From Proteomic Analysis to Biomarker Application
-Studies of Carbonyl Reductase in Fish

Akademisk Avhandling

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Abstract

Many anthropogenic substances are present in the aquatic environment, but there is limited information on how this combination of chemicals affects exposed wildlife. To assess the impact of chemicals, a biomarker approach is frequently applied in environmental biomonitoring programmes.

In this thesis, proteomic analysis was used as a hypothesis generator for biomarker development in fish. Two-dimensional gel electrophoresis was performed on a liver fraction from rainbow trout *(Oncorhynchus mykiss)* that were caged downstream of a sewage treatment plant, and it was found that a protein identified as carbonyl reductase was induced. This was confirmed with qPCR, indicating that carbonyl reductase mRNA expression levels were markedly induced by the same effluent and also by a second investigated sewage treatment plant effluent of a similar character. More advanced sewage treatment technologies reduced carbonyl reductase mRNA expression, indicating that fish that were exposed to effluents of conventionally treated sewage have induced hepatic carbonyl reductase expression.

To obtain more information regarding the possible inducers of carbonyl reductase, several different single-substance exposure studies were performed with rainbow trout in controlled aquaria systems. The results demonstrated that hepatic carbonyl reductase expression was not induced by the steroids that were tested but by an aryl hydrocarbon agonist and a pro-oxidant. Others have observed substances with the same mode of action in the effluents of Swedish sewage treatment plants.

To promote the use of carbonyl reductase as a biomarker, a method for measuring enzymatic activity was developed in the sentinel coastal fish species eelpout *(Zoarces viviparus)*, which is included in the Swedish national integrated fish-monitoring program. The protocol for carbonyl reductase enzymatic activity combined with qPCR analyses in eelpout were applied on a comprehensive field material, including sites with different grades of pollution among the Swedish, Danish and German coasts. Carbonyl reductase activity and mRNA did not show any site-specific differences between the polluted and reference sites. Therefore, it can be concluded that the induction of carbonyl reductase appears to be a promising biomarker for

assessing exposure and the possible impact of sewage treatment plant effluents in caged fish. However, more research is required before and if carbonyl reductase can be used in biomonitoring programmes as a biomarker to assess the impact of toxic chemicals in wild fish populations.

*Keywords: Fish, proteomics, biomarker, carbonyl reductase, Ah*R *agonists, oxidative stress, biomonitoring*

Populärvetenskaplig sammanfattning

Det här doktorandprojektets mål har varit att utveckla en ny biomarkör av tidig varningssignalskaraktär. Känns det för svårt? Vi tar det från början.

Människans framfart det senaste århundradet har orsakat många förbättringar för människan i kombination med många försämringar för vår omgivande miljö. I flertalet länder har man valt att övervaka dessa förändringar i olika miljöövervakningsprogram. När det kommer till att övervaka miljögifter, så kan man göra det på olika sätt: kemiskt eller biologiskt. Medan kemisterna mäter *halterna* av miljögifter övervakar vi biologer *effekterna* av dessa.

För att undersöka giftigheten av ett ämne kan man t.ex. undersöka vilken mängd av ämnet som krävs för att leda till att en organism dör. Denna information har dock begränsad relevans, då en organism sällan utsätts för endast en kemikalie i hög dos under kort tid i sin naturliga miljö. Den vanligaste exponeringsformen är istället mängder av ämnen i låga doser under en hel livstid. Dessutom önskar vi upptäcka effekter av gifter innan de har uppnått så höga koncentrationer att individer dör. Således finns det ett behov att kunna mäta effekter av låga koncentrationer av gifter, innan de hunnit orsaka allvarlig skada på individ eller population.

Av den anledningen arbetar många ekotoxikologer med att mäta biologiska markörer (biomarkörer) i individer exponerade för gifter. En biomarkör är en mätbar förändring hos den utsatta organismen, t.ex. en förändrad halt av ett protein i blodet. En klassisk biomarkör är proteinet vitellogenin i hanfisk. Vitellogenin finns normalt i höga halter hos könsmogna honfiskar, men kan öka kraftigt hos hanfiskar som utsätts för ämnen som liknar kvinnliga könshormoner. Detta kan ske nedströms våra avloppsreningsverk, där ämnen från p-pilleranvändning kommer ut i naturen. Höga halter av dessa ämnen kan leda till att hanfiskar blir tvåkönade och får en minskad möjlighet att fortplanta sig. Innan detta har skett ökar dock halten av vitellogenin, vilket alltså kan användas som en tidig varningssignal som uppträder innan fertiliteten blivit nedsatt.

Våra reningsverk är gjorda för att ta bort näringsämnen från vårt avloppsvatten, men de är dåliga på att rena det från många andra ämnen.

Fisk nedströms reningsverken lever därför i en miljö fylld av giftiga och icke-giftiga substanser. Avhandlingen utgår från studier vid Gråbo avloppsreningsverk. Först användes en storskalig molekylärbiologisk metod där flera hundra olika proteiner analyserades i levern hos fiskarten regnbågslax. Det visade sig att fisk nedströms reningsverket hade förhöjda halter av enzymet carbonyl reductase (CBR) i levern, jämfört med fiskar som var uppströms reningsverket. Liknande effekter sågs även vid Borås reningsverk. Sammantaget visade dessa studier att fiskar som exponerats för utgående renat avloppsvatten får en ökad nivå av CBR, troligtvis orsakat av kemikalier i vattnet.

I människa har man visat att CBR kan fungera som ett skydd mot oxidativ stress. Oxidativ stress uppstår av att reaktiva föreningar påverkar kroppen så att celler och organ skadas vilket kan leda till försämrad hälsa. De förhöjda CBR nivåer som vi sett i fisk är alltså inte skadliga, utan verkar som ett skydd. Men den förhöjda halten av CBR kan vara en indikation på att det finns ämnen i det renade avloppsvattnet orsakar oxidativ stress. Därför skulle CBR kunna fungera som en biomarkör och tidig varningssignal för en exponering av sådana typer av ämnen. Dessa oxidativa "stressare", vet vi finns i olika halter i en del akvatiska miljöer t.ex. nedströms avloppsreningsverk. Dom kommer från bland annat bekämpningsmedel och bildas även vid ofullständing förbränning. Sammantaget finns det många olika typer av ämnen som kan orsaka oxidativ stress.

För att ta reda på om oxidativa "stressare" kan inducera CBR i fisk utfördes ett antal kontrollerade försök i akvariesystem där fiskarterna regnbågslax och tånglake exponerades för olika typer av substanser. Det visade sig att fiskar som utsatts för ämnen med känd förmåga att orsaka oxidativ stress hade högre halter av CBR.

I den sista studien av det här doktorandprojektet mättes CBR i tånglakar som ingår i ett forskningsprogram som syftar till att förbättra miljöövervakning av fisk i kustmiljöer. CBR i fiskar från 16 olika lokaler med varierad föroreningsgrad i Sverige, Danmark och Tyskland analyserades. Studien visare att CBR halterna inte var högre i fisk som levt i förorenade områden jämfört med de som levt i områden med mindre industriell och mänsklig aktivitet. Detta skulle då kunna bero på att de undersökta stationerna inte hade olika halter av oxidativa "stressare". Men frånvaron av den förväntade ökningen av CBR i de mer förorenade områdena skulle även

kunna förklaras av att fisk som levt under konstant förorening inte får en kronisk höjning av CBR. Den ökning av CBR som vi sett i våra tidigare studier kan ha varit mer en akut effekt. Detta är dock inte fastställt.

Sammanfattningsvis har CBR egenskaper som gör den till en möjlig biomarkör för ämnen som kan orsaka oxidativ stress i vattenmiljö. Men vägen till att bli en etablerad biomarkör i miljöövervakningsprogram av vilda fiskpopulationer är fortfarande lång, och trots att jag spenderat halva delen av mitt vuxna liv med detta enzym så finns det fortfarande många frågor kvar att besvara.

Abbreviations

20β-HSD	20β-hydroxysteroid dehydrogenase
2DGE	Two-dimensional gel electrophoresis
AhR	Aryl hydrocarbon receptor
ARE	Antioxidant response element
ATSDR	Agency for toxic substances and disease registry
Balcofish	Integration of pollutant gene responses and fish ecology in Baltic coastal fisheries and management
BLAST	Basic logical alignment search tool
CBR	Carbonyl reductase
CBR l	Carbonyl reductase like
cDNA	Complementary deoxyribonucleic acid
cICAT	Cleavable isotope-coded affinity tags
Ct	Cycle threshold
CYP	Cytochrome p450
DDT	Dichlorodiphenyltrichloroethane
EROD	Ethoxyresorufin-o-deethylase
EST	Expressed sequence tag
FT-ICR	Fourier transform ion cyclotron resonance
GR	Glutathione reductase
GST	Glutathione *S*-transferase
GPX	Glutathione peroxidase
HNE	Hydroxynon-2-enal
i.p.	Intraperitoneal
iTRAQ	Isobaric tags for relative and absolute quantification
MIH	Maturing inducing hormone
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NADPH	Reduced β-nicotinamide adenine dinucleotide 2-phosphate
NQO1	NAD(P)H: quinone oxidoreductase
Nrf2	NF-E2 p45-related Factor
ONE	Oxonon-2-enal
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyls
PFAS	Perfluoroalkyl sulfonate
qPCR	quantitative polymerase chain reaction
REACH	Registration, evaluation, authorisation and restriction of chemicals
ROS	Reactive oxygen species
SOD	Superoxide dismutases
STP	Sewage treatment plant
TBARS	Thiobarbituric acid reactive substances
VTG	Vitellogenin
XRE	Xenobiotic response element

List of publications

This thesis is based on the following articles and manuscripts:

I. Proteomic analyses indicate induction of hepatic carbonyl reductase/20β-hydroxysteroid dehydrogenase B in rainbow trout exposed to sewage effluent. Albertsson, E., Kling, P., Gunnarsson, L., Larsson, D.G.J., Förlin, L., 2007. Ecotoxicology and Environmental safety 68, 33-39

II. Induction of hepatic carbonyl reductase/20β-hydroxysteroid dehydrogenase mRNA in rainbow trout downstream from sewage treatment works—Possible roles of aryl hydrocarbon receptor agonists and oxidative stress. Albertsson, E., Larsson, D.G.J., Förlin, L. Aquatic Toxicology 97, 243-249

III. Carbonyl reductase mRNA expression and activities in eelpout and rainbow trout treated with paraquat and β-naphthoflavone. Albertsson, E., Rad, A., Sturve, J., Larsson, D.G.J., Förlin, L. *Manuscript*

IV. Biomarker responses in eelpout from different coastal sites in Sweden, Denmark and Germany. Albertsson, E., Gercken, J., Strand, J., Asker, N., Bergek, S., Holmqvist, I., Kammann, U., Parkkonen, J., Förlin, L. *Manuscript*

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1. Introduction

1.1 Complex mixtures

Life today is hard to imagine without industrial man-made chemicals. Undoubtedly, many substances have increased the quality of human life in several ways. However, the increased global use of chemicals that has been seen in recent decades is also an increasing health concern for many organisms.

When contaminants are publicly discussed, the primary concern is the possible adverse effects on the human population. Baby bottles are one example that has been debated because they have until recently contained the hormone mimic compound bisphenol A. Another example is the release of contaminants from our clothes that sometimes have been treated with allergenic anti-mould-agents, antibacterial substances, toxic, brominated flame-retardants and carcinogenic formaldehydes. Although the amount of chemicals used is not always directly connected to the toxicity, it is quite astonishing that 3 kg of chemicals are required to make one t-shirt (KEMI, 2009). Then, of course, there are plenty of other sources of contaminants in our world, industry, traffic and agriculture to mention a few. In total, this has revealed the fact that the human body can contain a wide range of man-made substances even though living a life without big influences of chemical exposure (Houlihan et al., 2003). Unfortunately, data regarding toxicity and release are often limited or unknown for many chemicals that are dispersed into the environment. In some cases, substances known to be toxic are still used due to a lag in legislation, incomplete information and/or their illegal use.

Many substances will eventually end up in the aquatic environment where the exposed biota could suffer. While some aquatic environmental issues, such as acidification, eutrophication, ocean warming and overfishing, have been heavily debated, the widespread use and increase in man-made chemicals resulting in contaminated waters has been more overlooked during the last years. In this thesis, fish exposed to chemicals is the main focus. As fish both breath and eat in the same environment, they are likely to suffer from pollutants that are present in their habitat.

The sources of chemicals that are found in aquatic systems are many. Point sources from industries can influence the local area. Run off from land sources, atmospheric deposition, sewage treatment plants (STPs) and boat traffic are other examples of pollutant sources that influence the load of anthropogenic substances in the aquatic environment. The list of pollutants observed in Swedish water systems is long and includes household products (e.g., flame retardants, certain pharmaceuticals, phthalates, musk compounds sucralose, caffeine), industrial products (e.g., metals, phenols, dioxins, polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT), perfluoroalkyl sulfonate (PFAS), agricultural products (pesticides) and others (e.g., antifouling agents) (Adielsson et al., 2006; IVL, 2010a, b; SEPA, 2009). Polluted sites are often located in urban areas where there is not only a dense human population but also industries and harbours. Especially polluted areas are those with multiple pollution sources in combination with limited water exchange (e.g. the Baltic Proper). An organism living in a polluted site is likely to be exposed to a complex mixture of chemicals, which is defined as "A combination of so many chemicals that the composition of the mixture is not fully characterized, either qualitatively or quantitatively, and may be variable…." (ATSDR, 2004). There are concerns and problems with complex mixtures. First, mixtures can increase toxicity due to the effects of chemical interactions, which means that the toxicity of the mixture can be higher (or lower) than the estimated sum of the individual chemical components. Second, every polluted site consists of different components and changes constantly over time. Third, toxicology data are, in most cases, obtained from experiments on a single chemical, which results in limited information regarding the interaction of the chemical. Together, these obstacles make the complex mixtures very hard to predict, monitor and legislate.

1.1.2 Sewage treatment plant effluent

Releases from diffuse sources such as traffic, construction material and household products makes the chemical pollution high in urban areas (SEPA, 2009, 2010). Many of the releases reach the STPs where they sometimes mix with effluent water from local industries and storm water. Although the chemical load into many Swedish STPs is of a significant toxic level, STPs are not constructed for the degradation of all chemicals. Instead, the primary focus is to decrease the eutrophication load in the receiving water system (SEPA, 2008). There is plenty of evidence that STP effluents contain hundreds of chemicals (including pharmaceuticals)(IVL, 2010a, b;

Paxéus, 1996; Paxéus and Schroder, 1996; SEPA, 2008). STPs could be considered a point source of diffuse releases and contribute to the complex mixture of anthropogenic substances that are released to downstream living biota. The consequences for exposed populations can be significant. In fish, adverse health effects, such as oxidative stress and damage, an increased hepatosomatic index, decreased gonadosomatic index (GSI), alteration in immune system, genotoxic damage, endocrine disruption, intersex, reduced reproduction sucess, altered reproductive behaviour, reduced egg production and changes in the fish community have been observed due to STP effluent exposure (Almroth et al., 2008; Folmar et al., 2001; Hoeger et al., 2004a; Jobling et al., 1998; Lange et al., 2011; Larsson et al., 1999; Liney et al., 2006; Ma et al., 2005; Porter and Janz, 2003; Sebire et al., 2011; Sturve et al., 2008; Thorpe et al., 2009). Some of the measured effects are of such concern that new treatment technologies have been developed and evaluated. This includes ozone, activated carbon, TiO_2, ultra violet light, membrane bioreactors and moving bed biofilm reactors (Bundschuh et al., 2011; Chong et al., 2010; Gunnarsson et al., 2009; Lundström et al., 2010a). Although it has been observed that ozonation can create products that are more toxic than the parent compound (Gagné et al., 2007; Stalter et al., 2010), a meta-analysis that was performed by Bundschuh et al. states that there is an overall reduction in the ecotoxicity of ozone and activated carbon (2011). Estimations of the cost of these new technologies have been calculated for Sweden. As this is very hard to approximate, the price increase varies between 10% and 100% more than the current price (SEPA, 2008). Together, it seems likely that the treatment processes, in some cases, will be better in the future; however, there are still ecological and economical aspects that slows the decision progress.

1.2 Biomonitoring and biomarkers

As stated above, the water ecosystems are polluted in many regions. To assess and take action regarding environmental problems that are caused by pollutants, the issues of concern first need to be determined, preferably at an early stage before too much damage has occurred. This is a strong motivator for regular and comprehensive monitoring of the environment. Indeed, many countries run monitoring programmes where the environment is studied in different aspects. To monitor pollutants and their effects, chemical monitoring is often combined with biological monitoring (figure 1).

Chemical monitoring provides information about chemicals that are present in the investigated material (commonly water, sediment and biota). The presence of substances in water fluctuates in response to releases, precipitation and water movement, whereas the levels in sediment and biota can be more stable. Chemical analyses alone do not provide sufficient information about the health status of the exposed biota and the possible chemical interactions. Biomonitoring programmes in which the health status of different species in a certain geographical area is studied can aid in the assessment of pollutants and their effects. Commonly, data are collected yearly, which provide information for long-term trend analyses.

Because reduced health can be caused by many factors other than chemical pollution, specific markers are often used that provide information about the toxic compounds that are present. These are called biomarkers and are defined as "a biological response to a chemical or chemicals which gives measure of exposure and sometimes, also, of toxic effect"(Peakall and Walker, 1994). Van der Oost summarised the three classes of biomarkers that have been suggested by the National Research Council and the World Health Organisation (NRC, 1987; van der Oost et al., 2003; WHO, 1993) as follows:

- "biomarkers of **exposure**: covering the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism;

- biomarkers of **effect**: including measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease;

- biomarkers of **susceptibility**: indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, including genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure"

Even though these definitions exist in the literature, categorizations of the biomarkers that are in use are rather diffuse. Theoretically, many are

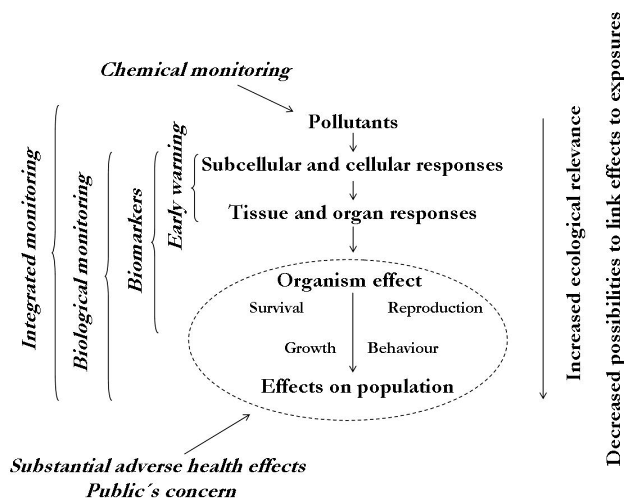

Figure 1. Chemical monitoring measures the concentration of pollutants while biological monitoring attempts to measure their effects. Biological monitoring can be operated at different levels of organisation. Generally, the ecological relevance increases with organisation level, whereas the ability to link the effects to exposure decreases. Often, the public's concern is not roused until the effects have been observed at higher levels. In this thesis, the aim was to develop an early warning signal to use as a biomarker in environmental biological monitoring.

useful as indicators of both exposure and effects depending on how the study is designed (Nikinmaa and Rytkönen, in press; van der Oost et al., 2003). But a good biomarker can predict exposure and/or adverse effects (or susceptibility) in a defined situation.

Because the effects at higher levels are always the result of changes in earlier biological processes, biomarkers at lower ecological levels can serve as an early warning system (figure 1) (van der Oost et al., 2003). Early warning biomarkers have been used since 1988 in the Swedish national integrated fish-monitoring program (Bignert et al., 2011; Hansson et al., 2006; Ronisz et al., 2005).

Biomarkers play an important role when it comes to monitoring waters with multiple chemical substances. This is commonly the situation for all aquatic environments that are located close to human populations. The flow from households and industries to the receiving effluents varies throughout

the day, and chemical analysis of the outgoing water does not accurately reflect the possible adverse effects on the biota of such a varied composition of chemicals. For example, biomarkers that have been used when studying the effects of effluent water from STPs have provided information about the estrogenic compounds (vitellogenin (VTG) induction), aryl hydrocarbon (AhR) agonists (Ethoxyresorufin-o-deethylase (EROD) activity), metals (metallothionein) and pro-oxidants (oxidative damage and antioxidants) that are present in outgoing STP effluents (Almroth et al., 2008; Burkhardt-Holm et al., 1999; Hoeger et al., 2004b; Larsson et al., 1999; Ma et al., 2005; Purdom et al., 1994; Sturve et al., 2008). In the Baltic proper, a yearly monitoring program has collected evidence that indicates a long-term trend of increasing EROD activity and a decreasing gonad somatic index in Perch *(Perca fluviatilis)* (Hanson et al., 2009a; Hansson et al., 2006).

Biomarker alterations in exposed populations might be as transient as the chemical analyses (Forbes et al., 2006). It is a difficult task to fully monitor and assess the contaminants and their possible effects on the aquatic environment. Because there are no universal tools, a monitoring programme should include a battery of chemical and biological analyses (integrated monitoring). The biomarkers used should ideally tell something about the exposure situation, however, a biomarker is not preferably a response or marker to one substance, instead, it could be a response to many substances giving a broader approach than chemical analyses.

1. 3 Proteomics

The advancement in macromolecule sequencing that began in the early nineties led to the development of broad-spectrum screening methods that contain the newly coined suffix "omics". Genomics, which can be described as large scale methods that are used to study the genome, were soon followed by proteomics, which is defined as "the large-scale characterization of the entire protein complement of a cell line, tissue or organism" (Graves and Haystead, 2002). In the beginning, the research was restricted to selected mammals. However, in the last 10 years, the effort to sequence environmentally relevant species has increased, which enabled "omics" studies for disciplines other than medicine.

To study the proteome or major parts thereof, proteins are extracted, which is followed by separation, quantification, identification and

characterisation. The most commonly used method for separation is two-dimensional-gel electrophoresis (2DGE) in which proteins are separated in two dimensions: by isoelectric point and size. Approximately 2 000 proteins can be monitored simultaneously depending on the gel size and the pH range (Görg et al., 2004). The relatively poor reproducibility between the technical replicates and the loss of quantification of low abundant proteins has driven the development of new methods, such as cleavable isotope-coded affinity (cICAT) and isobaric tags for relative and absolute quantification (iTRAQ) (Görg et al., 2004; Sanchez et al., 2011).

To monitor the effect of chemicals in the environment, "omics" methods can be used as a tool for the development of new biomarkers (Benninghoff, 2007; Fent and Sumpter, in press; Snape et al., 2004). While traditional biomarker research has been hypothesis driven, "omics" research can be a tool for generating hypotheses. This is of particular importance when it comes to ecotoxicology and complex mixtures in which hypotheses regarding the mode of action of multiple chemicals in exposed species is hard to predict. "Omics" could decrease the risk of unforeseen responses to chemicals.

The first articles that were published in the field that combined proteomics and ecotoxicology were not able to identify the regulated proteins because the sequence databases were too small (Shepard and Bradley, 2000; Shepard et al., 2000). The presentation of protein patterns alone as result from chemical exposure will not increase our understanding of the response to pollutants, which is one of the purposes of applying "omics" methods. Today, there are many studies that have identified some of the proteins that are regulated by chemical exposure (Apraiz et al., 2006; De Wit et al., 2008; Kling and Förlin, 2009; Kling et al., 2008; Lee et al., 2006; Ling et al., 2009; Lu et al., 2009; Malécot et al., 2009; Manduzio et al., 2005; Mezhoud et al., 2008; Rodríguez-Ortega et al., 2003; Sanchez et al., 2009; Shi et al., 2009; Wei et al., 2008; Zhu et al., 2006).

Some have applied ecotoxicoproteomics in field experiments (Amelina et al., 2007; Apraiz et al., 2009; McDonagh et al., 2005; Ripley et al., 2008; Wang et al., 2008). According to a recently published review article on proteomic approaches in aquatic toxicology by Sanchez et al., very few groups have used proteomics in fish field experiments (2011). Ripley and co-workers used proteomics to explain the extensive fish kill observed in the

Shenandoah River (VA, USA). The results compared the tissue of the anterior kidney collected from smallmouth bass (*Micropterus dolomieu*) captured at different sites along the river and suggested a site-associated change in the immune system. Wang investigated goldfish (*Carassius auratus*) in Gaobeidian Lake in China. This lake receives water from an STP and serves as a coolant for a power plant, which provides multiple stressors for the inhabitants. The results indicated that there was an alteration in lipid metabolism and an activation of the anti-oxidant response. In paper I of this thesis, the hepatic responses of rainbow trout that were exposed to STP effluents were investigated (2007). The most relevant outcome was the induction of carbonyl reductase 1 (CBR1).

1.4 Carbonyl reductase

The carbonyl group shown in figure 2 is a common group in many biomolecules; for example, it is found in many endogenous compounds, such as steroids, mediators (e.g., eicosanoids), cofactors and neurotransmitter precursors. Carbonyls are also found in food, pharmaceuticals and xenobiotics. They are parts of lipid aldehydes, which can be derived from oxidative stress. Some of the carbonyl containing compounds are reactive molecules that can be harmful to many cellular components and processes. Carbonyl reduction is a typical phase 1 reaction, which produces a less reactive alcohol which can be conjugated and excreted (Oppermann, 2007).

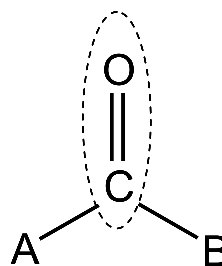

Figure 2. The carbonyl group

Because many of the carbonyls are highly reactive, there are plenty of carbonyl reducing enzymes in organisms. These enzymes are by far the most studied in humans in which at least 12 carbonyl reducing enzymes exist (Matsunaga et al., 2006). The promiscuous substrate capacity of carbonyl reducing enzymes has complicated the nomenclature of the enzymes, which is rather complex. For example, in rainbow trout *Oncorhynchus mykiss)* carbonyl reductase/20β-hydroxisteroid dehydrogenase (CR/20β-HSD) was

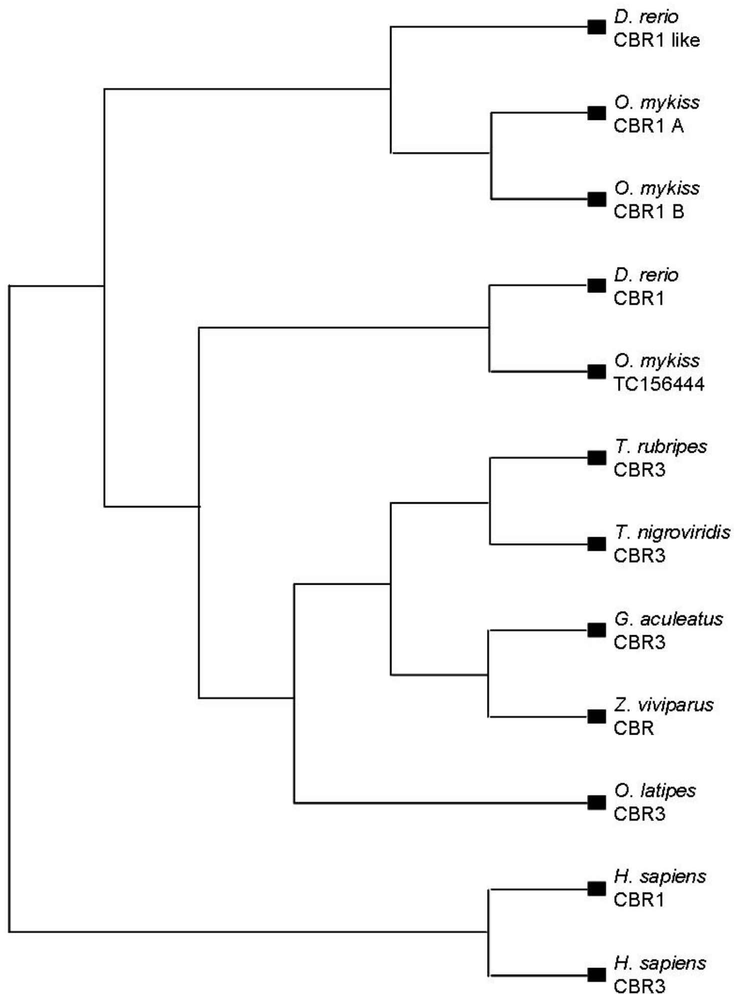

Figure 3. Six CBR proteins in five fully sequenced fish species *(Danio Rerio, Takifugu rubripes, Tetraodon nigriviridis, Gasterosteus aculeatus, Oryzias latipes)* are homologous to the human CBR1 and CBR3 proteins (http://www.ensembl.org). These eight CBR sequences were aligned with the three sequences from *Oncorhynchus mykiss* (CBR1 A, CBR1 B and a translated sequence of TC156444) and the *Zoarces viviparus* sequence (CBR) using the clustalw2 option in SeaVeiw (Gouy et al., 2010). The phylogenetic tree, rooted to the human CBR proteins was then created using the parsimony option with standard settings.

named after its two known enzymatic activities (Guan et al., 1999). The protein exists in two variants, A and B, and the B variant lacks enzymatic activity, even though it only differs from variant A by three amino acids (Guan et al., 1999; Guan et al., 2000). Today, the updated sequence of the rainbow trout CR/20β-HSD A is named carbonyl reductase 1 (CBR1). The name of the B variant has not been changed. However, within this thesis, the enzymes will be referred to as CBR1 A and CBR1 B. In paper I and II, the former names, CR/20β-HSD A and B, were used. The EST database indicates that there may be a third rainbow trout CBR, named TC156444. In eelpout *(Zoarces viviparous)*, which is also included in this thesis, there is currently only one carbonyl reductase sequence annotated, which will be referred to as CBR (Kristiansson et al., 2009).

When the sequences of rainbow trout CBR1 A and B (former CR/20β-HSD), rainbow trout TC156444 and eelpout CBR were compared to the fully sequenced genome of *Danio rerio*, two proteins that are annotated as carbonyl reductase 1 or carbonyl reductase 1-like (CBR1l) were most similar. According to homology predictions (ensembl.org/index.html) (ncbi.nlm.nih.gov/homologene), zebrafish CBR1 and CBR1l are paralogous, and both proteins are homologous to human carbonyl reductase 1 (CBR1) and carbonyl reductase 3 (figure 3) (Gouy et al., 2010). Because the literature regarding CBRs in fish is not comprehensive, part of the following information and discussion regarding regulation of fish CBR is based on comparisons with the mammalian homologues CBR1 or CBR3.

All the different names are used in fish literature, including carbonyl reductase, carbonyl reductase 1, carbonyl reductase 1-like, carbonyl reductase 3, 20β-HSD and combination of these names also exists. In most cases, these are likely to referring to homologs of human CBR1 and/or CBR3, but the exact evolutionary relationship is difficult to elucidate when information or links to specific sequences are not included in the papers.

1.4.1 Carbonyl reductase and oxidative stress

Oxidative stress is defined as "a serious imbalance between production of ROS/RNS and the antioxidant defence" (Halliwell and Guttridge, 1999). ROS (reactive oxygen species) are not only products of normal cell respiration but are also formed by several chemical substances, such as redox cycling compounds, PAHs, quinones, halogenated hydrocarbons and

metals (Livingstone, 2001). The generation of ROS can result in DNA lesions and mutations; protein modifications, such as protein degradation, misfolding and deposition; altered cytoskeleton and changes in signalling. The inactivation of ROS by antioxidants, such as superoxide dismutase (SOD), NAD(P)H: quinone oxidoreductase (NQO1), catalase and glutathione peroxidise (GPX), is a common defence strategy in cells (Oppermann, 2007; Sturve, 2005).

Quinones derived from aromatic hydrocarbons (e.g. PAHs) are carbonyl-containing groups found in both endogenous compounds and xenobiotics. Both the quinones and the parent aromatic compounds occur in STP effluent water (Paxéus, 1996). Quinones are toxic as they can induce DNA strand breaks, interfere with mitochondrial respiration and generate free radicals (Hoffmann and Maser, 2007). Quinones can undergo one or two step electron reductions. One step electron reduction by cytochrome P-450 reductase (CYP) results in a semi-quinone radical that, in turn, will oxidise the parent compound, a process that creates a superoxide radical, which is a precursor of reactive oxygen species (ROS). A two step reduction, however, is generally considered a detoxification step because the product hydroquinone can be conjugated followed by excretion (figure 4)(Hoffmann and Maser, 2007). CBR1 is considered the major two step reduction enzyme of the human liver, which is in contrast to rats in which NQO1 (DT-diaphorase) is the most influential enzyme in the reaction (Wermuth et al., 1986). The relationship between rainbow trout CBR and NQO1 has not been well investigated, but there is evidence that both are induced by PAH compounds (Paper II, Sturve et al., 2005b).

Lipid peroxidation is initiated by reactive oxygen species (ROS). ROS are capable of altering the lipid structure in membranes by extracting hydrogen atoms from the unsaturated bonds of the fatty acid chains. The damaged membranes then contain reactive aldehydes, such as 4-oxonon-2-enal (ONE), 4-hydroxynon-2-enal (HNE) and acrolein (Hoffmann and Maser, 2007). Reactive aldehydes can cause significant damage to macromolecules in the cell, and this damage can lead to cell death (Ellis, 2007). ONE is a potent genotoxin and is highly reactive towards proteins. HNE acts in a similar manner but not with the same potency (Lin et al., 2005). In 2004, Doorn et al. showed that human hepatic CBR can catalyse the reduction of ONE, which results in less toxic products (see figure 5).

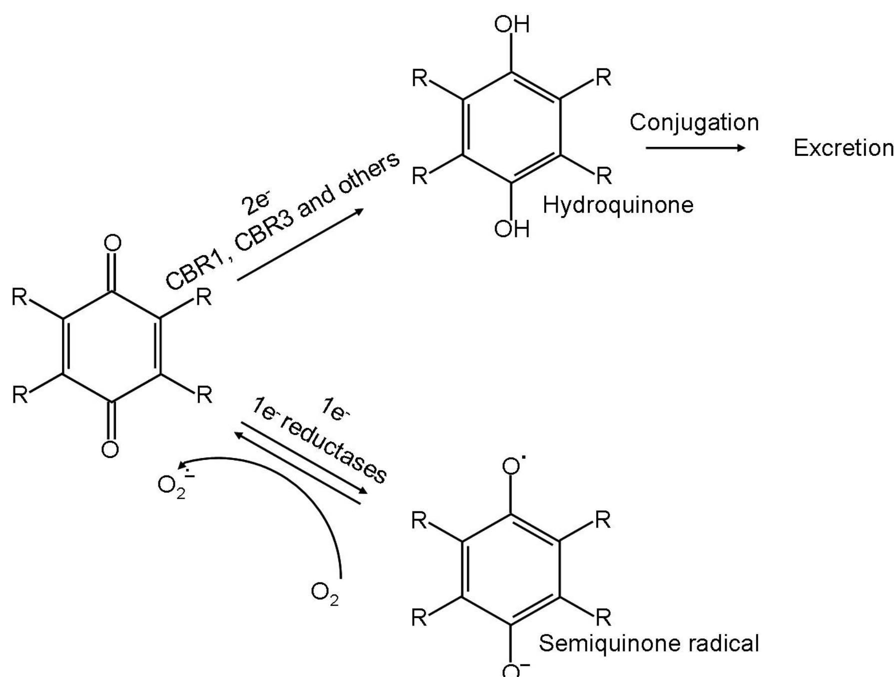

Figure 4. Quinone reduction by one-electron transfer results in a semiquinone radical that can be oxidised in the presence of molecular oxygen. This results in a superoxide anion (O_2^-), a reactive oxygen species that is highly reactive and can cause substantial damage to the cell. The two-electron reduction of quinones by CBR1 is considered a detoxification pathway and results in a hydroquinone that can easily be conjugated and excreted. Adapted from Opperman, 2007.

As stated in the above sections, CBR1 protects against oxidative stress and damage in different ways: first, via the reduction of the amount of ROS that are generated by quinone reduction and second, by reducing reactive toxic aldehyde derivatives that are derived from lipid peroxidation. To cope with oxidative stress, it is widely spread in the animal kingdom to increase oxidative protectors like certain enzymes and molecules. The relationship between oxidative stressors and increased CBR1 was observed by us in paper II and III and by others (Kelner et al., 1997; Lakhman et al., 2007). Over-expression of CBR1 is considered protective against oxidative stress in mammals (Rashid et al., 2010; Tak et al., 2011).

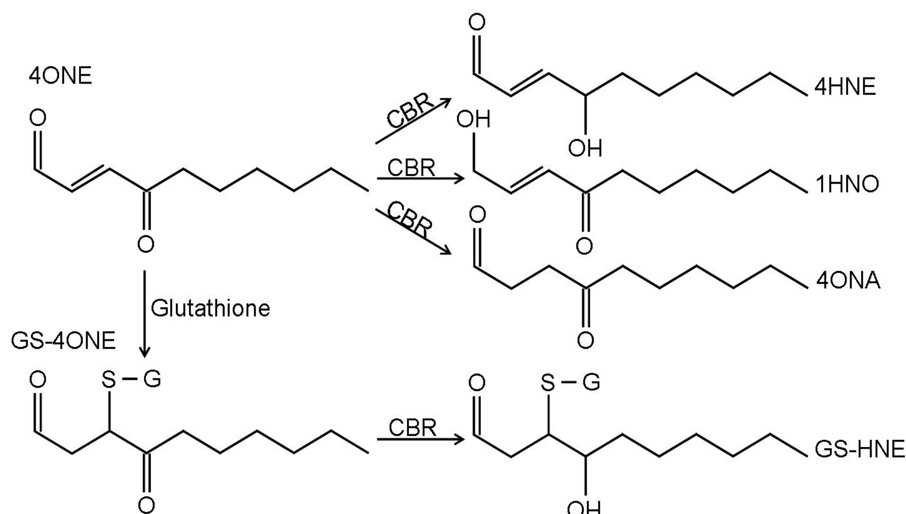

Figure 5. Reactive oxygen species can initiate membrane lipid peroxidation. This can result in highly reactive aldehydes, such as 4-Oxonon-2-enal (4ONE), which can modify DNA and proteins. Both 4ONE and its glutathione conjugate are substrates for CBR. Because the pathway leads to less reactive products, CBR is considered a protector against oxidative stress. Adapted from Doorn et al., 2004.

1.4.2 Carbonyl reductase as 20β-hydroxysteroid dehydrogenase

Many of the carbonyl reducing enzymes also play a role in the metabolism of steroids, which often contain a carbonyl group. Carbonyl reductase (CBR1 or CBR1-like) in the gonads of rats, pigs, rainbow trout and zebrafish have been shown to exhibit 20β-HSD activity (Guan et al., 1999; Takada et al., 2000; Tanaka et al., 1992). This is of particular interest in teleosts where the steroid catalysed by the enzyme converts into 17-alpha, 20-beta-dihydroxy-4-pregnen-3-one, which is considered as the the maturing inducing hormone (MIH) (figure 6) (Nagahama, 1997; Nagahama and Adachi, 1985; Nagahama and Yamashita, 2008). MIH is important for the maturation of oocytes and spermatozoa, and the increase in synthesised MIH is possible because of the gonadal increase in 20β-HSD during the late part of the vitellogenesis (Nakamura et al., 2005; Senthilkumaran et al., 2002; Senthilkumaran et al., 2004; Tanaka et al., 2002).

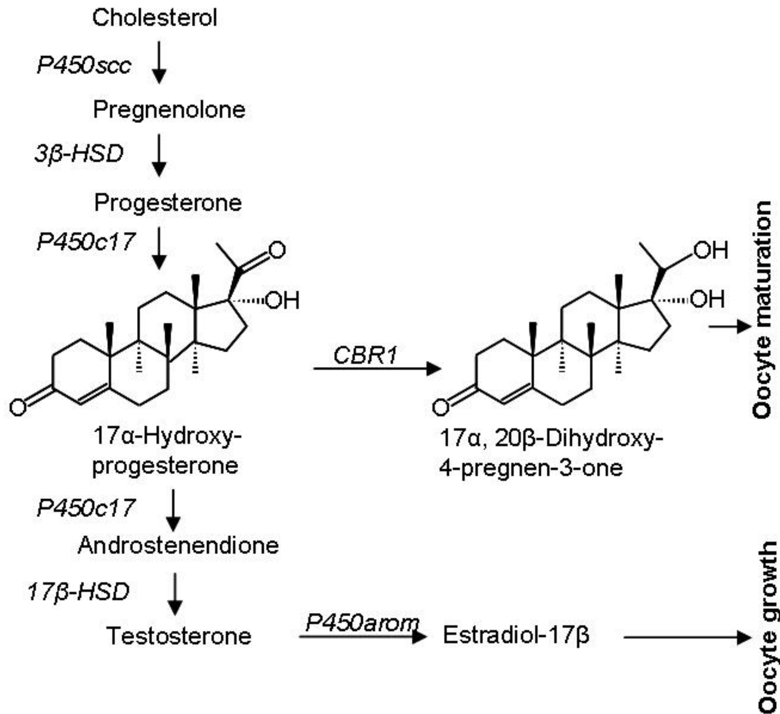

Figure 6. The role of CBR1 in the gonadal steroid synthesis. CBR1 converts 17 α-Hydroxysteroidprogesterone to 17α, 20β-Dihydroxy-4-pregnen-3-one which is considered as the maturing inducing hormone in salmonids. Adapted from Nagahama et al., 1997.

1.4.3 Regulation of CBR1 and 20β-HSD

Based on current knowledge of the enzymatic properties of carbonyl reductase, it seems likely that man-made chemicals may act on this enzyme. This has also been shown in several different studies, and gonadal 20β-HSD in fish has been shown to be regulated by nonylphenol, dicofol, din-butyl-phtalate, fenarimol, DDE, ketoconazole, benzo[a]pyrene and the androgens 11-ketotestosterone and testosterone (Hoffmann and Oris, 2006; Kortner et al., 2009; Thibaut and Porte, 2004; Villeneuve et al., 2007).

Few studies have investigated the regulation of CBR or 20β-HSD in the liver of fish. In the human liver, CBR1 catalyses the reduction of many quinones of polyaromatic hydrocarbons (Wermuth et al., 1986). *In vitro* studies of human hepatocarcinoma cells have shown that the PAH β-naptoflavone (β-NF) induces CBR1 mRNA expression, while the glucocorticoid receptor agonist dexamethasone and activators of the androstane receptor (clotrimazole and TCPOBOP) and pregnane X receptor (rifampicin) did not influence the CBR1 expression (Lakhman et al., 2007). Similar results were found in paper II, where hepatic mRNA expression in rainbow trout CR/20β-HSD was shown to be regulated a PAH (β-NF) and an oxidative stressor (paraquat) rather than by possible substrates in the steroid metabolism.

In humans, the pathways between PAH and/or pro-oxidant exposure and CBR1 and CBR3 expression is mediated by the arylhydrocarbon receptor xenobiotic response element (AhR-XRE) and the NF-E2 p45-related factor antioxidant response element (Nrf2-ARE) pathways (Ebert et al., 2010; Lakhman et al., 2007; Osburn and Kensler, 2008). β-NF is a bi-functional inducer that can activate the two above mentioned pathways in parallel. Miao et al., found that Nrf2 expression is partly regulated by the activation of multiple XRE elements at its promoter and that the Nrf2-ARE pathway is downstream of AhR-XRE activation in mice (2005). Paraquat is an oxidative stressor that only interacts with Nrf2-ARE. Both paraquat and PAH β-NF have been shown to increase the levels of CBR1 homologue in rainbow trout and eelpout in papers II (2010) and III.

In conclusion, human CBR1 is a promiscuous enzyme with broad substrate specificity (Pilka et al., 2009). Many of the CBR inducers that have been reported in both mammalian and fish literature for the liver and gonads have been found in the effluent waters from Swedish STPs (IVL, 2010a; Paxéus, 1996; Paxéus and Schroder, 1996; SEPA, 2008).

2. Aim

The general aim of this thesis was to explore the use of proteomics to generate hypothesis of potentially new biomarkers for pollutant exposure in fish, and to start evaluating the potential of one protein – CBR – as a biomarker. The specific aims for each paper are listed below:

Paper I

To use proteomics to identify regulated liver proteins between rainbow trout *(Oncorhynchus mykiss)* caged up- and downstream of an STP effluent. To validate possible responses with other methods. To initiate studies of possible inducers of the observed response.

Paper II

To investigate whether the increased expression of CBR1 seen in the first study could also be found in fish exposed to other STP effluents and whether more advanced sewage treatment could reduce the response in exposed rainbow trout. To perform several different intraperitoneal injection studies to gather information regarding what type of substances are able to induce the observed responses in rainbow trout. To conduct an *in vitro* study to investigate whether inducers act directly on the liver or if CBR1 induction requires the involvement of other organ(s).

Paper III

To set up protocols for measuring CBR enzymatic activity in rainbow trout. To set up protocols for mRNA and enzymatic activity measurements in the marine sentinel species eelpout *(Zoarces viviparus)*. To investigate if observed hepatic CBR mRNA induction correlates to CBR enzymatic activity in rainbow trout and eelpout.

Paper IV

To use CBR mRNA expression and activity measurement methods that were developed and tested in a controlled laboratory system on a comprehensive field material to compare CBR expression in eelpout collected at sites with different grades of pollutions.

3. Methodology

3.1 Fish

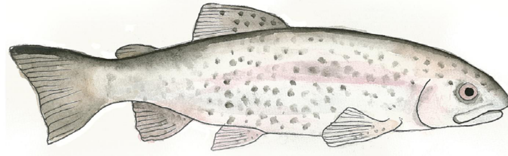

Figure 7. Rainbow trout, *Oncorhynchus mykiss*
Illustrated by Elisabeth Ledin

Fish are not a systematic group but are instead a paraphyletic group that contain approximately 32 000 species. Teleosts, the most abundant of today's fish, are an important source of food. They are also appreciated in recreational fishing. Being vertebrates, fish have a relatively closer evolutionary relationship to humans compared to many other water breathing animals, with the exception of juvenile (and some adult species of) amphibians. These different properties and uses have made fish health a public interest. They occupy many niches in the aquatic environment and exist at a high frequency compared to the many other vertebrate aquatic inhabitants. These are only some of the reasons why environmental monitoring and assessment with fish is common. My personal opinion is that public interest is important for a sustainable future and therefore should not be neglected. Several different model fish species are commonly used in ecotoxicology research. In this thesis, the two different species discussed below, rainbow trout (figure 7) and the common eelpout (figure 8), have been used because of their specific properties.

Rainbow trout is not a native species of the Swedish coastal or freshwater bodies, but have survived after its introduction from North America. It has been farmed for about 100 years, and the cultivation of the rainbow trout has given it both advantages and disadvantages as a research animal. It has a high tolerance to stressors occurring in exposure situation and is exceptional good on surviving during different experiment set-up including exposure to pollutants via injection studies, through water exposure and in field cage studies. The possibilities to use one species in both field cage and lab exposure studies have made the rainbow trout model successful in many studies. To the backside of using a cultivated species is the fact that selective pressure on growth will result in other physiological changes that could influence effects of exposures. Farmed fish are also often fed with food containing antioxidants, which may influence studies of

oxidative stressors. Overall, the extensive literature and on the lab knowledge of rainbow trout has made it a valuable part of this thesis (paper I, II and III) and for the other members of the group (Almroth et al., 2008; Larsson et al., 1999; Lennquist et al., 2010; Sturve et al., 2008)

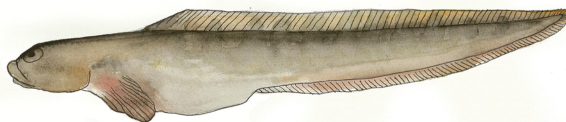

Figure 8. Eelpout, *Zoarces viviparus*
Illustrated by Elisabeth Ledin

The existing literature on our other model, the common eelpout, is not as comprehensive as for rainbow trout. However, it has some characteristics of interest in field ecotoxicological research. It is commonly found in the bottom of shallow waters on the coasts in Northern Europe. It has a limited migration pattern, which enables one to draw a correlation between local exposure and physiological changes (Hedman et al., in press; Jacobsson et al., 1986). In contrast to most other fish, the eelpout is viviparous where internal fertilisation gives rise to female "pregnancy" resulting in well-developed juveniles after several months. This can cause data to be hard to extrapolate to other teleosts where fertilised eggs most commonly are laid, developed and hatched outside the mother. Exposure during early life, which is a sensitive stage, is therefore rather different in eelpout compared to other fish because contaminants have to pass through the mother. Nevertheless, a species with viviparous behaviour enables the study of reproductive output, sex ratio and larvae malformation, which has frequently been performed in the eelpout (Gercken et al., 2006; Larsson and Förlin, 2002; Larsson et al., 2000; Napierska and Podolska, 2006; Strand et al., 2004; Vetemaa et al., 1997). The common eelpout is part of the Swedish national integrated fish-monitoring program where biometric data, the frequency of dead larvae and several different blood variables are studied yearly together with chemical and biochemical analyses (Bignert et al., 2011; Hedman et al., in press). In 2009, Kristiansson et al. sequenced the hepatic transcriptome of the eelpout, making it even more valuable as a model research species.

In this thesis, several types of fish experiments have been used: cage studies in effluent water, laboratory exposures and field studies involving the capture of wild individuals. Rainbow trout were used in the cage studies, the common eelpout was captured in the field and both species were used in

laboratory exposures. The different types of experimental setups provide different types of information. To investigate the effects on fish exposed to effluents from STPs, we used rainbow trout that were caged up- and downstream of the effluent outlet. As we were using the proteomic approach, it was important to use a species that had been sequenced to at least some extent. Few such species are commonly found in sufficient numbers downstream of Swedish STPs with the exception of roach *(Rutilus rutilus)*, in which sequence data are starting to accumulate (www.uniprot.org). At the start of this project in 2005, a cage study with rainbow trout was considered the most suitable option and capturing wild fish on site was not considered feasible. In a cage study, it is possible to control the exposure of fish to the effluent because they cannot swim away. Of course, such an experimental setup does not have the same significance as capturing wild fish, which reflects the real exposure situation in the natural fish population. However, it can provide us with information regarding the potential effects of STP effluents.

To link field experiments with laboratory exposure, it is possible to provide information regarding the possible components/chemicals in the chemical mixture that are responsible for the observed effects. Therefore, in this thesis, fish were exposed to chemicals via waterborne exposure or intraperitoneal (i.p.) injection. Fish exposed to a high dose of a single substance responding similarly to fish investigated in the field may provide information regarding the mode of action of the pollutants present in the field. This comparison between field and lab exposures has been used to a wide extent throughout this PhD project.

Field studies with captured wild fish are used in paper IV. Such a study will truly reflect the real exposure situation but what each individual has been exposed to during a life time is not known. This can to some extend be avoided by using a stationary species as the eelpout which makes the comparison of polluted and unpolluted sites feasible.

3.2 *In vitro* experiments

In Paper II, an *in vitro* study was conducted with a trout hepatoma cell line. Sewage treatment effluent water was extracted, and the cells were exposed to the extract. In this thesis, the method was not used further, but there are some advantages and disadvantages worth mentioning. First, the

amount of fish used could be reduced. Second, a cell study can provide information regarding the mode of action of pollutants compared to *in vivo* studies. Third, the screening of responses to chemicals can be very time consuming. Working *in vitro* can speed up such processes. Finally, when it comes to "omics" methods, reproducibility can be enhanced using a cell line. One drawback of the *in vitro* studies is its simplicity. Exposing only one cell type can produce results that are different from when an intact organism is exposed to a pollutant. This major disadvantage can cause the results from *in vitro* studies to be less physiologically relevant.

3.3 Two-dimensional gel electrophoresis

At the starting time for this thesis work, the most common method for proteomic work was still gel-based, i.e. the two dimensional gel electrophoresis (2DGE). Advancement in sequencing, identification and the development of soft wares able to match and simultaneously compare 2000 different spots resulted in a 2DGE era in medicine, followed by other field such as ecotoxicology. Many labs, including ours, have struggled with the most important aspect: reproducibility. During the first part of this PhD, most time was spent on making a robust protocol that is described further in paper I and in the discussion of the theses.

To confirm the identity of spots of interest usually a mass spectrometry (MS) with MALDI-TOF analyses of trypsin digested fragments is used. A good knowledge of sequences within the investigated species is crucial in order to get good identifications of the spots. At time being for this experiment set up, the core facility SweGene at University of Gothenburg, bought a Fourier Transform Ion Cyclotron Resonance MS (FT-ICR). This resulted in a higher accuracy of mass spectra on the whole fragment and most important, generation of peptide sequence information de novo. This makes is possible to BLAST (basic logical alignment search tool) identified peptides to the proteome of also other species then the studied which is helpful when working with species with limited genome information.

3.4 Quantitative PCR

Even though mRNA levels are not of such as physiological importance as protein levels, quantitative polymerase chain reaction (qPCR) analyses are commonly used to confirm the identity of regulated transcripts and proteins

following exploratory analyses. To find and measure a specific transcript of interest among all the existing mRNA is sometime compared with finding a needle in a haystack. In the PCR, specific primers to the transcript of interest are used together with DNA polymerase for amplification. The created double stranded cDNA is in qPCR linked to fluorescence that is measured in each doubling step. The advantages of this method; among those superior specificity, has made PCR popular, but seems to have decreased the amount of research into the translated products which are sometimes more demanding to develop.

3.5 Immunoblot

As proteins (in comparison to mRNA) better reflects physiological functions in the studied material, efforts were made to make an antibody towards CBR1 in rainbow trout. When this work was done, we had only data supporting an induction of mRNA CBR1 B variant in rainbow trout. Hence the peptide sequence NKGTGLAIC which is specific for the B variant were conjugated to Keyhole limpet hemocyanin, injected repeatedly with adjuvance in two rabbits and final bleeds were taken after 15 weeks. A similar setup was used for the peptide sequence CFGEQAEVTMRTNF which would be expected to bind both the A and the B variant

3.6 Enzymatic assays

To study the possible changes in functional expression, the activity was measured. Carbonyl reductase activity was measured according to a protocol developed by Wermuth (Wermuth, 1981), which has also been used by others (Gonzalez-Covarrubias et al., 2007; Guan et al., 1999; Wermuth et al., 1986). Menadion is used as a substrate, but can also be reduced by other enzymes than CBR1, for example CBR3 and DT-diaphorase (Lind et al., 1990; Pilka et al., 2009). Hence, rutin was used as an inhibitor and the carbonyl reductase activity is the rutin inhibitable NADPH dependent reduction of the menadione. Menadione is coupled to cytochrome c, which is reduced by the reduced menadione (Prochaska and Talalay, 1986; Sturve, 2005). The reduction of cytochrome c is followed spectrophotometrically at 550 nm.

4. Findings and Discussion

4.1 Wide screening methods in ecotoxicology

A few years ago, many review articles were published about how the new "omics" technologies would be applied and how they would improve ecotoxicology (Ankley et al., 2006; Calzolai et al., 2007; Denslow et al., 2007; Dowling and Sheehan, 2006; Iguchi et al., 2007; Snape et al., 2004; Travis et al., 2003; Watanabe and Iguchi, 2006). There was an optimistic view that many model species in the field would soon have fully sequenced genomes. Today, in 2011, when a large amount of data has been acquired, the question must be posed: has this resulted in an increased understanding of toxic exposure, safer hazard assessment and/or improved environmental monitoring?

There is no doubt that we have more information today regarding toxic responses to many contaminants compared to the "pre-omics" time. However, until now, many of the works published have been more proofs of concept of chemicals with already quite well known responses (Fent and Sumpter, in press). Pathways already known are highlighted and discussed and seldom are very new insights achieved. Still, it has to be stated that this has provided us with information that indicates the response to exposure at the gene and protein level is much more complex than most would have imagined. An organism exposed to a single chemical can respond by altering the expression of hundreds of genes. The time point and concentrations that result in different exposure patterns do not always reflect the "pre-omic" theories about dose response curves (Fent and Sumpter, in press). Taken together, the generated data has made the field of ecotoxicology more complex but has also given us a more realistic view of the response to chemical exposures. We still have very limited physiological knowledge that can be used to interpret all of the generated data, and basic bioinformatic gaps (fully sequenced relevant species, proper annotations) still exist. All together, it seems like we after a few years with the techniques still are in the developing phase and much exciting data are ahead of us.

There is a very high potential to use "omics" techniques in hazard assessment and in field studies because the methods have a better chance of decreasing the risk of overlooking responses compared to traditional toxicity tests and biomarker approaches. Although the methods have been in use for

some years they are rarely or never used for formal hazard assessment purposes (Carvan, 2007; EU, 2006), and they have not been included in any regulatory yearly biomonitoring programs, to my knowledge. This is likely due to the limitations that were previously mentioned. With more genomic information and researchers working from standardised protocols, one can hope that the methods may become an important part of hazard assessment and monitoring in the future, also outside of the universities.

In field studies, "omics" methods have been used by us and others(Falciani et al., 2008; Garcia-Reyero et al., 2008; Garcia-Reyero et al., 2011; Ings et al., 2011; Moens et al., 2007; Wang et al., 2008). With these types of experimental setups, hypotheses can be generated regarding the responses to multiple contaminants. The aim of this thesis was to use proteomics methods to discover new biomarker(s). Others have used similar methods (Fent and Sumpter, in press). For example, a study conducted by Gunnarsson et al. used a meta-analysis to search for sensitive and robust changes in gene expression in fish exposed to oestrogens (2007). Induction of zona pellucida protein 3 and nucleoside diphosphate kinase were suggested as possible biomarkers to detect low levels of estrogenic compounds. In paper I, we used a different approach; we started with a field study where the possible responses reflect the true exposure situation in the environment. After the results were generated, the focus was on further investigating the response and establishing other less time consuming methods to use when investigating possible biomarker characteristics. Later on, we tested these simpler methods in a biomonitoring program.

An ideal monitoring program should focus on what we want to monitor and protect. In many cases, it may instead be the opposite: driven by what we can observe with the present methods (i.e., technique driven) (Lehtonen and Schiedek, 2006). The question is then the following: what do we want to monitor? In a time where it is impossible to keep up to date with all of the chemicals that are produced and used, we might not always have the answer. If the same battery of biomarkers is always used in biomonitoring, there may be responses to compounds with possible adverse health effects that we do not measure. With the "omics" tools in our hands, at least we have a possibility to find responses earlier unknown for us. Hence, even though the large-scale methods are not incorporated in yearly monitoring, researchers connected to such programs can use these methods to improve them. To conclude the discussion of wide screening methods in ecotoxicology, I

would like to state that even though they can be accused of only being used as technical innovations (Fent and Sumpter, in press), technique-driven "omics" methods can lead to more focused-driven biomonitoring in the future.

4.2 mRNA expression, protein content and enzymatic activity

Throughout this thesis, different methods were used to investigate CBR expression (figure 9).

4.2.1 Protein content

In paper I, rainbow trout that were caged downstream of a local STP had decreased hepatic betaine aldehyde dehydrogenase, lactate dehydogrenase and one unidentified protein compared to fish that were caged upstream. Furthermore, one spot containing CBR1 and/or mitochondrial ATP-synthase alpha subunit was increased due to exposure.

Compared to other 2DGE publications in ecotoxicology, few, if any, have reported as few responses as we do in Paper I in either the lab or field exposure setups. The literature is often lacking information regarding biological/technical replicates and the total number of proteins on the gels, which makes it difficult to make proper comparisons. However, in general, many have used 3-6 biological replicates for each group, and the number of regulated proteins due to exposure varied between 18 and 80 (Apraiz et al., 2006; Kling and Förlin, 2009; Ling et al., 2009; Manduzio et al., 2005; Mezhoud et al., 2008; Sanchez et al., 2009; Shi et al., 2009; Wei et al., 2008; Zhu et al., 2006). The average number of observed spots on the gels in the mentioned publications was 580, and it is quite possible that false regulations were reported in some studies. When using this method to generate hypotheses, one might not be concerned because the regulations can be clarified by validating the observed responses with other molecular methods.

In our study, we initially had only four biological replicates, in which 15 spots were regulated by exposure to the effluent. Because we were searching for a robust response, we decided to increase the sample size to ten biological replicates. Then, we could confirm only four of the initial 15 regulated spots. For us, this demonstrated the need for comprehensive material for our purposes. It is possible that the strict approach increased the

risk of missing important regulations. As Swedish sewage treatment effluent water is well studied with the classical biomarker approach, the enzymes catalase, GR, GST, NQO1, CYP1A and the protein VTG, are known to be altered in fish exposed to STPs, either in activity or abundance (Almroth et al., 2008; Larsson et al., 1999; Sturve et al., 2008). Therefore, one could expect that we would have observed changes in at least a few of these proteins on the 2D gels. There are reasons other than the strict approach that might account for the absence of a response by these proteins. Some of them might only respond with alteration in catalytic activity and not on protein level. Also, the proteins may not be represented on the gels, or the level of protein of these biomarkers may not vary in this study. However, when working with large screening methods, there is always a balance between reporting false positives and missing important proteins. Therefore, the purpose has to be clear before the analysis is performed, and in some cases, it may be important to not miss any possible regulations. However, to only use three biological replicates within 2DGE analyses seems, in all cases, very little if one is interested in observing true responses.

As stated in section 3.3, the FT-ICR enhanced the process of identifying regulated spots from 2DGE systems. However, moving on to techniques where the mass of the individual peptides was obtained, we found that most spots on the 2D gel contained multiple proteins. Multiple proteins in each spot makes the comparison and quantification between gels difficult because in incompletely sequenced species, it is not possible to determine which of the proteins present at roughly the same location on the gel is responsible for the difference in intensity. Such results should be further investigated with other molecular methods, such as Western blot, protein activity or qPCR (the latter assuming a correlation between mRNA and protein levels). The increased spot in our results, containing two different proteins, were further evaluated.

To evaluate the protein abundance with immunoblot, we developed two antibodies (see section 3.5). The antibodies were used in two different experiments, and qPCR reactions were run in parallel. First, an immunoblot was run with liver homogenate from rainbow trout exposed to β-NF. This fish had, on average, altered CBR A and B mRNA expression of 3.6- and 45-fold, respectively, compared to the controls. Second, Western blot analyses were also performed on the livers of rainbow trout that were kept downstream of the Gässlösa STP and an mRNA induction of 2.8-fold (A-

variant) and 5.6-fold (B variant) was observed. Despite clear and strong regulation at the mRNA level and the possible induction of CBR1 on the 2DGE analysis (STP exposure), there was no observed induction by immunoblot analyses after exposure. We did not further evaluate if the antibodies were specific enough to measure CBR1 induction. Instead, we focused on qPCR and enzymatic activity analyses. However, an accurate method for measuring protein content is important to obtain a clear picture of CBR regulation. This can be addressed in future studies.

4.2.2 mRNA expression

Because the sequence of CBR in rainbow trout was known (Guan et al., 1999), it was convenient to investigate CBR with the qPCR method. As they provide data with a high grade of specificity, they were suitable for us to use in this part of the thesis work.

Differences in the starting material (cDNA) are likely to be obtained during cDNA synthesis. To control for the technical reproducibility, duplicates were used in the cDNA synthesis step. This is not commonly performed; most studies only use replicates in the final step, which is the PCR reaction. However, based on my data, there are more differences in cDNA synthesis than in the PCR reactions, and effort should be made to create replicates at an earlier step in the experiment, although it involves an increase in costs. It is also possible to replicate the analyses at an even earlier stage in the process, i.e., replicating the RNA extraction. However, as a known amount of RNA is used in each cDNA synthesis reaction and the concentration is measured after the RNA extraction, using technical replicates after the concentration measurements is, in my opinion, a reasonable compromise.

Even when using replicates at the cDNA synthesis step, there is a need to further control for true changes in the mRNA levels between samples. Often, reference genes are used for such purposes, even though other normalisation methods exists (Ellefsen et al., 2008). A set of reference genes that has been used by others and in our lab was tested for each experiment. In general, the most stable reference gene has been ubiquitin, which has been most frequently used. In the first three papers of this thesis, the results of qPCR analyses were quite clear. The results were similar with or without the reference genes, and there was a clear difference between a response and no response. qPCR data dealing with subtle results should always be handled

with care because a reference gene can influence the result, even when the reference gene is considered stable. In paper IV of this thesis, we did not manage to find a stable reference gene for all sites that were included in the statistical analyses. This makes the results less reliable because the observed differences in Ct values might reflect differences in the starting material (cDNA) instead of expression.

Differences in CBR mRNA levels have been frequently used in this thesis (figure 9). The qPCR method provided us with information regarding the possible inducers of CBRs in both rainbow trout and eelpout (see section 4.3.1, 4.3.2 and figure 9). As discussed below, it has its limitations, and if the aim is to study functional differences, protein abundance or enzymatic activity is generally preferred (Nikinmaa and Rytkönen, in press; Nikinmaa and Schlenk, 2009).

4.2.3 Enzymatic activity

Nikinmaa and Rytkönen stated in 2011 that "Toxicological responses of an organism are disturbances in function". To study functional differences, we optimised an assay to determine hepatic CBR enzymatic activity in paper III.

We used a method developed for human CBR by Wermuth (1981; 1986) with some minor modifications to the protocol CBR activity was estimated as the rutin inhibitable NADPH-dependent reduction of menadione. Rutin is only validated as an inhibitor for human CBR. Hence to control for specificity, we attempted to obtain the E. coli BL21 strains with inserted rainbow trout CBR1 A and CBR1 B genes that were reported by Guan et al. (1999). However, they lost the strains a few years ago (Guan, personal communication). Instead, we decided to look for possible correlations between the Ct values obtained by qPCR analyses, which is highly specific, with the results from the enzymatic assay in each individual. No correlation was observed in rainbow trout, and the enzymatic assay was not further used in this species. For eelpout, a correlation was observed, suggesting at least some level of specificity in the eelpout CBR enzymatic assay.

Even though the genomic information for rainbow trout and eelpout is limited, existing information indicates that rainbow trout has more forms of CBRs than eelpout (figure 3). CBR1 B has no confirmed enzymatic activity and hence is not expected to contribute to the activity in the enzymatic assay

(Guan et al., 1999). Thus far, we have not measured the third CBR transcript in rainbow trout, TC15644, at the mRNA level, and it is not known if it translates to an active protein. Therefore, we do not know if the sum of all of the active CBR transcripts is induced in any of our rainbow trout experiments. This could be one of the reasons for the poor correlation between mRNA and enzymatic activity in the species. Another reason may be an unspecific catalytic assay. Of course, one likely explanation is that induced CBR1 mRNA in rainbow trout does not lead to a corresponding change in the levels of active enzymes in rainbow trout.

As stated above, mRNA transcript and enzymatic activity do not always correlate and neither does protein abundance and measured activity. This is also true for other existing biomarkers. For example, a dose response curve of EROD activity can be biphasic while the CYP1A protein abundance is not (Celander et al., 1997). This is probably because a high dose of the inducer will inhibit and/or inactivate the EROD catalyst but not reduce the protein content. Other studies that included exposure to more than one chemical showed that certain substances can inhibit the EROD activity, but this will not be reflected by CYP1A protein abundance (Gräns et al., 2010; Hasselberg et al., 2004). To our best knowledge, there have been no such studies performed on hepatic CBR in fish, and we do not know if CBR activity is biphasic. Whether substances known to be present in the environment can inhibit hepatic CBR has not been investigated in any fish to my knowledge. Biphasic curves and inhibition can be problematic for biomarkers that are used to monitor sites with multiple stressors because the data can be difficult to interpret. This is an important aspect of CBR to study to be able to properly evaluate the results from monitoring studies.

To summarise my reflections on the molecular methods applied in this thesis, all steps of the molecular pathway from DNA to activity should be studied, when possible, as they provide us with complementary information. However, this is seldom performed. For monitoring, enzymatic activities are most frequently used because they reflect a change in function and are simple and rapid to measure.

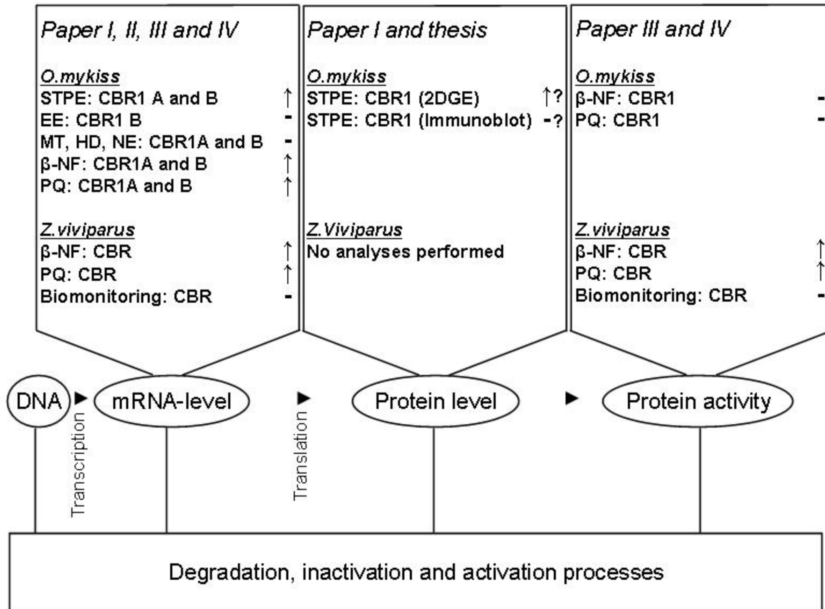

Figure 9. Several different processes can influence the molecular pathway from DNA to protein activity, hence, responses observed at one level may not be observed at the others. This picture aims to provide an overview of the CBR results measured at different levels of the pathway. Rainbow trout and eelpout have been exposed to either effluents or single substances. In the figure, the exposures are abbreviated as follow: β-NF = β-naphtoflavone, EE = ethinylestradiol, HD = hydrocortisone, NE = norethisterone, MT = methyltestosterone, PQ = paraquat, STPe = sewage treatment plant effluents.
↑ = Induction of exposure
- = No observed alteration of exposure
? = Result not totally reliable

4.3 Inducers of CBR expression

4.3.1 Effluents of STPs

In paper I and II, CBR expression was characterised in rainbow trout exposed to effluents from different STPs. The novel finding of CBR regulation on the 2DGE was followed up by mRNA expression in the same experimental setup and in two other experiments at different STPs. Downstream from the Gråbo STP, only CBR1 B was induced (paper I). The

B form has so far not been shown to have any enzymatic activity (Guan et al., 1999; Guan et al., 2000). At Gässlösa, both CBR1 A and B were induced at the mRNA level (paper II). Extraction of the same effluent resulted in induction of CBR1 A and B when trout hepatoma cells were exposed (paper II). In paper II, we were also able to include rainbow trout CBR1 A and B mRNA expression in an exposure setup from the Stockholm Water AB where the efficacy of different sewage treatment techniques were tested by several researchers in different aspects (Gunnarsson et al., 2009; Lavén et al., 2009; Lundström et al., 2010a; Lundström et al., 2010b; Samuelsson et al., 2011). It was clear that rainbow trout exposed to all modern technologies included in the study (moving bed biofilm reactors, ozone and membrane bioreactors) had decreased mRNA expression of CBR1 A and B compared to rainbow trout exposed to conventional treatment processes (paper II). Preliminary analyses of microarray data from the same experiment are in agreement with this conclusion (Cuklev et al., in preparation). Therefore, hepatic CBR1 in rainbow trout seems to frequently be affected by effluents from STPs at the mRNA level. The results regarding CBR1 protein content are less clear, as the possible regulation in the 2DGE was not observed on the immunoblot. However, as the antibodies were never validated regarding their specificity, this preliminary observation is not conclusive.

4.3.2. Exposure studies

We were interested in studying if any kind of compounds from the STP effluents caused the hepatic CBR1 response in rainbow trout. Studies on fish have mainly focused on gonadal CBR and/or 20β-HSD expression and activity, which are commonly induced by substances interfering with the steroid pathway. There is clear evidence of changes in the expression due to exposures to such substances (Kortner et al., 2009; Thibaut and Porte, 2004; Villeneuve et al., 2007). In livers, compounds interfering with the AhR-XRE and Nrf2-ARE pathways have been suggested to induce mammalian CBR (Lakhman et al., 2007; Pilka et al., 2009; Wermuth et al., 1986). Tilton et al. (2006)reported an induction of rainbow trout CBR1 B after exposure of hepatic tumour promoters, including 17β-Estradiol. All of these types of substances, i.e., PAHs, pro-oxidants and steroids, are present in the effluents of Swedish STPs (Paxéus, 1996; Paxeus and Schroder, 1996; SEPA, 2008). The collected information led us to perform several lab experiments with different model substances for steroids, PAHs and pro-oxidants (ethinylestradiol, norethisterone, methyltestosterone, hydrocortisone, β-naphthoflavone and paraquat). None of the steroids induced the studied

CBR expressions in rainbow trout. We also observed that the CBR responses did not parallel the induction of the estrogen responsive transcript of vitellogenin and zona pellucida when expression patterns were compared in the same individuals included in the experimental set up where effluent water treated with advanced and conventional treatment technologies were compared(Gunnarsson et al., 2009). Thus, this suggested that the observed induction of CBR in rainbow trout exposed to effluents from STPs could be caused by chemicals other than steroids present in the water.

In contrast to the steroids, β-naphthoflavone and paraquat strongly induced CBR mRNA expression in both rainbow trout and eelpout. Furthermore, the increase in mRNA levels was paralleled by a corresponding increase in enzymatic activity in eelpout. This is in line with previously published articles that investigated mammalian CBR regulation in the liver, where PAHs, quinones and oxidative stressors are known as inducers (Oppermann, 2007). Recently, Cuklev et al. showed that CBR was induced in the liver of rainbow trout exposed to waterborne diclofenac (2011). The primary mode of action of diclofenac is alterations in the prostaglandin synthesis (Gan, 2010). The transcription factor for ARE, Nrf2, is known to be under regulation of not only PAHs and pro-oxidants but also by prostaglandin (Itoh et al., 2010). All together, this suggests that there might be several substances that can induce CBR expression in the livers of fish. The question of how hepatic CBR is regulated in fish is clearly not simple and should be investigated further.

Collected results still demonstrate that CBR expression in rainbow trout and eelpout can be induced by compounds known to interfere with AhR-XRE and Nrf2-ARE. and others have shown that these elements can start the transcription of CBR1 and CBR3 in humans (Lakhman et al., 2007; Osburn and Kensler, 2008). There is also evidence from human studies that hepatic CBR1 reduce the amount of produced ROS and catalyze the reduction of reactive carbonyl compounds in lipid aldehydes (Oppermann, 2007). Our results, and the homology to the human CBR, imply that rainbow trout CBR1 can be induced by AhR agonists and/or pro-oxidants present in the STP effluents.

4.4 CBR biomarker application in biomonitoring

There is a constant need to improve the monitoring of chemical effects in the environment. Balcofish (Integration of pollutant gene responses and fish ecology in Baltic coastal fisheries and management) is a research program in which an integrated approach is applied to develop ideas and techniques to improve the tools that are used to assess the impact of toxic chemicals on coastal fish populations in the Baltic Sea. The viviparous eelpout is the species that was selected for these studies. To analyse the geographical differences in the biometric data and biochemical responses, eelpout were caught at 16 different stations that are more or less polluted at the Swedish, Danish and German coasts (Gercken, 2007; Gercken et al., 2006; Gercken and Sordyl, 2007; Hjorth and Josefson, 2010; Landner et al., 1994; Larsson et al., 2000; Ronisz et al., 2005; Schiedek et al., 2006; Strand et al., 2009; Sturve et al., 2005a; Vetemaa et al., 1997). In paper IV we decided to test eelpout CBR expression (mRNA and catalytic activity), which was measured in the liver of eelpout in this comprehensive field material. The CBR analysis was one of a set of biochemical analyses in paper IV.

In paper IV, hepatic CBR expression measured as mRNA content and enzymatic activity were not statistically different in eelpout from the polluted sites when compared to eelpout from the reference sites. This comparison was made separately within each of the four regions, i.e., two in Sweden (with 4 stations each) and one each in Germany (3 stations) and Denmark (5 stations). There may have been a small regulation of mRNA within the German sites. However, as mentioned in an earlier section in this thesis (4.2.2), we did not find stable reference genes for the CBR mRNA measurement for the German sites. Therefore, these results are not entirely conclusive.

The fish from Göteborg harbour, one of the polluted sites included in the study, had very high PAH metabolites (1-OH-Pyr) in their bile compared to fish caught at the four other sites on the Swedish west coast. The results from paper II and III indicated that CBR expression could be induced by a PAH model compound in rainbow trout (mRNA) and eelpout (mRNA and enzymatic activity). Since the caged rainbow trout exposed to effluents of STPs had altered CBR expression we also had data supporting release of CBR inducing compounds into the aquatic environment. This led us to hypothesise that we would be able to observe a difference in CBR

expression between more or less polluted sites on the Swedish west coast but, as stated above and shown in paper IV, such a pattern was not observed.

The fact that CBR expression is induced in fish exposed to STP effluents but not in fish caught at a PAH polluted field site can imply that fish downstream of STP effluents are exposed to substances of higher concentration and/or potency that induce CBR. However, species differences must also be considered; in the STP exposure experiment, e.g. rainbow trout were used while eelpout were used in paper IV. Additionally, the time of the exposure can be crucial. In the study measuring the effects of STPs, rainbow trout were caged for two weeks, while in paper IV, adult eelpouts were collected from coastal sites where, due to their stationary behaviour, most were likely exposed over a longer time period. Others have shown that some alteration or damage to antioxidant defence. For example, GST, GPX and lipid protein oxidation in tilapia *(Oreochromis niloticus)* and rainbow trout observed shortly after high dose exposures were not seen after a prolonged exposure time with lower doses (Miller et al., 2007; Prieto et al., 2007). CBR is also part of the antioxidant system, and so far, we have not studied any chronic exposures in controlled laboratory experiments with regard to CBR expression and hence do not know if it is an acute response or not.

In this thesis, the induction of carbonyl reductase appears to be a promising biomarker when assessing exposure and the possible impact of the sewage treatment plant effluents in caged fish. However, more research is needed before it may become a biomarker used to assess the impact of toxic chemicals in biomonitoring programmes using wild fish populations. As several different chemicals have been shown to induce CBR, its induction will not provide conclusive evidence for prior exposure to a specific substance. However, as investigated by us and others, induction caused by steroids (gonads), PAHs (liver) and oxidative stressors (liver) are all important and require biomarkers because these substances are commonly found in some aquatic environments and can result in adverse health effects in exposed individuals. Expression of CBR might give a very broad answer to the exposure questions; however, sometimes a promiscuous biomarker can serve its purposes well.

Hepatic CBR expression may be a good indicator of pollutants that can initiate transcription via Ahr or Nrf2. Such biomarkers already exist. Induction of CYP1A is a well-established and well-characterised biomarker. Its expression is mediated through Ahr-XRE. However, it is not induced by monofunctional inducers and hence will not parallel CBR1 expression when such substances are part of the exposure. Thus, analyses of CBR together with CYP1A may provide more information on the nature of the exposure than analyses of CYP1A alone. In contrast, many other enzymes often included as biomarkers in biomonitoring are mediated through ARE, for example enzymes for the glutathione synthesis, GST and NQO1 (Osburn and Kensler, 2008; Sykiotis et al., 2011). However, some have been shown to be age- and condition-related, and it appears that at least a few display fluctuating responses to exposure (Blom et al., 2000; Lushchak, 2011).

5.5 The biomarker approach - an aid in the monitoring of chemical impact?

The biomarker approach has been accused of resulting in very transient responses where one can not draw conclusions (Forbes et al., 2006). However, biomonitoring with biomarkers is useful and provides important information in addition to chemical monitoring. This is true, for example, in the Swedish national integrated fish-monitoring program where perch and eelpout have been sampled and analysed yearly since 1988 in coastal areas. These sites have been carefully chosen to represent areas with minimal influence by human activity. Fish have been collected at the same time each year, and long-term trends have been established. The transient nature of biochemical biomarkers could be less important because there is presumably no acute toxic exposure; instead, there are only slow increases/decreases in pollution. Fluctuating data can, after several years of monitoring, display trends. In the Baltic proper, long-term monitoring of perch has provided information regarding increased EROD activity and a reduced GSI (Hansson et al., 2006; Sandström et al., 2005). It has been concluded that individuals that lived during years of high EROD had lower GSI. Because bio banks are established there was an opportunity to backtrack PAH metabolites in bile banked from fish in the monitoring program. Indeed, it has been concluded that PAHs likely contributed to the increased levels of EROD (Hanson et al., 2009a; Hanson et al., 2009b). PAH measurements performed only yearly in fish bile would not have provided information regarding the reduced health status of the exposed populations. These long-

term trends illustrate the need for biomarkers when the chemical impacts on the environment need to be monitored.

As stated in the introduction, a good biomarker will predict exposure and/or effect. However, as mRNA, protein abundance or catalytic activity can be very transient, a biomarker might be good for one type of condition but not another. It is important to have comprehensive knowledge of the biomarker used and, for example, what factors other than chemical exposure may influence the results. However, efforts should also be put into developing new biomarkers that might add information regarding exposure and/or effect. This is especially important during a time when there is a constant addition of new chemicals in combination with limited information on their effects. It is often said that we should use a battery of biomarkers to monitor the environment. The battery should constantly be improved and increased. The work presented in the present thesis is a contribution in this direction.

5. Conclusions and future perspectives

In this thesis, different experimental setups and molecular methods were used in order to discover new biomarkers and characterise their usefulness. A wide screening method was initially applied using fish exposed to the effluents of one STP, and the results were followed up at other effluents of STPs as well as in controlled lab exposures. After some characterisation of what types of chemicals having the ability to regulate the expression of an identified candidate biomarker (CRB), the expression of this gene was applied in a comprehensive biomonitoring effort. The specific conclusions were as follows:

- 2DGE analysis revealed that rainbow trout exposed to the effluent downstream from a STP had increased hepatic CBR1 and or/mitochondrial ATP synthase alpha-subunit and decreased lactate dehydrogenase and betaine aldehyde compared to fish caged upstream. The higher CBR1 content was confirmed by higher hepatic CBR1 B mRNA levels in exposed fish compared to fish caged upstream the STP. Rainbow trout kept up- and downstream from a second STP effluent had induced hepatic mRNA expression of CBR1 A and B due to effluent exposure. An *in vitro* study performed on a trout hepatoma cell line that was exposed to extracts of STP effluents resulted in increased mRNA expression of both CBR1 A and CBR1 B. The collected results demonstrated that there is an impact of the outgoing STP water on hepatic CBR expression in rainbow trout and that the induction can be mediated directly in the liver.

- When rainbow trout were exposed to STP effluents treated with more advanced treatment technologies (moving bed biofilm reactors, ozone and membrane bioreactors), the hepatic CBR1 A and B mRNA expression was reduced compared to fish exposed to STP effluents with conventional treatment processes. It was hypothesised that more advance treatment technologies resulted in water with a decreased number and/or concentration of substances that may act on CBR expression.

- The CBR1 B mRNA was not induced in the livers of rainbow trout that were exposed to ethinylestradiol in a controlled water exposure

setup. Neither i.p. injections of norethisterone, methyltestosterone or hydrocortisone induced hepatic CBR1 A and B mRNA expression in rainbow trout. However, i.p. injections of β-NF and paraquat, which are known as stimulators of Ahr-XRE and/or Nrf2-ARE, induced hepatic mRNA expression in rainbow trout but the same substance did not alter enzymatic activity in rainbow trout. Additionally, i.p. injection of β-NF and paraquat in the sentinel coastal species eelpout induced CBR mRNA expression and enzymatic activity. This implies that chemicals that are known to be present in effluents of STPs might induce the expression of CBR.

- When CBR mRNA expression and enzymatic activity were analysed in eelpouts included in a comprehensive biomonitoring study in different coastal regions of Germany, Denmark and Sweden, there were no clear site-specific differences between fish that were caught at the polluted sites and fish caught in the reference areas of each region. This may indicate that concentrations of possible CBR1 inducers were too low to cause a response. It is also possible that CBR induction is not altered by chronic exposures and instead will only be induced at an acute phase; however, this has not been investigated.

In conclusion, the 2DGE system was useful for generating a hypothesis for a potential biomarker of PAHs and/or pro-oxidants. Future research with a recombinant CBR protein in parallel with knockdown methods (siRNA) would be valuable. With such tools, experimental setups to control for the specificity of CBR when using immunoblot and enzymatic assays can be performed. A recombinant protein could be used in studies to obtain more information regarding the different CBR inducers and possible inhibitors. This should be done in combination with chemical characterisation of effluent contaminates in the investigated STPs. Furthermore, a long-term exposure with a known inducer should be setup *in vivo* to measure whether hepatic CBR expression is an acute or chronic response. Moreover, the functional importance of having induced hepatic CBR has not been investigated in fish. This is of high interest and would increase our understanding of the enzyme and its possible role as a future biomarker.

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