DISSERTATIONS IN
FORESTRY AND
NATURAL SCIENCES

VICTOR CARRASCO NAVARRO

PAHs: Comparative biotransformation and trophic transfer of their metabolites in the aquatic environment

Fate of Polycyclic Aromatic Hydrocarbons in aquatic experiments

PUBLICATIONS OF THE UNIVERSITY OF EASTERN FINLAND
Dissertations in Forestry and Natural Sciences No 106

UNIVERSITY OF
EASTERN FINLAND

VICTOR CARRASCO NAVARRO

PAHs: Comparative biotransformation and trophic transfer of their metabolites in the aquatic environment

Fate of Polycyclic Aromatic Hydrocarbons in aquatic experiments

Publications of the University of Eastern Finland
Dissertations in Forestry and Natural Sciences
No 106
Academic Dissertation
To be presented by permission of the Faculty of Science and Forestry for public examination in the Auditorium N100 in Natura Building at the University of Eastern Finland, Joensuu, on June, 14, 2013, at 12 o'clock noon.
Department of Biology

Kopijyvä Oy
Joensuu, 2013
Editors: Profs. Pertti Pasanen,
Pekka Kilpeläinen, and Matti Vornanen
Distribution:
Eastern Finland University Library / Sales of publications
julkaisumyynti@uef.fi
www.uef.fi/kirjasto

ISBN: 978-952-61-1137-7 (nid.)
ISSNL: 1798-5668
ISSN: 1798-5668
ISBN: 978-952-61-1138-4 (PDF)
ISSN: 1798-5676 (PDF)

Author's address:
University of Eastern Finland
Department of Biology
P.O.Box 1111
80101 JOENSUU, FINLAND
email: victor.carrasco.navarro@uef.fi

Supervisors:
Senior Research Scientist Matti T. Leppänen, Ph.D.
Finnish Environmental Institute (SYKE)
Research and Innovation Laboratory
P.O.Box 35 (Survontie 9)
40014 JYVÄSKYLÄ, FINLAND
email: matti.t.leppanen@ymparisto.fi

Professor Jussi V. K. Kukkonen, Ph.D.
University of Jyväskylä
Department of Biological and Environmental Science
P.O.Box 35 (Survontie 9)
40014 JYVÄSKYLÄ, FINLAND
email: jussi.v.k.kukkonen@jyu.fi

Scientific officer Jani O. Honkanen, Ph.D.
European Chemicals Agency
P.O. Box 400
00121 Helsinki, FINLAND
email: jani.o.honkanen@gmail.com

Professor Valery Forbes, Ph.D.
University of Nebraska -Lincoln
School of Biological Sciences
348 Manter Hall
Lincoln, NE 68588-0118, USA
email: vforbes3@unl.edu

Reviewers:
Professor Anne McElroy, Ph.D
Stony Brook University
School of Marine and Atmospheric Sciences
Stony Brook, NY 11794-5000, USA
email: anne.mcelroy@stonybrook.edu

Professor Aimo Oikari, Ph.D
University of Jyväskylä
Section of Environmental science and technology
P.O. Box 35,
40014 University of Jyväskylä, FINLAND
email: aimo.o.j.oikari@jyu.fi

Opponent:
Adjunct Professor Pekka J. Vuorinen
Finnish Game and Fisheries Research Institute
PO Box 2
FIN-00791 Helsinki, FINLAND
email: pekka.vuorinen@rktl.fi

ABSTRACT

One of the most important and widespread groups of organic contaminants (OCs) are the polycyclic aromatic hydrocarbons (PAHs). It is well understood that their main source is anthropogenic (e.g. the incomplete combustion of organic materials). Certain PAHs exert their most toxic effects after their biotransformation. Toxicity caused by their biotransformation products (also called metabolites) includes mutagenicity and carcinogenicity. Therefore, their inclusion in studies that deal with PAHs as well as in chemical regulations is of vital importance. In the aquatic environment, PAHs tend to absorb to the sediment or particles, thus sediment dwellers can take up PAHs and thus introduce them into the trophic chain. Although the concentration of some OCs increases along the trophic chain (biomagnification), it is well known that this is not the case for PAHs and their metabolites. It is not expected that the trophic transfer of the latter is very important in higher levels of the trophic chain but their trophic transfer between organisms in the lower trophic levels may be important.

In the present thesis, the trophic transfer of the biotransformation products of the model PAH pyrene was studied in short aquatic food chains. *Lumbriculus variegatus* and *Chironomus riparius* were used as prey, while juvenile *Salmo trutta* and *Gammarus setosus* were used as predators. Additionally, a comparison of the biotransformation profiles of all the animals used was performed by using high performance liquid chromatography (HPLC) and liquid scintillation counting (LSC).

The trophic transfer was investigated by a mass balance that compared the pyrene ingested (measured by HPLC) and the metabolites known to be produced by predators (measured by a combination of LSC and HPLC). In addition, the comparison of the HPLC chromatograms of the animals was used.

The trophic transfer of the fraction tightly bound to tissues (called nonextractable fraction) found in *L. variegatus*, another

possible vector for the transfer of the metabolites, was measured by LSC.

The mass balance analyses revealed that the transfer of the metabolites occurred in all the experiments tested (*L. variegatus* to *S. trutta*; *L. variegatus* to *G. setosus* and *C. riparius* to *G. setosus*). One phase II metabolite of 1-hydroxy-pyrene (tentatively the glucose conjugate) produced by *L. variegatus* was transferred to *S. trutta* and two unidentified metabolites produced by *C. riparius* to *G. setosus* also occurred. On the contrary, the trophic transfer of the nonextractable fraction produced by *L. variegatus* did not occur.

Regarding the biotransformation of the test species, in general invertebrates biotransformed pyrene via the formation of glucose and sulfate conjugates (occasionally also double conjugates), meanwhile fish (*S. trutta*) mainly biotransformed via the glucuronidation pathway.

Overall, the data presented adds very valuable information about the biotransformation of PAH and underlines the vast field of research that still needs to be unveiled. Additionally, it raises concerns about the final fate of PAH metabolites in the aquatic environment and their inclusion in the risk assessment of PAHs.

Universal Decimal Classification: 502.51, 504.5, 574.5, 574.64, 615.015.4

CAB Thesaurus: pollutants; organic compounds; polycyclic hydrocarbons; aromatic hydrocarbons; aquatic environment; food chains; bioaccumulation; metabolism; metabolites; xenobiotics; aquatic organisms; Oligochaeta; Chironomus riparius; Salmo trutta; Gammarus; HPLC; liquid scintillation counting

Yleinen suomalainen asiasanasto: ekotoksikologia; saasteet; haitalliset aineet; vierasaineet; ympäristömyrkyt; PAH-yhdisteet; vesiekosysteemit; vesistöt; vesieläimisto; ravintoketjut; aineenvaihduntatuotteet; kertyminen

Preface

Joensuu, May 2013. The present thesis would not have come to an end without the help of many persons to whom I will be forever grateful.
First I would like to express my deepest gratitude to Dr. Matti T. Leppänen, who originally had the idea, applied and succeeded in getting the grant that lead to the completion of the first three years of the project. You made me see these results, experiments and writings from a more positive point of view compared to my initial negative thoughts. It would have been impossible to finish this dissertation without your help. I also would like to thank Professor Jussi Kukkonen, with whom it was a pleasure to work. Although you have been one of the busiest men I have ever met, I have had several interesting discussions about these small worms and their toxicokinetics. You encouraged me several times during this long process and I felt truly boosted again to continue after that.

Professor Valery Forbes supervised me during the first experiments and during my stay at Roskilde University during 2009. I would like to thank you for all the help offered during my visit and I feel obliged to say sorry for all the time spent, from both sides, it unfortunately was not rewarded as good results and corresponding papers. Still, I was able to include a little part of the research here as unpublished results. My gratitude also corresponds to Associate professor Annemette Palmqvist and Anne-Grete Winding for all the help offered in the lab and in the real life in Denmark.
Last but not least, Dr. Jani O. Honkanen introduced me to the HPLC world and always gave me comments on the things that seemed crystal clear to me, making a great impact in my –our– research and papers. I would also like to thank him for all the

other moments outside the Faculty, truly unforgettable experiences between friends.

My *"brother"* Stanley O. Agbo: all those moments of working like lab rats, struggling days writing the manuscripts, conference trips and the support given between us is a thing to remember for the rest of our lives. Maybe the best time ever. Well, let's just call it a *very special* time and hope that the best is still to come. What can I say? Thank you for everything.
All the members of the Ecotoxicology research group are to be acknowledged (present and departed): Jaska, Arto, Elijah, Paula, Sari A., Sari P., Kaisa, Kukka, Greta, Inna, Anita, Heikki, Juho, Suvi, Sebastian, Kristiina, José, Julio, Juuso, Henri, Petri, Joy… A big Family spread in Finland and some other parts of the World. Special thanks go to Marja and Julia, for being the best "helpers" in the lab (and Finnish language teachers), to Sergio, Heikki, Eija and Anna –Maija for the help during the different experimental setups and to Merja, Kimmo and Juha for comments and conversations about the manuscripts and experiments.

All my co-authors are here gratefully acknowledged, Jenny – Maria, Leif, Iris, JoLynn, Lionel and Sergio, you all made our articles better and allow me to save a precious time employed in some other analyses or writings. Thank you.
I also would like to thank the University of Eastern Finland, as well as the former University of Joensuu for providing excellent working facilities and location. The whole staff of the Biology department at the Faculty of Forestry and Natural Sciences have been great colleagues (loved those *Pikkujoulut*!). Special thanks go to Janne R., Matti S. for being so eager to help with informatics (and many other stuff), Matti V., Matti H. (how many Mattis?!!) and Eija R. Mervi, Tuula and Kaisa are also thanked for all the paper work.
The "EnSTe crew": always was a pleasure to share a beer with all of you once or twice a year. It felt relieving that we all were in the same situation.

The “Life after work” would have been impossible without all the friends that I had the pleasure to meet in Joensuu and around Finland & other parts of the World. Alfonso, Juha, Isabel, Albert, Jaume & Seija, Javi & Ulla, Jesús & Ewa, Sergio & Marisha, Blas & Mari, Antonio & Mar, Victor, Olalla, Cristobal, Del, Sari, Piia, Sanna, Julio, Alberto, Marcos, Tiina, Julia, Ursula, Kristina, Henri, Heikki, Arttu, Markku, Pirkko, Yasmin, Martin & Katja, Yuliya, Olga, Briana, Annika (Den), Guillaume (Den) and Tahina (Den) and may others. The JMPC photography Club in Joensuu is also acknowledged for entertain me during the last year; definitely there was photography after the *thesis writing*...
My friends in Spain also have been living this long process from the distance. Thanks for all these moments before coming here and during my vacations in Spain, when we all met again to tell old stories.

This work would not have been possible without the funding of the Maj & Tor Nessling foundation, The Academy of Finland (projects 123587 and 214545), the Finnish graduate school in Environmental Science and Technology (EnSTe) and dissertation grants from the Department of Biology of the Faculty of Forestry and Natural Sciences. These organizations are kindly and gratefully acknowledged.

I would like to thank my family: my father Julian, mother Maria del Carmen, sisters Sonia and Ana, dogs and my grandmother (*estoy seguro que estás viendo todo esto desde algún sitio*) for helping me achieving what I have achieved and for shaping who I am. Although in distance, you have been always with me. *Muchas gracias por toda esta vida juntos*.
Finally, I wish to express my never-ending gratitude to my beloved Johanna. It has been a year but it certainly looks as a century. Thanks for your patience, understanding and for taking care of me. Thanks for being who and how you are. *Oho!*

LIST OF ABBREVIATIONS

AFW: Artificial freshwater

ANOVA: Analyses of variance

ASW: Artificial seawater

B(a)P- 3OH: 3 hydroxy-benzo (alpha) pyrene

B(a)P: Benzo(alpha)pyrene

BCF: Bioconcentration factor

BMF: Biomagnification factor

CAS: Chemical Abstracts Registry

CoA: Coenzyme A

CYP: Cytochrome P-450

DAD : Diode array detector

DDD:metabolite of DDT, dichlorodiphenyldichloroethane

DDE: metabolite of DDT, dichlorodiphenyldichloroethylene

DDT: 1,1,1-trichloro-2,2'-bis(4-chlorophenyl)ethane or dichlorodiphenyltrichloroethane

DMSO: dimethyl sulfoxide

DW: Depurated worms

dw: dry weight

ECHA: European Chemicals Agency

EU: European Union

EVOS: Exxon Valdez oil spill

FLD: Fluorescence detector

GIT: Gastro-intestinal tract

GITC: Gastro-intestinal tract Content

HPLC: High performance liquid chromatography

HSP: heat shock protein

KOH: Potassium hydroxide

$LC_{50:}$ Environmental concentration causing 5o% of lethality in experimental animals

LOD: Limit of detection

LSC: liquid scintillation counter or counting

MeHg: Methylmercury

NDW: Not depurated worms

OECD: Organization for Economic Cooperation and Development

OC: Organic contaminants

PAH: polycyclic aromatic hydrocarbon

PBDE: polybrominated diphenyl ether

PBO: Piperonyl butoxide

PCB: Polychlorinated biphenyl

psu: practical salinity units

REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals

SULT: Sulfotransferase

TNT: Trinitrotoluene

t_R: retention time of a certain compound in HPLC

USEPA: United States Environmental Protection Agency

UV: Ultraviolet

wet wt: wet weight

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman numerals **I**-**IV**.

I Carrasco NavarroV, Brozinski J –M, Leppänen MT, Honkanen JO, Kronberg L, Kukkonen J V K (2011). Inhibition of pyrene biotransformation by piperonyl butoxide and identification of two pyrene derivatives in *Lumbriculus variegatus* (Oligochaeta). *Environmental Toxicology and Chemistry*, 30, Vol. 5, 1069 -1078.

II Carrasco Navarro V, Jæger I, Honkanen J O, Kukkonen J V K, Carroll, J L, Camus L (xxxx). Bioaccumulation, biotransformation and elimination of pyrene in the arctic crustacean *Gammarus setosus* (Amphipoda) at two temperatures. Manuscript.

III Carrasco Navarro V., Leppänen, M.T., Honkanen, J.O., Kukkonen J V K (2012). Trophic transfer of pyrene metabolites and nonextractable fraction from Oligochaete (*Lumbriculus variegatus*) to juvenile brown trout (*Salmo trutta*). *Chemosphere*, 88, 55 -61

IV Carrasco Navarro V, Leppänen M T, Kukkonen J V K, Godoy Olmos S (2013). Trophic transfer of pyrene metabolites between aquatic invertebrates. *Environmental Pollution*, 173, 61 -67.

Some **unpublished** results are presented and discussed.

AUTHOR'S CONTRIBUTION

The contribution of the author and co-authors in the different articles is as follows:

I the main idea of the experiment was planned by ML and JK.**VCN** performed the experiments and conducted the HPLC methods with the help of JH, who also suggested experiment 2. Toxicokinetics were performed by **VCN**, JH, ML and JK. Mass spectrometry analyses were conducted and interpreted in Åbo Akademi University by J –MB and LK, who also wrote the part corresponding to these analyses. The article was mainly written by **VCN** in collaboration with all the coauthors.

II IJ and JH were responsible for the design of the experiment. IJ conducted the exposure experiment and the extractions. **VCN** performed the HPLC analyses and interpreted the results. Additionally, **VCN** modeled the data to the equations (toxicokinetics) with the help of JK and JH. **VCN** also wrote the first versions of the manuscript, which was shaped with the ideas and comments of JH, JLC and LC.

III The experiment was designed by ML, JK, JH and **VCN**. **VCN** performed the feeding tests and all extractions, HPLC analyses and interpretation of the results. The sampling of the fish was conducted by **VCN**, ML and JK. **VCN** wrote the article with valuable comments and ideas by ML, JH and JK.

IV **VCN** and ML designed the experimental setup. **VCN** performed all the laboratory experiments, extractions and HPLC analyses with the help of SGO, who performed the extractions of *G. setosus* that fed on *L. variegatus*. The writing of the article and interpretation of the results was carried out by **VCN** with valuable comments and ideas from ML and JK.

VCN: Víctor Carrasco Navarro; ML: Matti Leppänen; JK: Jussi Kukkonen; JH: Jani Honkanen; J –MB: Jenny –Maria Brozinski; LF: Leif Kronberg; IJ: Iris Jæger; JLC: JoLynn Carrol; LC: Lionel Camus; SGO: Sergio Godoy Olmos

Contents

1 Introduction

1.1 GENERAL INTRODUCTION

The invention of substances that e.g.: preserve wood materials (chlorophenols), avoid the initiation of combustion in home appliances (flame retardants), protect us from certain diseases (pharmaceuticals) or help to prevent their spread (insecticides) has, on one hand, facilitated our daily lives and sometimes increased our life expectancy. On the other hand, some of these chemicals can be harmful to the environment in general and even to their creators, humans.

The massive growth in the number of anthropogenic chemicals is reflected in the world's most comprehensive list of substances, the Chemical Abstracts Registry service (CAS REGISTRY℠), where the number of chemicals registered increased by 10 million in just nine months, from November 2008 to September 2009. In May 2011, the 60 Millionth substance was registered (**www.cas.org**).

Some of these toxic chemicals may spread to remote locations, even if they are used only locally (Schwarzenbach et al., 1993).Two severe environmental episodes that have occurred within the last 50 years are good examples of what the spread of a chemical can cause. First, in 1962, the synthetic pesticide DDT was accused of poisoning the environment, wildlife and possibly humans in the book "Silent Spring" by Rachel Carson. DDT was banned in most developed countries starting in 1970 (Beard, 2006). Another well- known episode was the appearance of the Minamata disease, a neurodegenerative disease caused by the chronic methylmercury (MeHg) poisoning in Minamata bay (Japan). An acetaldehyde production plant discharged mercury continuously for 30 years, causing the contamination of the whole trophic web and affecting wildlife and humans (Harada et al., 1999). Although the number of officially recognized

affected individuals is 2264, it is suspected that there are around 200000 cases of MeHg poisoning (Ekino et al., 2007).
Fortunately, the establishment of regulations about the use of chemicals are becoming more common to avoid such dramatic episodes. As examples, REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) is an EU regulation that came into effect in June 2007 within the European Union (http://echa.europa.eu/regulations/reach/understanding-reach).
As a result, the European Chemicals Agency (ECHA) was also created and is implementing EU chemicals legislation on human health and environmental protection (http://echa.europa.eu/about-us).
For example, the US Environmental Protection Agency (USEPA) banned the manufacture and limited the use of PCBs in April 1979 (http://www.epa.gov/history/topics/pcbs/01.html) and in the EU their use and marketing were limited to a great extent in 1985 (http://ec.europa.eu/environment/waste/pcbs/index.htm), which was reflected in the decrease of the concentrations of PCBs in different environmental matrices, (Laender et al., 2012).

1.2 POLYCYCLIC AROMATIC HYDROCARBONS

One of the most important groups of contaminants that are widespread in the environment is the polycyclic aromatic hydrocarbons (PAHs), formed by two or more fused benzoic rings. The presence of these compounds in the environment has caused concern and some of them were included in the list of priority pollutants mentioned above and have been called the 16 priority PAHs by the US-EPA. Also the EU limited the concentrations of certain PAHs in surface waters (directive 2008/105/EC). Recently, they also have been identified as "emerging contaminants" in the Arctic (Laender et al., 2011). Therefore, they may be classified as priority pollutants but might not as POPs, as stated in **III** and **IV**. In all cases, it is very clear in my opinion, that they should be given priority, as they are ubiquitous and their discharge is continuous.

Alkyl PAHs also could be classified under the category of PAHs, being present at higher proportions than PAHs in crude oil (Wang et al., 2003). Although their nomenclature refers to their being solely made up of carbon and hydrogen, sometimes similar compounds with nitrogen (azaarenes), sulphur (thiophenes) and oxygen (furans) are included in this category of chemicals (McElroy et al., 1989) Also, Oxy-PAHs may belong to the PAH category, although they also may be produced during biotransformation processes (Lundstedt et al.. 2007).

1.2.1 Sources

Although PAHs are produced in nature by some bacteria, plants and fungi and released during natural marine seeps and forest fires (Neff, 1979; McElroy et al., 1989), it is widely accepted that anthropogenic activities are the main source of PAHs. Although several regulations have been implemented to limit or ban the emission of PAHs into the environment (e.g. the ban of creosote in wood treatment by REACH), emissions are still very high due to the incomplete combustion of organic materials such as in combustion engines, for example. In 2004, it was reported an estimation of 520 Gg y^{-1} of the 16 US-EPA priority PAHs pollutants (Zhang & Tao, 2009). Globally, the main sources of emissions were biomass burning (biofuel and wildfires), consumer products, traffic oil combustion, domestic coal combustion and some industrial activities such as coke production (Zhang & Tao, 2009). Every country has a distinct emission profile, that reflects their main activities, e.g.: in Brazil the PAHs emission originates mainly from forest fires (66%), in China from biofuel (66.4%) meanwhile emissions from the USA were mainly from consumer products use (35%) and traffic oil (23%).

PAHs are also emitted during mundane activities such as candle combustion (Orecchio, 2011), the use of traditional Chinese stoves (Shen et al., 2011) and Finnish sauna stoves (Häsänen et al., 1984), in cigarette smoke (Ding et al., 2005), in food such as smoked fish (Stolyhwo and Sikorski, 2005), and grilled meat

(Dyremark et al., 1995) and the manufacture of automobile tires (Sadiktsis et al., 2012).
PAHs emitted into the atmosphere may be directly precipitated into soils or the aquatic environment; the latter also receives PAHs via runoffs and/or sewage effluents (Neff, 1979). Another relevant source for the aquatic environment are oil spills. The estimated contribution of oils spills to the total PAH flow to the aquatic environment was estimated to be 170000 metric tons y^{-1} (Neff, 1979).

1.2.2 Fate in the aquatic environment

In the aquatic environment, PAHs tend to absorb into the sediment, particles or other substrates, reflecting their hydrophobicity (Neff, 1979). The more benzoic rings a PAH has, the lower its water solubility is, e.g. naphthalene (two rings) is more soluble in water than benzo(a)pyrene (five rings). Both positive and negative enhancement of the solubility of PAHs in water has been found with temperature and salinity, respectively (Neff, 1979)
A higher concentration of PAHs is normally found near the source point of contamination, e.g., harbours or oils spill sites. Reported PAH concentrations in sediments range widely (Table 1), from relatively clean locations (0.0012 mg PAHs kg dw^{-1} sediment; Baumard et al., 1998) to locations near industrial sites (20.5 mg PAHs kg dw^{-1} sediment; Baumard et al., 1998), harbours (3.2 and 13.7 mg PAHs kg dw^{-1} sediment; Savinov et al., 2003 and Bihari et al., 2006, respectively). The concentrations in the Elizabeth River (Virginia,USA), near two former wood treatment facilities were as high as 2500 mg PAHs kg dw^{-1} sediment in the subsurface sediment (Walker et al., 2004) and reached a maximum of 3300 mg PAHs kg dw^{-1} sediment at a creosote contaminated site in a Finnish lake (Hyötyläinen & Oikari, 1999a).
The concentrations of PAHs in sites where an oil spill has occurred have also been described. Neff et al. (2006) reported a

Table 1. *Reported total concentrations of PAHs in sediments and water column from different locations around the Globe. * Indicates that measures include particulate matter. [a] Indicates pore water concentrations. LOD: Limit of quantification.*

SEDIMENT(mg Kg dw^{-1}) Location	**Time**	**Range**	**Reference**
Elizabeth River, VA		2200	Vogelbein et al., 1990
Lake Höytiäinen, Finland		0.007	Cornelissen et al., 2004
Eccica Island, Corsica, Fr	1995	0.0012	Baumard et al., 1998
Ajaccio Harbour, Corsica	1995	20.5	Baumard et al., 1998
Guba Pechenga, Russia	1997	0,428 -3,2	Savinov et al., 2003
Rovinj, Croatia		0,032 -13,7	Bihari et al., 2006
Lake Jämsänvesi, Finland		8 -3294	Hyötyläinen & Oikari, 1999
Prince William Sound, AK, US	2001	21 -23000	Neff et al., 2006
WATER (ng L^{-1}) Location	**Time**	**Range**	**Reference**
S. Francisco bay, US	1993-2001	5-147	Ross & Oros, 2004
Three Gorges Dam, China	2008	14-97	Wang et al., 2009
Mississippi River, US	2004	63-145	Zhang et al., 2007
Hangzhou City, China	2002	990-9700	Chen et al., 2004
Bahía Blanca Est. (Arg)		<LOD-4900	Arias et al., 2009
Mountain lakes		0.7-1.1*	Vilanova et al., 2001
Lake Jämsänvesi, Finland		700 - 1.7*10^{6} [a]	Hyötyläinen & Oikari, 1999

mean concentration of total PAHs of 2.5 mg kg dw^{-1} sediment in the upper intertidal zone of Prince William Sound (Alaska, USA) thirteen years after the occurrence of the Exxon Valdez oil spill (EVOS) in 1989.

The highest PAH concentrations in the water column correlate positively to the proximity to industrial sites or human activities such as cities or populated river basins (Neff, 1979; Zhang et al., 2007). Some of the water concentrations of PAH that have been reported worldwide are shown in Table 1.

Some measures that have been used to try to restore the welfare of certain areas are capping (Myers et al., 2008), dredging

(Torres et al., 2009) and the development of directives that limit the use and emission of PAHs (e.g. EU directive 2005/69/EC that orders the use of low-PAHs oils in the manufacture of car tires). However, these techniques may not always work, as for example dredging may cause a measurable hazard to local organisms (Hyötyläinen & Oikari, 1999b).

1.2.3 Effects

Several studies have connected the environmental contamination with PAHs to certain negative effects on the local populations. For example, in the Elizabeth River (Virginia, USA), which is highly contaminated with PAHs (Table 1), 93% of the mummichogs examined had hepatic lesions, 33% of these having liver carcinomas (Vogelbein et al., 1990); in three British estuaries close to heavily industrialized areas, three species of fish presented hepatic alterations (Stentiford et al., 2003); and in the Black River (Ohio, USA) brown bullhead catfish had a high frequency of liver tumours directly associated with PAH contamination (Baumann & Harsbarger, 1995).

In places where the PAH contamination results from factories or mills, normally the closure of the plant produces a decrease in the sediment and tissue PAH concentrations, as well as a decline in liver tumour frequency (Baumann & Harsbarger, 1995).

Exposure of organisms to PAHs can cause different kinds of toxicity such as nonpolar narcosis and phototoxicity and additionally, they can alter vitellogenesis in fish (Nicolas, 1999), increase the lipid peroxidation and glycogen reserves in the digestive glands of clams (Frouin et al., 2007). A significant decrease in the filtration rate in the Pacific oyster was also found in response to exposure to concentrations of PAHs of 100 and 200 $\mu g\ L^{-1}$ (Kim et al., 2007). Additionally, Eertman et al. (1995) found an inverse correlation of the tissue concentrations of fluoranthene and benzo(a)pyrene with the clearance rate in the blue mussel. The same organism exposed to a mixture of anthracene, fluoranthene and phenanthrene showed a decrease in phagocytosis and damaged lysosomes (Grundy et al., 1996),

which may hamper the immune response of mussels in PAH contaminated areas. The LC_{50} values for water fleas, a model aquatic freshwater animal, exposed to PAHs were 10 μg/L (Benzo(a)anthracene), 5 μg/L (BaP), 3400- 4600 μg/L (naphthalene) (CCME, 1999). The exposure of the animals to PAHs and UV radiation generally decreased the LC_{50} values.

1.3 RELEVANCE OF BIOTRANSFORMATION PRODUCTS OF OCS

On most occasions, only the concentrations of parent OCs are measured in environmental matrices such as sediments or tissues of contaminated organisms. In order to completely understand their fate and toxicity, to know about their biotransformation (also called metabolism) is a necessary step. The term biotransformation refers to the organism-mediated transition of a certain chemical to product(s) that have different chemical and toxicological properties (McElroy et al., 2011). However, some authors use the term *metabolism* as a synonym of biotransformation and the term *metabolites* to refer to the biotransformation products of OCs (Stroomberg et al., 1999; Sepic et al., 2003; Ikenaka et al., 2007). The term *metabolism* may be incorrect or at least inaccurate, as it may be restricted to the endogenous biochemical reactions that involve normal cell molecules such as carbohydrates, proteins and lipids (Lech & Vodicnik, 1995) and in my opinion, it may overlap with the terminology used in the metabolomics studies. The same rationale applies to the term *metabolites*. In the present thesis, I will refer to them either as metabolites or biotransformation products. The final aim of biotransformation is to produce metabolites that are more soluble in water in order to facilitate their later excretion.

In addition, OCs may also be degraded in the environment by UV light (Yan et al., 2004), and also in water treatment plants (Fatone et al., 2011). Although these may not be the result of a biologically mediated transformation per sé, they are also relevant transformation products. Additionally, microorganisms

are also able to degrade or biotransform OCs (Neilson & Allard, 1998). Degradation does not always mean that the OCs are mineralized to CO_2 and H_2O; more persistent transformation products may be created and released into the environment.

Recently, there has been a growing concern about the risk from transformation products (including those of biological and chemical origin). The omission of transformation products from the risk assessment of a certain parent chemical can result in an incomplete estimation of its toxicity (McElroy et al., 2011). A classical example is vinyl chloride, an industrial chemical which biotransformation produced reactive metabolites that were found to bind proteins, DNA and RNA and cause hepatic tumors in humans (Bolt, 2005).

Taking into account the persistence, mobility and toxicity of parent and also transformation products, van Zelm et al. (2010) estimated the real impact of some chemicals on humans and ecosystems in a case study. Of the 16 chemicals studied, the impact of parent plus transformation products most likely increased 10- fold for four chemicals and might have increased by up to 100- fold for another five (van Zelm et al., 2010). There are several persistent transformation products that can result from selected persistent chemicals (Ng et al., 2011), for example the transformation products of the insecticide DDT (DDE and DDD; Menchai et al., 2008).

An enhanced toxicity is also produced by some biotransformation products of OCs, such as malaoxon that originates from the organophosphorus pesticide malathion (Aker et al., 2008), showing a lower LC_{50} than the parent compound in the Blue catfish *Ictalurus furcatus*. However, the most famous case of increased toxicity of the transformation products occurs with the polycyclic aromatic hydrocarbons (PAHs). Some of their metabolites are carcinogenic or exert a similar toxicity.

1.3.1 Biotransformation of PAHs

The most common biotransformation pathway of PAHs normally starts with the enzymatic introduction of a hydroxyl

group (Livingstone, 1998). This step (called phase I) is catalysed by the P-450 (CYP) family (Buhler & Williams, 1989), a large and complex family of proteins that, although well- known for the phase I biotransformation of xenobiotics (Rewitz et al., 2006) are also essential in endogenous functions such as testosterone metabolism (Thum & Borlak, 2002). A second step in biotransformation, called phase II, involves the conjugation of molecules such as glucoside, glucuronide, sulfate or glutathione to the hydroxyl(s) group. This step enhances the water solubility of the phase I product(s) with the ideal aim of excretion.

The contribution to toxicity caused by the biotransformation products of some PAHs can be significant when compared to the toxicity caused by parent compounds (Lee & Landrum, 2006). However, this is not the case for all PAHs. Some PAHs need an "activation" to exert their most toxic effects (Stegeman & Lech, 1991). This activation can be reached by the formation of (1) diol epoxides, (2) intermediate radical cations and (3) *o*-quinones that are prone to react with macromolecules, most commonly with DNA, forming DNA adducts (Cavalieri & Rogan, 1998).

Some examples of PAHs that can undergo these biotransformation pathways leading to increased reactivity and to the formation of DNA adducts are dibenzo (a, l) pyrene and the widely- known benzo(a)pyrene (Fig. 1A). Both have been found to induce the formation of DNA adducts, tumorigenicity and mutations (Prahalad et al., 1997). The most commonly-known route of activation is the formation of diol epoxides (Fig. 1A).

Additionally, some phase II metabolites such as sulfates can also lead to the formation of DNA adducts (Xue & Warshawsky, 2005). Although the main and original goal of the sulfonation of phase I metabolites and other xenobiotics is detoxification and excretion, sulfate metabolites can also be more toxic than the parent compound, although generally this is not the case. Some chemicals worth mentioning are the methyl PAHs, such as 7, 12 dimethyl- benzo (a, h) anthracene, 7 -methyl benzo anthracene, 5 -methyl chrysene and methyl pyrene. First, a hydroxyl group

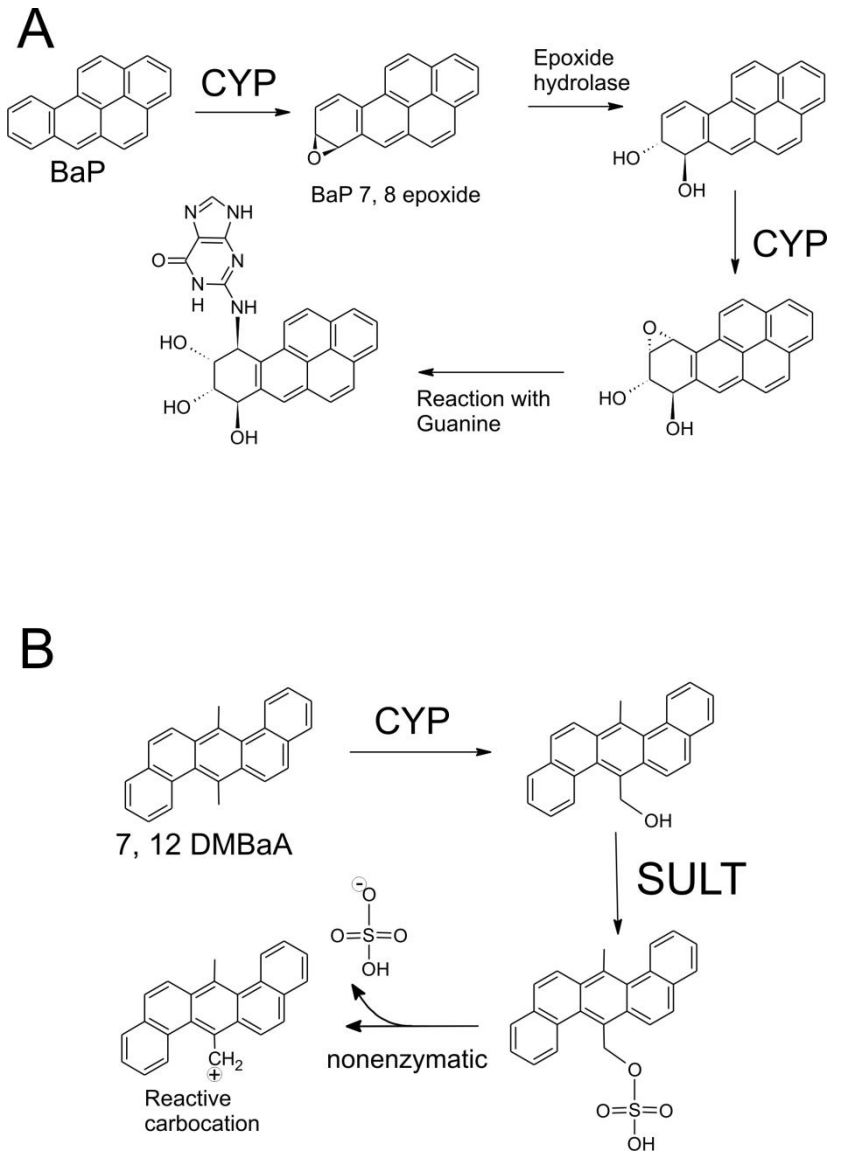

Figure 1. *Examples of biotransformation of PAHs that may lead to an enhanced toxicity. (A) shows the biotransformation of benzo (a) pyrene (BaP) that leads to the formation of a covalent bound with the DNA base Guanine. (B) shows the biotransformation of 7, 12 dimethyl benzo (a, h) anthracene (7, 12 DMBaA) that leads to the formation of its 7-hydroxymethyl sulfate metabolite, that is unstable and can eliminate the sulfate group, leading to the reactive carbocation. Adapted from Cavalieri & Rogan, 1998 and Schlenk et al., 2008. CYP = Cytochrome P-450, SULT = sulfotransferases.*

is introduced into one of the methyl groups, reaction catalyzed by CYP. Second, a sulfotransferase (SULT) introduces a sulfate molecule into the hydroxyl group. A spontaneous cleavage of the sulfate leaves a carbocation that is able to react with nucleophiles, possibly DNA bases (Fig. 1B; Watanabe, 1983; Xue & Warshawsky, 2005).
Furthermore, B(a)P metabolites formed covalent bonds with proteins in an in vitro studies with liver microsomes from rainbow trout and Lin et al. (2005) we able to determine that metabolites of naphthalene bound to several types of proteins, among them ß-actin, HSP 70 and mitochondrial proteins were suggested as targets.

These covalent bonds to DNA or proteins may form what has been called "nonextractable fraction" in the present thesis. To determine the animal body burden after exposure to PAHs, treatment of tissues with organic solvents are performed to extract the parent and metabolites present. Although these extractions methods have a good percentage recovery, still some PAH derivatives are found in the tissue residues. This nonextractable fraction has been suggested to be formed by some metabolites bound to macromolecules.

Despite this overall knowledge, the research about PAHs still focuses on the parent PAHs rather than adopting a wider perspective that would include biotransformation products as well.
When performing a search in the most common Internet literature databases of the words polycyclic aromatic hydrocarbons AND metabolites, the results only indicate between 7.5 and 10% of the total results for the search "polycyclic aromatic hydrocarbons" (Fig. 2). By substituting the word metabolites with biotransformation, this percentage was reduced to 1.5 -2 %. Thus, this is evidence for the lack of studies on PAHs that include their metabolites. This is likely to be caused by the absence of standards and other difficulties to measure their concentration.

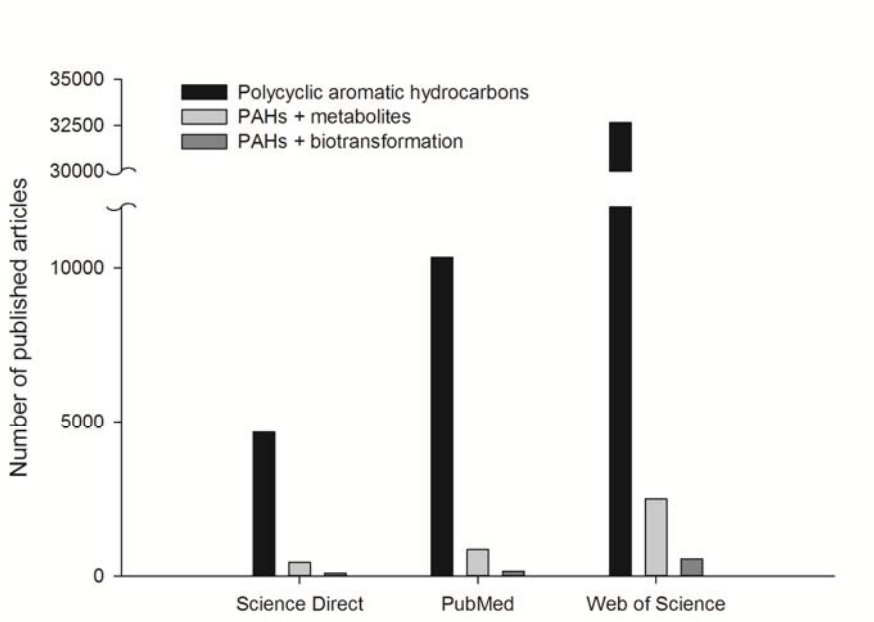

Figure 2. *Fig.2. Number of PAH related articles found using three different internet databases. The words used for the search were "Polycyclic aromatic hydrocarbons" and the results are in black bars, "PAHs" and "metabolites" in clear grey bars and "PAHs" and "biotransformation" in dark grey bars. Searches were performed in Environmental science, in abstracts, titles and keywords, from 1970 to present (Science Direct);, in abstracts and titles, from 1946 to present (Pub Med);and in topics, from 1975 to present in the SCI-EXPANDED database (Web of Science).*

1.3.2 Animals and their biotransformation capability

1.3.2.1 Lumbriculus variegatus

The freshwater annelid *Lumbriculus variegatus* (Oligochaeta; Fig. 3A) was used as a test organism in the article **I** and as a prey organism loaded with the chemical of study in articles **III** and **IV**. *L. variegatus* were cultured at the University of Eastern Finland (former University of Joensuu) as described thoroughly in **I**, **III** and **IV**. It is present widely in freshwater bodies in the Northern hemisphere, South Africa, Australia and New Zealand (Marshall, 1978). It is a recommended species for use in bioaccumulation tests (OECD, 2007) because of its flexibility to a variety of endpoints, its capability to bear long exposures

without feeding, it has a convenient tissue mass for chemical analyses and also it is easy to culture (Phipps et al., 1993; Brunson et al., 1998). In laboratory cultures, *L. variegatus* are usually smaller (4-6 cm) than those found in nature (5-10 cm) and are not sexually mature (Martinez, 2005). Besides, they reproduce by division, similar to worms in the wild in summer and fall. *L. variegatus* is a representative sediment burrowing organism capable of reaching a certain chemical dosing through water, sediment or both (Mount et al., 2006). Additionally, *L. variegatus* has been described as an excellent species to be used as a prey in dietary exposures with fish (Mount et al., 2006). Besides, a need for biotransformation was necessary for the success of the experiments reported in articles **III** and **IV**, a fact that was demonstrated in article **I**. However, there have been contradictory reports about its ability to biotransform PAHs. Not very long ago, biotransformation of OCs by this species was not even considered (van Hoof et al., 2001), or evidence of biotransformation was not found (Verrengia -Guerrero et al., 2002). These two facts favoured its use in tests that exclusively studied the bioaccumulation of substances. Additionally to experiment **I**, other articles have reported the ability of *L. variegatus* to biotransform PAHs and some other OCs, such as pyrene (Mäenpää et al., 2009; Lyytikäinen et al., 2007), benzo(a)pyrene (Leppänen & Kukkonen, 2000; Schuler et al., 2003), phenanthrene (You et al., 2006), bifenthrin and permethrin (You et al., 2009), perfluoroalkyl sulfonate (Higgins et al., 2007), ethinylestradiol (Liebig et al., 2005) and trinitrotoluene (Belden et al., 2005). As explained in **I**, these contradictory findings in the biotransformation capacity of *L. variegatus* may be explained if this species is in reality two or more species (Gustafsson et al., 2009). It is possible that sibling species have a radically different capacity to biotransform PAHs (Bach et al., 2005).

The terrestrial oligochaetes *Eisenia andrei* also have a limited capacity (0.1 to 1% of total pyrene in worms) to biotransform pyrene (Jager, et al., 2000) to three conjugates of 1-hydroxy-

pyrene (Stroomberg et al., 2004a).This may indicate that the Oligochaeta taxa in general are capable of biotransform PAHs.
All in all, *L. variegatus* has been used widely in ecotoxicological research (Airas et al., 2008; Ng and Wood, 2008; Verrengia Guerrero et al., 2002; Wiegand et al., 2007; Van Hoof et al., 2000; Higgins et al., 2007; Kukkonen & Landrum, 1994).

1.3.2.2 Chironomus riparius

The nonbiting midge *Chironomus riparius* (Diptera: Chironomidae; Fig. 3B) was used as a prey organism in **IV**. It is an organism whose life cycle is divided into four life stages (egg, larval stage with four instars, pupa and adult). It is also a recommended species for the testing of chemicals (OECD, 2010) and widely used in ecotoxicological research (Ristola et al., 1999; Paumen et al., 2008; Mäenpää & Kukkonen, 2006; Clements et al., 1994; Marinkovic et al., 2011) because its tolerance to a range of conditions and chemicals, and sometimes its use is convenient because the development of the larvae to adults can be followed in response to exposure to certain xenobiotics (Paumen et al., 2008).
The use of *Chironomus riparius* in the present thesis was of special interest, as it represents an organism with efficient biotransformation capacity, contrary to *L. variegatus*. It was able to biotransform 71-74 % of pyrene (Harkey et al., 1994) and a biotransformation rate of 3.2 nmols g^{-1} dwt h^{-1} was found for B(a)P (Leversee et al., 1982). *C. riparius* also biotransformed some other OCs such as chlorophenols, the fungicide fenpropidin and the herbicide trifluralin (Verrengia- Guerrero et al., 2002).

1.3.2.3 Capitella teleta

Capitella teleta (Annelida: Polychaeta; formerly named *Capitella capitata sp. I*; Fig. 3C) was exposed to sediment -bound fluoranthene and used as a prey species fed to *C. crangon* in **unpublished** experiments performed at the University of Roskilde (Denmark). This species was chosen because it is an organism present in marine sediments worldwide and

commonly found in disturbed and oil polluted sediments (Linke -Gamenick et al., 2000).
Due to its dense and ubiquitous presence, they may be important prey for larger invertebrates and some fish (Palmqvist et al., 2006), a fact that underlines the importance of this species in the trophic transfer of sediment bound contaminants.
C. teleta was known to biotransform fluoranthene very efficiently (Bach et al., 2005) to several metabolites (Forbes et al., 2001), and also pyrene via glucose and sulfate conjugation (Giessing et al., 2003). This was a reason to choose it as a prey.

1.3.2.4 Pallaseopsis quadrispinosa
Pallaseopsis quadrispinosa (Crustacea; Fig 3D) was also used as a test organism in a bioaccumulation and biotransformation study with pyrene (**unpublished data**). It is a glacial relict amphipod commonly found in freshwater lakes of Northern Poland, Germany, Scandinavia, the Baltic countries and even in low salinity waters from the Baltic Sea (Kolodziejczyk & Niedomagala, 2009). It has been declared as an important part of fish diet (Hill et al., 1990).
The information about its biotransformation capacity provided in my thesis is, to my knowledge, the only available so far. This is likely because *P. quadrispinosa* is not a common organism in water bodies nowadays, supporting its exclusion from ecotoxicological tests.

1.3.2.5 Gammarus setosus
Gammarus setosus (Fig. 3E) was used as a test organism in article **II** and as a predator in article **IV**. It is a common species of amphipod present in the benthos of subtidal and intertidal arctic areas and found as a predominant organism together with *Fucus distychus* and large *Oligochaeta* on the Svalbard coast line (Weslawski et al., 1993). It is abundant on sheltered beaches with loose stones. There are studies that suggest that *G. setosus* has reduced its habitat to inner fjord basins (Weslawski et al., 2010) due to the increase in water temperature during the past 20 years.

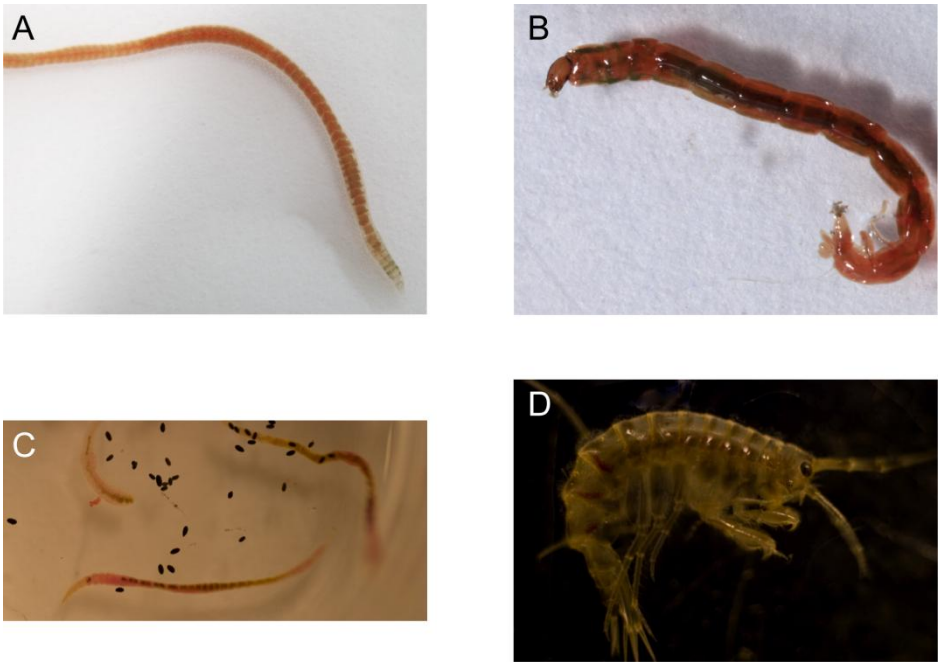

Figure 3.1. *Species studied in the thesis. A)Lumbriulus variegatus, B) Chironomus riparius, C) Capitella teleta, D)Pallaseopsis quadrispinosa, All photos by Victor Carrasco Navarro.*

As a predator, *G. setosus* is a scavenger that feeds on dead material present on the surface of the sea floor (Olsen et al., 2007), a circumstance essential in article **IV**. Additionally, the genus *Gammarus* is an important part of the aquatic ecosystems and a relevant food source for fish, crayfish and even some birds (Macneil et al., 1999). There was also a lack of information regarding the biotransformation capability of PAHs in *G. setosus*, what is covered in **II**.

1.3.2.6 Crangon crangon

The brown shrimp *Crangon crangon* (L) (Crustacea: decapoda; Fig. 3F) is a marine epibenthic organism found in a wide range of latitudes along the European coast (Campos & van der Veer, 2008). It is typical of estuaries, although also found at depths of 20-90 m. As it is a key organism in the trophic chains of these ecosystems, its choice as a predator in **unpublished** experiments

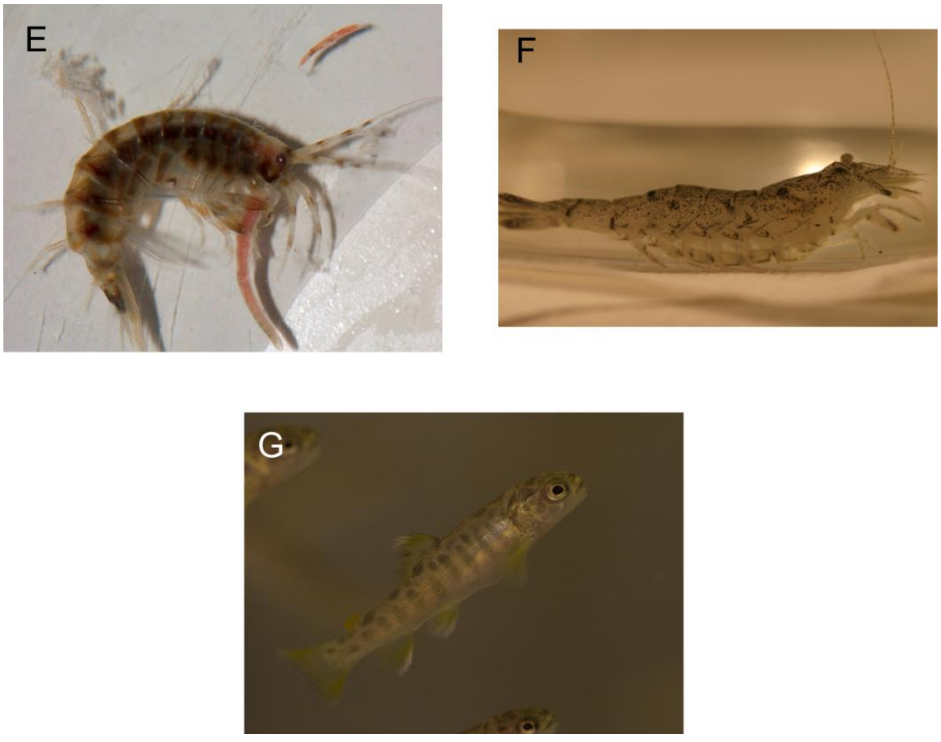

Figure 3 (cont). *Species studied in the thesis (cont). E) Gammarus setosus, F) Crangon crangon and G) juvenile Salmo trutta. All photos by Victor Carrasco Navarro.*

that tested the trophic transfer of PAH metabolites was logical. Due to its abundance, it is the prey for several predators such as fish, crustaceans and even birds (Campos & van der Veer, 2008). Additionally, it acts as a predator towards benthic organisms.Despite being a key organism in estuarine food webs and a relevant part of human consumption (Campos & van der Veer, 2008), as far as I know it has not been used in ecotoxicological studies, but it has been used among other organisms to monitor the presence of OCs in biota in an estuary from central Europe (Van Ael et al., 2012). Its biotransformation capacity remained so far unknown and the information gathered in the present thesis is as far as I know unique.

1.3.2.7 Salmo trutta

Brown trout (*Salmo trutta;* Fig. 3G) was used as a predator, representing a fish species in experiment **III**. It is a representative anadromous fish, member of the Salmonidae family, being one of its members (rainbow trout, *Oncorhynchus mykiss*) a recommended species for the testing of chemicals (OECD, 1992). *S. trutta* is native from the Eurasian and North African regions and the most commonly present freshwater fish in this territory (Bernatchez, 2001). It has also been found as an invasive species for example in New Zealand (Simon & Townsend, 2003).

Salmo trutta acts as a top consumer in the aquatic environment and it is an important subject of fishing (Gustafsson, 2011), facts that highlight its importance as a predator and as a vector between the aquatic environment and humans.

Juvenile brown trout were chosen as a predator species in article **III** mainly due to their small size, for the ease of analyses and setup (i.e. beaker size). Usually, juveniles fed on aquatic and terrestrial insects (Brelin, 2008), a fact that made *L. variegatus* a suitable species to be used as a prey.

S. trutta has been previously chosen as the test organism in some ecotoxicological tests (Brinkman & Hansen et al., 2007; Leland, 1983; Vermeirssen et al., 2005; Hoeger et al., 2008).

The biotransformation capacity of OCs by some members of the Salmonidae family has been reported; e.g. some PCB (Buckman et al., 2006) and the organo phosphate insecticides chlorpyrifos, parathion and fenthion (Lavado & Schlenk, 2011). Therefore, it was not surprising to find an efficient biotransformation capacity in *S. trutta*, reported as new information in **III**.

1.3.3 Model PAHs and their biotransformation

Pyrene is a four ringed PAH, included in the USEPA list of priority pollutants (USEPA, 2009). As it is found in all PAH mixtures (Jongeneelen, 2001), it is logical that it is commonly found in natural sediments contaminated with PAHs at a

relatively high concentration (Gosh et al., 2000, Frouin et al., 2007; Sanders et al., 2002). It also has been found as a predominant PAH in water (Wang et al., 2009). Pyrene is found or formed in everyday activities such as smoking (49 -138 ng cigarette^{-1}; Ding et al., 2005), during the roasting of coffee beans (3.3 -53 μg kg^{-1}; Houessou et al., 2008) and in automobile tires having an important percentage (Sadiktsis et al., 2012), which highlights its ubiquity. The measurement of it s main biotransformation product, 1-hydroxy-pyrene in urine has been used as a biomarker of occupational exposure to PAHs (Jongeneelen, 2001). The choice of pyrene as a model compound is logical, as 1-hydroxy-pyrene is its dominating phase I metabolite in eukaryotes (Giessing et al., 2003; Stroomberg et al., 1999), which reduces considerably the options for phase II metabolites. However, recent studies have also described other phase I metabolites and some of their phase II conjugated metabolites (Beach et al., 2009 & 2010; Beach & Hellou, 2011).
The biotransformation of pyrene continues with phase II, which conjugates the hydroxyl group(s) with molecules such as glucose, glucuronide acid or sulfate in order to increase the solubility of the phase I product(s) and, theoretically excrete them more rapidly. Additionally, double conjugates of 1-hydroxy-pyrene have been found (**I**, Ikenaka et al., 2007; Stroomberg et al., 2004a). Double conjugates refer to the conjugation of two molecules consecutively in the same position (C1 in the cases described).

Fluoranthene is a four ringed PAH sharing a high percentage of total PAH contamination in the environment (e.g. 11 ± 6% in sediments of the Savannah river; Sanders et al., 2002) and as pyrene, also present in the list of priority pollutants (USEPA, 2009).
Although fluoranthene was found to cause acute toxicity, it shown an enhanced toxicity in combination with UV light (Spehar et al., 1999), proving its phototoxicity.
Although its biotransformation pathway is more complicated than that of pyrene, it was proved to be biotransformed by the

fish *Solea Solea* to seven phase II metabolites, mainly glucuronide conjugates (Hillenweck et al., 2008). The existence of these metabolites implied the necessary formation of their phase I metabolites, including five mono- and di- hydroxylated metabolites (Hillenweck et al., 2008). The polychaetes *Capitella teleta* also biotransformed fluoranthene into numerous metabolites, most of them unknown (Forbes et al., 2001). Among them hydroxylated phase I metabolites were found. Fluoranthene has been also shown to cause DNA damage (Palmqvist et al., 2003 and 2006).

1.3.4 Trophic transfer of PAHs and their biotransformation products

The trophic transfer of parent PAHs was a subject of concern until it was proven that PAHs do not biomagnify along food chains or webs (Broman et al., 1990; van Brummelen et al., 1998; Nfon et al., 2008). Even considering that PAHs do transfer through diet from prey to predator (Filipowicz et al., 2007), predators such as fish and other vertebrates possess an efficient biotransformation and excretion systems, therefore PAHs are not normally found in e.g. fish (Beyer et al., 2010). For the same reason, the biomagnification of PAH and their metabolites does not occur, which is likely a reason field studies are focusing on the trophic transfer of other compounds such as PCBs (Cullon et al., 2012), perfluorinated compounds (PFCs; Houde et al., 2011), DDT (Strandberg et al., 1998) and PBDEs (Law et al., 2006; Wu et al., 2009).

The trophic transfer of PAH metabolites has not been studied extensively, despite that in the field prey organisms may have a body burden of both parent and metabolites as potential sources of toxicity for predators. As opposite examples, the Pacific oyster, *Crassostrea gigas*, contains a small amount of pyrene metabolites when exposed to the parent pyrene in seawater (Bustamante et al., 2012), whereas some invertebrates that

biotransform efficiently, such as *Capitella teleta*, may contain a higher percentage of metabolites than the parent compound (Bach et al., 2005). Therefore the differences in the body burdens of PAHs and their metabolites in prey organism can dictate the exposure of predators to such compounds.
This and the known enhanced toxicity of the metabolites are reasons that make the study of the trophic transfer of PAH metabolites worthwhile.

1.4 AIMS OF THE THESIS

One of the objectives of my thesis was the comparative toxicokinetics, covering the uptake, bioaccumulation, biotransformation and excretion of a model PAH. These concepts were covered in articles **I** and **II** using pyrene as a model chemical and *L. variegatus* and *G. setosus* respectively as test species. Additionally, the excretion of the PAHs and their metabolites was also studied more deeply in articles **II**, **III** and **IV**, as it is directly related to the trophic transfer. A special emphasis was given to the comparative biotransformation of pyrene among the species used in the thesis, a necessary step for the study of the trophic transfer of the pyrene biotransformation products.

The main objective of the present thesis is to ascertain the trophic transfer of the biotransformation products of model PAHs from prey to predators, tested in articles **III** and **IV**. The preys *Lumbriculus variegatus* and *Chironomus riparius* were exposed to the model compound (i.e. pyrene) and fed to the predators *Salmo trutta* and *Gammarus setosus*.

Another aspect of the trophic transfer of PAH metabolites was also in the scope of my thesis. The trophic transfer of the nonextractable fraction (formed by biotransformation products of PAHs bound to macromolecules) was investigated in article

III. This may also be a special vector of the trophic transfer of PAH metabolites that, to my knowledge, has not been directly investigated previously.

Finally, most of these concepts were also studied in **unpublished** experiments that are also reported in the present thesis.

2 Materials & methods

The summary of the experiments described in this section is presented in Table 2. A more detailed explanation of the methods used is provided in the single articles that form the present thesis. The description of the maintenance and culture of the organisms is also described in the corresponding articles.

2.1 ANIMALS

The animals used in experiments **I**-**IV** were sampled and cultured as described in the material and methods section of the correspondent articles. The methods used with the animals used in **unpublished** experiments are described below.

Pallaseopsis quadrispinosa were collected in the Lake Kuorinka (Finland; 62°37′48″N, 29°23′49″ E) in November 2008 and maintained at 2 ± 1 °C in lake water with a layer of sediment for approximately three months in our laboratory. During this period, the water was changed twice, aeration was provided constantly and Tetramin was added as a source of food. After this period, the water was substituted by Artificial Fresh Water (AFW) and the sediment removed almost completely. Two signs of the good health of amphipods in the culture were the observed mating and absence of mortality.
Crangon crangon were collected in Isfjord (55° 52' 00" N, 11° 49' 00" E), Roskilde (Denmark) in July, 2009 and kept in marine water (15 psu) in aquariums at the University of Roskilde, Denmark. They were fed commercial shrimp and aeration was provided constantly. Mechanical pumps (Eheim ecco, GmbH & CoKG, Germany) with internal substrate and gravel were used to keep the water cleaned.

Capitella teleta were grown in filtered sediment (about 10 cm) with marine water (32 psu) over it, with constant aeration and food provided. They originated from a permanent culture at the Roskilde University.

***Table* 2.** *Summary of the subjects covered in the present thesis.U indicates Unpublished and sed, sediment*

	Animals	**Exp. route**	**Type of experiment**	**Chemical**	**Aims**
I	*L. variegatus*	AFW	Bioaccumulation Biotransformation	Pyrene	Presence of CYP Toxicokinetics Id of metabolites Id of nonextr. fraction
II	*G. setosus*	ASW	Bioaccumulation Biotransformation	Pyrene	Toxicokinetics at different temp.
III	*L. variegatus* *Salmo trutta*	AFW and diet	Trophic transfer	Pyrene	Trophic transfer of metabolites and bound fraction
IV	*L. variegatus* *C. riparius* *G. setosus*	AFW and diet	Trophic transfer	Pyrene	Trophic transfer of metabolites biotransformation
U. I	*P. quadrispinosa*	AFW; sed.	Bioaccumulation Biotransformation	Pyrene	Presence of CYP Toxicokinetics
U. II	*C. crangon* *C. teleta*	diet	Biotransformation Trophic transfer	Flu	Biotransformation Trophic transfer

2.2 CHEMICALS

In all the experiments **I** -**IV**, pyrene (Fig. 4; CAS number: 129-00-0; purity 98%) was used. It is a relative water soluble compound (0.129-0.148 mg/kg at 25°C (McElroy et al., 1989) but still hydrophobic (log K_{ow} 4.92; DiToro & McGrath, 2000). The compound is stable in water.

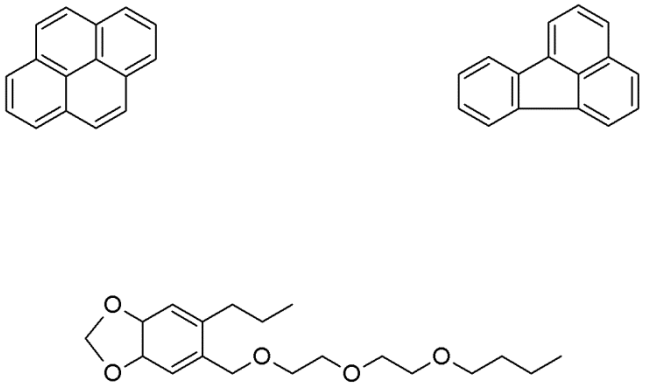

Figure 4. *Chemicals used in the present thesis. Pyrene, fluoranthene and piperonyl butoxide (PBO).*

It has been used in a large number of studies as a model compound (e.g.: Akkanen and Kukkonen, 2003; Giessing & Johnsen, 2005; Honkanen et al., 2008; Ikenaka et al., 2007; Jorgensen et al., 2005; Leppänen & Kukkonen, 1998; Stroomberg et al., 1999, 2004a and b) among other reasons, because of its simple main biotransformation pathway, as described above.

In **unpublished** experiments performed at the University of Roskilde (Denmark), the PAH fluoranthene (Fig. 4; CAS number 206-44-0; purity 98.5%) was used as a test compound. It is more soluble than pyrene in water (0.206-0.265 mg/kg; McElroy et al., 1989) and has similar hydrophobicity (log K_{ow} 5.1; DiToro & McGrath, 2000).
Fluoranthene (98.5%, HPLC) was purchased from Fluka and 3-^{14}C fluoranthene was received from the Midwest Research Institute. Its specific activity was calculated by HPLC and LSC and determined to be 56.5 mC_i $mmol^{-1}$.

Piperonyl butoxide (PBO; CAS n: 51-03-6; Fig.4; purity 98.2%) was used in experiments with *L. variegatus* (I) and *P. quadrispinosa* (**unpublished**), because of its characteristic inhibition of the cytochrome P-450 (CYP) enzyme. Its water solubility at 20 °C is 14.3 mg/L and its log K_{ow} is 4.75 (Amweg et

al., 2006). It is relatively stable in water and sediment systems in the dark and in anaerobic conditions but exposure to sunlight and oxygen accelerates its degradation.

2.3 BIOACCUMULATION AND TOXICOKINETIC EXPERIMENTS

The bioaccumulation of waterborne pyrene was studied in *Lumbriculus variegatus* (**I**), *Gammarus setosus* (**II**) and *Pallaseopsis quadrispinosa* (**unpublished** results). Additionally, the uptake and bioaccumulation from sediment was also studied in *P. quadrispinosa*.

In experiments **I** and **unpublished** experiment with *P. quadrispinosa*, the presence of a cytochrome P-450 (CYP) was studied by the addition of the known CYP inhibitor piperonyl butoxide (PBO).

The concentrations of PBO used (**I** and **unpublished**) were chosen to avoid any toxic effects produced by this chemical in the organisms based on values found in the literature (Ankley & Collyard, 1995).

A detailed description of the procedures followed is given in the material and methods section of article **I**. In experiment **II**, *G. setosus* were exposed to pyrene (~2 μg L^{-1}) in artificial seawater (ASW) at two different temperatures of 2 and 8 °C. All the experimental details are described in **II**.

P. quadrispinosa was exposed in water similarly to *L. variegatus*, with two water treatments consisting of (1) pyrene (nominal concentration 3 μg L^{-1}) and (2) pyrene (3 μg L^{-1}) + PBO (53μg L^{-1}). The proportion of radiolabeled pyrene in these exposures with *P. quadrispinosa* was 0.75% of the total chemical. Additionally, a *P. quadrispinosa* sediment exposure was conducted. The nominal concentration of the sediment was 1.5 μg pyrene g^{-1} wet wt. Approximately 700 g fresh wt of clean Lake Kuorinka sediment was spiked with 100 μl of a concentrated solution of pyrene, originating from the stock used for the water exposures. The sediment was mixed for 4 h with a rotating metal blade connected to a drill as described earlier (Leppänen & Kukkonen,

1998), and stored in the dark for four weeks at 5 ºC. The pyrene concentrations in sediment were measured by mixing fresh sediment samples (n=4; 0.34 ± 0.08 g) with Soluene®-350 (1 ml), incubation overnight at 50 ºC and an addition of Ultima Gold LSC cocktail (5 ml). The mixes were shaken and analyzed for radioactivity after 24 h.

For the experiment, approximately 20 g wet wt of sediment was added to 50 ml beakers and they were filled with 25 ml of AFW. The beakers were left to stabilize for approximately 14 h. One organism was then added per beaker and aeration was provided. Timepoints of 8, 24, 48, 72, 96 and 168 h were established in all three treatments. In the water treatment with only pyrene, a depuration time series was also set at 4, 8, 24, 48 and 96 h. In all treatments and timepoints, four replicates were used.

In all the experiments, water concentrations of pyrene were measured through liquid scintillation counting (LSC) before and after the water change in order to follow the decrease of pyrene in the water.

2.4 EXTRACTION OF PAHS AND THEIR METABOLITES

After exposures, tissues from all the pyrene experiments were extracted with different combinations of acetone, hexane and methanol in order to analyze their contents. The methods had small variations, their description is given in **I**, **II**, **III** and **IV** and compiled in Table 3.

The final tissue residues were stored at -20 °C. Most of these tissues residues were quantified using LSC, but in *L. variegatus* experiments (**I** and **III**) a proteinase K treatment was applied to liberate the tissue bound pyrene-related molecules.

In the experiments conducted at the University of Roskilde, fluoranthene and its metabolites were extracted from *C. crangon* with a method that divides the total body burdens into four parts: parent fluoranthene, polar metabolites, aqueous metabolites and bound fraction (Palmqvist et al., 2006; Selck et al., 2003).

Table 3. *Brief description of the extraction steps followed in the present thesis. The extractions were performed following the steps from left to right. Ac indicates acetone; Hex., hexane; Meth., methanol; Prot.K, proteinase K; TR, tissue residue; Hom., homogenization; Sonic., Sonication;LSC, Liquid scintillation counting*

Experiment & animal		**Pre-step**	**Ac:Hex (Sonic.)**	**Ac:Meth (Sonic.)**	**Meth (Sonic.)**	**Prot.K**	**TR**
I			2:2ml	2:2ml	3ml		
L.variegatus	Exp. 1	Hom.	(20min)	(20min)	(20min)	-	LSC
			4:4 ml	4:4ml	4ml	In	
	Exp. 2	Hom.	(20min)	(20min)	(20min)	text	LSC
II			4:4 ml	4:4ml	4ml		
G. setosus		Hom.	(30min)	(30min)	(30min)	-	LSC
			2.5:2.5				
III			ml	2.5:2.5 ml	4 ml	In	
L. variegatus	NDW	Hom.	(20min)	(20 min)	(20min)	text	LSC
				2.5:2.5 ml	4 ml	In	
	WAD	Hom.	-	(20 min)	(20min)	text	LSC
			2.5:2.5				
			ml			In	
III *S. trutta*	Liver	Hom.	(40min)	-	-	text	LSC
			2:2ml	2:2ml	3ml		
	GIT	Hom.	(20min)	(20min)	(20min)	-	LSC
			2:2ml	2:2ml	3ml		
	Gills	Hom.	(20min)	(20min)	(20min)	-	LSC
		Man.	2:2ml	2:2ml	3ml		
	carcass	Hom.	(20min)	(20min)	(20min)	-	LSC
			3:3ml	3:3ml	6ml		
	faeces		(60min)	(60min)	(60min)	-	LSC
			2:2ml	2:2ml	3ml		
	GITC		(60min)	(60min)	(60min)	-	LSC
			2.5:2.5				
IV			ml	2.5:2.5ml	3 ml		
G. setosus		Hom.	(20min)	(20min)	(20min)	-	-
			2.5:2.5				
IV			ml	2.5:2.5ml	3 ml		
L.variegatus		Hom.	(20min)	(20min)	(20min)	-	-
			2.5:2.5				
IV			ml	2.5:2.5ml	3 ml		
C. riparius		Hom.	(20min)	(20min)	(20min)	-	-
			2.5:2.5				
	P.		ml	2.5:2.5 ml	3 ml		
Unpublished	*quadrisp.*	Hom.	(20min)	(20min)	(20min)	-	LSC

Table 3 (cont.). *Extractions steps followed for the extractions of C. crangon. Chlor. indicates chloroform and Centr. centrifugation. Note the different solvents and steps compared to the rest of the animals.*

Crangon crangon	**Pre-step**	**Chlor:Meth (Sonic)**	**Chlor: H2O (Centr.)**	**Chlor. phase**	**Meth: H_2O phase**	**TR**
	Hom.+ 1ml H_2O	1:2ml (20min)	1:1ml (5min)	• Evaporate • Add DMSO (600µl) • 250 µl H2O • 150 µl KOH 1M • Mix • Add Hexane (2ml). Mix. • Centrif. (5min) • Separate both phases → LSC • Repeat 2x	2ml → LSC	-

Briefly, the carapaces were removed and the soft tissues were homogenized (Ultra –Turrax T8, IKA Werke, GMBH & Co KG, Staufen, Germany). A portion of the homogenized soft tissue was weighed to the closest 0.1 mg and used for the extractions. If the organism was not very big, all of the soft tissue was used.
A summary of the steps followed during the he extractions is shown in Table 3 and were based on methods already described in literature (Palmqvist et al., 2006; Selck et al., 2003).

2.5 TOXICOKINETICS

In the water experiments with *L. variegatus* and *P. quadrispinosa*, the concentrations of parent pyrene in tissues were measured by HPLC (methods described in the following section) with external standards and the experimental values were fitted to the first order equation (Landrum et al., 1992)

$$C_a = (k_u \cdot C_w)/k_e \cdot (1\text{-}\exp^{-ket}) \qquad (1)$$

where C_a is the concentration of parent pyrene present in the worms (mg g^{-1} wet wt), k_u is the conditional uptake rate constant (ml g^{-1} h^{-1}), C_w is the concentration in water (mg ml^{-1}), k_e is the conditional elimination rate constant (h^{-1}), and t is time (h). In all water treatments, C_w was the average value of all the water samples collected during all the uptake tests that were calculated by the LSC.

In experiment **II**, however, the total body burdens, including total metabolites and parent pyrene experimental values were fitted to a two -compartment model simultaneously using Scientist® software version 2.01 (Micromath® Scientific Software, St. Louis, USA). The variables k_u, k_{ep}, k_m and k_{em} were estimated and compared between the two treatments.

In the sediment exposure with *P. quadrispinosa* (**unpublished**), the total body burdens were fit to the equation (1), because the pyrene concentrations in tissues were only measured by the HPLC at the 168 h timepoint.

Once the toxicokinetic parameters were estimated, the bioconcentration factors (BCF) were calculated as described in Lydy et al. (2000)

$$BCF = k_u/k_e \qquad (2)$$

In articles **III** and **IV**, *L. variegatus* was exposed to pyrene for approximately two months and then depurated in clean Lake Höytiäinen sediment for 96 h. The decrease in the total body burdens of pyrene equivalents were followed by LSC and then fitted to the equation

$$C_a = C_f + (C_o - C_f) * e^{(-kd*t)} \qquad (3)$$

representing an exponential decay to a minimum and where C_a is the concentration of pyrene equivalents in organisms, C_f is the concentration in organisms after depuration, C_0 is the concentration at the start of the depuration (all in μg pyrene equivalents g wet wt^{-1}), t is time (h) and k_d is the depuration rate constant (h^{-1}).

In *P. quadrispinosa* depuration in clean AFW, the elimination kinetics of the parent pyrene experimental data points was fitted with the same equation (3).
In article **II**, the experimental values of total body burdens, total metabolites and pyrene during depuration were transformed to natural logarithm and fitted respectively to the generic equation

$$\ln C_{total,\,p,\,m} = \ln C_{total,\,p,\,m\,0} - k_{d,\,dp,\,m} \cdot t \qquad (4)$$

where $C_{total,\,p,\,m}$ is the concentration of total body burdens, pyrene or metabolites, $C_{total,\,p,\,m\,0}$ is the concentration of total body burdens, pyrene or metabolites at depuration time zero, $k_{d,\,dp,\,m}$ is the depuration rate constant for the total compound, parent or metabolites (h^{-1}) and t is time (h).

2.6 BIOTRANSFORMATION

The biotransformation of pyrene was studied for *L. variegatus* in articles **I** and **IV**, for *Gammarus setosus* in **II**, for juvenile *Salmo trutta* in **III** and briefly for *C. riparius* in **IV**. Additionally, evidence of the biotransformation of pyrene and fluoranthene was found in **unpublished** experiments with *P. quadrispinosa* and *C. crangon*, respectively. Initially, the additional experiments with *C. crangon* were meant to test the trophic transfer of fluoranthene metabolites produced by *Capitella teleta* to *C. crangon*. However, it was only possible to conduct an experiment where only the dietary transfer of parent fluoranthene was assayed. Although most of the samples still need to be analyzed, the finding of one possible phase II metabolite of fluoranthene is worth reporting.

Table 4. *HPLC methods used in the present thesis. The flow rate is indicated in ml min^{-1}. ACN indicates acetonitrile, amm. acetate ammonium acetate; TEA, triethylamine.*

Method	Used in	Column	Eluents	Flow rate	ACN Gradient
Pyrene A	**I, II**	Zorbax SB-C18 (100×3.0 ; 3.5mm) (Agilent)	H2O; ACN	0.5	Started at 5% ACN for 4 min, then was raised to 50% in 1 min, held in there for 4min and finally raised to 100% in 2min and held there for 7min.
Pyrene B	**I, II, IIIa**	Zorbax Eclipse XDB-C8 (150×4.6 ; 5mm) (Agilent)	0.01M amm. acetate (pH5); ACN	1	Started at 20% ACN and was raised to 82% in 23 min, then to 90 in 2 min and held there for 2 min. In experiments II & III, the method was shortened at 23 min (82% ACN).
Pyrene C	**IV, unpublished**	Zorbax Eclipse XDB-C8 + Xbridge C8 (150×4.6 ; 3.5mm) (Waters)	0.01M amm. acetate +0.1% TEA (pH9); ACN	0.7 (25min), ->1 (3min) kept there for 5 min	Started at 20% ACN and was raised to 55% in 25min, raised to 100% in 3 min and held there for 5 min.
Fluoranthene	**Unpublished**	Zorbax Eclipse XDB-C8 + Zorbax SB-C18 (100×3.0 ; 3.5mm) (Agilent)	0.01M amm. acetate (pH5); ACN	0.5	Started at 25% ACN and was raised to 100% in 55 min and held there for 10 min.

For the pyrene samples, three different HPLC methods were used, in order to get a better separation of the peaks of interest,

which was not optimal at first. The description of the methods used is given in Table 4 and in the single articles.
For the fluoranthene samples, a longer method with two columns was used due to the more complex biotransformation pathway for this compound when compared to pyrene. The diode array detector (DAD; set at 254 nm) was used for the detection of fluoranthene and its metabolites.
In the pyrene samples, the retention times (t_R) of the peaks not present in the controls were compared to the t_R of the available standards (pyrene, 1-hydroxy-pyrene and its glucuronide conjugate). Also, the ultraviolet (UV) absorption spectra of all peaks was surveyed and compared to those of the parent compounds, pyrene and fluoranthene.

The tissue concentrations of phase II metabolites in *P. quadrispinosa* were calculated by HPLC and LSC. The three peaks not present in controls that were found in the HPLC chromatograms were collected with the fraction collector and quantified by LSC. The phase II tissue concentrations were considered to be the sum of their concentrations. In *G. setosus* (**II**), the two main peaks found were collected together with the fractions collector and quantified as described above.
The *L. variegatus* extracts from experiment **I** were incubated with the deconjugation enzymes sulfatase, α- and ß-glucosidase, ß-glucuronidase and lactase. Additionally, the *C. riparius* extracts from experiment **IV** were incubated with sulfatase and ß-glucosidase (**unpublished** results).
Enzymatic deconjugations were also used to discern the total amount of 1-hydroxy-pyrene produced by the worms in **I**. Based on the results obtained with the individual enzymes the extracts were consecutively incubated with beta- glucosidase and sulfatase to deconjugate all phase II metabolites present.
The mass spectrometric techniques performed in Åbo Akademi University (Turku, Finland) that were used in experiment **I** to identify the glucose sulfate conjugate of 1-hydroxy-pyrene are described in the corresponding article.

2.7 TROPHIC TRANSFER EXPERIMENTS

Experiments **III** and **IV** were performed in order to study the trophic transfer of the previously described pyrene metabolites produced by *L. variegatus* and *C. riparius* to various relatively small predators.

2.7.1 Exposure of prey to pyrene

In experiment **III** and **IV**, the prey was *L. variegatus*. Briefly, they were exposed for approximately two months to a nominal concentration of pyrene of 20 μg L^{-1}, in order to gain sufficient mass of metabolites.

After the exposure, approximately half of the total number of worms was transferred to clean lake sediment, while the other half was kept in the same conditions for a week with no water change or feeding. By this, it was intended to decrease the parent pyrene body burdens, as the worms excrete it more rapidly than the metabolites (Leppänen & Kukkonen, 2000). The measurements were performed as described in **III** and **IV.**

Chironomus riparius was additionally used as a prey in experiment **IV**. The exposures needed to be different, as the life cycle and biotransformation capacity of this animal differ from *L. variegatus*. They were exposed to a nominal concentration of pyrene of 1.5 μg L^{-1} for 24 h in AFW at their fourth instar larval stage. Samples of 1-2 midges (8.05± 0.32 mg; n=5) were taken for extractions and analyses by HPLC and LSC.

2.7.2 Feeding of predators

Similar feeding experiments were conducted in **III** and **IV**, although the predator species was different, juvenile *S. trutta* in **III** and *G. setosus* in **IV**. The depuration of worms in clean sediment made possible the existence of different concentrations of parent pyrene and metabolites between depurated and non-depurated worms. However, the results of non- depurated worms in **IV** were not reported because they did not add anything new to the existing literature.

Additionally, as positive controls, fish were also fed pyrene- and 1-hydroxy-pyrene- spiked food pellets, in order to elucidate the biotransformation pathways of these chemicals in *S. trutta*. These treatments were not conducted in **IV,** as the biotransformation pathway of *G. setosus* was already determined by the studies in **II**.

As ingestion of prey by predators was a key issue in the experiments, the introduction of the prey in the beakers was performed taking into account the fact that the predator had to notice the prey. In the *S. trutta* experiments, all the worms were introduced vigorously in order that the fish noticed their presence. In the *G. setosus* experiment, the prey was introduced close to the predators.

2.7.3 Methods to determine the trophic transfer of metabolites

The methods included (1) a mass balance, comparing the estimation of pyrene ingested vs. the metabolites found in (a) the tissues of predators or (b) outside the tissues but produced by predators and (2) a comparison of the FLD-HPLC profiles of prey exposed to pyrene, predator fed on pyrene-exposed prey and predators exclusively exposed to pyrene.

Mass balance

By estimating the amount of pyrene ingested, the maximum amount of metabolites that can be produced from it was also estimated, as from one mol of pyrene a mol of metabolites is produced. Therefore, finding that the sum of metabolites (1) produced by predators and/or (2) produced by prey but found in predator's tissues is higher than the amount of parent pyrene ingested necessarily means that at least part of the metabolites were transferred from prey to predators.

The estimation of pyrene ingested by predators was calculated by multiplying the average amount of pyrene in worms (ng mg^{-1} wet wt; calculated by HPLC) by the estimated mass of worms ingested (mg) considering a mass of 5 mg per worm.

With the same methods, the amount of metabolites ingested was calculated. The estimation of metabolites present in tissues of

prey was calculated by subtracting the amount of pyrene (calculated by HPLC) from the total body burden (calculated by LSC).

Comparison of FLD-HPLC chromatograms

Knowing the specific metabolites that the prey and predator species produce as a result off their exposure to pyrene can help to distinguish between their different biotransformation pathways. By feeding a predator a pyrene exposed prey, it can be determined if the metabolites produced by the prey have been taken up by the predator. First, discarding the peaks produced by predator, these are determined by the chromatograms of predators exclusively exposed to pyrene; and second, comparing the prey and the predator -fed on that prey, checking for peaks eluting at the same t_R. If a given peak, produced by the prey but not by the predator, is found in the chromatograms of predators fed on pyrene-exposed prey, the trophic transfer from prey to predator has just been proven.

2.7.4 Calculations of biomagnification factors (BMFs) of the biotransformation products

Although not reported in the single articles, the biomagnification factors (BMFs) for the total amount of metabolites were calculated in the present thesis. It was not possible to calculate the BMFs for the single metabolites because of a lack of standards.

The BMFs were calculated by dividing the concentration of metabolites in predators by the concentration of metabolites in prey (both in μg of pyrene equivalents g wet weight^{-1}).

In experiment **III**, two calculations were made per treatment (NDW and DW). First, the calculations were made strictly considering the metabolites that are in fish tissues, i.e. carcass, gills, GIT and liver/bile. This provides the real BMFs that would be obtained if measured in the field. Second, the metabolites in faeces, water and GITC were considered for these calculations. These matrices were also considered in this case to reflect the

effect of excretion and to compare the BMFs with the invertebrates.

2.8 DIETARY EXPOSURE OF *CRANGON CRANGON* TO FLUORANTHENE

After collection, *C. crangon* were kept for several days in laboratory conditions before the feeding experiment with fluoranthene –spiked dead *C. teleta* was started. Approximately 520 mg of worms were sieved from the sediment and left in clean marine water to empty their guts for two hours. From that mass, 440 mg were homogenized and spiked with a solution of fluoranthene in acetone (500 μg ml^{-1}). The mix was shaken for 1.5 h and the acetone was evaporated. The 80 mg of worms left were used as controls.

As described in Palmqvist et al. (2006), the *Capitella* food to feed *C. cragon* was prepared. Briefly, a tiny part of worm homogenate was added to a drop of alginate and covered by another drop of alginate. The so -called food discs were solidified by adding a drop of a 2% solution of calcium chloride.

A food disc was added to individual beakers containing one *C. crangon* and the food was left in these beakers for 2 h. After the two hours, *C. crangon* were transferred to different beakers with clean marine water and the test was considered as started. At timepoints of 3, 15, 24 and 48 h, the animals (n=5) were collected, rinsed, weighed and frozen in plastic bags at -20 C.

Also water samples (10 ml) were collected, mixed with 10ml of Ultima Gold scintillation cocktail, mixed and measured for radioactivity after 24 h.

Due to the feeding behaviour of *C. crangon*, fluoranthene likely leaked into the water and therefore the exposure was not exclusively through diet.

2.9 STATISTICAL ANALYSES

Mainly univariate analyses of variance (ANOVA), t- and Mann-Whitney tests were used to compare the body burdens of pyrene, metabolites and tissue residues among different treatments. In experiment **I** and **II**, ANOVA was used to find whether differences exist between the concentrations of the different chemicals among the three treatments (SPSS v.16 and Sigma plot v. 11). To specify which treatments were different in **I**, a Games-Howell pos hoc test was applied (SPSS v.16), due to the unequal variances found in the experimental data. The statistical differences tested in experiments **III** and **IV** were surveyed using T or Mann-Whiney tests, depending on the equal or unequal variances of the experimental data (SPSS v. 17.0).

3 Results and discussion

3.1 BIOACCUMULATION AND TOXICOKINETICS

The experiments presented in this thesis represent three main different uptake routes for PAHs in aquatic organisms: water (fresh- in **I** and **unpublished** with *P. quadrispinosa* and marine in **II**), sediment (*P. quadrispinosa,* **unpublished**) and diet (**III**, **IV** and **unpublished** with *C. crangon*).

All the study animals were able to bioaccumulate pyrene, which is supported by McElroy et al. (1990), that detected the accumulation of benzo(a)anthracene despite the source of the PAH. Also, and essential for my thesis, all organisms were able to biotransform pyrene, though to a variable extent.

The ability to biotransform pyrene was directly related to the bioaccumulation of the parent compound: the organisms that had good biotransformation ability (*G. setosus*, *P. quadrispinosa*, *S. trutta*) had much higher tissues concentrations of metabolites than the organisms that biotransformed less efficiently (*L. variegatus*).

The toxicokinetic variables, BCFs and the r^2 of the model used are in Table 5. In the two experiments where the toxicokinetics were calculated based only on the parent pyrene (**I** and *P. quadrispinosa*), the estimated concentrations of pyrene at steady state ($[Pyr_{ss}]$), also included in Table 5, are much higher in *L. variegatus* that biotransforms less efficiently than *P. quadrispinosa*, although this is not only expected to depend on the biotransformation. In species like *G. setosus* (**II**) pyrene did not even reach steady state, the concentrations of metabolites being much higher than the concentrations of pyrene (Fig. 1 in **II**). This may be also the case in *P. quadrispinosa*, whose average tissues concentrations of pyrene (Fig. 5A) were approximately half than the concentrations of phase II metabolites in the treatment with pyrene and PBO (Fig. 5A and 5B) although variations were also high, probably due to the method used (section 2.6).

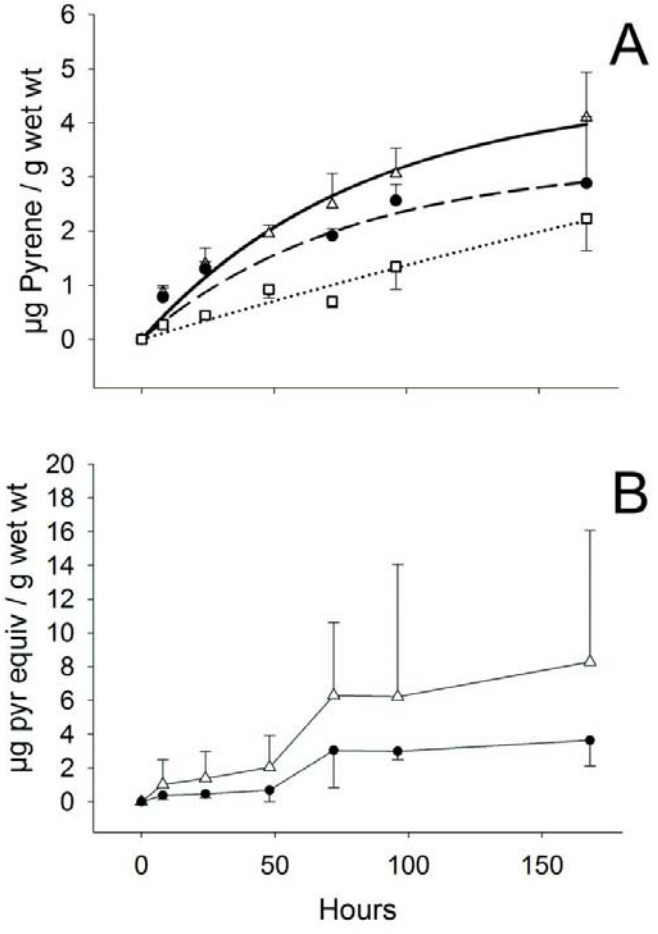

Figure 5. *Tissue concentrations of pyrene related molecules in P. quadrispinosa during uptake phase (0 -168 h). In A) the tissue concentrations of pyrene in the only-pyrene treatment (circles), in the pyrene + PBO treatment (triangles) and the total body burden in the sediment treatment (squares). The fits of equation (1) are continuous, dashed and dotted lines, in the same order. In B) the tissues concentrations of phase II metabolites in the only pyrene treatment (circles) and in the pyrene + PBO treatment (triangles) are shown.*

In the only pyrene treatment, concentrations of parent and phase II metabolites were similar. To illustrate the differences with the sediment treatment, the total body burdens of pyrene related- molecules are also shown in Fig. 5A.

Interestingly, the BCFs are in the same range despite the conditions and species used. Only an extraordinary high value was estimated in the *G. setosus* treatment at 2 °C, which could be explained by a low accuracy of the method used, as for example the SD of the k_e was as big as its own value. The values described in article I are comparable with previous experiments conducted in our laboratory (Mäenpää et al., 2009).

Table 5. *Toxicokinetic variables and steady state concentrations. Conditional uptake and elimination rate constants (k_u and k_e respectively), bioconcentration factors (BCF), averages of the experimental water concentrations (Cw; μg ml^{-1}) and estimated steady states for pyrene ([Pyr]$_{ss}$;μg g wet wt^{-1}) are shown. The adjusted coefficient of determination (Adj r^2) is shown as measure of goodness of fit for every model. k_u: ml g^{-1} h^{-1}; k_e: h^{-1}; C.I.: Confidence interval. [Pyr]$_{ss}$: μg g wet wt $^{-1.}$ #: Total body burden concentrations at steady state. *indicates the concentration in sediment(μg g wet wt^{-1}). In parenthesis the Standard deviation is provided.*

Exp.	**C_w**	**k_u**	**k_e**	**Adj. r^2**	**BCF**	**95% C.I.**	**[Pyr]$_{ss}$**
I NO PBO	0.0087 (0.002)	59.5 (20)	0.035 (0.015)	0.52	1715	593.5-4954	14.90
PBO 354	0.009 (0.002)	48.1 (9.7)	0.027 (0.007)	0.73	1763	919.9-3380	15.7
PBO 35.4	0.0091 (0.002)	55.6 (11.6)	0.035 (0.009)	0.71	1599	836.5-3055	14.50
II Cold 2°C	0.0013 ($4*10^{-4}$)	28.8 (7.3)	0.002 (0.002)	0.9	13913	2420 - 79964	18#
Warm 8°C	0.0011 ($5.3*10^{-4}$)	24.6 (8.8)	0.0085 (0.003)	0.88	2891	1540 - 5425	3.2#
P. quad.							
No PBO	0.0029 (0.011)	14.8 (3.2)	0.013 (0.005)	0.62	1158	490 – 2737	3.30
Pyr + PBO	0.0034 ($5.8*10^{-4}$)	16 (1.8)	0.012 (0.003)	0.85	1333	779.4– 2281.4	4.53
Pyr Sed.	1.58 (0.42)*	0.009 (0.0016)	0.0014 (0.003)	0.8	6.64	0.04– 24.4	10.50

Biotransformation processes can have a large influence over the BCFs (Akkanen & Kukkonen, 2003), but in my studies, this is only noticeable between species (Table 5). The BCFs are slightly lower in *P. quadrispinosa* than in *L. variegatus*, supporting the lower biotransformation capacity of the latter. Although differences in biotransformation were found among the PBO and no PBO treatments in article **I**, the BCFs were not statistically different, probably because metabolites accounted for a low share of the total body burden.
Additionally, the BCFs that were calculated with the total body burden concentrations (**II**) tended to be higher than the BCFs calculated only with the parent pyrene concentrations (**I** and **unpublished**).

3.2 BIOTRANSFORMATION

In all the experiments conducted (**I** to **IV** and the **unpublished** results with *P. quadrispinosa* and *C. crangon*), evidence of biotransformation was found. A comparison of the FLD- HPLC chromatograms that illustrate the different biotransformation pathways of pyrene in all the species studied is shown in Fig. 6.
The results are in agreement with reported literature that describes the ability of *L. variegatus*, *C. riparius* and salmonid fish to biotransform PAHs (Leversee et al., 1982; Leppänen & Kukkonen, 2000; You et al., 2006; Schuler et al., 2003; Kennedy & Tierney, 2008; Mäenpää et al., 2009). Additionally, the studies conducted added a comparison of the biotransformation pathways of pyrene in invertebrates that belong to different taxa (Oligochaeta, Crustacea and Diptera) and an important fish species. Additionally, evidence of biotransformation of fluoranthene by the brown shrimp *Crangon crangon* was found.

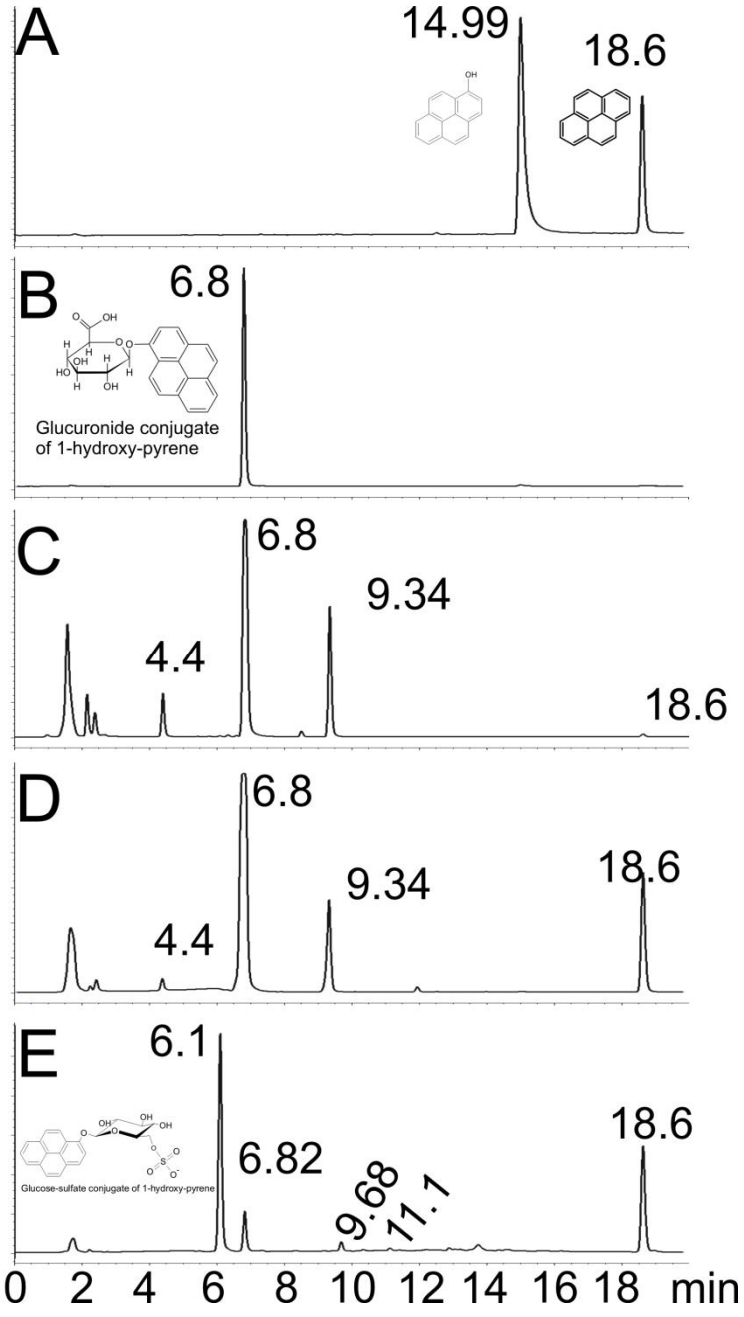

Figure 6. *Comparison of the FLD-HPLC chromatogram profile of the different species that were exposed to pyrene. A)Pyrene and 1-hydroxy-pyrene standards, B) Glucuronide conjugate of 1-hydroxy-pyrene standard, C) liver of S. trutta, D) GIT of S. trutta, E) L. variegatus.*

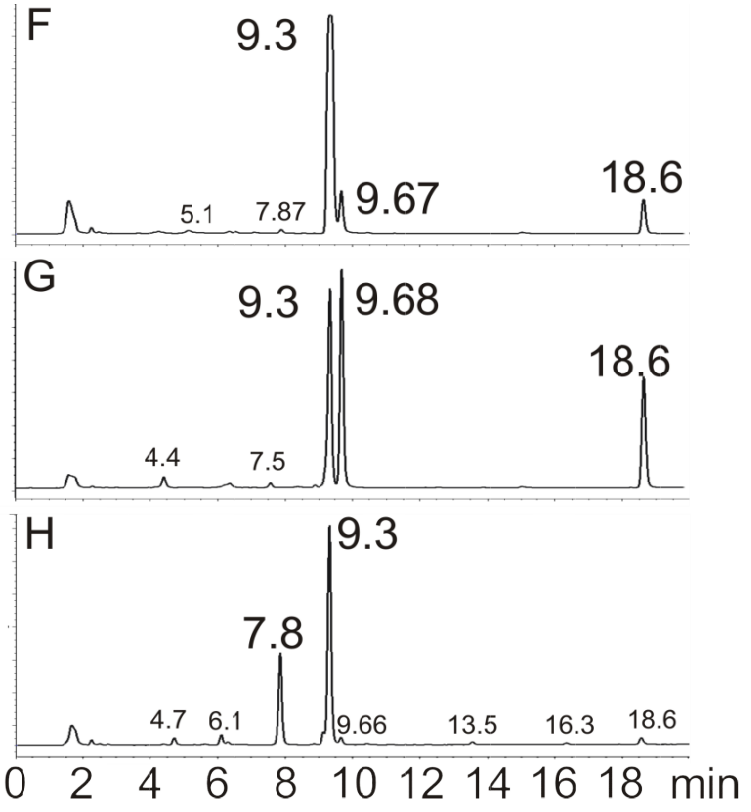

Figure 6 (cont). *F) G. setosus, G) P. quadrispinosa and H) C. riparius. The peaks of interest are indicated by their retention times (t_R) and the structures of the positively identified peaks are provided.*

3.2.1 Role of CYP

As the cytochrome P-450 (CYP) proteins are the main enzymes responsible for phase I oxidation reactions (Schlenk et al., 2008), their inhibition or inactivation will cause a decrease in the biotransformation rate of a certain compound. While there are very numerous CYP isozymes in vertebrates, e.g., fish (Schlenk et al., 2008) and humans (up to 57 isoforms; Redlich, 2008), their presence may not be that common in invertebrates, as their ability to biotransform PAHs varies widely (McElroy et al., 2000; Rust et al., 2004).

In experiment **I** and in the **unpublished** experiments with *P. quadrispinosa*, the presence of a CYP isoform was investigated indirectly by the addition of the known CYP inhibitor piperonyl butoxide (PBO). It is a compound commonly used as a synergist of some insecticides: it enhances the toxicity caused by the pesticide by blocking CYP and therefore inhibiting its biotransformation. The inhibition of CYP by PBO is a result of a stable bond between a carbene and the heme group of CYP (Hodgson & Levi, 1998), although it is not clear whether all or only some isoforms of CYP are the subject of this inhibition. PBO and some other similar compounds are extensively used in combination with active substances (that are biotransformed by CYP in insects) as a synergist to increase the mortality of these particular active substances (Hodgson & Levi, 1999).
PBO did not cause any statistical differences in the concentrations of parent pyrene in tissues among the treatments in **I** and *P. quadrispinosa* (ANOVA; square root transformed values; F [1, 46] = 2.046, p =0.159). Therefore, PBO may prevent the toxicity associated with PAH metabolites but it can enhance the toxicity caused by the parent compound directly, such as narcosis (Hawkins et al., 2002). On the other hand, my results are not in agreement with other studies that used PBO as a CYP inhibitor: the concentrations of pyrene in *Daphnia magna* (Akkanen & Kukkonen, 2003) and fluoranthene and benzo(a)pyrene in the larval grass shrimp *Palaemonetes pugio* (Weinstein & Garner, 2008) increased with the co-addition of PBO. These differences are likely due to the biotransformation capacities of the organisms: *L. variegatus* has a weak ability (**I**) while e.g. the efficient ability of *Daphnia magna* to biotransform pyrene was proved (Akkanen & Kukkonen, 2003; Ikenaka et al., 2006).

Furthermore, in *L. variegatus* tissues (**I**), statistically significant differences among treatments were found in the concentrations of solvent -extracted and total 1-hydroxy-pyrene, which is a clear sign of differences in biotransformation. In the treatments where PBO was added CYP would be blocked, preventing or

reducing the formation of 1-hydroxy-pyrene and thus its phase II conjugates which is precisely what was found. On the other hand, the differences in total 1-hydroxy-pyrene concentrations between *P. quadrispinosa* treatments with and without PBO were not significant.

The apparent species differences may be due to an insufficient concentration of PBO in *P. quadrispinosa*. Concentrations of PBO applied in other studies (Akkanen and Kukkonen, 2003; Filipowicz et al., 2007) were from two to 20 times higher than the concentrations applied in the *P. quadrispinosa* experiment (53 μg/L), achieving significant reduction in the biotransformation rate of pyrene and fluoranthene, respectively. This is similar to the results achieved in **I**, were only the higher concentration of PBO (354 μg/L) inhibited the biotransformation of pyrene significantly (**I**, Table S1).

Additionally, it is interesting to remark that the PBO mode of action may include a biphasic response that first causes an inhibition and then an induction of CYP (Hodgson and Levi, 1999). However, this has been observed after a single dose, while in our experiments PBO was constantly present and thus the phenomenon may occur through a different mechanism. This phenomenon has been observed in fish and lepidoptera (Hodgson and Levi, 1999). Additionally, PBO induced CYP 1A1 and 1A2 in mouse liver after a single intraperitoneal injection and CYP 1A1 in Wistar rats (Heder et al., 2001).To confirm the CYP -inducing phenomenon caused by PBO in *L. variegatus*, CYP activity should be measured and a single short exposure to PBO should be performed instead of a continuous exposure.

3.2.2 Phase I biotransformation

Pyrene is a compound that was chosen, among other reasons, because of its simple main biotransformation pathway, that starts with the introduction of a hydroxyl group to produce 1-hydroxy-pyrene (Fig. 7), although other alternative pathways are possible. *C. riparius*, *P. quadrispinosa*, juvenile *S. trutta* and *G. setosus* showed the ability to biotransform pyrene to 1-hydroxy-pyrene.

The production of 1-hydroxy-pyrene as a main phase I metabolite has also been reported in an extensive variety of organisms, such as the polychaetes *Nereis diversicolor* (Christensen et al., 2002; Tairova et al., 2009; Giessing et al., 2003) and *Nereis virens* (Jorgensen et al., 2005), the isopod *Porcellio scaber*, the oligochaetes *Eisenia andrei* and the springtail *Folsomia candida* (Stroomberg et al., 2004a), the marine whelk *Buccinum undatum* (Beach et al., 2010), *Salmo salar* m. *sebago* yolk-sac fry (Honkanen et al., 2008), speckled trout *Salvenius fontinalis* (Leonard & Helou, 2001), rat (Bouchard & Viau, 1998), among others.

Interestingly, in *C. riparius*, although its main phase I metabolite of pyrene is 1-hydroxy-pyrene, as the deconjugation results show (see next section), this organism may produce other phase I metabolites, as there are two peaks eluting close to 1-hydroxy-pyrene (Fig. 8). Therefore, it is possible that the conjugations also take place to these alternative phase I metabolites. The existence of alternative phase I metabolites in *C. riparius* may be supported by the recent description of uncommon phase I metabolites of pyrene in other animals (shown also in Fig. 7). Beach et al. (2010) tentatively identified 1, 6 –dyhydroxy-pyrene in the tissues of the whelk *B. undatum*, similar to the amphibian *Xenopus laevis* (Ueda et al., 2011).

It is precisely the poor sensitivity of some of these diols to the most common wavelengths (used to identify the other metabolites) which may have hampered their detection. Also their poor stability under the conditions used in my thesis may make their detection difficult (Beach et al., 2009), e.g. 1, 6 –dyhydroxy-pyrene may transform to 1,6 pyrenequinone (Wunder et al., 1994).

Another reason that hampers the detection and identification of phase I metabolites is that their concentrations in tissues tend not to be very high because they are not the final products of the biotransformation pathway. They are biotransformed to phase II metabolites very rapidly, which has been suggested in the literature (Selck et al., 2003). In fact, phase I reactions are a limiting step in the biotransformation and excretion of pyrene in

invertebrates (Beach et al., 2010; Jorgensen et al., 2005); this is also supported by the observations in article **I**. This is a general trend, as the concentrations of 1-hydroxy-pyrene found in tissues are generally much lower than the concentrations of phase II metabolites.

On the other hand, prokaryote organisms such as microbes usually biotransform pyrene through more diverse pathways, even reaching mineralization. I have included this information here, because e.g. in the GIT microbes can be responsible for the biotransformation of pyrene in the animals studied (also discussed in article **I**). Rather than biotransformation, a term that requires minor changes in the structure of the hydrocarbon, these pathways are usually classified as biodegradation, as they include the transformation of the skeleton of the molecule and also allow the organisms to grow on the hydrocarbon (Neilson & Allard, 1998). A *Mycobacterium sp.* strain was able to use pyrene as its sole energy and carbon source (Rehmann et al., 1998), and products of degradation included for example the three ring 4 -phenanthroic acid, the two ring 1-hydroxy-2-naphthoic acid and the one ring phthalic acid (Fig. 7; Rehmann et al., 1998). Besides also finding some of these metabolites, Heitkamp et al. (1988) also determined a degradation rate of pyrene of 60% by *Mycobacterium sp.* in four days. It has been reported that some bacteria strains are able to degrade pyrene and other PAHs down to acetyl-CoA and succinyl-CoA, which enter the Krebs cycle to produce energy (Kweon et al., 2011). Some biodegradation products of pyrene are shown in Fig. 7.
In experiment **I**, it was discussed whether the microbes that may be present in the gut of *L. variegatus* were responsible for the production of the described metabolites. As the worms were not fed during the whole experiment, the microbial community in the gut had limited accessibility to pyrene and, although the transformation may have happened, likely it is assumed to be not relevant. Besides, the small volume of microbes that may be in *L. variegatus* guts or the surrounding water are other factors to take into account. In pristine sea sediments, Giessing & Johnsen,

(2005) found that the mineralization of pyrene and 1-hydroxy-pyrene was negligible after 175 days, although a pre exposure to pyrene induced its microbial degradation (81 % mineralized after 95 days). The same authors determined that six strains of pyrene-degrading bacteria were not able to grow in wells covered by the major product of biotransformation of pyrene, 1-hydroxy-pyrene, and even a decrease in respiration was observed, suggesting a toxic effect (Giessing & Johnsen, 2005). The experiments in my thesis lasted few days, the water was changed every 24 h in most cases and there was not history of a pre exposure to pyrene that may have enhanced its degradation. Furthermore, it does not seem logical that microbes biotransform pyrene to 1-hydroxy-pyrene and its conjugates. Many bacteria use glucose and/or glucuronide acid as a carbon source (Giessing & Johnsen, 2005), and they would probably hydrolyze phase II metabolites of pyrene in order to obtain energy and carbon source. Additionally, the gut content of some fish has glucuronidase activity (Newman & Unger, 2003). Therefore, it is concluded that the metabolites described in the present thesis are produced by the animals studied in my experiments.

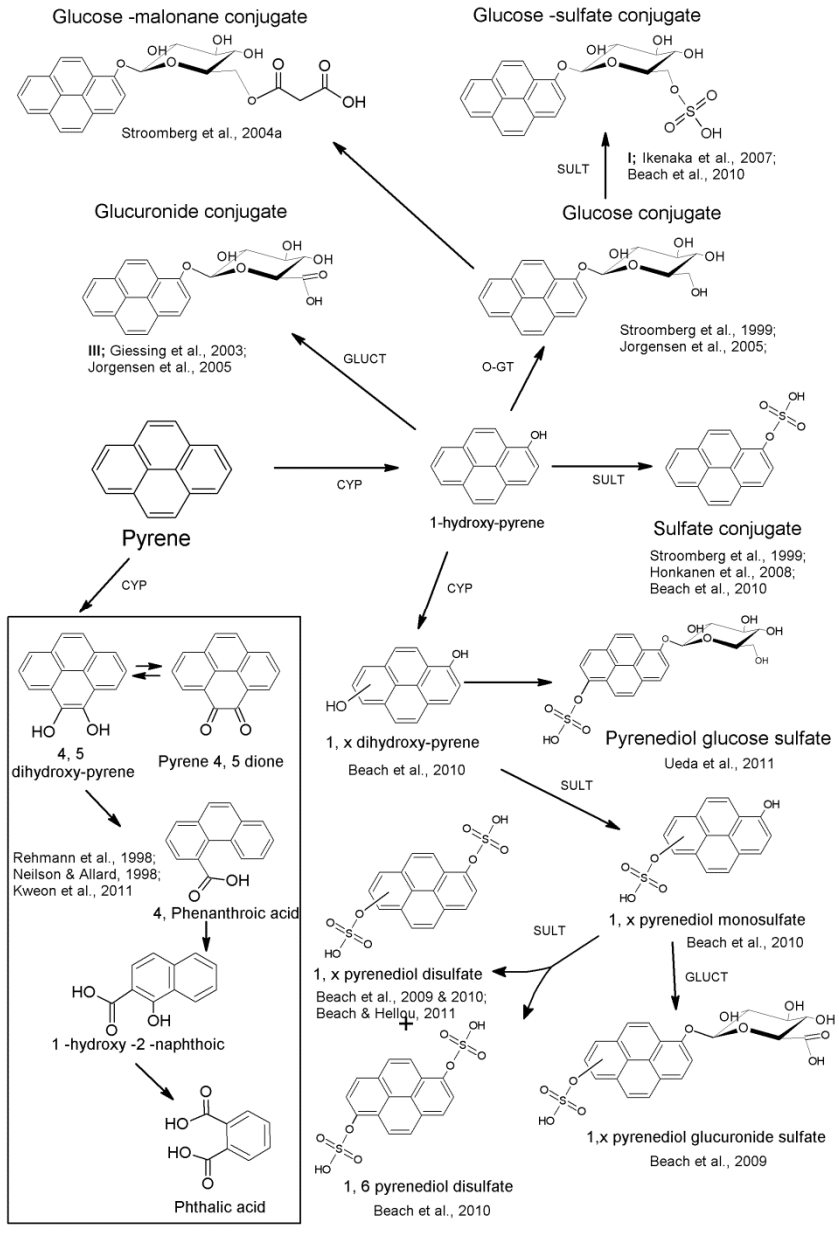

Figure 7*. Biotransformation and biodegradation pathways of pyrene described in literature. The names of the compounds are displayed near the structure, with the reference where it was reported. Some of the microbial biodegradation products of pyrene are shown in the box to distinguish them from the biotransformation products. CYP: Cytochrome P-450; SULT: Sulfotransferase; GLUCT: Glucuronosyltransferase; O-GT: Glucosyltransferase.*

3.2.3 Phase II biotransformation

As different HPLC methods were used in the articles (**I**-**IV**), a new HPLC method was performed where all the samples were run to compare the biotransformation pathways of pyrene in the different organisms. This comparison is presented in Fig. 6. A peak representing the parent pyrene is present in all the species previously exposed to it (t_R = 18.6 min) at different heights that reflect the biotransformation capacity of the species or specific organ.

The glucuronide conjugate of 1-hydroxy-pyrene was the main metabolite present in liver + bile and GIT of pyrene fed *S. trutta* (Fig. 6C and 6D), eluting at the same t_R as its standard (Fig. 6B; t_R = 6.8 min). Glucuronidation is normally the main conjugation pathway in fish (Leonard & Hellou, 2001; Luthe et al., 2002; Kennedy & Tierney, 2008; Schlenk et al., 2008), and in fact, it was also the main peak in extracts of GITC and faeces of trout (**III**, Suppl. information, Fig. A1). This conjugate was not present in any of the invertebrates studied in my thesis, although glucuronidation may sometimes appear in invertebrates (e.g. *Nereis diversicolor*, Giessing et al., 2003). Although *L. variegatus* seemed to produce this conjugate based on the t_R of this HPLC run (Fig. 6E; t_R = 6.82min), this is not the case as described thoroughly in article **I**. With other HPLC methods, the glucuronide conjugate and the unknown molecule in *L. variegatus* eluted at distinguishable t_R.

Another metabolite strictly produced by *S. trutta* among the species used eluted at t_R = 9.3 min. It was discussed in articles **II** and **III** that this molecule may be the sulfate conjugate of 1-hydroxy-pyrene. This is plausible, as sulfate conjugates are also important in the biotransformation of PAHs by fish (Luthe et al., 2002; Honkanen et al., 2008; Kennedy & Tierney, 2008). Additionally, the presence of glucuronide and sulfate conjugates in fish bile is expected, as it is common to use a ß-glucuronidase with arylsulfatase in order to hydrolyze the possible conjugates due to the lack of standards (Beyer et al., 2010). The sulfation of phase I PAH metabolites is catalyzed by the family of sulfotransferases enzymes (SULT), present in the liver of e.g.

rainbow trout (Schlenk et al., 2008). Additionally, *G. setosus*, *P. quadrispinosa* and *C. riparius* also shared this metabolite tentatively identified as sulfate (t_R = 9.3 min; Fig. 6F, 6G, and 6H, respectively).
All the invertebrates studied also have a common metabolite that eluted at t_R = 9.68 min, although it was only a major metabolite in *P. quadrispinosa*. It was discussed that this metabolite may be the glucose conjugate of 1-hydroxy-pyrene (**I** and **II**).

The other successfully identified molecule, the glucose sulfate conjugate of 1-hydroxy-pyrene produced by *L. variegatus* elutes at t_R = 6.1 min (Fig. 6E). Of the other species studied, only *C. riparius* may produce this conjugate when exposed to pyrene (Fig. 6H; t_R = 6.11), although it appeared to be a minor metabolite. To my knowledge, article **I** is the first study that describes a phase II metabolite of a PAH in *L. variegatus* (the glucose-sulfate conjugate of 1-hydroxy-pyrene). This metabolite was also produced by aquatic crustaceans (Ikenaka et al., 2007) and the whelk *Buccinum undatum* (Beach et al., 2010). The glucose-sulfate conjugation pathway is not exclusive to invertebrates and PAHs, as its metabolite has been found in humans for the naturally occurring drug ß-Lapachone (Savage et al., 2008), in cotton plants for 4-bromo 2-chlorophenol and 2,4-dichlorophenoxyacetic acid (Capps et al., 1996; Laurent et al., 2000, respectively). Besides glucose sulfate conjugates, other double conjugations have been found in invertebrates and plants. Terrestrial isopods and springtails have been reported to produce glucose malonate conjugate of 1-hydroxy-pyrene (Stroomberg et al., 2004a), *Lemna minor* was able to biotransform chlorophenols to glucose-malonate and glucose-apiose conjugates (Day & Saunders, 2004) and *Lupinus albus* biotransformed some flavonoids to their glucose- malonate conjugates (Bednarek et al., 2001). While the single conjugates such as glucuronide, sulfate or glucose are normally found to be produced by a high number of species, the overall occurrence and significance of the double conjugates is not clear. It has been

observed that the ultimate fate of glucose -malonate and glucose -apiose conjugates in plants are their sequestration into vacuoles and cell walls, respectively (Day & Saunders, 2004). Likely, the formation of the glucose sulfate conjugate would include three steps, phase I catalyzed by an isoform of CYP to introduce a hydroxyl, and two steps in phase II that include the introduction of a glucose in the hydroxyl group and the introduction of the sulfate molecule in the carbon 6 of the glucose (article **I**, Fig. S1). An interesting hypothesis is whether these double conjugates are produced by invertebrates as a result of the inability to excrete the single conjugates or as a signal for sequestration. In oligochaetes, a special type of cells called chloragogen is present around the gut and the blood vessels and they may be responsible for the detoxification of xenobiotics (Rodriguez & Reynoldson, 2011). These cells are also the main storage organ in Oligochaetes and they have been reported to immobilize metals (Morgan & Morgan, 1989), and the question is whether they would also sequestrate pyrene metabolites. This question already arose for ethinylestradiol conjugates in *L. variegatus* (Liebig et al., 2005).

Five phase II metabolites of pyrene were found in *Chironomus riparius* extracts (**IV**; Fig. 8A), suggesting an effective biotransformation capacity, already reported in literature (Harkey et al., 1994; Verrengia- Guerrero et al., 2002). Although not reported in **I**-**IV**, the deconjugations with ß- glucosidase and sulfatase were performed (Fig. 8B and 8C, respectively). The results showed that with ß- glucosidase the minor peaks (eluting between 5 and 7.5 min in Fig. 8B) disappeared, suggesting strongly that glucose conjugation is among the pathways used to biotransform pyrene by this species. The incubation with sulfatase yielded a peak with the same t_R as 1-hydroxy-pyrene, while the peak eluting at t_R 10.4 min decreased by 55% (Fig. 8C). These results indicate that this peak may be the sulfate conjugate of 1-hydroxy-pyrene, although its incomplete deconjugation possibly indicates that either another compound co-elutes at the same t_R or that the hydrolysis time or enzyme

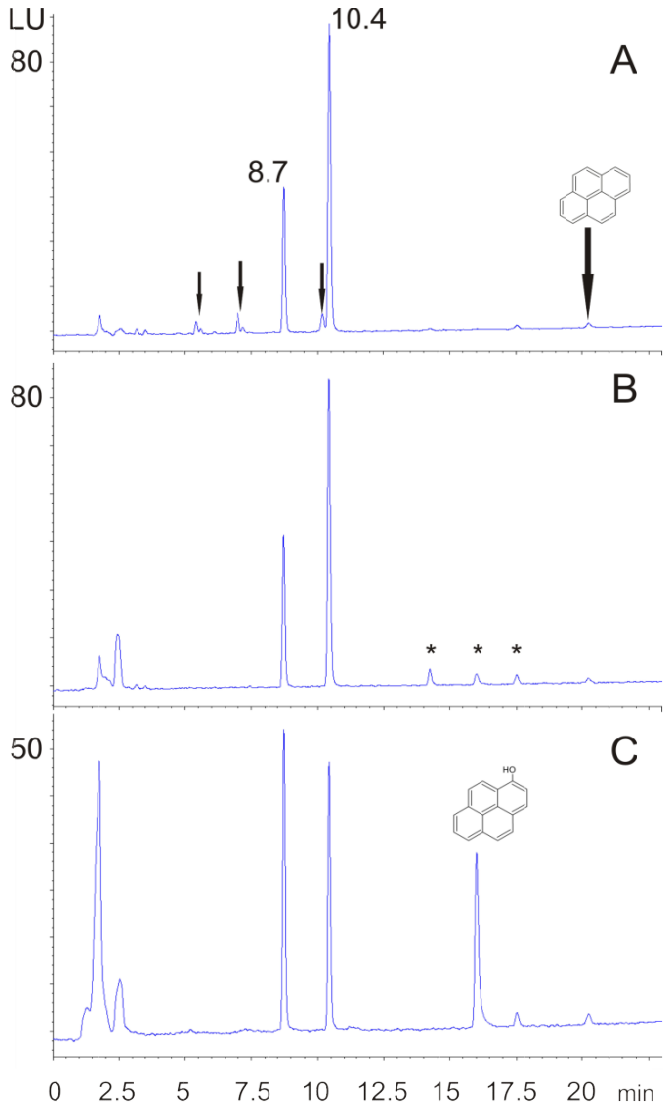

Figure 8. *Deconjugation of C. riparius tissue extracts to describe the biotransformation pathway. The panels show the HPLC-FLD chromatograms of A) C. riparius (blank), B) C. riparius extracts incubated with beta-glucosidase and C) C. riparius extracts incubated with sulfatase. In A, the two main peaks that do not appear in controls are pointed with their retention times and the secondary peaks with arrows. In B), asterisks indicate the possible three phase I metabolites produced by this organism. The peaks that represent pyrene and 1-hydroxy-pyrene are indicated by their structures.*

quantity applied was not optimal. Overall, the enzymatic incubations revealed that glucose and sulfate conjugates are the preferred pathways for the biotransformation of pyrene in *C. riparius*. It could be possible that these conjugates have different phase I precursors than 1-hydroxy-pyrene, as discussed in the previous section.

Pyrene was biotransformed into several metabolites by the marine *G. setosus* and the freshwater *P. quadrispinosa* (Fig. 6F and 6G), while we also found evidence of the biotransformation of fluoranthene by the marine *C. crangon* (Fig. 9). The UV spectra of the single peaks found in the organisms exposed to the parent compounds reveal a striking similarity to that of the parent compound, proving that the metabolites are derivatives from pyrene and fluoranthene.

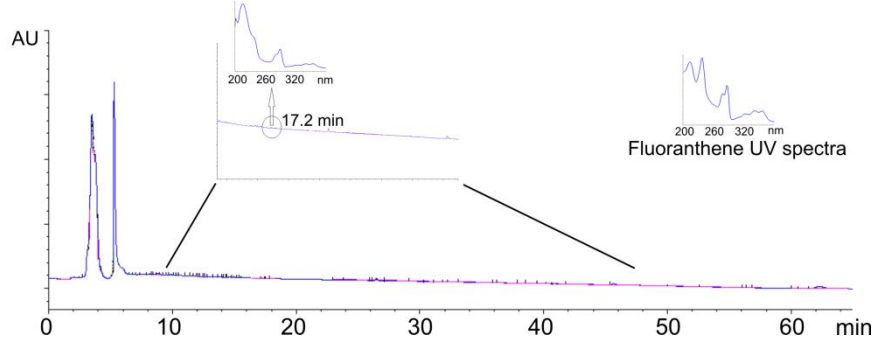

Figure 9. *HPLC-DAD chromatogram of the methanol-water phase generated during the extraction of Crangon crangon tissues. The area of interest is enlarged and the peak that is tentatively a fluoranthene metabolite produce by C. crangon is pointed with a circle and its retention time. The UV spectra of fluoranthene and the unknown peak are displayed to show the similarity.*

These results clearly suggest that the amphipods and shrimp chosen in the present thesis do have an active biotransformation system for model PAHs. This is also supported by the reported efficient PAH biotransformation capacity of three species of shrimp (Ikenaka et al., 2007), the amphipod *Rhepoxynius abronius* and the shrimp *Pandalus platyceros* (Varanasi et al., 1985).

Filipowicz et al. (2007) also suggested the biotransformation of fluoranthene by the grass shrimp.
Overall, several biotransformation pathways of pyrene, including those described in the present thesis are shown in Fig. 7.

3.2.4 Nonextractable fraction

A nonextractable fraction of pyrene was found in our experiments after extractions of tissues with organic solvents. This is a common finding after exposure of a given animal to PAHs, (McElroy and Sisson, 1989; Magnusson et al., 2000; Schuler et al., 2004; Palmqvist et al., 2006) and also other xenobiotics (Belden et al., 2005; Mäenpää et al., 2008).
The terminology used for this fraction is not fully consistent. For example bound metabolic products (McElroy & Sisson, 1989); tissue residues (Leppänen & Kukkonen, 2000); unextractable residues or portions (Conder et al., 2004; Belden et al., 2005); unextractable tissue residues (Palmqvist et al., 2006) or simply *unextracted* (Mäenpää et al., 2009). The most complete term may be "tissue bound non –extractable" (Honkanen et al., 2008). In the articles **I**-**IV**, the term "nonextractable fraction" was used, although we also used tissues residue in article **I**.
In the samples from the bioaccumulation experiments reported in **I** and **II**, the whole bound fraction was measured by scintillation counting, therefore preventing the identification of the chemicals that were forming it. However, the samples from experiment 2 (in **I**) and the *L. variegatus* samples and some fish samples from article **III** were extracted with an additional step where a proteolytic treatment with Proteinase K was used. This treatment was performed based on the extractions conducted by Stroomberg et al. (1999; 2004a & b) and Luthe et al. (2002), who used proteolysis to dissolve the hepatopancreas of the terrestrial isopod *Porcellio scaber*, in that way extracting several pyrene derivatives. The extracts were analyzed by HPLC without any pre- or post- extraction with organic solvents.
The proteinase K extracts of *L. variegatus* described in the present thesis (analyzed by HPLC) contained the same phase II

metabolites found after extractions just with organic solvents (Fig. 2 and Fig. 3 from article **I**). This fraction was called the Proteinase K fraction. Furthermore, there was still a part of the bound fraction that remained nonextractable, named residual nonextractable fraction and constituted averages of 1.8 and 3.3 % of the total body burden in NDW and DW worms, respectively (**III**; Table 6). As proteinase K is normally used during the isolation of DNA, it might be possible that the residual nonextractable fraction is formed by metabolites bound covalently to DNA, although this needs to be confirmed with additional studies.

Therefore, these results indicated that two types of bound fraction (proteinase K and residual nonextractable fractions) are possibly present, at least in *L. variegatus*. To my knowledge, this is the first time that this phenomenon has been described in literature (**III**).

However, it would be still possible that these phase II metabolites are not extracted at an acceptable recovery rate, although our results indicate otherwise, as the glucuronide conjugate of 1-hydroxy-pyrene is recovered at ~94% (article **III**, supplemental information), while in some cases the nonextractable fraction accounted for up to 35% (Table 6).

Therefore, the idea that suggests that some metabolites of pyrene or other PAH could bind to proteins sounds plausible.

The metabolites of certain PAHs have been found to bind proteins in previous studies, e.g. B(a)P- 3OH with albumin and bovine hemoglobin (Sugihara & James, 2003), some B(a)P metabolites were bound covalently to proteins in trout liver microsomes (Miranda et al., 2006), naphthalene metabolites targeted several proteins, including alpha- and beta- actin (related to the cytoskeleton), heat shock protein (HSP)70 and other chaperones (related to protein folding) in mouse lung epithelial cells (Lin et al., 2005). It is possible that these proteins are a dead end for some reactive metabolites, preventing other proteins from being damaged (Lin et al., 2005).

However, the metabolites that have been found to bind proteins are normally products of the phase I reactions, such as hydroxyl,

quinones or highly reactive intermediate products. Taking this into account, the findings reported in the present thesis about the possible binding of phase II metabolites to proteins are contradictory or a new finding.

Table 6. *Percentage of nonextractable fraction of total body burdens in the different treatments, species and tissues from* ***I-IV*** *and unpublished experiments with P. quadrispinosa.In* ***III****, total nonextractable is shown, with its Proteinase K (Prot.K) and residual fractions (Res. nonextr. fraction).*

L. variegatus **(I)**						
	No PBO	**PBO 354**	**PBO 35.4**	**Exp.2**		
	3.3 (2.1)	1.5 (0.61)	1.6 (0.6)	N.D.		
L. variegatus **(III)**	**NDW**			**DW**		
	Total	Prot. K fraction	Res. nonextr fraction	Total	Prot. K fraction	Res. nonextr. fraction
	8.8 (3.4)	7 (2.8)	1.8 (0.6)	34.1	30.8	3.3 (1.2)
G. setosus **(II)**	**2 °C** (168 h)	**8 °C** (168 h)				
	3.7 (0.97)	4.4 (0.8)				
S. trutta **(III)**						
	NDW					
	Liver/bile	GIT	GITC	Faeces	Gills	Carcasses
	1.74 (0.64)	10.2 (3.9)	10.5 (4.3)	38.1 (14.5)	23.6 (6.1)	9.9 (10.8)
	DW					
	Liver/bile	GIT	GITC	Faeces	Gills	Carcasses
	2.2 (0.8)	15.9 (1.7)	17.6 (7)	42.4 (6.5)	31.6 (3.6)	16 (14.7)
P. quadr.						
	PBO (96 h)	**No PBO** (96 h)	**Sediment** (168h)			
	2.8 (3.1)	6 (2.3)	5.2 (2.1)			
	At depuration (only No PBO)					
	4 h	8 h	24 h	48 h	96 h	
	6.3 (2)	4.9 (1.2)	8.4 (2.7)	5.9 (0.80)	6.4 (6.7)	

There are two additional points that support the idea of a metabolic fraction bound to proteins. First, in article **I**, in treatments where PBO was added, the nonextractable fraction share was smaller than in the treatment where PBO was not used after 168 h (Table 7). This was exactly the same result that can be observed with the shares of metabolites (more metabolites in the treatment without PBO), indicating that the pyrene derivatives forming the nonextractable fraction depended on the inhibition of CYP. Second, an increase in the share of the bound fraction in article **III** was found after worms were depurated in clean sediment for 96 h (from 9% in NDW to 34% in DW, Table 6). This pointed to an excretion of the body burden that was not tightly bound, while the nonextractable fraction remained bound to tissues. As it was discussed that the bound fraction may be formed of metabolites, this can be linked to the known slower depuration of PAH metabolites in invertebrates (Driscoll & McElroy, 1997; Leppänen & Kukkonen, 2000). These two points suggest that the bound fraction is formed by metabolites.

L. variegatus also appears to accumulate phase II metabolites of xenobiotics and to produce a nonextractable fraction also with other OCs such as benzo (a) pyrene and pentachlorophenol (Leppänen & Kukkonen, 2000; Mäenpää et al., 2008, respectively) and TNT (Belden et al., 2005). In the other animals studied in my thesis, a nonextractable fraction was also found.

3.3 EXCRETION

Conditions that need to be fulfilled for the occurrence of the trophic transfer of a contaminant are the bioaccumulation and the lack of (or slow) depuration in the prey. It is essential to know what molecules are excreted (i.e. parent or metabolites), what concentrations are present and what the elimination rates are.

The elimination of pyrene and its metabolites by *L. variegatus* in clean sediment was studied in experiments **III** and **IV** after a

two-month exposure to pyrene. The goal of this depuration stage was to lower the concentration of the parent pyrene, as it is excreted more rapidly than the metabolites by this organism (Leppänen & Kukkonen, 2000), in order to use these animals as prey for the analyses of the trophic transfer.

The body burden of total pyrene equivalents after the two-month exposure to pyrene were statistically higher (t-test, p = 0.002) in the *L. variegatus* that were fed to *S. trutta* than those fed to *G. setosus* (Fig 10A). This was likely due to the more frequent changes of experimental water and feeding performed in article **III**. Interestingly, the total body burden at the end of the 96 h depuration period were not statistically significant (t –test: p =0.118), which indicates that a fraction of the total body burden, common in the two experiments is eliminated very slowly. This possibly indicates that the elimination takes place from two different compartments, which has already been described in literature (Leppänen & Kukkonen 2004) and has also been demonstrated for the parent pyrene in *G. setosus* (**II**).

The depuration efficiencies of pyrene and metabolites were calculated based on their values at 0 and 96 h of depuration (Table S1 and Fig. S1 in **IV**). After those four days in clean sediment, *L. variegatus* excreted pyrene very efficiently (averages of 85 and 88 % in **III** and **IV**, respectively), supporting the reported faster elimination of parent PAHs compared to metabolites (Driscoll & McElroy, 1997). Regarding to the body burden of metabolites that were calculated by subtracting the concentrations of pyrene from the total body burden, the reduction was also significant (53 and 46 % as average, in **III** and **IV**).

On the contrary, the depuration of body burden, pyrene and its metabolites in clean water was not as evident as it was in sediment. It was studied in **unpublished** experiments with *P. quadrispinosa* (Fig. 10B) and in *G. setosus* (Fig. 2 in **II**). Although the concentrations of pyrene in tissues decreased, the total body burdens and the concentrations of phase II metabolites did not decreased significantly in *G. setosus* in any of the temperatures according to Eqn. (4) (Fig. 2 in **II**). However, the overall model

(Eqn. 1 -3 in **II**) predicted a slight decrease of total body burdens and metabolites but only at 8 ºC (Fig. 1B in **II**). This decreasing tendency may be supported by additional data, although only for total body burdens (discussed in article **II**).

Moreover, the concentrations of phase II metabolites in *P. quadrispinosa* even increased, being at the end of the depuration period more than twice their levels at the beginning of the depuration (Fig. 10B). This phenomenon has also been found in depuration periods in clean sediment with B(a)P metabolites produced by *L. variegatus* (Schuler et al., 2003). However, the increase in the concentrations of phase II metabolites of *P. quadrispinosa* is higher than the decrease of the concentrations of pyrene, what is contradictory. It can be possible that the method used to quantify the phase II metabolites was not the most accurate, as many steps were followed involving the collection of unknown peaks in the HPLC and their quantification by LSC.

Due to excretory organs like liver and kidneys, fish are able to eliminate PAH metabolites efficiently, which is also supported by **III** and other studies (Jonsson et al., 2004). Some metabolites that were produced by juvenile *S. trutta*, such as glucuronide conjugate of 1-hydroxy-pyrene, were found in the GITC. A peak produced by *S. trutta* (tentatively identified as the sulfate conjugate), was found in GITC and water thus assuming that it was mainly excreted through urine. In total, the share of total pyrene derivatives found in the water was approximately 60% of the total found in both NDW and DW treatments, what underlines the capacity of fish to excrete PAH metabolites.

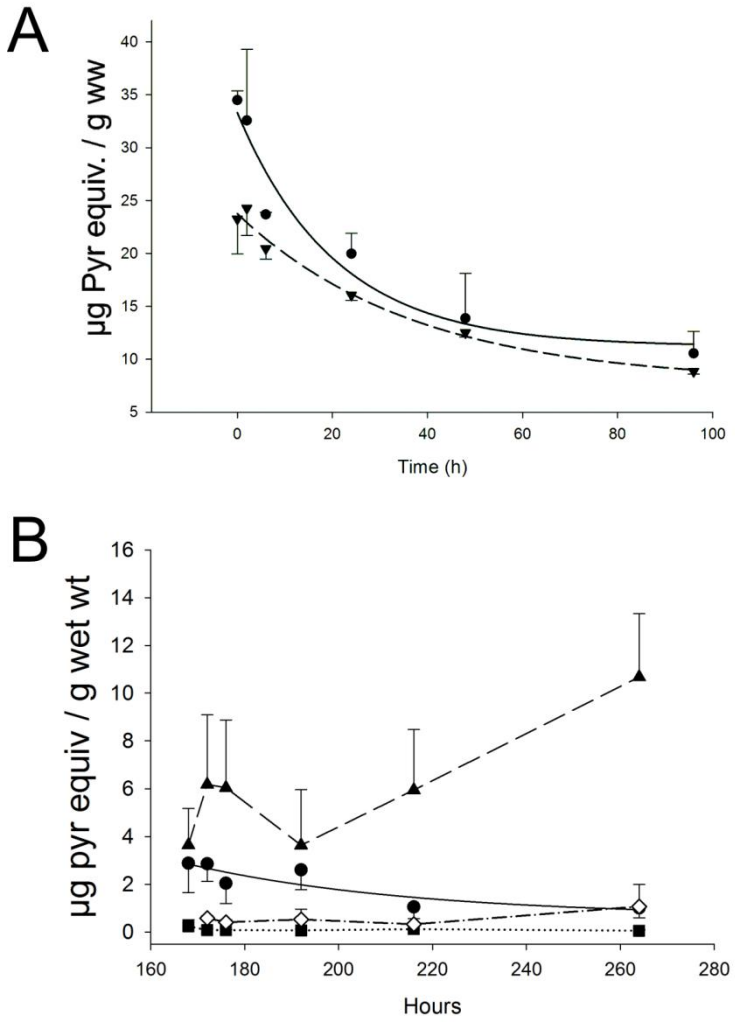

*Fig. 10. Elimination of pyrene and metabolites in different clean environments. A) Experimental values of the total body burdens of L. variegatus that were fed to S. trutta (**III**) are given by circles and their fit to Equ. (3) by continuous line. Experimental values of the total body burdens of L. variegatus that were fed to G. setosus are given by triangles and their fit to Equ (3) by a dashed line. B) Tissue concentrations of pyrene (circles, continuous line), phase II metabolites (triangles, dashed line), nonextractable fraction (diamonds, dashed-dotted line) and 1-hydroxy-pyrene (squares, dotted line) in P. quadrispinosa.*

3.4 TROPHIC TRANSFER

The overall potential impact that this lack of excretion of metabolites may have on ecosystems is partially unknown. These and other invertebrates that also may be affected by PAHs could be key species in the trophic chain and thus be the vectors for an eventual trophic transfer of PAHs or their metabolites (Leppänen & Kukkonen, 2000), a subject that is covered in the present thesis.

3.4.1 Trophic transfer of the single metabolites

A common way to assess the dietary uptake of a certain chemical (and also used with phase I metabolites of PAHs) is to introduce it into food pellets or the tissues of dead prey. Commonly, food is immersed in a solution of the PAH metabolite in acetone or ethanol and the solvent is later evaporated, leaving the food item impregnated with the metabolite.

If performed carefully, this is a straightforward method to test the availability and dietary exposure of single PAH biotransformation products. In article **III**, feeding of *S. trutta* with 1-hydroxy-pyrene-spiked food pellets was an alternative to test that at least this phase I metabolite can be taken up through diet. It is likely that ingested 1-hydroxy-pyrene was taken up through the GIT, as biotransformation was found in e.g. livers (Fig. A4 in the Supplemental information of **III**), and this phenomenon has been reported in literature (Beach et al., 2010). However, the undesired desorption of 1-hydroxy-pyrene into water (also suggested by the presence of phase II metabolites in gills) hampered distinguishing the uptake route. Palmqvist et al., (2006) prevented this desorption by completely covering the tissues with an alginate solution that solidified it. This method was applied in the **unpublished** experiments with *C. crangon* with not very successful results due to the *C. crangon* ingestion style.

Although this method proves the availability of the single metabolites through diet, it does not show an accurate value of

their dietary uptake in conditions where the prey has produced the metabolites. In order to evaluate the trophic transfer of PAH metabolites naturally produced by some prey, their exposure to the parent PAH is necessary. The biotransformation of the PAH by the prey will ensure that the metabolites produced are naturally distributed in its tissues, e.g.: bound to macromolecules and not artificially introduced into the tissues.

3.4.2 Dietary absorption of metabolites?

Both approaches used, the mass balance and the comparison of HPLC chromatograms, confirmed that PAH metabolites are transferred from PAH exposed prey to predators. The suggested phase II metabolites of pyrene produced by *L. variegatus* (eluting at t_R = 9.7 min; Fig. 3 in **III**) and *C. riparius* (t_R = 17.5 and 28.8 min; Fig. 1 in **IV**) were transferred to juvenile *S. trutta* (**III**) and to *G. setosus* (**IV**) respectively. It is discussed (**I**) that the phase II metabolite transferred from *L. variegatus* to *S. trutta* may be the glucose conjugate of 1-hydroxy-pyrene.

Due to the absence of standards of these metabolites, it was not possible to quantify either the share of single metabolites that was taken up through the GIT epithelium or the biomagnification factors. Although the exact amounts of phase II metabolites that were transferred were not available, the results pointed to the main transfer of phase I metabolites (1-hydroxy-pyrene mainly) from prey to predators. This has been already confirmed in literature (McElroy et al., 1989; McElroy et al., 1991). Despite that the trophic transfer of PAH metabolites occurred, to distinguish what kind of metabolites transfer through the GIT epithelium is difficult, especially in the case of phase I metabolites. The quantification is tedious, first because of their rapid conjugation to phase II metabolites and second because of the excretion of these conjugates, at least in fish and other aquatic vertebrates. To add a bit more complication, the phase II metabolites may also be deconjugated to their phase I metabolites in the GIT, absorbed to blood and again transformed in the liver into a phase II metabolite that is excreted through the bile and starts the cycle again. This

phenomenon is called enterohepatic recirculation (James, 1987) and has been observed also in fish (Tomy et al., 2008).

3.4.3 Biomagnification factors

The biomagnification is usually measured by the biomagnification factors (BMFs) that are defined as the concentration of the chemical in a predator divided by the concentration in its prey (Newman, 1998; Houde et al., 2011). Some studies have adjusted the BMFs for the trophic level of the animals studied (Cullon et al., 2012), although it was in a more complex field experiment. If the BMFs are greater than 1 (i.e the concentration in the predator is higher than in the prey), a biomagnification has occurred. Although the BMFs were not calculated in the articles that formed the present thesis, the BMFs (as averages) were calculated for the metabolites as a whole (Table 7), as it was not possible for the single metabolites. Although the BMFs are usually calculated and are of great significance in field food chain studies (Broman et al., 1990; Nfon et al., 2008), I consider as interesting and informative their calculation in the present thesis as a starting point for the study of whether the metabolites biomagnify or not (e.g. in field studies with invertebrates that do not excrete them efficiently).

Table 7. Biomagnification factors (BMFs) calculated for the trophic transfer experiments (**III** and **IV**). In **III**, the second BMF value (right box) indicates the BMF value calculated considering all metabolites produced by fish, despite their excretion, as an estimation in a scenario without excretion.

	III				**IV**		
Animals	*L. variegatus –> S. trutta*				*L. variegatus –> G.setosus*	*C. riparius –> G.setosus*	
Prey	**NDW as prey**		**DW as prey**		**Depurated worms as prey**	**40 h**	**65 h**
BMF	0.0158	0.15	0.0159	0.2	0.1	0.03	0.11

Our results confirm that a biomagnifications does not occur, as none of the BMFs exceeded 0.2. In fact, the highest values were found in experiment **III**, but in calculations that took into account all the metabolites that were not strictly inside the fish (i.e. water, faeces and GITC). Considering strictly the metabolites that were found in the certain organs of predators, the BMFs were low, with top values around 0.01. Although the metabolites that have been already excreted or are to be excreted (e.g. into GITC) must not be considered for the calculations, I included them in the calculations to illustrate a potential scenario without excretion and also to make a direct comparison with the real value.

Following this, an appropriate point for discussion is whether to consider the GITC contents as inside or outside the fish, as they potentially are to be expelled to the ambient medium. On the other hand, the GITC is inside the fish until it is excreted. This would be important in experiments where fish are used as prey.

Interestingly, there were differences in the BMFs between experiments **III** and experiment **IV** where predators were fish and invertebrates respectively. The BMFs values for the *L. variegatus –G. setosus* trophic chain are higher than the values for the *L. variegatus –S.trutta* trophic chain, reflecting the higher excretion capability of fish compared to invertebrates. It is also worth noticing that in the data from article **IV**, the BMFs increased in the *C. riparius -G. setosus* trophic transfer experiment. This raises questions about the trophic transfer and BMFs of PAH metabolites in longer feeding experiments or in the field, where predators can ingest contaminated prey constantly.

3.4.4 Trophic transfer of the nonextractable fraction

Another vector that may contribute to the trophic transfer of PAH metabolites and studied in the present thesis is the nonextractable fraction. As described above, part of the metabolites produced by a certain organism exposed to PAH form a fraction that is likely bound to macromolecules. However, the uptake of the nonextractable fraction from the

gastrointestinal tract (GIT) to internal organs of predators has not been extensively studied. McElroy & Sisson (1989) considered the possibility that the "bound metabolic products" produced by *N. virens* were taken up by the winter flounder through diet. Additionally, Belden et al. (2005) addressed the need for further research to confirm or deny the dietary uptake of the nonextractable biotransformation products of TNT in aquatic organisms.

As described in article **III,** two types of bound fractions were described: the proteinase K and the residual bound fractions. The availability of the residual bound fraction produced by *L. variegatus* to *S. trutta* was assayed. This fraction was chosen to make these calculations because it was assumed to be the fraction most strongly bound to tissue. Making a mass comparison of the amount the fish ingested as a bound fraction with the bound fraction found in fish faeces was considered as a reasonable method for the determination of the availability of the nonextractable metabolites.

The results of this comparison showed that the nonextractable fraction was not available for uptake from prey to predator, as the differences between the bound fractions ingested and excreted were not statistically significant in any of the treatments (Fig. 4B in **III**).

Additionally, taking a look at the proportions of the nonextractable fraction after ingestion (in faeces) and before it (in *L. variegatus*), the proportion of nonextractable fraction in faeces increased compared to its proportion in *L. variegatus* (Table 7).First, this indicates that the rest of the *L. variegatus* body burdens (extractable fraction) decreased their share, indicating that they were disrupted in the GIT; and second, that the bound fraction stayed intact or suffered small modifications while in the GIT.

It could be argued, however, that the extraction methods used for worms and faeces were not equally effective. Although the extra step with proteinase K was not used in the extraction of the faeces, gastric and pancreatic enzymes such as trypsins and

chymotrypsins are produced and released to GIT lumen during the normal digestion, hydrolyzing proteins to aminoacids and/or di- and tripeptides (Kleinow & James, 2001). Therefore, these proteolytic enzymes could perform the related action in the GIT of *S. trutta* that the proteinase K performed in vitro to *L. variegatus* tissues, therefore liberating the proteinase K fraction present in worms to the GIT lumen. Therefore, it would be possible that the pyrene metabolites present in the proteinase K fraction are available and also might be taken up through the GIT.

4 Concluding remarks

4.1 IMPORTANCE OF THE STUDY

Certain papers that I consider as key documents in my field raised the awareness about the contribution of the biotransformation products of organic contaminants to the overall toxicity caused by the parent compound (van Zelm et al., 2010; Schmidt et al., 2010; McElroy et al., 2011; Ng et al., 2011). The choice of the compounds of study was logical due to the ubiquity and constant discharge of PAHs and because the toxicity that certain PAHs can cause after biotransformation. Although PAHs were already long ago well known environmental contaminants, they have been recently discovered as "emerging contaminants" in the Arctic (Laender et al., 2011), what also makes article **II** more valuable.

The comparison of biotransformation profiles and capability among invertebrates is and has been a challenging task. The variability among the taxa is considerable (McElroy et al., 2000) and the small size of species hampers their proper use. This makes the comparison among the species performed in the present theis of great value. As invertebrates form a very big percentage of the total number of species and they belong to the first levels of the food chain, to gather information on their ability to uptake, biotransform and excrete contaminants is essential to protect the rest of the environment. The present thesis has contributed with some more pieces of the big puzzle directly dealing with questions raised during the conclusion of previous studies, such as the trophic transfer of the metabolites (Leppänen & Kukkonen, 2000) and the potential availability of the bound nonextractable fraction to animals in the next trophic level (McElroy & Sisson, 1989; Belden et al., 2005). Despite that the trophic transfer of PAH metabolites was investigated before (McElroy & Sisson, 1989; Palmqvist et al., 2006), to my knowledge articles **III** and **IV** are pioneers in determining the

trophic transfer of suggested phase II metabolites in vivo. However, the experiments failed to quantify toxicity, what has been covered in a recent paper (da Silva et al., 2012). It may be that the present work will be part of important data for the development of more studies and the inclusion of the metabolites in the risk assessment.

4.2 CONCLUSIONS AND MAIN FINDINGS

The trophic transfer of the metabolites was the main aim of the present thesis (**III** and **IV**).
Also, and essential part of the thesis, fundamental knowledge on comparative toxicokinetics and specially biotransformation was enhanced.
Using *L. variegatus* and *C. riparius* as prey species and *S. trutta* and *G. setosus* as predators, the transfer of the metabolites was proven by using LSC and HPLC. One phase II metabolite of 1-hydroxy-pyrene (tentatively the glucose conjugate) produced by *L. variegatus* was taken up by *S. trutta* and two unidentified metabolites produced by *C. riparius* were taken up by *G. setosus*. However, the metabolites did not biomagnify to *S. trutta* or *G. setosus*, as the BMFs were much lower than 1.
Additionally, the results suggested that the trophic transfer of the bound nonextractable fraction, another possible vector for the transfer of the metabolites, did not occur (**III)**, possibly because they were tightly bound to macromolecules. Therefore, the inclusion of the metabolites in the risk assessment of PAHs is suggested, as an addition to the already assessed parent compounds.

An Oligochaete worm, *Lumbriculus variegatus* biotransformed pyrene (7% of metabolites were found after 168h) to the glucose-sulfate conjugate of 1-hydroxy-pyrene and likely to the glucose conjugate, among other unidentified metabolites. The larval dipteran *Chironomus riparius*, and the amphipod crustaceans *G. setosus and P. quadrispinosa* were also able to biotransform

pyrene more efficiently than *L. variegatus* (respectively ~ 93, 84 and 60% of total body burden as metabolites) into the tentatively identified glucose and sulfate conjugates of 1-hydroxy-pyrene. Juvenile *Salmo trutta* efficiently produced the glucuronide conjugate of 1-hydroxy-pyrene as the major metabolite and possibly also the sulfate conjugate, among other unidentified metabolites. Also, the brown shrimp *Crangon crangon* biotransformed fluoranthene to at least one unidentified metabolite.

4.3 FUTURE STUDIES

Field studies with a longer duration would represent better a real scenario of the events that are occurring in the aquatic environment, what is a final aim of ecotoxicology. In long exposure periods lasting months, all processes related to biotransformation, depuration and the up regulation of some genes may alter the steady state of PAHs. This would be of special interest in invertebrates, as not much is known about how their toxicokinetics deal with long-lasting PAH contamination.

The biotransformation of PAHs is a field of research that has not received all the attention that it deserves. Regarding my thesis, the complete chemical identification and fate of all of the pyrene and fluoranthene metabolites in the species studied, at least in the common test species (i.e. *L. variegatus* and *C. riparius*) would be an important discovery. Also, it would be a first step to the development of standards of these compounds that would facilitate the measurement of such compounds in the environment and the performance of tests about their toxicity and overall fate. It would be ideal if studies also with other more toxic PAHs achieve these goals.

To know whether organisms keep the concentration of metabolites constant during longer periods in clean environments would help to reveal the animal body burden and to prevent the toxicity over these animals long after a

contamination episode (e.g. an oil spill). This would be of special importance in prey animals, to predict the appearance of toxic effects in predators and maintain the health of populations. It may be a field of research especially hard to confront, again reflecting the need for more information about biotransformation.

Other external factors besides temperature (studied in **II**) may also influence the fate of PAHs in animals. Such factors may be salinity, oxygen levels, pH, and hardness of ambient waters. Additionally, life cycle, which is highly diverse in taxa of invertebrates, may affect the different endpoints tested for PAHs like pyrene and fluoranthene.

A final question, in my opinion of great importance, deals with the fate of the metabolites once they are excreted from animals. Some organisms, such as fish do excrete PAH metabolites efficiently through urine and faeces (**III)**. It is possible that phase II metabolites are deconjugated and the resulting parent chemicals or phase I metabolites may sorb to the sediment or may be taken up by some organisms. As these hypotheses have not been studied deeply, the overall fate of excreted PAH metabolites and related contaminants in the aquatic environments should be expanded.

5 References

Airas S, Leppänen M & Kukkonen J V K (2008) Effects of temperature and oxygen concentration in sediment toxicity testing. *Ecotoxicology and Environmental Safety* 70: 475-482.

Aker W G, Hu X, Wang P & Hwang H M (2008) Comparing the relative toxicity of malathion and malaoxon in blue catfish Ictalurus furcatus. *Environmental Toxicology* 23: 548-554.

Akkanen J & Kukkonen J V K (2003) Biotransformation and bioconcentration of pyrene in Daphnia magna. *Aquatic Toxicology* 64: 53-61.

Amweg E L, Weston D P, Johnson C S, You J & Lydy M J (2006) Effect of piperonyl butoxide on permethrin toxicity in the amphipod Hyalella azteca. *Environmental Toxicology and Chemistry* 25: 1817-1825.

Ankley G T & Collyard S A (1995) Influence of piperonyl butoxide on the toxicity of organophosphate Insecticides to 3 Species of Fresh-Water Benthic Invertebrates. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* 110: 149-155.

Arias A H, Spetter C V, Freije R H & Marcovecchio J E (2009) Polycyclic aromatic hydrocarbons in water, mussels (Brachidontes sp., Tagelus sp.) and fish (Odontesthes sp.) from Bahía Blanca Estuary, Argentina. *Estuarine, Coastal and Shelf Science* 85: 67-81.

Bach L, Palmqvist A, Rasmussen L J & Forbes V E (2005) Differences in PAH tolerance between Capitella species: Underlying biochemical mechanisms. *Aquatic Toxicology* 74: 307-319.

Baumann P C & Harshbarger J C (1995) Decline in Liver Neoplasms in Wild Brown Bullhead Catfish After Coking Plant Closes and Environmental Pahs Plummet. *Environmental Health Perspectives* 103: 168-170.

Baumard P, Budzinski H & Garrigues P (1998) Polycyclic aromatic hydrocarbons in sediments and mussels of the

western Mediterranean sea. *Environmental Toxicology and Chemistry* 17: 765-776.

Beach D G, Quilliam M A & Hellou J (2009) Analysis of pyrene metabolites in marine snails by liquid chromatography using fluorescence and mass spectrometry detection. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 877: 2142-2152.

Beach D G, Quilliam M A, Rouleau C, Croll R P & Hellou J (2010) Bioaccumulation and Biotransformation of pyrene and 1-hydroxypyrene by the marine whelk Buccinum undatum. *Environmental Toxicology and Chemistry* 29: 779-788.

Beach D G & Hellou J (2011) Bioaccumulation and biotransformation of 1-hydroxypyrene by the marine whelk Neptunea lyrata. *International Journal of Environmental Analytical Chemistry* 91: 1227-1243.

Beard J (2006) DDT and human health. *Science of the Total Environment* 355: 78-89.

Bednarek P, Frański R, Kerhoas L, Einhorn J, Wojtaszek P & Stobiecki M (2001) Profiling changes in metabolism of isoflavonoids and their conjugates in Lupinus albus treated with biotic elicitor. *Phytochemistry* 56: 77-85.

Belden J B, Ownby D R, Lotufo G R & Lydy M J (2005) Accumulation of trinitrotoluene (TNT) in aquatic organisms: Part 2--Bioconcentration in aquatic invertebrates and potential for trophic transfer to channel catfish (Ictalurus punctatus). *Chemosphere* 58: 1161-1168.

Bernatchez L (2001) The evolutionary history of brown trout (Salmo trutta L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. *Evolution* 55: 351-379.

Beyer J, Jonsson G, Porte C, Krahn M M & Ariese F (2010) Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. *Environmental Toxicology and Pharmacology* 30: 224-244.

Bihari N, Fafandel M, Hamer B & Kralj-Bilen B (2006) PAH content, toxicity and genotoxicity of coastal marine sediments

from the Rovinj area, Northern Adriatic, Croatia. *Science of the Total Environment* 366: 602-611.

Bolt H (2005) Vinyl Chloride-A Classical Industrial Toxicant of New Interest. *Critical Reviews in Toxicology* 35: 307-323.

Bouchard M & Viau C (1998) Urinary and biliary excretion kinetics of 1-hydroxypyrene following intravenous and oral administration of pyrene in rats. *Toxicology* 127: 69-84.

Brelin, D (2008) Stress coping strategies inb trout (Salmo trutta): Ecological significance and effects of sea-ranching. PhD thesis n. 413. Faculty of Science and Technology. University of Uppsala.

Brinkman S F & Hansen D L (2007) Toxicity of cadmium to early life stages of brown trout (Salmo trutta) at multiple water hardnesses. *Environmental Toxicology and Chemistry* 26: 1666-1671.

Broman D, Naf C, Lundbergh I & Zebuhr Y (1990) An insitu study on the distribution, biotransformation and flux of polycyclic aromatic hydrocarbons (Pahs) in an aquatic food-chain (seston, Mytilus Edulis L, Somateria mollissima L) from the Baltic - An ecotoxicological perspective. *Environmental Toxicology and Chemistry* 9: 429-442.

Brunson E L, Canfield T J, Dwyer F J, Ingersoll C G & Kemble N E (1998) Assessing the bioaccumulation of contaminants from sediments of the upper Mississippi River using field-collected oligochaetes and laboratory-exposed Lumbriculus variegatus. *Archives of Environmental Contamination and Toxicology* 35: 191-201.

Buckman A H, Wong C S, Chow E A, Brown S B, Solomon K R & Fisk A T (2006) Biotransformation of polychlorinated biphenyls (PCBs) and bioformation of hydroxylated PCBs in fish. *Aquatic Toxicology* 78: 176-185.

Buhler, D R & Williams D.E.(1989) Enzymes involved in metabolism of PAH by fishes and other aquatic animal: Oxidative enzymes (or phase I enzymes). In Varanasi U (Eds) *Metabolism of Polycyclic aromatic hydrocarbons in the aquatic environment.* CRC Press*,* Boca Ratón, Florida, pp. 151-184.

Bustamante P, Luna-Acosta A, Clemens S, Cassi R, Thomas-Guyon H & Warnau M (2012) Bioaccumulation and metabolisation of C-14-pyrene by the Pacific oyster Crassostrea gigas exposed via seawater. *Chemosphere* 87: 938-944.

Campos, J & Van der Veer, H W (2008) Autecology of Crangon Crangon (L.) with an emphasis on latitudinal trends. In Gibson R N, Atkinson R J A & Gordon J D M (Eds) *Oceanography and Marine Biology: An annual review Vol. 46.* CRC Press*,* 65-104.

Capps T M, Barringer V M, Eberle W J, Brown D R & Sanson D R (1996) Identification of a unique glucosylsulfate conjugate metabolite of profenofos in cotton. *Journal of Agricultural and Food Chemistry* 44: 2408-2411.

Cavalieri E. & Rogan E.(1998) Mechanisms of tumor initiation by polycyclic aromatic hydrocarbons in mammals. In Neilson, A H (Eds) *The Handbook of Environmental Chemistry Vol. 3 Part J: PAHs and related compounds: Biology.* 81-118.

Canadian Council of Ministers of the Environment (CCME) 1999. Canadian water quality guidelines for the protection of aquatic life: polycyclic aromatic hydrocarbons (PAHs). In: *Canadian environmental quality guidelines, 1999*, Canadian Council of Ministers of the Environment, Winnipeg.

Chen B L, Xuan X D, Zhu L Z, Wang J, Gao Y Z, Yang K, Shen X Y & Lou B F (2004) Distributions of polycyclic aromatic hydrocarbons in surface waters, sediments and soils of Hangzhou City, China. *Water Research* 38: 3558-3568.

Christensen M, Andersen O & Banta G T (2002) Metabolism of pyrene by the polychaetes Nereis diversicolor and Arenicola marina. *Aquatic Toxicology* 58: 15-25.

Clements W H, Oris J T & Wissing T E (1994) Accumulation and food-chain transfer of fluoranthene and benzo[a]pyrene in Chironomus-riparius and Lepomis-macrochirus. *Archives of Environmental Contamination and Toxicology* 26: 261-266.

Conder J M, La Point T W & Bowen A T (2004) Preliminary kinetics and metabolism of 2,4,6-trinitrotoluene and its

reduced metabolites in an aquatic oligochaete. *Aquatic Toxicology* 69: 199-213.

Cornelissen G, Kukulska Z, Kalaitzidis S, Christanis K & Gustafsson O (2004) Relations between environmental black carbon sorption and geochemical sorbent characteristics. *Environmental Science & Technology* 38: 3632-3640.

Cullon D L, Yunker M B, Christensen J R, Macdonald R W, Whiticar M J, Dangerfield N J & Ross P S (2012) Biomagnification of polychlorinated biphenyls in a harbor seal (Phoca vitulina) food web from the strait of Georgia, British Columbia, Canada. *Environmental Toxicology and Chemistry* 31: 2445-2455.

da Silva A J, Santos T C A, Gomes V, Bicego M C, Barbosa A C R D, Passos M J D C, Hasue F M & Ngan P N (2012) Assessment of trophic transfer of benzo(a)pyrene genotoxicity from the post-larval pink shrimp F. brasiliensis to the juvenile Florida pompano T. carolinus. *Environmental Toxicology and Pharmacology* 34: 969-976.

Day J A & Saunders F M (2004) Glycosidation of chlorophenols by Lemna minor. *Environmental Toxicology and Chemistry* 23: 613-620.

Di Toro D M & McGrath J A (2000) Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbon criteria. II. Mixtures and sediments. *Environmental Toxicology and Chemistry* 19: 1971-1982.

Ding Y S, Trommel J S, Yan X Z J, Ashley D & Watson C H (2005) Determination of 14 polycyclic aromatic hydrocarbons in mainstream smoke from domestic cigarettes. *Environmental Science & Technology* 39: 471-478.

Driscoll S B K & McElroy A E (1997) Elimination of sediment-associated benzo[a]pyrene and its metabolites by polychaete worms exposed to 3-methylcholanthrene. *Aquatic Toxicology* 39: 77-91.

Dyremark A, Westerholm R, Overvik E & Gustavsson J A (1995) Polycyclic Aromatic Hydrocarbon (Pah) emissions from charcoal grilling. *Atmospheric Environment* 29: 1553-1558.

Eertman R H M, Groenink C L F M, Sandee B, Hummel H & Smaal A C (1995) Response of the Blue Mussel Mytilus-Edulis-l Following Exposure to Pahs Or Contaminated Sediment. *Marine Environmental Research* 39: 169-173.

Ekino S, Susa M, Ninomiya T, Imamura K & Kitamura T (2007). Minamata disease revisited: An update on the acute and chronic manifestations of methyl mercury poisoning. *Journal of the neurological sciences* 262: 131-144.

Fatone F, Di Fabio S, Bolzonella D & Cecchi F (2011) Fate of aromatic hydrocarbons in Italian municipal wastewater systems: An overview of wastewater treatment using conventional activated-sludge processes (CASP) and membrane bioreactors (MBRs). *Water Research* 45: 93-104.

Filipowicz A B, Weinstein J E & Sanger D M (2007) Dietary transfer of fluoranthene from an estuarine oligochaete (Monopylephorus rubroniveus) to grass shrimp (Palaemonetes pugio): Influence of piperonyl butoxide. *Marine Environmental Research* 63: 132-145.

Forbes V E, Andreassen M S H & Christensen L (2001) Metabolism of the polycyclic aromatic hydrocarbon fluoranthene by the polychaete Capitella capitata species I. *Environmental Toxicology and Chemistry* 20: 1012-1021.

Frouin H, Pellerin J, Fournier M, Pelletier E, Richard P, Pichaud N, Rouleau C & Garnerot F (2007) Physiological effects of polycyclic aromatic hydrocarbons on soft-shell clam Mya arenaria. *Aquatic Toxicology* 82: 120-134.

Ghosh U, Gillette J S, Luthy R G & Zare R N (2000) Microscale Location, Characterization, and Association of Polycyclic Aromatic Hydrocarbons on Harbor Sediment Particles. *Environmental Science & Technology* 34: 1729-1736.

Giessing A M B, Mayer L M & Forbes T L (2003) Synchronous fluorescence spectrometry of 1-hydroxypyrene: a rapid screening method for identification of PAH exposure in tissue from marine polychaetes. *Marine Environmental Research* 56: 599-615.

Giessing A M B & Johnsen A R (2005) Limited microbial degradation of pyrene metabolites from the estuarine polychaete Nereis diversicolor. *Chemosphere* 61: 1281-1287.

Grundy M M, Ratcliffe N A & Moore M N (1996) Immune inhibition in marine mussels by polycyclic aromatic hydrocarbons. *Marine Environmental Research* 42: 187-190.

Gustafsson D R, Price D A & Erseus C (2009) Genetic variation in the popular lab worm Lumbriculus variegatus (Annelida: Clitellata: Lumbriculidae) reveals cryptic speciation. *Molecular Phylogenetics and Evolution* 51: 182-189.

Gustafsson, P (2011) Forest - stream linkages: Brown trout (Salmo trutta) responses to woody debris, terrestrial invertebrates and light. PhD thesis. Karlstad University Studies.

Harada M, Akagi H, Tsuda T, Kizaki T & Ohno H (1999) Methylmercury level in umbilical cords from patients with congenital Minamata disease. *Science of the Total Environment* 234: 59-62.

Harkey G A, Landrum P F & Klaine S J (1994) Comparison of whole-sediment, elutriate and pore-water exposures for use in assessing sediment-associated organic contaminants in bioassays. *Environmental Toxicology and Chemistry* 13: 1315-1329.

Hawkins S A, Billiard S M, Tabash S P, Brown R S & Hodson P V (2002) Altering cytochrome P4501A activity affects polycyclic aromatic hydrocarbon metabolism and toxicity in rainbow trout (Oncorhynchus mykiss). *Environmental Toxicology and Chemistry* 21: 1845-1853.

Heder A F, Hirsch-Ernst K I, Bauer D, Kahl G F & Desel H (2001) Induction of cytochrome P450 2B1 by pyrethroids in primary rat hepatocyte cultures. *Biochemical Pharmacology* 62: 71-79.

Heitkamp M A, Freeman J P, Miller D W & Cerniglia C E (1988) Pyrene Degradation by A Mycobacterium Sp - Identification of Ring Oxidation and Ring Fission-Products. *Applied and Environmental Microbiology* 54: 2556-2565.

Higgins C P, McLeod P B, MacManus-Spencer L A & Luthy R G (2007) Bioaccumulation of Perfluorochemicals in Sediments by the Aquatic Oligochaete Lumbriculus variegatus. *Environmental Science & Technology* 41: 4600-4606.

Hill C, Furst M & Hammar J (1990) Introduction of the Amphipods Pallasea-Quadrispinosa and Gammaracanthus-Lacustris Into Lakes in Northern Sweden. *Annales Zoologici Fennici* 27: 241-244.

Hillenweck A, Canlet C, Mauffret A, Debrauwer L, Claireaux G & Cravedi J P (2008) Characterization of biliary metabolites of fluoranthene in the common sole (Solea solea). *Environmental Toxicology and Chemistry* 27: 2575-2581.

Hodgson, E & Levi, P E (1999) Interactions of piperonyl butoxide with cytochrome P450. In Jones D.G. (Eds) *Piperonyl Butoxide: The Insecticide Synergist.* Academic Press, London, UK, pp. 41-53.

Hoeger B, Dietrich D R, Schmid D, Hartmann A & Hitzfeld B (2008) Distribution of intraperitoneally injected diclofenac in brown trout (Salmo trutta f. fario). *Ecotoxicology and Environmental Safety* 71: 412-418.

Honkanen J O, Wiegand C & Kukkonen J V K (2008) Humic substances modify accumulation but not biotransformation of pyrene in salmon yolk-sac fry. *Aquatic Toxicology* 86: 239-248.

Houde M, De Silva A O, Muir D C G & Letcher R J (2011) Monitoring of Perfluorinated Compounds in Aquatic Biota: An Updated Review PFCs in Aquatic Biota. *Environmental Science & Technology* 45: 7962-7973.

Houessou J K, Goujot D, Heyd B & Camel V (2008) Modeling the formation of some polycyclic aromatic hydrocarbons during the roasting of Arabica coffee samples. *Journal of Agricultural and Food Chemistry* 56: 3648-3656.

Hyotylainen T & Oikari A (1999a) The toxicity and concentrations of PAHs in creosote-contaminated lake sediment. *Chemosphere* 38: 1135-1144.

Hyotylainen T & Oikari A (1999b) Assessment of toxicity hazards of dredged lake sediment contaminated by creosote. *Science of the Total Environment* 244: 97-105.

Häsänen E, Pohjola V, Pyysalo H & Wickstrom K (1984) Polycyclic Aromatic-Hydrocarbons in Finnish Sauna Air. *Science of the Total Environment* 37: 223-231.

Ikenaka Y, Eun H, Ishizaka M & Miyabara Y (2006) Metabolism of pyrene by aquatic crustacean, Daphnia magna. *Aquatic Toxicology* 80: 158-165.

Ikenaka Y, Ishizaka M, Eun H & Miyabara Y (2007) Glucose□sulfate conjugates as a new phase II metabolite formed by aquatic crustaceans. *Biochemical and Biophysical Research Communications* 360: 490-495.

Jager T, Sánchez F A A, Muijs B, van der Velde E G & Posthuma L (2000) Toxicokinetics of polycyclic aromatic hydrocarbons in Eisenia andrei (Oligochaeta) using spiked soil. *Environmental Toxicology and Chemistry* 19: 953-961.

James M O (1987) Conjugation of organic pollutants in aquatic species. *Environmental Health Perspectives* 71: 97-103.

Jongeneelen F J (2001) Benchmark guideline for urinary 1-hydroxypyrene as biomarker of occupational exposure to polycyclic aromatic hydrocarbons. *Annals of Occupational Hygiene* 45: 3-13.

Jonsson G, Bechmann R K, Bamber S D & Baussant T (2004) Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (Cyprinodon variegatus) Exposed to Contaminated Seawater. *Environmental Toxicology and Chemistry* 23: 1538-1548.

Jorgensen A, Giessing A M B, Rasmussen L J & Andersen O (2005) Biotransformation of the polycyclic aromatic hydrocarbon pyrene in the marine polychaete Nereis virens. *Environmental Toxicology and Chemistry* 24: 2796-2805.

Kennedy C J & Tierney K B (2008) Energy intake affects the biotransformation rate, scope for induction, and metabolite profile of benzo[a]pyrene in rainbow trout. *Aquatic Toxicology* 90: 172-181.

Kim M C, Cho S M & Jeong W G (2007) Short-term physiological response of the Pacific oyster, Crassostrea gigas, on exposure to varying levels of polycyclic aromatic hydrocarbon. *Aquaculture Research* 38: 1612-1618.

Kleinow, K M & James, M O(2001) Response of the teleost gastrointestinal system to xenobiotics. In Schelnk, D & Benson, W H (Eds)*Target organ toxicity in marine and freshwater teleosts: Volumen 1-organs.* Taylor & Francis, London & New York, pp. 299-394.

Kolodziejczyk A & Niedomagala W (2009) Relict Invertebrates in Lacustrine Habitats: Life History of Pallaseopsis Quadrispinosa (Sars, 1867) (Amphipoda: Gammaridae) in A Deep Mesotrophic Lake. *Polish Journal of Ecology* 57: 729-740.

Kukkonen J & Landrum P F (1994) Toxicokinetics and toxicity of sediment-associated pyrene to Lumbriculus variegatus (Oligochaeta). *Environmental Toxicology and Chemistry* 13: 1457-1468.

Kweon O, Kim S J, Holland R D, Chen H Y, Kim D W, Gao Y, Yu L R, Baek S, Baek D H, Ahn H & Cerniglia C E (2011) Polycyclic Aromatic Hydrocarbon Metabolic Network in Mycobacterium vanbaalenii PYR-1. *Journal of Bacteriology* 193: 4326-4337.

Laender D F, Hammer J, Hendriks A J, Soetaert K & Janssen C R (2011) Combining Monitoring Data and Modeling Identifies PAHs as Emerging Contaminants in the Arctic. *Environmental Science & Technology* 45: 9024-9029.

Landrum P F, Lydy M J & Lee H (1992) Toxicokinetics in aquatic systems: Model comparisons and use in hazard assessment. *Environmental Toxicology and Chemistry* 11: 1709-1725.

Laurent F, Debrauwer L, Rathahao E & Scalla R (2000) 2,4-dichlorophenoxyacetic acid metabolism in transgenic tolerant cotton (Gossypium hirsutum). *Journal of Agricultural and Food Chemistry* 48: 5307-5311.

Lavado R & Schlenk D (2011) Microsomal biotransformation of chlorpyrifos, parathion and fenthion in rainbow trout

(Oncorhynchus mykiss) and coho salmon (Oncorhynchus kisutch): Mechanistic insights into interspecific differences in toxicity. *Aquatic Toxicology* 101: 57-63.

Law K, Halldorson T, Danell R, Stern G, Gewurtz S, Alaee M, Marvin C, Whittle M & Tomy G (2006) Bioaccumulation and trophic transfer of some brominated flame retardants in a Lake Winnipeg (Canada) food web. *Environmental Toxicology and Chemistry* 25: 2177-2186.

Lech, J J & Vodicinik M.J.(1985) Biotransformation. In Rand, G M & Petrocelli, S R (Eds) *Fundamentals of Aquatic Toxicology*. Hemisphere, New York, pp. 526-557.

Lee J H & Landrum P F (2006) Application of multi-component damage assessment model (MDAM) for the toxicity of metabolized PAH in Hyalella azteca. *Environmental Science & Technology* 40: 1350-1357.

Leland H V (1983) Ultrastructural changes in the hepatocytes of juvenile rainbow trout and mature brown trout exposed to copper or zinc. *Environmental Toxicology and Chemistry* 2: 353-368.

Leonard J D & Hellou J (2001) Separation and characterization of gall bladder bile metabolites from speckled trout, Salvelinus fontinalis, exposed to individual polycyclic aromatic compounds. *Environmental Toxicology and Chemistry* 20: 618-623.

Leppänen M T & Kukkonen J V K (2000) Fate of sediment-associated pyrene and benzo[a]pyrene in the freshwater oligochaete Lumbriculus variegatus (Muller). *Aquatic Toxicology* 49: 199-212.

Leppänen M T & Kukkonen J V K (2004) Toxicokinetics of sediment-associated polybrominated diphenylethers (flame retardants) in benthic invertebrates (Lumbriculus variegatus, oligochaeta). *Environmental Toxicology and Chemistry* 23: 166-172.

Leppänen M T & Kukkonen J V K (1998) Relative importance of ingested sediment and pore water as bioaccumulation routes for pyrene to oligochaete (Lumbriculus variegatus,

Muller). *Environmental Science & Technology* 32: 1503-1508.

Leversee G J, Giesy J P, Landrum P F, Gerould S, Bowling J W, Fannin T E, Haddock J D & Bartell S M (1982) Kinetics and biotransformation of benzo(a)pyrene in Chironomus riparius. *Archives of Environmental Contamination and Toxicology* 11: 25-31.

Liebig M, Egeler P, Oehlmann J & Knacker T (2005) Bioaccumulation of C-14-17 alpha-ethinylestradiol by the aquatic oligochaete Lumbriculus variegatus in spiked artificial sediment. *Chemosphere* 59: 271-280.

Lin C Y, Isbell M A, Morin D, Boland B C, Salemi M R, Jewell W T, Weir A J, Fanucchi M V, Baker G L, Plopper C G & Buckpittt A R (2005) Characterization of a structurally intact in situ lung model and comparison of naphthalene protein adducts generated in this model vs lung microsomes. *Chemical Research in Toxicology* 18: 802-813.

Linke-Gamenick I, Forbes V E & Mendez N (2000) Effects of chronic fluoranthene exposure on sibling species of Capitella with different development modes. *Marine Ecology-Progress Series* 203: 191-203.

Livingstone D R (1998) The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology* 120: 43-49.

Lundstedt S, White P A, Lemieux C L, Lynes K D, Lambert L B, Oberg L, Haglund P & Tysklind M (2007) Sources, fate, and toxic hazards of oxygenated polycyclic aromatic hydrocarbons (PAHs) at PAH-contaminated sites. *Ambio* 36: 475-485.

Luthe G, Stroomberg G J, Ariese F, Brinkman U A T & van Straalen N M (2002) Metabolism of 1-fluoropyrene and pyrene in marine flatfish and terrestrial isopods. *Environmental Toxicology and Pharmacology* 12: 221-229.

Lydy M J, Lasater J L & Landrum P F (2000) Toxicokinetics of DDE and 2-chlorobiphenyl in Chironomus tentans. *Archives of Environmental Contamination and Toxicology* 38: 163-168.

Lyytikäinen M, Pehkonen S, Akkanen J, Leppänen M & Kukkonen J V K (2007) Bioaccumulation and biotransformation of polycyclic aromatic hydrocarbons during sediment tests with oligochaetes (Lumbriculus variegatus). *Environmental Toxicology and Chemistry* 26: 2660-2666.

Macneil C A L U, Dick J T A & Elwood R W (1999) The dynamics of predation on Gammarus spp. (Crustacea: Amphipoda). *Biological Reviews* 74: 375-395.

Magnusson K, Ekelund R, Ingebrigtsen K, Granmo A & Brandt I (2000) Tissue disposition of benzo[a]pyrene in blue mussel (Mytilus edulis) and effect of algal concentration on metabolism and depuration. *Environmental Toxicology and Chemistry* 19: 2683-2690.

Marinkovic M, Verweij R A, Nummerdor G A, Jonker M J, Kraak M H S & Admiraal W (2011) Life Cycle Responses of the Midge Chironomus riparius to Compounds with Different Modes of Action. *Environmental Science & Technology* 45: 1645-1651.

Marshall J W (1978) The first records of Stylodrilus heringianus (Oligochaeta: Lumbriculidae) from the Southern Hemisphere. *New Zealand Journal of Zoology* 5: 781-782.

Martinez, V G (2005) Cellullar and molecular correlates of neural morphallaxis in *Lumbriculus variegatus*. PhD thesis. Texas A&M University.

Mäenpää K & Kukkonen J V K (2006) Bioaccumulation and toxicity of 4-nonylphenol (4-NP) and 4-(2-dodecyl)-benzene sulfonate (LAS) in Lumbriculus variegatus (Oligochaeta) and Chironomus riparius (Insecta). *Aquatic Toxicology* 77: 329-338.

Mäenpää K, Sorsa K, Lyytikäinen M, Leppänen M T & Kukkonen J (2008) Bioaccumulation, sublethal toxicity, and biotransformation of sediment-associated pentachlorophenol in Lumbriculus variegatus (Oligochaeta). *Ecotoxicology and Environmental Safety* 69: 121-129.

Mäenpää K, Leppänen M T & Kukkonen J V K (2009) Sublethal toxicity and biotransformation of pyrene in Lumbriculus variegatus (Oligochaeta). *Science of the Total Environment* 407: 2666-2672.

McElroy A, Leitch K & Fay A (2000) A survey of in vivo benzo[alpha]pyrene metabolism in small benthic marine invertebrates. *Marine Environmental Research* 50: 33-38.

McElroy, A E, Farrington, J W & Teal, J M (1989) Bioavailability of polycyclic aromatic hydrocarbons in the Aquatic Environment. In Varanasi U (Eds) *Metabolism of Polycyclic aromatic hydrocarbons in the aquatic environment.* CRC Press*,* Boca Ratón, Florida, pp. 1-39.

McElroy A E & Sisson J D (1989) Trophic Transfer of benzo[a]pyrene Metabolites Between Benthic Marine Organisms. *Marine Environmental Research* 28: 265-269.

McElroy A E, Farrington J W & Teal J M (1990) Influence of mode of exposure and the presence of a tubiculous polychaete on the fate of benz[a]anthracene in the benthos. *Environmental Science & Technology* 24: 1648-1655.

McElroy A E, Barron M G, Beckvar N, Driscoll S B K, Meador J P, Parkerton T F, Preuss T G & Steevens J A (2011) A review of the tissue residue approach for organic and organometallic compounds in aquatic organisms. *Integrated Environmental Assessment and Management* 7: 50-74.

Menchai P, Van Zwieten L, Kimber S, Ahmad N, Rao P S C & Hose G (2008) Bioavailable DDT residues in sediments: Laboratory assessment of ageing effects using semi-permeable membrane devices. *Environmental Pollution* 153: 110-118.

Miranda C L, Chung W G, Wang-Buhler J L, Musafia-Jeknic T, Baird W M & Buhler D R (2006) Comparative in vitro metabolism of benzo[a]pyrene by recombinant zebrafish CYP1A and liver microsomes from beta-naphthoflavone-treated rainbow trout. *Aquatic Toxicology* 80: 101-108.

Morgan J E & Morgan A J (1989) Zinc sequestration by earthworm (Annelida: Oligochaeta) chloragocytes. *Histochemistry* 90: 405-411.

Mount D R, Highland T L, Mattson V R, Dawson T D, Lott K G & Ingersoll C G (2006) Use of the oligochaete, Lumbriculus variegatus, as a prey organism for toxicant exposure of fish through the diet. *Environmental Toxicology and Chemistry* 25: 2760-2767.

Myers M S, Anulacion B F, French B L, Reichert W L, Laetz C A, Buzitis J, Olson O P, Sol S & Collier T K (2008) Improved flatfish health following remediation of a PAH-contaminated site in Eagle Harbor, Washington. *Aquatic Toxicology* 88: 277-288.

Neff, J M (1979) *Polycyclic Aromatic Hydrocarbons in the Aquatic Environment.* Applied Science, London, UK.

Neff J M, Bence A E, Parker K R, Page D S, Brown J S & Boehm P D (2006) Bioavailability of polycyclic aromatic hydrocarbons from buried shoreline oil residues thirteen years after the Exxon valdez oil spill: A multispecies assessment. *Environmental Toxicology and Chemistry* 25: 947-961.

Neilson, A H & Allard, A-S (1998) Microbial metabolism of PAHs and heteroarenes. In Neilson, A H (Eds) *The Handbook of Environmental Chemistry Vol. 3 Part J: PAHs and related compounds: Biology*. Springer- Verlag, Berlin, pp. 1-80.

Newman, M C (1998) Bioaccumulation from food and trophic transfer. In Newman, M C (Eds) *Fundamentals of ecotoxicology*. Ann Arbor Press, Chelsea, MI, pp. 71-86.

Newman, M C & Unger M.A. (2003) *Fundamentals of Ecotoxicology*. Lewis Publisher, Boca Raton.

Nfon E, Cousins I T & Broman D (2008) Biomagnification of organic pollutants in benthic and pelagic marine food chains from the Baltic Sea. *Science of the Total Environment* 397: 190-204.

Ng C A, Scheringer M, Fenner K & Hungerbuhler K (2011) A framework for evaluating the contribution of transformation products to chemical persistence in the environment. *Environmental Science & Technology* 45: 111-117.

Ng T Y T & Wood C A (2008) Trophic transfer and dietary toxicity of Cd from the oligochaete to the rainbow trout. *Aquatic Toxicology* 87: 47-59.

Nicolas J M (1999) Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. *Aquatic Toxicology* 45: 77-90.

Olsen G H, Sva E, Carroll J, Camus L, De Coen W, Smolders R, Overaas H & Hylland K (2007) Alterations in the energy budget of Arctic benthic species exposed to oil-related compounds. *Aquatic Toxicology* 83: 85-92.

Orecchio S (2011) Polycyclic aromatic hydrocarbons (PAHs) in indoor emission from decorative candles. *Atmospheric Environment* 45: 1888-1895.

Organization for Economic Cooperation and Development (OECD), 1992. Guidelines for the Testing of Chemicals No. 203. Fish, Acute Toxicity Test. Paris, France.

Organisation for Economic Co-operation and Development (OECD), 2007. Sediment-water Lumbriculus toxicity test using spiked sediment, Guideline 225. Paris, France.

Organization for Economic Cooperation and Development (OECD), 2010. Guidelines for the Testing of Chemicals No 233: Sediment-water Chironomid Life-cycle Toxicity Test Using Spiked Water or Spiked Sediment. Paris, France.

Palmqvist A, Selck H, Rasmussen L J & Forbes V E (2003) Biotransformation and genotoxicity of fluoranthene in the deposit-feeding polychaete Capitella sp I. *Environmental Toxicology and Chemistry* 22: 2977-2985.

Palmqvist A, Rasmussen L J & Forbes V E (2006) Influence of biotransformation on trophic transfer of the PAK fluoranthene. *Aquatic Toxicology* 80: 309-319.

Paumen M L, Borgman E, Kraak M H S, van Gestel C A M & Admiraal W (2008) Life cycle responses of the midge Chironomus riparius to polycyclic aromatic compound exposure. *Environmental Pollution* 152: 225-232.

Phipps G L, Ankley G T, Benoit D A & Mattson V R (1993) Use of the aquatic oligochaete Lumbriculus variegatus for assessing the toxicity and bioaccumulation of sediment-associated contaminants. *Environmental Toxicology and Chemistry* 12: 269-279.

Prahalad A K, Ross J A, Nelson G B, Roop B C, King L C, Nesnow S & Mass M J (1997) Dibenzo[a,l]pyrene-induced DNA adduction, tumorigenicity, and Ki-ras oncogene mutations in strain A/J mouse lung. *Carcinogenesis* 18: 1955-1963.

Redlich G, Zanger U M, Riedmaier S, Bache N, Giessing A B M, Eisenacher M, Stephan C, Meyer H E, Jensen O N & Marcus K (2008) Distinction between Human Cytochrome P450 (CYP) Isoforms and Identification of New Phosphorylation Sites by Mass Spectrometry. *Journal of Proteome Research* 7: 4678-4688

Rehmann K, Noll H P, Steinberg C E W & Kettrup A A (1998) Pyrene degradation by Mycobacterium sp. strain KR2. *Chemosphere* 36: 2977-2992.

Rewitz K F, Styrishave B, Lobner-Olesen A & Andersen O (2006) Marine invertebrate cytochrome P450: Emerging insights from vertebrate and insect analogies. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* 143: 363-381.

Ristola T, Kukkonen J V K & Pellinen J (1999) Body residues and responses of the midge Chironomus riparius to sediment-associated 2,4,5-trichlorophenol in subchronic and chronic exposures. *Archives of Environmental Contamination and Toxicology* 37: 42-49.

Rodriguez, P & Reynoldson, T B (2011) Bioaccumulation and trophic transfer. In Rodriguez, P & Reynoldson, T B (Eds) *The Pollution Biology of Aquatic Oligochaetes.* Springer, London, pp. 159-199.

Ross J R M & Oros D R (2004) Polycyclic aromatic hydrocarbons in the San Francisco Estuary water column: Sources, spatial distributions, and temporal trends (1993-2001). *Chemosphere* 57: 909-920.

Rust A J, Burgess R M, Brownawell B J & McElroy A E (2004) Relationship between metabolism and bioaccumulation of benzo[a]pyrene in benthic invertebrates. *Environmental Toxicology and Chemistry* 23: 2587-2593.

Sadiktsis I, Bergvall C, Johansson C & Westerholm R (2012) Automobile tires-A potential source of highly carcinogenic dibenzopyrenes to the environment. *Environmental Science & Technology* 46: 3326-3334.

Sanders M, Sivertsen S & Scott G (2002) Origin and distribution of polycyclic aromatic hydrocarbons in superficial sediments from the Savannah River. *Archives of Environmental Contamination and Toxicology* 43: 438-448.

Savage R E, Tyler A N, Miao X S & Chan T C K (2008) Identification of a novel glucosylsulfate conjugate as a metabolite of 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione(ARQ 501, beta-lapachone) in mammals. *Drug Metabolism and Disposition* 36: 753-758.

Savinov V M, Savinova T N, Matishov G G, Dahle S & Naes K (2003) Polycyclic aromatic hydrocarbons (PAHs) and organochlorines (OCs) in bottom sediments of the Guba Pechenga, Barents Sea, Russia. *Science of the Total Environment* 306: 39-56.

Schmidt S N, Christensen J H & Johnsen A R (2010) Fungal PAH-Metabolites Resist Mineralization by Soil Microorganisms. *Environmental Science & Technology* 44: 1677-1682.

Schlenk D, Malin C, Evan G, Stephen G, Margaret J, Seth K, Peter v d H & Kristie W(2008) Biotransformation in Fishes. In Di Giulio R & Hinton, D E (Eds) *The Toxicology of Fishes.* CRC Press, 153-234.

Schuler L J, Wheeler M, Bailer A J & Lydy M J (2003) Toxicokinetics of sediment-sorbed benzo[a]pyrene and hexachlorobiphenyl using the freshwater invertebrates Hyalella azteca, Chironomus tentans, and Lumbriculus variegatus. *Environmental Toxicology and Chemistry* 22: 439-449.

Schuler L J, Landrum P F & Lydy M J (2004) Time-Dependent Toxicity of Fluoranthene to Freshwater Invertebrates and the Role of Biotransformation on Lethal Body Residues. *Environmental Science & Technology* 38: 6247-6255.

Schwarzenbach, R P, Gschwend P.M. & Imboden, D M(1993) Introduction. In Schwarzenbach, R P, Gschwend P.M. & Imboden (Eds) *Environmental organic chemistry*. John Wiley & Sons Inc., New York, USA, pp. 1-7.

Selck H, Palmqvist A & Forbes V E (2003) Biotransformation of dissolved and sediment-bound fluoranthene in the polychaete, Capitella sp I. *Environmental Toxicology and Chemistry* 22: 2364-2374.

Sepic E, Bricelj M & Leskovsek H (2003) Toxicity of fluoranthene and its biodegradation metabolites to aquatic organisms. *Chemosphere* 52: 1125-1133.

Shen G F, Wang W, Yang Y F, Ding J N, Xue M A, Min Y J, Zhu C, Shen H Z, Li W, Wang B, Wang R, Wang L, Tao S & Russell A G (2011) Emissions of PAHs from Indoor Crop Residue Burning in a Typical Rural Stove: Emission Factors, Size Distributions, and Gas-Particle Partitioning. *Environmental Science & Technology* 45: 1206-1212.

Simon K S & Townsend C R (2003) Impacts of freshwater invaders at different levels of ecological organisation, with emphasis on salmonids and ecosystem consequences. *Freshwater Biology* 48: 982-994.

Spehar R L, Poucher S, Brooke L T, Hansen D J, Champlin D & Cox D A (1999) Comparative toxicity of fluoranthene to freshwater and saltwater species under fluorescent and ultraviolet light. *Archives of Environmental Contamination and Toxicology* 37: 496-502.

Stegeman J J & Lech J J (1991) Cytochrome-P-450 monooxygenase systems in aquatic species - Carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environmental Health Perspectives* 90: 101-109.

Stentiford G D, Longshaw M, Lyons B P, Jones G, Green M & Feist S W (2003) Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. *Marine Environmental Research* 55: 137-159.

Stolyhwo A & Sikorski Z E (2005) Polycyclic aromatic hydrocarbons in smoked fish - a critical review. *Food Chemistry* 91: 303-311.

Strandberg B, Bandh C, van Bavel B, Bergqvist P A, Broman D, Näf C, Pettersen H & Rappe C (1998) Concentrations, biomagnification and spatial variation of organochlorine compounds in a pelagic food web in the northern part of the Baltic Sea. *Science of the Total Environment* 217: 143-154.

Stroomberg G J, de Knecht J A, Ariese F, van Gestel C A M & Velthorst N H (1999) Pyrene metabolites in the hepatopancreas and gut of the isopod Porcellio scaber, a new biomarker for polycyclic aromatic hydrocarbon exposure in terrestrial ecosystems. *Environmental Toxicology and Chemistry* 18: 2217-2224.

Stroomberg G J, Zappey H, Steen R J C A, van Gestel C A M, Ariese F, Velthorst N H & van Straalen N M (2004a) PAH biotransformation in terrestrial invertebrates - a new phase II metabolite in isopods and springtails. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* 138: 129-137.

Stroomberg G J, Ariese F, van Gestel C A M, van Hattum B, Velthorst N H & van Straalen N M (2004b) Pyrene biotransformation and kinetics in the hepatopancreas of the isopod Porcellio scaber. *Archives of Environmental Contamination and Toxicology* 47: 324-331.

Sugihara N & James M O (2003) Binding of 3-hydroxybenzo[a]pyrene to bovine hemoglobin and albumin. *Journal of Biochemical and Molecular Toxicology* 17: 239-247.

Tairova Z M, Giessing A M B, Hansen R & Andersen O (2009) 1-Hydroxypyrene as a biomarker of PAH exposure in the marine polychaete Nereis diversicolor. *Marine Environmental Research* 67: 38-46.

Thum T & Borlak J (2002) Testosterone, cytochrome P450, and cardiac hypertrophy. *Faseb Journal* 16: 1537-1549.

Tomy G T, Thomas C R, Zidane T M, Murison K E, Pleskach K, Hare J, Arsenault G, Marvin C H & Sverko E (2008) Examination of Isomer Specific Bioaccumulation Parameters and Potential In vivo Hepatic Metabolites of syn- and anti-Dechlorane Plus Isomers in Juvenile Rainbow Trout

(Oncorhynchus mykiss). *Environmental Science & Technology* 42: 5562-5567.

Torres R J, Abessa D M S, Santos F C, Maranho L A, Davanso M B, do Nascimento M R L & Mozeto A A (2009) Effects of dredging operations on sediment quality: contaminant mobilization in dredged sediments from the Port of Santos, SP, Brazil. *Journal of Soils and Sediments* 9: 420-432.

Ueda H, Ikenaka Y, Nakayama S M M, Tanaka-Ueno T & Ishizuka M (2011) Phase-II conjugation ability for PAH metabolism in amphibians: Characteristics and inter-species differences. *Aquatic Toxicology* 105: 337-343.

USEPA, U.S. Environmental Protection Agency. 2009. List of priority pollutants.Washington, DC.

Van Ael E, Covaci A, Blust R & Bervoets L (2012) Persistent organic pollutants in the Scheldt estuary: Environmental distribution and bioaccumulation. *Environment International* 48: 17-27.

van Brummelen, T C, van Hattum, B, Crommentujin, T & Kalf, D F (1998) Bioavailability and ecotoxicity of PAHs. In Neilson, A H (Eds) *The Handbook of Environmental Chemistry Vol. 3 Part J: PAHs and related compounds: Biology*. Springer- Verlag, Berlin, pp. 203-264.

Van Hoof P L, Kukkonen J V K & Landrum P F (2001) Impact of sediment manipulation on the bioaccumulation of polycyclic aromatic hydrocarbons from field-contaminated and laboratory-dosed sediments by an oligochaete. *Environmental Toxicology and Chemistry* 20: 1752-1761.

van Zelm R, Huijbregts M A J & van de Meent D (2010) Transformation products in the life cycle impact assessment of chemicals. *Environmental Science & Technology* 44: 1004-1009.

Varanasi U, Reichert W L, Stein J E, Brown D W & Sanborn H R (1985) Bioavailability and Biotransformation of Aromatic-Hydrocarbons in Benthic Organisms Exposed to Sediment from An Urban Estuary. *Environmental Science & Technology* 19: 836-841.

Vermeirssen E L M, Burki R, Joris C, Peter A, Segner H, Suter M J F & Burkhardt-Holm P (2005) Characterization of the estrogenicity of Swiss midland rivers using a recombinant yeast bioassay and plasma vitellogenin concentrations in feral male brown trout. *Environmental Toxicology and Chemistry* 24: 2226-2233.

Verrengia-Guerrero N R, Taylor M G, Davies N A, Lawrence M A M, Edwards P A, Simkiss K & Wider E A (2002) Evidence of differences in the biotransformation of organic contaminants in three species of freshwater invertebrates. *Environmental Pollution* 117: 523-530.

Vilanova R M, Fernandez P, Martinez C & Grimalt J O (2001) Polycyclic aromatic hydrocarbons in remote mountain lake waters. *Water Research* 35: 3916-3926.

Vogelbein W K, Fournie J W, Vanveld P A & Huggett R J (1990) Hepatic neoplasms in the mummichog Fundulus heteroclitus from a creosote-contaminated site. *Cancer Research* 50: 5978-5986.

Walker S E, Dickhut R M & Chisholm-Brause C (2004) Polycyclic aromatic hydrocarbons in a highly industrialized urban estuary: Inventories and trends. *Environmental Toxicology and Chemistry* 23: 2655-2664.

Wang J X, Bi Y H, Pfister G, Henkelmann B, Zhu K X & Schramm K W (2009) Determination of PAH, PCB, and OCP in water from the Three Gorges Reservoir accumulated by semipermeable membrane devices (SPMD). *Chemosphere* 75: 1119-1127.

Wang Z, Hollebone B P, Fingas M, Fieldhouse B, Sigouin L, Landriault M, Smith P, Noonan J, Thouin G, & Weaver J W (2003) Characteristics of spilled oils, fuels and petroleum products: 1. Composition and properties of selected oils. U.S. Environmental Protection Agency, Washington, D.C., EPA/600/R-03/072 (NTIS PB2004-101169).

Watanabe T (1983) Metabolic activation of 7,12-dimethylbenz[α]anthracene (DMBA) and 7-Methyl Benzo[α]anthracene (7-MBA) by rat liver P-450 and

sulfotransferase. *The Journal of toxicological Sciences* 8: 119-131.

Weinstein J E & Garner T R (2008) Piperonyl butoxide enhances the bioconcentration and photoinduced toxicity of fluoranthene and benzo[a]pyrene to larvae of the grass shrimp (Palaemonetes pugio). *Aquatic Toxicology* 87: 28-36.

Weslawski J M, Wiktor J, Zajaczkowski M & Swerpel S (1993) Intertidal Zone of Svalbard .1. Macroorganism Distribution and Biomass. *Polar Biology* 13: 73-79.

Weslawski J, Wiktor J, Jr. & Kotwicki L (2010) Increase in biodiversity in the arctic rocky littoral, Sorkappland, Svalbard, after 20-years of climate warming. *Marine Biodiversity* 40: 123-130.

Wiegand C, Pehkonen S, Akkanen J, Penttinen O P & Kukkonen J V K (2007) Bioaccumulation of paraquat by Lumbriculus variegatus in the presence of dissolved natural organic matter and impact on energy costs, biotransformation and antioxidative enzymes. *Chemosphere* 66: 558-566.

Wu J P, Luo X J, Zhang Y, Yu M, Chen S J, Mai B X & Yang Z Y (2009) Biomagnification of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls in a highly contaminated freshwater food web from South China. *Environmental Pollution* 157: 904-909.

Wunder T, Kremer S, Sterner O & Anke H (1994) Metabolism of the Polycyclic Aromatic Hydrocarbon Pyrene by Aspergillus-Niger Sk-9317. *Applied Microbiology and Biotechnology* 42: 636-641.

Xue W L & Warshawsky D (2005) Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. *Toxicology and Applied Pharmacology* 206: 73-93.

Yan J, Wang L, Fu P P & Yu H T (2004) Photomutagenicity of 16 polycyclic aromatic hydrocarbons from the US EPA priority pollutant list. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis* 557: 99-108.

You J, Landrum P F & Lydy M J (2006) Comparison of chemical approaches for assessing bioavailability of

sediment-associated contaminants. *Environmental Science & Technology* 40: 6348-6353.

You J, Brennan A & Lydy M J (2009) Bioavailability and biotransformation of sediment-associated pyrethroid insecticides in Lumbriculus variegatus. *Chemosphere* 75: 1477-1482.

Zhang S Y, Zhang Q A, Darisaw S, Ehie O & Wang G D (2007) Simultaneous quantification of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pharmaceuticals and personal care products (PPCPs) in Mississippi river water, in New Orleans, Louisiana, USA. *Chemosphere* 66: 1057-1069.

Zhang Y X & Tao S (2009) Global atmospheric emission inventory of polycyclic aromatic hydrocarbons (PAHs) for 2004. *Atmospheric Environment* 43: 812-819.

Victor Carrasco Navarro

PAHs: Comparative biotransformation and trophic transfer of their metabolites in the aquatic environment

Polycyclic aromatic hydrocarbons (PAH) are a group of widespread contaminants in the aquatic environment. They may biotransform once they enter an animal's tissue and the products of biotransformation may be more toxic than the parent compounds. In the present thesis, a comparison of the biotransformation of a model PAH among several taxa of invertebrates and fish is presented. The trophic transfer of some of the metabolites produced by black worms and nonbiting mosquito larvae to fish and shrimp is also covered and demonstrated.

UNIVERSITY OF
EASTERN FINLAND

Publications of the University of Eastern Finland
Dissertations in Forestry and Natural Sciences

ISBN: 978-952-61-1137-7 (nid.)
ISSNL: 1798-5668
ISSN: 1798-5668
ISBN: 978-952-61-1138-4 (pdf)
ISSN: 1798-5676 (pdf)