

# **Oxidative Damage in Fish Used as Biomarkers in Field and Laboratory Studies**

## **Akademisk Avhandling**

för filosofie doktorsexamen i zoofysiologi som enligt naturvetenskapliga fakultetens beslut kommer att försvaras offentligt fredagen den 9 maj 2008, kl.10.00 i föreläsningssalen, Zoologiska institutionen, Medicinaregatan 18, Göteborg

av

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## Dissertation Abstract

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Bethanie Carney Almroth (2008). **Oxidative damage in fish used as biomarkers in field and laboratory studies.** Department of Zoology/Zoophysiology, University of Gothenburg, Box 463, 405 30 Göteborg, Sweden.

Many toxic xenobiotics entering into the aquatic environment exert their effects through redox cycling. Oxidative stress, incorporating both antioxidant defences as well as oxidative damage, is a common effect in organisms exposed to xenobiotics in their environment. The studies included in this dissertation evaluate the effects of different types of environmental pollution on oxidative stress biomarkers in teleost fishes. Effects of aging, alone or in combination with oxidative stress, on protein carbonylation were also addressed.

Antioxidant enzyme activities were measured in rainbow trout (*Oncorhynchus mykiss*) caged in a river polluted by sewage treatment plant (STP) effluent as well as highly contaminated sediment, and in corkwing wrasse (*Symphodus melops*) collected at heavy metal contaminated and PAH contaminated sites. Antioxidant enzymes showed very few changes in these fish. Glutathione levels were affected by STP effluent exposure in rainbow trout and by PAH exposure in corkwing wrasse.

Protein carbonylation was elevated in plasma of the corkwing wrasse captured at the heavy metal site and in plasma of rainbow trout caged near the STP effluent. Lipid peroxidation was elevated in the livers of these rainbow trout. Oxidative damage biomarkers were also measured in eelpout (*Zoarces viviparus*) captured in a polluted harbor, before, during and after a dredging campaign, as well as following an oil spill. Protein carbonyl levels in livers of eelpout were affected by exposure to pollution in the harbor, though this was measured both as increases and as decreases, indicating a complicated relationship between prooxidant exposure and protein carbonyl accumulation. Lipid peroxidation in eelpout was unaffected by pollutant exposure. Western blot analyses of protein carbonylation in corkwing wrasse exposed to heavy metals and in rainbow trout exposed to paraquat (PQ) suggest that albumin may be the plasma protein most likely to undergo carbonylation in these fish.

Brown trout (*Salmo trutta*) were used in a laboratory study to investigate the effects of aging on oxidative stress parameters, i.e. protein carbonylation, 20S proteasome activity and glutathione levels. All parameters were affected by aging, as was the response to PQ exposure, where 1 year old fish were more sensitive than 0+ fish.

To conclude, the results presented here indicate that antioxidant enzymes may not provide a good biomarker of exposure to xenobiotics in the field. However, oxidative damage products, i.e. protein carbonyls and lipid peroxidation, seem to be useful as biomarkers. Care should be taken to consider age of the exposed individuals. Oxidative stress is a complex phenomenon to measure in the field. Antioxidant enzyme activities may be altered after an acute exposure and then return to normal levels but damage products can persist even after the initial stress has stabilized.

**Keywords:** fish, oxidative stress, antioxidant enzymes, glutathione, protein carbonyls, lipid peroxidation, biomarker, pollution, aging

## List of Papers

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

### Paper I

Carney Almroth, B., Sturve, J., Berglund, Å., Förlin, L. (2005). Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. *Aquatic Toxicology*, 73. 171-180.

### Paper II

Carney Almroth, B., Sturve J., Stephensen, E., Holth, T.F., Förlin, L. (2008). Protein carbonyls and antioxidant defences in corkwing wrasse (*Symphodus melops*) from a heavy metal polluted and a PAH polluted site. (Accepted, *Marine Environmental Research*).

### Paper III

Carney Almroth, B., Albertsson, E., Sturve, J., Förlin, L. (2008). Oxidative stress, evident in antioxidant defences and damage products, in rainbow trout caged outside a sewage treatment plant. *Ecotoxicology and Environmental Safety*, doi:10.1016/j.ecoenv.2008.01.023.

### Paper IV

Carney Almroth, B., Johansson, A., Förlin, L., Sturve, J. (2008). Aging and oxidative stress in brown trout, *Salmo trutta*. (manuscript).

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## Abbreviations

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<b>4-HNE</b> 4-hydroxynonenal	<b>GSSG</b> oxidized glutathione
<b>AhR</b> aryl hydrocarbon receptor	<b>GST</b> glutathione <i>S</i> -transferase
<b>ATP</b> adenosine triphosphate	<b>H<sub>2</sub>O</b> water
<b>BSA</b> bovine serum albumin	<b>H<sub>2</sub>O<sub>2</sub></b> hydrogen peroxide radical
<b>BEEP</b> Biological Effects of Environmental Pollution	<b>IP</b> intraperitoneal
<b>CAT</b> catalase	<b>MDA</b> malondialdehyde
<b>Cd</b> cadmium	<b>mRNA</b> messenger ribonucleic acid
<b>Cu</b> copper	<b>Msr</b> methionine sulfoxide reductases
<b>CDNB</b> 1-chloro-2,4-dinitrobenzene	<b>MT</b> metallothionein
<b>CYP1A</b> cytochrome P450 1A	<b>NADPH</b> nicotinamide adenine dinucleotide phosphate (reduced)
<b>DCIP</b> 2,6-dichloroindophenol	<b>NADP<sup>+</sup></b> nicotinamide adenine dinucleotide phosphate (oxidized)
<b>DNPH</b> 2,4-dinitrophenylhydrazine	<b>O<sub>2</sub></b> oxygen
<b>DTD</b> DT-diaphorase	<b>O<sub>2</sub><sup>•-</sup></b> superoxide anion radical
<b>DTNB</b> 5,5'-Dithio-bis-(2- nitrobenzoic acid)	<b>OH<sup>•</sup></b> hydroxyl radical
<b>EE2</b> 17 $\alpha$ -ethinyl estradiol	<b>PAH</b> polycyclic aromatic hydrocarbon
<b>EROD</b> ethoxyresorufin- <i>O</i> - deethylase	<b>PCB</b> polychlorinated biphenyl
<b>G6PDH</b> glucose-6-phosphate dehydrogenase	<b>PUFA</b> poly-unsaturated fatty acid
<b>GCS</b> $\gamma$ -glutamylcysteine synthetase	<b>ROS</b> reactive oxygen species
<b>GPx</b> glutathione peroxidase	<b>SOD</b> superoxide dismutase
<b>GR</b> glutathione reductase	<b>STP</b> sewage treatment plant
<b>GS</b> glutathione synthetase	<b>TBARS</b> thiobarbituric acid reactive substances
<b>tGSH</b> total glutathione	<b>UDPGT</b> UDP glucuronyl transferase
<b>GSH</b> reduced glutathione	





# 1. Introduction

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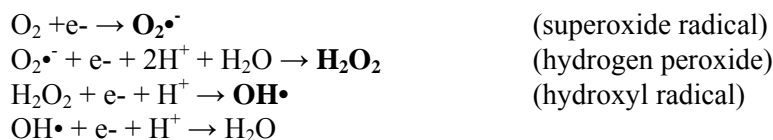
Marine and aquatic environments are home to a vast diversity of organisms ranging from prokaryotes to higher vertebrates. They are also of enormous direct importance to humans, providing essentials such as water and food as well as transportation, economic opportunities, recreation, and so on. Unfortunately, these environments also act as sinks for a great variety of anthropogenic pollutants, many of which are toxic. Sources include burning of fossil fuels, industrial waste water, sewage treatment plants, agriculture and non point source runoff, etc. It is important to have an understanding of the impact and effects of these chemicals on aquatic life forms. This knowledge will help us to identify sources of contamination, can provide governing bodies with information useful in management of aquatic ecosystems, information on which to base regulations concerning usage and handling of chemical compounds, and essentially help to protect aquatic ecosystems from the negative effects of anthropogenic activities.

Often times, xenobiotics entering into the environment exert their effects through their ability to redox cycle (one electron reduction and oxidation reactions). This may be intended in order to gain maximum effect for the xenobiotics' purposes or may just be a byproduct of their chemical structure. Negative side effects include the formation of reactive oxygen species with the ability to damage cellular molecules. These polluting compounds will often retain these qualities in the aquatic environment as well, with the ability to cause oxidative stress in organisms. The physiological systems of many aquatic organisms, which are in place to metabolise these compounds and resulting byproducts, are evolutionarily similar to those in humans. This thesis focuses on the effects of xenobiotics on oxidative stress in teleost fish.

## 1.1 Oxidative stress

The atmosphere of the Earth was originally highly reducing and the biota was dominated by anaerobic microbes. The abundance of carbon dioxide, water and solar energy allowed cyanobacteria to evolve and utilize photosynthesis, resulting in the production of molecular oxygen (Lesser 2006). When atmospheric oxygen became available to cellular organisms 2.5 billion years ago, it allowed for a more effective metabolic pathway. Aerobic respiration was more efficient and had greater energy yields but was, at the same time, more toxic. The molecular form of oxygen is a fairly unreactive molecule (dioxygen, O<sub>2</sub>). It can however be converted to free radicals, or reactive oxygen species (ROS), which are very reactive molecules possessing an unpaired electron. These are produced continuously in cells as byproducts of aerobic cellular metabolism, largely via leakage from the

electron transport chain in mitochondrial respiration. Fish, as all other aerobic organisms and their ancestors, use the reduction of molecular oxygen as the source of energy that will be stored in adenosine triphosphate (ATP) molecules. These will provide energy for the vast majority of cellular processes including biosynthetic reactions, motility and cell division. This reduction of molecular oxygen occurs in four steps in the mitochondrial electron transport chain, each of which results in the formation of an intermediate molecule, a free radical.



Other sources of ROS include irradiation, UV light, production of  $\text{H}_2\text{O}_2$ , NO and  $\text{O}_2^{\bullet -}$  by activated macrophages and phagocytes, metal catalyzed oxidation systems, air pollutants, and autooxidation of electron transport carriers (Stadtman and Levine 2000). ROS have historically been thought only to cause tissue damage and disease, but researchers are now beginning to recognize the importance of ROS in maintaining normal cellular activity including inter- and intracellular signalling (Halliwell and Gutteridge 1999, Dröge 2002, Hensley and Floyd 2002).

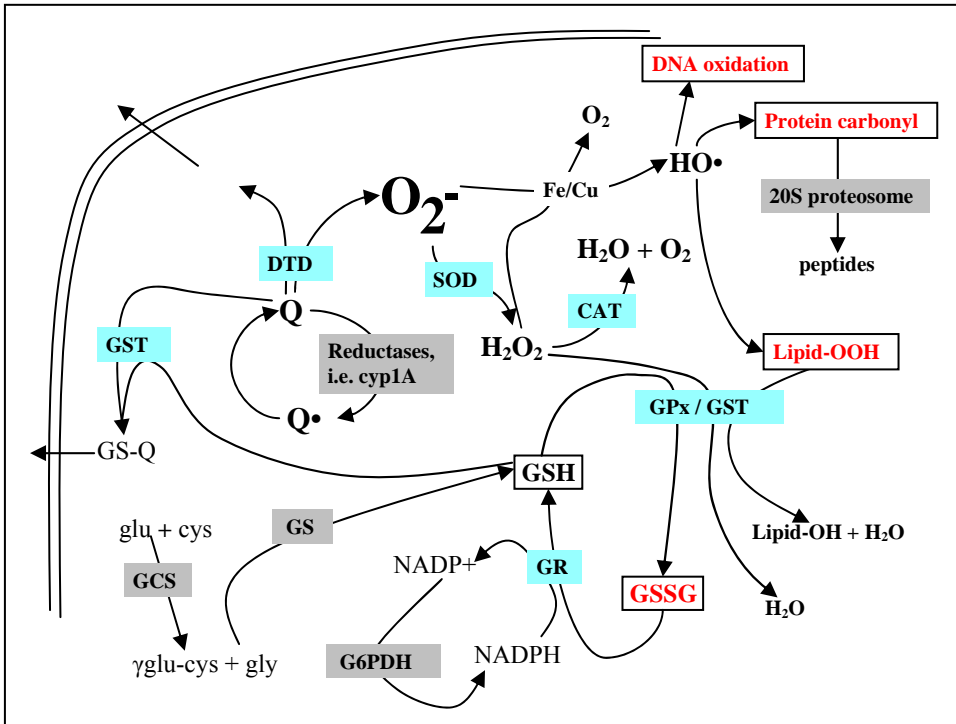
Aerobic organisms have developed a comprehensive antioxidant defence system, comprising both molecular and enzymatic defences, against the dangers of oxygen radicals (Halliwell and Gutteridge 1999), thereby preventing excess oxidation and damage. Mechanisms of defence include enzymatic inactivation of molecular peroxides and redox cycling compounds, enzymatic metabolism of ROS to water and molecular oxygen, molecules that scavenge free radicals and proteins that bind transition metals which could otherwise react with less deleterious ROS resulting in more damaging reactive oxygen species. A disturbance in the balance between the prooxidants and antioxidants leading to detrimental biochemical and physiological effects is known as **oxidative stress**. This is a harmful condition in which increases in free radical production, and/or decreases in antioxidant levels can lead to potential damage. Indicators of oxidative stress include changes in antioxidant enzyme activity, damaged DNA bases, protein oxidation products, and lipid peroxidation products. Oxidative stress is known to play a large role in the pathology of several human diseases, including atherosclerosis, pulmonary fibrosis, neurodegenerative diseases (i.e. Parkinson's, Alzheimer's) and cancer, as well as aging pathology (Thannickal and Fanburg 2000).

### 1.1.1 Antioxidant defences

#### *Enzymatic defences*

The removal of xenobiotics, and even some endogenous substances, from the cell is catalyzed by a number of different enzymes, so called phase I and II enzymes. Phase I enzymes are involved in xenobiotic biotransformation via the introduction of a polar moiety which renders a lipophilic contaminant more hydrophilic. The cytochrome P450 (CYP) family is the most well studied in fish, especially CYP1A. CYP1A serves to increase the solubility of hydrophobic molecules through a reduction reaction involving an oxygen molecule. It is inducible by a number of xenobiotics, for example PAHs and planar PCBs, via binding to the aryl hydrocarbon receptor (AhR) which acts as a transcription factor. Phase II enzymes are involved in conjugating metabolised xenobiotics to endogenous molecules, thereby easing excretion. Examples of phase II enzymes commonly used in biomonitoring programs involving fish include glutathione S-transferase (GST) and UDP glucuronyl transferase (UDPGT) (Halliwell and Gutteridge 1999).

The activity of phase I enzymes can lead to an increase in ROS production or the generation of reactive, redox cycling intermediates. Antioxidant enzymes facilitate the removal of these reactive chemical intermediates and resulting ROS molecules. The action of CYP1A can result in the production of  $O_2^{\bullet-}$  which in turn can be metabolized by superoxide dismutase (SOD) to  $H_2O_2$ . This hydrogen peroxide molecule can then be reduced to  $H_2O$  and  $O_2$  by catalase (CAT). Hydroxyl radicals ( $OH^{\bullet}$  ions) can form from both  $H_2O_2$  and  $O_2^{\bullet-}$  via reactions with redox cycling metal ions, for example iron and copper. This highly potent hydroxyl radical can attack both protein and lipid molecules to form oxidative damage products. Glutathione peroxidase (GPx) can reduce lipid peroxides to their respective alcohols and water. DT diaphorase (DTD) can react directly with redox cycling xenobiotics, i.e. quinones, thereby reducing them in a two electron reaction which stops the redox cycle and facilitates removal (Dinkova-Kostova and Talalay 2000). A schematic diagram showing oxidative stress, including the antioxidant defence system, is shown in figure 1. Glucose-6-phosphate dehydrogenase (G6PDH) is involved in the production of NADPH, the molecule that provides the reducing equivalents to many of the antioxidant enzymes mentioned above. G6PDH is often measured in studies focusing on antioxidant enzymes since NADPH is essential for their function (Halliwell and Gutteridge 1999).



**Figure 1:** Schematic diagram of oxidative stress. Antioxidant enzymes are shown in blue boxes, other enzymes in gray, oxidized molecules are shown in red text. Q represents a redox cycling compound.

### *Molecular defences*

There are a number of molecules that function as scavengers of free radicals. These include the well known dietary antioxidant molecules ascorbate (vitamin C), retinoic acid (vitamin A) and tocopherol (vitamin E) (Dalton et al. 1999). However, one of the most abundant and most important molecular antioxidants in cellular cytoplasm is glutathione (GSH). GSH, along with metallothionein (MT), are the two most well studied antioxidant molecules in fish. GSH is a low molecular weight thiol. It can react directly with ROS species, thereby detoxifying them. In addition, GSH is used as a conjugating molecule by GST to ease excretion of xenobiotics. GSH is also used as a reducing equivalent in the metabolism of reactive intermediates, for example reduction of lipid peroxides by the action of glutathione peroxidase (GPx). These reactions can result in the production of oxidized glutathione (GSSG). The balance between GSH and GSSG can be restored by glutathione reductase (GR). Levels of total glutathione (tGSH), GSSG,

%GSSG and GR activity have all been proposed as biomarkers of oxidative stress in fish (Stephensen et al. 2002).

Levels of glutathione within the cell are regulated via the activity of GR and via *de novo* synthesis of GSH, which occurs in a two step process involving glutathione synthetase (GS) and  $\gamma$ -glutamylcysteine synthetase (GCS). These are shown in figure 1. GSH can also be excreted from the cell either as a glutathione S-conjugate, i.e. a glutathione-bound intermediate metabolite from phase I reactions, or as GSSG, often produced during oxidative stress (Kepler 1999). Decreased levels of glutathione in the cytoplasm or an increase in the ratio of GSSG to GSH resulting from an increase in ROS can lead to transcription of redox sensitive genes. This occurs via initiation of a signal cascade resulting in assembly of transcription factor subunits in the nucleus. Initiation of transcription of such genes as SOD and CAT are known to occur in this manner (Thannickal and Fanburg 2000, Dröge 2002).

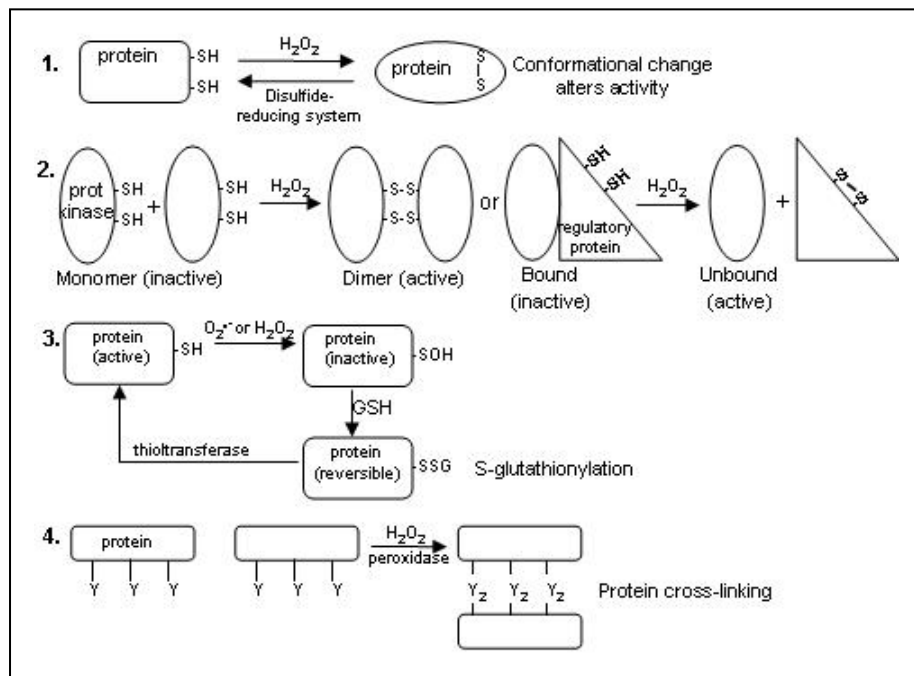
Metallothioneins (MTs) are small ubiquitous proteins known to be involved in metal homeostasis, including metabolism and detoxification. Free metals entering into the cell, generally via transport proteins or ion channels, can interact with ligands or MTs. Synthesis of MT is in part regulated by free metal ions, which bind to MT transcription factors which in turn bind to metal-responsive elements in the promoter region of the MT gene (Di Giulio et al. 1995). MT can exhibit antioxidant capacities indirectly through chelation of heavy metals that otherwise have the potential to interact with enzymes and respiratory protein complexes within mitochondria, thereby disrupting their function, uncoupling oxidative phosphorylation and causing oxidative stress (Di Giulio et al. 1995).

Metallothionein was first noted for its metal scavenging capacity, but has more recently been recognized as an important antioxidant (Sato and Kondoh 2002). This is in part due to its many cystein residues which can readily be oxidized (Palmiter 1998). MTs have been shown to scavenge a wide range of ROS including superoxide, hydrogen peroxide, hydroxyl radical and nitric oxide at high concentrations in mice. Evidence indicates that MT is important in regulating cell signaling in mice through interactions with these oxygen radicals (see Sato and Kondoh 2002).

### 1.1.2 Protein oxidation

Oxidation of proteins is of importance in regulating protein function within the cell. Many proteins undergo regulatory steps altering their oxidative status prior to release from the endoplasmic reticulum or Golgi apparatus. For example, proteins may undergo phosphorylation, acetylation and methylation of lysine residues, or thiolation and redox regulation of cystein and tyrosine residues, as important posttranslational modifications. Protein modifications can also take place in the

cytoplasm, either as a result of the action of regulatory enzymes or ROS.  $H_2O_2$  can induce intra-molecular disulfide linkages or protein dimerization via inter-molecular disulfide linkages. Protein kinases may dimerise or bind/unbind regulatory proteins, thereby altering activity (Thannickal and Fanburg 2000). See fig. 2.



**Figure 2:** Oxidation of protein thiols by ROS, adapted from Thannickal and Fanburg (2000). 1. Modification of protein by oxidation of cystein residues, 2. Formation of intra-molecular disulfide links, 3. Formation of inter-molecular disulfide links, 4. Dityrosine formation by interaction with hydrogen peroxide.

Oxidation of proteins via ROS, followed by inactivation and subsequent proteolytic degradation, was described in detail in glutamine synthetase from gram negative bacteria (Levine et al. 1981). This oxidation was catalyzed by two different model systems, mammalian CYP450 or ascorbic acid and oxygen. The reactions were inhibited by activity of the catalase enzyme. This article was the first, to my knowledge, that implicate CYP450 in protein oxidation and turnover within the cell. Levine (1983) later identified the alteration in glutamine synthetase as an oxidative modification on one histidine residue, which resulted in inactivation of the enzyme.

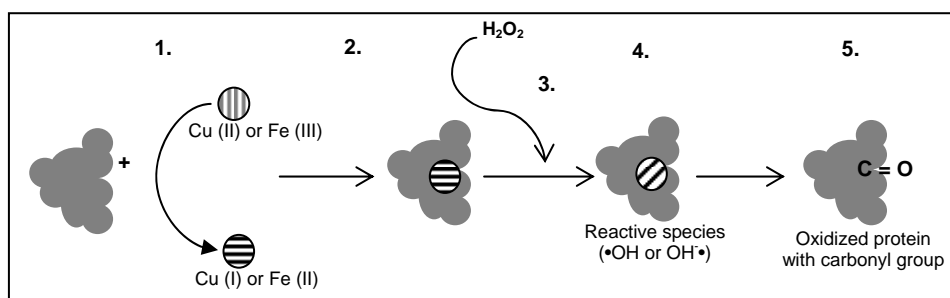
It is now well established that protein damage can occur as a result of oxidative stress. The balance between prooxidant and antioxidant factors will be mirrored in the amount of protein oxidation that occurs. The radical-mediated damage to proteins may be a result of electron leakage during normal metabolism, xenobiotic-induced activity of the CYP450 enzyme system, metal-catalyzed oxidation reactions or autooxidation of lipids and sugars as well as dietary and other environmental factors (i.e. radiation), which result in increased ROS production. ROS are known to convert amino groups of proteins and thereby alter protein structure or function, leading to changes in enzyme activity, receptor function, transport proteins and signal transduction pathways. This disruption can eventually lead to cell death.

### *Protein carbonylation*

Oxidation of proteins can occur in several manners, where protein carbonylation is the most widely studied and accepted as a marker of oxidative stress. This is largely due to the fact that carbonylation is an irreversible alteration, and therefore not transient, which requires proteolytic degradation of the protein. Protein carbonylation involves the formation of carbonyl moieties ( $-C=O$ ) at amino acid side chains. Amino acid residues most susceptible to this sort of oxidative damage include leucine, valine, isoleucine and proline (Grune 2000). Stadtman and Levine (2000) have also shown that lysine, arginine and proline are sensitive to carbonylation. An increase in the number of carbonyl groups correlates well to protein damage caused by oxidative stress (Shacter et al. 1994). Protein carbonylation has been shown to play an important role in a number of human diseases and much research has focused on these issues. Requena et al. (2003) showed that 55-100% of the carbonyl value obtained from measurements is derived from specific chemical structures resulting from, for example metal-catalyzed reactions or other biochemical pathways. This indicates that measurement of protein carbonyls via carbonyl-specific reagents such as 2, 4-dinitrophenylhydrazine is a valid means of assessing total protein oxidation.

Carbonyl groups can be introduced in proteins by a number of different pathways, predominantly via metal-catalyzed oxidation (MCO) but also via adduction of oxidized lipids or sugars containing carbonyls (Requena et al. 2003). In the case of MCO reactions, an electron donor system is needed to catalyze the reduction of  $O_2$  to  $H_2O_2$  and of Fe(III) to Fe(II). Depending on the system, these reactions may occur in one or two steps. Superoxide anions can react directly with Fe(III) to produce the reduced form of the metal. The Fe(II) ion then binds to the metal binding site on the protein followed by a reaction with  $H_2O_2$ . This reaction generates  $\bullet OH$ ,  $OH\bullet$  and Fe(III). Some amino acid residues will then form carbonyl groups. This mechanism is shown in figure 3. The active oxygen species

react locally with the amino acid side chains at the metal binding site (Stadtman and Oliver 1991). The resulting modified protein is susceptible to degradation, and may lose some or all of its function.



**Figure 3:** Metal catalyzed oxidation reaction resulting in carbonyl group on protein molecule. 1. Reduction by electron donor system, 2. Metal binds to metal binding site on protein, 3. Metal ion reacts with  $H_2O_2$ , 4. ROS is generated, metal is oxidized, 5. Protein is carbonylated.

Protein carbonyls can also form via secondary mechanisms resulting from reactions of free radicals with other cellular constituents, such as lipids, carbohydrates and nucleic acids (Grune 2000). For example, the lipid peroxidation product 4-hydroxynonenal (4-HNE) can react with the lysyl, histidyl or sulfhydryl groups of proteins to yield a derivative with aldehyde functions (Berlett and Stadtman 1997). Malondialdehyde (MDA) contains two aldehyde groups which can react with lysine  $\alpha$ -amino groups of proteins to produce carbonyl groups. Additionally, interaction with reducing sugars or dicarbonyl compounds derived from the sugars can lead to the formation of protein carbonyl derivatives (Stadtman and Levine 2000). In more recent years, protein carbonylation has been used as a biomarker of xenobiotic effects in fish. Bagnyykova et al. (2006, 2007) have measured this parameter in gold fish exposed to iron and arsenite and Craig et al. (2007) have measured protein carbonyls in zebrafish exposed to copper.

#### *Additional forms of protein oxidation*

Other oxidation products include protein S-thiolation, the formation of protein methionine sulfoxide derivatives, 2-oxohistidine formation from oxidized histidine, and conversion of tyrosine residues to dihydroxy derivatives (Stadtman and Levine 2000). Tyrosine residues can be modified by peroxynitrate (PN), a molecule that is produced endogenously by reaction of nitric oxide with the superoxide anion. This nitric oxide is a normal product of arginine metabolism (Berlett and Stadtman 1997). PN conversion of tyrosine residues interferes with the ability of the protein



molecules to undergo phosphorylation and dephosphorylation, a process that is essential in many signal transduction pathways (Stadtman and Levine 2000). PN can also oxidize methionine residues within proteins, thereby converting them to their adenylated form and changing regulatory functions. Thiol groups on proteins that can become oxidized may react with GSSG, thereby forming mixed disulphides which can inactivate the protein (Halliwell and Gutteridge 1999). Decreases in protein thiol groups have been correlated with decreases in enzyme activity and life expectancy (Agarwal and Sohal 1994).

The oxidation of proteins themselves has an antioxidant function within the cell. Methionine residues are particularly sensitive to oxidation by ROS. Met residues are converted to methionine sulfoxide. This type of protein oxidation is reversible, a reaction catalyzed by methionine sulfoxide reductase through the use of NADPH equivalents (Stadtman and Levine 2000). ROS-mediated oxidation of methionine residues results in an inactive form of ROS molecules (Stadtman 2006). Cystein residues within proteins can undergo oxidation/thiolation which plays a critical role in the regulation of protein function under conditions of oxidative stress. Cystein residues are converted to disulfides, even under conditions of mild oxidative stress, but can be restored through the action of disulphide reductases (Berlett and Stadtman 1997). Figure 2 shows oxidation of protein thiols by interaction with ROS.

### *Degradation of oxidized proteins*

The formation of carbonyl derivatives is non-reversible and increases the susceptibility of proteins to proteases. Some other forms of protein oxidation, for example *S*-thiolation and methionine sulfoxide derivation, are reversible (Stadtman and Levine 2000). There are also two known superfamilies of enzymes (aldo- keto reductases and short-chain dehydrogenases) that reduce carbonyl groups to corresponding alcohols (Maser 1995). The enzyme carbonyl reductase, for example, catalyzes the reduction of carbonyl compounds. The ubiquitous nature of this enzyme and its metabolism of activated carbonyl groups suggest that it play an important role in detoxification of endogenous as well as xenobiotic compounds.

The proteosomal system in the cytoplasm, nucleus, and endoplasmic reticulum is largely responsible for the breakdown of oxidized proteins. Mild oxidation of soluble proteins enhances their proteolytic susceptibility while it appears that severely oxidized proteins may be stabilized due to aggregation, crosslinking and/or decreased solubility (Grune et al. 2003). At moderate oxidant concentrations, the degradation of damaged proteins increases whereas higher oxidant concentrations can inhibit proteolytic degradation. This is especially prevalent in insoluble, globular proteins. As a result of the relationship between oxidant concentration and proteolytic activity and the very tight 'optimal oxidant

concentration', it can be difficult to interpret both *in vitro* and *in vivo* results from experimentation. Most protein degradation occurring in the cell is mediated by the 26S proteasome which consists of a 20S core proteasome with a 19S regulatory subunit at each end. This occurs after initial ubiquitination of the protein. Several enzymes are involved in 'tagging' lysine residues with a ubiquitin tail which is then recognized by the 19S subunit. The protein is de-ubiquitinated and unfolded, an energy demanding process, before being broken down by the 20S proteasome core, a process which requires no ATP hydrolysis.

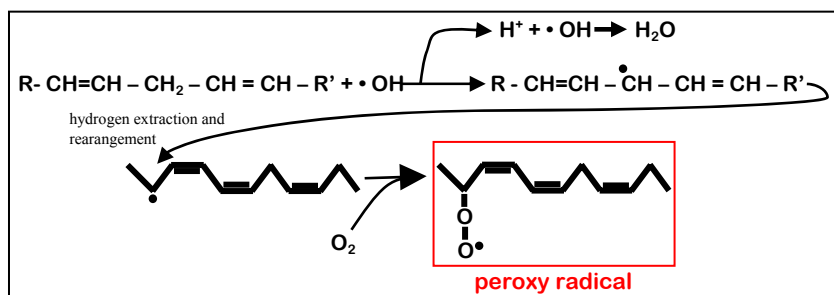
Oxidized proteins, however, appear to be relatively poor substrates for ubiquitination, and this system does not appear to be involved in the recognition or targeting of oxidatively damaged proteins for degradation. Studies of degradation of oxidized protein indicate a lack of ATP involvement, thereby indicating that the ubiquitination system is not involved. The hydrophobic patches on oxidized proteins, which are revealed as proteins sustain oxidative damage, can bind directly to the 20S proteasome core cylinder without ATP-demanding unfolding (Grune et al. 2003). This 20S core proteasome is also present at higher concentrations than the 26S proteasome within the cell; it exists in concentrations 2-3 fold those of the 19S or 26S subunits in living cells, and functions without binding regulatory proteins. Unlike the 26S proteasome, the 20S is relatively stable under conditions of mild oxidative stress (Reinheckel et al. 1998). Reinheckel et al. showed that activity of the 26S proteasome was diminished at lower H<sub>2</sub>O<sub>2</sub> concentrations than the 20S proteasome was. Regulation and stabilization of the 20S proteasome take place during oxidative stress, probably via the regulatory protein PA28 (Strack et al. 1996). This implicates 20S as a secondary defence mechanism against the effects of oxidative stress. Recognition of the damaged protein by the 20S proteasome is thought to be facilitated by partial unfolding of the protein following oxidation, thereby revealing previously covered hydrophobic moieties (Berlett and Stadtman 1997).

### 1.1.3 Lipid peroxidation

Lipid damage can occur as a result of oxidative stress or a disruption in the balance between prooxidant and antioxidant factors. Reactive oxygen species are known to extract hydrogen atoms from unsaturated bonds thereby altering lipid structure or function. This extraction process is easier in poly-unsaturated fatty acid (PUFAs) due to the close proximity of the unsaturated bonds, which allows for an easier abstraction of hydrogen atoms from a methylene group. Membrane fluidity, and thereby organelle function and cell health, can be affected (Halliwell and Gutteridge 1999). The lipid peroxidation process also affects biomolecules associated with the membrane, i.e. membrane bound proteins or cholesterol, and may be of importance in fish as their membranes contain a higher degree of PUFA

than other vertebrates. This is in part a result of homeoviscous adaptation during thermal acclimation, where an increase in PUFAs helps maintain membrane fluidity at lower temperatures (Monserrat et al. 2007).

Lipid radicals are formed with the abstraction of a hydrogen atom from an unsaturated fatty acid. Double bonds are rearranged to form dienes. Attack by molecular oxygen or hydroxyl radical produces a lipid peroxy radical which can in turn abstract a hydrogen atom from an adjacent lipid. This forms a lipid hydroperoxide in a chain reaction event. The extracted hydrogen reacts with a hydroxyl radical to form water. This mechanism is shown in figure 4. The resulting peroxy radical is then capable of reacting with adjacent lipids in a chain reaction event causing accumulation of damaged lipids. This reaction will continue until it is broken by interference of an antioxidant, for example tocopherol (vitamin E) or the enzymatic action of GPx.



**Figure 4:** Mechanism for the formation of lipid peroxide

Malondialdehyde (MDA) is a well characterized oxidation product of PUFAs. The TBARS (thiobarbitic acid reactive substances) method of quantifying lipid peroxidation in samples measures this end product. Another well known end product of lipid peroxidation is 4-hydroxynonenol (4-HNE), which is readily formed in the presence of H<sub>2</sub>O<sub>2</sub> via a reaction with iron. 4-HNE is a cytotoxic compound that results from oxidative stress, and is known to accumulate in several diseases, including Alzheimer’s disease, as well as in aging cells (Lovell et al. 1997). This lipid damage molecule is known to interfere with normal cell homeostasis in several ways including inhibition of 20S proteasome, thereby blocking protein metabolism (Grune et al. 2004), and interference in GSH pathways (Lu 1999).

Often, mitochondria will sustain oxidative damage to their lipid membranes over time, due largely to the high rate of production of ROS at the site of the electron transport chain. Old and damaged mitochondria are subsequently degraded in lysosomes. Lipofuscin is a cellular waste molecule composed mainly of lipids and proteins that originate from the incomplete lysosomal degradation of defective

mitochondria (Gray and Woulfe 2005). Lipofuscin is thought to result primarily from oxidation of unsaturated fatty acids in the mitochondrial membranes. These molecules are known to contain protein oxidation products, sugars and metals in addition to lipids. These molecules accumulate in a nearly linear fashion in post mitotic cells (Terman and Brunk 2006), with rapid accumulation in short lived species and slow accumulation in long lived species. Lipofuscin is known to accumulate at higher rates in fish and other aquatic organisms when they have been exposed to environmental contaminants (Winston 1991) and measurement could provide useful in situations of chronic exposure.

#### 1.1.4 DNA damage

There are many types of damage that can occur in DNA molecules as a result of the endogenous production of ROS, interactions with xenobiotics, irradiation, etc. The most prevalent causative agent of oxidative DNA damage is the  $\bullet\text{OH}$  ion, which can be produced as a result of a reaction between  $\text{O}_2\bullet^-$  and iron (Beckman and Ames 1997). Damage products include double strand breaks, single strand breaks, inter- and intra-strand crosslinks, adducts and DNA-protein crosslinks, to name a few (Wood et al. 2001). DNA damage by xenobiotics occurs in three basic steps, where the first is the formation of adducts with toxic molecules. The next stage, secondary modifications of DNA, includes single and double strand breakage, changes in DNA repair, base oxidation and crosslinks. Xenobiotics may induce these secondary modifications via ROS production. In the third stage, cells show altered function which can lead to cell proliferation and consequently cancer (Montserrat et al. 2007). Those DNA modifications most commonly used as biomarkers in studies addressing effects of xenobiotic exposure in fish include oxidative base damage and the formation of DNA adducts.

##### *Oxidative base damage*

More than 50 types of DNA oxidized base pairs have been identified, 8-oxoguanine (8-oxoG) being the one studied most. Some types of oxidative DNA products are mutagenic. The formation of 8-oxoG will result in replacement of a G:C base pair with a T:A base pair after two replication cycles since this type of damage allows guanine to aberrantly pair with adenine (Ohno et al. 2006). Major pathways involved in repair of DNA damage include DNA base excision repair, nucleotide excision repair and mismatch repair, all well studied in humans (Frosina 2001). Sussman (2007) showed that zebrafish have a much greater capacity to repair DNA damage induced by, for example, UV-irradiation, than humans and rats. This species would therefore be less sensitive to DNA damage induced by

exposure to environmental pollution. In most other fish species, however, the rate of DNA repair is slower than in mammals (Mitchelmore and Chipman 1998).

The prevalence of 8-oxoG in fish has been associated with oxidative stress, for example in gilthead seabream (*Sparus aurata*) (Rodriguez-Ariza et al. 1999). Fish that were exposed to urban and industrial pollution showed significant increases in 8-oxoG levels in liver, indicating that environmental pollution may have overwhelmed antioxidant defences, thereby resulting in damage (Rodriguez-Ariza et al. 1999). Xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs), can cause oxidative damage to DNA strands. PAHs are a byproduct of combustive processes and are almost ubiquitous in the environment, are initially activated *in vivo* via reductase systems, including for example CYP1A. They can produce ROS as a secondary effect, a consequence of the compounds' ability to redox cycling (Mitchelmore and Chipman 1998).

### *DNA adducts*

A DNA adduct is a form of DNA damage in which another molecule, for example a xenobiotic, is covalently bound to the DNA strand. This action is one of the central causes of tumorigenesis by carcinogenic compounds. PAHs are a very well studied group of carcinogenic compounds that are known to bind to DNA strands. Adduct formation leads to activation of DNA repair processes. If DNA adducts are not repaired/removed before replication, they can potentially result in the formation of site specific mutations, i.e. nucleotide substitutions or deletions, chromosomal rearrangement and potentially tumor growth (Mitchelmore and Chipman 1998).

DNA adducts have been studied in fish exposed to xenobiotics, as investigations of genotoxic compounds are deemed highly relevant. They can be quantified using the comet assay (or single cell gel electrophoresis) which is a method often used to measure DNA strand breakage in environmental studies. Increases in DNA adducts and subsequent strand breakage has been associated with decreased fitness in fish and other aquatic organisms (Mitchelmore and Chipman 1998) and are often measured in animals collected at polluted field sites. For example, eelpout collected in Göteborg harbor at contaminated sites, had higher levels of strand breakage in their erythrocytes than fish collected at reference sites (Frenzilli et al. 2004). DNA adducts have also been induced in, for example, brown trout exposed *in vivo* to benzo[a]pyrene under laboratory conditions (Mitchelmore and Chipman 1998).

### 1.1.5 Reactive oxygen species and cell signalling

The chemical environment within the cell is predominantly a reducing one. This is maintained via enzymatic action, an energy demanding process. ROS are utilized by the cell under normal conditions as signalling molecules (Dröge 2002). Much of this evidence comes from studies using prokaryotic models. For example, SOD has been shown to be inducible, via a number of protein oxidation and gene regulation steps, by the superoxide anion,  $O_2^{\bullet-}$  (Thannickal and Fanburg 2000). ROS have also been shown to be essential messenger molecules in higher organisms, including fish.  $O_2^{\bullet-}$  controls ventilation, muscle relaxation, immunological functions and oxidative stress responses (Dröge 2002). ROS can induce heat-shock carrier proteins, nuclear factors and the cell-cycle gene p53 (Lesser 2006). Nitric oxide ( $NO^{\bullet}$ ) is another essential reactive molecule involved in cell signaling, for example in control of vascular tone (Dröge 2002).

Cellular sources of ROS include both enzymatic and non-enzymatic reactions.  $O_2^{\bullet-}$  can be produced in higher organisms, via the action of oxidases present in the plasma membrane of phagocytic cells, a required function of host defence, or as a toxic byproduct of electron transfer reactions.  $H_2O_2$  is produced in high quantities in peroxisomes. Mitochondrial-derived ROS are thought to play a role in apoptosis through interactions with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 (IL-1). ROS derived from the reductase action of enzymes in the endoplasmic reticulum regulate protein folding and secretion (Thannickal and Fanburg 2000). A number of receptor molecules present in the cellular membrane, namely cytokine receptors, are involved in ligand-induced ROS production. Here, cytokines such as TNF- $\alpha$ , IL-1 and interferon- $\gamma$  are among the best studied. TNF- $\alpha$  activates the transcription factor nuclear factor (NF)- $\kappa$ B, but this activation can be inhibited by the action of SOD (Thannickal and Fanburg 2000).

As mentioned in section 1.1.1, glutathione plays an important role in the redox balance of the cell. The ratio between GSSG and GSH is a reflection of this. A decrease in intercellular GSH levels, or an increase in GSSG levels can initiate a series of phosphorylation/ dephosphorylation reactions in a protein kinase cascade pathway. This pathway results in the assembly of c-FOS and c-JUN in the nucleus, thereby building activator protein 1 (AP-1), a transcription factor that is redox-sensitive. AP-1 binds to a transcriptional control element on the DNA. Both ligand induced and exogenous oxidants have been implicated in AP-1 activation (Thannickal and Fanburg 2000). 4-HNE is known to induce oxidative stress, through depletion of GSH, induction of intracellular peroxide production and formation of 4-HNE-protein adducts. 4-HNE induces phosphorylation of c-Jun and consequently the transcription of redox-sensitive genes (Dalton et al. 1999).

Other ROS regulated transcription factors bind according to their specificity to a number of different sites on the DNA strand, for example the antioxidant

response element (ARE) or the electrophile response element (EpRE). The ARE/EpRE is a cis-acting regulatory element found in 5'-flanking regions of numerous genes including DTD, GSTs and GCS (Dalton et al. 1999, Dröge 2002).

Xenobiotics that affect ROS production in the cell, either directly through redox cycling or indirectly via leakage from reactions catalyzed by reductases, can interfere with signalling within the cell. In some cases this may be adaptive, i.e. in cases where SOD and/or CAT are induced thereby increasing antioxidant defences and counteracting ROS activity. In other cases, this can be detrimental, resulting in disturbances in cell cycle regulation and/or apoptosis.

## 1.2 Oxidative stress in aging

A lot of work in more recent years has been focused on cellular and organismal aging, but this research is often based on the free radical theory of aging put forth by Harman in 1957 (Harman 1957). This theory states that “the progressive accumulation of deleterious changes in the cells and tissues of an individual increase the risk of death and disease with increasing age and that these deleterious changes are a result of oxidative stress.” It is approximated that 1-2% of oxygen molecules utilized in the mitochondrial electron transport chain leak in the form of superoxide radicals. The free radical theory of aging is related to the rate of living theory of aging. The rate of living theory, stemming from ancient times, states that humans as well as other organisms have a finite number of breaths, heartbeats or other measures, and that the body deteriorates in direct proportion to its use. The more modern version of this rate of living theory focuses on the rate at which an organism metabolizes oxygen, i.e. standard metabolic rate determines maximum life span (Sohal et al. 2002).

Evidence has been accumulated to support the free radical theory of aging. This includes studies in *C. elegans*, where a mutation known as age-1, resulting in age related increases in SOD and CAT, extends the life span of the individual (Larsen 1993). These two enzymes are active in metabolizing the superoxide anion and hydrogen peroxide, two ROS that are known to induce oxidative damage. Similar evidence has been found in a mutant strain of *Drosophila melanogaster*, where again, over expression of SOD and CAT increase the life span of mutant individuals (Orr and Sohal 1994). Caloric restriction has been shown to increase life span in *D. melanogaster* as well as in a number of model vertebrates (mice and rats) (Sohal and Weindruch 1996, Harman 2003). This is thought to occur through a reduction in the rate of mitochondrial production of  $O_2\cdot^-$  and  $H_2O_2$ . Evidence also shows an age-related accumulation of transition metals, such as iron and copper, which could be responsible for converting less deleterious ROS to more biologically active radicals in older individuals (Hagen 2003).

Three types of aging have been identified in fish. These include 1) rapid - pacific salmon exhibit rapid senescence and sudden death after spawning, 2) gradual - i.e. guppies, Japanese medaka which continue to grow, though at decreased rates, throughout their life span, and 3) negligible - carp and rockfish shown indeterminate growth with no increased mortality. Therein differ fish from mammals (Patnaik et al. 1994, Kishi et al. 2003, Kishi 2004). The mechanisms controlling these differences are not fully understood.

### 1.2.1 Protein oxidation in aging

In accordance with the free radical theory of aging, protein carbonyls have been shown to accumulate with age in a large variety of organisms, ranging from prokaryotic yeast cells (Berlett and Stadtman 1997) and *E. coli* (Nyström 2002), to bivalves (Philipp et al. 2005a, 2005b), mice (Hernebring et al. 2006) and humans (Berlett and Stadtman 1997). These damaged cytosolic proteins constitute one of the factors contributing to the 'garbage' that accumulates in long-lived post-mitotic cells (i.e. myocytes and neurons) which can interfere with and lead to decreased cellular function, senescence and death (Davies and Shringarpure 2006, Terman and Brunk 2006). The accumulation of protein carbonyls with age occurs in an exponential manner (Davies 1987, Stadtman 2006), slowly to begin with and then more rapidly as the individual ages.

As mitochondria are the largest source of free radicals within in the cell, they are also the main target of oxidative injury and decay (Hagen 2003) which in turn leads to an increase in ROS leakage and an increase in oxidative damage to cellular molecules, i.e. protein carbonyls. Long lived arctic mud clams (*Laternula elliptica*) are able to maintain cellular redox status and avoid an increase in ROS leakage from mitochondrial membranes as they age, resulting in lower accumulation rates of protein carbonyls compared to temperate counterparts. The higher rate of oxidative protein damage accumulation in *Mya arenaria* is also correlated to a shorter life span (Philipp et al. 2005b).

Age related accumulation of protein carbonyls has been studied in zebrafish (Kishi et al. 2003), though to my knowledge, not extensively in other species of teleost. Different types of aging are necessary to consider when interpreting measurements of protein carbonylation in different fish species as different species may have different patterns of accumulation. In field sampling situations where protein carbonyls may be applied as a biomarker of xenobiotic exposure, age of the individuals is of importance. Age of the individual could not only affect steady-state protein oxidation levels, but also responses to oxidative stress.



### 1.2.2 The 20S proteasome

In addition to an increase in oxidized proteins, a decrease in 20S proteasome activity has also been associated with aging (Grune 2000, Davies and Shringarpure 2006). As stated above, this activity can be inhibited by agglomerates of oxidized proteins and lipids. So these two factors can potentially have confounding effects on one another, where an increase in protein carbonylation will result in an increase in protein aggregations that inhibit 20S activity; a decrease in 20S activity will result in increased accumulation of protein carbonyls. The same pattern is evident in the mitochondrial-lysosomal degradation axis. Oxidative stress can result in an abundance of defective mitochondria (Kowaltowski and Vercesi 1999) which can result in an increase in oxidation of cytosolic proteins through diffusion of mitochondria-produced-ROS from the lysosomes. An increase in damaged proteins can inhibit the proteosomal degradation pathway. This could lead to an accumulation of damaged proteasomes, which should be degraded via lysosomal-mediated autophagy, thereby overwhelming the lysosomal degradation pathway. In this sense, physiological age of an individual will affect and be affected by oxidative stress within the cell.

Moreover, carbonyl groups of proteins generated via interactions with reducing sugars of lipid peroxidation products may react further with the  $\alpha$ -amino groups of lysine residues to form protein cross-linking products. These products are resistant to degradation via the 20S proteasome and may inhibit the ability of this proteasome to degrade other damaged proteins (Stadtman and Levine 2000). This would also lead to further accumulation of cellular 'garbage' which can in turn further exasperate both the 20S proteasome and lysosomal degradation systems.

### 1.2.3 Effects of aging on cellular thiols

As stated, glutathione is one of the most important non-enzymatic antioxidants in the cell. It is the most abundant non-protein thiol in the cell and is important in regulating redox status of the cell and gene transcription. Total GSH levels decrease with age in a number of tissues, including the brain and liver, in a number of different organisms (Liu and Dickinson 2003). Levels are also decreased in disease patients (Alzheimer, Parkinson's) (Liu 2002). Liu (2002) proposed that decreases in the rate of *de novo* synthesis of GSH, rather than increased oxidative stress, cause the age-associated decrease in cellular GSH. This age associated decrease in GSH levels is controlled by down-regulation of  $\gamma$ -GCS regulatory subunit in brain tissue. An age-related decrease in GS was also found in kidneys and lungs (Liu and Dickinson 2003). A decrease in GSH levels with age reduces the cells capacity to reduce oxidants, i.e. via activity of GST and GPx, and affects

the transcription of genes that control cell cycle regulation, ultimately leading to increased damage to cellular molecules, degradation and death.

Protein thiolation is an important form of protein oxidation. Protein thiol groups can be lost due to oxidation during oxidative stress. Agarwal and Sohal (1994) have experimentally demonstrated that loss of membrane protein-SH groups is correlated to decreases in life expectancy in *D. melanogaster*. Methionine residues can be oxidized via reactions with ROS, filling an antioxidant function since the reaction results in an inactive form of the ROS molecule. Mutations leading to inactivation of methionine sulfoxide reductases shorten lifespan of organisms ranging from yeast to mammals while over-expression can extend lifespan via an increase in antioxidant defence mechanisms (Stadtman 2006).

### **1.3 Biomarkers in environmental monitoring**

A biomarker, according to Peakall and Walker (1996), is “any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status.” van der Oost et al. (2003) state that a biomarker should possess the following qualities: an assay that is reliable, cheap and easy; the biomarker should be sensitive to pollutant exposure and/or effects; mechanism of relationship between pollutant exposure and response should be understood; impacts of confounding factors to the biomarker response should be well established; and toxicological significance of biomarker and impact on organisms should be established. Another key issue that needs to be addressed before one can consider a specific parameter for use as a biomarker is determining baseline levels in the study organism.

Environmental effects can be measured at all organismal levels, from ecosystem, population, individual, tissue, cellular, biochemical to molecular levels. The first mentioned levels will have the most ecological relevance and will likely represent long term exposure, while the later mentioned levels (cellular, biochemical and molecular) can function as early warning signs of the effects of exposure.

#### **1.3.1 Biomonitoring**

Biomonitoring is the science of assessing the condition of an environment or ecosystem through observations conducted on the biota. Biomarkers, physiological or chemical responses to xenobiotics within the organisms, are used to assess trends over time, to monitor the effects of possible policy changes, i.e. regulations concerning crude oil usage and handling, or to discover ‘new’ pollutants or sources of pollutants. Even if our cumulative knowledge is growing, many cause-effect

relationships concerning xenobiotic exposure in aquatic organisms are still poorly understood, which thereby results in uncertainties for regulatory decision makers.

Biomonitoring programs have been used extensively in nations around the world, in an attempt to link xenobiotics in nature, their effects on fish health and their impact on populations or communities. This includes studies investigating not only xenobiotic body loads and detoxification enzymes, but also growth and reproductive parameters as well. Several well established biomarkers are used routinely in fish biomonitoring programs. Lysosomal membrane stability is a highly sensitive, non-specific biomarker that can function as an early warning sign indicating that organisms in a certain environment are stressed by pollutant exposure (Moore et al. 2006, Viarengo et al. 2007). Though this parameter is measured primarily in mussels, its use in fish is increasing. Vitellogenin, an egg yolk protein precursor normally produced in reproductive female fish, is used to detect effects of estrogenic compounds on aquatic organisms (Purdon et al. 1994, Larsson et al. 1999). VTG in male or juvenile fish indicates the presence of estrogenic endocrine disruptors in the environment. Several environmental pollutants, including oil hydrocarbons, PBCs and dioxins, are known to induce activity of cytochrome P450, measured as EROD, in fish (Goksøyr and Förlin 1992). EROD activity is a sensitive and reliable biomarker.

Some examples of biomonitoring campaigns include the studies addressing the impact of PAHs in Puget Sound, USA (Collier et al. 1992, Landahl et al. 1997), the monitoring of metals from mining activities in Canada (Munkittrick et al. 1991) and the monitoring of pulp and paper mill effluent in Sweden (Andersson et al. 1987, Balk et al. 1993, Förlin et al. 1995). Several studies also address changes in effect parameters over long periods of time. For example, perch (*Perca fluviatilis*) in the Baltic Sea and eelpout (*Z. viviparus*) on the west coast of Sweden were monitored during a 13 year period (Ronisz et al. 2005, Hansson et al. 2006).

Biomonitoring campaigns concerning fish health have been conducted in Sweden as well as jointly within the European Union (EU). Programs in Sweden have included cooperations between Fiskeriverket (Swedish Board of Fisheries), Gothenburg University, and pulp and paper mill industries as well as Naturvårdsverket (Swedish EPA). Swedish laboratories have also cooperated in an EU biomonitoring project entitled Biological Effects of Environmental Pollution (BEEP). This project was a collaboration between a number of EU member states and associated states. The primary concern of the BEEP project was contamination of coastal environments by chemicals such as hydrocarbons from, for example, oil spills and wastes, pesticides used in crop treatment, heavy metals as well as various organic pollutants in dredged sediments and waste waters. Biological effects monitoring was used to detect links between contaminants and ecological responses. These elements are important in monitoring and management of aquatic ecosystems, and the BEEP project aimed to prepare information and advice for user

groups and policy makers. One of the primary scientific goals of the program was to validate biomarkers used in biomonitoring campaigns and to apply additional biomarkers to environmental monitoring schemes. Another was to establish new biomarkers for use in monitoring campaigns (Pampanin et al. 2006). Two of the papers in this thesis, I and II, were conducted within the framework for the BEEP project. The study in paper III was conducted in cooperation with the county administration of Västra Götaland in western Sweden. Here, authorities were interested in investigating potential effects of contaminated sediment on fish, though the focus of this paper falls more on the sewage treatment plant releasing effluent into the river.

### 1.3.2 Oxidative stress and environmental pollution

Oxidative stress, as stated above, is an imbalance between the production of reactive oxygen species and the cells' ability to reduce ROS, detoxify reactive intermediates, and/or repair damage that may occur in cellular molecules. This imbalance may occur as a result of increased ROS production, a decrease in defence mechanisms, or both. ROS are endogenously produced from a wide number of sources within the cell. However, and very importantly, many environmental parameters are known to cause oxidative stress. Some of the most well documented in humans include irradiation, i.e. x-rays,  $\gamma$ -rays and UV light, air pollution, PAHs, drugs and their metabolites, and exposure to heavy metals (i.e. copper, mercury, cadmium) (Halliwell and Gutteridge 1999). Xenobiotics may cause oxidative stress via the action of enzymes i.e. CYP1A, NAD(P)H oxidases, and flavoprotein oxidases, via metal-catalyzed oxidation systems, or via redox cycling (Livingstone et al. 2000, Stadtman and Levine 2000, Livingstone 2001).

A large number of xenobiotics that are present in the environment as a direct result of human activity can cause an increase in ROS production in the cells of the exposed individual. As the amounts and diversity of chemicals entering into the aquatic environment increase, biological loads in fishes have also increased with deleterious effects. Many pollutants mediate their toxicity through oxidative stress, resulting in changes in antioxidant defences as well as damage to proteins, membrane lipids and DNA molecules. The result of such exposure leading to oxidative stress can impair cellular or biological function which can lead to disease.

#### *Biomarkers of oxidative stress*

Oxidative stress is more and more often used as a biomarker of the effects of exposure to environmental pollution in aquatic environments (see Livingstone 2001, van der Oost et al. 2003, Valavanidis 2006). The main reason to study oxidative stress in aquatic organisms is to understand not only whether animals are

detrimentally affected by exposure, but also to understand the mode of action of the toxicants. Oxidative stress as a result of environmental pollution has been documented in numerous fish species over the past decade (see van der Oost et al. 2003, Valavanidis 2006). Several studies have shown that antioxidants that are affected by reactive oxygen species show adaptive responses to xenobiotics that produce oxyradicals (Di Giulio et al. 1995) and are potential biomarkers for oxidative stress in fish (van der Oost et al. 2003).

Biomarkers of oxidative stress, such as changes in antioxidant enzyme activity or in degree of accumulation of damaged molecules, can offer an early warning sign for exposure to redox-active xenobiotics. These oxidative stress parameters have been associated with various disease pathologies and organism longevity in a number of species, thereby establishing ecological relevance in these cases. A large number of biomarkers of oxidative stress have been used in fish studies and these include both the antioxidant defense mechanisms possessed by the cell, both enzymatic and molecular, as well as oxidative damage products. However, the relationship between exposure, either in laboratory or field situations, and the antioxidant response, is unclear with regards to many antioxidant parameters (van der Oost et al. 2003). Knowledge is needed concerning the baseline levels of various antioxidant mechanisms and damage products in sentinel fish species. An expansion of this base of in-depth knowledge concerning cause-effect relationships in environmental toxicology, as well as information concerning mechanisms of action of specific toxicants in specific species, is required. Studies have been conducted on several oxidative stress related parameters, addressing species differences, nutritional status, annual variations, and life cycle variations (Linde et al. 1998, Ronisz et al. 1999, Meyer et al. 2005, Ruas et al. 2007).

### 1.3.3 Oxidative stress in fish exposed to pollution

In fish, oxidative stress has been documented in both field and laboratory exposure studies. Environmental contaminants present in complex mixtures in sewage treatment effluent and industrial harbor areas contain compounds capable of inducing oxidative stress in exposed individuals. This can be manifested in the form of upregulation of antioxidant enzymes as well as increases in oxidative damage, including protein carbonyls, TBARS and DNA damage. Laboratory studies have used both model substances such as paraquat or heavy metals, and controlled exposures to environmental contaminants such as bunker oil or sewage effluent. Effects in numerous different species have been investigated.

Mummichog (or killifish (*Fundulus heteroclitus*)) is a small estuarine teleost species that is often used in environmental studies. Oxidative stress has been studied in mummichogs living in environments polluted by PAHs, phenol and creosols. Bacanskas et al. (2004) found that both GPx and tGSH were increased in

fish collected in the polluted river, compared to a control river, while GR was unchanged. These same fish also had higher levels of hepatic lipid peroxidation. Weis (2002) has also investigated oxidative stress parameters in the same species and proposed that upregulated GST activity may be an adaptive response that is genetically based.

Another species commonly used in oxidative stress studies is the carp (*Cyprinus carpio*). Huang et al. (2007) have measured contaminant-induced oxidative damage in carp captured in the Yellow River, China, a river contaminated by phenols, oils, PAHs and ammonia. While SOD and GST were upregulated in all tissues investigated, CAT and GPx were decreased in both kidney and intestinal tissues, the same tissues which were also found to have higher levels of MDA, suggesting that a lack of antioxidant defences could result in oxidative damage. Common carp have also been caged in a river in Amsterdam in order to assess water quality. Individuals caged in the polluted area had increased activity of GST but not SOD, CAT, GPx or GR (van der Oost et al. 1998). A related species, the common goldfish (*Carassius auratus*), has recently been used in oxidative stress studies. Individuals exposed to iron sulphate had increased levels of protein carbonylation and lipid peroxidation and decreases in CAT, GST, GR and G6PDH activities (Bagnyukova et al. 2006). Goldfish exposed to arsenic had increased activities of SOD, CAT and GPx as well as increased levels of lipid peroxides and GSSG (Bagnyukova et al. 2007).

Several different species have been used to assess effects of harbor pollution on fish. Stephensen et al. (2000) compared antioxidant enzyme activity in shorthorn sculpin (*Myoxocephalus scorpius*) from clean and polluted harbors and found that fish captured in the polluted harbor had increased activity of CAT, GPx and GR. Eelpout (*Zoarces viviparus*) has also been used as a sentinel species in monitoring in a harbor, as mentioned below.

Teleost fish have also been used as model organisms in studies concerning the effects of heavy metal exposure on oxidative stress parameters. This has been done in both field exposure situations, as well as in laboratory exposures. Heavy metals have been demonstrated to induce MT which can function as an antioxidant in exposed fish. Red mullet (*Mullus barbatus*) dwelling near Cu contaminated sediment have higher intestinal MT levels (Filipovic Marijic and Raspor 2007) and brown trout (*Salmo trutta*) caged in a Cu contaminated river showed increased MT-transcription in liver and kidney tissue (Hansen et al. 2006). SOD and GR-transcription were increased in gill tissue of these same trout. Craig et al. (2007) showed that zebrafish (*Danio rerio*) exposed to copper had increased gene expression of SOD and CAT as well as COX-17 (a metal chaperone involved in assembly of COX, a key enzyme in the electron transport chain in mitochondrial respiration). Protein carbonylation was also increased in both liver and gill tissues. Three species of cichlid from a metal-contaminated river showed changes in SOD,

CAT and GPx activities, though these differences were species and seasonally variable. All species, however, showed increases in lipid peroxidation in the metal-contaminated river in both spring and autumn (Ruas et al. 2007).

Paraquat (N, N'-Dimethyl- 4, 4' -bipyridinium dichloride or PQ) is a redox cycling compound that is often used as a herbicide for spraying agricultural crops. This chemical is, however, highly toxic and causes acute respiratory distress, Parkinson's-like symptoms, heart and kidney failure and liver damage in humans and rats. It is also known to enter the aquatic environment where it has toxic effects on fish. PQ is often used to induce oxidative stress in controlled laboratory studies in organisms ranging from yeast to fish, and is known to be toxic in the aquatic environment. Stephensen et al. (2002) measured increases in GR, GST, and tGSH in liver of rainbow trout exposed to PQ for 5 days. Rodriguez-Ariza et al. (1999) found that PQ induced 8-oxodG in gills of exposed gilthead seabream, but not in livers. This was hypothesized to be due to the protection against oxidative damage offered by the observed increases in GR and tGSH in the livers, changes that were not observed in gill tissue.

Exposures to these various environmental toxicants can often times result in cancer, not only in humans but in fish as well (Kelly et al. 1998). However, the relationship between oxidative stress and the pathology of diseases is still unclear in many cases. The use of teleost species as model organisms for research concerning cancer development has risen in the past years, largely due to several factors: low costs of husbandry, ability to include very large numbers of individuals in studies, similarity between disease pathologies in human and fish, and similarities in toxicological and adaptive responses to oxidative stress (Bailey et al. 1996, Kelly et al. 1998). A number of studies have investigated occurrence of tumors in teleosts in the field and laboratory. A study concerning baseline differences in antioxidant defences between two related species, the channel catfish (*Ictalurus punctatus*) and brown bullhead (*Ameiurus nebulosus*) revealed that the bullhead, the species more prone to neoplasia, also had significantly lower antioxidant enzyme activities (Hasspieler et al. 1994). The brown bullhead also had higher rates of microsomal superoxide and hydrogen peroxide production as well as the DNA damage product, 8-hydroxydeoxyguanosine. These findings suggest a direct relationship between antioxidants, oxidative stress and tumor formation.

DNA damage in fish is used more and more often as a biomarker of the effects of pollutant exposure. Frenzilli et al. (2004) used the comet assay to show that eelpout (*Z. viviparous*) had increased levels of DNA damage following an oil spill in an industrial harbor. Fish from the most polluted regions of the harbor did not recover after a five month period, as did individuals caught in cleaner areas. Amando et al. (2006a, 2006b) have applied both the comet assay and micronuclei test as indicators of DNA damage in Brazilian flounder (*Paralichthys orbignyanus*) and croakers (*Micropogonias furnieri*) resulting from pollution exposure in the

field. The flounder captured at a polluted site suffered from oxidative stress and had much higher levels of DNA damage, possible resulting from inhibited or exhausted DNA repair mechanisms. Croakers from a polluted site also suffered genetic damage, and this was seasonally affected.



## 2. Aims

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The general purpose of this dissertation is to evaluate the effects of different types of environmental pollution of oxidative stress in teleost fishes. This work incorporates measurements of antioxidant defences but emphasis lies in studies conducted on oxidative damage, namely protein carbonylation and lipid peroxidation. Effects of aging and oxidative stress on protein carbonylation were also addressed. The specific aims of the papers included in this thesis are listed below.

To study how antioxidant defences in fish are affected by exposure to environmental contaminants, specifically heavy metals, PAHs and sewage treatment plant effluent

To investigate whether levels of protein carbonyls and lipid peroxidation in fish are affected by anthropogenic activities, including a harbor dredging campaign, an oil spill, sewage treatment plant effluent, PAHs and heavy metal exposure

To evaluate the use of protein carbonyls and lipid peroxidation as biomarkers in field studies using several different teleost fish species: rainbow trout, eelpout and corkwing wrasse

To measure changes in protein carbonyl levels and 20S proteasome activity in brown trout as they age, and to investigate how these parameters are affected by oxidative stress

### **3. Methodological Considerations**

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Protein carbonylation and lipid peroxidation are two biomarkers that have been used as indicators of molecular oxidative damage. Protein carbonyl measurements have been used primarily in humans and rats while lipid peroxidation, on the other hand, has been applied to a wider range of animals, including fish. In the current work in paper I, the levels of both parameters were measured in fish and I have been able to compare how they are differently affected by dredging and an oil spill. In paper II, a battery of antioxidant enzyme activities was measured as well as protein carbonylation. Paper III addresses oxidative damage markers (protein carbonylation and lipid peroxidation), glutathione amounts, antioxidant enzyme activities and mRNA levels, in order to determine effects of STP effluent and contaminated sediment on oxidative stress in rainbow trout. Paper IV investigates effects of aging on oxidative stress in brown trout, through measurements of protein carbonylation and 20S proteasome activity.

The laboratory exposure experiments in papers I and IV, and the additional laboratory studies discussed, were conducted at the Department of Zoology at Gothenburg University. All fish in all of the studies in this thesis were treated in accordance with national ethical requirements established by Centrala Försöksdjursnämnden (CFN) and guidelines determined by Gothenburg University, Department of Zoology.

#### **3.1 Fish in field and laboratory studies**

Fish are useful sentinel species in marine and aquatic monitoring campaigns because they are, with some 42,000 species, essentially ubiquitous in aqueous environments the world over, with species occupying the vast majority of niches. It is important to be able to assess not only levels of xenobiotics present in the environment, either in water or sediment phases, but also to assess what effects these chemicals may be having on biota. This thesis encompasses works focusing on four different species. These fish were used in several different types of experiments; field studies involving capture of wild individuals, laboratory exposure studies and a caging study in the field.

Field studies utilize fish that are captured at an exposed site, or several exposed sites, and reference site(s). Species that are known to be stationary are preferred, as they better represent an exposure pattern to be expected for individuals living in the affected area. Caging studies often utilize farmed fish whose age and nutritional background is better known, though wild captured fish can also be used. Caging allows for exposure of individual fish to conditions at a certain site, for of a known length of time. Laboratory studies can use fish that have either been farmed or

captured in the wild. Laboratory studies allow researchers to, under controlled conditions, expose individuals to a more exact amount of a certain xenobiotic for a known length of time. These experiments allow for controlling multiple variables which may exist in the field, i.e. water temperature, aeration, food availability, thereby by simplifying interpretation of results. The four species of fish used in the current thesis are discussed below.

Eelpout (*Zoarces viviparus*) is bottom dwelling and relatively stationary species of fish and is therefore suitable for use in environmental field studies. They inhabit the North Atlantic Sea, Barents Sea, the coasts of Scotland, England and Ireland as well as the Baltic Sea. They are common along the Swedish coast in both marine and brackish waters. They inhabit the littoral zone, and can even survive out of water under rocks and seaweed for periods of time. Eelpout feed on gastropods, chironomids, crustaceans, eggs and fry of fishes. They have internal fertilization of eggs and give birth to live young, thereby making them useful in reproductive studies as well. They have been used as sentinel species in several studies (Jacobsson and Neuman 1991, Frenzilli et al. 2004, Ronisz et al. 2004, Sturve et al. 2005a) and have been used in environmental monitoring campaigns by the Swedish Environmental Protection Agency for years. In paper I, eelpout were captured in the wild at three different locations for use in a field study. Additional fish were captured and used in a laboratory study. These fish were captured at the reference site, and then exposed to crude oil in several different treatment groups under laboratory conditions: 33 ‰ seawater under semistatic conditions, 10° +/- 1°C, 12 hr light:dark schedule. This study allowed for confirmation of effects observed in the field study.

Corkwing wrasse (*Symphodus melops*) is a species that is distributed in the east Atlantic Ocean from Norway to Morocco and the Azores as well as in the Mediterranean and Adriatic seas. Corkwing wrasse are schooling and territorial fish that lives in the littoral zone. They feed on mollusks, hydroids, bryozoans, worms and various crustaceans (Quignard and Pras 1986). Since they are territorial and widespread along the European coastline, this species has been chosen as a sentinel in biomonitoring projects (Aas et al. 2001, Aas et al. 2003). Corkwing wrasse were used as a sentinel species for the study conducted in paper II, a field study focusing on a heavy metal exposed site and a PAH exposed site.

Rainbow trout (*Oncorhynchus mykiss*) is a salmonid species commonly used in laboratory experiments. Much is known about its basic physiology and rainbow trout are easily obtained and thrive well in laboratory conditions. Those individuals used in paper III and the additional laboratory studies discussed in this thesis were purchased from a fish farm near Göteborg, Sweden, called Anten AB, for use in a caging study in the field. They were fed commercial food pellets before the experiments but not during. Fish were allowed to acclimate to laboratory conditions for at least one week before beginning laboratory exposures or being

introduced into the cages in a river. There they were exposed to both sewage treatment plant effluent as well as polluted sediment. In the laboratory, rainbow trout were kept in recirculating, aerated, filtered freshwater at 10°C +/- 1° with a light:dark cycle of 12 hours. Rainbow trout, as they are a hardy species, survived well under conditions in the field.

Brown trout (*Salmo trutta*) is another species of salmonid, one that lives in the wild in Swedish streams. The individuals used in paper IV were purchased from Vänneholms Fiskodling AB in Laholm. This species was chosen because we were able to obtain individuals of 4 known ages in good condition. In addition, the individuals obtained from the hatchery are genetically similar to those in the neighboring streams, making future comparisons of farmed and wild fish possible. The fish used in the current study were treated as follows: recirculating, aerated, filtered freshwater at 10°C +/- 1° with a light:dark cycle of 12 hours, commercial feed at 2-3% body weight before but not during the experiment.

Since antioxidant defense mechanisms and oxidative damage products are in large identical throughout evolution, they do not differ appreciably between these fish species. Protein carbonyls can be found in organisms ranging from yeast (*S. cerevisiae*) to humans. TBARS have been documented in a wide range of organisms. Antioxidant enzymes, including SOD and CAT, have been studied extensively in the nematode, *C. elegans*, and the same enzymes are present in higher vertebrates where they have been studied extensively in, for example, rats and humans. The 20S proteasome is well conserved from prokaryotes to humans, and extensive studies have been conducted in organisms ranging from *E. coli* to mice to humans. Variations have been found throughout the phylum on the gene sequence level as well as in the types or numbers of isoforms of a certain enzyme, but function has remained the same. While the genotypes of enzymes expressed in all four species of fish used here have not always been identified, as these animals are not prioritized in sequencing projects, the enzymes are present and functioning. The biochemical formation of oxidative damage products, including protein carbonyls, DNA damage and TBARS, is identical in all organisms. Variations may occur, however, in the extent of the antioxidant defences present, or in the individuals' ability to repair damage, for example to DNA.

### **3.2 Biochemical assays**

The activities of antioxidant enzymes were assessed through the use of enzyme kinetics. The methods described in the papers are well established, and have previously been applied in fish species. CAT, DTD, GST, GR and glutathione are all measured spectrophotometrically, where samples are diluted in a physiological buffer and a substrate, electron donor, and/or inhibitor are added. The accumulation of product or consumption of reagents was monitored spectrophotometrically over

time. This rate is compared to the total concentration of protein in the sample to quantify the enzyme activity.

Catalase is measured using hydrogen peroxide as a substrate (Aebi 1984). Here, a physiological buffer is added to samples and addition of hydrogen peroxide initiates the reaction. Catalytic decomposition of  $H_2O_2$  is observed as a decrease in absorbance at 240 nm.

DT-diaphorase enzyme activities were measured according to the method described by Sturve et al. (2005a). This protocol used 2,6-dichloroindophenol (DCIP) as a substrate. This is a dye that is usually used to determine ascorbic acid levels and can be reduced by a number of different substrates. Therefore, dicoumarol, a potent DTD inhibitor, is used to distinguish between DTD-catalyzed reduction and the total action of all relevant reductases. As DCIP is reduced, it becomes colorless and this can be measured spectrophotometrically over time at 600 nm.

The glutathione *S*-transferase method used here (Habig et al. 1974, Stephensen et al. 2002) measures multiple forms of this enzyme. This includes those that function as conjugating enzymes, attaching a glutathione molecule to target molecule to aid in excretion, as well as GST isoforms that function to reduce hydrogen peroxide or lipid peroxides. It is not possible to distinguish differences in activity due to changes in activity of conjugating forms and reducing forms, which is a limitation of the method. 1-chloro-2, 4-dinitrobenzene (CDNB) is used as a substrate in this reaction. The GST-catalyzed formation of CDNB-GSH can be detected by spectrophotometer at 340 nm.

Glutathione reductase is measured according to Cribb et al. (1989). GSSG is added to the reaction mixture in a physiological buffer. This molecule is a substrate for GR, which reduces GSSG to GSH, using reducing equivalents from NADPH. The reduced GSH can then react with 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB), forming a glutathione-TNB conjugate and free TNB, whose production is measured at 425 nm over time.

Molecular glutathione (GSH) is measured indirectly via a biochemical reaction using GR, according to Baker et al. (1990), later adapted to a plate reader by Vandeputte et al. (1994). Here GR converts all glutathione to GSH thereby oxidizing NADPH to  $NADP^+$ . DTNB in the reaction mixture acts as a conjugant to glutathione as described in the GR method. To measure the GSSG portion of the total glutathione pool in the sample, GSH is first derivitized by a reaction with 2-vinylpyridine.

### **3.3 Protein carbonyl assays**

Protein carbonyls were measured using three methods, a colorimetric method, described by Levine (1990) and Reznick and Packer (1994), an ELISA method

described by Winterbourne and Buss (1999), and a western blotting method (Levine et al. 1994). All methods are based upon a derivitization step in which 2, 4-dinitrophenyl hydrazine (DNPH) selectively binds to carbonyl groups on the proteins.

The colorimetric method used for measuring protein carbonyls is the more economical, though more labor intensive, quantification method. Samples must first be homogenized in a phosphate buffer containing a cocktail of antiproteases to prevent degradation of protein carbonyls via cytosolic or lysosomal proteases during the assay. Sample preparations are then reacted with DNPH, which selectively binds to carbonyl groups, followed by several precipitation and washing steps. The final pellet is resuspended and this solution is measured spectrophotometrically. This final pellet was difficult to redissolve, despite rigorous attempts using both heating and ultrasonication. Some amount of protein was always lost during the washing steps, and there was some concern about whether all proteins might not have the same solubility, thereby possibly resulting in under-representation of a certain protein or type of protein during the process.

The ELISA method (Winterbourn and Buss 1999) is much more time efficient, as up to 44 samples can be measured at once (a two day process). Some difficulties were encountered in starting up the method in our laboratory, due to various problems, but I was in the end able to produce reliable and reproducible results, using a BSA standard curve of oxidized and reduced proteins. These standards were quantified using the colorimetric assay by Levine (1990). In general, this assay is more appropriate for use with samples containing more simple mixtures of proteins, i.e. blood plasma or cell culture material. This is due to the fact that tissue samples may contain additional substances that can interfere with several steps of the assay. This includes binding of DNPH to protein carbonyls, in cases where there may be other sorts of carbonyls present, as well as in antibody binding and HRP labeling.

A western blot, used to locate and possibly identify carbonylated proteins, can be conducted in a number of ways, where the major difference between the methods lies in the step in which proteins are derivitized. This can be done prior to running the samples on the SDS page gel, while the proteins are in the gel, or after they have been blotted over to the membrane. In this thesis, the method described by Levine et al. (1994), in which proteins are derivitized directly prior to running the SDS page gel, was followed. While this could theoretically affect the rate at which proteins migrate, this method avoids detecting possible changes that may occur in proteins during electrophoresis and membrane preparation. This allows viewing carbonylated proteins as they would appear in the sample without viewing artifacts produced during electrophoresis or blotting.

### **3.4 Lipid peroxidation**

The TBARS method was used to measure lipid peroxidation in samples from fish. This method measures thiobarbitic acid reactive substances, or lipid peroxidation products, i.e. malondialdehyde (MDA). Liver samples were homogenized in acid, which precipitates lipoprotein fractions thereby preventing their interferences. Samples were then allowed to react with thiobarbitic acid in the presence of butylated hydroxytoluene (BHT), an antioxidant, at 100°C before extraction in butanol. Results are calculated using the differences in absorbance at 532 nm and 600 nm. Much controversy has appeared in the literature concerning the TBARS method. This is due to questions regarding the specificity of TBARS assays to compounds other than MDA. However, this assay remains the most widely used for assessing lipid peroxidation.

In addition to the traditional TBARS method, the Bioxytech LPO-586 kit was used to measure lipid peroxidation. The LPO-586 method is designed to assay either MDA alone, in hydrochloric acid, or MDA in combination with 4-HNE, in methane sulfonic acid. This is a colorimetric assay based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA or 4-HNE at 45°C.

Both the TBARS assay described in paper I, as well as the kit used in paper III (where both MDA and 4-HNE were measured), were sufficiently specific for my purpose, which was to elucidate potential differences in lipid peroxidation levels between fish caught or caged at different sites or placed in different treatment groups.

### **3.5 Real time PCR**

Measurements of mRNA levels are presented in paper III discussed in this thesis. Primers were designed using primer 3 or Beacon Design software. Gene sequences were based on data base searches using gene or gene-product names. In cases where no sequence data existed, available sequences from other teleost species were blasted and conserved regions were used for primer design for use in rainbow trout samples. The data bases used for these purposes were Kyoto Encyclopedia of Genes and Genomes (KEGG), National Center for Biotechnology Information (NCBI), the Salmon Genome Project (SGP) and the Gene Index Project. All primers were optimized for concentration and annealing temperature. PCR gene products were then isolated, purified and sequenced to confirm that the correct product was amplified.

## 4. Results and Discussion

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### 4.1 Effects of exposure to pollution on antioxidant defences

Effects of xenobiotic exposure on antioxidant enzyme activities as well as levels of antioxidant molecules were investigated in fish exposed in field situations in papers II and III.

#### 4.1.1 Effects of heavy metal and PAH exposure in corkwing wrasse (*S. melops*)

In order to evaluate the effects of heavy metals and PAHs on antioxidant enzyme activity in corkwing wrasse, a battery of enzyme activities was measured and reported in paper II as part of a field study conducted within the BEEP framework. Here, fish were collected at two polluted sites as well as at two reference sites near Stavanger, Norway. Visnes is a site located outside a disused copper mine, and is polluted by a number of heavy metals; mainly copper, cadmium and zinc. Fish were also collected outside an aluminium plant at Høgvarde in a body of water contaminated by PAHs.

Results indicated that enzymes involved in detoxification, i.e. EROD, the catalytic activity of CYP1A, were only slightly affected in male fish captured at the heavy metal and PAH contaminated sites. At Visnes, an increase was observed though this was not significant. At Høgvarde, a significant 2-fold increase was observed. PAHs are known to increase EROD activity in other fish species via interactions with the Ah receptor which induces transcription of CYP1A (Goksøyr and Förlin 1992). Induction and control of this enzyme activity is well conserved among teleosts (Siroka and Drastic ova 2004). Eelpout collected in a harbor during a dredging campaign showed an increase in both EROD activity and in PAH metabolites measured in the bile (Sturve et al. 2005a). Rainbow trout were shown to have cytochrome P450 enzymes that are inducible by model substances (Förlin 1980). Flounder (*Platichthys flesus*) from industrialized estuaries contaminated with PAHs and PCBs had induced EROD activity (Kirby et al. 1999) as did dab (*Limanda limanda*) exposed to hydrocarbon-contaminated sediments (Livingstone et al. 1993). Chronic PAH-induced stress, as may have occurred at Høgvarde, could induce adaptation in exposed populations. McFarland et al. (1999) hypothesize that bullheads chronically exposed to PAHs may undergo adaptation, as is evident in lower hepatic EROD activity in fish from a highly contaminated site and in lower levels of single strand DNA breakage. Lower EROD activity could be selected for since highly inducible CYP1A results in an increase in intermediate metabolites



and consequently an increase in DNA adducts and strand breakage, which lead to tumor formation.

Corkwing wrasse collected during the field study in Stavanger showed few responses in antioxidant enzyme activities. Though the metal exposure resulted in some insignificant increases and decreases, activities of GR, GST and levels of MT and GSSG were principally unchanged at Visnes. Only G6PDH was significantly affected, showing a decrease in activity in males. G6PDH is an enzyme involved in energy metabolism and the production of NADPH molecules, which are used as reducing equivalents by a number of other enzymes (Halliwell and Gutteridge 1999). This enzyme is also known to be susceptible to metal catalyzed reactions whereby it becomes carbonylated and inactivated (Fucci et al. 1983, Sohal et al. 1995). Since fish exposed to heavy metal contamination often take up metal ions via the gills, G6PDH in blood cells would be susceptible to reactions such as metal catalyzed oxidation. The resulting decrease in G6PDH activity indicated that G6PDH activity could provide a good biomarker for metal exposure in fish. However, the fact that this is the only antioxidant enzyme to show effects in this metal exposure study indicates that the fish may have acclimated to the exposure conditions. Fish can become more tolerant to acute challenges of metal exposure via regulation of uptake, detoxification, storage or excretion of metal ions (Kamunde and MacPhail 2007, Rainbow 2007).

At Høgvarde, the PAH contaminated site, tGSH significantly decreased in liver tissue. GSSG decreased as well, though not significantly. Conversely, Eufemia et al. (1997) found that tGSH levels were elevated in brown bullhead collected in a PAH contaminated river, though only during summer. The decrease observed at Høgvarde may be a result of the export of glutathione *S*-conjugates or GSSG from the cells (Keppler 1999). Chronic exposure to metal contamination has also been shown to affect glutathione status in fish species, resulting in an increase in hepatic GSH concentrations (Thomas and Wofford 1984). GSH has been shown to provide protection against cytotoxicity of Cu, Cd and Hg, but not Ni, Zn and Pb, in fish cells as these metals have a higher binding affinity for the sulfhydryl groups on the GSH molecule (Maracine and Segner 1998). This does not, however, appear to be the case in the wrasse measured in paper II as GSH levels were unaffected in fish captured at the heavy metal site.

Other studies have investigated effects of chronic heavy metal or PAH exposure on antioxidant defences in teleost fish, with differing results. For example, dab exposed to hydrocarbon-contaminated sediments had increased hepatic SOD activity (Livingstone et al. 1993) while sea bass (*Dicentrarchus labrax*) exposed to Cd *in vitro* showed inhibition of CAT activity (Roméo et al. 2000). Chub (*Leuciscus cephalus*) collected in metal-polluted rivers had increased hepatic GPx and GST activities (Lenártová et al. 1997). McFarland et al. (1999) collected brown bullhead (*Ameiurus nebulosus*) at two field sites that were

contaminated by PAHs. They found few differences in hepatic antioxidant defences when comparing the two groups of wild caught fish; activities of GPx, GR, GST, SOD were unchanged as was the total amount of GSH. CAT activity and GSSG levels were higher in fish exposed to PAHs originating from creosote contamination. Weis (2002) reported that populations of mummichogs (*Fundulus heteroclitus*) living in highly contaminated areas have developed tolerance to pollutant exposure via genetically based elevation of GST activity. Shorthorn sculpin (*Myoxocephalus scorpius*) caught in a PAH contaminated harbor had elevated CAT and GR activities compared to fish caught at a reference site (Stephensen et al. 2000).

These studies have in some cases indicated gender differences in antioxidant responses (McFarland et al. 1999, Stephensen et al. 2000). In paper II, we measured a decrease, though insignificant, in GR in male wrasse exposed to heavy metal contamination, but no change in females, and no change in GR in fish exposed to PAHs. Some studies report an increase in GR activity following exposure to PAHs, PCBs or DDE while others report decreases after PCB exposure (see review by van der Oost et al. 2003). However, many studies report no change in GR activity after exposure, either via laboratory exposures or in field studies.

The fact that antioxidant enzymes were in large unaffected in corkwing wrasse could indicate that the fish had undergone some degree of adaptation to the chronic exposure or that the degree of exposure was lower than anticipated. On the other hand, lack of induction coupled with exposure to xenobiotics that are known to cause an increase in ROS production, may in fact lead to higher levels of oxidative damage in this species. This includes protein carbonyls, as is discussed below.

The studies discussed here, including paper II, indicate that investigations into the effects of chronic exposure to pollution on antioxidant defences in different species of fish give differing results. Future studies should address mode of action of the pollutants and mechanisms controlling the observed effects. More of this sort of information in the literature would increase the usefulness of these antioxidant parameters in biomonitoring campaigns. The results from paper II indicate that measurements of antioxidant defences would not provide a suitable biomarker of exposure to heavy metals or PAHs in corkwing wrasse exposed under chronic conditions.

#### 4.1.2 Effects of sewage treatment effluent and contaminated sediment in rainbow trout (*O. mykiss*)

In paper III, farmed rainbow trout were used in a caging study to evaluate the effects of acute pollutant exposure on oxidative stress. The use of fish as model organisms in acute toxicity testing is wide spread, due in part to the need to investigate effects of xenobiotics on aquatic organisms (Bailey et al. 1996).

Rainbow trout are often used as model organisms to test effects of acute exposure to a xenobiotic compound (Lemaire et al. 1996, Otto et al. 1997, Stephensen et al. 2002, Sturve et al. 2005b) or complex mixture of xenobiotics (Sturve et al. 2008), on oxidative stress. These studies indicate that this species does have an antioxidant defence system that is inducible, as demonstrated in several laboratory exposure studies using both model substances (Stephensen et al. 2002, Sturve et al. 2005b) as well as the more complex chemical mixtures found in an STP effluent (Sturve et al. 2008). Viarengo et al. (2007) suggested that biomonitoring programs should use caging studies to obtain highly sensitive early warning signs of the effects of exposure to environmental pollution.

Rainbow trout were exposed to STP effluent and/or contaminated sediment in a 14 day caging experiment described in paper III. Individuals were caged at five sites in the river Viskan in Borås, a city in western Sweden. This river is polluted by both STP effluent from Gässlösa, as well as sediment contaminated with metals, dioxins, PAHs, PCBs, oil, pesticides and flame retardants, resulting mainly from textile and other industries active since the end of the 1800s. A site to the north of the city of Borås served as a reference.

CAT activity was significantly increased in rainbow trout caged at the STP, while DTD was significantly lower. DTD mRNA levels were also measured in fish caged at Gässlösa, the STP, revealing no differences in gene expression. This indicates inhibition of DTD at the protein level. DTD activity is known to be inhibited by coumarins, which occur naturally in at least 160 different plant families. Warfarin, an anticoagulant pharmaceutical, is a coumarin derivative that could be present in STP effluent (Kolpin et al. 2002). Inhibition of DTD in rainbow trout exposed to STP effluent under laboratory conditions has previously been shown to result in inhibition of DTD activity (Sturve et al. 2008). Inhibition of DTD could have confounding effects on oxidative stress in affected individuals in that the cells lose some of their ability to prevent redox cycling of xenobiotics which will ultimately result in an increase in ROS production (Cadenas 1995). None of the other antioxidant enzymes were affected by STP effluent, and fish caged further downstream at sites with contaminated sediment showed no responses at all. This is contrary to previous results. Hansen et al. (2007) caged brown trout in a river polluted with Cd and Zn and observed an increase in transcription of oxidative stress related genes, i.e. CAT, SOD and MT in various tissues.

STP effluent exposure caused an increase in tGSH, GSSG and %GSSG while fish exposed to contaminated sediment showed no changes in glutathione amounts or % GSSG. Several studies indicate that measurement of GSH status in tissues of exposed fish could be of vital importance in interpreting effects of xenobiotics on antioxidant biomarkers. GSH has been implicated as one of the most important antioxidants present in tissues (Sies 1999). GR has been suggested as a good

biomarker for oxidative stress in fish (Regoli et al. 2002, Stephensen et al. 2002, van der Oost et al. 2003) yet we see no differences between any of the groups. This lead us to investigate enzymes involved in synthesis of glutathione, an alternative means of maintaining levels of reduced GSH within the cell. Here we saw an increase in the mRNA levels of GCS ( $p=0.056$ ) but a significant decrease in GS mRNA levels. The catalytic subunit of GCS is known to be inducible by oxidative stress in rats and humans (Shi et al. 1994, Lu 1999), so the same may likely be the case in rainbow trout. GS mRNA levels were lower in fish caged at Gässlösa, indicating a possible inhibition at the transcriptional level. Little is known about transcriptional regulation of GS, but it seems that since GCS is the rate limiting step in the synthesis of glutathione, regulation of this enzyme alone may control GSH production within the cell (Lu 1999). Regulation of these genes in fish should be further investigated before implementing these parameters in biomonitoring campaigns. Glutathione can also be exported from hepatocytes, and transcription, activity and possible inhibition of the responsible pumps, known as multidrug or multixenobiotic-resistance proteins manner (Keppler 1999), could be of interest to study.

The importance of GSH and related enzymes has also been demonstrated in acute exposure studies in fish. European eel with upregulated GSH systems had the highest rates of survival when exposed to near lethal doses of an organophosphorus insecticide (Pena-Llopis et al. 2003). A decrease in GSH levels in gill tissue has been associated with acute exposure of large mouth bass to nanoparticles, fullerenes (Oberdörster 2004). Interestingly, teleosts are able to incorporate exogenous GSH into their tissues, as opposed to most mammalian models. This has been demonstrated in both rainbow trout and American eel (Otto et al. 1997). Treatment of these fish with GSH or GSH depletors as well as tetrachlorobiphenyl indicated that GSH status affected activity of antioxidant enzymes, i.e. SOD and CAT. SOD activity was lower in GSH depleted individuals and CAT was elevated in GSH enhanced individuals.

None of the parameters measured in paper III were affected in fish caged at sites with polluted sediment, indicating that these xenobiotics either did not affect the measured parameters, or, most likely, were not available to these fish. While rainbow trout is a good model for investigating the effects of STP exposure on aquatic animals, other organisms, for example those with bottom associated behavior, may provide more useful in future studies investigating effects of sediment bound toxins on biomarkers of oxidative stress.

Antioxidant responses can be important to include in biomonitoring studies, as many xenobiotics are prooxidants that exert their effects through induction of oxidative stress. However, antioxidant enzymes are often less responsive than other biomarkers (i.e. phase I enzymes) to exposures, may differ greatly in expression levels and inducibility in different species, and baseline knowledge may be lacking

for many exposure conditions in different species (van der Oost et al. 2003). Therefore, antioxidant biomarkers would be most useful as part of a larger battery of biomarkers when investigating effects of xenobiotics on biota. This may include oxidative damage products such as protein carbonyls, lipid peroxidation products, i.e. MDA and 4-HNE, or oxidative DNA damage.

## **4.2 Effects of exposure to pollution on protein carbonylation and lipid peroxidation**

Effects of xenobiotic exposure on protein carbonylation and lipid peroxide levels in fish in the field were investigated in papers I, II and III. These two types of oxidative damage can occur when antioxidant defences within the cell are overwhelmed and ROS, resulting either from endogenous sources or from xenobiotic exposure, can interact with cellular molecules.

### **4.2.1 Effects of harbor pollution in eelpout (*Z. viviparus*)**

Industrial harbors are subjected to large numbers and amounts of xenobiotics, originating from industrial effluent, runoff and sewage from the surrounding city and boat traffic. We investigated effects of a dredging campaign and an oil spill on protein carbonylation and lipid peroxidation in eelpout in paper I. Fish were captured in Göteborg harbor as well as at two different reference sites, Nordre Älv, the northern branch of the Göta River which forks before reaching the city of Göteborg, and at Fjällbacka, a national reference site on the Swedish west coast which is used by the Swedish EPA in monitoring campaigns. Sampling was conducted at 6 different time points over a two year period, before commencement of dredging, during dredging, after an oil spill of PAH-rich bunker oil, and after these events had been concluded. Eelpout were also collected from a clean site for use in a laboratory exposure study using the same bunker oil that was spilled into the harbor.

Results from the field study revealed that protein carbonyls were indeed affected by anthropogenic activities. Before commencement of the dredging campaign, protein carbonyl levels were lower in the harbor than at either of the two reference sites. During dredging and following the oil spill, protein carbonylation was significantly higher in fish from the harbor. Grune et al. (2003) have shown that at moderate oxidant concentrations, the degradation of damaged proteins increases whereas higher oxidant concentrations can inhibit proteolytic degradation. Mild oxidation of soluble proteins enhances their proteolytic susceptibility at the same time as 20S activity can be increased by oxidative stress

(Reinheckel et al. 1998). On the other hand, it appears that severely oxidized proteins may be stabilized due to aggregation, cross-linking and/or decreased solubility (Grune 2000, Grune et al. 2003). Therefore, both an increase and a decrease in protein carbonyl levels, compared to references, could serve as biomarkers, where an increase would appear to be the more severe of the two responses. Fish that were collected in the harbor before commencement of dredging may in fact have upregulated proteolysis due to oxidative stress induced by exposure to xenobiotics, thereby resulting in the lower protein carbonyls levels observed in November 2001 and May 2002. The contaminant exposure originating from the oil spill and dredging activity could increase the rate of protein carbonyl formation at the same time as activity of the 20S proteasome could have been inhibited by xenobiotic exposure. This could result in an increased rate in protein carbonyl formation, accumulation of insoluble aggregates, and decrease in proteolysis.

TBARS levels did not differ between reference and affected sites, neither during dredging activities nor the oil spill. Levels were, at all time points except May 2002, the same in hepatic tissue of fish from Göteborg harbor as in those fish caught at the two reference sites. May 2003 levels were higher at all three sites, compared to samples taken during the same month of the previous year. The highest TBARS levels were measured in July of 2003, following an oil spill in the harbor. However, there was no difference between the affected site and Fjällbacka, the reference site. This increase in TBARS may instead reflect seasonal variations, dependent upon water temperature, food availability, reproductive status, etc.

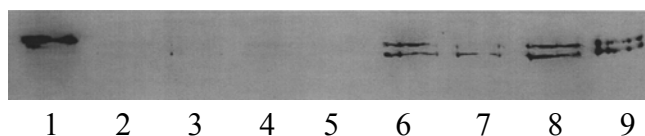
Laboratory exposure to crude oil induced protein carbonylation in a dose dependent manner in eelpout. On the other hand, no differences were seen in TBARS. The PAHs present in the crude oil may have increased production of ROS, i.e. via CYP1A activity, that were able to react with amino acid residues, thereby forming protein carbonyls, while lipid peroxidation did not occur. This supports observations from the field study, where TBARS levels were not elevated in eelpout collected in the harbor compared to the reference site while effects were seen in protein carbonyl levels.

#### 4.2.2 Effects of heavy metal and PAH exposure in corkwing wrasse (*S. melops*)

Protein carbonyls were measured in corkwing wrasse from two polluted sites in Norway (paper II). Both male and female fish collected outside the copper mine had increased levels of protein carbonyls in their blood plasma, compared to the reference site. No changes in protein carbonyl levels were found in fish collected at the PAH site. The increase in protein carbonylation measured at Visnes, the heavy metal-contaminated site, could be due to metal catalyzed oxidation reactions.

Transition metals are capable of interacting with ROS, i.e. H<sub>2</sub>O<sub>2</sub>, at metal binding sites on proteins, inducing site specific damage to amino acids (Stadtman and Oliver 1991). The fact that protein carbonylation was not affected at Høgvarde is in contrary to the results discussed in the previous section 4.2.1 (paper I) where eelpout exhibited increases in protein carbonylation in liver tissue after exposure to PAH rich oil. These differences may be due to biochemical differences in composition of the PAHs present at Høgvarde and Göteborg harbor, tissue differences (plasma vs. liver homogenate) or species differences.

A previous study has shown increases in protein carbonylation in goldfish exposed to heavy metals (Bagnyukova et al. 2006), which is in agreement with the findings presented in paper II. Figure 5 shows a western blot of plasma samples from corkwing wrasse caught at Visnes. Fish caught outside the copper mine showed two bands of oxidized proteins (lanes 6-9) while individuals caught at the reference site (lanes 2-5) did not show any discernable bands at all. A positive control of oxidized BSA was used when running the assay. Interestingly, the bands from the fish plasma were of the same size (approximately 66 kDa), indicating that protein carbonyls formed on fish blood albumin may be responsible for these results. This is highly likely in that albumin is by far one of the most abundant proteins present in plasma samples, and this protein contains a number of metal binding sites. This could easily lead to metal catalyzed oxidation reactions as a result of copper, cadmium or zinc exposure. Metal uptake from the water column occurs via gill membranes, so blood plasma and therefore albumin would be exposed to the metals prior to any potential metabolism that could take place, for example binding to MT in the liver.

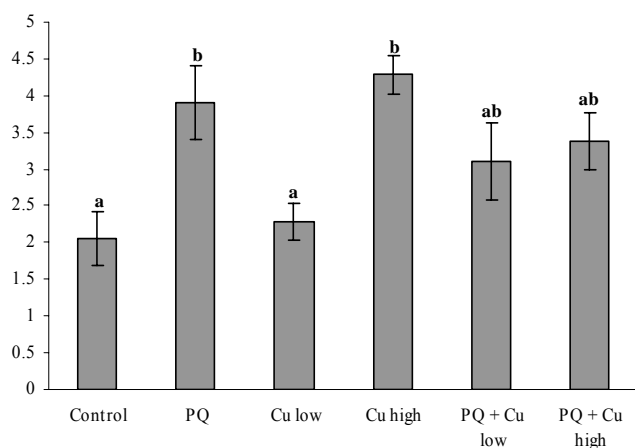


**Figure 5:** Western blot using plasma samples from corkwing wrasse captured in the field study. Lane 1 – positive control (oxidized BSA, 66.4 kDa), lanes 2-5 – plasma from individuals caught at Salvøy, lanes 6-9 – plasma from individuals caught at Visnes.

#### *Effects of copper and paraquat exposure on protein carbonyl levels in rainbow trout (*O. mykiss*)*

In addition to the field study from the heavy metal contaminated site, we conducted a laboratory exposure experiment in which juvenile rainbow trout ( $2.6 \pm 0.6$  g) were exposed to copper levels comparable to those found in the field study in paper II, and to paraquat (PQ) for a period of 14 days under semi-static conditions (own unpublished results). The fish were exposed to copper in two

doses, low (800  $\mu\text{g/ml}$ ) and high (4  $\text{mg/ml}$ ), with and without the redox cycling compound PQ (200  $\mu\text{g/ml}$ ). Measurement of protein carbonyls revealed a significant increase in this oxidative damage product in fish exposed to either PQ or Cu high dose (HD) alone. Fish that were exposed to both PQ and either the low dose (LD) or HD of copper did not differ from the control group. However, levels in the PQ + Cu LD group were higher than with copper LD alone while levels in the PQ + Cu HD group were lower than in the Cu HD or PQ alone groups, though these results were insignificant. Results are shown in fig. 6. These differences could be due to reactions involving the two substances within the cell, thereby affecting either the rate of protein carbonylation or the rate of proteolysis. We cannot, however, rule out chemical interactions between Cu and PQ in the water column which could change the chemical reactivity of one or both of the substances. Measurements of GR activity in these fish showed no differences between any of the groups, indicating that at least this portion of antioxidant defences was not induced, contrary to other studies where both Cu and PQ have induced GR levels in exposed fish. Stephensen et al. (2002) found an increase in GR activity in rainbow trout that were injected with PQ IP. Brown trout (*Salmo trutta*) exposed to water born-copper showed increased GR activity in gill tissue, and this increase correlated positively to Cu accumulation (Hansen et al. 2006). It is possible that the water exposure conducted in the current study also affected oxidative stress in gill tissue, though this was not measured here. On the other hand, the fish used here were juveniles, and younger fish are known to have greater antioxidant defences (Otto and Moon 1996, Beckman and Ames 1998, Harman 2001), so they might have been sufficient to counteract the effects of PQ exposure without the need for an additional increase.



**Figure 6:** Protein carbonyls (nmol/mg prot) in liver tissue of rainbow trout exposed to paraquat (PQ), 200  $\mu\text{g/ml}$ , copper (Cu) low dose, 800  $\mu\text{g/ml}$  water, or high dose, 4  $\text{mg/ml}$  water, or a combination. Letters indicate statistical difference between treatment groups ( $p < 0.05$ ).



#### 4.2.3 Effects of sewage treatment plant effluent and contaminated sediment in rainbow trout (*O. mykiss*)

Oxidative damage products which can result from ROS interactions, measured as protein carbonyls and lipid peroxidation, were investigated in paper III. Protein carbonylation was measured in plasma samples while lipid peroxides, both MDA and 4-HNE, were measured in liver tissue. Rainbow trout that were caged at GÄSSLÖSA STP, but not at the down stream sites, had increased levels of both protein carbonylation as well as lipid peroxides compared to the reference site. These results from the downstream sites with contaminated sediment are paralleled in the lack of induction of antioxidant enzyme activity, indicating no oxidative stress.

The observed increases in protein carbonylation and lipid peroxides at GÄSSLÖSA are in accordance with previous studies. Increases in protein carbonylation have been investigated in fish species exposed to xenobiotics (Parvez and Raisuddin 2005, Bagnyukova et al. 2006, Craig et al. 2007), though not in connection with STP effluent exposure. Oakes et al. (2004) found that gonadal TBARS levels increased in longnose sucker exposed to STP effluent, though this response was only measured during one sampling time point and not during successive years. Both protein carbonylation and lipid peroxidation can result in serious deleterious effects for the individuals. Protein function may be decreased (Davies 1987) and cellular 'garbage' accumulates (Grune et al. 2004).

20S proteasome activity, active in degradation of oxidized proteins, was also measured but no differences were observed. Little is known about regulation of this proteasome in fish, but studies have shown that the proteasome structure is well conserved throughout eukaryotic evolution and that expression and activity of the different  $\alpha$  and  $\beta$  subunits are controlled at the gene level as well as the protein level (Grune et al. 2004). Oxidative stress can induce proteolysis via the 20S proteasome (Grune et al. 1995). 4-HNE is a lipid oxidative damage product known to bind to and inhibit 20S activity (Esterbauer et al. 1991). It is possible that a regulatory increase in 20S may have been masked by inhibition of enzymatic activity via binding to 4-HNE, which increased in this exposure study, or via binding of oxidatively damaged protein aggregates. An accumulation of oxidatively damaged proteins has been shown to result in the formation of protease-resistant aggregates which in turn have been argued to bind to and inhibit 20S proteasome activity (Grune et al. 2004). Inhibition of 20S proteasome activity could in turn have confounding effects on the accumulation of oxidatively damaged proteins.

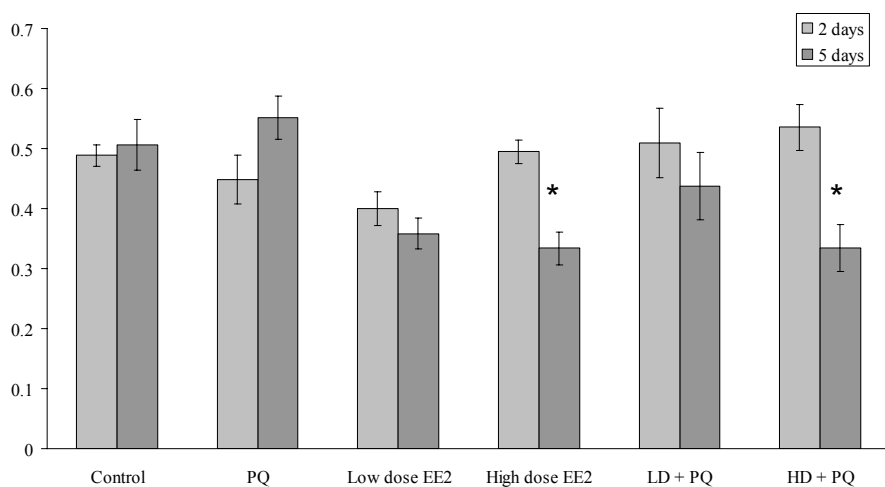
#### 4.2.4 Are specific proteins more readily carbonylated than others?

The research on protein carbonylation in fish species conducted within the current thesis has given rise to the question of whether one or a number of specific proteins might be more susceptible to protein carbonylation in fish as a result of xenobiotic exposure and induced oxidative stress. Agarwal and Sohal (1995) showed that high molecular weight proteins are more susceptible to oxidation, i.e. carbonylation, during aging and oxidative stress. I found, as discussed in section 4.2.2, that corkwing wrasse chronically exposed to heavy metals in the field in Norway had higher levels of protein carbonylation in their plasma than their cohorts from a reference site. The two bands of carbonylated protein visualized using Western blotting techniques may in fact be fish serum albumin, a conclusion based on the fact that the bands are of the same molecular weight as BSA. Albumin is normally the most abundant protein present in plasma samples and may undergo carbonylation in corkwing wrasse exposed to heavy metals.

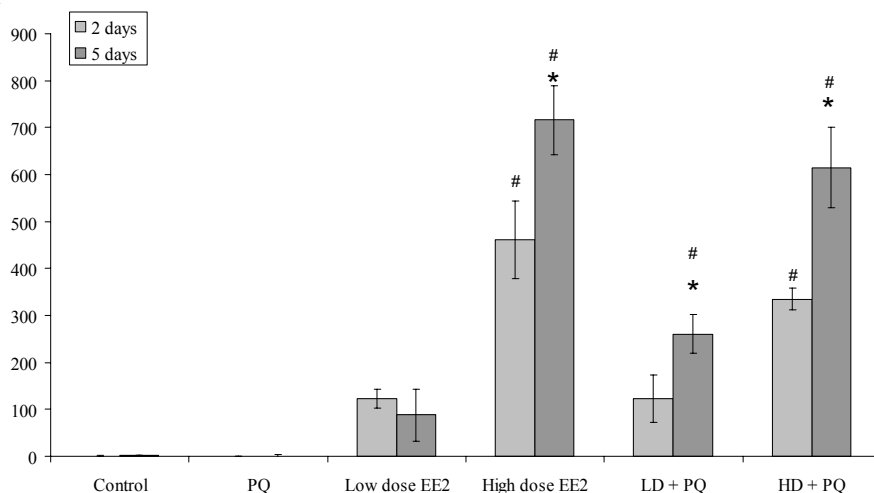
However, during reproductive life stages in female fish, vitellogenin (VTG) is one of the most abundant plasma proteins. VTG is a precursor to egg yolk proteins, produced in the liver of female fish and transported via the blood stream to the ovaries during vitellogenesis. Ando and Yanagida (1999) observed that VTG in the plasma of Japanese eel could have a protective effect against copper-induced damage to low density lipoproteins *in vitro*. We conducted a laboratory exposure study using juvenile rainbow trout to test whether VTG undergoes protein carbonylation *in vivo* (unpublished results from honors project, Eklöf et al. 2007). Fish were exposed IP to 17 $\alpha$ -ethinyl estradiol (EE2) in two doses (0.3  $\mu$ g/kg fish or 3  $\mu$ g/kg fish) in order to induce vitellogenesis, followed 5 days later by an IP injection of PQ (10  $\mu$ g/kg fish), a model substance known to induce oxidative stress. Fish were sampled 2 and 5 days following injection with PQ. Blood plasma was analysed for VTG levels and protein carbonylation using, in both cases, ELISA methods and Western blotting techniques (Silversand et al. 1993, Levine et al. 1994, Winterbourn and Buss 1999). Protein carbonyl levels did not increase in treatment groups compared to the control group. The fish used in this study were juveniles, less than 1 year of age, and other studies have indicated that protein carbonylation tends to accumulate as animals, including fish, age (paper IV, Harman 2001, Kishi 2004). Furthermore, younger animals normally have greater antioxidant defences than older counterparts (Otto and Moon 1996, Beckman and Ames 1998, Harman 2001), which may help them to reduce the amount of ROS-induced damage produced by a redox cycling compound such as PQ. Protein carbonyl levels did decrease from day 2 to day 5 in all groups exposed to EE2 while they increased in the fish exposed to PQ alone (fig. 7).

Levels of VTG (fig. 8) increased with increasing EE2 dose, as was expected. VTG also increased from day 2 to day 5 in fish receiving the high dose of EE2.

This was also the case in fish exposed to the low dose of EE2 and PQ, indicating that the PQ exposures may have had some effects on VTG metabolism. An analysis of 20S proteasome activity in these fish indicated no significant differences between groups (data not shown).

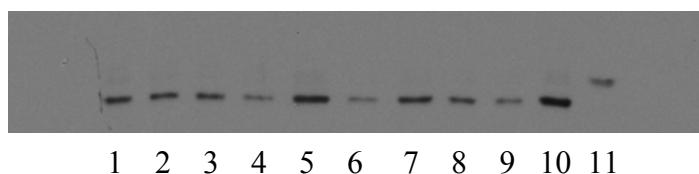


**Figure 7:** Protein carbonyls (nmol/mg prot) in plasma of rainbow trout treated with paraquat (PQ) (10  $\mu\text{g}/\text{kg}$  fish), 17 $\alpha$ -ethinyl estradiol (EE2) low dose (LD) (0.3  $\mu\text{g}/\text{kg}$  fish) or high dose (HD) (3  $\mu\text{g}/\text{kg}$  fish). \* represents statistical difference between 2 and 5 days,  $p < 0.05$ .



**Figure 8:** Vitellogenin levels ( $\mu\text{g}/\text{ml}$  plasma) in plasma of rainbow trout treated with paraquat (PQ) (10  $\mu\text{g}/\text{kg}$  fish), 17 $\alpha$ -ethinyl estradiol (EE2) low dose (LD) (0.3  $\mu\text{g}/\text{kg}$  fish) or high dose (HD) (3  $\mu\text{g}/\text{kg}$  fish). \* represents statistical difference between VTG levels at day 2 and day 5, within a treatment group. # represents statistical difference between treatment and control group, at either 2 or 5 days.  $p < 0.05$ .

A western blot analysis of plasma samples suggested again that albumin may be the most likely candidate for protein carbonylation, and not VTG as we suspected. In this case, the carbonylated proteins seen on the western blot are slightly smaller than BSA, which has a molecular weight of 66.4 kDa (fig. 9), but the other known abundant plasma proteins are larger, with the following approximate weights: VTG, 205 kDa, fibrogenin, 300 kDa and transferrin, 90 kDa. The difference in size observed in fig. 9 may be due to molecular differences in albumin structure between species, or may even be due to the effects of carbonylation. The oxidation of albumin has been the subject of many studies in human disease research, since this plasma molecule has been shown to be the major plasma protein target of oxidative stress while other plasma proteins, including transferrin and immunoglobulin, remain unaffected (Himmelfarb and McMonagle 2001). Some research has also identified fibrinogen as the major target protein for oxidative damage in plasma (Shacter et al. 1994). Amino acids in albumin molecules undergo carbonylation and thiolation and have the ability to scavenge ROS and bind transition metals. Albumin is a quantitatively important antioxidant in blood and extracellular fluids (Halliwell 1988). These unpublished results from studies in corksing wrasse and rainbow trout indicate that this may well be the case in fish plasma as well.



**Figure 9:** Western blot of protein carbonyls in plasma of juvenile rainbow trout treated with PQ and/or EE2. Lanes 1-3 – control, lanes 4-5 – PQ (10 µg/ kg fish), lanes 6-8, low dose EE2 (0.3 µg /kg fish) + PQ, lanes 9-10 high dose EE2 (2 µg /kg fish) + PQ, lane 11, positive control (oxidized BSA, 66.4 kDa).

### 4.3 Effects of age in fish on oxidative stress and damage

One of the major hypotheses of the free radical theory of aging is that oxidative damage products will accumulate within the cells of aging organisms as a result of free radical reactions (Harman 2001). Aging results in increased protein carbonylation as well as decreased antioxidant defences, including the secondary antioxidant, the 20S proteasome. These parameters were investigated in brown trout in paper IV.

#### 4.3.1 Effects of age and induced oxidative stress in brown trout (*S. trutta*)

Protein carbonylation and 20S proteasome activity were measured in two different tissues (liver and brain) of hatchery raised brown trout from 4 different age groups, 0+, 1, 2, and 3 years, in paper IV. Total glutathione, GSSG and %GSSG were measured in liver tissue of these same fish. The youngest 2 age groups of fish, 0+ and 1 year, were also exposed to PQ via IP injection to induce oxidative stress and were sampled after 4 days.

Protein carbonylation was significantly higher in the 3 year old fish and 20S proteasome activity was significantly lower in the 3 year old fish, compared to the 0+ group, in both liver and brain tissue. A Pearson correlation test revealed a significant correlation between both parameters and age in both tissues. Protein carbonylation was significantly higher in liver tissue than in brain tissue while 20S proteasome activity was significantly higher in brain tissue. These two parameters are interrelated in that an increase in protein carbonyls can lead to formation of agglomerates which are insoluble and can block activity of the 20S proteasome, which in turn leads to further accumulation (Grune et al. 2004). The results presented here are in accordance with observations in other species where there is an age-related accumulation of protein carbonyls paired with an age-related decrease in 20S proteasome activity (Grune 2000, Davies and Shringarpure 2006, Farout and Friguet 2006). However, only parameters measured in 3 year old fish differed significantly from the youngest, age 0+, according to a Bonferroni ANOVA test. It is possible that oxidatively damaged proteins accumulate in an exponential manner in this fish species, so that it is only possible to measure significant effects after a certain age. Brown trout are known to live up to 10 years, given favorable conditions, so 3 years may be a relatively young age. Oliver et al. (1987) have found that carbonyl content of human fibroblasts increases rapidly only when the age of the donor is 60 years or more, but that levels in diseased patients (i.e. progeria, Werner's syndrome) are much higher than age matched controls.

The ratio between GSSG and GSH (%GSSG) significantly increased with age. Total glutathione increased at 1 year of age and then decreased again at 2 and 3 years of age. This decreasing trend was significant from 1 to 3 years of age. GSSG shows an increasing trend with age, though this was not significant and is less clear than with tGSH. Otto and Moon (1996) conducted a study addressing effects of aging on antioxidant parameters in teleost fish and found that GSH levels increased in the majority of tissues in older rainbow trout, while they found no differences in GSSG levels. Black bullhead (*Ameiurus melas*), on the other hand, showed decreases in tGSH in gill and plasma samples. Otto and Moon's results from

rainbow trout are in contrast to those I found and to those reported in the majority of other organisms. See review by Beckman and Ames (1998).

Induced oxidative stress did not affect levels of glutathione within the liver tissues. No differences were seen in tGSH, GSSG or %GSSG when fish were treated with PQ. A significant increase in protein carbonyls in livers of 1 year old fish resulted from PQ treatment, compared to 0+ fish, while no such increase was seen in brain tissue. 1 year old fish also showed a significant decrease in 20S activity in brain tissue. A trend towards an increase in 20S activity in liver tissue after PQ treatment was seen in liver, but this was not significant. The fact that no effects were measured in 0+ fish may be due to the fact that younger animals tend to have better antioxidant defences (Otto and Moon 1996, Beckman and Ames 1998, Harman 2001) and may be better able to protect against oxidative stress induced by PQ.

#### **4.4 Oxidative damage as a biomarker of exposure in fish**

As discussed in papers I and III, protein carbonyl levels can be difficult to interpret. A decrease in protein carbonyl levels may indicate that the susceptibility to proteolytic degradation has been increased by mild oxidation of proteins (Grune et al. 2004). This may be paired with an increase in 20S proteasome activity. An increase in protein carbonyl levels could indicate that normal protein metabolism is disrupted, resulting in accumulation of damaged molecules. In order to gain a more complete picture of the effects of exposure to complex mixtures of pollutants in unknown amounts, such as is often the case in field studies, measurements of both protein carbonyls and 20S activity should be conducted. Additionally, as shown in paper IV, levels of protein carbonylation and 20S proteasome activity are affected by age in trout, as are responses to xenobiotic-induced oxidative stress. In the laboratory studies conducted here, an oil exposure study using eelpout (paper I) and a copper and PQ exposure study using rainbow trout, xenobiotic exposure resulted in dose dependent responses in protein carbonylation in liver tissue. This indicates that measurement of protein carbonylation is a potentially useful biomarker of oxidative stress in fish when exposure history is well known. Use of this oxidative damage product is already well established in other species, including humans, especially as a marker of disease pathologies (Berlett and Stadtman 1997, Stadtman 2002).

Several other studies have measured protein carbonylation in fish species as a biomarker of oxidative stress (Parvez and Raisuddin 2005, Bagnyukova et al. 2006, Craig et al. 2007). A freshwater fish, the spotted snakehead (*Channa punctata*), had increased levels of protein carbonyls in gills, liver and kidneys after acute exposure

to various pesticides including PQ (Parvez and Raisuddin 2005). Goldfish (*Carassius auratus*) exposed to iron ions at different pHs displayed increased protein carbonyl levels in both liver and kidney tissue (Bagnyukova et al. 2006). These authors also measured an indirect correlation between G6PDH activity and protein carbonylation in liver tissue. As this enzyme is known to be susceptible to MCO reactions (Fucci et al. 1983, Sohal et al. 1995), it is probable that it sustains oxidative damage and loses catalytic function.

These studies measuring protein carbonylation in fish, as well as the vast literature concerning formation of protein carbonyls in other model organisms (Sitte et al. 2000, Stadtman and Levine 2000, Grune et al. 2001, Dalle-Donne et al. 2003, McDonagh et al. 2005), indicate that this biomarker has good potential for use in biomonitoring campaigns. However, evidence points to the need for establishing age-related changes in protein carbonylation in different species (paper IV, Berlett and Stadtman 1997, Biesalski 2002, Harman 2003). Measurement of protein carbonyls seems to be a useful biomarker in studies addressing oxidative damage accumulation with increasing age in teleost species, as described here in paper IV. However, both the identification of specific proteins that are more susceptible to oxidative damage as well as effects of age-related changes in antioxidant defences would help create a clearer depiction of the effects of xenobiotic-induced protein carbonylation on teleost fitness and the ecological relevance of this biomarker.

The use of TBARS as a biomarker in fish studies is better established than the use of protein carbonylation (Ploch et al. 1999, Roméo et al. 2000, Oakes and Van Der Kraak 2003, van der Oost et al. 2003, Oakes et al. 2004). The work discussed in paper III shows that exposure to sewage treatment plant effluent can cause an increase in lipid peroxidation in rainbow trout. STP effluent often contains a number of contaminants that can act as prooxidants (Paxeus 1996, Livingstone 2001). We could not, on the other hand, show lipid peroxidation in eelpout exposed to compounds released from sediment during a dredging campaign, or exposed to crude oil rich in PAHs (paper I). Eelpout may not be susceptible to this form of oxidative damage by the xenobiotics investigated here.

Ploch et al. (1999) treated two different species of fish, brown bullhead and channel catfish, with a prooxidant (t-BOOH), with and without induced glutathione depletion. Here, glutathione depletion alone caused an increase in lipid peroxidation in brown bullhead, a response which was augmented following treatment with t-BOOH. Channel catfish had elevated levels of TBARS following both glutathione depletion, t-BOOH exposure and treatment combining both glutathione depletion and t-BOOH. TBARS have also been used as a biomarker in white sucker (*Catostomus commersoni*) exposed to pulp mill effluent (Oakes and Van Der Kraak 2003) and longnose sucker (*Catostomus catostomus*) exposed to either pulp mill effluent or sewage treatment effluent (Oakes et al. 2004). Wild

caught white suckers exposed to pulp mill effluent had increased levels of lipid peroxidation in liver tissue. Longnose sucker, also caught during a field study, showed elevated levels of TBARS in both gonadal and hepatic tissue after exposure to pulp mill effluent while those fish captured downstream from an STP did not, with the exception of one sampling time point. These results are contradictory to those presented in paper III, but differences may lie in the types of chemicals present in the effluent water of the two STPs or in differing responses between rainbow trout and longnose sucker. Roméo et al. (2000) measured the effects of *in vitro* cadmium and copper exposure on lipid peroxidation in sea bass (*Dicentrarchus labrax*) kidney microsomes. Cu resulted in dose related increases in lipid peroxidation while Cd did not. The authors suggest that these differences in effect may be due to the fact that the kidney is the main storage organ of Cd where exposure leads to increased MT expression in this organ, thereby offering protection, while Cu is stored in the liver. Cu, on the other hand, is able to redox cycle, resulting in increased ROS production and lipid peroxidation.

Some controversy exists concerning the use of and specificity of the TBARS method, which measures MDA. This is in part due to the fact that lipid peroxidation can occur both as a result of xenobiotic-related effects as well as a result of other cellular damage injuries. Additionally, the TBARS method will also include reactions with non-MDA substances. This can be reduced by the inclusion of the antioxidant BHT in reaction mixtures (Oakes and Van Der Kraak 2003). Despite these problems, measurements of lipid peroxidation are considered to be of great importance in environmental risk assessment (van der Oost et al. 2003).



## 5. Summary and Conclusions

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The general aim of this dissertation was to evaluate the effects of different types of environmental pollution on oxidative stress in teleost fishes. Four different species were used in field studies, a caging study and laboratory exposure studies, in order to assess the effects of exposure to sewage treatment plant (STP) effluent, PAHs, metals and paraquat (PQ) on antioxidant defences and oxidative damage, namely protein carbonylation and lipid peroxidation. Effects of aging, alone or in combination with oxidative stress, on protein carbonylation were also addressed.

Activities of several antioxidant enzymes were affected in rainbow trout caged in a river polluted by STP effluent. CAT activity increased, indicating the presences of compounds that can cause oxidative stress, while DTD activity decreased. Measurement of DTD mRNA using qPCR showed no effects, indicating that exposure to STP effluent resulted in inhibition of DTD at the protein level. This is of interest because of the possibility that some xenobiotics which could be present in the STP effluent, for example the pharmaceutical wafarin, could inhibit this enzyme thereby by decreasing the fish's ability to metabolize redox cycling compounds. This could have hazardous effects on the fish.

On the other hand, antioxidant enzyme activity was on the whole not affected in the corkwing wrasse caught at a PAH and a metal contaminated site, nor were they affected in rainbow trout caged in a river with polluted sediment. Antioxidant enzyme activities do not seem to provide good biomarkers for oxidative stress caused by exposure to pollution in a field situation in the species investigated here.

GSH is an antioxidant molecule often used as a biomarker of oxidative stress. Rainbow trout exposed to STP effluent had increased levels of tGSH, GSSG and %GSSG in their liver tissues. tGSH was decreased in male corkwing wrasse captured at a PAH contaminated site while those individuals captured at the heavy metal contaminated site showed no effects.

Oxidative damage products seemed to be better biomarkers of oxidative stress in fish exposed to pollutants. Lipid oxidation products, TBARS and 4-HNE, were elevated in rainbow trout caged at the STP effluent. Protein carbonyls were increased in corkwing wrasse exposed to metals, in rainbow trout exposed to STP effluent and in eelpout exposed to PAH rich oil and dredging material from the harbor basin. Results from eelpout exposed to PAHs and dredging material indicated, however, that the relationships between formation, proteolytic degradation, and accumulation of protein carbonyls could be important parameters affecting the amount of damaged proteins that can be measured in a tissue sample from a fish in an exposure study. Also, lipid peroxides can interfere with protein metabolism during oxidative stress, and should be measured.

Albumin was suggested as the plasma protein molecule most likely to undergo carbonylation in corkwing wrasse exposed to heavy metals in the field under chronic conditions, as well as in rainbow trout exposed to PQ in a laboratory exposure study. Vitellogenin did not seem to be damaged in these rainbow trout, as hypothesized.

Changes in protein carbonylation and 20S proteasome activity were also measured in brown trout of different age classes. As a decrease in antioxidant defences and an accumulation of oxidative damage products are two parameters associated with aging in a variety of other organisms, it is important to consider these age effects when measuring oxidative stress parameters as biomarkers in fish. Results from paper IV clearly show an increase in protein carbonyls and a decrease in 20S proteasome activity in rainbow trout as they age. Also, the youngest age group, 0+, treated with PQ did not show any effects while 1 year old fish had an increase in protein carbonyls in their livers and a decrease in 20S activity in their brains following PQ exposure. This indicates that fish of different ages show different responses in oxidative stress parameters when exposed to a redox cycling compound.

In addition to changes in protein carbonyls and 20S activity, GSH levels were affected as fish aged. tGSH levels in livers of brown trout declined after the age of 1 year, and %GSSG increased from 0+ to 3 years of age. While this antioxidant molecule was not affected by PQ treatment in 0+ and 1 year old fish, it is clearly affected by aging.

## 6. Future Perspectives

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The work conducted in this thesis has focused largely on the use of biomarkers of oxidative stress in different fish species. Here, biomarkers are used to identify effects of xenobiotics on fish species in marine and aquatic ecosystems by comparing individuals from polluted and reference sites. Though these biomarkers may function well in projects designed as such, there are large gaps in our knowledge concerning base line levels of antioxidant enzyme activity and regulation of GSH levels in several of the species used here. Though more information is available concerning these parameters in rainbow trout, which are more extensively used in oxidative stress studies, baseline knowledge could also be expanded in this species. Studies should be designed to address effects of annual variations, temperature differences, nutritional status, and life and reproductive stages in all of these species if they are to be used in biomonitoring. Also, research focusing on regulation of both gene transcription as well as protein activity could prove invaluable.

Future research should also focus on the effects of age on both antioxidant defences as well as on oxidative damage products. A number of studies have addressed changes in antioxidant enzyme activities as fish age. However, these studies are few, and the results presented in this thesis suggest that both enzymatic defences as well as molecular antioxidants may be affected by aging in fish. Studies addressing these changes should investigate how the accumulation of oxidative damage products is affected by aging. As oxidative stress, specifically protein carbonylation and DNA damage, is viewed as one of the most important factors affecting cellular aging, these parameters are essential to consider when using oxidative stress as a biomarker of xenobiotic exposure in fish.

Protein carbonylation could provide a useful biomarker of oxidative stress resulting from xenobiotic exposure, as is demonstrated both in the current thesis as well as in a few other recent publications (Parvez and Raisuddin 2005, Bagnyukova et al. 2006, Craig et al. 2007). However, the relationship between accumulation of protein carbonyls and 20S proteasome activity is vital to address in oxidative stress conditions. Mechanisms affecting the formation of protein carbonyls as well as those controlling 20S proteolysis need to be elucidated. Lipid oxidation products are also essential to address here, as they can affect transcription of antioxidant enzymes as well as activity of the 20S proteasome.

Finally, future research should focus on identifying which specific proteins are damaged via carbonylation during oxidative stress, as there may be age or sex differences in this regard. This information could provide knowledge on the toxicological relevance of protein oxidation, as damaged proteins may be involved in any number of cellular processes which could affect organismal health and therefore have implications on the population level.

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## 9. Swedish summary

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Oxidativ stress, som kan mätas som förändringar i antioxidanta skyddet och oxidativa skador, är vanlig hos organismer som exponeras för föroreningar i miljön. I denna avhandling studeras effekterna av olika typer av miljöföroreningar på oxidativ stress hos fiskar. Effekter av åldrande och åldrande i kombination med oxidativ stress, har också undersökts.

Antioxidantenzymaktiviteter mättes i regnbåge (*Oncorhynchus mykiss*) som hölls i burar i en å med kontaminerade sediment samt utsläpp från ett kommunalt reningsverk. Antioxidantenzym mättes även i skärsnultra (*Symphodus melops*) fångade vid två platser förorenade av antingen tungmetaller eller PAH'er. Antioxidantenzym visade få effekter hos fiskarna i dessa studier.

Däremot var proteinkarboxylnivåerna förhöjda i plasma hos skärsnultror fångade vid den metallförorenade lokalen och i regnbåge burade vid avloppsvattnet från reningsverket. I regnbåge konstaterades även förhöjd lipidperoxidering. Biomarkörer för oxidativa skador mättes också i tånglake (*Zoarces viviparus*) fångade i en förorenad hamn, innan, under och efter en större muddring, samt efter ett oljeutsläpp. Proteinkarboxylnivåerna i leverna från tånglakarna varierade med säsong och var påverkade av muddringen och oljeutsläppet. Både förhöjda och sänkta nivåer uppmättes, vilket indikerar att relationen mellan exponering för prooxidanter och akumulering av proteinkarboxyl är komplicerad. Western blot analyser av proteinkarboxyleringen i skärsnultra exponerade för tungmetaller samt i regnbåge exponerade för paraquat (PQ) tyder på att albumin är det plasmaprotein som skadas mest via karboxylering i dessa fiskar.

Öring (*Salmo trutta*) användes i ett laboratorieförsök för att utvärdera effekter av åldrande på proteinkarboxylering, 20S proteosomaktivitet samt nivåer av antioxidanten glutation. Alla parametrar påverkas av ålder. 20S aktiviteten och glutation minskade med åldern medan protein karboxyl och oxiderat glutation ökade. Parametrarna påverkades även efter PQ-exponering, där ettåriga fiskar var känsligare än 0+ fiskar.

Oxidativ stress är ett komplext fenomen att mäta i fältstudier. Ändringar i antioxidantaktiviteterna verkar indikera akuta stress responser till en exponering; aktiviteter kan återgå till normala nivåer när djuren har aklimatiserat sig. Däremot, tycks oxidativa skador som proteinkarboxyl och lipidperoxider bestå. Slutsatsen av resultaten presenterade i denna avhandling är att antioxidantenzym inte fungerar väl som biomarkörer vid exponering av fisk fångade i förorenade lokaler medan oxidativa skador kan vara användbara biomarkörer i vildfångad fisk. Det är också viktigt att ta hänsyn till åldern av individerna som undersöks eftersom oxidativ skador ökar med åldern medan antioxidanter minskas.