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Toxicity of retene and pyrene on fish at different light conditions

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ABSTRACT

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Aquatic environments act as sinks of numerous chemicals, many of which are toxic to aquatic species, including ubiquitous polycyclic aromatic hydrocarbons (PAHs). The effects of some environmental chemicals, including PAHs, on aquatic organisms may be enhanced by ultraviolet radiation (UVR). In this work, two PAHs, retene (RET) and pyrene (PYR), were studied at different nominal concentrations (at range of 1-100 µg/l), as separate compounds. In the presence of RET or PYR, juvenile rainbow trout and eleoutheroembryo stages of whitefish and northern pike were exposed to three different light conditions: experimented without additional UV radiation, with authentic solar light, and with artificial UV radiation. The mortality and behavioral changes were monitored and the sublethal biochemical effects were measured. cDNA microarray and real-time quantitative PCR were used to study the effects on transcriptome, and Western blot was used to analyze proteins, as two main important ones as cytochrome P450 (CYP1A) and one heat shock protein (Hsp70). Also the activity of glutathione reductase (GR) was measured. In results, studied PAHs were not acutely lethal to fish at these concentrations. RET was phototoxic at 100 µg/l and PYR extremely phototoxic even at the lowest concentration (less than 1 µg/l). Only RET induced CYP1A in studied salmonidae at all light conditions. Both PAHs, toxicity or phototoxicity, were associated with cellular stress formation, measured as heat shock protein 70, PYR also with oxidative stress, in fish. We conclude that both PAHs affected lethally only when enhanced by solar light or UV radiation, and sublethally even at low concentrations (1 µg/l). RET and PYR had some similar and dissimilar modes of actions measured with different endpoints, and PYR effects were stronger than RET ones at same equimolarities on transcriptome. The responses against these substances varied between different species, tissues, and light conditions. In conclusion, additional solar light and UV radiation are important environmental factors, enhancing the ecotoxicological risks of PAHs in aquatic environment.

Keywords: eleutheroembryos; environmental chemicals; fish; juvenile; microarray; northern pike; phototoxicity; protein endpoints; rainbow trout; transcriptome; qPCR; toxicity; whitefish.

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LIST OF ORIGINAL PUBLICATION

This thesis is based on the following manuscripts which are referred to in the text by their Roman numerals I-III. I carried out a large part of the conducting the experiments and analyzes in each paper. I am the main author in each paper which were completed in co-operation with the other authors.

I Kati Räsänen, Tiina Arsiola, Aleksei Krasnov, Sergey Afanasyev and Aimo Oikari. 2011. Transcriptomic effects of retene and pyrene in liver of juvenile rainbow trout (*Oncorhynchus mykiss*). M*anuscript.*

II Kati Räsänen, Tiina Arsiola and Aimo Oikari. 2011. Effects of solar light on toxicity of retene and pyrene in eleutheroembryos of whitefish (*Coregonus lavaretus*). *Manuscript.*

III Kati Räsänen, Eeva-Riikka Vehniäinen and Aimo Oikari. 2011. Different sensitivities of whitefish (*Coregonus lavaretus*) and northern pike (*Esox lucius*) eleutheroembryos to photoinduced toxicity of polycyclic aromatic hydrocarbons. *Polyc. Arom. Comp.* 31: 65-83.

ABBREVIATIONS

AS	action spectrum
ANOVA	analysis of variance
BiP	bichaperone
cDNA	complementary DNA (deoxyribonucleic acid)
CIE-AS	Commission Internationale de l'Eclairage (International Commission on Illumination), i.e. the action spectrum specific for human erythema
CYP	cytochrome P450
DMSO	dimethylsulfoxide
DNA-AS	the action spectrum specific for DNA (deoxyribonucleic acid)
ERA	environmental risk assessment
GC-MS	gas chromatography – mass spectrometry
GR	glutathione reductase
GRP	glucose regulated protein
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HSF	heat shock factor
Hsp	heat shock protein
HSE	heat shock promoter element
LC50	lethal concentration for 50 % of the members of a tested population
log K_{ow}	logarithmic of octanol-water-partition-coefficient
NADPH	nicotinamide adenine dinucleotide phosphate
PAH	polycyclic aromatic hydrocarbon
PEC	predicted environmental concentration
PNEC	predicted no effect concentration
PYR	pyrene
RET	retene
ROS	reactive oxygen species
qPCR	quantitative polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polycrylamide gel electrophoresis
sHSP	small heat shock proteins
SOD	superoxide dismutase
SOL	solar radiation
UV	ultraviolet light
UVR	ultraviolet radiation, consisting of 280-400 nm
UV-A	ultraviolet A radiation (wave lengths between 320-400 nm)
UV-B	ultraviolet B radiation (wave lengths between 280-320 nm)
UV-C	ultraviolet C radiation (wave lengths between 200-280 nm)
VIS	visible light wave lengths between 400-760 nm
US-EPA	United States Environmental Protection Agency

1 INTRODUCTION

1.1 Polyaromatic hydrocarbons (PAHs) as environmental contaminants

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous chemicals in and pollutants of the environment (Douben 2003). They are hazardous to the environment and human health, and commonly mutagens, carcinogens, or reproductive toxicants (US-EPA 2010a, b). The physical and chemical characteristics of PAHs vary, affecting their distribution and fate in air, soils, and aquatic environments (Douben 2003).

1.1.1 Basic chemical characteristics of PAHs

Polycyclic aromatic hydrocarbons include at least three aromatic carbon rings, the only exception being naphthalene which includes two rings. The molecular weight of most of the PAHs is between 128-278 g/mol. They are lipophilic compounds, their volatility varies a lot, and their octanol-water-partition-coefficient (log K_{ow}) can be between 3.37-6.75 (Douben 2003).

PAHs can be formed in three ways: by high-temperature pyrosynthesis of organic materials, by low- to moderate-temperature diagenesis of sedimentary organic material in fossil deposits, and by direct biosynthesis by microorganisms and plants (Douben 2003). Today, they are mainly formed as the result of incomplete combustion of organic material (Martin 1999). The sources of PAHs in the environment can be petroleum spills, chronic leakages, leachings, surface water run-offs, sewage effluents, or industrial processes (Cerniglia & Heitkamp 1989). The environmental fate processes of PAHs are evaporation, photochemical oxidation, microbial degradation, and sedimentation (Cerniglia & Heitkamp 1989). The total concentration of PAHs varies in city air 0.2-500 ng/m^3, sewage water 1-625 µg/l, sludge 1.2-5.3 µg/g, sea water 0.01 to over 47 µg/l, and sea sediment 0.32-170 000 ng/g (Douben 2003).

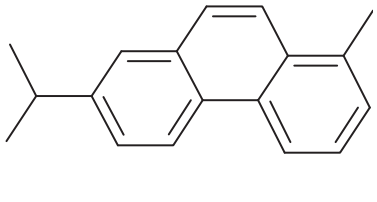
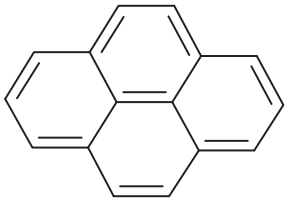
In aquatic ecosystems, the highest concentrations of PAHs are in sediments, medium in aquatic biota, and the lowest in water column (Tuvikene 1995). As relatively stable and lipophilic organic compounds they can bind to humic substances in aquatic environments, i.e. be relatively persistent in the environment (Haitzer et al. 1998). Hydrophobic compounds may bind to sediments which form a sink for individual xenobiotics. However, a significant fraction of compounds can be bioavailable, i.e. they can be taken up by an organism, while desorbed from sediments (Tomson & Pignatello 1999). Finally, they can easily penetrate biological membranes and accumulate in organisms (Varanasi 1989). Therefore, PAHs can be bioavailable by multiple mechanisms and bioconcentrate into organisms (Haitzer et al. 1998).

1.1.2 Retene and pyrene

Retene (7-isopropyl-1-methyl-phenanthrene) is produced from recin acids by anaerobic processes (Ramdahl 1983, Tavendale et al. 1997). It can also originate from algal and bacterial precursors (Wen et al. 2000). In sediments of pristine lake areas, its concentration ranges from nanograms to micrograms per g dry weight (Boulabassi & Saliot 1993, Leppänen & Oikari 1999), but can exceed from a few dozen micrograms to over one milligram per g dry weight in pulpmill sludges and sediments downstream of wastewater discharges (Leppänen & Oikari 1999, Leppänen et al. 2000, Leppänen & Oikari 2001, Meriläinen et al. 2006, Rämänen et al. 2010). Retene is very hydrophobic (log K_{ow} 6.4; Basu et al. 2001), which is why it can bind for example to carbon pool of sediments. Sediment-bound retene can, however, be dissolved in the water phase (e.g. in a water elutriate study, its concentration was 13 µg/l; Meriläinen et al. 2006). The predicted solubility of retene to water ranges from 17-34.2 µg/l (Kiparissis et al. 2003, SRC – PhysProp Database 2011, Turcotte et al. 2011). Sediment-bound retene can therefore be bioavailable to aquatic organisms (Leppänen & Oikari 1999, Oikari et al. 2002). The basic chemical characteristics of retene are shown in Table 1.

Pyrene (benzo-phenanthrene) is usually considered to be a representative of many other PAHs. It belongs to the list of priority pollutants of the United States Environmental Protection Agency (US-EPA). The list includes 16 PAHs from a total of 126 priority pollutants (US-EPA 1990, Tuvikene 1995, US-EPA 2010a, b). Pyrene occurs in the environment as a byproduct of the incomplete burning of wood treated with creosote and of a multitude of oil-based products, including gasoline (Burgess et al. 2003). It is also relatively hydrophobic (log K_{ow} 4.92; Di Toro et al. 2000) and can bind to aquatic humic substances, decreasing their bioavailability (Akkanen et al. 2001). Pyrene's water solubility is 135 µg/l (Mackay & Shiu 1977), and aqueous oil-originated pyrene can be bioavailable to fish (Nahrgang et al. 2009). The characteristics of pyrene are shown in Table 1.

TABLE 1 Basic chemical characteristics of retene and pyrene.

	Retene	Pyrene
Molecular name	7-isopropyl-1-methyl-phenanthrene [a, d]	benzo-phenanthrene [b, d]
Model compound for	alkyl-PAH	unsubstituted PAH
Molecular formula	$C_{18}H_{18}$ [a]	$C_{16}H_{10}$ [b]
Molecular weight (g/mol)	234.34 [c, d]	202.26 [c, d]
CAS -register number	483-65-8 [a, d]	129-00-0 [b, d]
Water solubility (µg/l at 25 °C)	17 [h] 29 [j] 34.2 [d]	135 [i, d]
log K_{ow}	6.4 [e]	4.84 or 5.14 [g] 4.92 [f] 4.88 [d]
Melting point (°C)	99 [a] 101 [d]	156 [b] 151.2 [d]
Boiling point (°C)	390-394 [a] 390 [d]	393 or 404 [b] 404 [d]
Vapor pressure (mm Hg at 25 °C)	$2.64*10^{-6}$ [d]	$2.5*10^{-6}$ [b] $4.5*10^{-6}$ [d]
Henry's law constant (atm m^3/mol at 25 °C)	$11*10^{-5}$ [d]	$1.14*10^{-5}$ [b]
Molecular structure	 [c]	 [c]

References:

[a] Oxford University 2010
[b] US 1995
[c] made by ISIS/Draw-program
[d] SRC – PhysProp Database 2011
[e] Basu et al. 2001
[f] Di Toro et al. 2000
[g] Helweg et al. 1997
[h] Kiparissis et al. 2003
[i] Mackay and Shiu 1977
[j] Turcotte et al. 2011

1.2 Solar energy, atmospheric ozone layer, and UV radiation

The main source of energy for Earth's ecosystem comes from solar electromagnetic radiation (Simon 1997). Solar radiation consists of different wavelengths, and part of them comes through the ozone layer in Earth's atmosphere, an area called stratosphere. There the ozone (O_3) is formed from the oxygen (O_2) by the solar ultraviolet radiation (UVR) (van der Leun 2004). Radiation at shorter wavelengths is absorbed completely by the ozone layer. That is why UVC (200-280) and shorter wavelengths cannot reach Earth. Part of UVB (280-320 nm), UVA (320-400 nm) and wavelengths up to that reach Earth (Simon 1997).

UV doses that reach Earth can be measured as irradiation fluence rates (mW/m^2), and calculated for doses depending on total irradiation time (kJ/m^2), and also for unweighted or weighted for the action spectrum specific to human erythema, called CIE (McKinlay & Diffey 1987), or the action spectrum specific for DNA (Setlow 1974). For instance, the CIE-weighted dose was 1.5 kJ/m^2 for 3-h period in May in Lake Palosjärvi, Central Finland (Häkkinen & Oikari 2004).

In boreal aquatic systems, UVB radiation can penetrate into a few meters depth in the clearest oligotropic lakes and a few centimeters in humic lakes in Finland (Huovinen et al. 2000). UVR can penetrate from a few centimeters in lake waters with high concentrations of dissolved organic carbon (DOC) to over 10 m or more in low concentrations of DOC (Kirk 1994, Morris et al. 1995, Williamson et al. 1996, Huovinen et al. 2000).

1.2.1 Changes in UV-climate

Over the past few decades (since early 1970s), studies have been made to predict the depletion of the ozone layer in the stratosphere. The changes in the ozone layer are diminished thickness and the Antarctic ozone hole (Stolarski et al. 1992), which are consequences of pollution influenced by human activities (van der Leun 2004). In addition, UVB radiation is predicted to increase on Earth particularly in the northern latitudes due to stratospheric ozone depletion (Taalas et al. 2000).

1.2.2 Phototoxicity

The shorter the solar spectrum wavelength is, the more energetic it is, and the more harmful it is for organisms. On the other hand, UVR can damage organisms indirectly through photosensitization (Foote 1987) or photomodification (Ren et al. 1996) of chemicals. In photosensitization, singlet-state oxygen radicals are formed in tissues of organisms (Foote 1987, Larson & Berenbaum 1988), whereas photomodified compounds differ structurally from their parent structures (Ren et al. 1996, McConkey et al. 1997).

1.3 Toxicity of PAHs on fish

Polycyclic aromatic hydrocarbons are xenobiotics that can be toxic by inducing sublethal effects or even lethality on organism. Measured sublethal and lethal effects are also called endpoints (e.g. biomarkers). A biomarker can be briefly defined as a change in a biological system, affected by exposure, or an effect of an environmental substance (Peakall & Shugart 1989). Sublethal biomarkers of effect can be divided into three main classes: biochemical and physiological, behavioral, and histological. Sublethal biomarkers are also early signals that may represent first signs of biological stress, which can lead to individual's death or even destruction of the population (Rand 1995). Among other chemicals they can be used to study PAH toxicity (van der Oost et al. 2003).

Stress can be a physiological response, expressed as changes of factors that could result in cellular damage. These factors include energy depletion, elevated body temperature, oxygen deprivation, generation of reactive oxygen species (ROS), decreased intracellular pH, and physical damage to the tissue (Iwama et al. 2004). Besides potentially detrimental consequences, cellular stress responses protect organisms from further damages by a wide variety of stressors, including elevated temperatures, ultraviolet (UV) light, and xenobiotics (Sanders 1993).

While PAHs are taken up by fishes, they can disturb their development, immunity, reproduction, growth, and survival, and they may also induce blue sac disease (Gravato & Santos 2001, Vehniäinen et al. 2003, Häkkinen et al. 2004, Colavecchia et al. 2006, Reynauda & Deschaux 2006). Fish have several mechanisms to protect themselves in exposure to PAHs (Tuvikene 1995). Sublethal toxicity of PAH can be studied by biotransformation enzymes, oxidative stress parameters, biotransformation products, stress proteins, genotoxic parameters, and histological and other morphological parameters (van der Oost et al. 2003).

The movement of a xenobiotic in an organism is called toxicokinetics, and it can be absorption, distribution, biotransformation, or elimination. In absorption, a xenobiotic needs to contact a biological membrane (bioavailability) and pass it passively, e.g. by diffusion, or actively, e.g. via cell membrane receptors. In an organism, the xenobiotic is distributed around the body and encounter the receptor of the target tissue or cells, where it is activated and affecting (Klaassen 2007). After a xenobiotic has entered the body, higher organisms have some capacity to get rid of the compound by elimination. Hydrophilic compounds can be readily excreted in the urine via the kidneys (Boesterli 2007, Klaassen 2007). Lipophilic compounds can be handled by the body in two ways. They can be stored into the body or they can be enzymatically converted into a more hydrophilic species through phase I and II, called biotransformation (Boesterli 2007). However, biotransformation is not always a detoxication process; xenobiotics can also be converted into more toxic species (Boesterli 2007). Biotransformation system can be divided into four categories based on the catalyzed reactions: hydrolysis, reduction, and oxidation belonging to the phase I, and conjugation to the phase II (Klaassen 2007).

Cytochrome P450 (CYP) works as metabolizing enzymes in oxidation reactions, and plays a key role in the initial step of phase I biotransformation of PAHs and other xenobiotics to phenols and oxides. These heme-containing CYP-proteins insert activated oxygen into the substrate and use nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor (Klaassen 2007). The oxygenated products may be further metabolized to more polar conjugated products by phase II enzymes. The metabolites are usually more soluble in water than the parent compounds and can be excreted from the body (Varanasi et al. 1989, Boesterli 2007).

Bioaccumulation kinetics can be used to estimate the bioavailability of a xenobiotic. It describes the proportion of a xenobiotic in the surrounding environment taken up by an organism, simultaneously including uptake and elimination. It can be studied by measuring xenobiotic concentration or absorption rate between environment and organism, or by detecting metabolites. Bioconcentration factor (BCF) is the quotient of the compound's concentration in the organism and the environment. Partition coefficient (log K_{ow}) can be used to predict BCF using different equations (Rand 1995, Klaassen 2007).

1.3.1 Sublethal effects of PAHs in fish

CYP, being a large superfamily (Klaassen 2007) in animals, is composed of several families like CYP1, CYP2, CYP3, CYP4, CYP5, CYP6, CYP7, CYP8, CYP9, CYP10, CYP11, CYP17, CYP19, CYP24, CYP26, and CYP27 (Lewis 2001). These families can be divided into subfamilies. In addition, plants, fungi, and bacteria have their own nomenclature (Lewis 2001). CYPs are regulated via ligand-activated receptors, the major ones being the aryl hydrocarbon receptor (AHR), the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the peroxisome proliferator activated reseptor-alpha (PPARα). AHR regulates e.g. CYP1A1, CYP1A2, and CYP1B1, PXR e.g. CYP3A, CAR e.g. CYP2A, CYP2B, and also CYP3A, PPARα e.g. CYP4A (Boelsterli 2007, Klaassen 2007).

CYP1A is perhaps the most commonly known family member of CYP and is expressed in most tissues, especially in liver, located in the smooth endoplasmic reticulum (ER) (Lewis 2001, Klaassen 2007). It has been widely used as a biomarker for PAH exposure (Whyte *et al*. 2000). CYP1A is known to be induced by TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) (Rifkind 2006) and retene, e.g. in whitefish (Vehniäinen et al. 2003). CYP1A induction is a consequence of the activation of aryl hydrocarbon receptor (AhR), mediated after ligand binding in cytosol (Klaassen 2007). The other CYP, CYP3A, has wide substrate specificity for physiological ligands, but may also metabolize many drugs. It is also expressed in cytosol and microsomes, and especially in the small intestine and liver where it contributes to the first-pass metabolism (Klaassen 2007). CYP3A has a significant role in metabolism of numerous xenobiotics and can be used as another biomarker with CYP1A (Hegelund & Celander 2003).

In almost all organisms studied, in the cases where there are protein damages (unfolded or misfolded proteins), the stress response entails the rapid synthesis of a suite of proteins referred to as stress proteins, or heat-shock proteins, (family of heat shock proteins, Hsp) (Iwama et al. 2004). They are involved in the folding and translocation of cellular protein under normal conditions, but under stress conditions they inhibit irreversible protein aggregation or misfolding (Sanders 1993, Morimoto 1998). That is why they also serve as an indicator of stressed condition of tissues. It is also known that Hsps are part of the various physiological functions of fish, including development and aging, stress physiology and endocrinology, immunology, environmental physiology acclimation, and stress tolerance (Basu et al. 2002). Patterns of Hsp induction have been well studied in fishes, but a complete understanding of the mechanisms of stress and the regulation of Hsps is far from clear (Iwama et al. 2004).

Vertebrate heat shock proteins can be divided into five main groups based on the molecular mass: families of Hsp90 (85–90 kDa), Hsp70 (68-73 kDa), Hsp60, Hsp40, and small heat shock proteins (sHsp) (Basu et al. 2002, Kampinga et al. 2009, Heikkila 2010). Hsp70 superfamily includes e.g. Hsp70, Hsc70, and BiP (bichaperone), the last one also called Grp (glucose regulated protein) 78. Hsp90 includes Hso 90α and Hsp 90β, and Grp94 is also called Gp96. An Hsp cycle in cells covers the activation of heat shock promoter element (HSE) and heat shock factor (HSF) (Morimoto 1998).

Hsp30, Hsp70, and Hsp90 are cytoplasm proteins, but Grp78 and Grp94 are associated with ER (endoplasm reticulum) (Klaassen 2007). Hsp30 is induced by environmental stressors, has a chaperon function, and can have a function of prevention of apoptosis (Heikkila 2004, Kondo et al. 2004). Hsp70 is generally known to be part of protein folding (Iwama et al. 2004). Hsp70 is induced when organisms are exposed to various types of stressors that generate denatured proteins, e.g. UVR and chemicals, and they are believed to have a protective role against tissue damage (Suzuki & Watanabe 1992, Sanders 1993, Lewis et al. 1999). Furthermore, Hsp70 is known to have some protective function in cell apoptosis (Mosser et al. 2000). In fish, RET can induce Hsp70 as expressed of protein levels (Fragoso et al. 1999, Vehniäinen et al. 2003). Hsp90 is a chaperone function protein, and it has also a role in apoptotic and immune processes (Roberts et al. 2010). It is a component of the AhR-complex in the absence of ligand (Klaassen 2007). Hsp90 is expressed also in fish (Pan et al. 2000, Sathiyaa et al. 2001).

Oxidative stress can be defined as an imbalance between the formation of reactive oxygen species (ROS) and the cellular antioxidant defence system, which may lead to cellular damage. During oxidative stress, free oxygen radicals (e.g. singlet oxygen (O2•) or radical hydroxyl (OH•)) are formed in a cell (Boelsterli 2007). Normally, a cell uses oxygen aerobically, which also produces possibly harmful reactive oxygen radicals. There are some cellular systems which degrade the harmful free radicals. Superoxide-dismutase (SOD) transforms a radical to hydrogen peroxide (H_2O_2). However, catalase can transform hydrogen peroxide back to water and oxygen. Glutathione peroxidase transforms reduced

glutathione (GSH) to glutathione disulfide, also called oxidized glutathione (GSSG), and also then it reduces free hydrogen peroxide to water. At the same time, glutathione reductase (GR) catalyses the reduction of GSSG back to GSH in the presence of NADPH, thus maintaining adequate levels of GSH in cells and tissues. Oxidative stress can be formed if there is imbalance between the formation of reactive oxygen species (ROS) and the cellular antioxidants. Antioxidants prevent the oxidation of other compounds, and protect cells from the effects of free radicals. All in all, oxidative stress can lead to over-formation of free radicals, which can lead to serious cellular damages, such as degradation of proteins and lipids (Boelsterli 2007, Müller et al. 2007).

Some links around the oxidative stress, e.g. GR activity, can be used as parameters for oxidative stress (Meister & Anderson 1983, Boelsterli 2007). Selenoproteins, e.g. 15 kDa selenoprotein, can also be part of oxidative stress being part of some glutathione peroxidases (Rotruck et al. 1973, Gladyshev 2006). UVR is generally considered to induce oxidative stress (Birch-Machin & Swalwell 2010), also in fish (Lesser et al. 2001). Some PAHs can induce oxidative stress in fish (Timme-Laragy et al. 2009). Similarly, some PAHs can induce GR activity (e.g. benzo[a]pyrene, Cheung et al. 2001; and anthracene, Richardson et al. 2008).

1.3.2 Phototoxicity of PAHs in fish

Direct UV radiation (UVR) is harmful to organisms and can cause e.g. mortality and a neurobehavioral disorder (spiral swimming) to post-hatched fish embryos (Häkkinen et al. 2004, Vehniäinen et al. 2007a). Fish larvae have some ways to can cope with enhanced UV irradiation: DNA photorepair mechanism, skin melanin pigmentation, and avoidance behavior (Ahmed & Setlow 1993, Häkkinen et al. 2002, Vehniäinen et al. 2007b).

Many PAHs exert phototoxicity in the presence of UV radiation, including retene and pyrene (Arfsten et al. 1996, Vehniäinen et al. 2003, Häkkinen et al. 2004). PAHs can be transformed into being phototoxic also by visible light (400-760 nm) (Wang et al. 2007). Phototoxicity has been shown to become induced in fish, e.g. in vendace and whitefish post-hatched embryos (Vehniäinen et al. 2003). Small, transparent organisms, such as fry or juveniles, and infaunal animals seem to be particularly UV-PAH sensitive (Pelletier et al. 1997, Weinstein 2001).

1.3.3 Effects of retene and pyrene on fish

Retene has not been shown to cause acute lethality on fish, but it is chronically lethal to rainbow trout (*Oncorhynchus mykiss*) larvae (Billiard et al. 1999, Hawkins et al. 2002). Its chronic median lethal concentration (LC50) for early life stage of trout was 177 µg/l (Billiard et al. 2000). Instead, retene has been shown to be acutely phototoxic to aquatic organisms such as whitefish and vendace (Vehniäinen et al. 2003), or pike (Häkkinen et al. 2004) post-hatched embryos. Subchronic exposure to retene in visible light (no UVR) causes blue sac disease (BSD), with pericardial and yolksac edema, craniofacial malformations, hemorrhaging, decreased growth, and increased mortality in early developmental stages of zebrafish (*Danio rerio*) (Billiard et al. 1999) and rainbow trout (Billiard et al. 1999, Brinkworth et al. 2003, Bauder et al. 2005). Retene induced stress at acute exposure, measured as stress indicators as significant decrease in plasma cortisol and a rise in plasma glucose (Teles et al. 2003).

Retene's metabolites have been found from rainbow trout, but they have not been structurally identified (Fragoso et al. 1999, Hodson et al. 2007). Sublethal effects or photoeffects of retene can include changes in proteins of CYP1A and Hsp70 in whitefish (*Coregenus lavaretus*), vendace (*Coregenus albula*) (Vehniäinen et al. 2003), and pike (*Esox lucius*) (Häkkinen et al. 2004) post-hatched embryos, and in the activity of CYP1A in juvenile rainbow trout (*Oncorhynchus mykiss)* (Fragoso et al. 1999). Recent findings concluded that retene toxicity is associated with the function of AhR2-reseptor and CYP1A metabolism (Scott et al. 2011), which lead to formation of retene's metabolites that finally cause the toxicity (Hodson et al. 2007). Some evidence has been shown about retene's genotoxicity in eel (*Anguilla anguilla*) (Maria et al. 2005).

Pyrene is acutely lethal to fishes. LC50 to rainbow trout in 4-day exposure ranged 125-250 µg/l (Krasnov et al. 2005b). Pyrene induced 100 % mortality to zebrafish embryos exposed for 4 days at 150 µg/l concentration in 6-7 days after fertilization (Incardona et al. 2004). In a similar experiment with Sheepshead minnow (*Cyprinodon variegatus*), pyrene induced 100 % mortality at same concentration as earlier (150 µg/l) in 8 days after fertilization (Hendon et al. 2008). There is no doubt about the acute lethal phototoxicity of pyrene. It has been shown in microbes, plants, invertebrates, and vertebrates (Oris and Giesy 1985, Huang et al. 1995, Arfsten et al. 1996, Boese et al. 1997, Hatch & Burton 1998).

In metabolic studies on fish the main product of pyrene in phase I was 1-hydroxypyrene (Luthe et al. 2002, Honkanen et al. 2008), and the phase II metabolite was pyrene-1-sulphate (Honkanen et al. 2008). Pyrene is considered to act via AhR pathway and exhibit pericardial edema, failed swim bladder inflation, anemia in early life stages of zebrafish and sheephead minnow (Incardona et al. 2004, Hendon et al. 2008), but it is a weaker AHR agonist than other more potent PAHs such as benzo(a)pyrene (Barron et al. 2004). Pyrene induced CYP1A mRNA and protein in Nile tilapia (*Oreochromis niloticus*) (Zapata-Pérez et al. 2002) and mRNA in sheepshead minnow (*Cyprinodon*

variegatus) (Hendon et al. 2008). There are some conflicts concerning the induction of CYP1A; it was not induced in yolk-sack fry of salmon (*Salmo salar*) (Honkanen et al. 2008). On transcriptomic studies, pyrene caused changes in genes involved in genetic apparatus, immune response, glycolysis, and metabolism of ions, especially iron in liver and kidney of rainbow trout (*Oncorhynchus mykiss*) (Krasnov et al. 2005b), and immune and stress responses in liver of brown trout (*Salmo trutta lacustris*) (Krasnov et al. 2007). Pyrene is known to be genotoxic *in vitro* (Cerniglia & Heitkamp 1989, Woo et al. 2006), but it is not carcinogenic (Cerniglia & Heitkamp 1989). Visible light and UVA radiation can enhance the genotoxicity of pyrene *in vitro* (Botta et al. 2008).

1.4 Experimental toxicology with fish

1.4.1 Organisms: rainbow trout, whitefish, and northern pike

Rainbow trout (*Oncorhynchus mykiss*) belongs to the family of Salmonidae. It lives in boreal freshwaters, originated from North-America, and spawns in November to May. Their diet ranges from planktons and crustacea to small fish (Muus & Dahlstrøm 2005). Rainbow trout is widely used as a fish model in cold-water research, including genomics, and they are economically important to aquaculture and fisheries (Thorgaard et al. 2002).

Whitefish (*Coregonus lavaretus* s.l. Svärdson) is also a member of the family of Salmonidae. They are found both in fresh and brackish waters, and are widely distributed across the northern hemisphere. Whitefish eat plankton and crustacean and lay their eggs on lake bottom in fall, but hatch in spring. After hatching they swim near the surface for the first month (Shkorbatov 1966, Viljanen et al. 1995). Thereafter whitefish develop quite fast, and their yolk wears away in three or four days (Koli 1998, Muus & Dahlstrøm 2005).

Northern pike (*Esox lucius* L.), also distributed in boreal areas, is a top predator as an adult, which means that it is exposed to lipophilic xenobiotics both from the surrounding water and via the food chain. They are stationary sit-and-wait predators with a strong homing behavior (Karås & Lehtonen 1993, Eklöv 1997). Northern pikes lay their eggs to shallow lake bottoms in spring, and they hatch after 10-15 days. The yolk wears away in two weeks, during which time pike larvae are very immobile (Koli 1998, Muus & Dahlstrøm 2005).

1.4.2 Developmental stages of fish

The developmental stages of fish, juvenile (I) and eleutheroembryos (II, III), studied in the present work, are two discrete stages of the life cycle of these animals. Reproduction is one of the most sensitive indicators of the exposure of fishes to sublethal concentrations of environmental chemicals. Reproductive toxicity may be defined as an adverse effect on reproductive success. With the knowledge of the changes in reproduction, predictions can be made for the large scale of ecology: the effects from individuals to the population level (Schlenk et al. 2001).

In experiments without additional UV radiation (I), the effects of RET and PYR were studied in liver of juvenile half-year-old rainbow trout. The liver is the most important organ in biotransformation, being first in processing compounds before they enter the systemic circulation (Klaassen 2007). Young juvenile fish were chosen to exclude the effects of early development as well as sexual maturation, both being the life stages with many functional changes in gene expression. E.g. gender-related differences are exhibited in physiological (cortisol) and cellular (Hsp70 expression) stress responses in salmonid fish (Afonso et al. 2003).

In experiments with authentic solar light (II) and artificial UV radiation (III), the effects of RET and PYR were studied in post-hatched embryos, less than one day old, referred to as eleutheroembryos (free swimming forms of fish with yolk sac unresorbed, used e.g. in the study of Howarth et al. 2010) of whitefish and northern pike. Besides their sensitivity towards ambient stressors, eleutheroembryos were chosen to replace, reduce, and refine the use of live animals, mammals in particular, in toxicity testing. This goal is also demanded in the REACH legislation of the European Union since 2008 (EU-directive 2010/63/EU. 2010). Embryos as well as eleutheroembryos, which are not regarded as laboratory animals, may prove viable alternatives (OECD 1998, Lammer et al. 2009).

1.4.3 Aquatic toxicology and risk assessment

To begin with, a few words about a cellular life, i.e. biochemistry, needs to be discussed. The smallest level in a cell is a genome, and genetic information is the same in every cell, but expressed mRNA and protein content called transcriptome and proteome, respectively, vary between the cells and tissues. Both environmental and developmental conditions affect the expression of genes in animals. In general, only some specific genes are expressed at a certain time in any cell type or tissue. Thus, gene expression studies detect the abundance of certain mRNA molecules produced at a specific time or in specific circumstances. Protein levels, on the other hand, reflect larger scales in a cell, i.e. ongoing functional processes (Nelson & Cox 2000, Benson & Di Giulio 2007). From these perspectives also aquatic toxicology can be approached. This young field of

science studies aquatic systems from an ecotoxicological view, and today biochemical and molecular studies occupy an important position. These low-level studies also serve as a foundation for practical applications and needs for risk analysis, as well as regulatory and management decisions which are ultimately the aim of ecotoxicology. An understanding of biochemical mechanisms of contaminant metabolism, adaptation, and toxicity is useful for assessing environmental contamination or quality and aquatic ecosystem health (Rand 1995). The changes in several individuals, i.e. a low level study, can give us information for predictions for higher level studies, i.e. the effects on populations, and the whole ecosystem (Rand 1995, Wenning et al. 2005).

In risk assessment, a series of important issues needs to be approached: what are the target species and substances of interest, what are the key environmental factors, and what are the biological and societal relevancies? These matters need to be studied from various perspectives (Rand 1995, Wenning et al. 2005). In short, risk assessment can be directed to be a cross comparison, where PEC is predicted to have environmental concentration and PNEC is predicted to have no effect concentration. In its simplest comparison, when PEC > PNEC, there is a risk in a studied system.

1.4.4 Experiments in environmental perspective

Fish, boreal species in this research (Koli 1998), may be exposed to aquaeous retene originated from contaminated sediment (Leppänen & Oikari 1999, Oikari et al. 2002), and aqueous pyrene originated from several sources (Nahrgang et al. 2009). UV radiation can penetrate into water in lakes (e.g. Huovinen et al. 2000). To conclude, fish can be exposed to UV radiation, and in the case of the environment containing aqueous retene or pyrene, them or their photoproducts.

2 OBJECTIVES

The aim was to study how two polycyclic aromatic hydrocarbons, retene and pyrene, affect fish at different light conditions. Effects of retene and pyrene were studied as separate compounds on juvenile-stage of rainbow trout and post-hatched embryo-stages of whitefish and northern pike. Shortly, a question arose what kinds of biochemical effects these PAHs have and what the functional mechanisms before individual's death could be. We were interested in identifying the hazard and choosing endpoints of effects (sublethal and lethal) studied in the laboratory. This molecular toxicology data could help environmental risk assessment (ERA) of these PAHs.

The more detailed questions studied were the following, reported in articles I-III and as combined in this thesis summary

1) experiments without additional UV radiation, when the studied subject was liver transcriptome of juvenile rainbow trout (I)
2) experiments with authentic solar light, when the studied subject was whole individual of whitefish at its post-hatched stage (II)
3) experiments with artificial UV radiation, when studied subjects were whole individuals of whitefish and northern pike at their post-hatched stages (III)
4) visible light when all studied species are compared (I, II, III).

3 MATERIALS AND METHODS

3.1 Fish species exposed and analysis of PAHs in experimental waters

Rainbow trout exposures (I) were conducted in two different time periods: one and ten days. One day exposure gives basic information about acute toxic effects of studied chemicals, whereas ten days experiment was suggested to reveal data on subacute effects, and represents a more ecotoxicological perspective.

Whitefish (II, III) and northern pike (III) exposures lasted three days, giving information about acute toxicity. In addition, the same procedures have been used earlier in our studies with whitefish and northern pike (Häkkinen et al. 2003, 2004, Vehniäinen et al. 2003), being thus comparable with experiments conducted in this research.

Six-month old juvenile rainbow trout (*Oncorhynchus mykiss*) were used in experiments without additional UV radiation (I, Fig. 1). Fish were obtained from the hatchery of Hanka-Taimen Inc. (Venekoski, Finland, stock KLF-STR-07) in September 2007. Length was 9.2 cm (± SD 2.9) and weight 9.5 g (± SD 1.0). Fish were transferred into the 500 liter all-steel tank and acclimatized in flow-through water (flow 1-2 l/min, oxygen concentration over 8 mg/l, pH 7.6 (± SD 0.1), temperature 12.0 °C (± SD 0.1), photoperiod light:dark 16:8 h) for two and three weeks before the initiation of 1-d and 10-d exposures, respectively. Fish were fed every day with pellet fish food (Royal Plus, 3.5 mm, Raisio, Finland) ad libitum with 0.25 % of fish biomass until four days before the start of exposures. During the 10-day experiments, fish were fed ad libitum with 0.5 % of fish biomass on the 4th and 7th days.

Less than one day old post-hatched embryos of whitefish (*Coregonus lavaretus* s.l. Svärdson) were used in experiments with authentic solar light (II, Fig. 1). Fish were obtained from hatchery of Hanka-Taimen Inc. (Hankasalmi, Finland, stock VS-KOK-08) in April 2008. Wet weights and lengths, measured from 30 fish before the exposures, averaged 9.5 mg (± SD 2.2) and 11.8 mm (± SD 0.6), respectively. The dry weight was about 11 % from the wet weight. The fish were not fed.

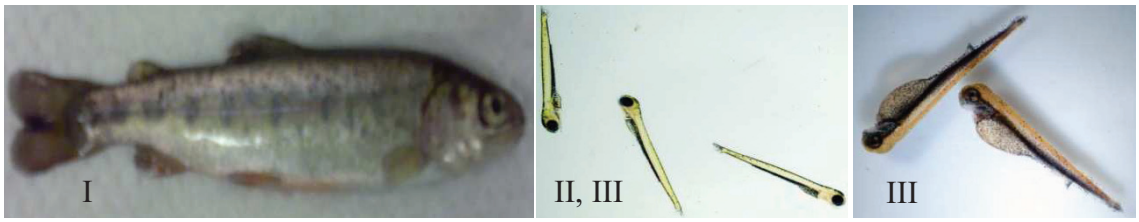


FIGURE 1 Six months old juvenile rainbow trout used in experiment without additional UV radiation (I), less than one day old eleutheroembryos of whitefish used in experiment with authentic solar light (II) and with artificial UV radiation (III), and less than one day old eleutheroembryos of northern pike used in experiment with artificial UV radiation (III).

Less than one day old post-hatched embryos of whitefish (*Coregonus lavaretus* s.l. Svärdson) (III, Fig. 1) and northern pike (*Esox lucius* L.) were used in experiments with artificial UV radiation (III, Fig. 1). Whitefish were obtained from hatchery of Hanka-Taimen Inc. (Hankasalmi, Finland, stock VS-KOK-07) in April 2007. Northern pike, caught from Lake Päijänne, Finland, in April 2007, were obtained as fertilized embryos, and they were incubated and developed in hatchery cones in our laboratory at the University of Jyväskylä. Wet and dry weights and lengths, measured from 30 animals before exposures, were 6.1 mg (± SD 0.9) and 1.5 mg (± SD 1.0) and 11.5 mm (± SD 0.7), for whitefish, and 7.3 mg (± SD 1.7) and 1.9 mg (± SD 0.5) and 8.2 mm (± SD 0.4), for pike, respectively. The dry weight of both species was about 25 % from the original wet weight.

In experiments without additional UV radiation (I), the water samples were collected from nominal 3.2 µg/l concentration of RET and 2.9 µg/l of PYR after 2 h and 24 h. Three replicates were measured separately. There was 9.5 µg internal standard per 200 ml sample. In experiments with authentic solar light (II), additional water experiment was carried out without fish and solar radiation. Water samples were collected from nominal 10 µg/l concentrations of RET and 8.7 µg/l of PYR after 2, 24, 48, and 72 h, just before partial (50 %) replacements of exposure waters. Three replicates were pooled into one analysis. There was 2.5 µg internal standard per 200 ml sample. In experiments with artificial UV radiation (III), water samples were collected from all concentrations of RET treatments after 24 and 72 h. The samples after 24 h were taken before the exposure to UV radiation, and those after 72 h from both VIS and UVR treatments. Three replicates were pooled into one analysis. There was 6 µg internal standard per 200 ml sample.

Pooled samples of 200 ml were extracted immediately with 50 ml hexane three times, using d10-anthracene as the internal standard (I, II, III). The hexane was evaporated with rotavapor (VV 2000, Heidolph) in 10 ml. The extracts were stored at –20 °C until analysed. Following this, the hexane was evaporated with nitrogen gas, the sample dissolved again in 25 µl of hexane, and measured with a gas chromatograph-mass spectrophotometer (GS model Hewlett Packard 6890, MS detector Hewlett Packard 5973). The method for the extraction of PAHs was applied from our previous studies (Rämänen et al. 2010).

3.1.1 Experiments without additional UV radiation (I)

Rainbow trout (I) were transferred into the 50-l all-glass aquaria, water volume was 45 l, three fish per aquarium replicated three times. In the aquarium room, the treatments received routine visible light (TLD 36 W/950 daylight, Philips, Holland), and light intensity was quantified with lux-radiometer (HD 9221, Electronor, Finland). Also, UV radiation (less than 400 nm) was quantified with UV-radiometer (RM 21, UV-elektronik GmbH, Dr. Gröbel, Germany). The water to biomass ratio was approximately 2 l/g fish/d. Aquaria were covered with black plastic on each side to protect fish from unnecessary stimuli. Water oxygen concentration was over 8 mg/l, temperature 12.3 °C (± SD 0.1), and photoperiod light:dark 16:8 h.

Fish were exposed to three nominal concentrations of PAHs (low, medium, high) in waters, with three replicates. The concentrations for RET were 1.0 (low), 3.2 (medium) and 10.0 (high) µg/l, which were equimolar for PYR 0.9 (low), 2.9 (medium) and 8.7 (high) µg/l, respectively. The stock of PAHs was dissolved in DMSO, forming stock solutions of 1 mg/ml, accordingly the concentration of 0.01 % DMSO was used in control treatments.

Fish were exposed to PAHs for 1 and 10 days in October 2007 in Jyväskylä. At the end, fish were randomly netted from the aquarium and quickly stunned with a blow to the head. Fish weight and length were 8.6 g (± SD 2.7) and 9.3 cm (± SD 1.1) in 1 d experiment, and 9.1 g (± SD 3.1) and 9.9 cm (± SD 1.0) in 10 d experiment, respectively. The animals were then decapitated, and their livers were divided into two parts and snap frozen in liquid nitrogen. A timescale and a picture of the experiment are shown in Figures 2 and 3, respectively.

Exposure timescales

Start End

0 24 h

‖-*----------*‖

PAH-exposure

Start End

0 10 days

‖----------‖

PAH-exposure

FIGURE 2 A scheme depicting a timescale in experiments without additional UV radiation (I). Rainbow trout were exposed to substances (RET and PYR) for one and 10 days (photoperiod 16:8 h). Half of the water was changed daily, and water samples were collected as marked to figure by *.

FIGURE 3 A picture of the experiment without additional UV radiation in the laboratory (I). Only standard working lights were hold in the aquarium room, with the period of 16 h light each day.

3.1.2 Experiments with authentic solar light (II)

Whitefish (II) as groups of forty animals were exposed in a glass bowl, 1-l water volume and water depth of 40 mm, as three replicates. The water to biomass ratio was approximately 7.9 l/fish g/d, and the water pH 8.1 (± SD 0.1) and temperature 10.5 °C (± SD 0.5). Unexpectedly, pH in water treatment system was higher than usually. Half of the water was changed daily, ensuring that the oxygen concentration remained at approximately 10 mg/l and no aeration was required. The light:dark cycle in the laboratory was 16:08 h.

Fish were exposed to three nominal concentrations of PAHs (low, medium, high) in waters, with three replicates. The concentrations for RET were 1.0 (low), 3.2 (medium) and 10.0 (high) µg/l, which were equimolar for PYR 0.9 (low), 2.9 (medium) and 8.7 (high) µg/l, respectively. The stock of PAHs was dissolved in DMSO, forming stock solutions of 1 mg/ml, accordingly the concentration of 0.01 % DMSO was used in control treatments.

Experiments were made in late April – early May 2008 in Jyväskylä (Finland, W 62° 13' 43.981'' and E 25° 44' 43.127''). During the 72 h treatment with PAHs, fish were exposed outdoors to the authentic solar light twice, 24 h and 48 h (on 30th April and 1st May 2008) after the beginning of the chemical exposures. The bowls were carried outdoors into a tank serving as a water bath with a cooler to provide stable temperature in all treatments. Each solar exposure (named as SOL+) lasted for three hours. Control fish, exposed to PAHs but not to solar light, were also transferred outdoors into a tank, but covered with black textile material (named as SOL–). Solar exposures for RET groups took place before noon, and for PYR groups in the afternoon. In spite of the coolers used in the tanks, the water temperatures did not stay equal between the two SOL+ groups due to solar heat energy, on average being 12.4 °C (± SD 1.8) for RET and 14.5 °C (± SD 2.9) for PYR. Solar light data was obtained from Lake Päijänne research platforms (LTER 2008) on the lake Jyväsjärvi, about 300 m from the experiment site. Routine visible light was quantified in the laboratory as described in the section 3.1.1.

Dead fish were removed daily and, after 72 hour-exposures to PAHs, the live specimens were sampled. After quick anaesthetization by carbon dioxide, five fish were pooled into one analytical sample, frozen in liquid nitrogen, and stored at –80 °C. A timescale and a picture of the experiment are shown in Figures 4 and 5, respectively.

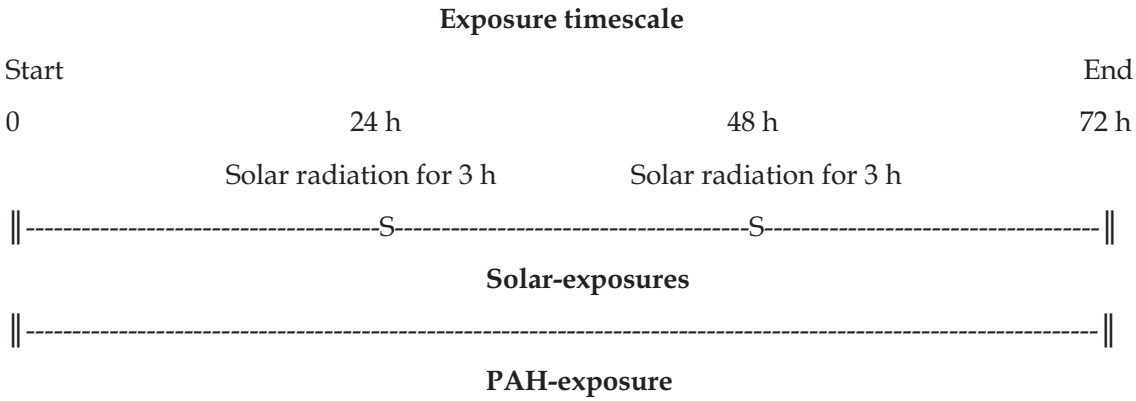


FIGURE 4 A scheme depicting a timescale in experiments with authentic solar light (II). Whitefish were exposed to substances (RET and PYR) for 72-h (photoperiod 16:8 h). Solar light was given outdoors on two days, after 24 h and 48 h from the beginning of the chemical exposures, three hours each time, marked by S. Half of the water was changed daily.

FIGURE 5 A picture of the experiments with authentic solar light outdoors (II). Solar light was given on two days, after 24 h and 48 h from the beginning of the chemical exposures, three hours each time.

3.1.3 Experiments with artificial UV radiation (III)

Whitefish and northern pike (III) as groups of forty animals were exposed in a glass bowl, 1-l water volume and water depth of 40 mm, as three replicates. The water to biomass ratio was approximately 12-13 l/g/day for whitefish and 10 l/g/day for pike. The water temperature was 8.0 °C (± SD 1), pH 7.9 (± SD 0.4). Half of the water was changed daily, ensuring that the oxygen concentration remained at approximately 10 mg/l and no aeration was required. The light:dark cycle in the laboratory was 12:12 h.

Animals were exposed to three nominal concentrations, as three replicates, to RET or PYR at 10, 32, and 100 µg/l. The stock of PAHs was dissolved in DMSO; accordingly the concentration of 0.01 % DMSO was used in control treatments.

Fish were exposed to PAHs during 72 hours. Exposures took place in April-May 2007 in Jyväskylä. Whitefish exposures to RET and PYR were carried out in two phases at different times, whereas all of the pike exposures were carried out at the same time. In addition, fish were irradiated with UVR for three hours at around noon on two consecutive days. Each UVR exposure (named as UVR+) lasted for three hours. Control experiments without UVR exposures (but exposed to PAHs) were also conducted (named as UVR–).

UVA (315-380 nm) and UVB (280-315 nm) were provided with fluorescent lamps (UVA 40W/05, Philips, Holland, and UVB 313, Q-Panel, Cleveland, OH, USA). Any UVC radiation (less than 280 nm) was blocked with a cellulose diacetate filter (Clarifoil, Derby, UK). The treatments without UVR received visible light (TLD 36 W/950 daylight, Philips, Holland), which was also present in the UV treatments. Lamps with a maximum intensity in the UVB area (denoted as UVB lamps) were applied for the RET exposures, whereas lamps with maximum intensity in the UVA area (UVA lamps) were used in the PYR exposures. Thus, both lamps contained UVA and UVB, but at different ratios. UVR was quantified using a Hamamatsu Photonic Multichannel Spectral analyzer (model PMA-11, No. SSCS1040E07, made in Japan), measuring the area 280-380 nm at every 1 nm, and the UVR doses were calculated as unweighted.

The behavioral changes were monitored and the mortality counted after 72 hours of PAH exposure. Behavioral changes described herein were defined as fish laying on their side on the bottom of the treatment bowl and exhibiting spiral swimming (described in the study of Häkkinen and coworkers 2004). Dead fish were removed daily and, after 72 hours, the live specimens were sampled. After fast anaesthetization by carbon dioxide, five fish were pooled into one analytical sample, frozen with liquid nitrogen, and stored at –80 °C. A timescale and a picture of the experiment are shown in Figures 6 and 7, respectively.

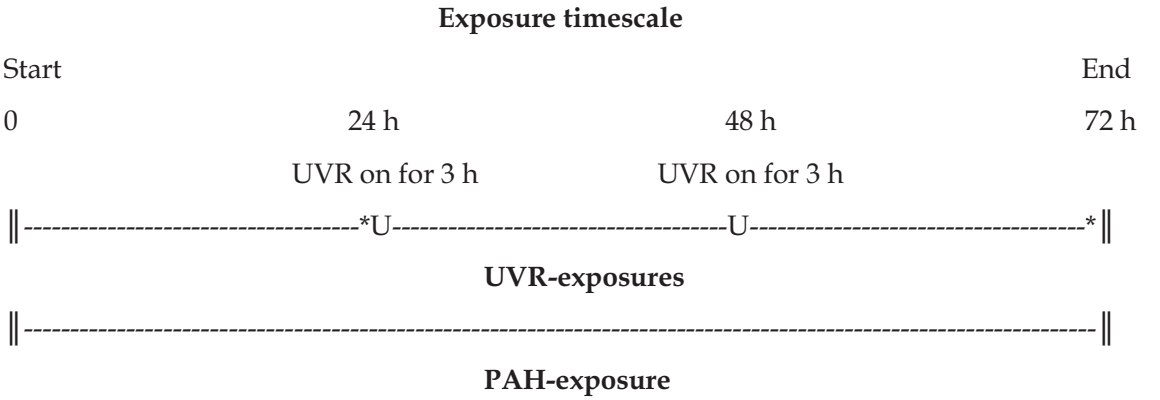


FIGURE 6 A scheme depicting a timescale in experiments with artificial UV radiation (III). Whitefish and pike were exposed to substances (RET and PYR) for 72 h (photoperiod 12:12 h). UV radiation was given in the laboratory on two days, after 24 h and 48 h from the beginning of the chemical exposures, three hours each time, marked by U. Half of the water was changed daily, and water samples were collected just before giving UV radiation as marked to figure by *.

FIGURE 7 A picture of the experiments with artificial UV radiation in the laboratory (III). UV radiation was given on two days, after 24 h and 48 h from the beginning of the chemical exposures, three hours each time.

3.2 Biochemical analysis

3.2.1 Extraction of RNA and proteins

For RNA extraction, the samples obtained from rainbow trout liver (I) and whitefish (II) where processed. For microarray analyses of rainbow trout livers, one sample was obtained only from one aquarium of each treatment, after one and 10-day experiments; one sample included a pool of three fish livers. For qPCR analyses one sample was obtained from each three replicated aquaria in each treatment, but from one-day experiments only; one sample included a pool of three fish livers (I). From whitefish for qPCR analyses, one sample was composed of five individuals (approximate wet weight 55 mg/sample, including three replicates) (II). Total RNA was extracted with TRI-reagent (Sigma-Aldrich, MO, USA), 1 ml/sample. The homogenates were centrifuged for ten minutes at 12 000 g at 4 °C. Chloroform 200 µl/sample was added, and RNA was precipitated from the aqueous phase with 500 µl of isopropanol. RNA pellets were washed and stored in 75 % ethanol at –80 °C. Diluted RNA was purified with PureLink™Micro-to-Midi total RNA purification system (Invitrogen, CA, USA). During the purification, traces of genomic DNA were removed with DNase I treatment (Invitrogen, CA, USA) for 15 min at room temperature, using 1 U for each µg of RNA for 15 min at room temperature. Purified RNA was quantified with NanoDrop 1000 spectrofotometer (CA, USA) and RNA quality checked with agarose-gel electrophoresis (1.2 % agarose, 1 % formaldehyde).

For protein extraction, the samples were obtained from whitefish (II, III) and pike (III), and used for Western blot analyses and for the activity of GR measurements (II, III). Total proteins were extracted from pooled samples of five whitefish and pike. Fish were homogenized for approximately one minute with a plastic stave in plastic tubes (Sarstedt, Germany) at 4 °C on ice with 50 µl potassium gluconate buffer (5 mmol/l of $MgSO_4$, 5 mmol/l of NaH_2PO_4, 40 mmol/l of *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 70 mmol/l of potassium gluconate, and 150 mmol/l of sorbitol, buffer pH 7.8). The homogenates were centrifuged for twenty minutes at 12 000 g at 4 °C.

Total protein concentrations of supernatants were determined by Lowry method adapted for 96-well plates (II, III). Five µl of standard and diluted samples were analysed in triplicate, by using Bio-Rad DC Protein Assay. The plate was incubated for 15 minutes, and the absorbances read at 690 nm by Labsystems iEMS Reader (Type MF 1401, Labsystem, Finland).

3.2.2 Analysis of transcriptome by cDNA microarray (I)

cDNA salmonid fish microarray (SFA 2.0, Geo Omnibus GPL6154), containing 1 804 unique clones, each printed in six replicates, was used (I; Karsnov et al. 2005a). The genes had been selected based on their functional roles including immune response, cell communication, protein folding, and response to oxidative stress, cell cycle, metabolism of xenobiotics and others. RNA of control and experimental groups were labeled with fluorescent dyes Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia, Little Chalfont, UK), respectively, incorporated in cDNA using the SuperScriptTM Indirect cDNA Labelling System (Invitrogen, CA, USA), respectively. Synthesis of cDNA was performed at 46 °C for 3 hours in a 21 µl reaction volume, following RNA degradation with 2.5 M NaOH at 37 °C for 15 min and alkaline neutralization with 2 M Hepes. Labelled cDNA was combined and purified with Microcon YM30 filter system (Millipore, MA, USA).

Microarray slides were pretreated with 1 % BSA fraction V, 5 x saline sodium citrate buffer (SSC) and 0.1 % sodium dodecyl sulfate buffer (SDS) for 30 min at 50 °C, and then washed with 2 x SSC (3 min) followed by 0.2 x SSC (3 min) at room temperature and hybridized over-night at 60 °C in a cocktail containing 1.3 x Denhardt's solution, 3 x SSC, 0.3 % SDS, 0.67 µg/µl polyadenylate and 1.4 µg/µl yeast tRNA. After hybridization, slides were washed at room temperature in 0.5 x SSC and 0.1 % SDS (15 min), 0.5 x SSC and 0.01 % SDS (15 min), 0.06 x SSC (3 min) and 0.06 x SSC (3 min).

Scanning was performed with MicroArray ScanArray 5000 Analysis System (PerkinElmer, MA, USA). The measurements in spots were quantified with ScanArrayExpress Version 3.0 program (PerkinElmer, MA, USA). The spots were filtered by criterion (I-B)/(SI+SB) ≥ 0,6, where I and B are the mean signal and background intensities, and SI, SB are the standard deviations. Low-quality spots were excluded from analyses, and genes with less than 3 high-quality spots on a slide were discarded. After subtraction of median background from median signal intensities, the expression ratios (ER) were calculated. Lowess normalization was performed first for the whole slide and then for twelve rows and four columns per slide. Technical accuracy was assessed by difference of log2-ER from zero in six spot replicates (Student's t-test, $p < 0.05$). The mean values were calculated and a single value per fish was used in subsequent analyses.

The search for the groups of co-regulated genes or transcription modules (TM) began with hierarchical and K-means clustering of genes with differential expression ($p < 0.01$) in at least one sample. Next, correlation to mean expression profile was analyzed and genes with Pearson $r < 0.7$ were discarded. Dose-dependence and time course of responses were analyzed in TM with ANOVA.

3.2.3 Analysis of transcription by quantitative PCR (qPCR) (I, II)

Seven specific genes were designed for real-time quantitative PCR (I, II); heat shock protein 30 (Hsp30), heat shock 70 kDa protein 1 (Hsp701), heat shock 90 kDa protein 1 (Hsp90), 78 kDa glucose-regulated protein precursor (Grp78), 15 kDa selenoprotein, glutathione peroxidase 1 (GP1), and cytochrome P450 1A (CYP1A). The primers were designed with OligoPerfect™ Designer (2010) software (Invitrogen, USA) available on the Internet. Primers were designed to amplify 50-200 bp fragments, and each primer pair showed specific amplification in melting curve analyses.

Out of the four candidates, NADH-ubiquinone oxidoreductase (NUOR) was used as the reference gene for rainbow trout study (I) and 18SrRNA for whitefish study (II), showing superior invariability between different runs. The two other candidates were elongation factor 1 (EF1a), and RNA polymerase 2 (RPL2). Reference genes of 18SrRNA, EF1a, and RPL2 were previously validated in Atlantic salmon (Jørgensen et al. 2006). NUOR was a candidate, but not used instead of 18SrRNA in Atlantic salmon (Jørgensen *et al.* 2008). 18SrRNA was used as a reference gene also for early life stages of *Cyprinodon variegatus* (Hendon et al. 2008). More widely, β-actin and GAPDH are commonly used reference genes in human studies, among others (Mori et al. 2008).

The purified and pooled RNA was subjected to 20 µl vol. cDNA synthesis with SuperScript® VILOTM cDNA Synthesis Kit (Invitrogen, CA, USA) using 2.5 µg RNA for each sample. Several dilutions of cDNA were tested in order to find the optimum amount of template in qPCR reactions. For validation of gene expression, Express SYBR® GreenERTM qPCR SuperMix Universal (Invitrogen, CA, USA) was used in 15 µl vol. qPCR reactions of 1:25 diluted cDNA (1.4 µl). Reactions were run in duplicates with ABI Prism 7700 Sequence Detector (Perkin Elmer, MA, USA). Standard curves for each gene were produced by using a set of cDNA dilutions as templates to ensure that the efficiency of amplification was within the acceptable limits ($0.8 < Ex < 1.1$). Consequently, standard curve method was used for quantification of gene expression, and expression of each target gene was normalized to the expression of reference gene in the same sample.

In addition, CYP1A, Hsp30, Hsp70, and Hsp90 were sequenced to ensure that the amplicons solely represent their expression (II). Therefore the amplified PCR fragments were run into gel, extracted with GeneJET Gel Extraction Kit (Fermentas, Canada) and sequenced with MegaBACE 750 (GE Healthcare, CT, USA). The DNA sequences were submitted to BLAST and significant alignments were obtained to various salmonid CYP1A, Hsp30, Hsp70, and Hsp90 sequences in GenBank and to EST sequences in Rainbow Trout Gene Index database (Rexroad et al. 2003).

3.2.4 Analysis of protein endpoints by Western blotting (II, III)

The method for Western immunoblot analysis (II, III) was based on the method of Laemmli (1970). Sodium dodecyl sulfate-polyacryl-amide gel electrophoresis (10 % SDS-PAGE) with a chemiluminescent detection system was used. The 12 000 g-supernatant samples were diluted in a buffer solution (0.125 mol/l of Tris-HCl, 2 % w/v SDS, 20 % v/v glycerol, 0.02 % w/v bromophenol blue, and 5 % (v/v) 2-mercaptoethanol) and loaded onto gels after heating (4 min, 95 °C). There was 50 µg protein/lane for CYP1A and CYP3A, and 20 µg protein/lane for Hsp70. Proteins were run for 45 minutes (V = 100 V for 5 min and 200 V for 45 min), the gels soaked in blotting buffer (25 mmol/l of Tris, 192 mmol/l glycine, and 20 % (v/v) methanol, pH 8.3), and transferred to a nitrocellulose membrane (300 mm x 3 m, pore size 0.45 µm, Protran®, Whatman, Schleicher and Schuell, Germany) using Mini-Protean II apparatus (Bio-Rad, CA, USA) at 100 V for 90 min. The blot was probed with primary antibodies (600 ng/ml anti-CYP1A, MAb 1-12-3, provided by Dr. John Stegeman (Park et al. 1986); 1:1000 anti-CYP3A provided by Prof. Malin Celander (Celander et al. 1996), and 1:10000 anti-Hsp 70, MA3-006, BioReagents, USA) in blocking buffer for 2 h and, after washing in TBST, probed with 1:3000 secondary antibodies (HRP-labeled anti-mouse IgG, A9044, Sigma-Aldrich Chemie, Steinheim, Germany, for CYP1A and Hsp 70; and 1:1000 HRP-labeled donkey anti-rabbit IgG, SA1-200, BioReagents, USA, for CYP3A) for one hour.

The chemiluminescent substrate used for the detection of CYP1A and Hsp70 (II, III) was Immobilon™ Western HRP substrate (Millipore, MA, USA). The blots were scanned with the ChemiDoc XRS, and the pictures analysed using the Quantity One 4.6.3. program (Bio-Rad, CA, USA). For CYP3A (III) the chemiluminescent substrate was LumiGLO® (Upstate, NY, USA). The exposed films were scanned, and the pictures analysed using the Scion Image 4.0.2. program. Sample intensities were compared to each other and to positive controls, with each positive control fixed at a value of 100 %.

The antibodies used were not fish-specific, but detect their specific proteins in many vertebrate species. For this reason, juvenile rainbow trout livers expressing the proteins of interest were used as internal laboratory reference in all blots in order to make the blots comparable also between species. For CYP1A and Hsp70 our own internal laboratory references were livers of juvenile rainbow trout (*Oncorhynchus mykiss*) injected with *β*-naphtoflavone dissolved in DMSO-almond oil (1:1, 28 mg/kg). The fish were kept at 12 °C for four days, anesthetized with MS-222 (50 mg/l) and sacrificed. For CYP3A our reference was the liver of untreated rainbow trout, abundantly expressing this protein. The pooled livers were divided into parts and frozen immediately in liquid nitrogen. Once thawed on ice, the pieces were homogenized and 1 000 g supernatants were prepared and used the same way as the other samples.

3.2.5 Analysis of glutathione reductase activity (II, III)

The method (II, III) to measure glutathione reductase (GR) was based on studies of Massay and Williams (1965) and Rodriguez-Segade and coworkers (1978). In short, regarding the reaction of GSSG + NADPH + $H^+ \leftrightarrow$ 2 GSH + $NADP^+$, the consumption of NADPH was monitored as a decrease in the absorbance at 340 nm (A340) for six minutes. The samples were diluted in a buffer with pH 7.5 (50 mmol/l K_2PO_4, 1 mmol/l EDTA and 1 mg/ml albumin). The assay was performed on 96-well plates. The buffer containing GSSG had pH 7.5 (125 mmol/l K_2PO_4, 2.4 mmol/l of GSSG and 2.5 mmol/l EDTA). The buffer containing 0.543 mmol/l NADPH with pH 7.5 was made of 2.86 mmol/l mannitol and 0.143 mmol/l Trizma base. Auto-oxidations in the assay system were subtracted in order to control the specificity. Positive control was 0.05 U/ml glutathione reductase purchased commercially (Sigma Aldrich, MO, USA). The results of GR activities were calculated as µmol/min/mg/protein.

3.3 Statistical analysis

SPSS (version 15 for Windows) was used in statistical analyses. For the fold-data from qPCR (results as fold-units) (I, II), the logarithm ($\log_{10}$) was used to make the response scale linear (Hedges et al. 1999). The data from protein measurements (results as % -units) (II, III) were first transformed to homogenize their variance using the following equation (Piegorch & Bailer 2005): $z = \sin^{-1}(\sqrt{(y/100)})$, where z is the transformed result and y percent result. The normality was tested using the Shapiro-Wilk-test. The differences between the treatments were tested using two-way ANOVA. If an interaction was found for light treatment and PAH dose, one-way ANOVA with the Bonferroni test was used to test the effect of PAH concentration within a radiation group (=SOL– and SOL+ or UVR– and UVR+ groups were tested separately). Non-normally distributed groups of values were tested with the Mann-Whitney non-parametric test. A probability level of $p < 0.05$ was considered to be statistically significant.

4 RESULTS

4.1 Effects of retene and pyrene in rainbow trout liver without additional UV radiation (I)

In this experiment (I), the measured water concentrations after 2 h were 0.9 µg/l (± SD 0.2) for RET and 2.5 µg/l (± SD 0.2) for PYR, when the nominal values were 3.2 µg/l for RET and 2.9 µg/l for PYR. After 24 h exposure the measured concentrations from the same treatments were 0.5 µg/l (± SD 0.04) for RET and 1.4 µg/l (± SD 0.2) for PYR. Thus, after 2 and 24 h from the start of exposures, the measured concentrations of RET were 28 % and 16 % from the nominal (3.2 µg/l), respectively. For PYR, they were 86 % and 48 % from the nominal (2.9 µg/l), respectively.

The experiments received routine visible light with the period of 16 h each day, and on average light intensity was 699 lux (± SD 55). In those treatments, no UV radiation (less than 400 nm) was detected.

In this study, no mortality occurred, and there was no behavioral difference in comparison with control group of rainbow trout, except that in the ten-day experiment the fish appeared to be more sluggish at the highest concentrations of both PAHs.

By microarray, the results showed that sublethal effects were seen at all concentrations in both time periods. The two short term exposure times did not differ from each other very much. The changes were often the greatest at the medium dose of PYR and at the highest dose of RET. Out of 1800 genes, 99 genes showed technically significant expression changes in at least 2 of 3 exposed groups to PYR or RET at one of two exposure time points (1 or 10 days). Most genes responded to either PYR (68 %) or to both compounds (22 %), and only 9 % of the genes responded to RET (Fig. 8). Compounds induced genes as part of functions on protein folding, inflammation and metabolic toxicity. 15 of these 99 genes showed differential expression, meaning that markers responded to PYR only or to both PYR and RET, but not to RET alone. These genes are involved in inflammation, cellular and oxidative stress, metabolic toxicity, and regulation of cell cycle.

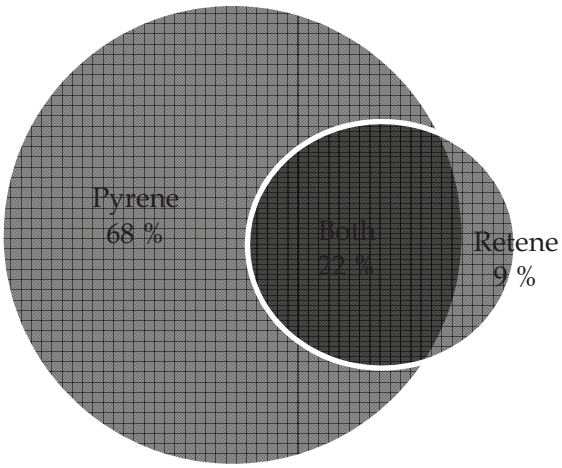


FIGURE 8 Proportions (%) of 99 genes that showed differential expression in rainbow trout exposed to retene and pyrene (I). The microarray contained totally 1 804 unique clones.

By qPCR, CYP1A showed dose-dependent induction by the exposure to RET (65-fold at the highest level, $p < 0.01$), meaning that CYP1A was a good biomarker for RET. CYP1A did not respond to PYR at these concentrations (Fig. 9).

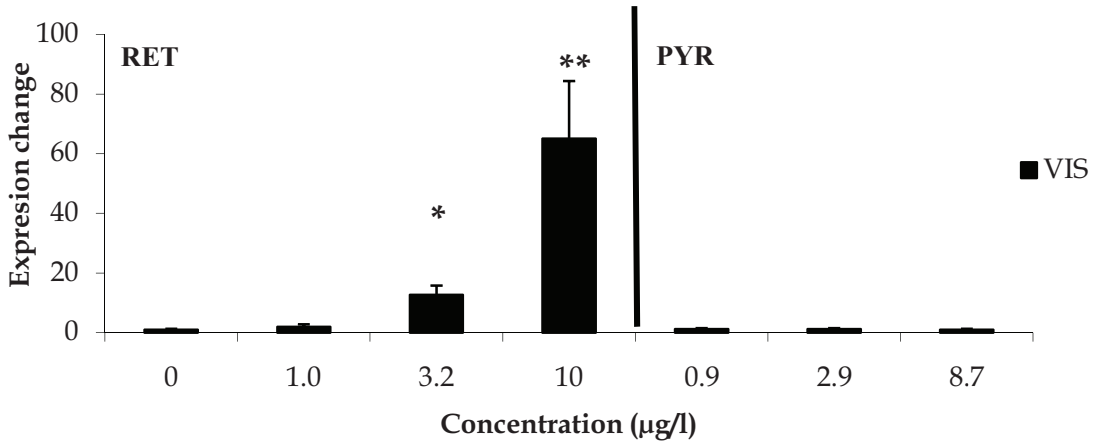


FIGURE 9 Expression change of CYP1A mRNA analyzed by qPCR in rainbow trout liver (mean ± SEM, I). Fish were exposed to RET and PYR in three equimolar nominal concentrations: low (1.0 and 0.9 µg/l), medium (3.2 and 2.9 µg/l), and high (10.0 and 8.7 µg/l), respectively, for one day. Control group (0) denotes treatment with the carrier (0.01 % DMSO). The expressions are normalized to corresponding NADH-ubiquinone oxidoreductase expressions. One analyzed sample was a pool from three livers, with three replicates per treatment. Each sample was run in two replicates in qPCR. Asterisk denotes significant difference from the respective control (* for $p < 0.05$, and ** for $p < 0.01$).

4.2 Effects of retene and pyrene in whitefish eleutheroembryos with and without solar light (II)

Besides the exposures with animals, in this experiment (II) the PAH concentrations were also measured from the treatments without fish. After 2 h, the highest measured concentrations were 6.2 µg/l for RET and 5.7 µg/l for PYR, when the nominal values were respectively 10 µg/l for RET and 8.7 µg/l for PYR. After 24, 48, and 72 h exposure, the measured concentrations from the same treatments dropped on average to 1 µg/l for RET and 2.4 µg/l for PYR. No PAHs were found in control aquaria. Thus, after 2 h and later the measured RET concentration averaged 60 % and 10 % from the nominal (10 µg/l), respectively. Corresponding values for PYR were averaged 65 % and 30 %, from the nominal (8.7 µg/l), respectively.

Solar light data was obtained from Lake Päijänne research platform (LTER 2008). For RET groups, exposed for 24 h and 48 h, the average three-hour exposure doses for total solar irradiation were 5965 and 4943 kJ/m^2. It corresponds with UV radiation band and CIE weighted UV doses 0.6 and 0.5 kJ/m^2, respectively. For PYR groups, the values were 6181 and 5677 kJ/m^2, and CIE weighted UV doses 0.7 and 0.5 kJ/m^2, respectively. In the laboratory the experiments received routine visible light with the period of 16 h each day, and on average light intensity was 333 lux (± SD 69). In those treatments, no UV radiation (less than 400 nm) was detected.

The cumulative mortality of fish in 72 hours was zero or close to zero among all treatment groups, on average 3.0 % in RET SOL– group, 2.8 % in PYR SOL– group, and 5.6 % in RET SOL+ group. Instead, SOL+ combined with PYR increased lethality dramatically, being over 90 % in low PYR, and up to 100 % in other higher concentrations.

Specific amplification of CYP1A and Hsp30, Hsp70, and Hsp90 were confirmed with amplicon sequencing. CYP1A and Hsp30 amplicons showed high homology to several comparable mRNA sequences in Rainbow Trout Gene Index database, and Hsp70 and Hsp90 in salmonid species in NCBI BLAST database.

Proteins of interest, CYP1A and Hsp70, were detected with immunoblotting. Furthermore, in order to study oxidative stress, activity of GR was analyzed. Due to high lethality among fish exposed to medium and high concentrations of PYR with SOL+, the analyses could not be performed in all these groups. In addition, only fish exposed to RET were subjected to gene expression analyses. Because of the great variability in one replicate of the three controls, probably because of mistakes in cDNA synthesis, the data from that sample was left out from the results.

By qPCR, CYP1A showed a dose-related induction by RET, both without and with solar treatment (Fig. 10). However, solar radiation enhanced the induction of CYP1A by RET, the difference between SOL+ and SOL– groups was statistically significant ($p < 0.05$). In SOL– the average fold difference between

control and the highest concentration was 6.1, in SOL+ it was as high as 22.9 (p < 0.05).

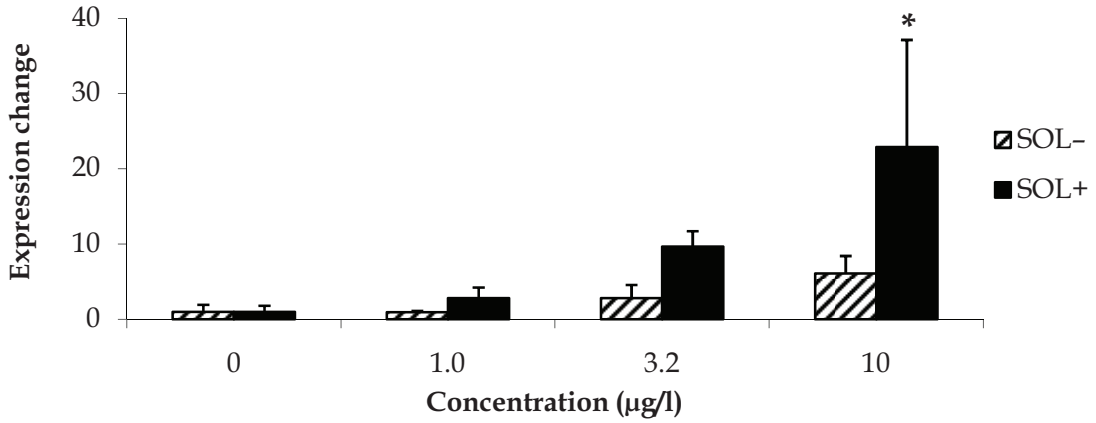


FIGURE 10 Expression change of CYP1A mRNA analyzed by qPCR in whitefish eleutheroembryos (mean ± SEM, II). Fish were exposed to RET in three nominal concentrations: low (1.0 µg/l), medium (3.2 µg/l), and high (10.0 µg/l), without (SOL–) and with (SOL+) solar light for 72-h. Control groups (0) denote treatment with the carrier (0.01 % DMSO). The expressions are normalized to corresponding 18S rRNA. One analyzed sample was a pool from five eleutheroembryos, with three replicates per treatment. Analyzes were done twice by qPCR. The control represents data only from two replicates. Asteriks denotes significant difference from the respective control (* for p < 0.05). Overall, solar radiation (SOL+) increased CYP1A levels in RET treatments compared to corresponding SOL– groups (p < 0.05).

By immunoblot, CYP1A showed an increasing dose-dependency by RET both in SOL– and SOL+ treatments. There was about two times more CYP1A at the highest concentration than in the control and low RET in both SOL– and SOL+ exposures (p < 0.01 when comparing high with all other treatments in SOL– groups; p < 0.01 when comparing high with control, and p < 0.05 when comparing high with low in SOL+ groups). Overall, differences in CYP1A between SOL+ and SOL– RET treatments were highly significant (p < 0.01). Among samples analyzed, PYR did not induce CYP1A protein, or treatments did not differ from control.

By qPCR, Hsp30 showed induction in SOL+, and the difference between SOL+ and SOL– groups was statistically significant (p < 0.05). The average induction was highest at the medium concentration of RET with SOL+ treatment, the difference being 3.7-fold that of control. Among RET treatments without solar radiation (SOL–), no statistically significant increase in Hsp30 expression was detected.

By qPCR, Hsp70 showed a dose-related induction by RET with solar treatment, and the difference between SOL+ and SOL– groups was statistically significant (p < 0.01). Solar radiation enhanced the cellular stress response as

expressed as Hsp70 by RET. In SOL– the average difference between the control and the highest concentration was 1.3-fold, whereas in SOL+ it was as high as 14-fold ($p < 0.05$).

By immunoblot, RET seemed to increase the levels of Hsp70 both with and without solar exposure. There were 1.4- and 1.7-fold more Hsp70 at the highest concentration that of the control in RET SOL– and SOL+ exposures, respectively. PYR also seemed to induce Hsp70 levels in an increasing dose-related manner both with and without solar exposure. There was 1.4-fold more Hsp70 at the highest concentration that of the control in SOL– treatment. However, the differences were not statistically significant in any of the analyzed treatments with RET or PYR. Even though there seemed to be more Hsp70 induction in SOL+ in both RET and PYR treatments, the differences between SOL+ and SOL- groups were not significant.

By qPCR, Hsp90 showed a dose-related induction by RET with solar treatment, and the difference between SOL+ and SOL– groups was statistically significant ($p < 0.001$, Fig. 11). Thus, solar radiation enhanced the cellular response as expressed as Hsp90 by RET. In SOL+ the highest average induction was between control and medium concentration, the difference in relation with the control group being 7.9-fold ($p < 0.01$). The highest and lowest concentrations of RET in SOL+ also induced Hsp90 significantly, with $p < 0.01$ and $p < 0.05$, respectively, compared to control.

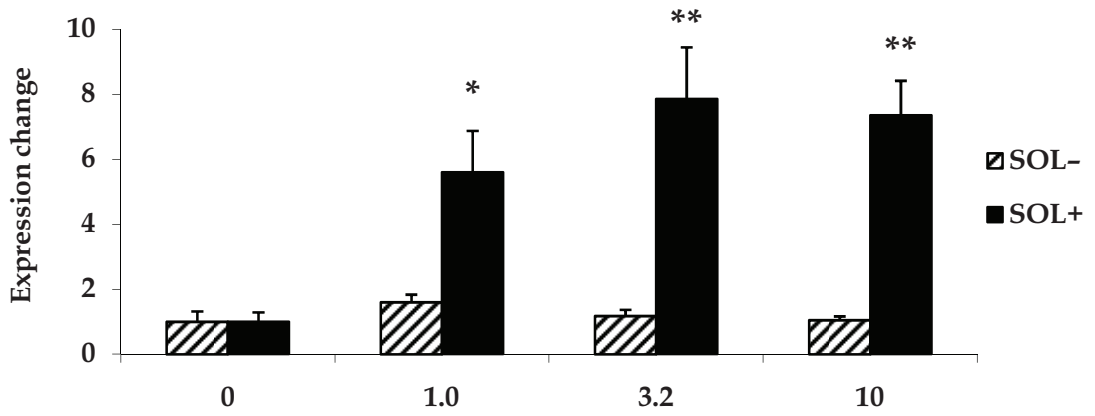


FIGURE 11 Expression change of Hsp90 mRNA analyzed by qPCR in whitefish eleutheroembryos (mean ± SEM, II). Fish were exposed to RET in three nominal concentrations: low (1.0 µg/l), medium (3.2 µg/l), and high (10.0 µg/l), without (SOL–) and with (SOL+) solar light for 72-h. For the details, see legend to Fig. 10. Asteriks denotes significant difference from the respective control (* for $p < 0.05$ and ** for $p < 0.01$). Overall, solar radiation (SOL+) increased Hsp90 levels in RET treatments compared to corresponding SOL– groups ($p < 0.001$).

In the activity measurement, there were no differences between the various RET groups in the whole-body activity of GR in whitefish (Fig. 10). However, GR increased due to solar exposure in fish exposed to RET ($p < 0.05$). In the PYR-

treated groups, GR activity increased in a dose-dependent manner in SOL–. It was significantly higher when exposed to high PYR compared to lower concentrations and control ($p < 0.01$ when comparing high to control and medium, $p < 0.05$ when comparing high to low). Finally, there was 1.4-fold more GR at the highest PYR concentration that of the control.

4.3 Effects of retene and pyrene in whitefish and pike eleutheroembryos with and without artificial UV radiation (III)

In this experiment (III), after 24 h the measured RET concentration in waters averaged 60 % from the nominal concentration of 100 µg/l. After 72 h, despite two partial (50 %) replacements of exposure waters the measured RET concentration in UVR– treatment was on average 40 % and in UVR+ treatment 10 % from the nominal (100 µg/l). The water concentration of PYR was more stable than that of RET, and after 72 h the measured PYR levels from UVR- and UVR+ treatments were 80 % and 65 % from the nominal (10 µg/l), respectively.

UVR+ was quantified using a Hamamatsu Photonic Multichannel Spectral analyzer. The total three-hour irradiance dose for UVA treatment was 28 kJ/m^2, and for UVB 30 kJ/m^2. In addition, CIE-weighted dose was calculated only for UVB treatment and was 3.3 kJ/m^2. UVR– treatments received standard visible light with the period of 12 h each day.

The cumulative mortality after 72 hours in clean and solvent (DMSO) controls was low. In whitefish, it was 0 % in all cases, except for one control in which it was 0.8 %. In pike, excluding three control groups with mortalities from 4.2 to 5.8 %, all the other treatments revealed values between 0.8 and 2.5 %. While the UVR+ treatment alone or the exposures to RET and PYR in visible light did not change the mortality in relation to controls in either species, UVR+ dramatically increased this response when combined with PAH.

In whitefish irradiated with UVR+, over 80 % of the specimens died in the highest RET treatment (100 µg/l), but the lower concentrations (10 and 32 µg/l) did not cause mortality. All whitefish died in the PYR UVR+ treatments. The mortality of pike was lower than that of whitefish, pike receiving visible light only similar to controls (maximally on average 3.3 %). Likewise, in the UVR+ treatments with RET the mortality was similar between the treatment and control groups (average 2.2 %). In the UVR+ treatments with PYR, the two highest concentrations (32 and 100 µg/l) caused 100 % mortality.

The behavioral changes were monitored only after 72 hours. In whitefish, PYR caused behavioral changes even under visible light at concentrations 32 and 100 µg/l. Simultaneous exposure to UVR+ at the two highest concentrations of RET (32 µg/l and 100 µg/l) caused behavioral abnormalities in a dose-related manner. In pike, there were abnormally behaving fish in the UVR+ treatment with the highest concentration of RET (100 µg/l).

Overall, the sublethal results from the clean-water controls were similar to those of the solvent controls, the latter ones being used for statistical comparisons. Due to almost complete mortality in the high exposure concentration of RET with UVR+, sublethal data from high RET UVR+ are missing.

By immunoblot, in whitefish RET seemed to increase levels of CYP1A protein in association with dosed chemical, both in the presence and absence of UV– radiation (Fig. 12); however, this difference was not statistically significant. There was 4.4-fold CYP1A at medium concentration that of the control in UVR+ and 1.8 times more at the highest that of the control in UVR– exposure. Again, PYR did not induce CYP1A protein. In pike, only very faint CYP1A bands were detectable in the specimens exposed to retene in all treatments, making quantification impossible.

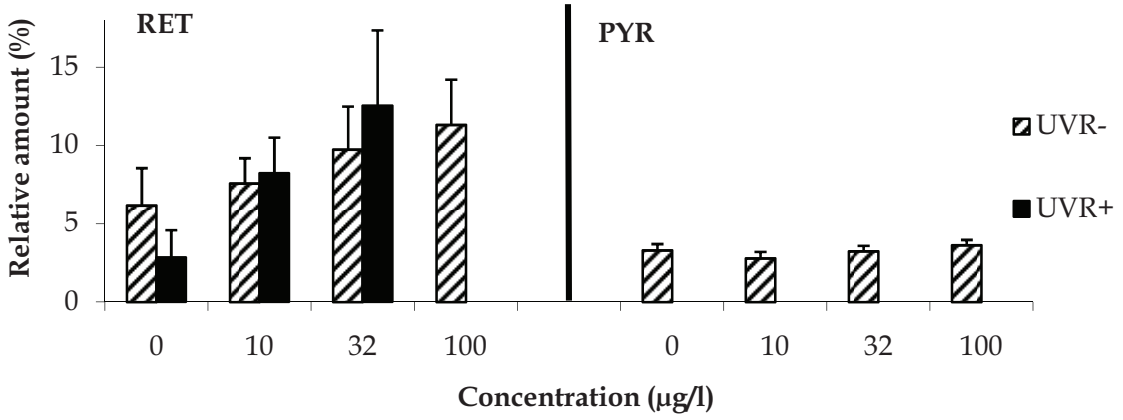


FIGURE 12 Relative amount (band intensities vs. positive control) of CYP1A protein by immunoblot in whitefish eleutheroembryos (mean ± SEM, III). Fish were exposed to RET and PYR in three nominal concentrations: 10, 32, and 100 µg/l, without (UVR–) and with (UVR+) UV radiation for 72-h. Control groups (0) denote treatment with the carrier (0.01 % DMSO). One analyzed sample was a pool from five eleutheroembryos, with three replicates per treatment.

By immunoblot, CYP3A in whitefish was detected in all the exposures, but there were no significant differences between the treatments or when comparing the treatments with UVR– to UVR+. CYP3A was not detected in pike by the technique used.

By immunoblot, the Hsp70 protein was detected in the eleutheroembryos of all the treatments in both species. In whitefish, UVR+ increased Hsp70 in RET experiment ($p < 0.001$), being 1.5-fold Hsp70 at medium concentration that of the control. The highest RET concentration (100 µg/l) induced Hsp70 in the absence of UV radiation ($p < 0.05$), being 3.1-fold Hsp70 at the highest concentration that of the control. In whitefish in visible light, the amount of Hsp70 was significantly higher at 10 µg/l PYR than in the other two treatments ($p < 0.05$, when comparing the control with 10 µg/l, and $p < 0.001$, when comparing 10 µg/l with

32 µg/l or 100 µg/l), being 1.9-fold Hsp70 at the lowest concentration that of the control.

By immunoblot, in pike UVR+ had a significant effect by RET on Hsp70 ($p < 0.01$). The two highest concentrations of RET induced Hsp70 ($p < 0.05$, for 32 µg/l and 100 µg/l), expressing on average 3.4-fold Hsp70 that of the control. However, UVR+ attenuated this response: exposure to RET under UVR+ did not induce Hsp70.

The total body activity of GR was also measurable in the post-hatched embryos of both species, yet it was approximately 90 % lower in pike than in whitefish. In whitefish, neither RET nor UVR+ influenced the activity of GR. Instead, the highest concentration of PYR (100 µg/l) significantly decreased the GR relative to control in the treatment with visible light ($p < 0.01$), expressing half of Hsp70 that of control. In pike, UVR+ increased GR activity ($p < 0.001$), and the medium (32 µg/l) and highest (100 µg/l) concentration of PYR significantly increased the GR relative to the lowest concentration in the treatment with visible light ($p < 0.05$ when comparing 10 µg/l to 32 µg/l and 100 µg/l). There was 6.1- and 3.2-fold GR at medium and the highest concentrations, respectively, that of the control.

5 DISCUSSION

5.1 Experimental conditions in fish exposures

Water volume-to-biomass ratios (2 l/g/day with rainbow trout, I, 7.9 l/g/day with whitefish, II, and 10-13 l/g/day with whitefish and pike, III) were high in all the experiments. Therefore it was supposed that PAH absorption only into fish was low compared to the total amount of PAH, even though absorption into fish is a precondition for the biological responses.

The tendency of PAH concentration in water was decreasing over time in all the studies, also in the additional water experiment (II), even though no fish was used there. In conclusion, relative concentrations of RET in water were lower than PYR ones (maximum and minimum concentrations of RET and PYR were 60 and 10 %, and 86 and 30 %, respectively). The decrease of PAH concentration in water was probably due to the relatively rapid absorption on the glass walls of the experimental bowls and all the other laboratory materials used. The absorption can be explained by the high hydrophobicity of RET with log K_{ow} 6.4 (Basu et al. 2001) and the relatively high hydrophobicity of PYR with log K_{ow} 4.92 (Di Toro et al. 2000). It appears that decreasing levels of RET under exposures conducted in glass-walled aquaria have been common also in several previous studies. For instance, measured retene concentrations in water averaged 83 % of the nominal concentration (100 µg/l) within two hours following the addition, being 24 h dropped to 24 % of the nominal in studies with rainbow trout embryo (Scott et al. 2009). Secondly, light may also change these compounds via photomodification (Ren et al. 1996, McConkey et al. 1997). UVR photomodified retene and decreased its concentration by 63 % in 3 h (Häkkinen et al. 2004). Pyrene concentrations in water were also measured to be 19-26 % lower than the nominal concentrations (20-150 µg/l) after 18 days (Hendon et al. 2008).

All experiments contained routine visible light cycle (photoperiod 16:8 h light:dark in I and II, and 12:12 h light:dark in III). The experiment without additional UV radiation (I), and control groups for additional light conditions (i.e. SOL– in II, and UVR– in III) received only standard visible light cycle. The light intensity ranged between 300-600 lux, and no UV radiation (less than 400 nm) was detected. Two series of experiments provided additional light conditions, the solar light (i.e. SOL+ in II) and artificial UVR (UVR+ in III), in 3 h periods on two consecutive days.

Many PAHs exert phototoxicity in the presence of UV radiation (Arfsten et al. 1996). Also single UVA (Diamond et al. 2008), or even visible light (Wang et al. 2007a), can cause PAHs to become phototoxic. We suggest that the standard visible light condition, included in all experiments (I, II, III), represented the base line for the assessment of toxicity of these PAHs, and that other additional light conditions (II, III) could change the effects from the base line condition.

The average irradiation doses and CIE-weighted doses for total 3-h periods in the additional light conditions (II, III) are shown in Table 2. Total energy was much stronger in solar light condition (II, on average 5692 kJ/m^2) than only in artificial UVR condition (III, on average 29 kJ/m^2), which was as presumed. Solar light is more energetic than UV radiation area only; total solar spectrum consists of different wavelengths above 1000 nm, including UV radiation area (less than 400 nm, Simon 1997, Sasha & Flocke 1997). However, CIE-weighted dose was much stronger in UVR+ condition (III, on average 3.3 kJ/m^2) than in solar light condition (II, on average 0.6 kJ/m^2). CIE-weighted dose is calculated for the UVB area, which affects the erythema for organism's skin (McKinlay & Diffey 1987).

TABLE 2 The average irradiation doses and CIE-weighted doses for total 3-h periods in solar light (SOL+ in II) or artificial UVR (UVR+ in III) conditions.

	SOL+ (II)	UVR+ (III)
Irradiation dose (kJ/m^2)	5692	29
CIE-weighted dose (kJ/m^2)	0.6	3.3

Solar light experiment was carried out in May in Central Finland. Outdoors irradiation doses vary substantially depending on day and season, being less in spring than in midsummer in Finland. For example, the maximum irradiation doses for 3-h period were 11 545 kJ/m^2, 9180 kJ/m^2, and 7430 kJ/m^2, and CIE-weighted doses 1.4 kJ/m^2, 1.1 kJ/m^2, and 0.7 kJ/m^2 in July, May, and April 2008, respectively, in Lake Jyväsjärvi, Central Finland. This data was obtained from the same source as the measurements done in our solar light experiment (II, LTER 2008).

In conclusion, the measured PAHs concentrations in water were lower than the nominal ones, and as the more lipophilic compound RET concentrations were even lower than PYR. All in all, despite the unstable conditions in experimental waters, the differences between the three dose levels and replicates were maintained throughout the experiment. In this thesis, the results are discussed as nominal water concentrations. From the additional light conditions, solar light treatment represents a more natural situation than the artificial UVR-treatment only. Solar light included more energy, but CIE-weighted dose was less than in artificial UVR, as calculated only for UVB-wavelength area. In addition, irradiation doses outdoors can vary between seasons, so it is always important to specify the quality of light, irradiation dose, and weighted dose.

5.2 Effects to individuals

Effects to individuals have been documented as mortalities and behavioral changes of fish.

5.2.1 Mortality

RET was not acutely lethal at these concentrations to any of the studied fish species (I, II, III). On the other hand, RET is known to be lethally phototoxic to aquatic organisms, e.g. post-hatched embryos of fish (Vehniäinen et al. 2003, Häkkinen et al. 2004). However, RET was not lethally phototoxic to whitefish by solar light (CIE dose 0.6 kJ/m^2, II), or to pike by UV radiation (CIE dose 3.3 kJ/m^2, III) within used concentrations (range 1-100 µg/l) applied nominally. (Nevertheless, RET was lethally phototoxic to whitefish by artificial UVR at 100 µg/l, where less than 20 % of fish survived (CIE dose 3.3 kJ/m^2, III).

In comparison to previous studies, RET induced some differences in mortality due to photoinduced toxicity in whitefish (II, III). Some mortality was seen already with UVR+ treated (CIE dose 2.8 kJ/m^2) RET at 10 µg/l in whitefish (Vehniäinen et al. 2003, Häkkinen et al. 2004). The reasons for this can simply be lower concentrations of RET (maximum nominal concentration of 10 µg/l) and lower CIE-weighted UV dose in experiment with solar light (II) than in previous experiments (Vehniäinen et al. 2003, Häkkinen et al. 2004). Another reason for the lesser mortality might have been the variable source and taxonomy of the whitefish stocks, *Coregenus lavaretus pallasi,* used in previous studies (Vehniäinen et al. 2003, Häkkinen et al. 2004), whereas *Coregenus lavaretus lavaretus* was used in these studies (II, III). It was also noted earlier that there are differences in tolerance to UVR between populations (Vehniäinen et al. 2007b).

In visible light without UVR, PYR was not acutely or subacutely lethal at these concentrations to any of the studied fish species (I, II, III). Pyrene's acute LC50 in 4-day exposure was more than 125 µg/l in rainbow trout (Krasnov et al. 2005b), but with concentration of 100 µg/l it did not affect acute lethal toxicity in rainbow trout fry (Koskinen et al. 2004). Pyrene is known to be phototoxic to a number of aquatic organisms, including microbes, plants, invertebrates, and vertebrates, in the presence of UV radiation (Oris and Giesy 1985, Huang et al. 1995, Arfsten et al. 1996, Boese et al. 1997, Hatch & Burton 1998). Also in our studies PYR was extremely phototoxic (II, III). It induced over 80 % mortality to whitefish already at low concentration (less than 1 µg/l) by solar light (II). Additionally, 100 % mortality occurred with whitefish, and over 80 % with pike even at concentration of 10 µg/l by artificial UVR+ (III).

Whitefish eleutheroembryos were used in two experiments (II, III). Figures 13 and 14 show the total mortality of whitefish at different light conditions exposed to RET or PYR, respectively. The data is a combination of both experiments.

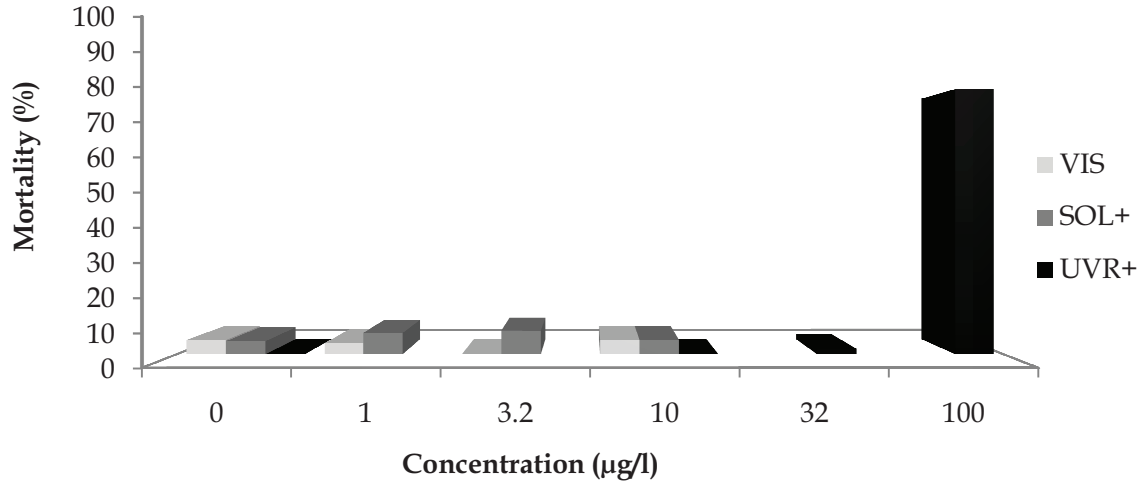


FIGURE 13 Total mortality (%) of whitefish to retene at different light conditions (VIS in II and III, SOL+ in II, UVR+ in III). Fish were exposed to RET with nominal concentrations at range of 1 to 100 µg/l. VIS = visible light, SOL+ = solar light, UVR+ = artificial UV radiation. Visible light data is a combination of mortality from two series experiments without additional light (i.e. SOL– in II, and UVR– in III).

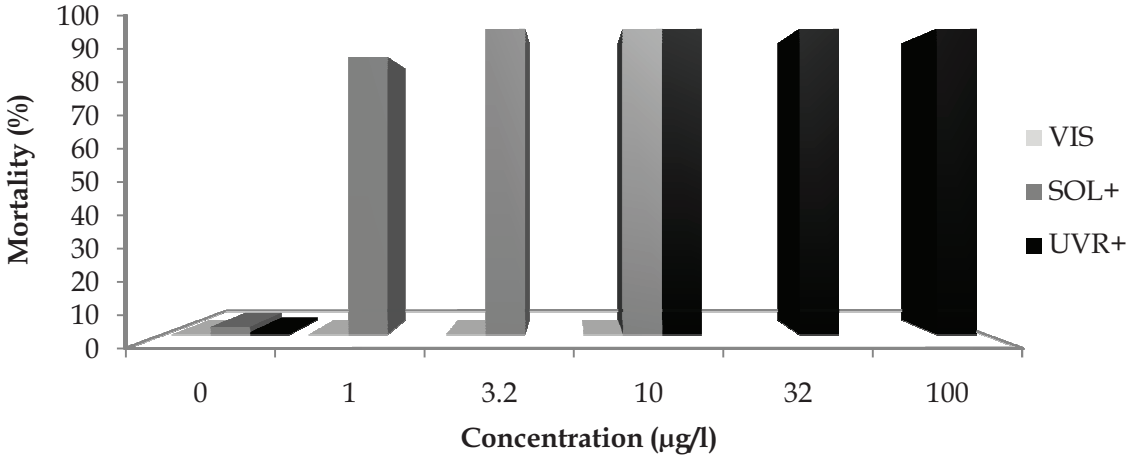


FIGURE 14 Total mortality (%) of whitefish to pyrene at different light conditions (VIS in II and III, SOL+ in II, UVR+ in III). Fish were exposed to PYR with nominal concentrations at range of 0.9 to 100 µg/l. VIS = visible light, SOL+ = solar light, UVR+ = artificial UV radiation. Visible light data is a combination of mortality from two experiments without additional light (i.e. SOL– in II, and UVR– in III).

5.2.2 Behavioral changes

Both PAHs caused behavioral difference (sluggishness) in rainbow trout at 10 µg/l concentrations in comparison with control group in 10-d experiment (I). PYR caused behavioral changes (fish lying on their side at the bottom of the treatment bowl or exhibiting spiral swimming) in whitefish at concentrations of 32 µg/l and above without UVR (III). In whitefish, RET with UVR+ caused behavioral abnormalities at concentration of 32 µg/l and more severe ones at 100 µg/l (III). In pike, behavioral changes were seen at 100 µg/l of RET with UVR+ (III). In previous studies, retene with UVR (Häkkinen et al. 2004) and only UV radiation (Vehniäinen et al. 2007a) induced behavioral changes, i.e. spiral swimming, in pike larvae.

5.2.3 Conclusions on the effects to individuals

In conclusion, these PAHs were not lethally toxic at the studied concentrations to fish in visible light conditions, but RET was acutely phototoxic by solar light to whitefish, and PYR extremely phototoxic to whitefish and pike. All these results confirm the previous studies. Overall, phototoxicity exists in whitefish and pike, and whitefish were more sensitive to it than pike, as previously documented (Vehniäinen et al. 2003, Häkkinen et al. 2003, 2004). Thus, wide variation may be expected among species in the sensitivity to the toxicities and photoinduced toxicities of PAHs.

5.3 Effects to cells and tissues

Effects to cells have been documented as measurements of protein and transcriptomic endpoints.

5.3.1 Cytochrome P450 (CYP1A) as a biomarker

CYP1A plays an important part in cellular biotransformation of PAHs and other xenobiotics (Klaassen 2007). RET is known to induce CYP1A in fish (Fragoso et al. 1999, Hawkins et al. 2002, Vehniäinen et al. 2003, Häkkinen et al. 2004).

RET induced CYP1A mRNA and protein in dose-related manner in all analyses (I, II, III), but significantly only in the following cases: as mRNA in rainbow trout liver when comparing control to 3.2 µg/l ($p < 0.05$) and to 10 µg/l ($p < 0.01$) (I), as mRNA in whitefish when comparing control to 10 µg/l ($p < 0.05$) in SOL+ treatment (II), and as CYP1A protein in whitefish when comparing high (10 µg/l) to all other concentrations ($p < 0.01$) in SOL– treatment, and when comparing high to control ($p < 0.01$), and high to low ($p < 0.05$) in SOL+ treatment (II). Dose-dependent induction of CYP1A protein also occurred in vendace and whitefish (Vehniäinen et al. 2003) and pike (Häkkinen et al. 2004) embryos. This confirms again CYP1A's status as a good biomarker for RET. In addition, this could indicate that retene toxicity and metabolism correlate by CYP1A (Fragaso et al. 1999, Hawkins et al. 2002) in retene's half life 14-h in fish (Fragaso et al. 1999). Furthermore, in our study the fold difference was very strong in CYP1A mRNA in rainbow trout liver, being 65-fold between control and the highest (10 µg/l) concentration, indicating also increased capacity of hepatic biotransformation via CYP1A.

PYR did not induce CYP1A mRNA or protein at used concentrations in rainbow trout liver (I) or whitefish eleutheroembryos (II, III). Similarly, CYP1A was not induced by PYR in mice (Shimada et al. 2006), or its activity in yolk-sack fry salmon (*Salmo salar*) (Honkanen et al. 2008). However, there exist also contrary observations where PYR induced CYP1A in Nile tilapia (*Oreochromis niloticus*) (Zapata-Pérez et al. 2002) and in sheepshead minnow (*Cyprinodon variegatus*) (Hendon et al. 2008). Also, high concentrations of pyrene induced CYP1A activity in zebrafish embryos (Incardona et al. 2005, 2006.) In conclusion, one may suggest that there are differences in CYP1A function between species.

Dose-dependent results of CYP1A also indicated the different dose levels of RET in all water treatments. In others words, even though PAH concentrations were not always stable in water, increased induction of CYP1A would be used to prove the different dose levels in the treatments.

Light had an effect on CYP1A induction as contributor of PAH phototoxicity. CYP1A mRNA and protein induction was stronger in whitefish exposed to RET with solar light (CIE dose 0.6 kJ/m^2) than in treatment without it (SOL+ vs. SOL– $p < 0.05$ and $p < 0.01$, respectively, II), and with UVR+ (CIE dose 3.3 kJ/m^2) than in treatment without it (III). When comparing only controls, there

was more CYP1A protein in solar-treated (II) control, but less CYP1A protein in UVR-treated (III) control whitefish than control without additional light. In the previous study, CYP1A induction was stronger in UVR+ treated (CIE dose 2.8 kJ/m^2) control whitefish than controls without UVR (Vehniäinen et al. 2003). However, CYP1A protein inductions were nearly the same in whitefish between treatment with UVR (CIE dose 5.4 kJ/m^2) and without it (Vehniäinen et al. 2003). In other studies, UV radiation induced CYP1A activity in mouse liver (Goerz et al. 1983), and CYP1A mRNA in human cell lines when comparing to control (Wei et al. 1999). We suggest that solar light and UVR have an effect on CYP1A induction, but it can vary between different light conditions.

In conclusion, CYP1A mRNA and protein were induced in studied salmonidae by RET in increasing dose-related, even in some cases dose-dependent, manner at all light conditions. This confirms CYP1A as a good biomarker for RET. Interestingly, PYR did not induce CYP1A at these concentrations, an important difference between these PAHs. Even though CYP1A has been widely used as biomarker for PAH exposure (Whyte et al. 2000), we suggest that it cannot be generalized to all PAHs or species. In addition, solar light with RET induced CYP1A protein, suggesting that it can be used as a biomarker for RET phototoxicity.

5.3.2 Heat shock proteins (Hsp) as stress biomarkers

Hsp30 has been found from fish (Norris et al. 1997, Currie et al. 2000, Stensløkken et al. 2010) but has not yet been commonly used as endpoint at research of environmental toxicity. The present study showed that Hsp30 mRNA did not respond to RET in SOL–, but was induced in whitefish by RET in SOL+ (II), suggesting some role for the solar light in enhancing toxicity of RET.

Hsp70 induction can be used as a marker for organism's stress (Lewis et al. 1999). Hsp70 is generally known to be part of protein folding (Iwama et al. 2004). It helps other proteins to fold correctly by keeping their conformation, transferring proteins into their right compartment in the cell, and tagging incorrectly folded proteins for degradation (Hartl 1996, Bukau and Horwich 1998). Moreover, Hsp70 proteins are part of many multi-protein complexes in tissues, and they also have a role in endo- and exocytosis and have some protective function in cell apoptosis (He & Fox 1997, Mosser et al. 2000, Zinsmaier & Bronk 2001).

RET induced Hsp70 protein in dose-related manner in whitefish and pike (II, III), except in exposure to RET with UVR+ in pike (III), where UVR+ diminished Hsp70 amount. Hsp70 was induced significantly only in the following cases: as mRNA in whitefish when comparing control to 10 µg/l ($p < 0.05$) with solar light (II), as protein in whitefish when comparing control to the highest RET concentration (100 µg/l, $p < 0.05$) without UVR (III), and as protein in pike when comparing control to two highest RET concentrations (32 and 100 µg/l, $p < 0.05$) without UVR (III). Hsp70 was induced also in whitefish by RET

and RET with UVR (Vehniäinen et al. 2003). This suggests Hsp70 as a good biomarker for RET in whitefish and pike.

PYR appeared to induce Hsp70 protein in dose-related manner in whitefish eleutheroembryos (II, III), but only up to 10 µg/l concentration. The only significant difference occurred when comparing the control to 10 µg/l (p < 0.05, III). There was no dose-dependent effect towards increasing concentrations, which can be a signal for PYR toxicity in whitefish eleutheroembryos (III). In pike, PYR did not induce Hsp70 (III).

As mentioned, Hsp90 is part of the AhR-complex. Observations reveal that inhibitors of Hsp90 suppress the activation of AhR-dependent gene expression induced by PAH (Hughes et al. 2008). The present study shows that this is true also by RET phototoxicity (II). Hsp90 showed a dose-related induction by RET with solar treatment. Interestingly, Hsp90 was induced strongly already with low (nominal 1 µg/l) concentration of RET. In contrast, Hsp90 was not induced without solar light by RET.

Light had an effect on several types of Hsp as markers of PAH phototoxicity. UVR is known to induce Hsp70 (Lewis et al. 1999). In our studies, solar light with RET increased Hsp70 mRNA (SOL+ vs. SOL– p < 0.01) and protein induction (II), and also UVR+ with RET increased Hsp70 protein (UVR+ vs. UVR- p < 0.001, III) in whitefish eleutheroembryos. However, UVR instead decreased Hsp70 (UVR+ vs. UVR– p < 0.01, III) in pike, substantiating earlier observations (Vehniäinen et al. 2003, Häkkinen et al. 2004). Solar radiation with RET increased also Hsp30 (SOL+ vs. SOL– p < 0.05) and Hsp90 (SOL+ vs. SOL– p < 0.001) mRNA induction in whitefish (II), meaning that also these Hsps can be used as a biomarker for RET phototoxicity.

In conclusion, RET and PYR toxicities or phototoxicities affect the cellular stress response as expressed as heat shock proteins, and mainly as Hsp70, on fish. Also, wide variations can be expected in cellular Hsp inductions between species and environmental conditions.

5.3.3 Glutathione reductase (GR) and oxidative stress

In our studies GR activity was used as another parameter of cellular stress (Meister & Anderson 1983, Boelsterli 2007). Some PAHs can induce oxidative stress in fish (Timme-Laragy et al. 2009). Also UVR is generally known to induce oxidative stress (Birch-Machin & Swalwell 2010).

RET induced some oxidative stress parameters, glutathione peroxidase and 15 kDa selenoprotein, by qPCR in rainbow trout liver (I) but not significantly. However, in whitefish eleutheroembryos there were no significant differences in GR activities between any RET treatments (II, III). Instead, GR activity was increased by RET in pike in visible light (III) but not significantly. SOD (superoxide dismutase, also a parameter of oxidative stress) was increased by retene in pike but not significantly (Häkkinen et al. 2004). In addition, retene did not change whole body lipid peroxide concentrations but had minimal effect

as reduction of glutathione status in rainbow trout (*Oncorhynchus mykiss*) (Bauder et al. 2005). These findings concluded that there is no clear evidence that RET could induce oxidative stress. Bauder and coworkers (2005) concluded that retene toxicity is not induced by oxidative stress but by some other stress function.

PYR induced genes related to oxidative stress (glutathione peroxidase and two selenoproteins) by microarray, and induced oxidative stress genes (glutathione peroxidase and 15 kDa selenoprotein) by qPCR in rainbow trout liver (I) but not significantly. It also induced GR activity in dose-dependent manner in the following cases: in whitefish when comparing the highest (8.7 µg/l) concentration to the other groups ($p < 0.05$) without solar light (II), in pike when comparing 32 and 100 µg/l concentrations to lower concentration, and to control ($p < 0.05$) without UVR (III). By comparison, PYR induced some oxidative stress genes in rainbow trout fry (*Onchorhynchus mykiss) (*Koskinen et al. 2004). It also significantly induced one oxidative stress enzyme, catalase, in Atlantic salmon fry (*Salmo salar*) (Honkanen et al. 2008). Pyrene can induce oxidative stress *in vitro* (measurement of 8-oxodeoxyguanosine) (Botta et al. 2008). In conclusion, all these indicate oxidative stress induced by PYR.

Light conditions had an effect to GR activity as revealed as part of PAH phototoxicity. GR activity was increased in whitefish by RET with solar light (SOL+ vs. SOL– $p < 0.05$, II), and in pike by PYR with UVR+ (UVR+ vs. UVR– $p < 0.001$, III). These increased GR activities indicate oxidative stress connected to phototoxicity of certain PAHs.

In conclusion, GR was induced in whitefish and pike eleutheroembryos, even though there was less GR activity in pike compared to whitefish. PYR toxicity was associated with oxidative stress in all species.

5.3.4 Transcriptional changes of gene activity (I)

Both PAHs, studied at the same equimolar concentrations, PYR induced some similar gene expressions as seen in our previous studies (Koskinen et al. 2004, Krasnov et al. 2005b, 2007), but the data from RET at transcriptomic changes was new (I). By microarray, RET and PYR changed expressions of many genes, but mainly they were part of functions on protein folding, inflammation, and metabolic toxicity. An interesting difference between RET and PYR was that only RET induced CYP1A in dose-dependent manner by qPCR. At the range of 0.9-10 µg/l-concentrations, PYR caused very small, and RET even smaller responses in gene expressions in juvenile rainbow trout liver.

5.3.5 Concluding remarks on the effects to fish tissues

RET and PYR under routine UVR-free lighting conditions induced sublethal transcriptomic effects in rainbow trout liver studied concentrations (at range of 0.9-10 µg/l, I), transcriptomic and protein effects in whitefish eleutheroembryos in all concentrations (at range of 0.9-10 µg/l, II), and protein effects in whitefish and pike eleutheroembryos in all concentrations (at range of 10-100 µg/l, III). Sublethal photoeffects were studied in whitefish (II, III) and in pike (III) eleutheroembryos.

RET toxicity and phototoxicity or additional light were linked to CYP1A biotransformation studied on mRNA and protein levels, and cellular stress response as expressed as Hsp70. RET did not induce significantly any oxidative stress parameter, and thus there is no clear evidence that RET could induce oxidative stress. Some behavioral changes, and decreased Hsp70 and GR activity at concentration of 100 µg/l with UVR treatment in whitefish and pike eleutheroembryos (III), can be early warning signals for RET lethal phototoxicity.

PYR was not biotransformated by CYP1A in studied fish species. Cellular stress response as expressed as Hsp70 was induced by PYR toxicity and phototoxicity or additional light. PYR induced oxidative stress. Some behavioral changes, decreased Hsp70 at concentration of 32 and 100 µg/l, and decreased GR activity at concentration of 100 µg/l without UVR treatment in whitefish eleutheroembryos (III) can be early warning signals for PYR lethal toxicity.

In conclusion, sublethal responses in liver may differ from the responses in whole body, and responses appeared to be stronger in whitefish than in pike eleutheroembryos. In addition, sublethal responses varied depending on exposure to PAHs or light condition. Thus, wide variation may be expected among tissues, species, and light conditions in the sensitivities to different PAHs.

6 CONCLUSIONS

Both environmental and developmental conditions affect the expression of genes in a cell. In general, only some specific genes are expressed at a certain time in any cell type or tissue. In this study, normal environmental conditions were disturbed with two different environmental chemicals, PAHs (RET or PYR), at different light conditions with juvenile rainbow trout and post-hatched embryo stages of whitefish and northern pike. Light conditions were experimented without additional UV radiation (I), with solar light (II), or with artificial UV radiation (III). We suggest that all visible light conditions were providing the base line for the toxicity of these PAHs.

We tried to find answers to the questions: how RET or PYR, as separate compounds, affect individuals and cells of fish at different light conditions, what could be the mechanisms of biochemical toxicity in an individual, and what could be the reason for the death? First of all, in this study we could give suggestions only for a piece of the whole biochemical world and life in a cell. The sublethal responses, as the signals of toxicity in an individual, are predictions of the death of individual, which can act ultimately on populations. An understanding of the toxicity and phototoxicity on fish is an anchor for the environmental risk assessment (ERA) of these PAHs.

In the results, studied PAHs at the near environmental concentrations were not lethally toxic to fish, but both PAHs were lethally phototoxic even in the short term, PYR much more phototoxic than RET. Whitefish were more sensitive than pike eleutheroembryos to phototoxicity of both PAHs. In addition, sublethal effects were stronger in whitefish than in pike. While both PAHs caused sublethal effects in visible light, in additional light conditions the effects were generally stronger. In sublethal studies on transcriptome, RET and PYR had some similar and dissimilar modes of actions measured with different endpoints, and PYR effects were stronger than those of RET at same equimolarities. In conclusion, the mode of action of these PAHs was that cellular RET toxicity was associated with CYP1A induction at used concentrations in studied salmonidae in all light conditions, thus contrasting with PYR. Toxicities of both PAHs were part of the cellular stress response as expressed as heat shock proteins, and PYR also of oxidative stress.

To conclude, these PAHs induced different lethal and sublethal effects at different light conditions (visible light, solar light, and artificial UV radiation).

The responses differed depending on tissue, species, light condition, and even PAH. Additional light could change, generally enhance, the toxicity of these PAHs on fish. Solar light and UV radiation are important environmental factors when concerning risk assessment. These PAHs and their phototoxicity may affect risks to fishes in the environment.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Reteenin ja pyreenin toksisuus kaloilla erilaisissa valoympäristöissä

Polysykliset aromaattiset hiilivedyt (PAH) ovat ympäristöistä löytyviä yhdisteitä, jotka voivat olla haitallisia eliöille. Ne voivat muuntua valon tai UV-valon vaikutuksesta ja siten myös uusien muuntuneiden tuotteiden vaikutukset eliöihin voivat erota alkuperäisistä.

Työn tarkoituksena oli tutkia kahden PAH:n, reteenin (RET) ja pyreenin (PYR), vaikutuksia kaloihin erilaisissa valoympäristöissä. Tutkimuskohteina olivat nuori kirjolohi sekä siian ja hauen vastakuoriutuneet poikaset. Kokeet tehtiin ilman lisättyä UV-valoa, auringon valossa ja lisätyssä UV-valossa. Jokainen koe sisälsi myös näkyvän valoympäristön ilman lisättyä UV-valoa.

Tulokset osoittivat, että molemmat PAHt eivät olleet letaalisti toksisia millekään tutkitulle kalalle, mutta molemmat PAHt aiheuttivat käyttäytymismuutoksia kirjolohelle 10-vuorokauden kokeessa sekä pyreeni aiheutti käyttäytymismuutoksia 32 µg/l pitoisuudessa siialle. Reteeni oli valotoksinen UV-valon vaikutuksesta siialle, jolloin se aiheutti kuolleisuutta 100 µg/l pitoisuudessa ja käyttäytymismuutoksia jo pitoisuuksissa 32 ja 100 µg/l sekä 100 µg/l pitoisuudessa hauelle. Pyreeni oli erittäin valotoksinen jo hyvin pienessä pitoisuudessa, 1 µg/l siialle ja 32 µg/l hauelle. Valotoksisuutta siis esiintyi molemmissa lajeissa, mutta siika oli herkempi kuin hauki. Molemmat PAHt aiheuttivat subletaaleja transkriptiotason vaikutuksia kirjolohelle kaikissa pitoisuuksissa 0,9 - 10 µg/l, subletaaleja transkriptio- ja proteiini-tasojen vaikutuksia siialle pitoisuuksissa 0,9 - 10 µg/l, ja subletaaleja proteiinitason vaikutuksia siialle ja hauelle pitoisuuksissa 10 - 100 µg/l. Tärkein ero reteenin pyreenin välillä oli, että reteeni lisäsi CYP1A:n määrää annosvasteisesti mRNA ja proteiinitasoilla kaikissa valoympäristöissä, mutta pyreeni ei indusoinut CYP1A:ta tutkituissa pitoisuuksissa. Tämä ilmeisesti tarkoittaa reteenin metaboloitumista CYP1A:n kautta. Reteeni ja pyreeni vaikuttivat annosvasteisesti Hsp70-proteiinin lisääntymiseen, kertoen myös stressin syntymisestä kudoksissa. Lisäksi pyreeni aiheutti oksidatiivista stressiä.

Molemmat PAHt olivat akuutisti letaaleja kaloille vasta auringon valon tai UV-säteilyn seurauksena, minkä seurauksena niitä voidaan pitää valotoksisina. Ne olivat subletaaleja hyvin pienissä pitoisuuksissa (alle 1 µg/l) kaikissa valoympäristöissä. Tutkitut PAH:t aiheuttivat joitain samoja, mutta myös monia eri vasteita kaloissa. Vaikutukset erosivat toisistaan eri kudoksissa, kalalajeilla ja valoympäristöissä. Auringon valo ja UV-säteily muuttivat ja usein voimistivat PAH:ien vaikutuksia. Valo on tärkeä ympäristötekijä, jotka tulee ottaa huomioon riskinarvioinnissa. Nämä PAHt ja niiden valotoksisuus voi aiheuttaa riskin kaloilla vesiympäristöissä.

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