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RANDEL KREITSBERG

Using biomarkers in assessment of environmental contamination in fish – new perspectives

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Using biomarkers in assessment of environmental contamination in fish – new perspectives

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PRESS

Department of Zoology, Institute of Ecology and Earth Sciences
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> *“The ideal scientist thinks like a poet and only later works like bookkeeper,”*
> *Edward O. Wilson in “Letters to a Young Scientist”.*

Autor (vasakul) Taani väinades Kopenhaageni Ülikooli uurimislaeval traalpüügil.

The author (the one on the left) on board a Copenhagen University research vessel in the Danish straits during monitoring trawling.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I R. Kreitsberg, I. Zemit, R. Freiberg, M. Tambets, A. Tuvikene. 2010. Responses of metabolic pathways to polycyclic aromatic compounds in flounder following oil spill in the Baltic Sea near the Estonian coast. *Aquatic Toxicology*, 99: 473–478.

II R. Kreitsberg, A. Tuvikene, J. Baršiene, N. F. Fricke, A. Rybakovas, L. Andreikenaite, K. Rumvolt, S. Vilbaste. 2012. Biomarkers of environmental contaminants in the coastal waters of Estonia (Baltic Sea): effects on eelpouts (*Zoarces viviparus*). *Journal of Environmental Monitoring*, 14: 2298–2308.

III R. Kreitsberg, J. Baršiene, R. Freiberg, L. Andreikenaite, T. Tammaru, K. Rumvolt, A. Tuvikene. 2013. Biomarkers of effects of hypoxia and oil-shale contaminated sediments in laboratory-exposed gibel carp (*Carassius auratus gibelio*). *Ecotoxicology and Environmental Safety*, 98, 227–235.

IV R. Kreitsberg, K. Broeg, A. Räägel, A. Tuvikene. Liver colour of Baltic Sea flounder (*Platichthys flesus trachurus*) as a biomarker of environmental pollution with PAHs. *Submitted to Environmental Monitoring and Assessment.*

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The author contributed to the design of all the studies, took an active part in developing the methods and collecting the field data, was responsible for analysing the data, and is the leading author of all four publications. The original idea for paper IV was proposed by Katja Broeg, Alfred Wegener Institute for Polar and Marine Research, Germany.

1. INTRODUCTION

It is generally known that there are numerous toxic chemicals in almost every type of environment. These include heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs and dioxins), pesticides and medical drugs. Many of those chemicals also have natural sources, e.g. heavy metals leach from rocks and PAHs emerge from volcanoes and ocean hydrothermal vents, whereas others (PCBs, dioxins) have strictly anthropogenic origins. In recent years the effects of many new types of chemicals have been described, toxic effects of food plastic (Muncke, 2011) and microplastic (plastic debris) (Hammer et al., 2012) among them. Therefore, it is evident that our environment contains a mixture of different pollutants (many of them unknown), affecting living organisms at all levels of biological organization (genes to communities).

One of the most widespread groups of toxic chemicals is polycyclic aromatic hydrocarbons. PAHs can originate from natural as well as from anthropogenic sources. Anthropogenic sources of PAHs in the aquatic environment include petroleum spills and discharges, industrial and municipal waste water, refuse incineration and internal combustion engines (atmospheric deposition). It has generally been assumed that the aromatic components of petroleum, particularly polycyclic aromatic hydrocarbons, are the principal determinants of the toxicity of oil and oil products to aquatic organisms (Clark, 2001). Other sources of PAHs can be important regionally, such as the oil-shale industry in North-East Estonia (Tuvikene et al., 1999). When released into the aquatic environment, PAHs can be rapidly absorbed by suspended matter and sediment and become bioavailable to aquatic organisms through the food chain (Perugini et al., 2007). Moreover, under certain circumstances, the contaminants can be released back into the water column from the sediment, rendering them more readily available to the biota (Chapman, 2007).

In fish, organic pollutants are easily absorbed via passive diffusion through the gills, but also intestine and skin, and accumulate in lipid-rich tissues (Varanasi, 1989). During the spawning season, one of the most lipid-rich tissues is the gonad, so problems with reproductive success are among the many possible associated disorders. Other problems include lesions in the hepatic tissue, damaged erythrocytes and nuclei, reduced body condition, etc. Because of the natural sources of PAHs, which have existed for millions of years, fish and other organisms have developed various metabolic pathways to transform the lipid-soluble PAH molecules into water-soluble metabolites that are removed from the body (Hahn et al., 1988). Cytochrome P450 enzyme complexes have been found in all branches of the “tree of life” that catalogues the diversity of life forms. In the broadest terms, these oxygenases have two main functions. One is the metabolism of xenobiotics (compounds exogenous to the organism), protecting the organism from them by degradation or by providing polar handles for solubilising them in preparation for excretion. The other is the biosynthesis of critical signalling molecules used in the control of development and homeostasis (Denisov et al., 2005). Almost 4000 identified P450 genes had been identified by the time the Denisov et al. (2005) paper was written.

In areas of high anthropogenic pressure such as the Baltic Sea, man-made accidents such as oil spills can make considerable contributions to environmental pollution. Hundreds of thousands of tons of crude oil have been released into the aquatic environment through the world's largest oil spills (Galt et al., 1991; Gonzalez et al., 2006). The intensity of shipping in the Baltic Sea has increased enormously during recent years and is predicted to increase even further. There are around 2000 sizable ships at sea at any one time in the Baltic (HELCOM, 2010). In 2000–2008, 61 accidents were reported to have resulted in some pollution, ranging from 0.015 m^3 to 150 m^3 of oil spillage (HELCOM, 2009a). Luckily, the number of deliberate, illegal oil discharges has been successfully reduced over the past twenty years; from 763 in 1989 to 210 in 2008 (HELCOM, 2009b). The extent of such spillages has also decreased: the total estimated volume of oil spills in 2008 was 64.3 m^3. Nevertheless, in 2008, 20% of vessels that entered or left the Baltic Sea via the Kattegat were tankers, carrying as much as 170 million tonnes of oil (HELCOM, 2010). Although large-scale ecological catastrophes still make headlines (e.g. Deepwater Horizon in the Gulf of Mexico, 2010), the trend in oil pollution is changing towards long-term persistent leaks from pipelines and rigs (Jernelöv, 2010). Spills from ageing, ill-maintained or sabotaged pipelines have increased, and places such as Arctic Russia, the Niger Delta and the north-western Amazon have become sites of recurring oil pollution (Jernelöv, 2010).

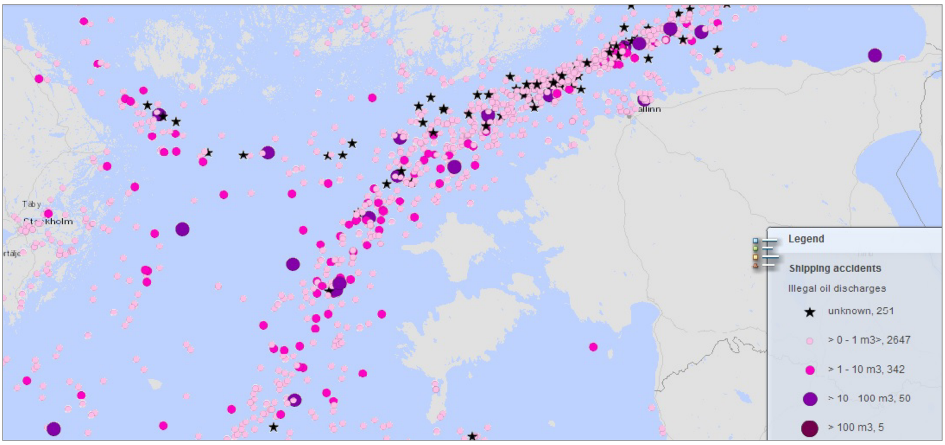

Figure 1. Illegal oil discharges in the Baltic Sea close to Estonia observed by aerial surveillance activities 1998–2012 (HELCOM, 2013).

All these data indicate that appropriate chemical pollution monitoring of marine areas is indispensable and the effects of pollution on the biota must be assessed. Because the aquatic environment contains a mixture of different pollutants (many of them unknown) it is difficult if not impossible to detect and measure each and every one of them. Therefore, it is advisable in such a multi-stressed system to assess the effect of toxicants on living organisms (environmental health). Those effects can occur at any level of biological organization (from genes to communities) and are called biomarkers: by definition a biomarker is "a change in a biological response which can be related to exposure to or toxic effects of environmental chemicals" (Peakall, 1994). Examples of biomarkers include 7-ethoxyresorufin-O-deethylase (EROD) activity, lysosomal membrane stability, PAH metabolites in bile, nuclear abnormalities, liver and gonad somatic indices, changes in population structure, etc. Biomarkers can be more or less specific to a certain chemical or group of chemicals and can reveal effects from short-term to chronic exposure.

Biomarker responses in fish cells and tissues are widely used by ecotoxicologists around the Baltic Sea, providing valuable information on fish health, environmental stress and anthropogenic pressure on fish habitats. Flounder (*Platichthys flesus*), Baltic herring (*Clupea harengus membras*), and eelpout (*Zoarces viviparus*) are most frequently used in such studies (e.g. Broeg and Lehtonen, 2006; Gercken et al., 2006; Schiedek et al., 2006). Eelpout is also used as a bioindicator of potentially hazardous substances by the German Environmental Specimen Bank, the Baltic Marine Environment Protection Commission (Helsinki Commission) and the BONUS Joint Baltic Sea Research Programme.

Nevertheless, disagreements and questions have arisen about the reliability of several biomarkers. For example, EROD activity has been found to be highly influenced by environmental and seasonal factors (Hedman et al., 2011); a high liver somatic index (LSI) can demonstrate good nutritional status but also contamination (van der Oost et al., 2003). In addition, reliable biomarkers for rapid assessment of environmental health are still lacking.

In my thesis I have focused on assessment of environmental contamination and other stressors (hypoxia, parasites) using biomarkers, in the context of oil-spill, diffuse contamination of the coastal Baltic Sea and oil-shale-contaminated sediments. I used two approaches, laboratory experiments and field sampling. In January 2006 an oil-spill that involved approximately 40 tons of heavy fuel oil affected more than 30 km of the north-west coast of Estonia (Kreitsberg et al., 2010). According to the International Tanker Owners' Pollution Federation (ITOPF) classification, this amount of hazardous substance is classed as middle-sized pollution. Assessing the effects of the oil-spill and monitoring contamination of the coastal Baltic Sea included a total of six sampling locations close to heavy shipping routes as well as pristine sea areas. Meanwhile, the Baltic Sea close to north-eastern Estonia is affected by contamination brought in by rivers that start from oil-shale industrial areas

(Tuvikene et al., 1999). Sediments from these river mouths were used in laboratory exposure experiments to examine the effects of contamination coupled with hypoxia.

As study subjects, three fish species were chosen for biomarker analyses: eelpout and flounder in assessments of the Baltic Sea, and gibel carp (*Carassius auratus gibelio*) as the experimental model species. Eelpout or viviparous blenny and flounder are both common and abundant species and have many characteristics that make them appropriate for biomarker monitoring: they do not avoid harbours and other human-affected areas and they feed on benthic organisms (gastropods, chironomids, crustaceans, eggs and small fish) and are therefore exposed to toxicants accumulated in sediments. Flounder is also important for human consumption. Gibel carp was chosen for the experimental part because *Carassius* spp. are widely used as model organisms for evaluating potential risks associated with aquatic pollution (e.g. Zhang et al., 2004; Falfushynska et al., 2012). The gibel carp is known for its ability to withstand adverse environmental conditions (including high nutrient load, extreme temperatures, hypoxic events, etc.), which has facilitated colonization of most water bodies throughout Europe (Perdikaris et al., 2012), including the coastal waters of Estonia. Stamina at this level is necessary in an experiment with such high contaminant levels in sediments, such as the ones we collected in the mouth of River Purtse, North-East Estonia.

The objectives of my thesis were to examine the effects of oil pollution **(I)** and of oil-shale contaminated sediments **(III)** on a wide set of fish biomarkers, and to examine the effect of dispersed environmental contamination on fish in the coastal Baltic Sea **(II)**. Furthermore, two new biomarkers of pollution are proposed in addition to the traditional ones **(I, IV)**, and the effects of hypoxia on fish biomarkers are analysed.

2. MATERIALS AND METHODS

Eelpout and flounder specimens were collected with the help of the Estonian Marine Institute and local fishermen from coastal Baltic Sea areas – Treimani, Kihnu, Pärnu, Sõrve, Vilsandi, Nõva, Saarnaki and Käsmu (**I, II, IV**) – using gillnets and fish traps. Gibel carp were collected from the pond in the Botanical Garden of Tartu University (**III**) using seine. Altogether 455 fish specimens were used in these studies.

2.1. Biomarkers

A multi-biomarker approach was chosen to enable the combined effects to be assessed comprehensively and integratively. Our set of biomarkers reveals the different effects of environmental pollution from genetic changes to alterations in fish size.

- Indices of fish size and visual assessments of parasites (mainly *Pomphorhynchus laevis,* in our study) are regular tools in fish monitoring and can also provide information about environmental conditions, including contamination. All collected fish specimens were measured using a standard protocol: total length, total and gutted weight, sex, and liver and gonad masses were recorded. The metrical indices calculated included liver somatic index (LSI = liver weight in grams per total weight in grams * 100), gonadosomatic index (GSI = gonad weight in grams per total weight in grams * 100) and condition factor (CF = total weight in grams per total length in cm^3 * 100).
- Lesions and abnormalities in fish liver (2 mm thick central piece) were identified by histological examination. The histological sampling and processing techniques used in this study accorded with a standard protocol for flatfish species (Feist et al., 2004), modified with regard to the demands of eelpout liver tissue (Fricke and Lang, unpublished). Four to six stained liver sections from each sample were examined by light microscopy and any alterations were documented.
- Fish liver also provided information about the activities of biotransformation processes induced by environmental contaminants. Measurement of 7-ethoxyresorufin-O-deethylase (EROD) in homogenized liver demonstrates cytochrome P450 enzyme activity, which represents the ability to metabolize a wide range of aromatic xenobiotics, e.g. PAHs and PCBs (Pikkarainen, 2006). Livers were homogenized and EROD was measured using a method described by Kirby et al. (2000). EROD activity was normalized to protein content and expressed as pmol resorufin/min/mg protein.

- Liver colour was determined, after the peritoneal cavity was opened, using a colour table developed within the framework of the project “Fish diseases in the Wadden Sea”, supported by the Umweltbundesamt (Wahl et al., 1995). During this project, a correlation was established between the pathological deposition of neutral lipids in flounder liver and the discoloration of the liver from dark brown to pale brown and white.
- Fixed wavelength fluorescence (FF) measurements on diluted bile and urine are the most rapid and technically undemanding ways to obtain meaningful PAH exposure data. Bile and urine from fish exposed to PAH mixtures provide a concentrated multifluorophoric sample, the fluorescence being attributable primarily to the PAH metabolites present. Bile samples were diluted in 48% ethanol (Aas et al., 2000) and measured using fixed wavelength pairs (excitation/emission) to detect PAH metabolites: 290/380 nm representing naphthalene (2-ring PAH); 256/380 nm representing phenanthrene (3-ring PAH); 341/383 nm representing pyrene (4-ring PAH); and 380/430 nm representing benzo(a)pyrene (5-ring PAH) (Lee and Anderson, 2005). These wavelengths also provide information about the PAH source: a complex mixture of non-alkylated and alkylated naphthalene metabolites is typically found in fish exposed to petrogenic contamination (crude oil or marine fuel related), whereas combustion-type sources (traffic, domestic heating, industry) give rise to a stronger FF signal at wavelengths characteristic of 4- and 5/6-ring PAHs (Beyer et al., 2010). Bile and urine samples were collected directly from the bladders using 1 mL disposable syringes after the abdominal cavity was opened.
- Peripheral blood erythrocytes were chosen to measure the endpoints of environmental genotoxicity and cytotoxicity since they are the carriers of pollutants in the circulation. The genotoxicity effects investigated are of high priority since they indicate damage to genetic material in organisms caused by different hazardous substances and their mixtures (Moore at al., 2004). Induction of micronuclei (MN), nuclear buds (NB) and binucleated cells with nucleoplasmic bridges (BNb) in erythrocytes were used as genotoxicity endpoints; and induction of fragmented-apoptotic (FA), binucleated (BN), blebbed (BL) and 8-shaped cell nuclei were assessed as cytotoxicity endpoints. To identify different nuclear abnormalities in fish erythrocytes, blood smears were stained with 5% Giemsa in phosphate buffer, pH 6.8. The stained slides were examined under an Olympus BX51 light microscope at a final magnification of 1000*. The final results were expressed as the mean (‰) of the sums of the individual nuclear abnormalities scored in 1000 cells per fish (a total of 4000 immature erythrocytes with intact cytoplasm were examined for each specimen) (Baršiene et al., 2004).

Figure 2. Fish biomarkers include a wide variety of indicators that represent the animal's condition and the effects of environmental contamination. The indices of fish liver and body size, PAH metabolites in bile and urine, liver colour and parasites are among them (eelpout, Zoarces viviparus, in the picture).

2.2. Chemical concentrations (HPLC)

Reversed phase high performance liquid chromatography (HPLC) was used to measure the content of polycyclic aromatic hydrocarbons in fish muscle, liver and sediment. The method described by Tuvikene et al. (1999) was modified for extraction of the parent PAHs: the muscle and liver samples were homogenized and rotor-extracted with KOH (for fat hydrolysis) and n-hexane for 24 h. The sediment samples were also rotor-extracted with n-hexane for 24 h, then concentrated and transferred to 1 ml acetonitrile for HPLC separation. Reversed-phase HPLC was accomplished using a Shimadzu Prominence (Japan) series system with a photodiode array (PDA) and fluorescence detectors to separate the sixteen PAHs listed by the US Environmental Protection Agency (EPA). Absorbance spectra were recorded at wavelengths from 200 to 800 nm and used to identify the PAHs. A pre-programmed fluorescence detector with different excitation and emission wavelengths (Wegrzyn et al., 2006) was used to confirm the presence or absence of trace amounts of fluorescent PAHs.

Most of the analyses and exposure studies were conducted at the Estonian University of Life Sciences and University of Tartu; geno- and cyto-toxicities were investigated at the Institute of Ecology of the Nature Research Centre, Lithuania; and liver histology was studied at the Johann Heinrich von Thünen-Institut in Germany.

2.3. Statistical analysis

Analysis of variance (one-way/two-way ANOVA, or Kruskal-Wallis ANOVA) together with the *post-hoc* Tukey test was used for comparisons among most of the study groups (metrical data, PAH metabolites, EROD activity in fish). The non-parametric Mann–Whitney U-test was used to test the frequency of nuclear abnormalities among groups, where appropriate. Pearson's correlation coefficient was used to demonstrate relationships between the studied biomarkers and biometrical measurements on the fish, together with Spearman correlations for small sample sizes. Principal components (PC), calculated from the concentrations of the four types (2-, 3-, 4- and 5-ring PAH metabolites) of fluorescent aromatic compounds, were used. Where appropriate, normality and variance homogeneity were checked using the Kolmogorov–Smirnov test. The Statistica 7 and GraphPad PRISM 5.0 packages were used for statistical analyses.

3. RESULTS AND DISCUSSION

3.1. Biomarkers and toxicants in coastal Baltic Sea

Eelpout and flounder specimens for the studies were collected from eight areas in the coastal Baltic Sea: Treimani, Kihnu and Pärnu, representing the Gulf of Riga; Sõrve and Vilsandi, close to the Baltic Proper; Saarnaki in the Moonsund straits; and Nõva and Käsmu, representing the Gulf of Finland.

In the study with eelpout, Nõva and Sõrve were the sites that seemed most affected by environmental contaminants according to almost every biomarker comparison, whereas Pärnu and Treimani in the Gulf of Riga were the least affected. The results demonstrated that fish from the Gulf of Riga emitted lower levels of fluorescence in fixed wavelength analyses (representing equivalents of PAH metabolites in bile and urine), and accordingly they exhibited less geno- and cyto-toxicity and parasite infection, a higher liver somatic index (LSI) and a higher condition factor (CF) than fish inhabiting areas close to the Baltic proper and in the Gulf of Finland **(II)**.

Overall, alterations were found at all biological levels studied, from genes to fish size parameters. There were many non-specific inflammatory liver lesions (19), which co-existed with erythrocyte abnormalities. The most intriguing correlations were between 3-ring PAH metabolites (together with low CF) and the abundance of the parasitic acanthocephalan worm *Pomphorhynchus laevis*, and a negative correlation between LSI and PAH metabolites in bile. In this study, in contrast to numerous others (Williams and Iatropoulus, 2002; van det Oost et al., 2003; de la Torre et al., 2007), high LSI is revealed as an indicator of good fish condition. Earlier studies demonstrated a positive relationship between high LSI and xenotoxic pollution. In our study, LSI correlated negatively with parasitism and with PAH metabolites in bile (2-, 3- and 4-ring molecules). De la Torre et al. (2007) described adverse effects on hepatic function (increased LSI values) of fish living in a contaminated river. Liver enlargement accompanied by induction of biotransformation enzymes is elicited by numerous xenobiotics, notably PAHs, barbiturates, butylated hydroxytoluene, and polychlorinated biphenyls (PCBs). According to the review by van der Oost (2003), LSI increased significantly in line with contaminant (PCBs, OCPs, BKME, PCDDs, and PAHs) exposure in 38% of laboratory studies and in 43% of field studies; a decrease of LSI was described in fewer than 20% of cases. Liver size is believed to reflect the organ's ability to accommodate an increased functional load due to active cell proliferation (Williams and Iatropoulos, 2002), but it also reflects the dietary status of the fish owing to the role of the liver as a fat storage organ. Therefore, the high LSI values in eelpouts from the relatively pristine environments, Pärnu and Treimani, are most likely to be caused by an exceptionally good feeding area (which would also give high CF values) and are not a result of contaminants **(II)**. Similar results were found in another study with Baltic Sea flounder **(IV)**:

we saw that LSI is correlated with the condition factor, which also supports the hypothesis that a high LSI is the result of very good feeding conditions.

It is interesting to note that the two potentially polluted sea areas, Nõva and Sõrve, are closest to the Baltic proper, being situated close to the open sea. Such areas are hydrodynamically active, which implies that they are exposed to wind, waves and currents, all of which facilitate the dispersion of pollutants. However, Napierska and Podolska (2005) described the opposite situation on the southern Baltic coast, where flounders (*Platichthys flesus*) from the open sea areas close to Leba had lower enzyme activities (GST and EROD) than those of fish from the Gulf of Gdansk. When discussing such results as we found, it is important to note that the Baltic is believed to be one of the most contaminated seas in the world: Witt (1995) estimated that the PAH concentrations in the water column (including seston) of the Baltic Sea were 2–10 times higher than those in the North Sea. Possible routes for contamination include atmospheric deposition, river runoffs, shipping activity, sediment disturbances, and spills. Since there is no significant industry or dense population along the Estonian west coast, long-range atmospheric contamination and shipping activity are possible explanations. Both Sõrve and Nõva are close to active international shipping routes; cargo ships and oil tankers pass these waters daily. During 2010 a huge seabed disturbance occurred in the Gulf of Finland because of North Stream gas pipeline construction work, and resuspension of contaminated sediments is a possibility. Sediment particles can relocate tens of kilometres away from their original site (Lips, *personal communication*) and could therefore affect biota close to the Estonian coastline. Baršiene (2011) found a similar induction of nuclear buds in fish from the southern Baltic in areas affected by North Stream construction. Nevertheless, the waters of the eastern Baltic Sea seem to be rather moderately polluted (PAH metabolites and EROD), especially the Gulf of Riga – which gives a good background for future Baltic studies **(II)**.

3.1.1 Difficulties with interpretation of the EROD assay

Our study demonstrates a gradient of EROD activity (average values: pmol per min per mg protein): Treimani, 2009 (8.7) < Pärnu, 2010 (11.6) < Nõva, 2009 (16.4) < Nõva, 2010 (22.8) < Pärnu, 2009 (26.2) < Sõrve, 2010 (28.7). Compared to other studies in the Baltic Sea our EROD values remain relatively moderate; they are much lower than those presented in the review by Hedman et al. (2011). EROD is a semi-specific biomarker that represents the amount of the active P450 enzyme CYP1A in fish liver. Measurement of EROD has been traditionally used as an in vivo biomarker of exposure to PAHs and structurally similar compounds such as some PCBs and dioxins, which are able to bind and activate the Ah-receptor. For example, Hanson et al. (2009) suggested that high enzyme activities in fish are caused by PAH pollution, and demonstrated a correlation between PAH metabolites in perch bile (*Perca fluviatilis*) and

EROD, though other studies have found no correlation (Hedman et al., 2011). In our work **(II)**, there was no correlation between EROD activity and PAH metabolites. Types of pollution other than PAHs could cause high enzyme activities; high EROD activities could be caused by algal toxins, PCBs or dioxins, or be the result of seasonal enzyme activity fluxes (Hansson et al., 2006; Hedman et al., 2011). Hedman et al. (2011) found variability in EROD activity between and within (seasonal) years: there were seasonal differences in female eelpouts but no variation in males. Also, there was no correlation with temperature. Furthermore, Fonseca et al. (2011) discussed the effects of temperature and salinity on enzyme activities (e.g. EROD and GST) and suggested that variations in these biomarkers are more specific to chemical exposure rather than environmental dynamics **(II)**.

Nevertheless, no elevated enzyme activity was found in the EROD assays during the aquarium exposure experiment with oil-shale-contaminated sediment **(III)**. In fish, cytochrome P4501A (CYP1A) plays a key role in the biotransformation and bioactivation of chemicals and is induced by a variety of environmental pollutants: any compound that can bind and activate the aryl hydrocarbon receptor (Ah-r). In liver homogenates, EROD activity reflects cytochrome P450 activity, which represents the ability to metabolize this wide range of xenobiotics (Pikkarainen, 2006). Therefore, any association between EROD activity or CYP1A induction and the presence of a particular chemical or group of chemicals (mainly PAHs) must be interpreted with great care (Valdehita et al., 2012). In our previous studies, we found no correlation between PAHs and EROD activity **(II)**, and in the experimental study **(III)** we found little EROD activity in any of the exposure groups. Industrially contaminated sediments are complex mixtures of chemicals and it is difficult to predict their biological effects: reciprocal interactions, cascade and indirect mechanisms can either enhance or suppress the expected responses (Benedetti et al., 2007). For example, Benedetti et al. (2007) described the effects of trace metals on the metabolism of benzo(a)pyrene (BaP). They found that EROD activation after BaP injection was suppressed by more than 80% in organisms co-exposed to cadmium and copper, while EROD activity in those treated with BaP and other metals (Hg, Ni, Pb) was comparable with organisms exposed only to BaP. Moreover, biliary BaP-type metabolites were detectable in fish exposed to BaP, alone or in combination with various metals, and with no significant differences between those exposure groups. These results confirm that bile metabolites can indicate some metabolism of PAHs even at low levels of EROD induction (Benedetti et al., 2007). A recent study has also demonstrated hypoxia-induced down-regulation of CYP1A (Rahman and Thomas, 2012). Exposure to hypoxia (dissolved oxygen: 1.7 mg/L for 2–4 weeks) led to significantly lower hepatic CYP1A mRNA and protein levels than in fish kept under normoxic conditions. The results suggested that the observed down-regulation is due to alterations of nitric oxide (NO) and oxidant status and hepatocellular interleukin-1α and hypoxia-inducible factor-1α (HIF-1α) levels

(Rahman and Thomas, 2012). It is therefore suggested that induction of the CYP1A gene can be modulated by hypoxia as well as by a selection of heavy metals **(III)**.

3.1.2 PAH metabolites in fish urine

In fish, PAH levels in bile determined by the semiquantitative fixed wavelength fluorescence (FF) technique have been used as a biomarker of exposure. FF measures the fluorescence emitted by all compounds present with the same number of benzene rings, giving an indication of PAH-type accumulation (Beyer et al., 2010). This method was also used to screen PAH metabolites in several other tissues including fish liver, muscle and brain, and was successfully adapted to measuring these compounds in the haemolymph, urine, digestive gland and muscle of crab species (Silva et al., 2013). In our studies **(I, II)**, fish urine was used as a possible deposit of PAH metabolites and for the first time the FF technique was used to detect PAH metabolites in the urine. Relying on the analogy between enzyme complexes in liver and kidney (Pesonen et al., 1987), the same wavelength pairs and dilutions were used for urine samples as for bile samples (Lee and Anderson, 2005).

In assessing sediment re-suspension and the effects of oil-spill on the North-West Estonian coast, both bile and urine were analysed for PAH metabolites. The principal component scores of four types of fluorescent aromatic compounds (FACs) differed at various time-points (June 2006 to August 2008) in the polluted area. At the same time, PAHs with different molecular sizes had different metabolite concentrations in bile and urine. The significant interaction term indicates that PAH metabolites with different molecular sizes were disproportionally distributed among tissues; there were relatively higher concentrations of low-molecular PAH metabolites in bile. In contrast, the amounts of 4- and 5-ring PAH metabolites remained at approximately the same level in the bile and urine samples **(I, II)**.

Previous studies using several extra-hepatic tissues of different fish species revealed that almost every tissue or organ examined had detectable enzyme (monooxygenase) activity towards PAH, with maximum activity in the liver (Varanasi,1989). Two metabolic pathways excrete biotransformed PAH compounds via the liver and bile into the faeces or through the kidney and into the urine. Metabolites from bile can be further biotransformed by intestinal microbes before final excretion (Karasov and Martínes del Rio, 2007) **(I)**.

We used a novel method to compare the activities of these two metabolic pathways (liver-bile and kidney-urine) in oil-polluted fish. FF measurements have not previously been used to detect PAH metabolites in fish urine. The results demonstrated that PAH metabolite concentrations differ between bile and urine. Furthermore, their distribution is based on the metabolite's molecular size. We found that metabolic reactions in kidneys were notably high, particularly for 4- and 5-ring molecules, causing approximately the same transformation level as in liver. However, metabolic activity for 2- and 3-ring PAHs was higher in the liver, as previously described (Pesonen et al., 1987; Nakayama et al., 2008). Earlier studies demonstrated that the main transformation enzymes for PAHs are components of cytochrome P-450 (Varanasi, 1989) and the levels of P-450 are approximately 5-fold higher in liver than in kidney (Pesonen et al., 1987; Nakayama et al., 2008). Therefore, higher concentrations of metabolites in bile would be expected. This was the case for 2- and 3-ring PAHs in this study but not for the levels of 4- and 5-ring PAH metabolites. Our results therefore suggest that kidneys are much more important in PAH metabolism than has hitherto been assumed **(I, II)**.

3.1.3 Liver colour

We found the content of the final PAH conjugates to affect the colour of Baltic Sea flounder livers. In particular, fish with pale-coloured livers (colour no. 4 – bright orange) contained more PAH metabolites in the bile than fish with dark-coloured, brown (colour no. 2) livers. This trend is particularly evident in the analyses with 3-, 4- and 5-ring metabolites, representing molecules of polycyclic aromatic hydrocarbons of similar size (Beyer et al., 2010). Similar results have been found in studies with North-Sea flounder (*Platichthys flesus* Linne): in Broeg et al. (1999), liver colour was positively correlated with total PCB and DDT metabolite residues measured in fish muscle and liver tissues. We have reason to believe that the liver is decoloured from dark brown to pale brown and white because of pathological deposition of neutral lipids. In an extensive review of the effects of pesticides on fish livers, Couch (1975) reported that the most commonly encountered liver lesion was abnormal fat accumulation. More recently, in a study of Colorado River fish biomarkers in the United States, discoloration of the liver was described as an indicator of poor health (Hinck et al., 2007). Liver discoloration, granular spleen and kidney, pale gills, and other lesions accounted for the highest HAI (necropsy-based health assessment index) scores **(IV)**.

In conclusion, the liver colour table is potentially very useful as a toxicological biomarker, applicable for rapid use in the field. Nevertheless, further validation with larger sample sizes and liver lipid content analyses is required. Since the liver is responsible for the production of vitellogenin, the yolk protein precursor, seasonal variation has to be taken into consideration when the liver lipid content is used as a biomarker. Several factors such as food supply, feeding behaviour and reproduction, especially in females, can influence the levels of liver lipids (Broeg et al., 1999) **(IV)**.

3.2. Effects of oil-spill

In January 2006 an oil spill that involved approximately 40 tons of heavy fuel oil affected more than 30 km of the north-west coast of Estonia. The aquatic pollution of the oil-spill area of the Baltic Sea was monitored by measuring the content of selected polycyclic aromatic hydrocarbons (PAHs and PAH metabolites) in flounder (*Platichthys flesus trachurus* Duncker). Two different methods were used during our study; a relatively fast and inexpensive fixed wavelength fluorescence analysis, and a time-consuming and more expensive HPLC method. This work demonstrates that using both methods gave more detailed results (quantitative HPLC method) and made the throughput of samples quicker (FF analysis) **(I)**.

The dominant PAHs in Nõva liver samples were fluoranthene and pyrene and the highest concentrations in liver reached 25 µg/g dry weight (DW). The dominant PAHs were identical in oil and in liver samples from the polluted area of Nõva, indicating that the pollutants had a common origin. HPLC analyses revealed detectable levels in some muscle samples too and demonstrated that benzo(b)fluoranthene was one of the most abundant PAHs in fish muscle tissues (highest concentration 1.4 µg/g DW). The results of both methods, total PAH concentrations in liver, and the principal components of PAH metabolites in bile and urine, differed significantly at different sampling times during the summer of 2006 in the oil-spill area. The PAH content of the liver decreased significantly during this period; from June to October the PAH levels fell 83% in liver tissues (means 30.2 and 5.1 µg/g DW, respectively). Also, remarkable decreases in PAH metabolite concentrations in bile and urine were evident: the mean concentrations of PAH metabolites decreased by 82% in bile and by 113% in urine samples **(I)**.

The results reveal that PAH concentrations remained relatively high in the livers of fish even five months after the oil spill, indicating that flounder were exposed to highly polluted sediments (re-suspension processes). Recovery from oil pollution ranges from a few months (Lee and Anderson, 2005) to several years (Hayakawa et al., 2006; Boehm et al., 2007). We conclude that in the case of Nõva most the pollution was buffered by a hydrodynamically active sea (heavy waves, currents and upwelling area), and this was evident in October when the concentrations of PAHs were close to the reference level. The total level of PAHs in fish livers from the polluted area decreased six-fold between June and October, an effect similar to those found in analogous studies in Spain, Scotland and Alaska (George et al., 1995; Lee and Anderson, 2005; Gonzalez et al., 2006). No toxicological studies had been carried out in this area before the accident, so there is no direct information about natural PAH concentrations in these waters. Nevertheless, total liver PAH content reached 5.1 µg/g DW in October, which is comparable to the level in our control area, Saarnaki. Baršiene et al. (2006) reported an approximately 66% decrease in PAH metabolites in the bile of flounder seven months after the Butinge oil-spill of

November 27, 2001 in Lithuania (Baltic Sea). Compared to the latter measurements, the present data demonstrate a more gradual decrease in pollutants (82% over two years), which could be due to sedimentary effects in Nõva and Keibu Bay. Martin (2006) reported a large amount (8 m^2) of oil buried under a slight layer of sediment in underwater observations **(I)**.

In conclusion, 40 tons of heavy fuel oil caused moderate harm to fish and there was no evidence of large-scale deaths in the polluted area. Nevertheless the level of contaminants was highest in early spring, the time of spawning and maturation of eggs and larvae. Flounders in this part of the Baltic have short migration routes, and according to Ojaveer and Drevs (2003) the spawning areas in the Gulf of Finland partly overlap the oil-spill areas. Long-term studies have demonstrated that early stages of the reproductive process in female fish (e.g. English sole (*Parophrys vetulus*)) can be disrupted by contaminant exposure, and female fish exposed to substantial levels of pollutants can be partially excluded from the spawning population (Collier et al., 1992). Although our data do not show that, the oil spill could have exerted an impact on the population of coastal spawning flounder in north-west Estonian sea areas **(I)**.

3.3. Effects of hypoxia

The natural oxygen level can vary widely owing to changes in water temperature and salinity, mixing of surface and deep water masses, eutrophication, vertical stratification of the water column, photosynthetic activities of algae and intensity of respiration of organisms (Lushchak and Bagnyukova, 2006). Nevertheless, many of these factors can also be influenced by human activities. In fish, ventilation frequency is increased during hypoxia (Cannas et al., 2012), and the rate of entry of toxins through gill respiration is therefore affected by the level of dissolved oxygen. In the context of fish physiology and survival, it is therefore necessary to consider the effects of contamination and hypoxia in concert, as we did in our laboratory exposure study with gibel carp (*Carassius auratus gibelio*, Bloch 1782) and oil-shale contaminated sediment **(III)**.

The most abundant PAHs in the oil-shale-contaminated sediment were benzo(a)pyrene, pyrene, fluoranthene, benzo(a)anthracene, phenanthrene and anthracene. The total PAH concentrations at the end of exposure were lower in the sediments from the oxygen-saturated tanks than in those from the hypoxic tanks. Nevertheless, mortality occurred in the former. This unexpected result can be explained by the behaviour of the fish: they were less active under hypoxic conditions than under oxygen saturation (swimming around and exploring the tank, searching for food), and this could have contributed to the release of the chemicals from the sediments and increased the exposure of the fish to them. Indeed, the effect of sediment disturbance has been described as outweighing that of many other abiotic factors: e.g. a study on a metal-contaminated sediment demonstrated that metal release and sequestration rates were much more affected by changes in overlying water pH and sediment disturbance (by physical mixing) than by changes in dissolved oxygen concentration (3–8 mg/1) or salinity (Atkinson et al., 2007). Another study associated mortality during exposure to a PAH-contaminated sediment with increased bioturbidity caused by the burrowing and feeding activities of the oligochaete *Lumbricus variegatus* (Pang et al., 2012). These activities caused more re-suspension of sediment particles, so contaminants associated with those particles entered the overlying water column (Pang et al., 2012). It is also known that many pollutants or their metabolites can exert toxicity by causing oxidative stress (Livingstone, 2001) **(III)**.

Moreover, the fluorescence from all four types of PAH conjugate (2-, 3-, 4- and 5-ring molecules) in fish bile was significantly higher in the tank with contaminated sediment and hypoxia (in some cases dozens of times higher) than all other experimental groups. In addition, for all experimental sediments, the mean total PAH concentrations in the sediment were lower in the tanks with oxygen saturation than under hypoxic conditions (remember that no mortality occurred under hypoxia). Oxygen saturation affects the bioavailability of contaminants, while hypoxia is known to increase the ventilation rate in fish (Cannas et al., 2012), which in turn increases the water flow rate through the gills and hence exposure to dissolved chemicals. Passive diffusion through gill lamellae is the main route by which lipophilic chemicals enter the fish body (Varanasi, 1989), so an increased body burden due to an elevated ventilation rate (especially in the groups exposed to contaminated sediment) is to be expected. This could explain the high total genotoxicity in the hypoxic exposure group with contaminated sediment (more than double the control level), and the significantly higher level of PAH conjugates in the bile in these fish. Owing to the gibel carp's ability to cope with adverse environmental conditions, including chronic stress induced by toxic pollution, the fish in the hypoxic exposure groups survived **(III)**.

Our study demonstrates that the bioavailability of sediment-bound contaminants changes continually and the effects on aquatic organisms depend on physiological state (season, maturity, hibernation), aquatic chemistry (temperature, dissolved oxygen, pH) and biotic interactions (food availability, biodegradation). Our study demonstrated the importance of those interactions, which outweigh individual effects, and remind us that even for gibel carp, a species resistant to such environmental conditions, some combinations of the abovementioned parameters are lethal **(III)**.

* * *

In conclusion, this thesis demonstrates that alterations were found at all studied biological levels in fish from the Baltic Sea, representing biomarkers from genes to fish size parameters. In almost every biomarker comparison, Nõva and

Sõrve (closest to the Baltic proper) were the sites that seemed most affected by environmental contaminants, whereas Pärnu and Treimani in the Gulf of Riga were the least affected. The most intriguing correlations of biomarkers were between 3-ring PAH metabolites and the abundance of the intestinal parasite *Pomphorhynchus laevis*, and the negative correlations between LSI and PAH metabolites, and CF and PAH metabolites in bile. However, there was no observable correlation between EROD activity and PAH metabolites. Also, two novel biomarkers for assessing environmental pollution are proposed: measuring PAH metabolites in fish urine by FF, and using liver colour in flounder as an indicator of contamination. The results of the thesis demonstrate that the effect of interactions among environmental factors supersedes individual effects.

SUMMARY

The environment contains a mixture of different pollutants (many of them unknown), affecting living organisms at all levels of biological organization (from genes to communities). In fish, organic pollutants are absorbed easily via passive diffusion through the gills, intestine and skin, and accumulate in lipid-rich tissues. Because natural sources of PAHs have existed for millions of years, fish and other organisms have developed metabolic pathways to transform lipid-soluble toxicants into water-soluble metabolites for excretion from the body. Fish in the Baltic Sea are under constant stress from environmental contamination due to long-range transportation of toxicants and heavy shipping activity. In 2000–2008, 61 shipping accidents were reported to have resulted in some pollution in the Baltic Sea, ranging from 0.015 m^3 to 150 m^3 of oil spill.
All these data indicate that appropriate chemical pollution monitoring in marine areas is indispensable and the effects of pollution on biota must be assessed. It is difficult if not impossible to detect and measure each and every one of those chemicals in the sea. Therefore, assessing the effects of toxicants on living organisms (environmental health) is advisable. Those effects can occur at any level of biological organization (from genes to communities) and are called biomarkers. Nevertheless, various contradictions and questions have arisen about the reliability of several biomarkers.

The objectives of my thesis were to examine the effects of oil pollution **(I)** and oil-shale contaminated sediments **(III)** on fish biomarkers, and to examine the effect of dispersed environmental contamination in fish in the coastal Baltic Sea **(II)**. Furthermore, two new biomarkers of pollution are proposed **(I, IV)**, and the effects of hypoxia **(III)** on fish biomarkers were studied. Two approaches were used, a laboratory experiment and field sampling. Assessing the effects of oil-spill and disperse environmental contamination of the coastal Baltic Sea included a total of six sampling locations close to heavy shipping routes as well as pristine sea areas. As the study subjects, three fish species were chosen for biomarker analyses: eelpout (*Zoarces viviparus*) and flounder (*Platichthys flesus trachurus*) for assessment of the Baltic Sea, and gibel carp (*Carassius auratus gibelio*) as an experimental model species.

The thesis demonstrates alterations at all studied biological levels in fish from the Baltic Sea, representing biomarkers from genes to fish size parameters. In almost every biomarker comparison, Nõva and Sõrve were the sites that seemed most affected by environmental contaminants, whereas Pärnu and Treimani in the Gulf of Riga were the least affected. Since there is no industry or dense population along the Estonian western coast, long-range atmospheric contamination and shipping activity are the most plausible explanations **(II)**.

In our work there was no observable correlation between EROD activity and PAH metabolites **(II).** Types of pollution other than PAHs could have caused the high enzyme activities; high EROD activities could be caused by algal toxins, PCBs or dioxins, or be the result of seasonal enzyme activity fluxes.

Therefore, any association between EROD activity or CYP1A induction and the presence of a particular chemical or group of chemicals (mainly PAHs) must be interpreted with great care. Moreover, no enzyme activity was found in the EROD assays during the aquarium exposure experiment with oil-shale contaminated sediment **(III)**.

In our studies, fish urine was used as a deposit of PAH metabolites and for the first time the FF technique was used for to detect PAH metabolites in urine **(I, II)**. Two metabolic pathways excrete biotransformed PAH compounds: via the liver and bile into faeces, and through the kidney into urine. We found that metabolic reactions in kidneys were notably high, particularly for 4- and 5-ring molecules, causing approximately the same transformation level as in liver. This suggests that kidneys are much more important in PAH metabolism than has hitherto been assumed.

We found the content of final PAH conjugates to affect the colour of Baltic Sea flounder livers: fish with pale-coloured livers (bright orange) contained more PAH metabolites in the bile than those with dark-coloured, brown livers **(IV).** This trend was particularly highlighted in the analyses of 3-, 4- and 5-ring metabolites, representing molecules of polycyclic aromatic hydrocarbons of similar size. We believe that liver colour has potential for use as a toxicological biomarker, suitable for rapid deployment in the field.

The aquatic pollution of the oil-spill area of the Baltic Sea was monitored by measuring the content of selected polycyclic aromatic hydrocarbons (PAHs and PAH metabolites) in flounder **(I)**. The PAH content of livers decreased significantly during this period; there was also a remarkable decrease in PAH metabolite concentrations in bile and urine. The results revealed relatively high PAH concentrations in the livers of fish even five months after the oil spill, indicating that flounder were exposed to highly polluted sediments via re-suspension processes.

In fish, ventilation frequency is increased during hypoxia, and the rate of entry of toxins through gill respiration is therefore affected by the level of dissolved oxygen. Our study demonstrates that the bioavailability of sediment-bound contaminants changes continually and the effects on aquatic organisms depend on physiological state (season, hibernation), aquatic chemistry (dissolved oxygen) and biotic interactions (food availability, biodegradation) **(III)**. The total PAH concentrations at the end of exposure were lower in the sediments from the oxygen-saturated tanks than in those from the hypoxic tanks. Moreover, the fluorescence from all four types of PAH conjugates in fish bile was significantly higher in the tanks with contaminated sediment and hypoxia (in some cases dozens of times higher) than in all other experimental groups.

The knowledge gained in this thesis could be useful from the perspective of biomarker monitoring and environmental health assessment. The eastern Baltic Sea has been poorly studied in the context of contamination biomarkers and many interactions between environmental factors and fish biomarkers are not clearly understood. The coastal sea of Estonia is under continuous threat from major oil-spills and at the same time eutrophication and cyanobacteria blooms are occurring in different Baltic areas every summer. The results of the thesis demonstrate that interactions among environmental factors outweigh individual effects in importance, so some well-known and widely used individual biomarkers (e.g. EROD) are not necessarily good for monitoring. At the same time, the thesis demonstrates that there are still well-studied aspects of fish physiology that are potentially useful as biomarkers. Fish of the Eastern Baltic Sea are among the most important links between the aquatic environment and people living by the sea: a healthy sea environment also means a higher quality of life on shore.

KOKKUVÕTE

Keskkonnas leiduvad toksilised ühendid mõjutavad elusloodust igal selle organiseerituse tasemel (geenidest kooslusteni), samas kui mitmed sellised ained on teadusele seni tundmatud või rasked tuvastada. Kalade puhul toimub toksiliste ainete kehasse sisenemine valdavalt passiivse difusiooni teel: läbi lõpuste, sooltoru ja naha. Enamuse orgaaniliste reostusainete rasvlahustuva iseloomu tõttu toimub nende akumuleerumine rasvarikastes kudedes: maksas, gonaadides, ajus jm. Tänu asjaolule, et paljud toksilised ained (sealhulgas polütsüklilised aromaatsed süsivesinikud ehk PAHid) on olnud loodusliku keskkonna komponentideks juba sadu miljoneid aastaid, on elusorganismidel evolutsiooni käigus tekkinud võime neid aineid kahjutuks muuta ja/või kehast väljutada. Läänemerd peetakse üheks maailma reostunuimaks mereks ning siin elavad kalad on seetõttu pidevas füsioloogilises stressis. Aastatel 2000 kuni 2008 tuvastati kokku 61 laevaõnnetust, millest igaühe tulemusena sattus merre 0,015 m^3 kuni 150 m^3 naftat või kütteõli. Selle info baasil tuleb tõdeda, et merekeskkonna reostatuse ja elustiku käekäigu pidev seire on vajadus, millest ei saa mööda vaadata. Kuna kõigi meres leiduvate reostusainete kontsentratsioone on pea võimatu pidevalt määrata, on soovitatav keskenduda nende ainete mõjude ehk biomarkerite analüüsile. Biomarkerid kirjeldavad elustiku seisundit ning samaaegselt aitavad hinnata antropogeensete ühendite koormust keskkonnale. Samas kaasneb biomarkerite kasutamisega ja tulemuste interpreteerimisega ka mitmeid vastuolulisi aspekte ja küsitavusi.

Minu doktoritöö eesmärk oli hinnata naftareostuse **(I)**, põlevkivitööstuse poolt reostatud setete **(III)** ja Läänemere rannikuvetes leiduva hajureostuse **(II)** mõju kalade biomarkeritele. Lisaks eelnevale pakume me välja ka kaks uut biomarkerit **(I, IV)** ning uurime hapnikupuuduse **(III)** mõju kalade biomarkeritele. Töös on kasutatud kahte metoodilist lähenemist: nii eksperimendi käigus saadud andmeid kui ka välitöödel kogutud materjali. Läänemere rannikuvetest koguti proove kokku kuues piirkonnas ning biomarkereid analüüsiti kolmel kalaliigil: emakalal (*Zoarces viviparus*), lestal (*Platichthys flesus trachurus*) ja hõbekogrel (*Carassius auratus gibelio*).

Biomarkerite analüüside käigus leiti kaladel mitmeid kahjustusi alates geneetilistest kõrvalekalletest kuni muutusteni üldises konditsioonis ja kasvukiiruses. Erinevate Eesti rannikumere proovipunktide võrdluses joonistusid selgelt välja Nõva ja Sõrve uurimisalad, kus elavatel kaladel leiti kõige rohkem keskkonnareostusest tulenevaid kahjustusi. Samal ajal lubavad Pärnu ja Treimanni proovipunktidest saadud andmed arvata, et Riia lahe idaosa keskkond on kaladele soodsam. Võimalike reostuse vektoritena on kõne all toksiliste ainete kaugkanne, sissevool jõgedest, aktiivne laevaliiklus ja õlireostused **(II)**.

Biomarkerite omavaheliste korrelatsioonide analüüsimisel oli olulisemaks tulemuseks korrelatsiooni puudumine EROD aktiivsuse ning PAHide metaboliitide vahel **(II)**. On teada, et lisaks PAHidele võivad ensüümiaktiivsuste tõstjateks olla ka mitmed teised kemikaalid: dioksiinid, sinivetikate toksiinid ja polüklooritud bifenüülid (PCB). Töö tulemustest lähtuvalt soovitamegi vältida ERODi ensüümiaktiivsuste tõusu sidumist konkreetsete kemikaalide grupiga. Seda ka seetõttu, et põlevkivireostuse katses ei toimunud EROD aktiivsuse tõusu üldse **(III)**, mis võis olla tingitud nii hapnikupuudusest kui ka raskmetallide inhibeerivast mõjust.

Lisaks kala sapis leiduvatele PAHide metaboliitide uurimisele õnnestus PAHide ainevahetuse jääke esmakordselt fluorestsentsi abil ka mõõta ka lestade uriinis **(I, II)**. PAHide biotransformatsioon toimub kaladel kahte paralleelset ainevahetuse rada pidi: kas maksa (ainete eritamine sapi kaudu) või alternatiivselt neerude (uriini) kaudu. Meie andmed näitasid neerudes toimuva ainevahetuse oluliselt suuremat osatähtsust, võrreldes varasemates teadustöödes leiduva infoga – ning seda eriti 4- ja 5-tuumsete PAHide puhul.

Töö näitab lesta sapis leiduvate PAHide metaboliitide seost maksa värvusega **(IV)**: täpsemalt seda, et heledama-kahvatuma värvusega kalade maksa leiame sagedamini kaladelt, kes on tugevamalt mõjutatud keskkonnareostuse (PAHide) poolt. Samal ajal pärinevad tumeda pruuni maksaga kalad suurema tõenäosusega puhtast keskkonnast. Seos on eriti tugev 3-, 4-, ja 5-tuumsete PAHide metaboliitide puhul. Me usume, et maksa heledus võiks olla kasutatav reostuse biomarkerina.

2006. aastal toimunud Nõva naftareostuse järelmõjude uuringus analüüsisime PAHide kontsentratsioone ning biomarkereid reostusala lesta kudedes **(I)**. Uurimisperioodi käigus nägime kalades arvestatavat toksiliste ainete kontsentratsiooni langust. Tulemustest lähtub, et isegi 5 kuud pärast naftareostust sisaldavad kalade koed arvestataval määral toksilisi PAHe, kuid juba 9 kuud pärast reostust on toimunud oluline kontsentratsioonide langus. Naftareostus avaldas piirkonna elustikule pikaajalist mõju tänu osaliselt setete alla mattunud õlile, mille resuspenseerumine mõjutas elustikku pikka aega.

Lisaks kontsentratsioonidele sõltub toksilise aine mõju ka füsioloogilistest protsessidest. Nii sõltub vees lahustunud toksiliste ainete kaladesse sisenemine kalade hingamisaktiivsusest: mida rohkem kala "õhku ahmib", seda suurem on lõpuseid läbiva vee hulk ning seda aktiivsem on toksiliste ainete difusioon lõpuse kapillaarides voolavasse verre. Meie laborieksperiment näitab, et ka mitmed keskkonnamõjurid (hapnikusisaldus, toidu kättesaadavus) ning kalade füsioloogiline seisund (sigimisaeg, hiberneerumine) mõjutavad toksiliste ainete kehasse sisenemist ning mõjude avaldumist. PAHide kontsentratsioonide analüüs reostunud settega akvaariumieksperimendis näitas, et võrreldavatest gruppidest olid madalamad kontsentratsioonid nendes, kus keskkond oli hapnikuga küllastunud. Samal ajal toimus hapnikupuuduse olukorras toksiliste ainete bioloogiline lagundamine aeglasemalt.

Käesoleva doktoritöö tulemusel saadud uute teadmiste kasutuspõld on lai, alates üksikute biomarkerite hindamisest seire kontekstis ja lõpetades üleüldise keskkonnaseisundi hindamise strateegiate kujundamisega. Läänemere idaosa on biomarkerite osas halvasti uuritud ning biomarkerid ise sageli raskesti interpreteeritavad. Samal ajal on Läänemerel jätkuvalt naftareostuste toimumise oht ning igasuvised eutrofeerumisega kaasnevad sinivetikate õitsengud ja lokaalsed hapnikupuuduse ilmingud suurendavad ökoloogilist stressi siinsetele elus-organismidele veelgi. Doktoritöö tulemused kinnitavad eri keskkonnafaktorite interaktsioonide ülemuslikkust võrreldes eraldiseisvate mõjudega. Läänemeres elavad kalad on üheks olulisemaks ühenduslüliks merekeskkonna ja inimese vahel – puhas meri tähendab paremat elukvaliteeti ka meile siin mere kallastel.

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I made my first fishing trip at the age of seven, on the shores of the Pärnu River, using an old-fashioned bamboo rod. My first fish was a stickleback. I was deeply disappointed, hearing from my father that this fish was unsuitable for eating and had to be released. However, my second fish was a small perch and so a fisherman was created. Most of the fish that I caught during childhood were eaten, and so are they today – even after discovering so much about environmental toxins and pollution. We are part of the environment, whether we like it or not – and I have still found no argument to persuade me to give up eating fish.

During my first school years it was my mother who sat up long hours with me, looking over my homework, and thereby creating the habit of study. Thank you for this support! Without this habit I wouldn't gone so far. Further thanks to my first class teacher who supported me. I believe that those early years play a significant role in the making of a future scholar.

I thank my father, who had the will to bring home an incredible variety of dead stuffed fish, shark teeth and mysterious sea creatures, while working as a seaman on the Atlantic Ocean. Sea urchins and -stars, fangs of a sea lion, sword of a swordfish were my toys and tools during those years. Together with nature films by National Geographic and Discovery, they creased a deep interest in this underwater world. Even now, 20 years later, I have the shark jaws he brought here by my desk in the University of Tartu.

Years later I ended up in Sütevaka Private High School of Humanities, and thanks to its credo – freedom, creativity, and responsibility – I learned how to work and think on my own. Some of my best friends come from those years, and we still meet, do sports and go hiking together. This has a truly remarkable effect of my sanity! To wake up in the morning in a tent in a forest, make pancakes over the fire, sing songs and walk for hours (while seeing really nothing), makes all the small things count in life.

I had the great luck to meet Arvo Tuvikene at the University of Tartu (although he mainly works in Estonian University of Life Sciences). I had the privilege of enjoying his broad knowledge, fatherly advice, selfless help – and sometimes in the middle of a cold winter night even a taxi service – for all of those years. Doing science together with Arvo, whether it is a monitoring fishing in Lake Võrtsjärv in December, or long hours of laboratory work, has been more of a hobby than work for me. Thank you for that! Many thanks to his wife Lea – all your hospitality and lunches helped me to survive during my first student years. The Centre for Limnology of Estonian University of Life Sciences has been a most lovely place with kind and welcoming people.

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Thank you!

TÄNUAVALDUSED

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Osalemine naftareostuse tõrje vabatahtlike koolitusel, Eestimaa Looduse Fond, Tartu (2007)

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