/m^2/day had no lethal effects to larval whitefish or vendace (the survival was > 90 % in all treatments), although the cumulative CIE dose in two-weeks experiment was as high as 31.4 kJ/m^2 (I). Similarly, neither in whitefish nor vendace did UV-B cause significant acute mortality during 48 h experiment, even though daily doses applied were considerable (up to 5.4 kJ/m^2; II, III).

On the other hand, a two-week experiment (Ylönen & Karjalainen 2003) demonstrated 30 and 60 % mortality in whitefish at CIE-weighted doses of 3.65 and 6.15 kJ/m^2/day, respectively. However, when compared to solar UV-B, the cumulative dose in the two-week laboratory exposure was much higher than the difference seen in Fig. 2, because in nature there rarely exist episodes of several days with high UV doses in a row. Further, laboratory exposures took place in shallow water depths (5 cm and 15 cm), with low DOC (2.8 mg/l) and high UV-B penetration (65 % of 310 nm UV-B radiation penetrated to a depth of 15 cm). In nature, instead, even in oligotrophic waters UV-B intensity is more significantly attenuated (Smith & Baker 1979). Overall, this thesis shows that larvae of vendace and whitefish are very UV-B tolerant and, in the near future, we may not expect any dramatic mortality or recruitment failure related to increased UV-B radiation.

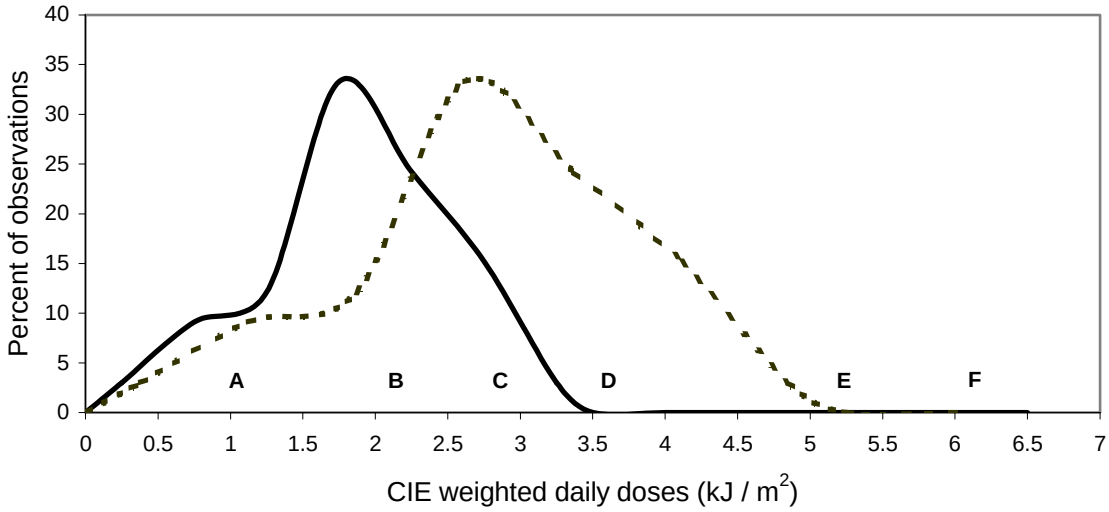

FIGURE 2 Today's (the solid line) and future's (the dotted line) frequency distributions of daily UV-B doses based on measurements made in Southern Finland (Jokioinen, Finnish Meteorological Institute) in May 1998-2000, and the scenarios of future UV-climates suggesting 50 % increases in UV-B doses to the earth (Taalas et al. 2000, 2002). With alphabeticals are denoted the most important biological endpoint responses in fish larvae evoked by experimental UV-B doses (I-V): A) Neurobehavioral disorders in pike both in the laboratory and field. B) Increased pigmentation of vendace and whitefish by ca. 30 % in two weeks. C) UV-radiation increased dramatically the lethality of vendace and whitefish to retene; behavioral disorders also developed. D) UV-B doses causing over 30 % mortality of whitefish in two weeks (Ylönen & Karjalainen 2003). E) UV-photoinduced toxicity of retene showed skin and liver damages in whitefish. F) UV-B doses causing over 60 % mortality of whitefish in two weeks (Ylönen & Karjalainen 2003). Picture redrawn and extended from Oikari et al. (2002b).

4.2.2 Sublethal effects of UV-radiation

Growth and development

UV-B radiation may interfere with the development of larval fish. This can be indicated as impairment of respiratory control (Freitag et al. 1998), reduced growth rate, axial malformations, and eye or brain damage (Hunter et al. 1979, 1981, Dethlefsen et al. 1996, 2001). However, there was no evidence of developmental abnormalities among larvae of either coregonid species (I). Minor growth effects were observed both in vendace and whitefish after a one-week exposure; however, these effects were reversible and exposed larvae caught up with the controls after two weeks, even when they were further exposed (I). Winckler & Fidhiany (1999) demonstrated the effect of UV-A irradiation on the total metabolism of a subtropical cichlid *Cichlasoma nigrofasciatum*, evoking a general metabolic depression in early life stages of this species. In our coregonid larvae, there was a slight, but not statistically significant difference in the average mass-specific maximum, minimum and routine metabolic rates between UV-B irradiated and control larvae (I).

Sunburn reactions

The skin of the healthy larvae of whitefish was uniform and consisted of epidermis of one or two cell layers, lying over the melanophores and dorsal muscle. The skin of the newly hatched whitefish was partly undifferentiated without a distinct dermis zone. Whitefish at this developmental stage have no scales in the skin (II). Many studies have demonstrated that UV-B at high dose rates causes skin lesions and sunburn reactions (Bullock & Coutts 1985, Berghahn et al. 1993, Little & Fabacher 1994, Blazer et al. 1997). Accordingly, minor sunburn reactions, resembling the classical sunburn response (Bullock & Coutts 1985), were seen in larvae exposed to high daily dose of UV-B irradiation (5.4 kJ/m^2). In exposed animals the integrity of the skin was not lost, but the epidermis had a few cells with nuclear droplets or necrotic nuclei in it. It is hypothesized that these so called sunburn cells may be apoptotic (Noceda et al. 1997), but so far there is no direct evidence supporting that hypothesis.

In newly hatched whitefish, differentiation of mucous (goblet) cells could be observed, and their numbers were counted from histological sections targeted to the dorsal side of animals. The suggested functional significance of fish epidermal mucus includes osmoregulation, protection from abrasions, entanglement of particulate materials, defence against pathogens, UV radiation and parasites, reduction of swimming drag or friction, and protection against environmental contaminants (McKim & Lien 2001). In contrast to earlier studies (Little & Fabacher 1994, Blazer et al. 1997, Noceda et al. 1997), larvae of whitefish that were exposed to UV-B irradiation had more mucous cells than controls, but the difference was not statistically significant (II).

4.3 Other effects of UV-B

Effects of enhanced UV-B via trophic interactions on fishes are also possible, although not studied in this thesis. These effects may arise from changes in the planktonic food web changing both availability and quality of food. Protozoa and zooplankton, harvested by larval fishes, are also sensitive to UV radiation (Häder et al. 1998). Further, production of UV-absorbing compounds as a tolerance mechanism takes carbon and energy away from cell growth and these compounds might also be less useful as food for higher trophic levels (Scott et al. 1999). Besides these, UV-B irradiation reduces total lipid content (Arts & Rai 1997), including the polyunsaturated fatty acids (PUFA; Goes et al. 1994, Hessen et al. 1997) of some microalgae. For zooplankton and fish larvae the only source of PUFAs is from diet, since those organisms cannot synthetize these fatty acids. PUFAs are essential to normal development and growth of fish (Reitan et al. 1997, Sargent et al. 1997).

In addition, UV-B is an immunosuppressive agent in adult fish (Salo et al. 1998, 2000a, 2000b, Jokinen et al. 2001). However, it is not yet known whether

this is the case also in fish embryos or larvae, but in studies with larval amphibians UV-B radiation markedly amplifies the impact of pathogens (Kiesecker & Blaustein 1995). Further, for species that spawn near the surface, UV-B may affect sperm quality (Don & Avtalion 1993).

4.4 The healing or damaging UV-A?

Most of UV studies have concerned the harmful effects of UV-B. This is logical because UV-B irradiance coming to earth is continuously increasing. However, Bass & Sistrun (1997) demonstrated that in medaka (*Oryzias latipes*) hatching success decreased in a cumulative manner when exposed to UV-A. Similarly, Winkler & Fidhiany (1999) showed that exposure to UV-A light produced a significant decrease in metabolic rate of a cichlid fish (*Chiclasoma nigrofasciatum*). UV-A can also modulate immune defence of adult roach (Salo et al. 2000b). In addition, it has been demonstrated in fish that UV-A may play a major role in induction of melanomas (Setlow et al. 1993). However, Kouwenberg et al. (1999) found no effects when fish larvae were exposed to UV-A. Usually, UV-A is considered as a necessary factor to organisms exposed to UV-B, because it induces photorepair mechanisms (Shima & Setlow 1984).

4.5 Protective responses against UV-B

Fishes have several protective strategies by which they try to adapt to high UV-B stress. First of all, the screening substances of the skin such as mycosporine-like amino acids (MAAs) and melanin pigmentation protect inner organs from harmful UV radiation by absorbing UV-B (I, Zamzow & Losey 2002). Also other UV-B screening substances have been isolated from the skin of fish (IV, Fabacher & Little 1995) and other aquatic vertebrates (Hofer & Mokri 2000).

As a second line of defence, fish also produce quenching agents such as superoxide dismutase (SOD) that scavenges oxygen radicals induced by UV (Charron et al. 2000). In addition, fish have photoinducible photorepair mechanisms and light-independent excision repair that repair UV induced DNA damage (Shima & Setlow 1984, Applegate & Ley 1988, Ahmed & Setlow 1993). Some species are able behaviorally to avoid high UV-B dose rates or light intensities (Kelly & Bothwell 2002, Ylönen et al. 2003).

In this section the protective role of UV-B screening substances is discussed. The roles of second line defenses i.e. SOD, HSP70 and CYP1A against UV mediated oxidative stress are discussed in more detail in Chapter 4.7.

4.5.1 Melanin pigmentation

The melanin pigment produced in melanocytes has protective functions against sun damage and sunburn in several aquatic organisms (Hobaek & Wolf 1991, Hessen 1996, Cummins et al. 1999). Not only does melanin absorb and scatter light, but it also effectively scavenge reactive oxygen species (ROS) (Sarna et al. 1984, Bustamante et al. 1993). Further, increased melanin content may be connected with decreased numbers of dermal DNA-dimers, caused by UV radiation (Ahmed & Setlow 1993). Laboratory exposures of larval whitefish and vendace to UV-B revealed that exposure to enhanced UV-B radiation induced production of total melanin (I). These color changes involve real alterations in the quantity of pigment within the animal or its integument (Baker et al. 1986). As a difference between species vendace had ca. 50 % more pigmentation than whitefish (I). Compared to controls, larvae of whitefish and vendace that were irradiated with the highest UV-B (2.24 kJ/m^2) dose induced their melanin content significantly, by over 30 % (Fig. 3).

However, the role of melanin to fish is actually controversial. Translucent 1-d-old larvae of *Clupea pallasi* are more sensitive to UV-B than pigmented 7- and 14-d-old larvae (Speekmann et al. 2000). Similarly, studying several *Daphnia* species, Rhode et al. (2001) showed that the extent to which daphnids tried to avoid ultraviolet radiation was inversely associated with their pigmentation. Few studies have suggested, however, that melanin has only negligible protective influence or no influence at all against UV-B radiation (Blazer et al. 1997, Armstrong et al. 2002). As matter of fact, some studies strongly indicate that light absorbed by melanin damages DNA, causing melanomas (Setlow et al. 1993), i.e., there might be a long-term trade-off of a short-term adaptive trait. Coregonid larvae were UV-B tolerant and induced their melanin content (I), these two facts could not be directly linked to each other.

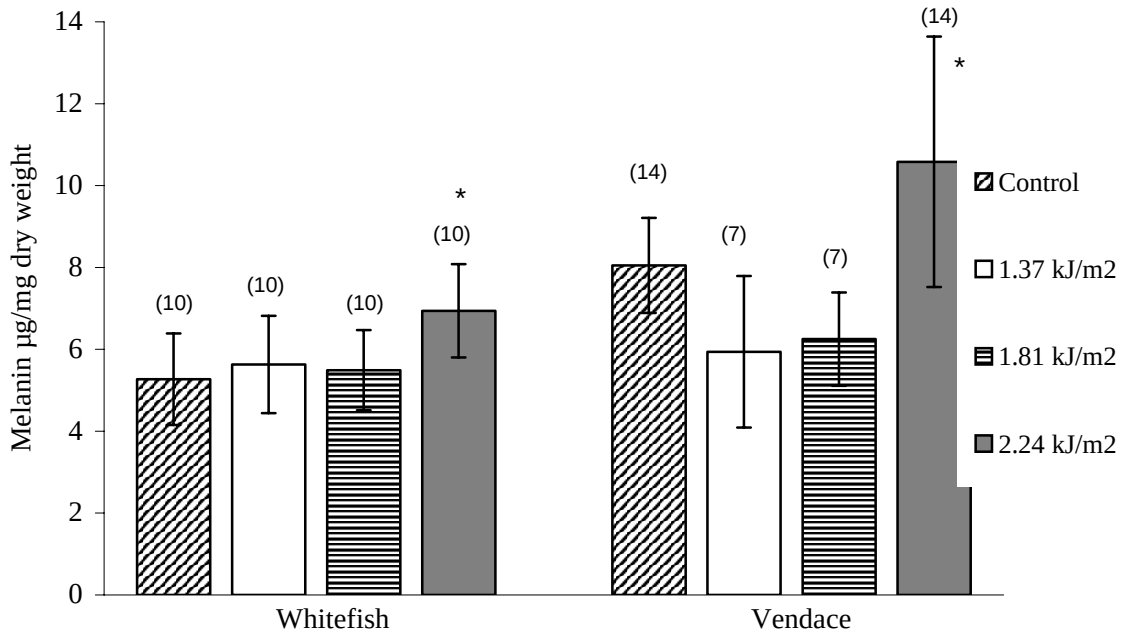

FIGURE 3 Total melanin concentration in whitefish and vendace larvae after two-week exposure to various doses of UV-B radiation. The bar denotes SD and the stars a statistically significant difference ($P < 0.05$) compared to the control. Number of samples analyzed in parentheses, each sample consisting of two (whitefish) or three (vendace) animals.

Melanin as a population marker of lakewater UV-quality?

The objective was to examine if fish pigmentation is associated with the depth of UV-B penetration in lakes or with the color properties of water. Melanin concentration was also analyzed from vendace and whitefish larvae, sampled in May from lakes with different UV attenuation depth, water color, and visibility. When comparing pigmentation of larvae to optical properties of lakes, it can be suggested that UV-B radiation at ambient levels is insufficient to determine the degree of melanin pigmentation of coregonid larvae in Finnish lakes (IV). Actually, the melanin content in larvae correlated negatively with UV-B penetration. However, there was a significant positive correlation between water color and larval pigmentation in both vendace and whitefish (IV). This might be due to adaptation to the color of the background, i.e. to the water color. However, the most heavily pigmented individuals came from the littoral zone of Lake Konnevesi, not from Lake Pyhäselkä where the water was darkest (IV). The number of melanophores may increase or decrease, if fish stay long enough in the same environment with a certain background, depending on the color of that substrate (Latey & Rangneker 1982, Stepien 1987). The actual concentration of melanin may also change, and our results with pelagic vendace and also with whitefish support this hypothesis.

The data allow also comparison between the littoral and pelagic zones in three lakes (IV). In Lake Paasivesi, the vendace larvae from the pelagic zone had ca. 90 % more melanin than those from the littoral zone (*t*-test, $p < 0.05$). In the

other lakes, Puruvesi and Konnevesi, there was no difference in larval melanin concentration between horizontal zones (*t*-test, $p > 0.05$). Thus no consistent difference between horizontal zones was observed.

Further, the comparison between laboratory-acclimated larvae and larvae sampled from nature (both originating from the same lake) provides strong evidence for the influence of environment on pigmentation (IV). One possible explanation for the correlation between pigmentation and water color can be that dark coloration in dark water and a transparent body in clear water protect against visually hunting predators (Hairston 1979). Predation can be an even more powerful selective factor than UV radiation (Hansson 2000). On the other hand, predators may have contrast-increasing mechanisms such as polarization vision, colored ocular filters, offset visual pigments and UV vision (Lythgoe 1984, Bowmaker & Kunz 1987, Loew et. al. 1993, Browman et al. 1994, Shashar et al. 1998, Losey et al. 1999).

In Lake Paasivesi a strong negative correlation was observed between sampling depth and melanin concentration of the vendace larvae (Spearman's correlation coefficient, $r = -0.413$, $p < 0.01$). However, no generalization was possible, because in Lake Puruvesi ($p > 0.05$) there was no correlation. Still, the most heavily pigmented individuals were swimming near the surface both in Lake Paasivesi and Lake Puruvesi, implying color adaptation (IV). Possibly, in lakes Paasivesi and Puruvesi, there is a trade-off between pressures due to predation and UV radiation. This suggestion agrees with the observation of Hansson (2000) that pigmentation of copepods was higher in clear waters than in humic ones; however, in the presence of a fish predator pigmentation of copepods decreased.

4.5.2 UV-B absorbing substances

Another group of protective substances is mycosporine like aminoacids (MAAs) and related gadusols that have been found in numerous organisms (Shick & Dunlap 2002), including many fish species (Zamzow & Losey 2002). MAAs have strong absorbance in the UV-B range and in some cases may act as antioxidants as well (Shick & Dunlap 2002). Although different MAAs were not identified, wavelength scans of methanol-extracted larvae of vendace revealed high absorbance in the UV-B range (peak in 280-282 nm); in contrast no absorbance was demonstrated in whitefish samples in the UV-B range (IV). The observed absorbance peak in vendace was not equal to any known MAAs (Shick & Dunlap 2002). Unknown UV-B absorbing compound, possibly MAA-compounds, was recently found in skin extractions of freshwater fishes and the concentration of the substance correlated with increasing UV-B resistance (Fabacher & Little 1995, Kaweewat & Hofer 1997). It is apparent that, besides melanin, other protective compounds may be important for high UV tolerance of coregonid species.

4.6 UV-B as a risk factor for chemical toxicity

Phototoxicity of PAHs has been demonstrated in a multitude of aquatic species including algae (Gala & Giesy 1992), plants (Huang et al. 1995, Mallakin et al. 1999), zooplankton (Wernesson & Dave 1997, Nikkilä et al. 1999), crustaceans (Boese et al. 1997), amphibians (Hatch & Burton 1998, Monson et al. 1999) and fishes (Oris & Giesy 1985, McCloskey & Oris 1991, 1993, Little et al. 2000). In this thesis, phototoxicity of PAH to boreal fishes was evaluated using retene as a model compound (II, III, V).

4.6.1 Retene as a risk chemical

In natural waters, retene (7-isopropyl-1-methylphenanthrene) is mainly formed via the action of anaerobic microbes from resin acids, oleoresinous constituent in coniferous trees (Tavendale et al. 1997). In lake areas contaminated by treated pulp and paper mill effluents, high retene concentrations have been found in sedimenting particles (highest observed concentration 54 µg/g d.w.) as well as in the sediment surface (up to 500-1600 µg/g - Leppänen & Oikari 1999, Leppänen et al. 2000) (Table 3). Further, preliminary elutriation trials show that sediment retene is dissolved from this matrix (up to 13 µg/l) (Oikari et al. 2001). Besides water as a retene source, it can be accumulated by eggs from the sediment, or by larvae from particulate material (Leppänen et al. 2000, Oikari et al. 2002a). In laboratory exposures retene appears to accumulate in the liver and muscles of fish (Brumley et al. 1997). Further, Leppänen & Oikari (1999) demonstrated that retene could be found in the bile of fish, demonstrating bioavailability.

TABLE 3 Highest measured retene concentrations in Finnish lakes.

Site	Measured from	Highest conc. of retene µg/g d.w.	References
Southern Lake Saimaa	Surface sediment	1600	Leppänen & Oikari 1999
Southern Lake Saimaa	Sedimenting particles	54	Leppänen et al. 2000
Lake Päijänne	Surface sediment	1300	Lahdelma & Oikari 2003
Lake Lievestuoreenjärvi	Subsurface sediment	3300	Leppänen & Oikari 2001
Lake Lievestuoreenjärvi	Surface sediment	100	Leppänen & Oikari 2001

4.6.2 Toxicity of retene alone

Based on results of this thesis, retene was not acutely lethal to boreal fish at water-soluble concentrations (II, III, V). On the other hand, it is known that

retene is teratogenic to fish embryos in subchronic exposures, most distinctly indicated as blue-sac disease, at low water concentrations (~ 32 µg/l or below) (Billiard et al. 1999, 2000). No signs of blue-sac disease were visible neither in whitefish nor vendace exposed for three days posthatch to retene, with or without UV-B. It could be expected that the sensitivity of whitefish larvae would further increase with longer-term exposures. In fact the characteristic effect of retene, without UV-B, to cause symptoms of blue-sac disease (yolk edema) was observable during the latter half of the period from hatch to complete absorption of yolk (Billiard et al. 2000). However, with larval pike 9 d of retene exposure, produced no signs of edema (unpublished data). Biochemical responses to retene are discussed in chapter 4.7.

4.6.3 Synergistic toxicity with UV-B

Many polycyclic aromatic hydrocarbons (PAHs), being relatively non-toxic, may reveal enhanced toxicity because of UV-induced structural changes of the chemical (photomodification) or through photosensitization of animals, the mechanisms caused by activation of chemicals bioaccumulated in tissues (Ankley et al. 1994, Arfsten et al. 1996, Mallakin et al. 1999, Little et al. 2000). In the case of retene, whether the establishment of enhanced phototoxicity requires bioaccumulation and activation inside tissues by UV or just photomodification externally, is not well known. However, paper V demonstrated that after retene was exposed to UV-B two unknown photoproducts appeared as concentration of retene decreased (Fig. 4). This indicates that retene-sourced UV-B phototoxicity (II, III) includes chemical photomodification of precursor retene. However, photosensitization in tissues could not be excluded. In studies with *Daphnia magna*, accumulation of retene in animal before UV exposure was essential for induced toxicity (Huovinen et al. 2001).

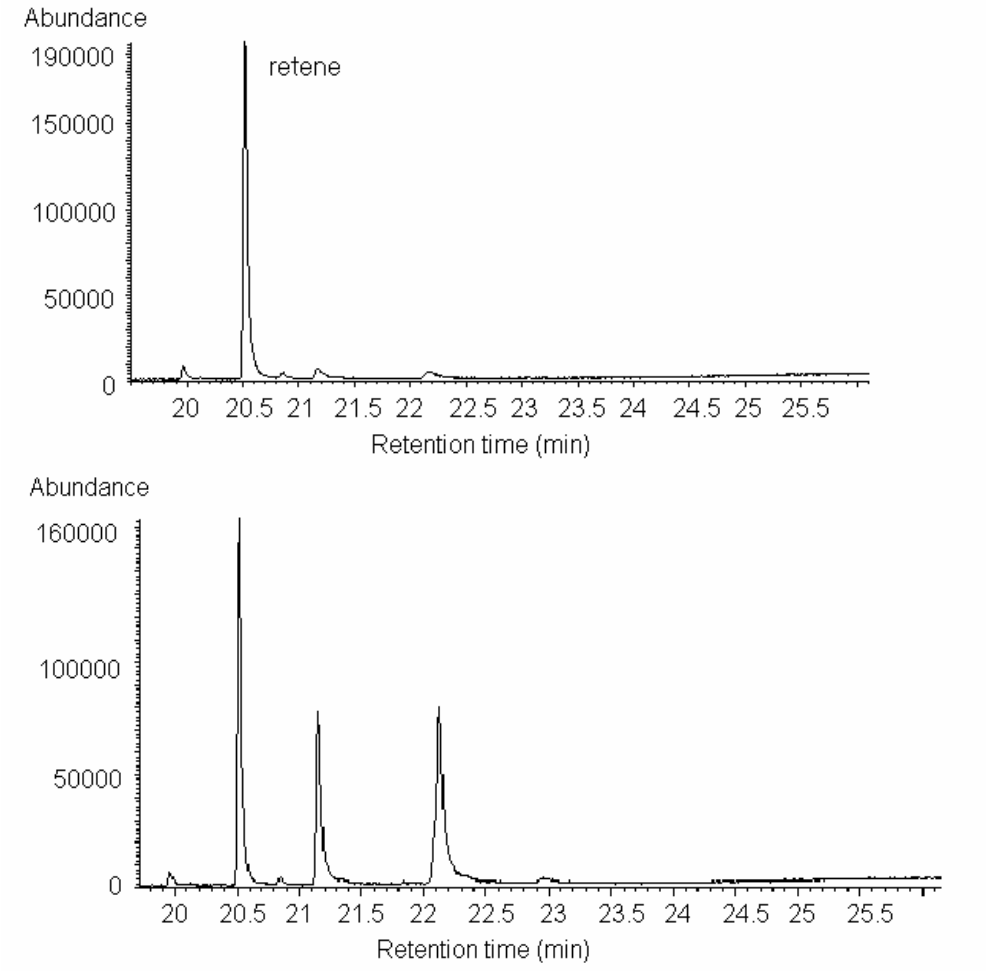

FIGURE 4 Appearance of two photomodification products of retene after exposure to UV-B radiation (analysed with MS-GC system). Retene in water at concentration 100 µg/l was irradiated for 3 h by 2.7 kJ/m^2. Retention times of photoproducts were 21.15 and 22.12 min., but no chemical identification was found in the MS-library (Wiley 275).

Lethality

Whereas retene alone was not acutely toxic to larval coregonids, simultaneous UV-B and retene exposure raised toxicity of retene by 2-3 orders of magnitude (II, III), as retene was acting as the precursor. Both in vendace and whitefish, dramatic mortality in low retene (µg/l) concentrations was demonstrated (Fig. 5). The LC50 of retene as a precursor was 41 µg/l for vendace and, depending on UV dose, 15-16 µg/l for whitefish (III). In pike no retene-mediated phototoxicity was observed (V), even though UV-B doses applied as well as retene concentrations were comparable with coregonid studies (II, III). Except experiments in this thesis (V), there are only a few reports where phototoxicity of PAH has not been taken place (McConkey et al. 1997, Verrhiest et al. 2001).

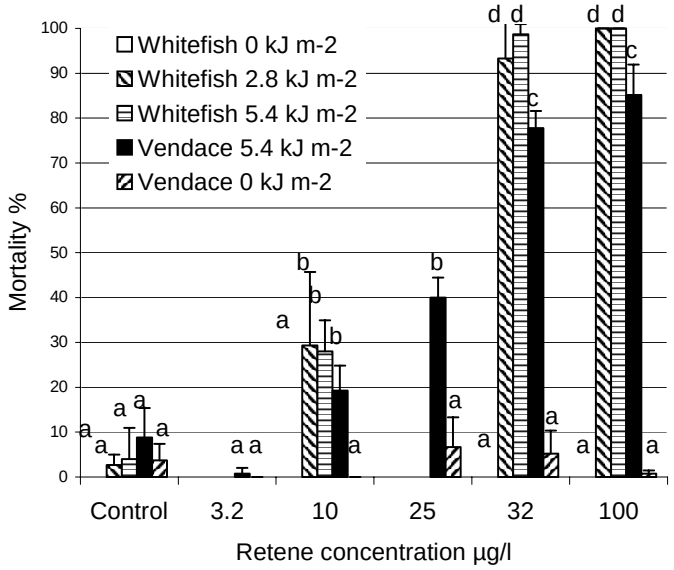

FIGURE 5 Retene-based lethality of newly hatched vendace and whitefish. After a 24-h accumulation period, larvae were irradiated once a day (altogether twice) with either UV-B (2.8 or 5.4 kJ/m^2) or visible light for 3 h. Bar gives standard deviation. Groups denoted by the same letter do not differ significantly from each other ($p \geq 0.05$, Tukey).

There are several possible reasons for this difference between species. One reason might be behavioral as coregonids swim continuously whereas pike larvae hold onto the substrate (plants most of the time) and therefore require less oxygen than coregonids. Another possibility is that larvae of whitefish and vendace have more efficient PAH metabolism, indicated as higher CYP1A levels, than larvae of pike. Retene is readily excreted to fish bile (Leppänen et al. 2000), which in turn can reduce concentrations interacting with UV-B (Ankley et al. 1994). However, it might be that the metabolites of retene are more toxic than the parent compound or that the metabolites are activated by UV light. In my work, pike's pigmentation was not studied as extensive as in coregonids, and it is possible that the mucus in skin of pike gives protection against the photomodification products of retene.

Behavioral response

The first sign of coregonid larvae having problems was the change in behavior of whitefish and vendace larvae (II, III), an observation, which can be related to respiratory stress. Retene caused clear signs of irritation and an apparent hypoxia during simultaneous UV-B exposure. Hypoxia was suggested by a tendency to swim to the water-air interface. The symptoms were observed during the first irradiation and immediately thereafter. Later, fish expressed uncontrolled spiral swimming and had tremors in the cranial area. The most severely affected fish remained at the bottom of the bowl. Practically all larvae had these symptoms in the highest retene concentration (100 µg/l), as did most fish in the other retene concentrations. After the first UV-B exposure, however,

the response in coregonids was reversible and most larvae recovered within 5-6 h. After the second irradiation next day, these symptoms recurred, but this time they were irreversible and the larvae died. Coregonid larvae exposed to retene or UV-B alone had no behavioral symptoms (I). Similar to the results of this thesis, increased ventilation rate and coughing were also noted in juvenile sunfish (*Lepomis macrochirus*) (Oris & Giesy 1985) when exposed to PAH and UV in combination.

In pike larvae combined UV and PAH exposures had no additive effect to neurobehavioral symptoms caused by UV-B alone. UV-B alone had so dramatic effects to larvae of pike that any combined effects would surely be masked.

Histopathological observations

Overall, our results also support the suggestion (Oris & Giesy 1985) that the primary site of phototoxic action is at the respiratory or skin surface, where UV exposure is maximal. In the case of larval whitefish, the mechanism of UV-induced phototoxicity of retene appears to be the disruption of integrity of the skin epithelium - indicated as histopathological alterations - of larvae that rely on skin respiration in early development (II). Observations showed structural phototoxicity of retene induced by simultaneous UV-B, causing extensive and multiple skin damage in three-d-old posthatch larvae of whitefish (II) (Fig. 6). Supporting earlier UV studies with histological analysis (Bullock & Coutts 1985, Berghahn et al. 1993, Noceda et al. 1997), some of the lesions resembled the classical sunburn response, appearing as foci of granular nuclei, sloughing and vacuolization of the skin of larvae (II). Cellular damage - mainly in the gills - caused by oxidative stress, has been suggested to lead to PAH induced phototoxicity in fish (Oris & Giesy 1985, 1987, McCloskey & Oris 1993, Weinstein et al. 1997, Choi & Oris 2000). Because of direct exposure to UV, skin can be the primary target organ in aqueous exposures of larval fish that utilize skin respiration during early development (McKim & Lien 2001).

Changes in dermal mucous cells were also monitored (II), their structural elements considered as protective in the skin against UV + retene exposure. In this study the number of mucous cells increased significantly after simultaneous retene and UV treatments (II), as it did after sole UV-B exposure. Somewhat surprisingly, the result was opposite to previous studies indicating decreased density of mucous cells after exposure to UV-B (Little & Fabacher 1994, Blazer et al. 1997, Noceda et al. 1997).

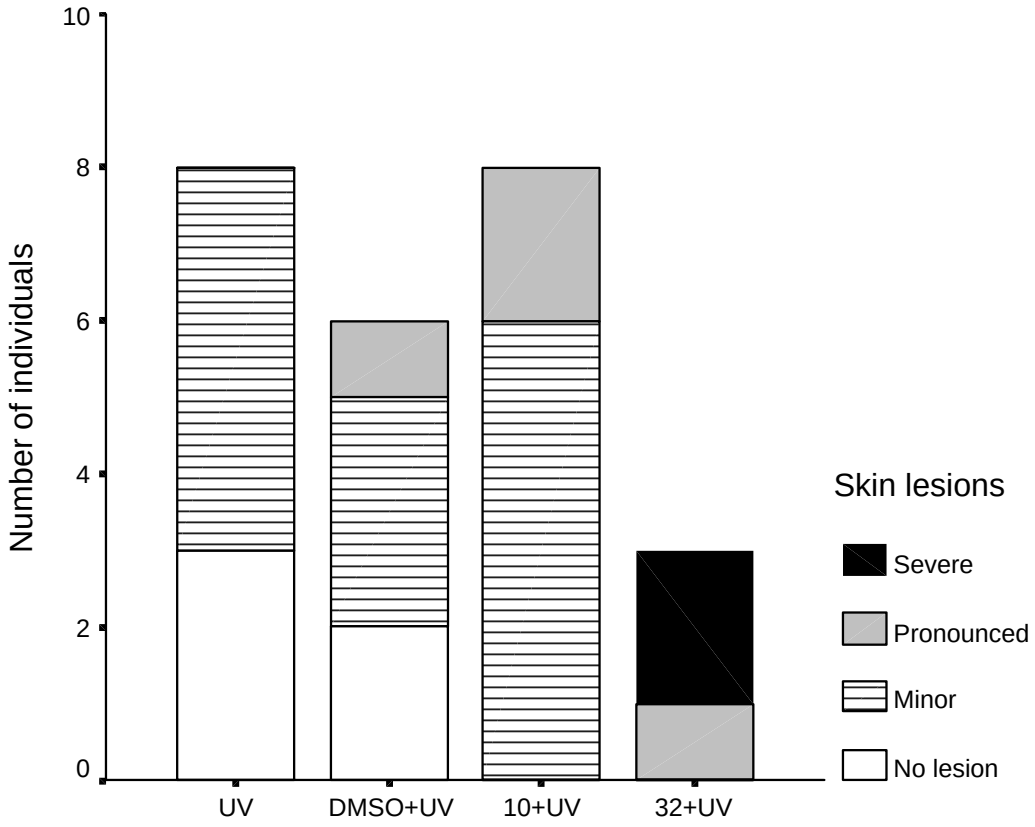

FIGURE 6 The number and severity of skin lesions observed in different UV-B plus retene treatments. The severity of skin lesions was classified to four groups, depending on how large percentage of the dorsal skin was affected (see Materials and Methods 3.7.4).

UV-B irradiated retene was also acutely hepatotoxic to young whitefish. The newly hatched larvae, exposed to 10 µg/l or 32 µg/l retene with UV-B, showed hepatocytes with necrotic nuclei (II). The mechanism of retene-induced phototoxicity in the whitefish liver may be oxidative stress. Choi & Oris (2000) using fish liver microsomes *in vitro*, obtained evidence that lipid peroxidation is an important mechanism in PAH phototoxicity. Liver is a potential target organ due to its large blood supply and great metabolic capacity (Hinton et al. 2001) and retene is known to accumulate in the liver of fish (Brumley et al. 1997). Therefore the hepatotoxicity in larval whitefish is not surprising. Further, the existence of an apparently remote site of action, the liver, is not necessarily unexpected due to circulatory transfer and possible accumulation of retene's photo-oxidation products originally formed in ambient water or in the skin. Fish skin observations suggest that UV affects fish and their reactions to photoproducts, but toxic compounds could be formed outside as well (Fig. 4). In all, it is not clear if the hepatotoxic agent had been created outside or inside the fish and transferred to their liver.

4.7 Sublethal biomarker responses to UV-B and retene alone and in combination

In order to test the concept of early warning of biochemical responses in relation to lethality to posthatch stages of boreal fishes, we examined the effects of UV-B and retene alone and together on the levels of CYP1A and HSP70 (III, V, VI). Further, in pike the activity of SOD was determined, acting as protection against oxidative stress and indicating indirectly the formation of the superoxide anion (V, VI).

4.7.1 Stress protein HSP70

The heat shock protein 70 (HSP70) is responsible for correct folding of cell proteins under normal or stressful conditions (Beckmann et al. 1990, Gething & Sambrook 1992). HSP70 expression has been demonstrated in numerous organs of fish (Dyer et al. 1991). Whole body HSP70 concentrations were determined, since it is induced by exposures revealing oxidative stress, including UV-B (Sanders 1993). In this work, pronounced differences between species were observed when stress protein (HSP70) reactions to different stressors were compared (III, V) (Table 4).

In pike both UV-B and retene decreased HSP70 concentrations significantly in a dose related manner (V). Surprisingly simultaneous UV-B and retene exposure tended to increase HSP70 concentrations when compared to UV controls, although not statistically (V). However, in vendace there was no observed effects on HSP70 levels by UV-B, retene alone or together with UV-B. On the other hand, in whitefish both UV-B radiation and retene alone increased HSP70 concentration, however, without any significant additive effects of simultaneous exposure (III). In whitefish, it can thus be suggested that HSP70 acts as an early warning signal of effect due to exposure of UV-B or retene alone, and can be considered as one protective mechanism helping to tolerate high UV-B irradiance. It seems, however, that this sublethal response cannot be used as a specific biomarker of combined retene and UV-B exposure.

TABLE 4 HSP70 Stress protein responses (compared to controls) in fish species exposed to different stressors (III, V, VI): + symbol indicates a positive response ($p < 0.05$), - symbol a negative response ($p < 0.05$) and ± no response was observed. In the case of combined UV plus retene exposures the additive responses are presented in parenthesis, indicated as symbols mentioned above.

Species	Type of exposure			
	UV-B	Retene	UV-B+retene	UV exposure in field
Vendace	±	±	± (±)	Not exposed
Whitefish	+	+	+ (+, but $p > 0.05$)	Not exposed
Pike	-	-	± (+, but $p > 0.05$)	±

In pike a possible causal relationship with decreased HSP70 and observed neurobehavioral disorders was proposed (V). It is well known that HSP70 give protection against programmed cell death i.e. apoptosis (Sharp et al. 1999, Beere & Green 2001, Weber & Janz 2001). While UV-B exposure in northern pike led to neurobehavioral disorders and late mortality with decreased HSP70 concentration, earlier studies have demonstrated that UV-B can be detrimental to fish larvae causing brain lesions (Hunter et al. 1979). As HSP70 may be high in nervous tissue, especially in the central nervous system, we suggest that apoptotic cells in nervous tissues are related to decreased HSP70 levels. Supporting this idea, adult northern pike exposed to the high mercury (neurotoxicant) had lower HSP70 levels in their tissues (Duffy et al. 1999). However, UV-B radiation had no influence on HSP70 levels in our in the field experiment (Table 4). The apparent reason for the difference is that highest measured UV-B dose rate in on-field experiment corresponded with the lowest dose rate in the laboratory study.

However, the same neurotoxicological explanation for a significant drop of HSP70 levels is not directly valid with retene, since it did not cause mortality or neurobehavioral disorders in larval pike. However, HSP70 was assayed as total body analysis. One option is that retene-mediated oxidative stress, while induces cytochrome P450 system (CYP1A) in the liver, revealed by a negative correlation between HSP70 and CYP1A concentrations (Weber et al. 2001). An alternative explanation for the observation, retene and UV-B both decreasing the amount of HSP70, is that larval pike rely on some other protective mechanism.

While UV-B and retene abolished whole body HSP70 in pike, in contrast to coregonids, it is most difficult to explain why simultaneous UV and retene exposure in pike did not decrease HSP70 concentrations even more. In pike, retene also had no additive influence on the mortality or behavioral disorders caused by UV-B. However, clear photomodification of retene in water was observed and slight HSP70 induction might be a response to these unknown substances. On the other hand, in whitefish it was possible that when animals were stressed either with UV-B or retene alone HSP70 induction was maximal without any further capacity for induction by an additional stress. Vendace possibly relies on some other protective mechanisms.

4.7.2 Cytochrome P450 (CYP1A)

The majority of phase I detoxification reactions, involving unmasking or adding reactive functional groups, are catalyzed by microsomal monooxygenase enzymes (Goeptar et al. 1995). Induction of cytochrome P450 enzyme is probably the best-studied biomarker for environmental pollution in aquatic ecosystems (Goksøyr & Förlin 1992, Van der Oost et al. 2003). The content and activity of induced CYPIA are related to levels of PAHs in a dose-dependent manner (Stegeman & Hahn 1994).

In my thesis, CYP1A concentration in larval fishes was analyzed because of a possible link between the CYP enzyme system and oxidative stress. When

oxidizing its substrate, the CYP system loses electrons, which in turn results in ROS formation (Klotz et al. 1984, Choi & Oris 2000). Retene strongly induces the CYP system in fishes (Fragoso et al. 1998, Billiard et al. 1999, 2000). Supporting recent studies, retene evoked a substantial induction of CYP1A in all studied species and can be considered as a biomarker of exposure to retene even at larval stages (III, V) (Table 5).

TABLE 5 CYP1A induction (compared to controls) in fish species exposed to different stressors (III, V, VI): + symbol indicates a positive response ($p < 0.05$), - symbol a negative response ($p < 0.05$) and ± no response was observed. In the case of combined UV plus retene exposures the additive responses are presented in parenthesis, indicated as symbols mentioned above.

Species	Type of exposure			
	UV-B	Retene	UV-B+retene	UV exposure in field
Vendace	±	+	+ (±)	Not exposed
Whitefish	+	+	+ (±)	Not exposed
Pike	±	+	+ (±)	Not measurable

UV-B radiation induces CYP1A gene expression in mammalian cells (Goerz et al. 1983) and even in human skin UV-B radiation can induce CYP in a dose related manner (Afaq & Mukhar 2001). Irradiation of the amino acid tryptophan with UV causes the formation of formulated indolocarbazoles, which are very potent Ah-receptor agonists (Wei et al. 1999). Although no CYP1A induction by UV-B could be detected in vendace and pike, a slight induction (10 % of the maximum induction detected, $p < 0.05$) was observed in whitefish exposed to the lower UV-B dose (2.8 kJ/m^2). In whitefish larvae exposed to the higher dose (5.4 kJ/m^2), however, no induction by UV-B was detected. It can be presumed 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 histopathological observations (II). Overall, however, the response of CYP1A in animals exposed to combined UV-B + retene equalled the response to exposure to retene alone (III, V).

4.7.3 Superoxide dismutase (SOD)

Oxidative radicals (superoxide anion $O_2^{-\bullet}$, hydrogen peroxide H_2O_2 and the hydroxyl radical $OH^\bullet$) may react with macromolecules, leading to enzyme inactivation, lipid peroxidation and DNA damage (Winston & Di Giulio 1991). As it is plausible that the oxidative stress resulting from formation of reactive oxygen species (ROS) is responsible for the damaging effects of UV-B (Ahmed & Setlow 1993, Jurkiewicz & Buettner 1994, Renzing et al. 1996, Charron et al. 2000) it appears to be an important mechanism also regarding UV-induced phototoxicity of PAHs in aquatic animals (Oris & Giesy 1987, Ankley et al. 1994, Choi & Oris 2000).

The activity of SOD was determined from studies with fish larvae (V, VI), indirectly indicating formation of superoxide anion and oxidative stress (Fridovich 1978, Van der Oost et al. 2003). In the present work, both UV-B and retene increased whole body SOD activity in pike ($p > 0.05$) at low dose levels (V). When compared to controls, the lowest UV-B dose induced SOD activity by 43 %. Supporting the observation in paper (V), Charron et al. (2000) showed the biochemical impact of UV-B on antioxidant status of zebrafish (*Danio rerio*) indicated higher SOD activities. Many xenobiotics have potency for causing oxidative stress and numerous studies have shown that PAHs, PCBs and some pesticides induce SOD activity in fish species (Vig & Nemcsok 1989, Palace et al. 1996, Peters et al. 1996), whereas there are also studies showing no induction (Lemaire et al. 1996, Sole et al. 2000) or even inhibition in SOD activity (Otto et al. 1994, Pedrajas et al. 1995). When compared to the solvent control, retene alone induced SOD activities slightly (24 and 29 %, respectively) in larval pike in low exposure concentrations (9 and 30 µg/l; $p > 0.05$). Instead, in fish that were exposed to higher UV-B doses and retene concentrations the SOD activity was at the level of controls, as it was also when larvae were exposed simultaneously to both stressors. It can be suggested that in higher exposure levels the SOD already had scavenged the superoxide anion together with other mechanisms and was no longer in active form, or the capacity of SOD had run out (V). Another possibility is cellular damage making it unable to respond. Further, when pike were exposed outdoors to ambient or artificially enhanced UV-B levels no induction of SOD levels could be seen (VI), probably because of low underwater dose rates. It can be concluded that relatively large intragroup variability and small relative change make SOD less attractive as a sole biomarker for use regarding ROS stress.

4.8 Relevance of phototoxicity for ecological risk assessment

Interestingly, our results do not follow the trend that sensitivity of species to UV-B alone or UV induced phototoxicity of PAHs correlates with the possibility of the species being exposed to episodes of UV in nature. Species that cannot be exposed to UV-B in real life are known to be very sensitive to photoinduced toxicity of PAHs (McDonald & Chapman 2002) or UV-B alone. This is well demonstrated as immunodeficiency of roach, a benthic fish, due to UV-B (Salo et al. 1998). All three species studied in this thesis have equal opportunity to be exposed to episodes of UV radiation in early life stages. While larvae of coregonids are phototactic, pike spawn in shallow water areas. On the other hand, larval pike are usually attached to vegetation in nature, which offer at least some cover against harmful UV-B irradiance. Larvae of pike were not capable of actively seeking cover from UV-B, when the opportunity was offered under laboratory conditions (Häkkinen unpublished data). However, newly hatched larvae of pike were much more sensitive to UV-B alone than were

whitefish and vendace, whereas strong phototoxicity of PAHs was demonstrated only in coregonids.

Recently McDonald & Chapman (2002) raised the question as how ecologically relevant a phenomenon PAH phototoxicity is and if it could happen in nature. The suggested exposure scenario (II, III, V) can be considered as ecologically relevant to certain fish species, at least on a local scale and in shallow lake areas chemically contaminated by pulp and paper mills, where high concentrations of retene are found in sediments (Leppänen et al. 2000). Further, dramatic phototoxic effects were demonstrated, even at concentrations that were below concentrations likely occurring in the nature. It has to be noted that two days simultaneous exposure to UV-B and retene concentration of 10 µg/l was enough to cause 20-30 % lethality. Of course, when comparing laboratory experiments to nature, it has to be admitted that exposure conditions are extreme: shallow water depth with high UV penetration, organic solvent increasing bioaccumulation rates (Duxbury et al. 1997), no chance to avoid UV or PAH exposure. In nature, several environmental factors, such as dissolved organic carbon (DOC), colored humic substances and light conditions may have a strong influence on photoinduced toxicity of PAHs. DOC, besides reducing the bioavailability of PAHs to fish and other aquatic organisms (Kukkonen et al. 1989, Weinstein & Oris 1999), may strongly reduce the penetration of UV-B radiation to the lake (Williamson et al. 1996, Huovinen et al. 2000). Since most of these uncertainties can be applied to toxicity studies of almost any compounds, rejecting the principle of precaution where phototoxicity studies is concerned, would be unwise.

McDonald & Chapman (2002) suggested there are no direct evidence that PAH phototoxicity have caused problems in field populations, but this does not necessarily have to mean that it is not possible. However, it could be the most difficult to demonstrate, requiring a long-term campaign of field observations that has never been done. Still, the when impact of phototoxicity is local and sudden, how can you tell what was the cause of it? Or we might wonder why in certain species annual recruitments vary without known reason in waters near pulp and paper mills (Hakkari 1992, Karels & Niemi 2002), and not even think of the potency of PAH photoxicity to early life stages. More research is needed, especially under field conditions to resolve the potency of UV induced phototoxicity of PAHs and related effectors to cause harm in boreal lake ecosystems.

4.9 Future's research needs

Whereas this thesis answered to many important questions it also raised new interesting questions and hypotheses to be solved. As it was clearly demonstrated that UV-B had negative influence on pike larvae, however, the mode of UV-B action remained largely unknown. A hypothesis presented in my

thesis proposing the correlations between HSP70 concentration and brain damage both possible correlating with behavioral disorders should and can be studied in the future with immunohistochemical methods from brain sections. More research both in laboratory and field is needed to give answer whether the cumulative UV-B dose or episodically high short-time UV-B irradiance or some mixed type of model of action should to be suggested as the basis for risk assessment. Further, as stated in Oikari et al. (2002b), *in situ* experimentation in the field, including crosstransfer design of experiments between lakes and populations with different characteristics, would increase our knowledge on the margin of safety in boreal lake ecosystems over the current more preliminary assessment of ecological risks.

More contribution is also needed to give answer the question whether the phototoxic actions of retene are due to external chemical photomodification or internal modification of bioaccumulated retene or its metabolites. Further, more research is warranted to specify how the observed differences in capacity of different species to adapt to UV-B exposure can be explained and what is the relative importance of different protective mechanisms. In the future for example the role of DNA-repair mechanisms should be investigated by using ecologically relevant models.

As proposed recently in Oikari et al. (2002b) more detailed scenarios for extreme occasions of UV-climate should be modeled for boreal latitudes. These could include hour, day, week and month timescales, most importantly for May. In addition, campaigns of underwater measurement should be continued in order to spatially estimate and model the exposure risks in different natural waters.

5 CONCLUSIONS

Ongoing ozone depletion has drawn attention to possible harmful effects to organisms in lake ecosystems. In this thesis the aim was to evaluate whether or not increased UV-B radiation is a risk factor for freshwater fishes at current doses or at those predictable for coming decades. Using retene as a model compound, the risk of UV-B alone or combined with a PAH-compound was assessed for boreal fish.

The most remarkable result from this work was the sensitivity of larval pike to UV-B radiation, even at lower doses than currently exist episodically or more commonly in the nature, indicated as severe neurobehavioral disorders leading eventually to death. A correlation between decreased HSP70 concentration and neurological disorders was suggested. Field experiments with pike suggest that only minor increase in ambient UV-B to the earth's surface may cause sublethal effects to larval fish specimens. Fortunately, the frequency of behavioral disorders was lower in the field than under laboratory conditions. However, it can be concluded that in pike the margin of safety is very narrow.

In contrast, the impact of UV-B on both whitefish and vendace is negligible at most, indicated as a minor sunburn reaction of the skin, reversible growth response and increased pigmentation. However, certain biological endpoints such as development and survival of coregonid larvae appear to fall beyond current intensities of UV-B in May, as well as the predicted scenarios in coming decades. In addition, UV-B itself is an insufficient factor to modify the degree of melanin pigmentation of coregonid larvae in Finnish lakes. The risk is even lower than this since natural water in lakes attenuates UV-B efficiently and irradiances in underwater are much lower than in laboratory experiments.

Besides abiotic protection, fish rely on different protective mechanisms that mitigate the harmful effects of UV-B radiation. Obviously, even close relatives such as vendace and whitefish have very different protective mechanisms against radiation stress. Vendace have more efficient absorptive pigmentation that directly prevents UV-B penetration to inner organs. Whitefish rely more on second lines defense such as the repairing effect of

HSP70. In both species the defense probably involves other mechanisms, not yet studied, as well.

Newly hatched larvae of whitefish, vendace and northern pike differed substantially in their reactions to UV-photoinduced toxicity of retene. By itself, retene practically has no toxic effects to the larvae of boreal fishes. Further, retene had no phototoxic effects to larval pike. However, in coregonids combined chemical and UV-B exposures increased the ecotoxicological risks of retene by two orders of magnitude at least. The mode of action behind this phototoxicity in coregonid larvae seems to be related to the functional disruption of mucous cells eventually expressed as respiratory stress. Overall, it can be suggested that for post-hatch larvae of whitefish and vendace the synergism of UV-B and PAHs such as retene can be a key factor of potential ecotoxicological risk to be taken into consideration in lake areas chemically contaminated by the pulp and paper industry or other related sources of PAHs.

Acknowledgements

This study was carried out at the University of Jyväskylä, Department of Biological and Environmental Sciences during the years 2000-2003. I am grateful to this institute to providing excellent working facilities.

Naturally, my special thanks belong to my supervisor, Professor Aimo Oikari. First of all, he gave me the opportunity to do my thesis. Further, his guidance and support acted a large role during this study. I am also very grateful to my other supervisor Professor Juha Karjalainen. His not just theoretical but also practical contribution at the beginning of our project was very important. Dr. Markus Soimasuo and Dr. Pirjo Huovinen contributed in the planning of the project.

My warmest thanks belong to my colleagues MSc Eeva Vehniäinen and MSc Olli Ylönen for very smooth and supportive cooperation. Especially, I would like to thank Ms. Mervi Koistinen for skillful, humoristic and very careful assistance in conducting experiments and laboratory analysis.

I thank MSc Tuula Sinisalo and MSc Hennamaarit Korhonen for assistance in melanin analyses. In addition, MSc Jami Saarnivaara is acknowledged for assistance in on-field experimentation and MSc Jarno Heikkilä for his contribution in first laboratory experiment. Further, Ms. Kaisa Malmberg, Dr. Heikki Penttilä, Dr. Markus Soimasuo and professor Kalevi Salonen are appreciated for helping with UV measurements. Dr. Tarja Hyötyläinen is acknowledged for MS-GC consultation. I wish to thank Dr. Hannu Huuskonen for obtaining samples from Lake Saimaa and Lake Pyhäjärvi. Finnish Meteorological Institute is thanked for the use of UV radiation data of Jokioinen. This thesis was improved through the critical comments of professor James T. Oris and Dr. Peter M. Chapman.

I would also like to thank my wife Maria for optimisms and encouragement and my family and friends for support during this work. This research was funded by the Academy of Finland (Figare/Solar-project), the Maj and Tor Nessling foundation and the Finnish Graduate School in Environmental Science and Technology.

YHTEENVETO (Résumé in Finnish)

Maapallon elämää suojaava yläilmakehän otsonikerros on ohentunut ihmistoiminnan takia, minkä seurauksena maanpinnalle tulevan UV-B säteilyn määrä on lisääntynyt huomattavasti. Otsonikadon ennustetaan jatkuvan aina vuoteen 2050 saakka, ja tämän seurauksena UV-B säteilyn (CIE-painotetun) odotetaan kasvavan Suomen leveysasteilla 20-50 prosentilla. Työn tarkoituksena oli arvioida, voiko UV-B säteilyä pitää nykyisillä tai ennustetuilla annostasoilla potentiaalisena riskitekijänä taloudellisesti ja ekologisesti tärkeimmille makeanveden kalalajeillemme (siika, muikku ja hauki) niiden herkimmissä kehitysvaiheissa heti kuoriutumisen jälkeen. Siikakalojen poikaset uivat kuoriutumisensa jälkeisinä parina kuukautena lähellä pintaa, jolloin ne voivat altistua UV-säteilylle. Hauki puolestaan kutee matalaan rantaveteen, eivätkä kuoriutuneet poikaset yleensä poistu alueelta ensimmäisten elinviikkojensa aikana. Lisäksi ensimmäistä kertaa pohjoisten alueiden kalalajeilla tutkittiin myös polyaromaattisten hiilivetyjen (PAH) UV-valossa aktivoituvaa myrkyllisyyttä, käyttäen malliaineena reteeniä (7-isopropyyli-1-metyylifenantreeni), jota esiintyy runsaasti sellu- ja paperiteollisuuden alapuolisissa sedimenteissä ja leviävissä jätevesipäästöissä.

UV-B-säteilyn tunkeutuvuus järviveteen riippuu veden humuspitoisuudesta ja vaihtelee paljon järvien välillä. Kuitenkin kirkkaimmissa Suomen järvissä UV-B-säteilyä voi tunkeutua merkittävästi jopa metrin syvyyteen. Siialla ja muikulla UV-B-säteily aiheutti vain vähäisiä biologisia vaikutuksia, huolimatta pitkästä altistusajasta tai korkeista annoskertymistä. Siialla korkea annostaso aiheutti klassisen, mutta lievän ihon palamisreaktion. Molemmilla lajeilla ihon melaniinipitoisuus lisääntyi pitkäkestoisessa altistuksessa. Kuitenkaan pysyviä haitallisia vaikutuksia kasvuun, kuolleisuudesta puhumattakaan, ei voitu havaita. Mielenkiintoinen huomio oli se, että nämä lähisukuiset lajit ovat sopeutuneet ympäristöönsä hyödyntäen osaksi eri suojamekanismeja. Molemmilla lajeilla oli runsaasti melaniinia, jonka määrä lisääntyi UV-säteilyn vaikutuksesta, mutta vain muikulla esiintyi metanoliuutteessa absorptiota UV-B alueella. Siialla sitä vastoin olivat aktiivisina solunsisäiset suojamekanismit: vaurioita korjaava stressiproteiini eli HSP70 sekä mahdollisesti antioksidanttina toiminut CYP1A. Kaikkiaan tulokset osoittavat, että UV-B säteilyn aiheuttama riski muikulle tai siialle on vähäinen tai olematon.

Sitä vastoin hauen ruskuaispussipoikaset ovat erittäin herkkiä lyhytaikaisellekin UV-B altistukselle jopa nykyistä alhaisemmilla annostasoilla. Nykytiedon valossa hauella havaittu vakava neurologinen käyttäytymishäiriötila onkin yksi herkimmistä UV-B säteilyn aiheuttamista biologisista haitoista. Neurologisia häiriöitä esiintyi jopa 80 prosentilla poikasista, jotka altistuivat alhaiselle 1.0 kJ/m^2 päiväannokselle, mikä on selvästi luonnossa keväällä vallitsevia UV-B säteilyannoksia alhaisempi säteilytaso. Jatkoseuranta osoitti, että tämä häiriö kehityksessä johti lopulta viivästyneeseen kuolemaan, mikä

lisää havainnon merkittävyyttä. Myös maastoaltistuksessa nykyistä korkeammilla tasoilla esiintyi neurologisia oireita, mutta vain 20 prosentilla. Kuitenkin tulokset osoittavat, että jo pieni muutos maahan tulevan UV-B säteilyn määrässä riittää aiheuttamaan haitallisia vaikutuksia hauen varhaiskehitykselle.

Muikun, siian ja hauen herkkyydet reteenin valotoksisuudelle erosivat huomattavasti toisistaan. Itsessään reteeni ei ollut haitallista minkään tutkitun lajin poikasille (suurin testattu pitoisuus 100 µg/l). Massakromatografisesti voitiin osoittaa reteenistä UV-B säteilyn vaikutuksesta syntyvän kaksi tuntematonta valotuotetta. Muikulla ja siialla samanaikainen altistus UV-B säteilylle ja reteenille aiheutti dramaattisen kuolleisuuden 72 tunnin aikana, vaikka käytetyt reteenipitoisuudet olivat alhaisia sedimentissä esiintyviin pitoisuuksiin verrattuna. Histopatologinen tarkastelu osoitti, että reteenin valotoksinen vaikutus siialla liittyi vaurioon ihon pintakerroksessa, jota varhaiskehityksen aikana käytetään mm. hengitykseen. Toisaalta hauen-poikasille reteenillä ei ollut valotoksisia vaikutuksia. Voidaan todeta, että muikun ja siian poikasille reteenin ja sen kaltaisten aineiden valotoksisuus voi olla yksi tärkeimmistä ekotoksikologisista riskitekijöistä järvialueilla, jotka sijaitsevat sellu- ja paperiteollisuuden tai muiden PAH-aineita ympäristöönsä tuottavien lähteiden vaikutuspiirissä.

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