Universiteit Antwerpen

Defense mechanisms of fish with different sensitivities to Copper: interaction and dynamics of proteins and hormones

Verdedigingsmechanismen van vissen met verschillende stressgevoeligheden voor zware metalen: interactie en dynamiek van eiwitten en hormonen

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de

Biomedische Wetenschappen aan de Universiteit van Antwerpen

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Contents

Abbreviations

96h LC50	Lethal concentration for 50% of the population within 96 hours
Asc	Ascorbate
BAM	Biologically Active Metal
BIM	Biologically Inactive Metal
Ca	Calcium
CA	Carbonic Anhydrase
CAT	Catalase
Cl	Chloride
Cu	Copper
DNA	Deoxyribonucleic Acid
Dige	Differential In Gel Electrophoresis
GO	Gene Ontology
GR	Gluthatione reductase
GSH	Glutathione
iTRAQ	isobaric Tags for Relative and Absolute Quantification
MRC	Mitochondria Rich Cell
mRNA	messenger Ribonucleic Acid
MT	Metallothionein
Na	Sodium
PNA	Peanut lectin Agglutinine
PRL	Prolactin
PVC	Pavement Cell
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
T3	Triiodothyronine
T4	Thyroxine

Chapter 1

General introduction

1.1 Problem Statement.

Historically, toxic metals in the aquatic environment contributed substantially to water pollution in Belgium, despite the recent improvements in water quality. Although some of these toxicants are essential for cellular processes in small quantities and thus for the ability to live, they can jeopardy ecosystems when the concentration reaches a certain threshold value (Ahmad et al., 2005; Pyle and Wood, 2008). For instance copper (Cu), which has uses in agriculture and the industry, is known to be vital in cellular biochemistry (Burke and Handy, 2005), however can cause deleterious effects when concentrations are high (Kaùr and Dhawan, 1994; Taylor et al., 2003). An excess of free Cu-ions will induce hydroxyl radicals and subsequent oxidative stress, a disturbed ion homeostasis as well as DNA damage (Bury et al., 1998; Grosell et al., 2002; Wood, 2001), and will eventually lead to cardiac arrest (Evans et al., 1999).

Initially, Cu rapidly accumulates in gill tissue, initiating ionoregulatory disturbances such as disruption of the active uptake mechanisms for Na^+ and Cl^- (mainly through reduction of Na^+/K^+-ATPase activity) and an increase in gill permeability (De Boeck et al., 2001; Grosell et al., 2003). Secondly, accumulation and storage in liver tissue and other organs, together with increased stress hormone production, introduces additional challenges (Grosell and Wood, 2002; Laurén and McDonald, 1987). Nevertheless, among species, different sensitivities and handling strategies of Cu exist to protect the organism for the Cu induced toxicity. Since this defense network contains several aspects of metabolism and is intertwined with the concentration of metal transporters, ion homeostasis, protein production, intracellular partitioning and many others, unraveling the exact processes and steps in these mechanisms is intriguing and necessary to understand differences in metal sensitivity. Moreover, Cu resistance or acclimatization mechanisms are dynamic processes and some species show better developed ones than other. Brazilian tambaqui, for instance, are equipped with natural defenses such as an unique Na^+-channel and Na^+ -transport system which are less sensitive to Cu (Matsuo, et al., 2005). Adaptation occurs as well, such as in the Australian black striped rainbow fish which gained an increased resistance after 40 years of adaptation to mine disposal (Gale et al., 2003). Besides these natural obtained defense mechanisms, studies also provided evidence for plasticity to develop an increased Cu tolerance in laboratory settings (Grosell et al., 2001; Hashemi et al., 2008).

To study Cu and the different responses it elicits, 3 fish species which showcase clear differences in Cu bioaccumulation patterns and toxicity: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio*), were chosen based on previous research (De Boeck et al., 2004). Interestingly, the sensitive rainbow trout showed low uptake rates and elaborate negative effects, while the most resistant fish species, the gibel carp, displayed opposite characteristics. Together with the intermediate sensitive common carp, these fish species form a solid base to study involved hormone regulation, intracellular Cu distribution, oxidative stress reactions and dynamics of specific metal binding protein, chaperones and transporters. The resulting knowledge will hopefully reveal the main players in the resistance of gibel carp and the vulnerability of rainbow trout to Cu exposure.

1.2 Research goals.

Since gills are the first and largest exchange surface in fish, they are also the first target for metal toxicity. Therefore we chose for an in depth comparison of effects and processes in this tissue.

- ✓ Firstly, since Cu disrupts the ion regulation we looked at plasma ion concentrations, ion transport (gill Na^+/K^+-ATPase activity), concentrations of ionoregulatory hormones (plasma T3/T4) and the expression of some receptors of ion-regulatory hormones (prolactine receptor and thyroid hormone receptor β in gill). This study will give us an insight in how these fish species manipulate their ionoregulatory important hormones and ion osmoregulatory processes when exposed to Cu. (Chapter 3)

- ✓ Secondly, since a well performing anti-oxidant defense system is essential for any organism to protect itself against the adverse effects of Cu exposure, superoxide dismutase activity, catalase activity, glutathione reductase activity and reduced glutathione and ascorbate concentrations after Cu exposure were determined in gill tissue. As such, we can assess if differences in anti-oxidant defenses in the gills of these fish are contributing to the differences in sensitivity to Cu exposure. (Chapter 4)

- ✓ Thirdly, intracellular differences in protein expressions, transporters, carriers and metal binding proteins,…might as well be key components in adequately diminishing negative effects opposed by Cu exposure. By studying the gill proteome of the three fish species, we can evaluate the dynamics of these protein patterns and glance at existing (dis-) advantages in the protein apparatus. (Chapter 5)

- ✓ Finally, with the last study, we wanted to evaluate the dynamics of the subcellular accumulation patterns in three different fish species under a similar scenario of sublethal copper exposure. The aim of this Cu analysis in subcellular compartments is to assess whether gill and liver cells of fish with a different sensitivity to copper exposure show intracellular differences in accumulation and handling of copper and whether this can help explain their differences in sensitivity to metal exposure. (Chapter 6)

Our ultimate goal is to be able to get a clear picture of the differences in intracellular physiological and molecular responses in gills of our model species

1.3 Copper accumulation and toxicity.

1.3.1 Sources and bioavailability of Cu in the environment.

Sadly, while increasing modernization and civilization means growing comfort and knowledge, it also leads to augmented concentrations of pollutants in the environment. Cu has been used by humanity for more than 7000 years, first as an implement in weapons, jewelry and tools, and later on the ductility and conductivity of heat and electricity of this metal was discovered. It was mined intensively and therefore uses in electrical wiring and metal pipes expanded (Hong et al., 1996; Shrivastava, 2009). Additionally, nowadays Cu compounds are commonly used in agriculture as pesticides, for water treatment and in many other fields (Kiaune and Singhasemanon, 2011).

Unfortunately, Cu is not biodegradable, on the contrary, it is very persistent and as such, easily builds up in the ecosystem. Releases of Cu in water can be natural (rock weathering and atmospheric deposition) or anthropogenic (urban and agricultural) (Nirel and Pasquini, 2010). Soil, sediment, water and organic materials in urban and industrial areas are known to contain higher, or even toxic, concentrations. However, the environmental concentration of a metal does not relate directly to toxicity in aquatic organisms. Of outmost importance for the occurrence of toxicity is the bioavailability of the metal. This indicates which part of the total concentration is available for incorporation into biota. Bioavailability is a complex issue relying on many factors including pH, concentration and speciation, total organic content (particulate and dissolved), water hardness, salinity and more (Erickson et al., 1996). Dissolved organic matter is one of the factors that can decrease negative physiological impact by complexation with Cu ions acutely but foremost chronically (McGeer et al., 2002; Richards et al., 1999; Kim et al., 1999). The organic matter concentration can greatly exceed Cu ion concentrations in the water column and is therefore able to provide a large buffering capacity by forming copper complexes (Kiaune and Singhasemanon, 2011). The speciation of trace metals is related to the chemical conditions of the surroundings and can consist out of free metal ions, inorganic and organic complexes and organometallic compounds. In reducing aquatic conditions, metals are often associated with sulfide minerals which have limited negative effects as long as they are kept in those reducing conditions (Luoma, 1989). The protective effects (and other) of different parameters were brought together in the Biotic Ligand Model for Cu, resulting in more accurate predictions of the free metal concentration in

the aquatic environment and estimations of metal-biotic ligand interactions (Niyogi and Wood, 2002; Paquin et al., 2002).

1.3.2 Cu homeostasis, handling and subcellular distribution.

Cu is an essential metal of which the concentration is strictly regulated within a narrow range (Grosell et al., 1997). The delicate balance between metal deficiency and metal excess is controlled by a well coordinated uptake and excretion (Bury et al., 2003). Unlike other vertebrates, fish have two uptake routes: through the diet and directly from the water across the gill epithelium. For freshwater fishes, the later is the most important, since they hardly drink. As such, in this PhD, the main focus was placed on Cu uptake at the gill in freshwater fishes. For reviews on Cu uptake in the intestine, we refer to Clearwater et al. (2002) and Burke and Handy (2005).

Homeostatic mechanisms are able to buffer an excess of Cu for a certain amount of time and up to a certain concentration. However, when this period of time or concentration is exceeded, toxicity can occur. The organism can for instance, decrease the metal accumulation, increase metal excretion, differentially allocate the metal in different organs or change the intracellular distribution in a cell. In an environment with high Cu concentrations, rapid accumulation of this metal in gill tissue, followed by a steady state (albeit with elevated Cu concentrations), occurs (De Boeck et al., 2004; Grosell et al., 1996, 1997, 1998a.). Furthermore, such a waterborne Cu exposure can result in a transient increased plasma Cu concentration in freshwater fish and increased Cu concentrations in a number of other tissues such as liver, kidney, gut and muscle (Grosell et al., 1997; Kamunde and McPhail, 2008). In plasma, the majority of Cu is bound to albumin or other low molecular weight proteins and is quickly cleared from the plasma by the liver. The liver is known to be essential in Cu homeostasis (Grosell et al., 2000). The majority of Cu is accumulated in this organ, in normal and in excess conditions (Giguère et al., 2004). Moreover, the liver produces ceruloplasmin, which can distribute Cu to extrahepatic tissues for incorporation in metal dependent enzymes or proteins (Bury et al., 2003; Harris, 2000).

Metal uptake and accumulation greatly depends on extrinsic environmental factors such as water chemistry and temperature, and on intrinsic factors which determine how the metal is processed in the organism/cell (Kraemer et al., 2005). Furthermore, the Cu acquisition via the gills is adjusted to the environmental Cu concentrations. In normal conditions, the uptake is relatively low, however, when dietary Cu concentrations are reduced, the uptake can increase

dramatically (Kamunde et al., 2002b). In contrast, when waterborne Cu concentrations are increased, uptake at the gills can decrease (Grosell et al., 1998a; Kamunde et al., 2002a).

More into detail, the metal binds to the mucus layer on the gills upon exposure. This mucus can provide a temporary protection and can decrease Cu accumulation for a certain amount of time (Kraemer et al., 2005). Cu moves most likely across cell membranes as the Cu^{+} ion via a Na-insensitive high specific Cu- channel (CTR1) and a Na-sensitive leak through epithelial Na Channels (ENaC) (Craig et al., 2010; Grosell and Wood, 2002; Handy et al., 2002; Harris, 1991) (Fig.1 Bury et al., 2003). The Cu^{2+} ion is probably transported via a Na-insensitive divalent metal transporter (DMT1) or reduced to Cu^{+} at the gills facilitated by a metal reductase (Bury et al., 2003).

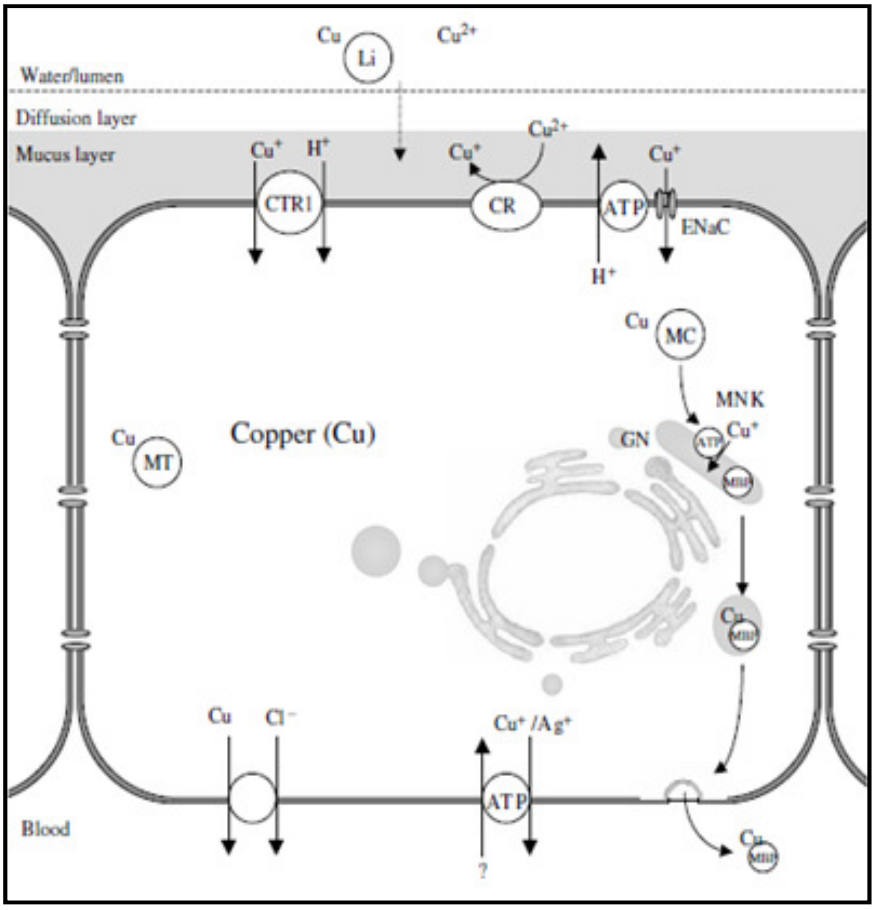

Fig.1 Represents the hypothetical generic epithelial copper uptake pathways in teleost fish. CTR1: Cu Transporter; ENac: epithelial Na channel; MC: metallochaperones; MNK: Menkes-like Cu ATPase; MT: Metallothionein; Li: ligand, CR: Cu reductase (Bury et al., 2003). Cu can enter the cell through sodium insensitive and sensitive channels. Once within the cell, Cu is associated with glutathione and sequestered by metallochaperones which deliver Cu to sites of incorporation, to MT or to detoxification vacuoles. Furthermore, through a pathway across the golgi apparatus, it can be excreted at the basolateral side of the cell.

Once within the cell, Cu is associated with the most abundant intracellular Cu ligand, glutathione (GSH) (Banci et al., 2010). Thereafter, Cu can be sequestered to metallochaperones (MC) which deliver Cu to sites of incorporation, to metallothionein (MT) or to vacuoles for detoxification (Horn and Barientos, 2008). These MCs guide and protect the metal ion while

they promote correct interactions for instance with Cu containing enzymes such as superoxide dismutase and mitochondrial cytochrome oxidase (Horn and Barientos, 2008; O'Halloran and Culotta, 2000). Furthermore, gills are thought to have an important role in Cu homeostasis by adapting the concentration of Cu transport proteins according to the availability of Cu in the water (Kamunde et al., 2002a). MCs bind Cu and transport it to the Golgi network (GN) where it is incorporated in metal binding proteins (MBP) via a Menkes-like Cu^{+}-ATPase (MNK). Subsequently, vesicles from the Golgi apparatus are transported to the basolateral membrane, where exocytosis can take place (Fig.1 Bury et al., 2003). This Cu^{+}-ATPase will relocate to the plasma membrane in the presence of excess Cu (Camakaris et al., 1999; Grosell and Wood, 2002). MTs on the other hand, are capable of metal detoxification and can protect the organism for oxidative stress and toxicity (Dos Santos Carvalho et al., 2004). Moreover, the concentration of MT can increase rapidly upon exposure albeit not to the same extend in all tissues or fish species (De Boeck et al., 2003). Metals bound to MT or stored in metal rich granules or lysosomes, are considered metabolically unavailable and are consequently not bioavailable to more sensitive cellular fractions (Laflamme et al., 2000; Kraemer et al., 2005; Wallace et al., 2003).

Having the knowledge of the internal association of Cu with specific subcellular compartments can shed light on the homeostasis, the different accumulation strategies and the tolerance to Cu (Kamunde and MacPhail, 2008; Vijver et al., 2004; 2006). Most importantly, metal toxicity is often discussed in terms of distribution of the excess Cu to sensitive compartments of the cell, which are the organelles and enzymes. The internal compartmentalization of metals is mostly studied by a differential centrifugation protocol to isolate several fractions (Wallace et al., 1998; Vijver et al., 2004). The resulting subcellular fractions can be divided into biologically active (BAM) and biologically inactive metal (BIM) pools (Steen-Redeker and Blust, 2004). Rainbow (2002) indicated that the onset of toxic effects is highly dependent of the concentration of metals in the biologically available (or active) form. Therefore, when the metal concentration in BAM exceeds a certain threshold value, toxicity can occur which can have effects on an array of physiological processes. The BAM is comprised of the organelles fraction, the heat denaturable fraction and the nuclei-cell debris fraction. The enzymes and proteins in these fractions are vulnerable to increased Cu concentrations since Cu can induce alterations in enzyme and protein conformations, and as such inhibit their function. The metal rich granule fraction and the heat stable fraction are appointed to the BIM since these fractions are known to be part of certain metal detoxification

pathways (Wallace and Lopez, 1997), mostly by binding the excess Cu. Consequently, if more Cu is stored in the BIM, less Cu is available and increased tolerance can occur. Furthermore, Rainbow (2002) and Wallace et al. (2003) believe that the BAM is effectively protected when the rate of excretion and detoxification exceeds the uptake and accumulation rate.

The most important excretion route of Cu is via the bile (Grosell et al., 2000) and its excretion is stimulated by waterborne and dietary Cu exposure (Grosell et al., 2004a; Kamunde et al., 2001). Due to tightly associated plasma Cu with larger proteins such as albumin and ceruloplasmin, Cu is unavailable for glomerular filtration. This results in extremely low Cu concentrations in urine of fresh water fish (Grosell et al., 1998b). Finally, branchial excretion of Cu is at present not fully understood.

1.3.3 Cu Toxicity.

As previously mentioned, the degree of Cu toxicity highly depends on its bioavailability. Generally, Cu^{2+} is believed to be the main toxic form of Cu, followed by $CuOH^{-}$ and $Cu(OH)_2$ complexes (Eisler, 1998). When Cu establishes complexes with carbonate, the bioavailability decreases vastly (Blanchard and Grosell, 2005). Furthermore, the amount of dissolved organic matter (DOM or dissolved organic carbon (DOC)) in the environment can influence toxicity to a great extent as well since the complexation of Cu with DOM reduces Cu uptake (McGeer et al., 2002; Matsuo et al., 2005). Additionally, dissolved cations, such as Na^{+}, and in a lesser extent H^{+}, Mg^{2+} and Ca^{2+}, will provide a physiological protection at the gill site and are therefore able to decrease Cu uptake and reduce the toxicity (Di Toro et al., 2001; Grosell and Wood, 2002; Kjoss et al., 2005; Niyogi and Wood, 2004; Tao et al., 2001).

When fresh water fishes encounter highly toxic and acute Cu concentrations in their environment, the gill epithelia display acute oedema and epithelial lifting as a result of the osmotic influx, caused by the inhibition of the branchial Na^{+}/K^{+}-ATPase and displacement of Ca^{2+} from the membranes by Cu and subsequent ion accumulation in the cells. The ionoregulatory control of the gill is quickly lost, which causes an efflux of electrolytes from the blood over the gill epithelium. Finally, this can result in cardiovascular collapse and death (Handy, 2003; Laurén and McDonald, 1985; Li et al., 1998; Mazon et al., 2002; Taylor et al., 1996).

Since differences in physiology occur in acute and chronic exposure conditions, some fish can withstand higher Cu concentrations than others. Especially in chronic exposure conditions these differences can be responsible for increased acclimatisation or adaptation. McDonald and Wood (1993) described the events leading to Cu acclimation during chronic sub lethal waterborne exposure as a 'damage-repair' model. Three phases are being recognised: an initial shock phase where the gill and ion-regulatory mechanisms are targeted, a recovery phase where biosynthetic processes are increased to repair the damage and finally, an acclimatisation phase where the internal physiology returns to pre-exposure conditions or where a new equilibrium is established (McGeer et al., 2002). For instance, according to Schjolden et al. (2007), the crucian carp is one of the least Cu sensitive freshwater fish, since they are able to avoid the Cu induced hypoxia and can survive with extreme loss of ions.

Effects of chronic Cu exposure are found in a number of physiological processes including respiratory distress, the inhibition or induction of enzymes/metabolism, changed cell types and cell turnover in tissues, modified immunity, disruption of endocrine function, changes in swimming speed, reduced growth and altered reproductive strategy (De Boeck et al., 1995; Handy et al., 2003; Hansen et al., 2002; Hontela et al., 1995; Jönsson et al., 2006; Laurén and McDonald, 1987; Manzl et al., 2003; McGeer et al., 2000; McKim and Benoit, 1971; Wilson and Taylor, 1993) as well as in toxicological damage to the structural and biochemical integrity of organs. Another important Cu induced impairment is the inhibition of the olfactory system by Cu's interaction with the olfactory epithelia, influencing an array of behavioural aspects of fish biology (Green et al., 2010; Johnson et al., 2007; Tilton et al., 2008). The target tissues of Cu accumulation during exposure are predominantly gill, liver, gut and kidney (Bopp et al., 2008; De Boeck et al., 2004; Grosell et al., 1996; Handy, 2003). Fish gills have a large surface area and critical ionoregulatory functions, and are therefore considered a prime target for Cu (Matsuo et al., 2004; Van Heerden et al., 2004; Wood, 2001).

According to Gagnon et al. (2006), Cu can disrupt cortisol secretion in rainbow trout through a direct toxic effect on the adrenocortical cells when exposed to high Cu concentrations over a longer period of time. Nevertheless, De Boeck et al. (2001) argue against a major role for cortisol as an intermediate for the toxic effects of Cu. However Cu can induce necrosis directly and apoptosis indirectly via cortisol (Bury et al., 1998). The exposure to Cu mobilized the glycogen and glucose metabolism as well, possibly due to an increased demand

for glucose by Cu challenged tissues (Gagnon et al., 2006). Other known effects of Cu exposure are the influences on a number of hormones such as prolactin and thyroid hormones (Alturfan et al., 2007; Dang et al., 2000b; McDonald and Wood, 1993).

Nonetheless, the most common physiological effect noticed in waterborne Cu exposure is definitely ionoregulatory failure ((Laurén and McDonald, 1985; 1986; Monteiro et al., 2005). Normally, fish will not survive when they have lost up to 20-40% of their Na^+ to the environment (Taylor et al., 2003). As mentioned earlier, the Na^+/K^+-ATPase enzyme appears very vulnerable to Cu although differences in the degree of acclimation to Cu influence its activity (Laurén and McDonald, 1987). Research has shown that Cu is able to covalently bind to SH-groups of this enzyme and interact specifically with its Mg^{2+} binding site. Hereby, conformational protein changes are induced (Kone et al., 1990; Li et al., 1998) and pump activity is inhibited (Li et al., 1996). Concomitant with Na^+ loss, Cl^- loss is observed, possibly by Cu's action on CA which causes a reduction of the amount of HCO_3^- available for exchange (Blanchard and Grosell, 2006; Ditusa et al., 2001; Perry et al., 2003). Moreover, the inhibition of CA has been linked to a disturbed acid-base balance which in turn can inhibit ammonia excretion. Additionally, the elevated plasma cortisol correlated with Cu exposure stimulates protein catabolism and thus metabolic ammonia production as well (Grosell et al., 2002; Grosell and Wood, 2002). This would explain the elevated plasma ammonia/ammonium in fresh and seawater fish exposed to sub lethal Cu (Beaumont et al., 2003; Grosell et al., 2004b; Wilson and Taylor, 1993). Recently, rhesus proteins in fish have also been appointed an important role in ammonia excretion (Nawata et al., 2007; Wright and Wood, 2009). Furthermore, the observed hyper-ammonaemia, together with an increased O_2 demand due to increased metabolic cost, are possibly involved in the reduced swimming capacities of Cu stressed fishes (Beaumont et al., 2003; Taylor et al., 1996).

Due to its specific chemical speciation, Cu can undergo Fenton reactions, which form the basis of its pro-oxidant capacities, resulting in the formation of reactive oxygen species (ROS), inhibited (anti-oxidant) enzymes, depleted glutathione levels, changed/ inhibited proteins lipid peroxidation and DNA damage (Ahmad et al., 2005, Hansen et al., 2006a; Sampaio et al., 2008; Sanchez et al., 2005; Stohs and Bagchi, 1995; more information in chapter 2.6 Oxidative stress). As such, a main mechanism by which Cu exerts its effects is through non specific binding of the Cu^{2+} to biologically important proteins, DNA or other molecules (Bogdanova et al., 2002; Bopp et al., 2008). More generally, as discussed in the

previous section (1.3.2) Rainbow (2002) indicated that the onset of toxic effects is highly dependent of the concentration of metals in the metabolically available form, on an intracellular level. In fish, the ability to withstand toxic metal concentrations might therefore be associated with the allocation of metal to detoxifying compartments (Klerks and Bartholomew, 1991; Wallace et al., 2003) since metals sequestered or stored are not bioavailable to the metal sensitive/biologically active cellular fractions such as organelles and enzymes (Hogstrand et al., 1991; Kraemer et al., 2005; Rainbow, 2002).

1.4 Focus on ion regulation.

Since the three studied fish species belong to the freshwater fishes, they are hyper-osmotic to the surrounding environment and face osmotic gain of water and diffusional loss of NaCl across the gill epithelium. Consequently, these freshwater fishes need to have highly efficient ion/osmoregulatory mechanisms to ensure body fluid homeostasis and subsequent normal function of all physiological processes (Hwang and Lee, 2007). To compensate for this water absorption and ion loss, large volumes of dilute urine are excreted via de kidney while active uptake of NaCl occurs at the gill (Evans, 2008). Due to the passive water gain, freshwater teleosts are also not obliged to drink water (Hwang and Lee, 2007). Although fishes do have kidneys and these are equipped to minimize renal salt loss by tubular reabsorption, it is actually the gill that performs most of the ion regulatory functions (Evans et al., 2005).

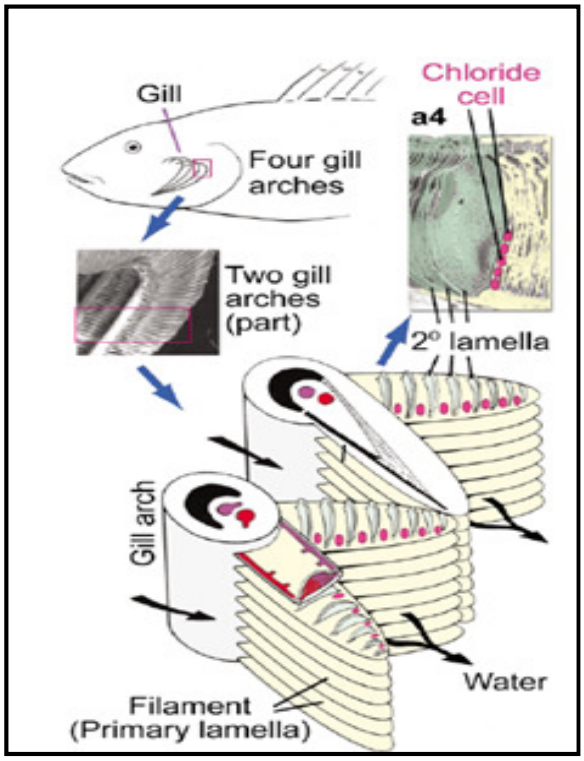

Fig. 2 Gill structure (Hirose et al., 2003). The gills contain 4 gill arches which consist out of primary lamella, the filaments. These filaments are subdivided into secondary lamella. The gill epithelium is mostly made of pavement cells and mitochondria rich cells (chloride cells).

To fulfill all of these (and other) functions, gills must be highly specialized. Indeed, their structure is designed in such a way that their highly complex vasculature and their high surface area are optimized to ameliorate gas and ion exchange. Individual gill arches (usually 4 on each side) are supported by vertical elements of the cartilaginous branchial skeleton (gill rays) (Fig 2. Hirose et al., 2003). They run through the medial portion of each arch and are partially calcified in teleosts (Wilson and Laurent, 2002). The connective tissue between the gill rays forms an interbranchial septum. In teleosts, the septum is usually limited to the base of the filaments, making a more freely moving filament possible. Externally, the gill arches

are protected by a thin bony flap, the operculum. Water, which enters the pharynx from the mouth and passes the filaments, exits the caudal opening of the operculum (Evans et al., 2005). The gill filaments form the functional basis of the gills and are composed out of two sheets of individual pillar cell series, the lamellae. Blood from an afferent filamental artery crosses the lamellae, countercurrent to the water flow, and drains into an efferent filamental artery (Evans et al., 2005; Hornich and Tomanek, 1983). The gill filament and arches themselves are covered with stratified epithelium which consists out of unspecialized cells. The actual specialized cells are distributed related to their function (Morgan and Tovell, 1972). Furthermore, according to Rombough (2007), the ion exchange appears to occur mainly in the proximal arches while the elements of gill responsible for most of the oxygen uptake are distal.

The gill epithelium consists out of an array of different cells of which pavement cells (PVC) are the predominant ones (>90%) and mitochondria rich cells (MRC or "chloride cells") the second in line (<10%) (Evans et al., 2005). Apart from PVCs and MRCs, the gill epithelium shows the presence of pilar, mucus and neuro-epithelial cells as well (Perry, 1997). Previously it was assumed that PVCs did not contribute substantially to the ion transport (Perry, 1997), although recent evidence points to a subdivision of PVCs which are involved in ion uptake and acid-base transport (Evans et al., 2005). These particular PVCs appear to have a higher number of mitochondria present as well as V-ATPases (Galvez et al., 2002; Goss et al., 2001). All PVCs are linked with intercellular tight junctions to adjacent cells, relatively impermeable for ions (Evans et al., 2005). Furthermore, they are thought to be important in gas exchange since they are thin cells with an extensive apical surface area and microvilli (Perry, 1997).

MRCs are believed to be responsible for ion uptake and are mostly located in the afferent edge of filaments and the inter-lamellar region. In freshwater fishes, they often occur singly in the epithelium. MRCs have high densities of mitochondria in their cytoplasm and are round or ovoid shaped cells. Additionally, Na^+/K^+-ATPase is as well present in large quantities in these cells (Hirose et al., 2003). In literature, these cells are also often referred to as "chloride cells" which is derived from their NaCl secretory function in seawater teleosts (Evans et al., 2005). Moreover, not just one type of MRC exists; several researches have defined various morphological MRC subtypes. Firstly, Pissam et al. (1988, 1991, 2000) detected distinct morphological characteristics based on the degree of osmium staining resulting in light α-MRCs and dark β-MRCs. Apparently, when acclimated to seawater, the α-MRCs differentiated into the typical seawater teleost MRC. The β-MRCs on the other hand

degenerated. Although some characteristics of these subtypes are defined, the specific functional roles have yet to be identified (Evans et al., 2005). More recently, Galvez et al. (2002) used magnetic cell separation techniques and binding to peanut lectin agglutinin (PNA) to identified PNA^- and PNA^+ MRC subtypes. PNA^- cells are thought to be the PVCs involved in ion uptake and acid base secretion (as previously mentioned). The PNA^+ cells on the other hand show similar characteristics to a typical teleost MRC (Evans et al., 2011).

An important focus in this PhD regards plasma ion concentrations and their uptake via gill tissue in freshwater fish. In a figure (Fig.3) of Evans et al. (1999) a model of ion transport is discussed. Besides Hirose et al. (2003), Evans and co-workers (1999; 2005; 2011) have reviewed this subject thoroughly. In freshwater, the gills actively take up salt, to counteract the diffuse loss of ions to the dilute hypo-osmotic surroundings, predominantly through mechanisms in the mitochondrion rich cells (Grosell et al., 2002).

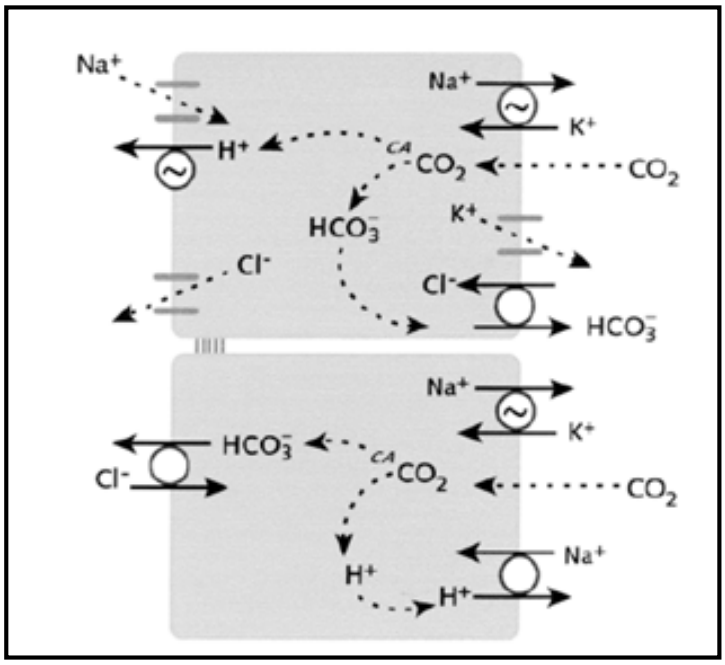

Fig 3. Model for NaCl uptake by fresh water fish gill epithelium. (Evans et al., 1999). The basolateral Na^+/K^+-ATPase actively transports intracellular sodium in exchange for extracellular potassium ions, inducing an influx of sodium at the apical site. This uptake can occur via exchange for protons or ammonia as well as through an apical Na channel coupled to an apical H-ATPase The hydration of carbon dioxide by carbonic anhydrase (CA) generates the protons used for the exchange with sodium. This reaction provides bicarbonate as well, which is a substrate for the chloride-bicarbonate exchange at the apical membrane in favor of chloride uptake.

Briefly, the basolateral Na^+/K^+-ATPase actively transports intracellular sodium in exchange for extracellular potassium ions (in a 3:2 ratio) (Cutler and Cramb, 2001), inducing an influx of sodium at the apical site. This uptake can occur via exchange for protons (first proposed by Krogh 1937; 1938) or ammonia as well as through an apical Na channel coupled to an apical H-ATPase (Grosell et al., 2002). The hydration of carbon dioxide by carbonic anhydrase (CA) generates the protons used for the exchange with sodium. This reaction provides bicarbonate as well, which is a substrate for the chloride-bicarbonate exchange at the apical membrane in

favor of chloride uptake (Boisen et al., 2003; Grosell et al., 2002). Carbonic anhydrase appears to be absent from the basal regions of the gill epithelium (Randall and Brauner, 1998) nevertheless, it is important in the acid base regulation and chloride uptake (Gilmour and Perry, 2009). Pathways of chloride extrusion on the basolateral side might occur via a chloride channel although this is still uncertain (Evans et al., 2011).

In an adapted figure of Bianchini and Wood (2007) (Fig. 4), known adverse actions of Cu on gill cells are shown.

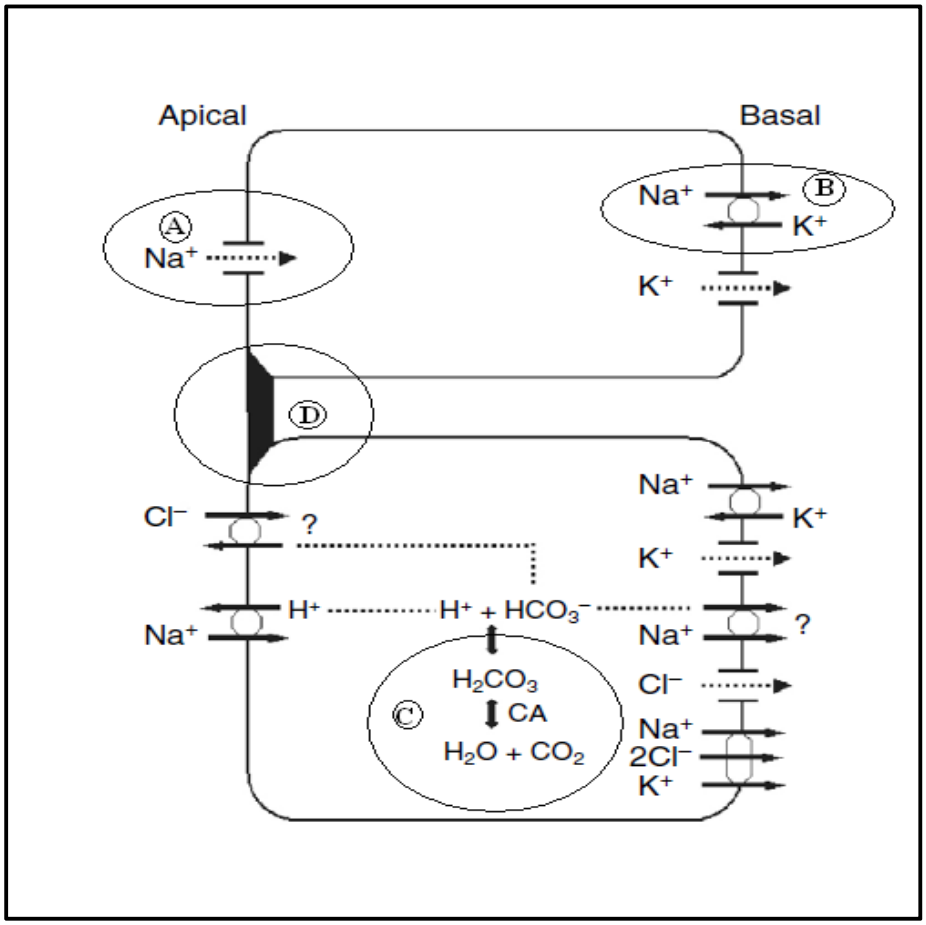

Fig. 4 Adverse Cu actions on the gill cells (adapted figure of Bianchini and Wood, 2007). A: Cu can compete for uptake with Na. B: Cu inhibits Na^+/K^+-ATPase activity. C: Cu disturbes the function of carbonic anhydrase. D: Cu can replace calcium in the tight junctions, causing a changed permeability.

As discussed in the section concerning Cu toxicity (1.3.3), a key target for copper is the sodium homeostasis. Cu can compete with Na to enter the cell at the apical side (A) and can inhibit Na^+/K^+-ATPase activity at the basolateral side (B), causing a decreased Na uptake and a decreased Na concentration in the plasma. Cu also disrupts the tight junctions (D) through the replacement of Ca^{2+} by Cu^{2+}, which leads to leaky membranes and even more ion losses. Furthermore, Cu can disturb the CA mechanism (C), compromising the uptake of Na and Cl. All these events result in a disturbed osmotic balance, one of the most important toxic effects of excess Cu concentrations in the aquatic environment (Handy, 2003; McGeer et al., 2000).

Studying differences in handling the excess Cu in gill tissue of different fish species can therefore yield a better understanding of the various defence mechanisms in place.

1.5 Focus on ion-regulatory hormones.

Fish ion-osmoregulation is a result of integrated transport activities of the gill, gut and renal system (Foskett et al., 1983) and regulates internal water balance and plasma metabolites (Sangiao-Alvarellos et al., 2006; Van der Linden et al., 1999) to ensure necessary water and ion gain or loss, depending on the environment (salt or fresh water). However, a number of pollutants are capable of endangering the delicately balanced ion-osmoregulation. For instance, Cu is known to cause ion-osmoregulatory effects in fish and recovering from this impact is of great importance for its survival. A multitude of hormones, such as cortisol, prolactin, growth hormone, thyroid hormones and many more, are involved in the regulation of this very important process (Review Evans et al., 2005).

Cortisol is the most substantial corticosteroid produced by the activation of the hypothalamic-pituitary-adrenal axis in stress situations (Peruzzi et al., 2005). Synthesis and secretion of this hormone, which has mineralocorticoid and glucocorticoid functions, is carried out by interrenal cells and is primarily controlled by the adrenocorticotrophic hormone which is in turn produced and secreted by the pituitary. The proliferation and differentiation of the chloride cells of the gills and the Na^+/K^+-ATPase expression in these cells are stimulated by cortisol (Dang et al., 2000a; Flik and Perry, 1989; Sloman et al., 2001) which makes cortisol a crucial mediator during ion-osmolar stress or seawater acclimatization. Furthermore, cortisol appears to interact with NaCl to initiate salt secretion or uptake and is involved in carbohydrate metabolism as well (Laurent and Perry, 1990; McCormick, 2001; Wendelaar Bonga, 1997). According to McCormick (2001), high growth hormone concentration and low prolactin concentrations promote salt secretion. In contrast, low growth hormone concentration and high prolactin concentration will result in ion uptake. Part of the interaction between growth hormone and cortisol is thought to be growth hormones ability in up regulating the number of gill cortisol receptors (Shrimpton and McCormick, 1999). The majority of the actions of cortisol are most likely conducted via glucocorticoid receptors (Dang et al., 2000b) since gill tissue sensitivity to cortisol has been correlated positively with the concentration of these receptors in for instance, rainbow trout. The number of gill cortisol receptors is strongly linked with the capacity of cortisol to stimulate gill Na^+/K^+-ATPase and the regulation of these receptors is therefore of outmost physiological importance (Shrimpton and McCormick, 1999). On the other hand, since cortisol can rapidly inhibit prolactin release,

it is believed that steroids, such as cortisol, can alter physiological processes through membrane-associated mechanisms (Borski et al., 2001; Christ et al., 1999).

The antagonism of prolactin (PRL) toward cortisol induction of salt secretory processes is an important aspect of the numerous functions of prolactin (McCormick, 2001; Tse et al., 2008). PRL is involved in growth and development, water and electrolyte balance, reproduction, endocrine metabolism, brain and behavior, immunoregulation and protection (Bole-Feysot et al., 1998; Manzon, 2002; Sakamoto and McCormick, 1999; Sangiao-Alvarellos et al., 2006) and has more than 300 different functions in higher vertebrates (San Martin et al., 2007). A large proportion of these actions seem to be associated with cell proliferation and/or apoptosis, either directly or indirectly (Sakamoto et al., 2005).

PRL-secreting cells are responsible for the secretion of the prohormone of PRL (a polypeptidic hormone) and are situated in the pars distalis of the pituitary (Manzon, 2002). The mature hormone is formed by the cleavage of the N-terminal signal peptide after biosynthesis. In several teleost, ectopic production of PRL, which suggests a paracrine function, has been described (Power, 2005; Santos et al., 1999). The mRNA expression of PRL can be influenced by the PRL releasing peptide (Fujimoto et al., 2006).

PRL exerts its effect via PRL receptors which are members of the cytokine class I receptor superfamily and have multiple isoforms due to alternative splicing of the mRNA transcript of one single gene (Cavaco et al., 2003; San Martin et al., 2007). Generally, gill, kidney and intestine have the highest PRL receptor levels. This receptor is a single-pass transmembrane receptor and by interacting with PRL, epithelial barriers are influenced. This interaction mainly promotes ion retention and prevents hydration in hyper-osmoregulating fish (Hirano, 1986; Kelly and Wood, 2002; Lee et al., 2006). When the environment has low concentrations of ions, expressions of mRNA PRL and plasma levels of PRL tend to increase to augment ion uptake and prevent ion loss and water uptake (Manzon, 2002; Sangiao-Alvarellos et al., 2006), making PRL the most important fresh water adapting hormone in euryhaline teleosts (Uchida et al., 2004). Nevertheless, differences of the importance of PRL in fresh water adaptation and in the action of PRL exist between and within species (Bern, 1983; Manzon, 2002).

Furthermore, PRL is a member of the same protein family as growth hormone (GH) (McCormick, 2001; San Martin et al., 2007), which is as well secreted by (extra-) pituitary

tissue and has similar class I receptors (Pierce et al., 2007). Comparable to PRL, the GH receptor has at least 2 isoforms which are differentially expressed among tissues of various fish species (Ma et al., 2007; Very et al., 2005). GH receptors have been reported in liver, gill, gut and kidney (Kajimura et al., 2004; Lee et al., 2001; Sakamoto et al., 2005; Tse et al., 2003).

GH is involved in the regulation of growth by the stimulation of insulin growth factor in liver and other tissues (Wood et al., 2005), osmoregulation, metabolism, reproduction, immunity (Lee et al., 2001; Yada, 2007) and many more (Very et al., 2005; Wong et al., 2006). The role of GH in osmoregulation can be different among species (Sakamoto and McCormick, 2006). In salmonids for instance, GH facilitates seawater acclimation through chloride cell proliferation and hence enhanced branchial Na^+/K^+-ATPase activity (Sakamoto et al., 1993). In contrast, depending on the species, GH can enhance the capacity to hyper- or hypo-osmoregulate in non-salmonid species (McCormick, 2001). Among teleosts, insulin growth factor is partly responsible for the osmoregulatory actions of GH (McCormick et al., 1991; McCormick, 1996; McCormick, 2001), however a similar interaction between insulin-like growth factor (IGF) and cortisol (as there is for growth hormone and cortisol) has not been found yet (McCormick, 2001). On the other hand, the functional role of IGF as feedback signal for GH production is well conserved. The effects of steroids and thyroid hormones on the feedback mechanisms of GH are more variable in and between species (Wong et al., 2006). Reversibly, action of GH on the conversion of thyroxine to thyronine was absent in salmonids (Gomez et al., 1997). Thyroid hormones play critical roles in differentiation, growth, development, aerobic energy metabolism and reproduction (Swapna and Senthilkumaran, 2007). The primary thyroid hormone (TH), tetraiodo-L-thyronine (thyroxine, T_4) is produced in a single layer of specialized epithelial cells, the thyroid follicles, which are dispersed in the connective tissue along the afferent artery (Swapna and Senthilkumaran, 2007; Yamano et al., 2005). The necessary iodine is predominantly absorbed through the gills from the environment. The pituitary produces a thyroid stimulating hormone (TSH) which regulates the production of the TH via TSH receptors. First, iodide that has been concentrated in the thyroid follicles is incorporated into the glycoprotein thyroglobulin. This molecule is subsequently through endocytosis and proteolytic digestion in phagolysosomes hydrolyzed to release thyroid hormones, mostly T_4 (Iseki et al., 2000; Swapna and Senthilkumaran, 2007).

After secretion, T_4 is metabolized to triiodo-L-thyronine (T_3) by outer ring deiodination or to the inactive reveres T_3 (rT_3) by inner ring deiodination (Orozco and Valverde, 2005). Furthermore, both T_3 forms can be metabolized into diiodothyronine (T_2). These modifications predominantly take place in the liver, are catalyzed by at least three iodothyronine deiodinases and contribute to the regulation of the thyroidal status by adjusting T_3 availability in plasma and tissues (Orozco et al., 2003; Van der Geyten et al., 1998; Walpita et al., 2007; Yamano et al., 2005). Besides deiodination, a small portion of the TH can also be utilized by glucuronide or sulfate conjugation, deamination or decarboxylation (Power et al., 2001; Yamano et al., 2005). TH are upon release in circulation, for 99% bound to thyroid binding globulin, thyroid binding prealbumin, transthyretin and plasma lipoproteins (Power et al., 2001, Yamano et al., 2005). This hormone interacts with nuclear receptors as well as membrane-associated receptors to exert their biological effect (Swapna and Senthilkumaran, 2007). Moreover, similarly to PRL and GH, at least 2 receptor isoforms have been identified in teleosts; TRα and TRβ. The expression patterns share some common features (high expression in pituitary and brain), however, TRβ shows higher expression in the muscle and gill compared to TRα (Filby and Tyler, 2007; Power et al., 2001; Swapna and Senthilkumaran, 2007). Since the binding affinity of T_3 to their hormone receptors is much greater than for T_4, it is assumed that T_3 is the most biologically active form of TH. Although Sandler et al. (2004) showed results of T_4 activity in the nucleus when present in high enough concentrations. Interestingly, during production of thyroglobulin, H_2O_2 is required, showing an associating of thyroid hormones with the oxidative status of the organism (Alturfan et al., 2007; Iseki et al., 2000). Furthermore, during stress situations, the produced cortisol might decrease plasma T3 concentrations, although clear cut conclusions of the interaction between cortisol and thyroid hormones cannot be made (Shrimpton and McCormick, 1999; Walpita et al., 2007).

As described in the Cu toxicity section (1.3.3), Cu causes a series of physiological changes and can disrupt endocrine functions in fish (Handy, 2003). Since stress responses can be stimulated by numerous factors, including metal exposure, an effect on the stress-axis is very likely. Indeed, the vast majority of research on hormones during Cu exposure investigates the effects of cortisol. In a number of studies, activation of cortisol has been demonstrated in fish (De Boeck et al., 2001; Dethloff et al., 1999; Teles et al., 2005). Since an increased cortisol concentration could protect cells against Cu toxicity, the protective effect of cortisol during Cu exposure has been studied as well. However, the results were not straight forward since

contradicting results were obtained (Bury et al., 1998; De Boeck et al., 2001; Gagnon et al., 2006; Manzon et al., 2002). In case of thyroid hormones and prolactin, Cu related research is scarce and often ambiguous (Gagnon et al., 2006; Teles et al., 2005).

1.6 Focus on oxidative stress.

2.6.1 Definition of oxidative stress and sources of ROS.

Oxidative stress can be described as the occurrence of a disproportioned balance between reactive oxygen species (ROS) production and anti-oxidant defenses, leading to an inherent impairment of cellular processes and survival in general (Almroth et al., 2008; Sies, 1991). This imbalance can be a result of an increased ROS production itself, an insufficient anti-oxidant defense system or an incapability to repair the elicited oxidative damage. Alterations of cellular macromolecules, DNA and proteins can be induced by ROS, causing disrupted cell functions and eventually cell death (Dorval and Hontela, 2003).

Most of the molecular oxygen is reduced to water, coupled to the oxidation of food and production of energy. 1-3% of the consumed oxygen is however only partially reduced, creating oxygen metabolites or ROS (Jena et al., 2009; Livingstone, 2001) such as hydroxyl radicals, peroxyl radicals, alkoxyl radicals, hydroperoxyl radicals, hydrogen peroxide, hypochlorous acid, singlet oxygen and peroxynitrate (Halliwel and Gutteridge, 1993). These unwanted by-products can arise from various endogenous sources and processes and it is postulated that mammalian cells are subjected to 10000 oxidative hits per day resulting from the formation of ROS (Lloyd and Phillips, 1999). The mitochondrial electron transport chain, oxidative processes at the endoplasmatic reticulum membranes and a variety of cellular oxidases are considered main sources of ROS generation (Bagnyukova et al., 2005), whereas some enzymatic reactions (for instance xantine oxidase, aldehyde oxidase, glucose oxidase, cytochrome P450 system …) and auto-oxidation are of importance as well (Hidalgo et al., 2002; Livingstone, 2001).

2.6.2 Inducers and effects of oxidative stress with a focus on Cu.

Despite some positive functions attributed to ROS, such as their use in the immune system and signal transduction pathways, their concentrations are, in normal circumstances, kept very low to prevent oxidative damage. Various xenobiotics, hormonal and/or temperature changes, hypoxia-normoxia transitions and metal accumulations appeared to be capable of inducing an increase in ROS production in aquatic animals (Bagnyukova et al., 2006; Sampaio et al., 2008; Radi and Matkovics, 1988). Generally, protein carbonylation, lipid peroxidation and DNA damage are results of the opposed oxidative stress. Protein carbonyl formation is irreversible, can lead to enzymatic degradation of proteins and is as well a subject of age related deterioration (Almroth et al., 2008; Grune, 2000). Lipid peroxidation is the oxidative

deterioration of the membrane lipids (Elstner, 1991) which can alter the fluidity, permeability and receptor alignment of biomembranes (Jena et al., 2009). Furthermore, oxidative damage probably constitutes the most varied class of DNA damage with at least 20 different lesions identified (Lloyd and Phillips, 1999).

Transition metals, such as Cu, have characteristic mechanism for causing cellular damage through the increase of ROS concentrations or the reduction of anti-oxidant capacities (Atli et al., 2006). Cu can catalyze the formation of hydroxyl radicals derived from the superoxide anion and hydrogen peroxide under a process called Fenton reaction (Formula 1) (Craig et al., 2007; Moriwaki et al., 2008), it can disrupt the proper mitochondrial function and it can alter the activity of enzymes by binding to their functional group or by displacing the metal associated with the enzyme (Viarengo, 1985).

$$\mathrm{Cu\ (II) + H_2O_2 \rightarrow CuOOH^+ + H^+}$$
$$\mathrm{CuOOH^+ \rightarrow Cu\ (I) + O_2^- + H^+}$$
$$\mathrm{Cu\ (I) + H_2O_2 \rightarrow Cu\ (II) + OH^- + OH^{\cdot}}$$

Formula 1: Cu fenton reactions (Moriwaki et al., 2008)

The subsequent alterations in important biotransformation enzymes may lead to a disrupted endogenous metabolism and invasive decreases in animal fitness (Gravato et al., 2006). Additionally, Cu is all together a major contributor to DNA degradation since it acts as a genotoxic pollutant that changes the integrity of DNA by the induction of single-and double-stranded DNA breaks (Bopp et al., 2008; Gutteridge and Halliwell, 1989). Interestingly, since the lifetime of the produced hydroxyl radical is very short (Moriwaki et al., 2008), adverse reactions occur almost instantaneously at the site where the radical is produced.

2.6.3 Anti-oxidant mechanisms.

The role of anti-oxidants consists out of the disposal, scavenging and suppression of ROS formation and consequently regulating and/or diminishing the (toxic) action of ROS (Arabi and Allaedini, 2005). Anti-oxidant defenses are present in various molecular (glutathione (GSH), ascorbate (Asc) and enzymatic (anti-oxidant enzymes) forms.

Enzymatic mechanisms include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These enzymes deal directly with radical species and are thought to form the first line of defense. The metalloenzyme SOD reduces the superoxide anion whereas CAT or GPx degrade the resulting hydrogen peroxide (Bagnyukova et al., 2005; Hansen et al.,

2006a). There are three main isoforms of SOD; Cu/Zn-SOD (located in cytosol and nucleus), Mn-SOD (mitochondria) and extracellular SOD (EC-SOD), all important in reducing ROS concentrations. To ensure proper function of SOD (and GPx as well), some micronutrients such as Cu, Zn, Mn and Se, should be readily available as well (Hidalgo et al., 2002).

According to some researchers, CAT has a relatively low H_2O_2 affinity and a high number of CAT turnover, so certain amounts of H_2O_2 will remain in the cell and possibly generate highly reactive hydroxyl radicals (Arrigoni and Di Tullio, 2002). Whereas other studies claim that CAT is the main hydrogen peroxide detoxifying enzyme and not GPx (Bagnyukova et al., 2005), which makes this anti-oxidant response probably very species specific. Moreover, it is known that superoxide radicals can directly inhibit CAT activity. Therefore, if the initial Cu effect is an increase in superoxide radicals, CAT activity might be inhibited (Craig et al., 2007).

From the molecular defense mechanisms against oxidative stress, glutathione is by far the most important and abundant cellular nonprotein thiol. The cysteine in glutathione is capable of rapidly reducing Cu (and other heavy metals or ROS) with GS-Cu complexes as a result, preventing further redox cycling and ROS generation. GSH participates additionally enzymatic as a substrate in detoxification reactions of organic and inorganic peroxides and xenobiotics catalyzed respectively by GPx and GST (Canesi et al., 1999; Elia et al., 2006; Gravato et al., 2006; Stohs and Bagchi, 1995). The oxidized GSH can be reduced to GSH through the action of glutathion reductase (GR) which controls the cellular levels of GSH, together with glutamyltranspeptidase, glutamylcysteine synthetase, amino acid transporters and other multiple enzyme systems (Wild and Mulcahy, 2000). The ratio between the oxidized and reduced form of GSH is of outmost importance for the redox balance as well as cell signal transduction (Sies, 1999). Dorval and Hontela (2003) even suggested that the GSH redox cycle can effectively detoxify H_2O_2 when CAT is compromised. Heavy metal accumulation can therefore result in decreased availability of reduced GSH, due to GSH binding and oxidation (Canesi et al., 1999). According to Elia et al. (2003), GSH depletion may reduce the cellular ability to scavenge free radicals raising the general oxidative potential in the cells.

Ascorbic acid (AA) not only scavenges a variety of toxic free radicals efficiently (Fig. 5), it is present in large amounts in cells and is suitable for regeneration as well, making it an added value in the anti-oxidant defense mechanism (Arrigoni and Di Tullio, 2002; Bogdanova et al.,

2002) as seen for instance in a rainbow trout gill cell line where negative effects of Cu exposure were suppressed by the use of ascorbic acid (Bopp et al., 2008).

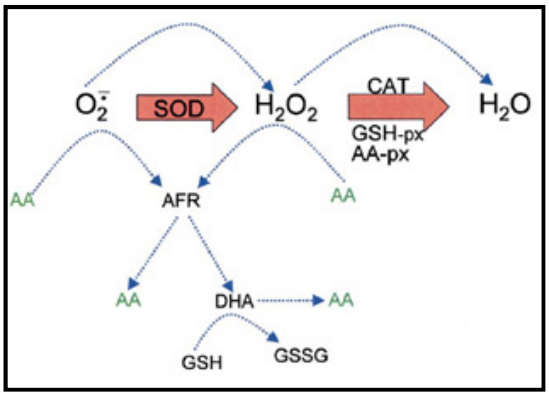

Fig.5. Free radical scavenging action of ascorbic acid (AA) (Arrigoni and Di Tullio, 2002).

And finally, DT-diaphorase is an enzyme that protects against redox-cycling xenobiotics, reduces quinones in a two-electron reaction and thereby prevents redox cycling and facilitates excretion from the cell (Almroth et al., 2008).

The responses of the anti-oxidant mechanisms under an array of circumstances, including Cu exposure, have been intensively studied by a number of researches (Bagnyukova et al., 2005; Hoyle et al., 2007; Oruc and Uner, 2004; Pandey et al., 2008; Stephensen et al., 2002). All this data indicated an influenced anti-oxidant system with a significant basal capacity and a possibility of induction upon oxidative stress (Livingstone, 2001).

For instance, alterations in the GSH/GSSG ratio have been observed in exposures to waterborne Cu; a decrease in reduced glutathione (GSH) levels in European eel possibly arose due to an increased use of GSH and an inefficient GSH regeneration. In previous studies on fish copper exposure, glutathione was shown to inhibit free radical formation through its ability to stabilize copper in its oxidative state, preventing redox cycling and free radicals generation (Ahmad et al., 2005; Pandey et al., 2001; Parvez et al., 2003). Canesi et al. (1999) showed a decreased GSH content in gill and digestive gland, as quickly as after one day of exposure to Cu. Fernandes et al. (2008) indicated an increase in metabolic level related to Cu induced oxidative stress, suggested by a positive relationship between Cu and CAT activity in liver of Portuguese wild adult mullet. Furthermore, Jena et al. (2009) concluded that comparatively low dose of Cu (0.5 ppm) induced mild oxidative stress in the in the muscle of a widely consumed freshwater fish *Labeo rohita* with concomitant elevation of GSH and AA content of the muscle. However, high concentration of $CuSO_4$ in the ambient water caused

severe oxidative stress. Cu induced a rapid and transient increase of anti-oxidant enzymes and a depletion of glutathione content during the first 8 days of exposure in three-spined stickelbacks (Sanchez et al., 2005). These few examples of responsiveness in the anti-oxidant mechanism of fish during Cu exposure give an indication of the differences in response to ROS in a variety of species and are not meant to be covering all research conducted on the anti-oxidant mechanism.

1.4 Focus on proteins.

The entirety of proteins (large molecules made up of strings of amino acids) found in a particular cell /organism under a particular condition, is referred to as proteome. Proteomics is therefore the large-scale search for information about proteins by the means of studying a whole proteome of a sample at a certain point in time. Proteomics identifies proteins, modifications of proteins, interactions between proteins, and allows further unravelling of biological processes. Unlike the relatively static genome, the proteome changes continuously in response to a large number of intra- and extracellular environmental signals, on which environmental stressors can have a negative impact.

In aquatic toxicology, mussels were one of the first species to undergo proteome analysis, followed quickly by fish and other aquatic invertebrates such as crab (López Barea and Gómez-Ariza, 2006; Shepard and Bradley, 2000). These early studies were performed with all kinds of stressors, among which metals are still of outmost importance since they easily react with proteins. Furthermore, a vast number of proteins contain a metal as a cofactor. These metalloproteins are mostly enzymes or transcriptional factors involved in electron transport, oxygen storage, metal transport, chemical bond hydrolysis, redox processes, and synthesis of biochemical compounds. Moreover, since these metals are essential to the organism however toxic when in excess, regulatory processes are present (metallothionein, regulatory and signaling proteins, membrane-bound transporters, …), which makes metal metabolism very exciting to investigate within the field of proteomics (Kulkarni et al., 2006).

Due to the complexity of the proteome, the proteomics technology used should integrate the separation of proteins, analytical sciences for identification and quantification of the analytes and bioinformatics for data management and analysis, to meet the requirements of the field of proteomics (Gygi and Aebersold, 2000; Martyniuk and Denslow, 2009).

The typical workflow used in proteomics includes three main processes: First, extraction; second, separation and quantification; and third, identification and characterization (Sanchez et al., 2005). Historically, high resolution 2-D polyacrylamide gel electrophoresis (2DE) has been used to separate complex protein mixtures (iso-electric focusing and sodium dodecyl sulfate polyacrylamide gel) (Patton, 2002). Subsequently, mass spectrometry and sequence database searches are typically used for identification of the excised (i.e. from the gel) protein spots (Görg et al., 2000; Gygi and Aebersold, 2000). Typically, up to 100 mg of proteins are used during 2D gel electrophoresis and up to 11000 proteins can be separated. Important for a

successful processing of the results is an accurate detection of these proteins, which is mostly done by colloidal coomassie staining or silver staining. Next, the alignment of the multiple gels should be perfect as well (Figeys et al., 1998).

Commonly, trypsin is used to proteolytically digest a protein sample prior to analysis by mass spectrometry (Smith and Figeys, 2006) which identifies the proteins by measuring the *m/z* ratio of the peptides. Sequences from a protein sequence database can be used to generate predicted fragmentation spectra that can be matched against experimental spectra. High confidence protein identification is attained by matching one or more tandem mass spectra from peptides in the same protein to the databases; this is often called peptide mass fingerprinting. Different software packages have been developed for the identification of proteins based on the accurate measurement of peptide masses. For example, Mascot, which originated from the software called MOWSE (Pappin et al., 1993) (*http://www.matrixscience.com/*), takes into account the protein size and the relative abundance of peptides in the databases. It further incorporates probability scoring for the probability that the match between the data and the entry in the database will be a random event. The identification is then established by ordering the proteins with a decreasing probability of being a random match (Figeys et al., 1998).

Since various proteins are separated on their iso-electric point and their molecular weight, they migrate on the gel in many different ways. As such, 2DE proved itself very useful in the past (Gharbi et al., 2002; Karp et al., 2004; Spandidos and Rabbits, 2002), especially for the analysis of posttranslational modifications. Nonetheless 2DE has to face up to important disadvantages: it is very time consuming, labor intensive and is known to produce large variations in the quality of the gel. Furthermore, it can mask expressions of less abundant proteins which can result in potential misidentifications and inaccurate quantification and cannot be applied globally to the analysis of membrane proteins (Sanchez et al., 2005). Researches succeeded in eliminating the large intergel variability by the introduction of techniques such as fluorescent 2D difference gel electrophoresis (2D-Dige: Amersham Biosciences, Inc.) which simplifies the analysis and provides higher sensitivity and a larger dynamic range (Gygi and Aebersold, 2000; Van den Bergh et al., 2003) (Fig.6). In this technique, different protein digests (control and treatment samples) are linked with mass- and charge-matched *N*-hydroxy succinimidyl ester derivatives of the fluorescent cyanine dyes Cy3 and Cy5, which possess distinct excitation and emission spectra (Cy3 and Cy5) whereas an

internal standard is labeled with Cy2, before iso-electric focusing and electrophoresis separation. The dyes differentially label lysine residues of the proteins in the samples. Only 20% of molecules of a particular protein are covalently modified with one Cy dye molecule, which is referred to as 'minimally' labeled. This procedure decreases dramatically the variability across gels and allows detection of as little as 125 pg of a single protein (Ünlü et al., 1997). Afterwards, the gel is scanned and resulting fluorescent images are overlaid while differentially expressed proteins are identified with software such as DeCyder$_{tm}$ software (GE healthcare) or Decodon. De Wit et al. (2008) have successfully used this approach to examine the protein expression changes in zebrafish exposed to environmental stressors such as tetrabromobisphenol 1. 2D-Dige has as well been used as a high-throughput tool for large-scale proteome analysis by Hu et al. (2003). This research team investigated various metal exposures on *Saccharomyces cerevisiae.* It's especially the combination with mass spectrometry which makes 2D-Dige an excellent tool for biomarker research as well (Zhou et al., 2002)

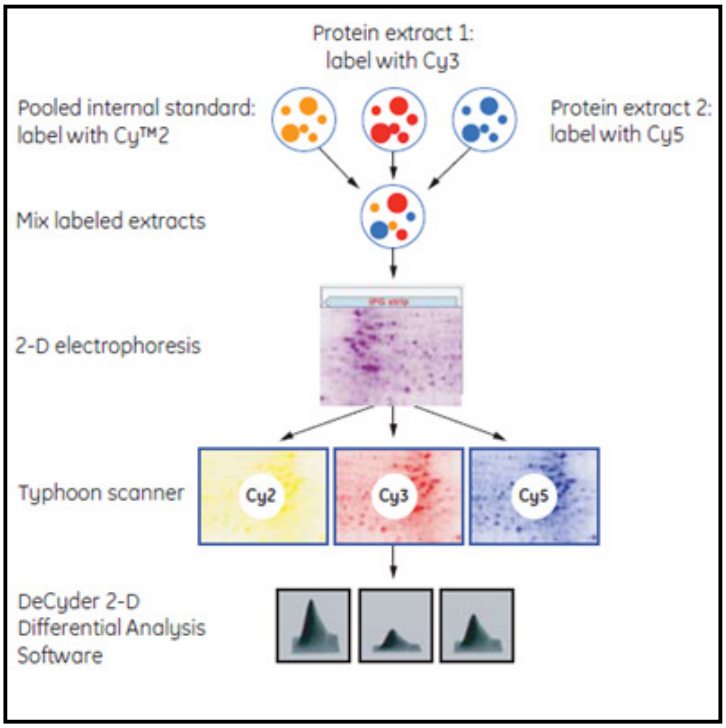

Fig. 6. 2D-Dige workflow. Protein samples are separately labeled with Cy Dye and combined before performing 2D electrophoresis. Afterwards, the gel is scanned and DeCyder or Decodon software analysis is applied to identify different protein patterns.

On the other hand, researches have used LC as a separation method as well, followed by MS/MS which typically resulted in higher numbers of proteins identified per sample. One of these gel free techniques, iTRAQ (isobaric tags for relative and absolute quantification), can use up to 8 reporter tags (113-119 and 121 *m/z*) in one analysis, opening up a whole new

array of possibilities (Unwin et al., 2005) (Fig. 7). Each label includes a reporter tag with varying mass, a balance to assure equal masses of peptides from different samples, and a peptide reactive group that chemically tags amine groups of the digested protein sample (Martyniuk and Denslow, 2009). After labeling, samples are combined, fractionated using LC (commonly Reversed phase and Strong Cation eXchange) and analysed by tandem mass spectrometry. When fragmented in MS/MS, different signature ions are produced which can be used for quantitative analysis and similar as in 2D-Dige, protein identification is obtained by the use of databases (Aggarwal et al., 2006; Zieske, 2006). Interestingly, post translation modifications are as well detectable. iTRAQ has been successfully used in cancer (Seshi, 2006), Alzheimer's disease (Melanson et al., 2006) and toxicological research (Glückmann et al., 2007).

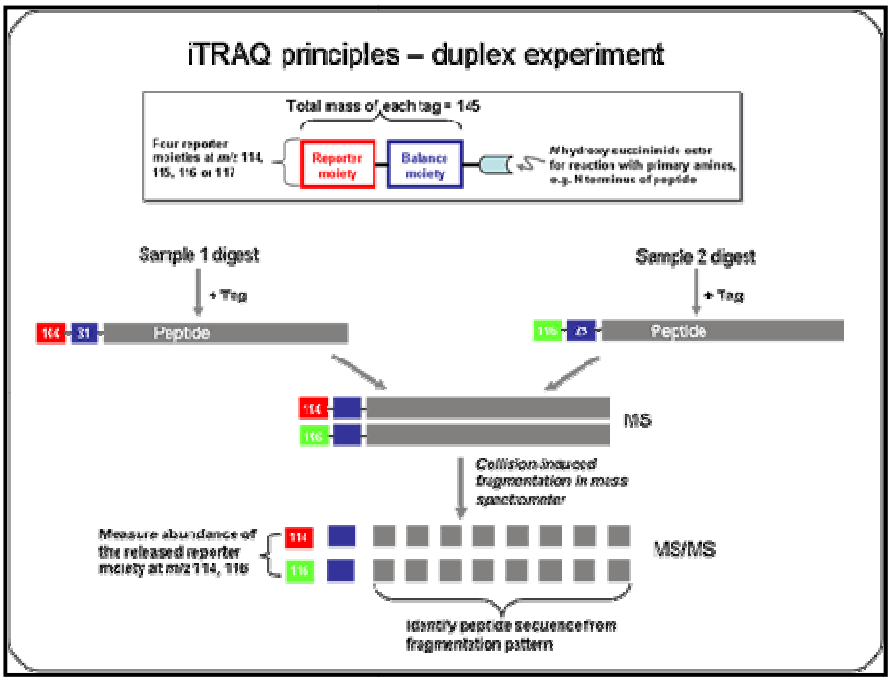

Fig. 7. iTRAQ workflow. Samples are separately labeled with an iTRAQ label, combined, fractionated with liquid chromatography and analyzed with Tandem mass spectrometry.

Although each proteomics technique has its advantages and disadvantages, the best approach continues to be the utilization of several techniques simultaneously since this normally produces a more complete picture of the proteome studied and offers a great potential as a biomarker discovery tool.

Chapter 2

Introduction to experimental work

2.1 Test species.

Introduced throughout the world for its sport and commercial uses, rainbow trout (Animalia Chordata Actinopterygii Neopterygii Teleostei Salmoniformes Salmonidae *Oncorhynchus mykiss*) originates from western North America. They live primarily in freshwater and need well oxygenated, clean fast flowing streams or rivers to thrive in (McDowall, 1990). They feed on insects, molluscs, fish eggs, crustaceans and small fish. Common carp (Animalia Chordata Actinopterygii Cypriniformes Cyprinidae *Cyprinus carpio*) on the other hand prefers mainly plants and water insects and aquires them by scavenging the bottom of the river, stirring the substrate around (Parkos and Wahl, 2000). Although common carp's precise origin is uncertain, he probably originated in Asia. Besides as a food source for humans, they are often used as ornamental fishes. They have more robust features compared to rainbow trout. As for body shape, gibel carp (Animalia Chordata Actinopterygii Cypriniformes Cyprinidae *Carassius auratus gibelio*) leans more towards common carp and are native to Asia as well. Of all the three test species, gibel carp is the most versatile in its habitat and nutrition, furthermore is it capable to tolerate low levels of dissolved oxygen for several hours to weeks (Nilsson and Renshaw, 2004). (Fig. 8 shows an example of the three fish species).

Fig. 8. Rainbow trout-Common carp-Gibel carp.

2.2 Preliminary research.

Fundamental for this PhD was the research performed by De Boeck et al. (1997, 2001, 2003, 2004, 2006, 2007, 2010) who first noticed the differences in sensitivity to Cu in our test species: the sensitive rainbow trout, the intermediate common carp and the more resistant gibel carp each differ with a factor 2 to 3 in their tolerance to Cu exposure (De Boeck et al., 2004). Although rainbow trout showed low Cu accumulations and uptake in gill tissue, no respiratory distress or use of anaerobe metabolism and limited gill damage, it still appeared to be extremely vulnerable for Cu partly due to the lack of MT induction in gill tissue and the massive plasma Na^+ loss in the beginning of the exposure. Furthermore, the liver, who acts as the main Cu accumulating organ, displayed high Cu concentrations even in control situations. During exposure, the liver burden increased even more, since Cu accumulated quickly in this

organ. The liver of common carp was targeted as well, indicated by an increased Cu concentration correlated with an increased MT induction in the same organ. However, Cu accumulation in gill tissue presented a greater challenge to this fish species since accumulation was fierce and gill damage showed a shock and repair pattern. Not correlated with the latter, but nevertheless important is the compromised gas exchanged experienced by common carp in the beginning of the exposure. The plasma Na^+ loss on the other hand, was limited in the first days, just as was seen in gibel carp. Gibel carp seemed to be confronted with a respiratory acidosis and moderate to severe gill damage as exposure continued. The accumulation of Cu happened mainly in gill and kidney, whereas the role of liver tissue was diminished. An enhanced MT induction coincided with the increase of Cu concentration in gill and kidney, showing an efficient capturing of free Cu ions and possibly an important role for kidney in Cu excretion.

These studies provided a first glance at the different physiological mechanisms used in the studied fish species and formed a solid base to investigate other important processes to unveil a more complete picture of the distinctive confrontations and threats these fish species endure during Cu exposure.

2.3 Research Tissues.

Fish gills serve as an important target for waterborne metals and represent a major uptake route of metals (Tao et al., 2006) whereas liver is known to regulate circulating Cu concentrations, Cu excretion and is the major Cu accumulating organ (Grosell et al., 1996; Handy, 2003). Therefore, these were the main tissues analyzed within the framework of this PhD.

2.3.1 Plasma and Gill.

The importance of fish plasma lies in its transportation function. It is responsible for transporting gases, ions, nutrients, hormones, waste products and carrier molecules. Furthermore, plasma proteins regulate the osmotic pressure (Andreeva et al., 2007) which (for teleosts) means generally a concentration of about one-third of that of seawater (McCormick, 2001). Besides plasma, gills are of outmost importance to ensure osmoregulation (see 1.4 Focus on ion regulation). Moreover, the gills serve many other purposes such as aquatic respiration, ammonia excretion and acid-base balance (Evans et al., 2005; Grosell et al., 2002). Hormone production, modification of circulating metabolites and immune defense are

other functions appointed to the gills (Dos Santos et al., 2001; Olson, 1998; Rombough, 2007; Zaccone et al., 1996). Compared to the three gases involved in gas exchange (O_2, CO_2, NH_3), ion movements do have a direct impact on the osmoregulatory status, on the acid-base status and on the ammonia excretion (Rombough, 2007). The gill and all its functions are prime targets during a Cu exposure (see Chapter 2.4) and therefore this organ is an ideal tissue to study the effects of Cu on the ion regulation, occurring oxidative stress, protein profile and subcellular distribution.

2.3.2 Liver.

Fish liver has reasonably similar functions compared to other vertebrates. It has a key role in many physiological processes such as detoxification, digestion, and storage of glycogen and lipids (Channa and Mir, 2009). The teleost liver, a bi or tri lobed dense organ, is located in the cranial region of the visceral cavity, between other visceral organs (Brusle and Anadon, 1996; Mir et al., 2011) and the structure can be significantly different between fishes (Robertson and Bradley, 1992; Rocha et al., 1994). Two cellular plates surrounded by sinusoids make out the hepatic parenchyma. Furthermore, between two adjacent sinusoids, the hepatocytes are arranged as anastomic cords (Hinton et al., 1972; Vicentini et al., 2005). Ultra structurally, the hepatocytes show one rounded nucleus, mostly located in the center of the cell. The liver comprises several other cell types, such as biliary epithelial cells, macrophages, fat-storing cells and endothelial cells (Rocha et al., 1994). A number of fish species, such as carp, have characteristic high content of glycogen in their hepatocytes. In this case, this glycogen fills most of the cytoplasm (Vicentini et al., 2005). On the other hand, Rocha et al. (1994) investigated liver ultra structure of brown trout and noticed relatively small contents of glycogen in the hepatocytes, apparently a trait of salmonids liver. Under certain toxic circumstances, the biochemical and physiological functions of the liver can be altered. As such, the fish liver appears well suited to serve as a model for analysis of the interaction between natural environmental changes and hepatic morphology at the subcellular level (Mir et al., 2011). Furthermore, as previously mentioned, the liver is the most important Cu accumulating organ and therefore perfectly suited to investigate differences in subcellular Cu distribution.

2.4 Outline study.

All experiments in this PhD were carried out in a climate chamber at 17°C at the University Of Antwerp, Belgium. Fish were kept in green 200l aquaria (Fig. 9) during acclimatization (4 weeks prior to exposure) and exposure periods. From a fish farm (Luc and Patrick Bijnens , Zonhoven, Belgium) rainbow trout (*O. mykiss*), common carp (*C. carpio*) and gibel carp (*C. auratus gibelio*) were obtained and kept in filtered tap water; an open trickling filter, consisting of filter wadding, activated charcoal and lava stones. The lava stones in the filters provided a place for nitrifying bacteria which removed ammonia and nitrites from water. Water temperature was kept at 17±1 °C in a flow through system that replaced total water volume of the aquaria each 7 hours. Fish were fed *at libitum* once a day and a light/day cycle of 16 hours of light and 8 hours in the dark was maintained. Water quality was checked every day: oxygen concentration stayed well above 90% saturation, hardness was 250 mg CaCO3/l and pH 7.6± 0.2. Ammonia, nitrate and nitrite levels were kept below toxic concentrations (<0.1 mg/l). Before exposure, lava stones and charcoal were removed from the filter to prevent Cu absorption in the filter. Cu was added manually at the start of the experiment as a copper nitrate solution (Cu(NO3)2·2H2O, Merck, Darmstadt, Germany) to the exposure aquaria. The desired concentration was maintained by using a peristaltic pomp (Watson Marlow 505 S) connected to a stock solution of Cu, for the duration of the entire experiment.

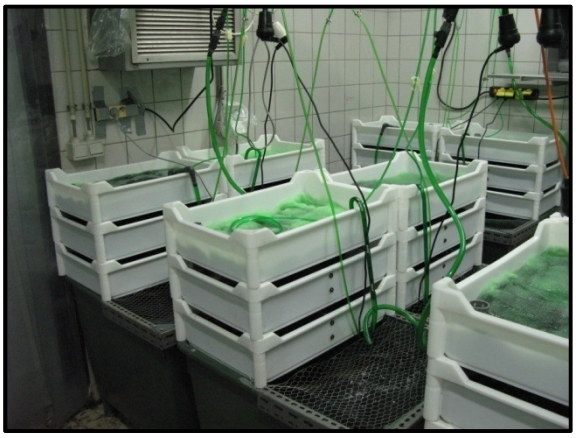

Fig. 9 Exposure aquaria with trickling filters at the University of Antwerp.

The three different fish species were challenged with 2 different sub lethal Cu concentrations. In a first series of experimental exposures, the Flemish water quality criterion for surface waters was used. This 50µg/l Cu concentration was therefore the same for rainbow trout, common carp and gibel carp. In contrast, the second series of experimental exposures was conducted with a copper level 10 times lower than their 96h LC50 value. Rainbow trout was

exposed to 20µg/l, common carp to 65 µg/l and gibel carp to 150µg/l. These values were obtained from previous research by De Boeck et al. (2004). All experiments (these studies and the study of De Boeck et al., 2004) were set in the same experimental test conditions and water quality using the same fish species which increased reliability and reduced the number of experimental fish needed for our experiment. With the exposure of 10% of the 96hLC50 value of each fish species, we can create a similar toxic load for our test species and ensure similar impact levels for the three fish species.

During each exposure, fish were intensively followed during the first hours to days after the start of the exposure: sampling occurred at 1, 12 and 24 hours, 3 days, 1 week and 1 month. Eight fish of each fish species at each time point and condition (control, 50µg/l exposure and 10% of the 96h LC50 value exposure) were quickly netted and anaesthetized in a buffered MS222 solution (100mg/l Sigma Chemical, St. Louis, MO, USA), weighed and measured.

Afterwards, a blood sample was collected from the caudal blood vessel using Li-heparinised 1ml syringes. Blood was immediately centrifuged for 3 min in Li-heparinised 1.5 ml bullet tube and plasma was transferred to a fresh cryovial and flash frozen in liquid nitrogen. Next, gill tissue was dissected on ice, rinsed and flash frozen in liquid nitrogen as well. Finally, a piece of the liver tissue was as well dissected on ice and flash frozen in liquid nitrogen. All samples were stored in -80°C until analysis. The outline of the study is represented in fig. 10. After a pre-exposure acclimation of 4 weeks, the 3 fish species were exposed to 50µg/l Cu and the 10% of the 96h LC50 value of each fish species specific. The hormonal and ion-regulatory response and oxidative stress response were investigated in both these exposures and 6 time points (1 hour, 12 hours, 24 hours, 3 days, 1 week and 1month) (Chapter 3 and 4). The subcellular distribution was examined in gill and liver of all three fish species exposed to 50µg/l for 24 hours, 3 days, 1 week and 1 month (Chapter 6). And finally, this exposure concentration was as well used to study the changes in proteome profiles at 3 days of Cu exposure (Chapter 5).

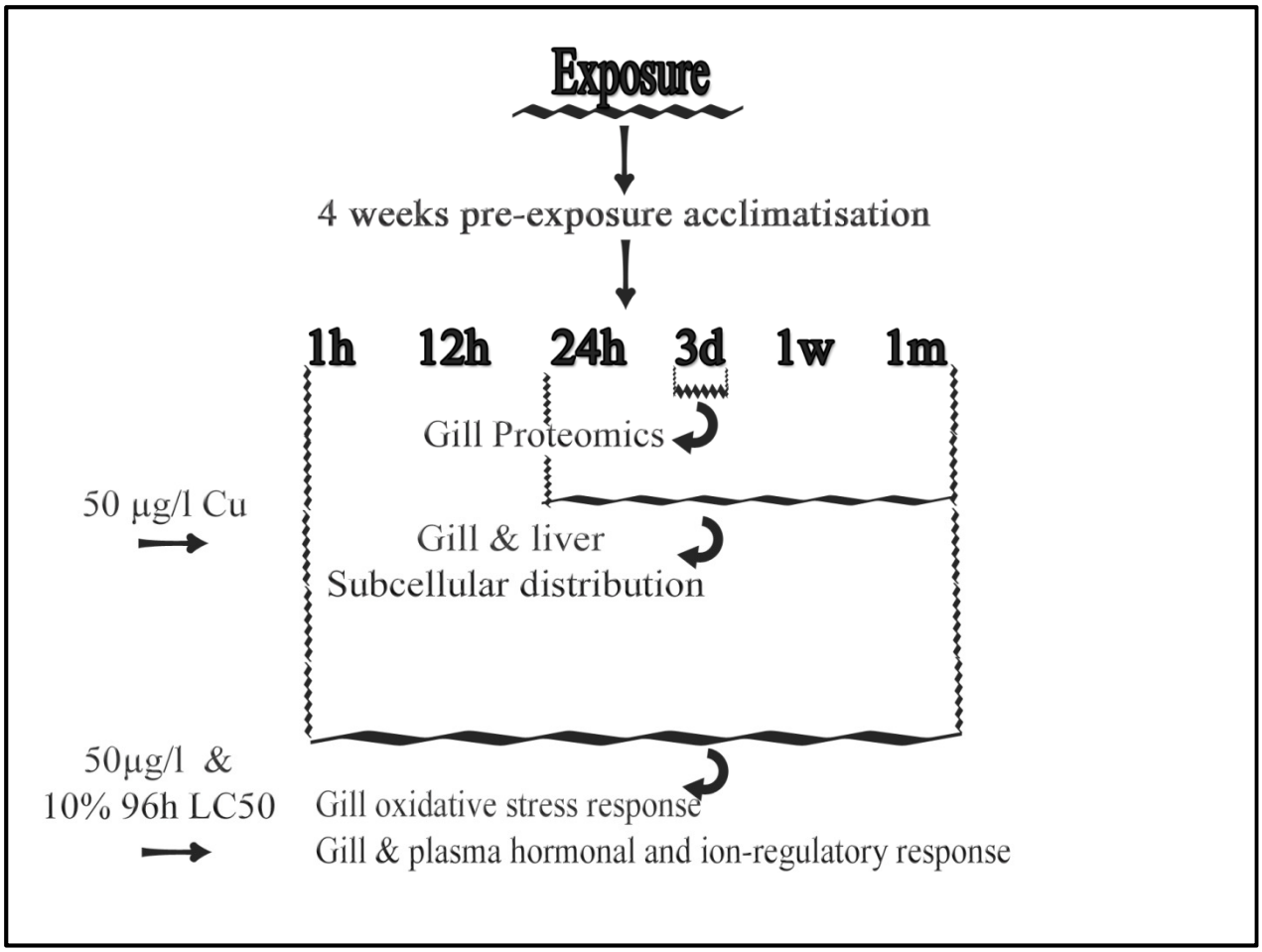

Fig. 10 The hormonal and ion-regulatory response and oxidative stress response were investigated in both these exposures and 6 time points (1 hour, 12 hours, 24 hours, 3 days, 1 week and 1month) (Chapter 3 and 4). The subcellular distribution was examined in gill and liver of all three fish species exposed to 50µg/l for 24 hours, 3 days, 1 week and 1 month (Chapter 6). And finally, this exposure concentration was as well used to study the changes in proteome profiles at 3 days of Cu exposure (Chapter 5).

Chapter 3

Hormonal and ion regulatory response in three freshwater fish species following waterborne copper exposure

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3.1 Abstract.

We evaluated effects of sublethal copper exposure in 3 different freshwater fish: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio*). In a first experiment we exposed these fishes to an equally toxic Cu dose, a Cu level 10 times lower than their 96h LC50 value: 20, 65, and 150 µg/l Cu. In a second series we exposed them to the same Cu concentration (50 µg/l). Na^+/K^+-ATPase activity in gill tissue was disturbed differently in rainbow trout then in common and gibel carp. Rainbow trout showed a thorough disruption of plasma ion levels at the beginning of both exposures, whereas common carp and gibel carp displayed effects only after 3 days. Rainbow trout and common carp thyroid hormones experienced adverse effects in the beginning of the exposure. The involvement of prolactin in handling metal stress was reflected in changes of mRNA prolactin receptor concentrations in gill tissue, with an up regulation of this mRNA in rainbow trout and a down regulation in gibel carp, which was more pronounced in the latter. Overall, rainbow trout appeared more sensitive in the beginning of the exposure, however, when it overcame this first challenge, it handled copper exposure in a better manner then common and gibel carp as they showed more long term impacts of Cu exposure.

Keywords: common carp, copper exposure, gibel carp, gill Na^+/K^+-ATPase activity, plasma ion concentration, prolactin, rainbow trout, thyroid hormones

3.2 Introduction.

Long and frequent use of metals by mankind, have led to a widespread release of these toxicants in aquatic systems. As the exposed organisms try to cope with this pollution, they develop species-specific defense mechanisms, which are rarely fully apprehended. For instance, fish are able to develop a copper (Cu) tolerance after sublethal Cu exposure in laboratory settings (Grosell et al., 2001; Grosell et al., 2002; Hashemi et al., 2008) as well as in their natural habitat (Gale et al., 2003). However, not all fish species handle elevated Cu concentrations equally adequately. For example, rainbow trout appeared to be three times more sensitive to Cu exposure than common carp, and almost seven times more sensitive than gibel carp (De Boeck et al., 2004).

Considering Cu is an essential element, organisms build up homeostatic mechanisms and strictly regulate free Cu concentrations. When Cu concentration augments to a toxic range, oxidative stress, DNA damage and a disrupted ion osmo homeostasis will occur (Arabi, 2004; Arabi and Alaeddini, 2005; Bopp et al., 2008; Bury et al., 1998; Gravato et al., 2006; Grosell et al., 2002; Handy, 2003; Wood, 2001). Previous research revealed that Cu possibly targets sodium and chloride transport systems in the gills of freshwater animals. A reduction in branchial sodium uptake occurs by inhibiting the Na^+/K^+-ATPase by nonspecific binding to thiol groups on the subunits of the transporter (Laurén and McDonald, 1987; Li et al., 1998), and by binding to the Mg^{2+} binding site, as well as competitive inhibition at the apical Na^+-channel (Grosell and Wood, 2002; Pyle and Wood, 2008). Increases of sodium loss caused by a displacement of calcium by copper in the tight junctions, leading to changes in permeability, is another effect caused by Cu exposure in freshwater fish. These changes result in a net loss of Na^+, an increase in blood viscosity and blood pressure, a compensatory tachycardia and, when exposed to acute toxic concentrations, cardiac failure (Evans et al., 1999; Handy et al., 2002; Jorgensen, 2008; Laurén and McDonald, 1985; Lingwood et al., 2005; Lingwood et al., 2006; Niyogi et al., 2006; Pyle et al., 2003; Wilson and Taylor, 1993). Within these processes, it seems that the rate of Na^+ loss is more important in determining the sensitivity of a species than the amount of Na^+ lost (Grosell et al., 2002; Pyle and Wood, 2008).

Controlling ion osmoregulation, hormones such as cortisol, prolactin (PRL), growth hormone and thyroid hormones, are of outmost significance in dealing with the effects of these toxicants. PRL, produced in the pituitary gland, is active in a broad spectrum. This hormone is

most characterized by its function in water and electrolyte balance (Sangiao-Alvarellos et al., 2006), yet it is equally essential for metabolism, growth, development, reproduction, behavior and immunoregulation (Power, 2005). In a freshwater environment, fish are forced to prevent loss of ions to the external hypo osmotic conditions and inhibit the influx of water. PRL 's ability to increase plasma ion concentrations and decrease the permeability of osmoregulatory surfaces to water, plays an important role in adapting to these circumstances (Manzon, 2002). PRL initiates its actions through binding to a specific cell surface PRL receptor (PRLR). Generally, PRLR mRNA is found to be heavily expressed in osmoregulatory organs such as gills, kidney and intestine of different fish species (Lee et al., 2006; Le Rouzic et al., 2001; Sandra et al., 1995; Tse et al., 2000).

In teleosts, thyroid hormones are synthesized, stored, and released from thyroid follicles, which are distributed in a diffuse pattern on the surface of the ventral aorta and other sub- and parapharyngeal areas (Swapna and Senthilkumaran, 2007). The major hormone synthesized in these follicles is L-thyroxin (T4), which is metabolized to the more biologically potent 3,5,3'-triiodo-L-thyronine (T3) by outer ring deiodination via deiodinases, mostly in peripheral tissues. Extensive evidence indicates that most thyroid hormone effects are mediated at the genomic level via binding to the nuclear thyroid hormone receptors (TRs), of which there are two forms (TRα and TRβ). The expression patterns share some common features (high expression in pituitary and brain), however, TRβ shows higher expression in muscle and gill compared to TRα (Filby and Tyler, 2007; Power et al., 2001; Swapna and Senthilkumaran, 2007). Thyroid hormones are involved in development and metabolism (Hontela et al., 1995; Nelson and Habibi, 2008; Zoëller et al., 2007).

Since the gill is one of the major sites for control of ion and water exchange and the first organ to accumulate metals under metal exposure due to its extensive surface area and direct contact with the external environment (Manzon, 2002), it is the best suited tissue to investigate species specific differences in handling Cu exposure. The present study aimed at unraveling relationships between plasma ion concentrations, plasma T3/T4 concentrations, gill Na^+/K^+-ATPase activity and gill mRNA concentrations of PRL receptors and thyroid hormone receptor β in rainbow trout (*Oncorhynchus mykiss)*, common carp (C *Cyprinus carpio)* and gibel carp (*Carassius auratus gibelio)* after exposure to either the Flemish standard of surface water (50µg/l Cu) or an equally toxic dose of 10% of the LC50 96h value, previously determined (De Boeck et al., 2004). These diverse exposures will give us an

insight in how these fish species manipulate their hormones and ion osmoregulatory processes when introduced to a similar concentration of a toxicant or a concentration corresponding with a similar toxic load.

3.3 Materials and Methods.

3.3.1 Animal maintenance and copper exposure.

Rainbow trout (*O. mykiss*), common carp (*C. carpio*) and gibel carp (*C. auratus gibelio*) were obtained from a fish farm at Luc and Patrick Bijnens, Zonhoven, Belgium. Average size of rainbow trout was 296 ± 46g, of common carp 231± 85g, and of gibel carp 224 ± 96g.

Fish were kept at the University of Antwerp in aquaria (200l) for at least a month before the exposure started. Tap water was filtered with an open trickling filter, consisting of filter wadding, activated charcoal and lava stones. Those lava stones provide a place for nitrifying bacteria that remove ammonia and nitrites from water. Experiments were conducted at 17±1 °C in a flow through system that renewed total water volume of the aquaria each 7 hours. Fish were fed *at libitum* once a day. Water quality was checked every day: oxygen concentration stayed well above 90% saturation, hardness was 250 mg CaCO3/l and pH 7,6± 0,2. Ammonia, nitrate and nitrite levels were kept below toxic concentrations (<0.1 mg/l). Cu was added manually at the start of the experiment as a copper nitrate solution (Cu(NO3)2·2H2O, Merck, Darmstadt, Germany) to the exposure aquaria. The desired concentration was maintained by using a peristaltic pomp (Watson Marlow 505 S) connected to a stock solution of Cu, for the duration of the entire experiment. Each fish species was exposed to the Flemish standard of surface water (50µg/l) and to 10% of the 96hLC50 value (20µg/l for rainbow trout, 65µg/l for common carp and 150µg/l for gibel carp). Cu concentrations were measured using atomic absorption spectroscopy.

3.3.2 Sampling procedures.

Eight fish of each fish species and each condition were sampled after 1h, 12h, 24h, 3days, 1 week and 1 month. Fish were quickly netted, anaesthetized in a buffered MS222 solution (100mg/l Sigma Chemical, St. Louis, MO, USA) weighed and measured. A blood sample was collected from the caudal blood vessel using Li-heparinised 1ml syringes. Blood was immediately centrifuged for 3 min in Li-heparinised 1.5 ml bullet tubes. Plasma was transferred to a fresh cryovial, flash frozen in liquid nitrogen and stored at -80°C until [Na^+],

[Cl^-], [Ca^{2+}], [T3/T4] analysis. Gill tissue was dissected on ice, rinsed and flash frozen in liquid nitrogen followed by storage in -80°C for determination of Na^+/K^+-ATPase activity and mRNA concentration of PRL and TH receptors.

3.3.3 $Na^{+/}K^+$-ATPase activity in gill tissue.

Frozen gill filament samples were thawed and homogenized for determination of Na^+/K^+ ATPase activity using the method of McCormick (1993). Briefly, homogenization was carried out in a SEID buffer (10 mM EDTA, 150mM sucrose, and 50mM imidazole, with 0.1% w/v sodium deoxycholate). The samples were centrifuged for 1 min at 4°C and 5000g. The supernatant was used for further determination. Using a 96-well microplate, 10µl of the resultant supernatant was run in duplicate with assay A mixture (400U Lactate hydrogenase, 500U pyruvate kinase, 2.8 mM phosphoenolpyruvate, 0.7 mM ATP, 0.22 mM NADH, 50mM imidazole) and in parallel, 10µl of the supernatant in duplicate with assay B mixture (contains assay A with 0,4mM ouabain). Using a spectrophotometer (Ultra microplate reader $ELX808_{IU}$ Bio-Tek instruments inc., Vermont, USA) at 340nm, absorbance was measured. By subtracting the oxidation rate of NADH to NAD in the presence of ouabain from that in the absence of ouabain, the Na^+/K^+-ATPase activity was determined. The associated protein content was assayed using the technique of Bradford (1976).

3.3.4 Ion concentration in plasma.

Plasma [Na^+], [Cl^-] and [Ca^{2+}] were analyzed using an AVL 9180 Electrolyte Analyser (AVL, Roche Diagnostics, Belgium).

3.3.5 Radioimmunoassay for thyroid hormones in plasma.

Radioimmunoassays were performed to determine plasma [T_3] and [T_4] as previously described by (Van der Geyten et al., 2001). All plasma samples were measured within a single assay and thyroid hormone levels were expressed as pmol/ml.

3.3.6 Real Time Reverse Transcriptase PCR analysis.

3.3.6.1 Homogenization and mRNA extraction

For disruption, frozen gill tissue of all fish species and exposure conditions was grinded to a fine powder in liquid nitrogen, using mortar and pestle. The tissue was kept in frozen state

using liquid nitrogen. The suspension was transferred into a cooled tube, allowing the liquid nitrogen to evaporate without allowing the sample to thaw. Lysis buffer was added and homogenization was performed using the homogenization kit of Qiagen (Qiashredder Cat no. 79654). Subsequently, Rneasy Mini kit from Qiagen (Cat.no 74104) was used to extract mRNA. Concentration was determined by measuring absorbance at 260 nm using a nanodrop spectrophotometer (Thermo Scientific). The quality of RNA was verified with gel electrophoreses.

3.3.6.2 cDNA synthesis

1µg RNA was used to synthesise 1 µg cDNA via the Revert Aid H Minus First strand cDNA synthesis kit (Fermentas Cat. No K1632).

3.3.6.3 Real Time Reverse Transcriptase PCR analysis

Real Time Reverse Transcriptase PCR was carried out on a Roche Molecular Biochemicals LightCycler 3.5 with the Maxima Sybr green qPCR Mastermix (2x) (1000 reactions, Fermentas, Cat no K0222) according to the manufacturer's instructions. Primers were designed using Roche Molecular Biochemicals Lightcycler Probe Design Software 1.0 (table 1. Primer Sequences). Relative expression levels were calculated using a housekeeping gene, selected from previous runs. β-actin was chosen as housekeeping gene for rainbow trout and gibel carp, whereas EF1α was selected as housekeeping gene for common carp, as those genes were not differentially expressed during the entire experiment. To normalize between runs, a universal sample was made out of cDNA of all exposure conditions and time points. The Ct values of this sample were used for normalization. The relative expression of the target transcript sequences was calculated as reported by Pfaffl et al. (2002) including efficiency corrections for each transcript. The formation of specific PCR products was checked by melting curve analysis.

3.3.7 Statistical analysis.

Data are presented as mean ± S.E.M. Statistical analysis of the differences between control, fish exposed to the Flemish standard of surface water and fish exposed to their 10%LC50 96h value (for each fish species separately) was performed by two way analysis of variance (ANOVA) followed by Tukey HSD post hoc test. Data were considered significant if $p<0.05$. All tests were performed using Statistica.

Gene specification	Species	NCBI GenBank accession number	Primer sequences sequence written 5' to 3'
glyceraldehyde-3-phosphate dehydrogenase	Oncorhynchus mykiss	NM_001124246.1	Fwd: TCC-ACC-GGA-GTC-TTC-CT
			Rev: CGT-GGA-TGA-CCT-TAG-CC
	Cyprinus carpio	AJ870982.1	Fwd: ATC-TGA-CGG-TCC-GTC-T
			Rev: CCA-GCA-CCG-GCA-TCA-AA
	Carassius auratus	AY641443.1	Fwd: CAC-TAA-CTG-CTT-GGC-TCC
			Rev: CTT-GGC-AGC-ACC-AGT-TG
elongation factor EF1 alpha	Oncorhynchus mykiss	AF498320.1	Fwd: AAC-GGT-GTG-ACT-CTG-CTG
			Rev: GGC-GAA-GGT-GAC-GAT-CAT
	Cyprinus carpio	AF485331.1	Fwd: TGG-AGA-TGC-TGC-CAT-TGT
			Rev: TGC-AGA-CTT-CGT-GAC-CTT
	Carassius auratus	AB056104.1	Fwd: GGA-TGG-CAT-GGT-GAC-AAC
			Rev: ACA-CCA-GTC-TCC-ACA-CGA
beta-actin	Oncorhynchus mykiss	AF157514.1	Fwd: ATC-GGA-GGC-TCC-ATC-TTG
			Rev: TCT-GGT-GGC-AGA-GCT-GAA
	Cyprinus carpio	M24113.1	Fwd: AGC-TAG-GCC-TTG-AGC-TAT
			Rev: CCT-GCT-TGC-TAA-TCC-ACA
	Carassius auratus	AB039726.2	Fwd: ATC-CAG-GCT-GTG-CTC-TC
			Rev: CAG-ATC-ACG-ACC-AGC-CA
thyroid hormone receptor beta	Oncorhynchus mykiss	AF302246.1	Fwd: AGG-ACA-TTG-GTC-AAG-CG
			Rev: ACA-AGG-CAG-CTC-ACA-GA
	Danio rerio	BC163106.1	Fwd: GAC-ATT-GGA-TCG-GCA-CCT
			Rev: ACT-GCT-GCT-CGA-AGA-GAC
	Carassius auratus	AY973630.1	Fwd: CAT-CAA-CGC-ACC-AGA-GG
			Rev: ACT-GCT-GCT-CGC-AGA-GA
prolactin receptor	Oncorhynchus mykiss	NM_001124599.1	Fwd: GCA-GTG-TGA-AGC-ACT-GT
			Rev: CAC-CAG-CAG-ATC-CTC-GT
	Cyprinus carpio	AY044448.1	Fwd: GTT-CAT-GGT-GCA-GGT-TCG
			Rev: GCA-ACA-GGA-GGC-AGC-AA
	Carassius auratus	AF144012.1	Fwd: GCG-CGT-GAT-AAG-TCA-TCG
			Rev: GCG-GTT-GCT-GGA-TCT-TG

Table 1. Primer sequences used in RT-Real Time PCR.

Rainbow trout	Condition		Time point		condition*time point	
	F value	P value	F value	P value	F value	P value
$[Na^+]$ in plasma	56.3460	0.0000	7.0070	0.0000	7.4290	0.0000
$[Cl^-]$ in plasma	12.8370	0.0000	20.5600	0.0000	15.8280	0.0000
$[Ca^{2+}]$ in plasma	38.5650	0.0000	6.7080	0.0000	9.7360	0.0000
Na^+/K^+ ATPase in gill	5.4486	0.0065	4.6078	0.0007	3.7843	0.0002
[T3] in plasma	0.9748	0.3804	6.9250	0.0000	0.8467	0.5849
[T4] in plasma	29.9970	0.0000	7.0190	0.0000	3.0130	0.0021
mRNA PRLR in gill	9.0540	0.0002	30.8870	0.0000	9.3330	0.0007
mRNA THRβ in gill	1.5820	0.0210	63.9500	0.0000	1.0910	0.3744
Common carp	Condition		Time point		condition*time point	
	F value	P value	F value	P value	F value	P value
$[Na^+]$ in plasma	22.4650	0.0000	7.7120	0.0000	2.0710	0.0315
$[Cl^-]$ in plasma	22.7440	0.0000	10.6710	0.0000	3.9530	0.0001
$[Ca^{2+}]$ in plasma	2.3000	0.1045	15.1680	0.0000	2.5934	0.0278
Na^+/K^+ ATPase in gill	5.3384	0.0060	4.4332	0.0009	1.0031	0.4456
[T3] in plasma	6.7260	0.0017	20.6000	0.0000	4.9690	0.0000
[T4] in plasma	21.8100	0.0000	29.8430	0.0000	8.4940	0.0000
mRNA PRLR in gill	0.7240	0.4870	15.9980	0.0000	2.3080	0.0167
mRNA THRβ in gill	2.2256	0.0113	9.0987	0.0000	1.7361	0.0810
Gibel carp	Condition		Time point		condition*time point	
	F value	P value	F value	P value	F value	P value
$[Na^+]$ in plasma	45.9080	0.0000	12.5490	0.0000	6.7950	0.0000
$[Cl^-]$ in plasma	50.1520	0.0000	12.7830	0.0000	11.2200	0.0000
$[Ca^{2+}]$ in plasma	0.4554	0.6353	3.8914	0.0026	1.6637	0.0965
Na^+/K^+ ATPase in gill	13.0620	0.0000	7.4670	0.0000	2.5130	0.0087
[T3] in plasma	39.3910	0.0000	6.0170	0.0001	3.8160	0.0002
[T4] in plasma	0.3247	0.7236	3.0237	0.0134	3.1155	0.0015
mRNA PRLR in gill	6.3785	0.0023	7.8008	0.0000	4.8447	0.0000
mRNA THRβ in gill	9.0620	0.0002	19.3290	0.0000	1.2180	0.2862

Table 2. F values and their significance for all measured parameters, analysed with three way anova, for rainbow trout, common carp and gibel carp.

3.4 Results.

Measured total water [Cu] in each exposure was on average 98.4 ± 4.67 % of nominal Cu during the entire experiment and no mortality occurred.

3.4.1 $Na^{+/}K^{+}$-ATPase activity in gill tissue.

In this experiment, $Na^{+/}K^{+}$-ATPase activity was affected by copper exposure (fig.11, table 2). In rainbow trout a diminished activity occurred after 12 hours of exposure to 50µg/l Cu, however no changes were seen when exposed to 20µg/l. In common carp $Na^{+/}K^{+}$-ATPase activity was not affected. In gibel carp, a decrease in activity appeared in both exposure conditions. A decrease in activity after 12hours was seen when exposed to 50µg/l and when exposed to 150µg/l Cu, a prolonged decrease was seen with a fall in activity from 3 days of exposure to 1 week. We did notice a tendency of decreased activity after 12hours when exposed to 150µg/l Cu and after 3days and 1 week when exposed to 50µg/l Cu, however, those data were not statistically significant.

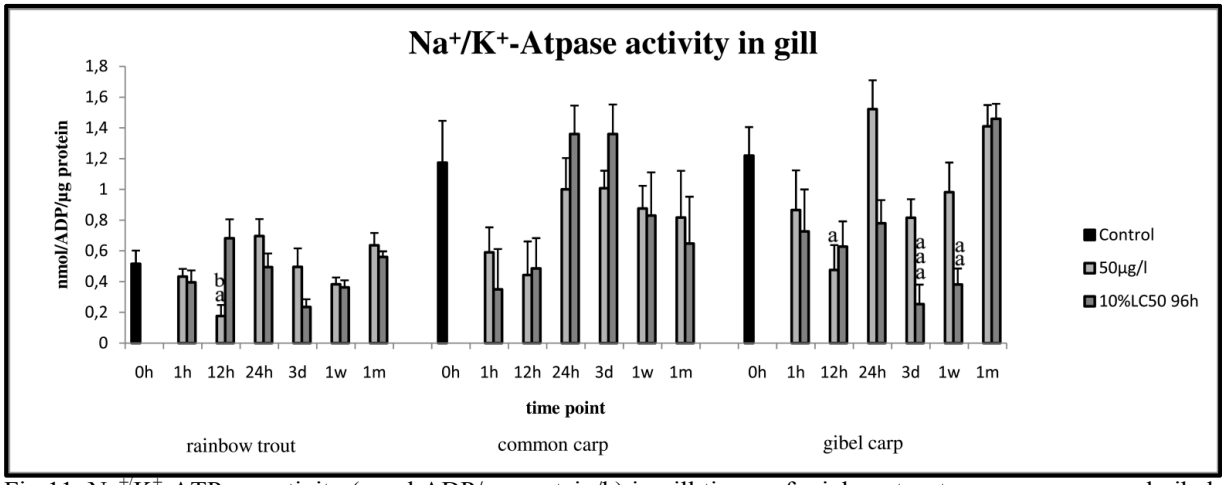

Fig 11. $Na^{+/}K^{+}$-ATPase activity (nmol ADP/µg protein/h) in gill tissue of rainbow trout, common carp and gibel carp after 1hour, 12hour, 24hour, 3days, 1week and 1 month of exposure to either control conditions, 50µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. An a indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), b indicates a significant difference between exposed to 50µg/l and exposed to the 10%LC50 96h value (b: $p<0.05$; bb: $p<0.01$; bbb: $p<0.001$).

3.4.2 Ion concentration in plasma.

The ion concentration in plasma showed differences between the rainbow trout and the Cyprinidae (Fig.2a, b, c, table 2). When considering $[Na^{+}]$ in plasma of rainbow trout (Fig. 12a), we observed a decline in both exposure conditions ranging from 1 hour of exposure, for 20µg/l Cu and from 1 hour to 24 hours of exposure, a more severe effect, when exposed to 50µg/l Cu. $[Cl^{-}]$ followed the same pattern as $[Na^{+}]$ in rainbow trout (Fig. 12b). $[Cl^{-}]$

decreased after one hour of exposure to 50µg/l and 20µg/l Cu and this decrease was maintained until 24 hours of exposure when exposed to the Flemish standard value of surface waters (50µg/l). Plasma [Ca^{2+}] of rainbow trout was affected by exposure to the Flemish standard (from 1 hour to 24 hours of exposure) as well as to 10% LC50 96h value (1 hour of exposure) (Fig. 12c). At the end of the exposure, all three ion concentrations recovered to their normal status.

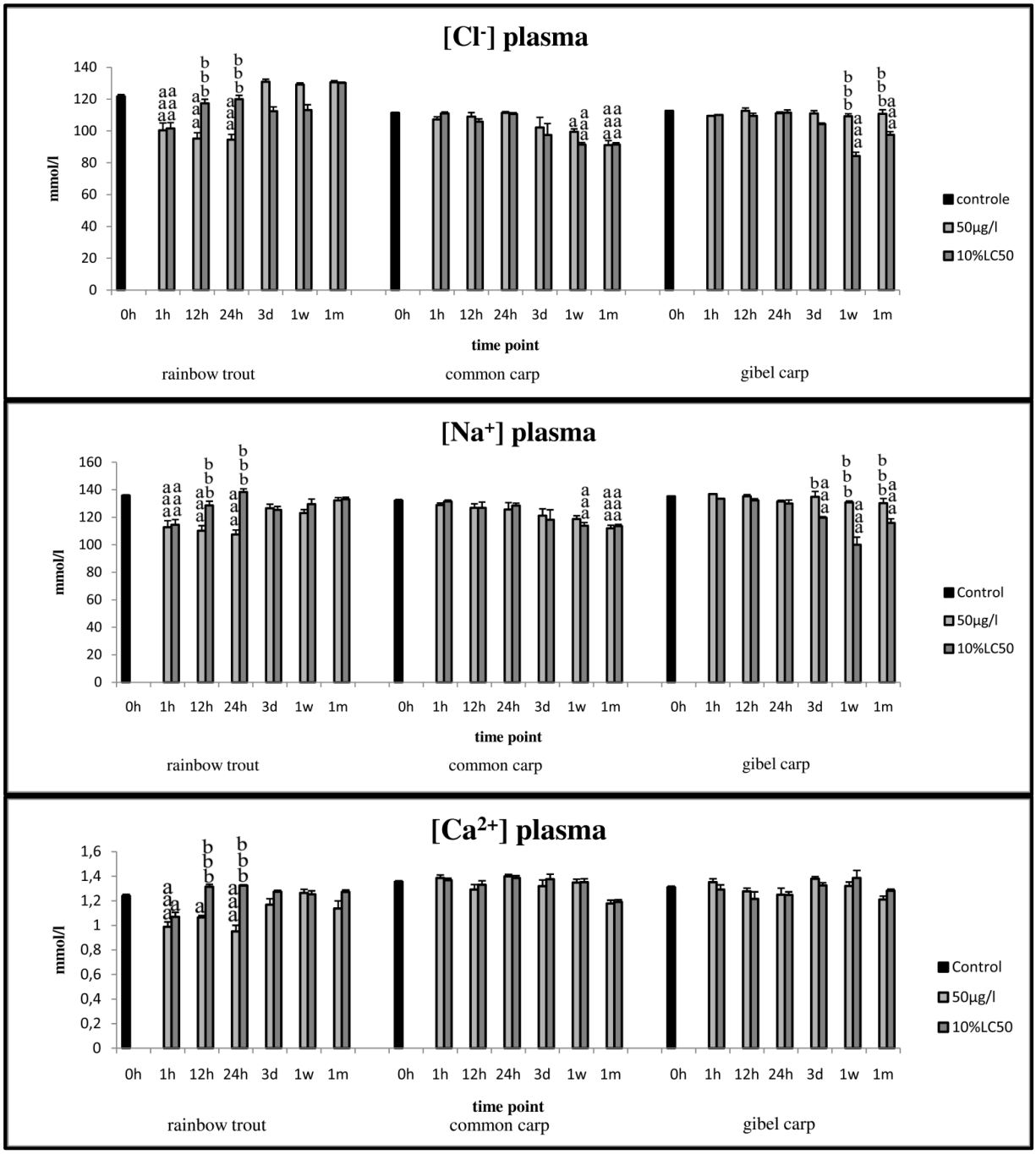

Fig 12. [Na+] (Fig. 12a), [Cl-] (Fig. 12b), [Ca2+] (Fig. 12c) in plasma of rainbow trout, common carp and gibel carp after 1hour, 12hour, 24hour, 3days, 1week and 1 month of exposure to either control conditions, 50µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. An a indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), b indicates a significant

difference between exposed to 50µg/l and exposed to the 10%LC50 96h value (b: p<0.05; bb: p<0.01; bbb: p<0.001).

A decrease in [Cl^-] and [Na^+] in common carp was seen after 1 week and 1 month of exposure to 65µg/l Cu and exposure to 50 µg/l caused a decrease after 1 month of exposure. The ions in plasma of gibel carp were not disturbed when exposed to 50µg/l Cu, however they exhibited a decline of [Na^+] after 3 days, 1 week and 1 month and of [Cl^-] after 1 week and 1 month of exposure to 150µg/l Cu.

3.4.4 Thyroid hormone concentration in plasma.

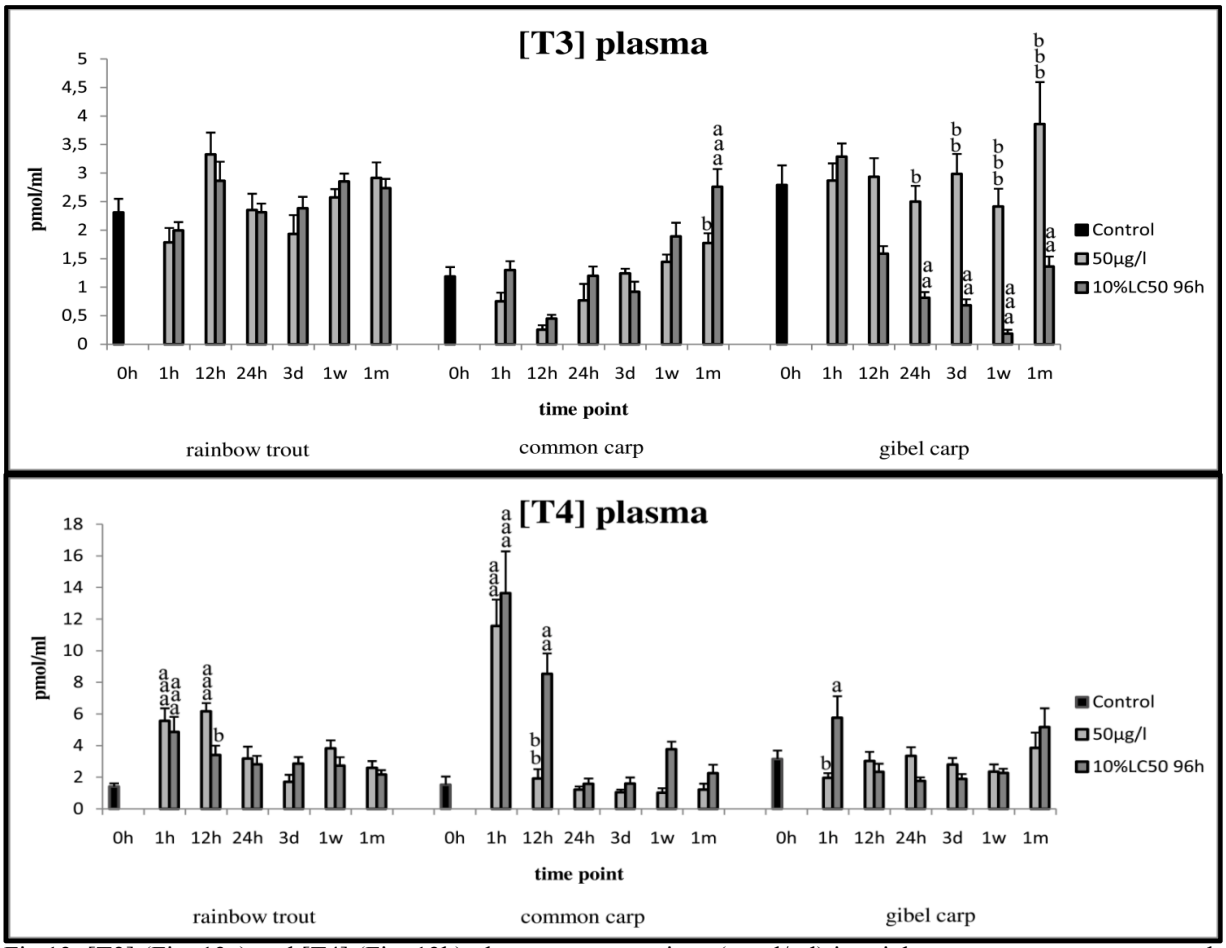

Fig.13. [T3] (Fig. 13a) and [T4] (Fig. 13b) plasma concentrations (pmol/ml) in rainbow trout, common carp and gibel carp after 1hour, 12hour, 24hour, 3days, 1week and 1 month of exposure to either control conditions, 50µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. An a indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: p<0.05; aa: p<0.01; aaa: p<0.001), b indicates a significant difference between exposed to 50µg/l and exposed to the 10%LC50 96h value (b: p<0.05; bb: p<0.01; bbb: p<0.001).

Triiodothyronine (T3)

Cu exposure induced different changes in plasma [T3] in the studied fish species. Our results (Fig. 13a, table 2) showed no changes in rainbow trout. Common carp and gibel carp [T3] responded differently during the experiment. In common carp's plasma, we observed an

increase in [T3] in the highest exposure group (65µg/l) after 1 month. [T3] in plasma of gibel carp, showed no effect when exposed to 50µg/l Cu, although exposure to 150µg/l Cu generated a thorough drop in [T3] plasma levels after 24h, which continued until 1 month of exposure and appeared most severe after 1 week of exposure.

Thyroxin (T4)

Contrary to [T3], we saw more effects in [T4] at the moment the exposure started (Fig. 13b, table 2). Gibel carp reacted with a single peak after 1 hour when exposed to 150µg/l Cu. Common carp followed this same trend with a distinct increase of [T4] after 1 hour of exposure to 50µg/l Cu and a prolonged rise in [T4] from 1 hour till 12 hours of exposure to 65µg/l Cu. Rainbow trout showed an immediate elevation as well after 1 and 12 hours of exposure to 50µg/l Cu and an increase after 1hour when exposed to 20µg/l.

3.4.5 Real time Reverse Transcriptase PCR: mRNA concentrations of Prolactin receptor and thyroid hormone receptor β in gill tissue.

mRNA concentrations are expressed relative to a housekeeping gene and shown as percentage of control values (Fig. 14 for THRβ, Fig.15 for PRLR, table 2). THRβ mRNA in gill tissue of rainbow trout increased after 1 month of exposure to 20µg/l and 50µg/l Cu. In common carp, a considerable decrease was seen after 1 month of exposure to both concentrations. THRβ mRNA in gill tissue of gibel carp displayed effects after 3 days and 1 week of exposure, concentrations dropped when exposed to 150µg/l Cu.

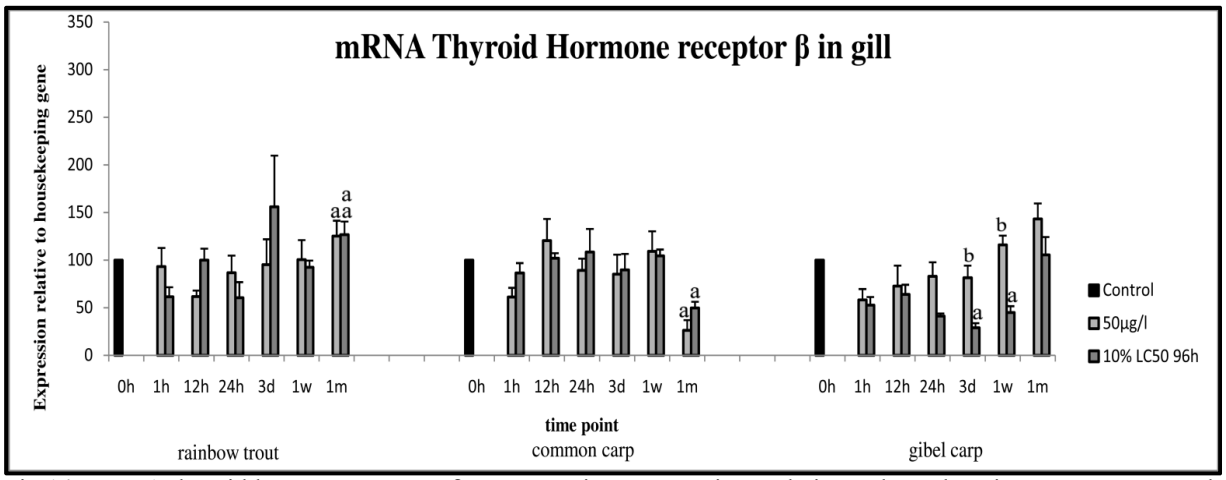

Fig.14. mRNA thyroid hormone receptor β concentrations expressions relative to housekeeping genes, compared to control values of rainbow trout, common carp and gibel carp after 1hour, 12hour, 24hour, 3days, 1week and 1 month of exposure to either control conditions, 50µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. An a indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), b indicates a significant difference between exposed to 50µg/l and exposed to the 10%LC50 96h value (b: $p<0.05$; bb: $p<0.01$; bbb: $p<0.001$).

Exposure of rainbow trout to 20µg/l Cu, tended to increase PRLR mRNA expression in gill tissue with significant effects after 1 month (Fig.15), however, exposure to 50µg/l Cu did not appear to exert any effect on PRLR mRNA. As for common carp no effects were seen either. PRLR mRNA levels in gill tissue of gibel carp dropped after 3 days of exposure to 150µg/l Cu until 1 week of exposure. Concentrations of PRLR mRNA recovered thereafter.

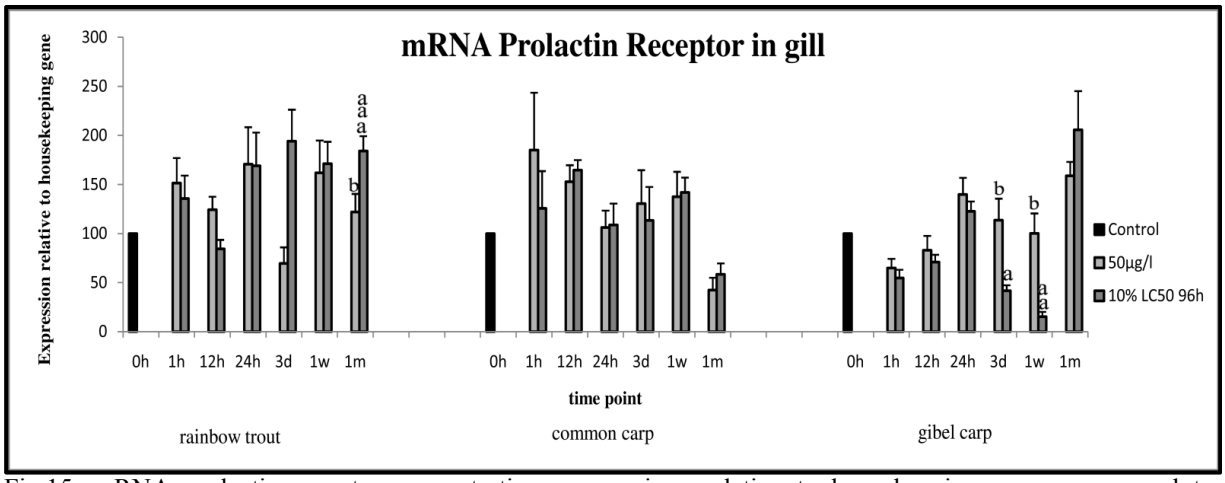

Fig.15. mRNA prolactin receptor concentrations expressions relative to housekeeping genes, compared to control values, in rainbow trout, common carp and gibel carp after 1hour, 12hour, 24hour, 3days, 1week and 1 month of exposure to either control conditions, 50µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. An a indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), b indicates a significant difference between exposed to 50µg/l and exposed to the 10%LC50 96h value (b: $p<0.05$; bb: $p<0.01$; bbb: $p<0.001$).

3.5 Discussion.

Both the 10% of the 96h LC50 as well as the Flemish standard of surface water (50µg/l Cu) were sublethal during a one month exposure of rainbow trout, common carp and gibel carp. However, clear responses were observed in these fish species at different time points, such as changes in Na^+/K^+-ATPase activity in gill, changes in the levels of ions in plasma and changes in the studied hormones.

In rainbow trout the results showed a thorough decrease in $[Na^+]$ plasma in the first day after exposure to 50µg/l Cu. The effect of Cu exposure on plasma ions of the Cyprinidae appeared later: the reduction in $[Na^+]$ only started after three days of exposure. This drop in Na^+ concentration, as mentioned in the introduction, can be caused by a decrease in Na^+/K^+-ATPase activity in gill tissue. Such decline in activity was seen at 12 hours of exposure in rainbow trout and gibel carp when exposed to the Flemish standard of surface water. Gibel carp exposed to the highest concentration of copper (150µg/l) displayed a loss in activity of this enzyme from 3 days of exposure to one week. Despite the fact that we detected only a transient reduction in Na^+/K^+-ATPase activity, we observed a more pronounced decrease in

[Na^+]. As such, the inhibition of Na^+/K^+-ATPase activity is not likely to be the sole ionoregulatory effect exerted by Cu. Possibly different systems are influenced depending on the duration of the exposure. In previous research, apical Na^+/H^+ exchangers, Na^+ channels and intracellular carbonic anhydrase were shown to be affected by Cu exposure (Blanchard and Grosell, 2006; Handy, 2003; Laurén and McDonald, 1985). An inhibition of these components can threaten Na^+ transport across the gills. Not only would a decrease of carbonic anhydrase activity reduce branchial Na^+ transport by reducing proton concentration (Ditusa et al., 2001; Vitale et al., 1999), the hydration of carbon dioxide by cellular carbonic anhydrase also produces bicarbonate (Grosell et al., 2002; Perry, 1986). Sodium and chloride are indirectly linked by the same carbonic anhydrase since bicarbonate is a substrate for the chloride bicarbonate exchange across the apical membrane and thus determines partly chloride uptake (Perry et al., 1984). Our results showed indeed a decrease of chloride concentrations, for rainbow trout in the beginning of the exposure, and for common carp and gibel carp, after 3 days or 1 week of exposure. These declines in chloride concentration showed strong similarities with sodium concentration in plasma of the exposed fish (Crespo and Karnaky, 1983; Grosell et al., 2002; Laurén and McDonald, 1985; Wilson and Taylor, 1993). In addition, ion leakage caused by changed membrane permeability can attribute to the decreased ion concentrations in plasma, as well (Kurilenko et al., 2002). As rainbow trout is really susceptible to severe ionoregulatory failure (Beaumont et al., 2003), we found that when exposed to 50µg/l Cu, calcium concentration in plasma of rainbow trout is also affected in the beginning of the exposure.

With compensatory mechanisms, such as stimulation of Na^+/K^+-ATPase synthesis and enhanced chloride cell turnover, fish can compensate for the damage caused by Cu exposure, thus following a damage-repair model. Three phases are being recognized in this model: an initial shock phase, a recovery phase and an acclimation phase (Grosell et al., 2002; Hogstrand et al., 1995; McDonald and Wood, 1993; McGeer et al. 2002; Wendelaar Bonga, 1997). Laurén and McDonald (1987) and Tate-Boldt and Kolok (2008) have also shown this temporary nature of Cu-induced osmoregulatory disturbance with return to control levels. In rainbow trout, ion losses were reversed subsequent to the initial shock phase; however common carp and gibel carp did not re-obtain their control levels after one month of exposure. It might be that rainbow trout, after it survives the initial shock phase, is more capable of restoring homeostasis whereas common carp and gibel carp are better equipped to survive and acclimate to their reduced plasma ion levels in this one month experiment.

However, more research is needed to investigate if carp can restore ion homeostasis when exposed for a longer period of time. The ability to cope with this ionosmoregulatory stress was also noticed in the related crucian carp, which displayed a high tolerance for Cu exposure (Schjolden et al, 2007). The rate of the diffuse loss of ions from extracellular fluids to the surrounding dilute environment appears to be more important for the occurring mortality as the total loss of ions (Grosell et al., 2002; Pyle and Wood, 2008); once rainbow trout overcomes the fast loss of ions in the early hours of exposure, it is more likely to survive afterwards. Additionally, as a migrating species, rainbow trout is probably better equipped with mechanisms to compensate for changing ion concentrations in contrast to the Cyprinidae. Prolactin receptor mRNA levels are universally expressed in teleost fish in gill, kidney and gut (Lee et al., 2006; Manzon, 2002; Tse et al., 2000). The majority of research concerning prolactin focuses on the adaptation of fishes from seawater to freshwater and vice versa (Lee et al., 2006; Seale et al., 2006). To survive this transition, the prolactin cell is influenced by a low pH and a decrease of plasma monovalent ions (Flik et al., 1989; Wendelaar Bonga, 1988) which leads to a decrease of branchial sodium loss, a reduction in the number of chloride cells exposed to the extracellular environment, and an influenced Na^+/K^+-ATPase activity. This last mentioned activity can be decreased (Madsen and Bern, 1992), increased (Boeuf et al., 1994) or not influenced at all (Herndon et al., 1991; Kelly and Wood, 2002). Similar to a transition of fish from seawater to freshwater, a decrease in plasma ions occurs during exposure to Cu (Grosell et al., 2002; Handy et al., 2002). During salinity transition, this is a result of differences in ion concentrations between the fish and a hyper osmotic environment (Fujimoto et al., 2006), and when exposed to Cu, due to an inhibition of Na^+/K^+-ATPase activity in gill chloride cells and leakage of ions (De Boeck et al., 2007). A stimulation of prolactin release could therefore be a good defense mechanism to cope with the first, and often the most, toxic adverse effects of copper exposure. Since we investigated mRNA receptor concentrations and not actual receptor numbers, no assured conclusions can be drawn regarding the link between gene expression and protein concentration considering that there are various complicated post transcriptional and translational mechanisms involved in the process of turning mRNA into protein (Greenbaum et al., 2003). It should be mentioned as well that the correlation between PRL concentration in plasma and PRLR mRNA in gill tissue (as well as for mRNA THRβ and thyroid hormone receptor β) is still under investigation. Pierce et al. (2007) could not find a significant relationship between those parameters in tilapia. However, the number of hormone receptors

is an important parameter in control of physiological mechanisms, since the responsiveness of a cell is greatly dependent on the receptor concentration in that cell (Vanderbilt et al., 1987). As the PRLR mRNA in rainbow trout rises after 1 month of exposure to 20µg/l Cu, this could indicate an increase of circulating prolactin in plasma. However, when exposed to 50µg/l Cu, there was no statistical effect on PRLR mRNA in rainbow trout. Possibly, effects occurring when exposed to this higher copper concentration are beyond repair by adaptation of prolactin. Nevertheless, an affected sodium concentration in plasma can lead to an increased release of prolactin, which results in an activation of the proton ATPases and a better uptake of sodium. In gibel carp, an opposite reaction occurs; PRLR mRNA declines after three days and one week of exposure to 150µg/l Cu. As seen in previous research (De Boeck et al., 2007), gibel carp and in lesser extent, common carp, can decrease their ventilation rate when faced with toxic environmental parameters, reducing the loss of ions through gill epithelia and perhaps thus preventing the activation of the release of prolactin. A reduction of gene transcription in gill tissue can be a result of epithelial gill damage occurring from the exposure conditions as well. A previous study on Cu toxicity conducted by De Boeck et al. (2007) revealed relatively little gill damage in rainbow trout and a shock and repair pattern with recovery after one week in common carp. However in gibel carp, gill damage appeared only after a few days of exposure to Cu and continued until the end of exposure time, so for gibel carp, this alternative conclusion of reduced gene transcription caused by gill damage might be an explanation for the observed decline in mRNA PRLR in gill tissue. Despite this gill damage, gibel carp's ventilation rate returned to normal within a day and remarkably, they used their anaerobe metabolism from the start of the exposure (De Boeck et al., 2007). We also investigated thyroid hormone concentrations in the plasma and the expression of THRβ mRNA in gill tissue. A number of studies report on thyroid concentrations of plasma in fish (De Groef et al., 2006; Gagnon et al., 2006; Kühn et al., 1998; Laflamme et al., 2000; Monteiro et al., 2005). Teles et al. (2005) exposed European eel (*Anguilla anguilla* L.) short-term to low concentrations of copper and noticed a decrease in plasma [T4] without influencing [T3] in plasma. When eels were exposed for a longer period of time (7 days), Oliveira et al. (2008) noted an unaltered [T4] in plasma and a [T3] decrease. A variety of mechanisms can result in a change of the thyroid status, such as an alteration in the hypothalamus and / or pituitary status, biosynthesis and secretion steps of T3 and T4, uptake by peripheral tissues, or hormone catabolism and clearance rates (Hontela et al., 1995; Oliveira et al., 2008). Gomez et al. (1997) suggested that the plasma concentration of T3 is

not influenced (short-term) by plasma concentrations of T4. Rather, thyroid stimulating hormone stimulates T4 production, and T3 concentration is regulated in peripheral tissues by T3-producing and -degrading deiodinases (Darras et al., 1998; Orozco and Valverde, 2005; Van der Geyten et al., 2001, Van der Geyten et al., 2005, Walpita et al., 2007). Our results indicate both differences in T3 and in T4 plasma concentrations. In the beginning of the experiment all fish species show an extensive peak in T4 plasma concentrations. As T3 is the bioactive form of T4, T3 binds THRβ with higher affinity than T4 does. According to Sandler et al. (2004), T4 can stimulate THR at appropriate concentrations in humans. Since T3 and T4 are identical in all vertebrates, it might be that the peak of plasma T4 concentration can cause an initial augmentation in metabolism, in order to handle the first stress response. In the articles mentioned above, no thyroid hormone concentrations were measured after one hour, so it is possible that this first and acute reaction to Cu appeared in their studied fish species as well. Looking at the [T3] in plasma reveals differences between the rainbow trout and the Cyprinidae. While no effect is seen in rainbow trout T3 plasma concentrations, common carp reacts with an increase of [T3] after 1 month of exposure to 65µg/l and gibel carp keeps its T3 plasma concentration low from 24hours of exposure to the end of the exposure to 150µg/l Cu. The altered T3 concentrations in plasma can be a product of a deprived transformation of T4 into T3. As seen in rats, depression of the metabolism by hypothyroidism can cause a decreased oxidant production and hereby protect the organism for oxidant damage (Alturfan et al., 2007). Looking at the results of THRβ mRNA expressions and [T3], especially gibel carp (and in a lesser extent, common carp) tends to decrease this parameters causing hypothyroidism and thereby protecting its vital functions. On the other hand, the reduction in THRβ mRNA expressions might be as well partly due to the gill damage caused by the opposed Cu exposure. However, if thyroid hormones alter, this can cause a reduced fish physiological competence and survival (Oliveira et al., 2008). It appears that gibel carp clearly tries (and succeeds) to protect itself by changing its metabolism. These findings suggest that the effects on the thyroid hormones and their receptors are not directly caused by the ion osmoregulatory stress elicited by Cu exposure, but are a result of the altered metabolism. Results from De Boeck et al. (2006), support this theory since they observed an increase of the energy metabolism in rainbow trout and a decrease of the energy metabolism in both common and gibel carp after one day of exposure to Cu.

We can conclude that exposure to the Flemish water quality criterion for surface waters gives rise to a number of hormonal changes in some of the fish species studied. As rainbow trout

experienced more effects, in the beginning of the exposure than common carp, it demonstrates once more the sensitivity of this fish species and the need to survive the severe ion loss in order to cope with the opposed Cu concentrations. Only gibel carp hardly showed any effects when exposed to this Flemisch standard. However, when exposed to the 10% of their LC50 96h value, it appears that they need to use their abillity to depress their metabolism in order to survive the copper toxicity. Furthermore, they (and common carp as well) are not able to regain their normal ion homeostasis within one month and are as follows, more sensitive to long term impact of Cu exposure.

3.6 Acknowledgements.

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Chapter 4

Exposure to waterborne copper reveals differences in oxidative stress response in three freshwater fish species

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4.1 Abstract.

Among species, various strategies in metal handling can occur. Moreover, the same metal concentration, or even the same metal dose, does not always seem to exert the same effect in different species. Here, we have investigated differences in a copper induced oxidative stress response between rainbow trout (Oncorhynchus mykiss), common carp (Cyprinus carpio) and gibel carp (Carassius auratus gibelio). Fish were exposed to two sub-lethal Cu concentrations, being an identical concentration of 50µg/l for all fish species and an identical toxic dose which was 10% of the concentration lethal to 50% of the fish within 96 hours of exposure (LC50 96h value) for each of the 3 species (20µg/l for rainbow trout, 65µg/l for carp and 150µg/l for gibel carp). Different anti-oxidative enzymes (superoxide dismutase, glutathione reductase and catalase) and anti-oxidants (reduced glutathione and reduced ascorbate) were determined in gill samples collected after 1h, 12h, 24h, 3days, 1 week and 1 month of Cu exposure. Changes in the measured parameters were present in all 3 species, yet a clear differentiation between fish species could be made before and during the exposure. Gibel carp showed to have ascorbate levels that were twice as high as in common carp or rainbow trout. In contrast, rainbow trout showed to have more than twice the level of glutathione than the two other species, in combination with increased glutathione reductase activities. In rainbow trout a decrease of reduced ascorbate and reduced glutathione was observed in the beginning of the exposure, indicating that ROS scavenging molecules were under pressure; only followed by an increase in the activity of superoxide dismutase after 3 days of exposure. In contrast, common carp and especially gibel carp enhanced their anti-oxidant enzymes activities as quickly as in the first day of exposure. Furthermore, our research seems to confirm that some fish rely more on glutathione as a first line of defence against metal exposure, while others rely more on metallothionein in combination with anti-oxidant enzymes.

Keywords: anti-oxidants; carp; catalase; glutathione reductase; rainbow trout; superoxide dismutase

4.2 Introduction.

In recent years, there has been an ever increasing emphasis on metal contamination in freshwater ecosystems. The biological impact, so it seems, is not solely dependent on the concentration of a contaminant, but also water chemistry and interactions with co-pollutants do exert important influences on uptake, metabolism and toxicity (Glover and Hogstrand, 2003; Pagenkopf, 1983). Species specific sensitivity to a pollutant is especially of interest, since it is a crucial factor determining toxicity as well. Several studies on freshwater organisms were able to document some of these species specific sensitivities to a number of pollutants (Ahmad et al., 2005; Croke and McDonald, 2002; Poléo et al., 1995; Poléo et al., 1997; Pyle and Wood, 2008; Schjolden et al., 2007; Taylor et al., 2003). In the case of copper (Cu) for instance, yellow perch seemed less sensitive to waterborne Cu exposure than rainbow trout (Taylor et al., 2003; Pyle and Wood, 2008) and gibel carp (*Carassius auratus gibelio*) appeared considerably less sensitive to aqueous Cu than common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (De Boeck et al., 2004).

Cu is one of the most abundant transition metals in nature. Although this metal is an essential nutrient and has numerous functions in cellular biochemistry (such as a cofactor for many different enzymes) (Burke and Handy, 2005), it may become inhibitory and ultimately toxic at higher concentrations (De Boeck et al., 2004; Deshmukh and Marathe, 1980; Kaùr and Dhawan, 1994). The mechanism of Cu toxicity in freshwater fish involves ionoregulatory disturbances at the gills, with a rapid Cu accumulation in this organ followed by accumulation in other organs, such as liver, kidney and muscle (Grosell et al., 1997; Grosell et al., 1998b; Grosell and Wood, 2002; Laurén and McDonald, 1985; Laurén and McDonald, 1987). The gills are in continuous contact with the external environment and are thus a primary target for waterborne pollutants such as Cu (Dang et al., 2000a; Pandey et al., 2008; Perry and Laurent, 1993). This can lead to a number of adverse effects in the gill tissue, such as, in case of Cu exposure, disruption of the active uptake mechanisms for Na^+ and Cl^- (mainly through reduction of Na^+/K^+-ATPase activity), an increase of gill permeability and oxidative stress. Ultimately, the resulting net ion loss can cause death by cardiac arrest (De Boeck et al., 2001; Grosell et al., 2002; Laurén and McDonald, 1985; Matsuo et al., 2004; Wilson and Taylor, 1993).

Cu can affect anti-oxidants in tissues as they induce redox cycles through Fenton reactions, causing severe peroxidative damage to cell membranes, DNA and other macromolecules (Atli

et al., 2006; Craig et al., 2007; Pandey et al., 2008). Furthermore, the positive Cu^{2+} ion can act directly, through binding to negatively charged protein -SH groups and denaturation of enzymes, or indirectly via generation of reactive oxygen species (ROS) (such as hydrogen peroxide, superoxide radicals, hydroxyl radicals), resulting in oxidative stress (Ahmad et al., 2005; Bopp et al., 2008). This oxidative stress is an imbalance between oxidative and reductive processes in the cell (Almroth et al., 2008) and can be induced by an increase in ROS, an impairment of anti-oxidant defence systems, or an incapacity to repair oxidative damage (Dorval and Hontela, 2003). ROS are also naturally formed during normal metabolic processes and therefore, molecular (glutathione (GSH), ascorbate (Asc)) and enzymatic defences (various anti-oxidant enzymes) are present preventing ROS from causing oxidative cellular damage.

The enzyme superoxide dismutase (SOD) forms the first line of defence against oxidative stress by scavenging the superoxide anion, whereas catalase (CAT) eliminates the hydrogen peroxide originated from the former reaction, (Ates et al., 2008; Atli and Canli, 2007; Elia et al., 2006; Ken et al., 2003) by converting it into water en oxygen (Dorval et al., 2003; Lloyd and Philips, 1999; Lui et al., 2006). SOD and CAT deal directly with radical species whereas other enzymes, including glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH), contribute to the renewal of the reductive capacity of a cell (Bagnyukova et al., 2005; Bagnyukova et al., 2006). A simultaneous induction of SOD and CAT activity has been observed in several fresh- and saltwater teleosts when exposed to oxidative stress (Fernandes et al., 2008; Livingstone, 2001; Pandey et al., 2003). This induction is a common mechanism of the adaptive oxidative stress response in fish that can vary among tissues (Stephensen et al., 2002). Consequently, these anti-oxidant enzymes contribute to the maintenance of a relatively low level of ROS in cells (Hidalgo et al., 2002).

The intracellular fate of both essential and non essential metal ions strongly depends on thiol containing molecules, particularly GSH and metallothioneins (MT) (Lange et al., 2002). GSH, the most abundant cellular thiol, is one of the most prevalent anti-oxidants and is converted into oxidized glutathione (GSSG). The balance in favour of the latter is an indication of oxidative stress (Arabi and Alaeddini, 2005; Arabi, 2004; Remião et al., 2000). Cellular glutathione homeostasis depends on a complex process of precursor amino acid uptake, synthetic enzymatic capacity and redox cycling of GSSG to GSH by a NADPH dependent reaction catalyzed by glutathione reductase (Kaplowitz et al., 1985; Kuhn et al., 2000; Saeij et

al., 2003). GSH rapidly traps Cu by forming a stable GS-Cu(I) complex (Canesi et al., 1999; Gravato et al., 2006, Sanchez et al., 2005) but also plays a role in the regeneration of other anti-oxidants, such as ascorbic acid (Kand'ár et al., 2006). Ascorbate (Asc), an ion of ascorbic acid, is an important cellular metabolite with various described functions, its potent anti-oxidant activity amongst one of them (Arrigoni and De Tullio, 2002; Borsook and Keighley, 1993; Deutsch, 1998; Fraga et al., 1991; Parvez et al., 2003; Parvez and Raisuddin, 2006).

A well performing anti-oxidant defence system is essential for any organism to protect itself against the previously mentioned adverse effects of Cu exposure. Therefore we studied SOD, CAT and GR activities together with reduced GSH and Asc content in gill tissue of Cu exposed rainbow trout, common carp en gibel carp. These fish species showed a different response toward Cu exposure (De Boeck et al., 2004) and as such can be used to identify underlying mechanisms involved in determining Cu sensitivity. The aim of this study was to assess if differences in anti-oxidant defences in the gills of these fish contributed to the differences in sensitivity to Cu exposure.

4.3 Materials and methods.

4.3.1 Animal maintenance and copper exposure.

Rainbow trout (*O. mykiss*), common carp (*C. carpio*) and gibel carp (*C. auratus gibelio*) were obtained from a commercial fish farm (Luc & Patrick Bijnens, Zonhoven, Belgium). Average size of rainbow trout was 296 ± 46 g, of common carp 231 ± 85 g and of gibel carp 224 ± 96 g. A month before starting the experiments, 18 groups of 8 fish per species were allowed to acclimate to lab conditions in 200 l tanks with filtered tap water. As controls, 6 groups of 8 fish (of each fish species) (N=48 controls for each fish species) were used and each copper exposure consisted out of 6 groups of 8 fish (of each fish species) as well (N= 48 exposed to 50 µg/l Cu of each fish species and N=48 exposed to 10%LC50 96h value of each fish species). Water temperature was kept at 17 ± 1 °C in a flow through system that renewed total water volume of the aquaria each 7 hours. Additional open trickling filters, consisting of filter wadding, activated charcoal and lava stones were used in order to maintain an overall good water quality. Fish were fed *at libitum* once a day in their experimental tanks. This could possibly lead to an additional Cu exposure through the digestive tract, especially for the groups who were exposed for one month. Nonetheless, the same food was used for controls and exposed fish and it was always consumed rapidly (within 5 minutes) so that soaking of

the food by the exposure water was limited. Water quality was checked every day before and during the exposure: oxygen concentration stayed well above 90% saturation, hardness was 250 mg ± 11 $CaCO_3$/l and pH 7.6± 0.2. Ammonia, nitrate and nitrite levels were kept below toxic concentrations (<0.1 mg/l).

Before exposure, lava stones and charcoal were removed from the filter to prevent Cu adsorption. Cu was added manually at the start of the experiment as a copper nitrate solution ($Cu(NO_3)_2 \cdot 2H_2O$, Merck, Darmstadt, Germany) to the exposure aquaria. The desired concentration was maintained by using a peristaltic pomp (Watson Marlow 505 S) connected to a stock solution of Cu, for the duration of the entire experiment. Cu concentrations were measured using atomic absorption spectroscopy. Each fish species was exposed to the Flemish standard of surface water (50 µg/l) and to 10% of the 96hLC50 value (20 µg/l for rainbow trout, 65 µg/l for common carp and 150 µg/l for gibel carp). The latter concentrations were based on experiments conducted by De Boeck et al., (2004). Both experiments (this study and the study of De Boeck et al., 2004) were set in the same experimental test conditions and water quality using the same fish species which increased reliability and reduced the number of experimental fish needed for our experiment. With the exposure of 10% of the 96hLC50 value of each fish species, we can create a similar toxic load for our test species and investigate the occurring differences in effects.

4.3.2 Sampling procedures.

Eight fish (in 1 tank) of each fish species and each condition (control group, exposure to 50 µg/l Cu group, exposure to 10%LC50 96h Cu group) were sampled after 1h, 12h, 24h, 3days, 1 week and 1 month. Fish were quickly netted, anaesthetized in a buffered MS222 solution (100 mg/l Sigma Chemical, St. Louis, MO, USA) weighted and measured. Gill tissue was dissected on ice, rinsed and flash frozen in liquid nitrogen followed by storage at -80°C for determination of SOD, CAT and GR activity as well as reduced GSH and Asc content.

4.3.3 Enzyme activity measurements in gill tissue.

Frozen gill filament samples were grinded in liquid nitrogen using a mortar and a pestle. The fine powder was then thawed in a 100mM sodium phosphate buffer at pH 7. After a short centrifugation step, enzyme activities were determined in the supernatant. The associated protein content was assayed using the technique of Bradford (1976). All enzymatic assays

were quantified spectrophotometrically, with an Ultra micro plate reader $ELX808_{IU}$ (Bio-Tek instruments Inc, Vermont, USA).

The SOD activity was measured with a commercial colorimetric assay kit (19160 SOD determination kit, Fluka analytical, Sigma-Aldrich, Switzerland) with minor adjustments. The protein amount giving 50% inhibition of maximum colour development contained 1 unit (U) of SOD (McCord and Fridovich, 1969). The results are accordingly given as U SOD/mg protein.

Catalase activity was assayed according to Johansson and Borg (1988). This method utilizes the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colourless to purple. The formaldehyde concentrations of the samples were calculated using the linear regression curve from the formaldehyde standards. The catalase activity derived from these formaldehyde concentrations was calculated in terms of μmol formaldehyde formed/min/mg protein.

The assay used for determination of glutathione reductase was based on the following reaction: ß-NADPH + GSSG is converted into ß-NADP + 2 GSH by glutathione reductase (Mavis and Stellwagen, 1968). Briefly, a mixture of MQ (Millipore, Bedford, MA, USA), 0.1 M phosphate buffer at pH 7.4 with 3.4 mM EDTA, 1 mM GSSG, 0.1 mM ß-NADPH, 1% BSA and a sample was kinetically measured at 340 nm (a continuous spectrophotometric rate determination). Activity was expressed as μmol NADPH/min/mg protein.

All chemicals used in these assays were purchased from Sigma-Aldrich, Missouri, USA.

4.3.4 HPLC measurements of reduced GSH and ASC content in gill tissue.

High-performance liquid chromatography with electrochemical detection was applied to the estimation of GSH and Asc in gill tissue. Simultaneous determination of Asc and GSH was carried out by a Reversed-Phase HPLC of Shimadzu (Hai Zhonglu, Shanghai). Gill tissue was homogenized using a mortar and pestle under liquid nitrogen. The resulting powder was thawed on ice in a 6% meta-phosphoric acid (MPA) solution (0.5 ml MPA/ 100 mg wet tissue). After homogenization, the samples were centrifuged for 12 min at 12000 *g*. 100 μl of

the supernatant was combined with 300 µl eluens (2 mM KCl, adjusted to pH 2.5 with ortho phosphoric acid). Anti-oxidants were separated on HPLC by injecting 10 µL onto a Polaris C18-A column (3 µm particle diameter, 100 mm, 4.6 internal diameter from Varian), with a 1 ml/min flow rate (Shimadzu auto-injector SIL-10ADVP, Shimadzu isocratic pump LC 10-ADVP, 90 bar pressure, *Shimadzu degasser DGU-14A*). The different components were identified and quantified using a home-made amperometric detection system (glassy carbon working electrode, calomel reference electrode, reference potential 500 mV) in series with a diode array detector (SPDM10AVP, Shimadzu, 's Hertogenbosch, Netherlands) as well as connected to a personal computer via an SS420 board (Shimadzu). Chromatogram analysis was performed with the ClassVP software package from Shimadzu.

The concentrations of Asc and GSH were calculated using a standard curve generated by known concentrations of Asc and GSH and expressed in terms of nmol/g fresh weight. The standards were prepared immediately before use (Peak Asc: 242 nm, peak GSH: 196 nm; Retention time of Asc was 1.7-1.8 min and of GSH 2.2-2.3 min).

4.3.5 Statistical analysis.

Data are presented as mean ± S.E.M. All data passed a Kolmogorov-Smirnov test and each compared data set had equal standard deviations (method of Bartlett). Statistical analysis of the differences between control and Cu exposed fish (for each fish species separately) was therefore performed by two- way analysis of variance (ANOVA) followed by Tukey post hoc test using Statistica 5.1 for Windows. Statistical analysis of the differences between controls of rainbow trout, common carp and gibel carp was performed by one-way ANOVA, followed by Tukey post hoc test using Graph pad Intstat. Data were considered significant if $p<0.05$.

In control fish, no significant differences were observed over time except at one time point in gibel carp where control levels of reduced ascorbate were significantly lower after one month. Because of the limited change in controls and for clarity of the graphs, graphs show pooled control data for each species.

Rainbow trout	Condition		Time point		condition*time point	
in gill tissue	F value	P value	F value	P value	F value	P value
SOD	10.937	0.000	6.583	0.000	3.722	0.000
CAT	2,2341	0,1115	5,189	0,0002	1,0451	0,4103
GR	2,3584	0,0992	2,2048	0,0586	3,1611	0,0013
ASC	3,979	0,0214	12,555	0.000	2,663	0,0059
GSH	10,985	0.000	6,53	0.000	2,265	0,019
Common carp	Condition		Time point		condition*time point	
in gill tissue	F value	P value	F value	P value	F value	P value
SOD	6.586	0.0020	10	0.000	5.536	0.000
CAT	3,5708	0,0313	2,8133	0,0195	6,7586	0.000
GR	1,1239	0,3284	5,546	0,0001	5,4441	0.000
ASC	6,175	0,0029	11,269	0.000	4,686	0.000
GSH	6,565	0,002	23,449	0.000	4,728	0.000
Gibel carp	Condition		Time point		condition*time point	
in gill tissue	F value	P value	F value	P value	F value	P value
SOD	58.921	0.000	11.676	0.000	10.653	0.000
CAT	22,598	0.000	6,714	0.000	5,92	0.000
GR	10,532	0,0001	3,831	0,003	10,024	0.000
ASC	6,9611	0.0014	4,2403	0.0014	2,9173	0.0027
GSH	8,4416	0.0004	8,3785	0.000	1,5795	0.1220

Table 3. F values and their significance for all measured parameters, analyzed with two way Anova.

4.4 Results.

Measured total [Cu] in each exposure was on average 98.4 ± 4.7 % of the nominal [Cu] during the entire experiment. No mortality was observed during the 1 month exposure period.

4.4.1 Superoxide dismutase activity in gill.

SOD activity in rainbow trout increased after three days in both the exposure conditions. Exposure to 50 µg/l Cu yielded an increase of 184% and exposure to 20 µg/l Cu an 160% increase of the enzyme activity (Fig.16, table 3) (mean control value: 18.2 ± 0.8 Units SOD/mg protein). The activity of this enzyme was up-regulated (250% increased enzyme activity) after 12h in common carp as well, when exposed to 65 µg/l Cu. In contrast to the rainbow trout and common carp, the gibel carp showed a steeper increase of SOD activity. Exposure to 150 µg/l Cu gave rise to an activation of SOD after 1h (183 % increased enzyme activity) and 12h (380% increased enzyme activity), and again after 1 month of exposure

(224% increased enzyme activity). A significant difference existed ($p<0.05$) between mean control values from common (20.0 ± 1.2 Units SOD/mg protein) and gibel carp (16.5 ± 0.9 Units SOD/mg protein) where the activity of SOD was slightly lower in gibel carp compared to common carp.

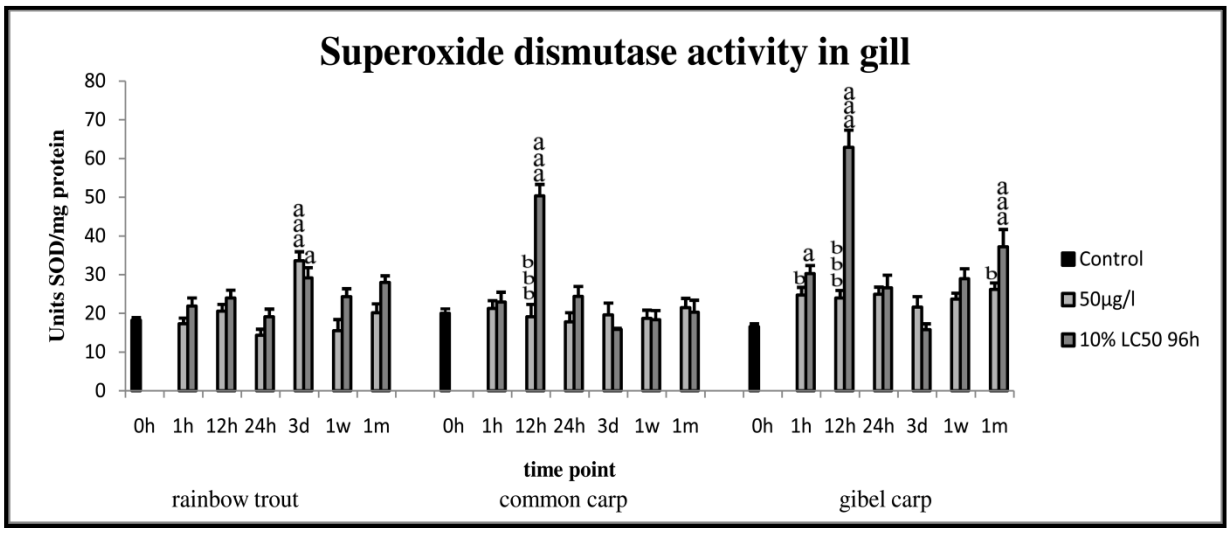

Fig.16. Superoxide dismutase activity (units SOD/ mg protein) in gill tissue of rainbow trout, common carp and gibel carp after 1h, 12h, 24h, 3days, 1week and 1 month of exposure to either control conditions, 50 µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. 'a' indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), 'b' indicates a significant difference between exposed to 50 µg/l and exposed to the 10%LC50 96h value (b: $p<0.05$; bb: $p<0.01$; bbb: $p<0.001$).

4.4.2 Catalase activity in gill.

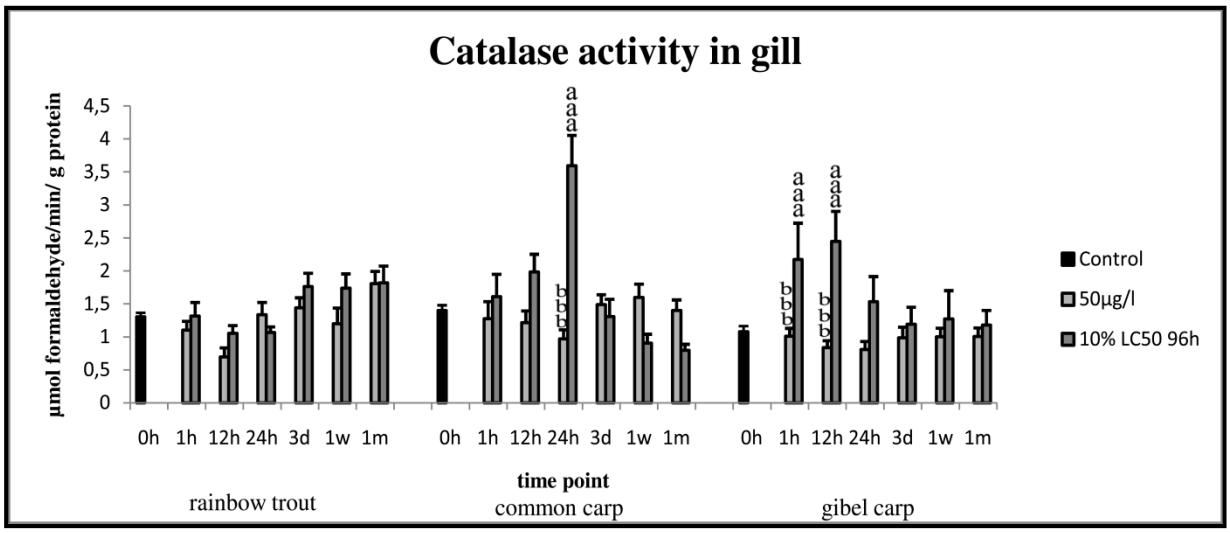

Fig.17. Catalase activity (µmol formaldehyde/min/g protein) in gill tissue of rainbow trout, common carp and gibel carp after 1h, 12h, 24h, 3days, 1week and 1 month of exposure to either control conditions, 50 µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. 'a' indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), 'b' indicates a significant difference between exposed to 50 µg/l and exposed to the 10%LC50 96h value (b: $p<0.05$; bb: $p<0.01$; bbb: $p<0.001$).

Figure 17 (table 3) shows no differences in catalase activity in rainbow trout (mean control value: 1.3 ± 0.1 µmol formaldehyde/min/g protein). In contrast, common carp displayed an

increase of 256% after 24h when exposed to 65 µg/l Cu, and gibel carp after 1h and 12h when exposed to 150 µg/l Cu (200% and 226% increase respectively). As well as in SOD activity, mean control values of gibel carp (1.1 ± 0.1 µmol formaldehyde/min/g protein) were lower compared to common carp (1.4 ± 0.1 µmol formaldehyde/min/g protein) ($p<0.01$).

4.4.3 Glutathione reductase activity in gill.

Glutathione reductase activity (fig. 18, table 3) was not affected by Cu exposure in rainbow trout and common carp. However, in gibel carp, a 2 fold enhanced activity was seen after 12h of exposure to 150 µg/l Cu. Mean control values differed between the three fish species studied ($p< 0.001$). Rainbow trout showed a higher GR activity in control conditions (3.1 ± 0.1 µmol NADPH/min/mg protein) compared to common carp (1.4 ± 0.1 µmol NADPH/min/mg protein) and gibel carp (1.9 ± 0.1 µmol NADPH/min/mg protein). The latter being significantly higher ($p<0.001$) compared to common carp as well.

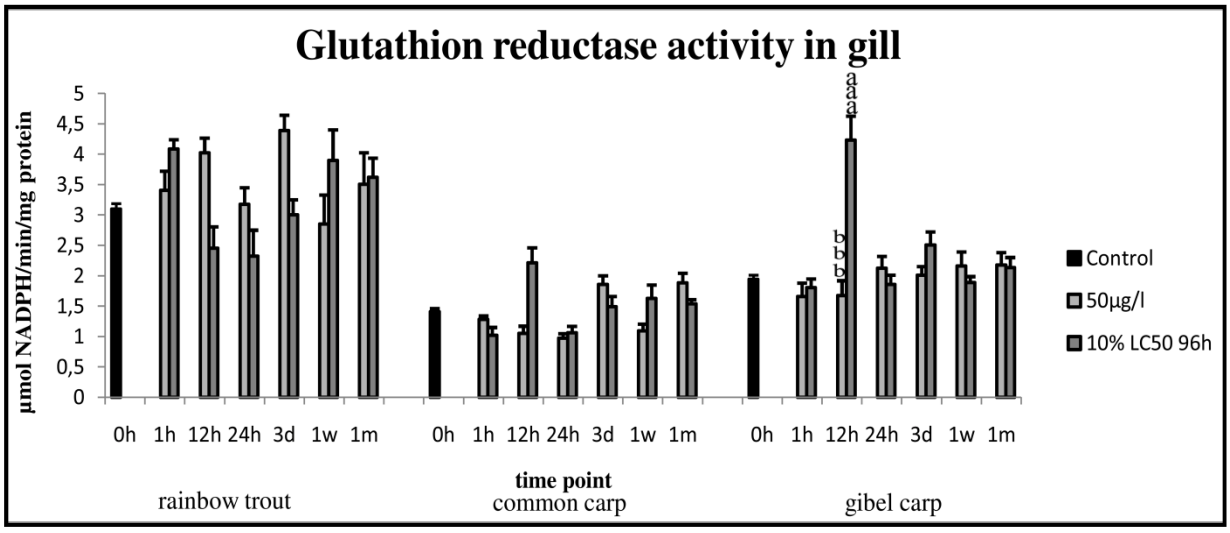

Fig.18. Glutathione reductase activity (µmol NADPH/min/mg protein) in gill tissue of rainbow trout, common carp and gibel carp after 1h, 12h, 24h, 3days, 1week and 1 month of exposure to either control conditions, 50 µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. 'a' indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), 'b' indicates a significant difference between exposed to 50 µg/l and exposed to the 10%LC50 96h value (b: $p<0.05$; bb: $p<0.01$; bbb: $p<0.001$).

4.4.4 Reduced ascorbate and reduced glutathione concentration in gill.

In rainbow trout, reduced [Asc] decreased after 24h of exposure to 50 µg/l (Fig. 19, table 3) with 35%. Exposure of common carp to 50 µg/l Cu also induced an initial decrease after 1 hour of exposure (47%), with a recovery thereafter followed by an increase of [Asc] at the end of the exposure (143%). In contrast, when exposed to 65 µg/l Cu, common carp reacted with an increase in reduced [Asc] after 24h (133%). Gibel carp appeared to have a higher reduced

[Asc], however, no significant increase or decrease occurred when compared with the control concentrations. Important to mention is that there was a decrease in control values of [Asc] after 1 month of exposure, and due to this fact, the difference between controls and exposed fish was significant at this time point only. Strikingly, reduced [Asc] concentrations in gibel carp (mean control values 913.2 ± 223.0 nmol/g fresh weight) are more than 2 times higher ($p<0.001$) compared to common carp and rainbow trout mean control values (resp. 309.1 ± 78.6 nmol/g fresh weight and 375.0 ± 135.6 nmol/g fresh weight).

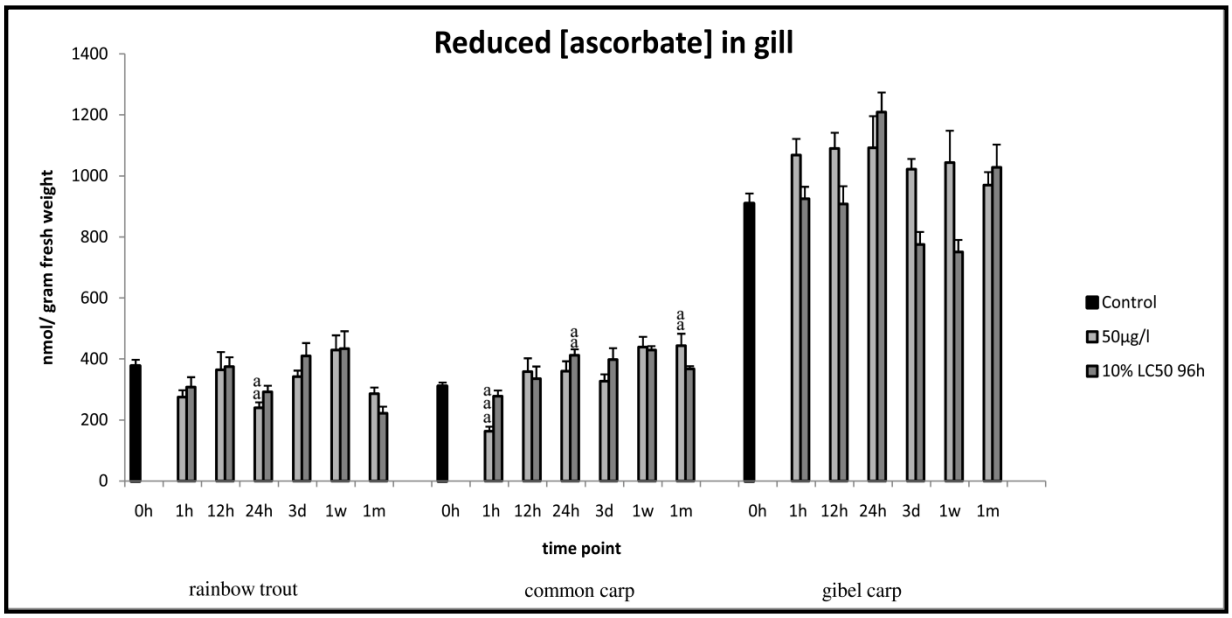

Fig.19. Concentration of reduced ascorbate (nmol/g fresh weight) in gill tissue of rainbow trout, common carp and gibel carp after 1h, 12h, 24h, 3days, 1week and 1 month of exposure to either control conditions, 50 µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. 'a' indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), 'b' indicates a significant difference between exposed to 50 µg/l and exposed to the 10%LC50 96h value (b: $p<0.05$; bb: $p<0.01$; bbb: $p<0.001$).

Similar to the reduced [Asc] in rainbow trout, there was a decrease (22%) in reduced [GSH] in gill tissue after 24h of exposure to 50 µg/l Cu (Fig. 20, table 3). In common carp, such a decrease in reduced [GSH] occurred after a 1h exposure to 50 µg/l Cu (57%) and 65 µg/l Cu (39%). In contrast, gibel carp showed no decrease in activity in reduced [GSH] when exposed to 50 µg/l or 150 µg/l Cu, instead, an increase was seen after 12h when exposed to 50 µg/l Cu (130%) and after 24h when exposed to 150 µg/l Cu (140%). Remarkably, mean control values for common carp (624.7 ± 115.8 nmol/g fresh weight) and gibel carp (459.9 ± 104.8 nmol/g fresh weight) are significantly ($p<0.001$) lower compared to rainbow trout mean control values (1250.4 ± 271.0 nmol/g fresh weight).

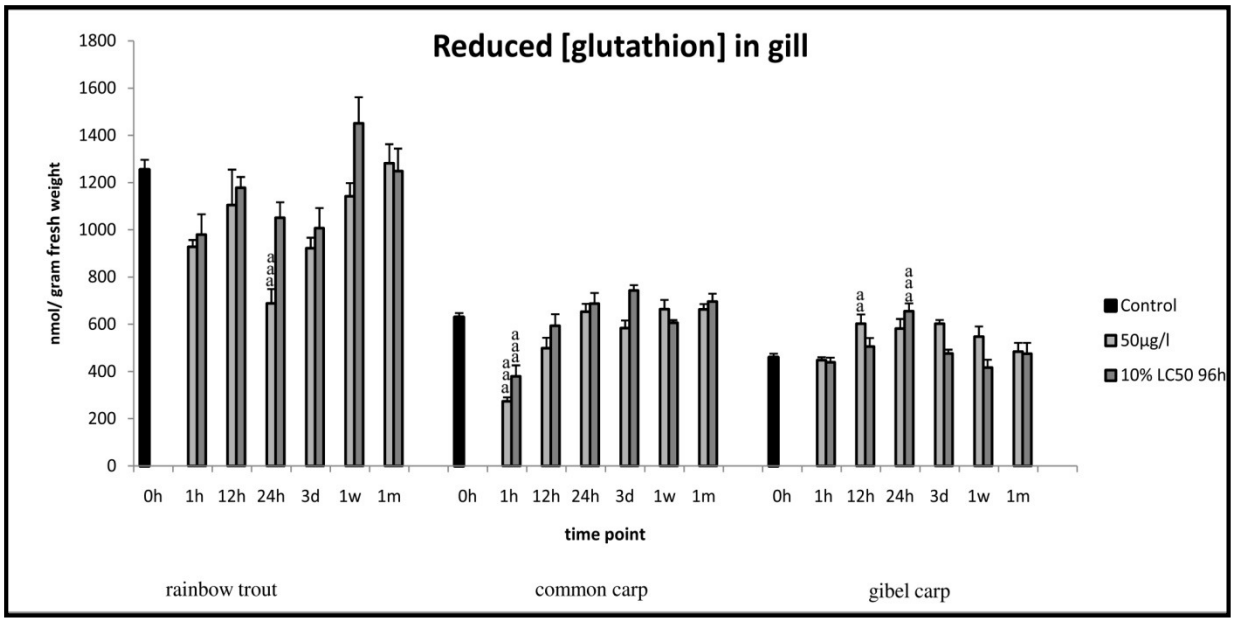

Fig.20. Concentration of reduced glutathione (nmol/g fresh weight) in gill tissue of rainbow trout, common carp and gibel carp after 1h, 12h, 24h, 3days, 1week and 1 month of exposure to either control conditions, 50 µg/l Cu or 10% of the respective Cu 96h LC50 value for that species. 'a' indicates a significant difference between exposed (N=8) and control fishes (N=48) (a: $p<0.05$; aa: $p<0.01$; aaa: $p<0.001$), 'b' indicates a significant difference between exposed to 50 µg/l and exposed to the 10%LC50 96h value (b: $p<0.05$; bb: $p<0.01$; bbb: $p<0.001$).

4.5 Discussion.

From our results, it is obvious that anti-oxidant defence mechanisms were activated during Cu exposure. The metal toxicity observed here can therefore partly be explained through oxidative stress (Ates et al., 2008). But even without Cu exposure, some striking interspecies differences became apparent. First, gibel carp have more than twice the level of reduced [Asc] in their gills compared to the other species, and secondly, the same is true for reduced [GSH] in rainbow trout, with matching higher glutathione reductase activities as well.

When considering the assumption that an increased Asc pool may be an adaptive response to limit the oxidative damage in gills (Jena et al., 2009), the high levels of reduced [Asc] can mean an advantage for the gibel carp when opposed to oxidative stress. Most likely this is related to their capacity to survive anoxia which is similar to that of crucian carp, *Carassius carassius*, a close relative of the gibel carp. Anoxic events, and especially re-oxygenation afterwards, are known to induce ROS (Lutz et al., 2003), and thus organisms that survive anoxia should posses well developed anti-oxidant defences. It is known that crucian carp can survive up to 100 days of anoxia at 5°C with a depressed metabolism and use of their large glycogen reserves in liver as part of their survival strategy (Lutz and Nilsson, 1997; Van den

Thillart and Van Waarde, 1985). Studies of the response of the anti-oxidant mechanisms during anoxia in goldfish, in goby and pacu, revealed species and tissue dependant reactions of the studied parameters (Lushchak et al ., 2001; Lushchak and Bagnyukova, 2007; Sampaio et al., 2008) Freshwater turtles that are hypoxia tolerant show remarkably high levels of ascorbate in their brains, and these levels remain surprisingly constant when challenged with ROS (Rice and Cammack, 1991; Rice et al., 1995). Possibly due to the large buffer capacity of the high [Asc] levels, gibel carp's reaction to the opposed metal stress was somewhat different compared to the other investigated species. There was a short increase in reduced [GSH] rather than a decrease at the beginning of both exposures, and there was no significant effect on the reduced [Asc]. The small increase in gill [Asc] when exposed to 150 µg/l Cu appeared to be a decrease in control [Asc] instead of an actual increase in reduced [Asc]. Ascorbate can act as a pro-oxidant as well by it's interaction with free catalytically active metal ions which could lead to production of hydroxyl and alkoxyl radicals in vitro (Carr and Frei, 1999). Suh et al. (2003) nonetheless discovered that ascorbate acted as an anti-oxidant by preventing lipid peroxidation in human plasma in presence of Cu and Fe. The concentration of ascorbate is, according to Xie et al. (2007), one of the important factors which determine the pro- or anti-oxidant capacity. Further investigations would be helpful to determine the anti-oxidant capacities of ascorbate during metal exposure.

What is true for gibel carp and its high reduced [Asc] concentrations in gill tissue, also appears genuine for rainbow trout and its high reduced [GSH] in gill tissue. This pool of GSH is almost 2 times higher in rainbow trout compared to the carp species studied. Concomitant, also GR levels are higher. GSH might be the defence molecule of choice in fish gills (Hansen et al., 2006a). This thiol containing molecule is an important Cu carrier although this only plays a small role in GSH's function since it is the most abundant cellular thiol, protecting cells against the toxic effects of ROS (Hoyle et al., 2007). Just like the anti-oxidant enzymes, cellular GSH levels have been reported to decrease, increase or remain unchanged after metal exposure (Berntssen et al., 2000; Canesi et al., 1998; Canesi et al., 1999; Lange et al., 2002; Parvez and Raisuddin, 2006).

Despite the high concentrations of reduced GSH in gill tissue, rainbow trout exposed to 50 µg/l Cu, showed a drop in reduced [Asc] and [GSH] after 24h indicating an elevated use of these molecules. In common carp, a similar decrease in [GSH] appeared as quickly as 1h after exposure, and a drop in reduced [Asc] was also seen at this time. As common carp gill tissues

accumulated Cu much quicker than rainbow trout upon exposure (De Boeck et al., 2003), the depletion of GSH after 1 hour in both exposure conditions might be caused by the elevated binding of GSH with Cu. A depletion of GSH content reduces the cellular availability to scavenge free radicals and can lead to more oxidative stress related damage (Elia et al., 2003). This depletion can be a result of an increased binding of Cu (stabilization of Cu in oxidative state), an enhanced use of GSH's oxidizing ability (conversion into GSSG, the oxidized form of glutathione) or an ineffective GSH regeneration (Ahmad et al., 2005; Pandey et al., 2001; Parvez et al., 2003; Parvez and Raisuddin, 2006). It is actually very interesting that several researchers suggested that GSH is very important in handling metal stress as a first line of defence, since some fish species, such as Cu exposed trout, do not seem to rely on metallothionein (MT) production to handle Cu toxicity in gills (Ahmad et al., 2000; Hansen et al., 2006b; Lange et al., 2002). The high reduced [GSH] in rainbow trout gill tissue compared to common and gibel carp, can be indicative for its intensive use in metal binding, besides its function in counteracting the effects of oxidative stress. Metallothioneins are involved in metal regulation and detoxification tasks since they buffer changes in free metal ion levels in the cell (De Boeck et al., 2003; George and Olsson, 1994; Muto et al., 1999). Previous research by De Boeck et al. (2003) showed a good correlation between gill MT induction and Cu accumulation in gills of gibel carp. However no MT induction in gills of rainbow trout and common carp were found. In the latter two species, GSH becomes therefore even more important in addressing the Cu toxicity in gill cells. So it seems that some fish species rely on GSH, while others rely more on MT's, as a first line of defence against metal exposure.

Critical in considering GSH, is the enzyme GR. This enzyme is responsible for maintaining the GSSG/GSH ratio (Almroth et al., 2008) and when activity enhances, more GSSG is converted in GSH, hence increasing the free radical scavenging ability of a cell. Interestingly, the reduced [GSH] in gill tissue (after 1h of exposure to 65 µg/l Cu) in common carp seemed to elicited a tendency to enhance GR activity after 12h of exposure. In gibel carp, the rise in GR activity after 12h of exposure to 150 µg/l Cu was clearly significant despite the fact that no differences were seen in reduced [GSH]. This could indicate that any increased use of reduced [GSH] in gibel carp was counteracted efficiently with an increased activity of GR, or perhaps that a brief reduction in [GSH] occurred at a not investigated point during the exposure, and was counteracted by the next sampling point. In rainbow trout, however, no effect on GR occurred. As mentioned before, reduced [GSH] and GR activity were

remarkably higher in rainbow trout controls compared to common and gibel carp, giving rainbow trout more scope to buffer changes in the GSH status.

When we consider the other anti-oxidant enzymes studied, rainbow trout responded with an enhanced activity of SOD after 3 days of exposure. From 1 week until one month of exposure, all anti-oxidant enzymes in gill tissue returned to normal levels. Looking at the graph of CAT activity, there appeared to be a decrease of CAT activity after 12 hours of exposure, however this is only a trend. Such decrease in CAT activity in rainbow trout gill tissue could have been caused by a direct binding of Cu to –SH groups on this enzyme or by the elevated ROS concentrations due to Fenton like reactions of Cu (Ates et al., 2008; Vutukuru et al., 2006). Reaction of some lipid peroxidation products with amino acid residues of the enzyme can change its protein function causing a decreased enzyme activity as well (Bagnyukova et al., 2006; Stadtman and Levine, 2000). Additionally, carbonylation of proteins can also result in loss of enzyme activity (Stadtman et al., 1991; Starke et al., 1987). Either one of these would reduce the capacity of rainbow trout to cope with oxidative stress. A less severe exposure of 20 µg/l Cu induced no effects in the activities of the anti-oxidant enzymes studied. Intriguingly, a study has shown that rainbow trout accumulated more Cu in liver tissue than in their gills, where the accumulation rate is much slower compared to accumulation rates in gill tissue of common and gibel carp (De Boeck et al., 2004), hence rainbow trout might have a reduced need of anti-oxidant responses in gill tissue.
Common carp exposed to 50 µg/l Cu did not seem to suffer much from oxidative stress since no anti-oxidant enzymes were induced or inhibited. However, when common carp were exposed to 65 µg/l Cu, there was an increase in SOD (after 12h of exposure) and CAT (after 24h of exposure) activities. Despite the fact that common carp's SOD and CAT base activities are higher compared to gibel carp, they respond slower to the opposed metal stress.

Cu is not the only transitional metal element that can alter anti-oxidant enzymes activities (Hu, 2000). Iron, cobalt, chromium and vanadate are involved in Fenton (-like) generations of the superoxide and hydroxide radicals, while lead, mercury, cadmium and nickel deplete glutathione storages and bind to sulfhydryl groups of proteins (Stohs and Bagchi, 1995; Valko et al., 2005). Most of these metals are also capable of inducing responses of the anti-oxidant mechanisms (Pandey et al., 2008; Soares et al., 2008; Velma and Tchounwou, 2010). Furthermore, Cu^{2+} and Fe^{2+} are able to unwind the double helix by interacting in between the

bases of the DNA. In contrast, Cr^{3+} can form stable adducts with DNA and Ni^{2+} and Co^{2+} form complexes with phosphate groups on the DNA backbone (Lloyd and Philips, 1999). This interaction between the metal and the DNA, together with the valence of the transition ion, are key components of the formation of oxidative DNA damage and oxidative stress in general (Moriwaki et al., 2008; Valko et al., 2005).

When exposed to Cu, species specific differences in gill damage appeared to have an influence on enzyme activity as well (Fernandes et al., 2008). De Boeck et al. (2007) noted a difference in the pattern of gill damage after exposure to Cu. Little gill damage was seen in rainbow trout, while common carp showed a shock in the first 24h of exposure and repair followed recovery after one week. Gibel carp experienced less gill damage during the first hours to days of the exposure but after three days, loose epithelia occurred and gill damage increased. It seemed that gibel carp embedded secondary lamellae in an interlamellar cell mass possibly as an attempt to reduce ion loss (Sollid et al., 2003) and decrease Cu uptake. This mechanism, the high reduced [Asc] and the enhanced SOD, CAT and GR activities in the beginning of the Cu exposure, suggests that gibel carp reacts the most efficient to the opposed metal stress. Rainbow trout on the other hand do not seem able to readily initiate protective responses in their gill tissue via anti-oxidant enzymes. They might use their high reduced [GSH] as a first defence.

A balance between the activities of all anti-oxidant enzymes and free radical scavenging molecules, such as GSH and Asc, is necessary to achieve optimal protection. A decrease in these parameters suggests a compromised anti-oxidant defence of gills by metal exposure (Pandey et al., 2008). From the studied fish species, gibel carp showed high Asc levels and immediate and clear defence responses of anti-oxidant enzymes in gill, the first target organ during Cu exposure. Common carp responded fast in the beginning of the experiment although to a lesser extent. Their fast response might correlate with the rapid accumulation of Cu observed in a previous study (De Boeck et al., 2003). Rainbow trout activated less of its anti-oxidant mechanisms, and this occurred much slower i.e. after several days of exposure. They possessed high levels of GSH and GR, which offers some capacity for metal binding and defence against ROS, but the lack of response in anti-oxidant responses as well as MT induction (De Boeck et al., 2003) might explain their sensitivity to Cu exposure. Apparently, gibel carp, which showed the most pronounced effects in anti-oxidant enzyme activities and

in MT induction (De Boeck et al., 2003) is the fish species that can withstand the highest Cu exposures.

4.6 Acknowledgments.

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Chapter 5

Comparative proteomics of copper exposure and toxicity in rainbow trout, common carp and gibel carp

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5.1 Abstract.

Species specific differences in transporters, chaperones, metal binding proteins and other targets are important in metal toxicity. Therefore, we have studied the effects of copper exposure on the proteome of gill tissue from *Oncorhynchus mykiss*, *Cyprinus carpio* and *Carassius auratus gibelio*, which have different sensitivities towards copper. Fish were exposed to the Flemish water quality standard for surface waters, being 50µg/l, for 3 days. Sampled gill tissue was subjected to a 2D-Dige and an iTRAQ analysis. While gibel carp showed more positive responses such as increased apolipoprotein A-I, transferrin and heat shock protein 70, common carp's gill tissue on the other hand displayed a changed actin cytoskeleton, and indications of a changed metabolism. These last two traits were evident in rainbow trout as well, together with decreased expressions of transferrin and albumin. Furthermore, the Weighted Gene Co-Expression Network Analysis of rainbow trout's data revealed a network of 98 proteins related to Cu accumulation in gill, of which the occurrence of proteins related to oxidative stress, such as, superoxide dismutase and cytochrome c, were promising. Additionally, the outcome of the different proteomics techniques demonstrates the usefulness of iTRAQ analysis compared to 2D-Dige.

Keywords: 2D-Dige ; carp; copper ; gill ; iTRAQ ; oxidative stress ; proteome profiling; rainbow trout

5.2 Introduction.

Understanding the accumulation, handling and toxicity of pollutants such as metals, are key aspects in the study of environmental toxicology. Insights in these mechanisms are helpful to predict risks and effects of pollution. However, species specific differences in those particular processes are making it difficult to generalize sensitivity or resistance to metals. For example, De Boeck et al., (2004) observed differences in copper (Cu) toxicity and Cu bio-accumulation patterns in three freshwater fish species: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio*). Since Cu is an essential element, homeostatic mechanisms are available to regulate its concentration and free Cu in cells is therefore maintained at very low concentrations ($< 10^{-18}$M) (O'Halloran and Culotta, 2000). Intriguingly, this regulation of Cu can be very different among fish species and is one of the most important factors in fish survival when confronted with excess Cu (Taylor et al., 2003). Differences in accumulation and excretion rates, the nature and number of target sites, induction of metallothionein (MT) and induction of anti-oxidant systems may have a strong effect on metal sensitivity, although the relationship between these processes is not always that straightforward. The most pronounced effect of Cu toxicity was, for instance, seen in rainbow trout, despite a low Cu gill accumulation rate and high MT concentration (De Boeck et al., 2003; 2007, Eyckmans et al., 2010, 2011). The gill is one of the first organs in direct contact with the surrounding environment and one of the first organs to accumulate metals (Manzon, 2002). Studying the gill proteome in the three previously mentioned freshwater fish species during Cu exposure, may reveal characteristic metallochaperones and transporters responsible for these distinctions. This could ultimately lead to more insights in the underlying mechanisms of metal toxicity since the abundance or lack of occurrence of different types of metal binding proteins can result in increased resistance or sensitivity. As Eyckmans et al. (unpublished results) showed in their research concerning subcellular distribution of Cu in gill tissue, a Cu exposure of three days demonstrated differences in subcellular handling between the studied fish species. Therefore, the same exposure period was chosen to investigate changes in protein expression in gill.

In this study, we are using a gel based proteome platform, 2D-Dige (2 dimensional differential gel electrophoresis) (Brewis and Brennan, 2010; Gygi et al., 2002), and a gel free one, iTRAQ (isobaric tags for relative and absolute quantification) (Unwin et al., 2010). Two dimensional electrophoresis has been widely used to separate and compare complex protein

mixtures (Patton, 2002). With the introduction of fluorescent dyes to label samples, three samples could be run on a single gel, eliminating the gel-to-gel variation. On the other hand, certain groups of proteins are poorly represented on a 2D gel (membrane proteins, proteins with high molecular weights, hydrophobic membrane proteins,…) (Lilley and Dupree, 2006), making the use of a gel free platform interesting. Because each method has its own unique set of advantages, the combined use of both techniques can aid to accomplish the objectives and detect changes in protein abundance.

5.3 Materials and Methods.

5.3.1 Experimental fish.

The average weight/size of rainbow trout (*O. mykiss*) used in this experiment, was 295.7 ± 9.4 g/ 26.6 ± 0.3 cm, of common carp (*C. carpio*) 234.1 ± 21.7 g/ 18.6 ± 0.7 cm and of gibel carp (*C. auratus gibelio*) 227.3 ± 20.4 g/ 17.6 ± 0.6 cm. These three fish species were obtained from a commercial fish farm (Luc & Patrick Bijnens, Zonhoven, Belgium). All fish species were acclimated for a month to lab conditions in 200 l tanks filled with filtered tap water prior to the experiments. Water temperature was kept at 17 ± 1 °C in a flow through system that replaced total water volume of the aquaria each 7 hours. In addition, open trickling filters, consisting of filter wadding, activated charcoal and lava stones were used in order to maintain an overall good water quality. Fish were fed *at libitum* once a day in their tanks. Water quality was checked every day before and during the exposure, oxygen concentration stayed well above 90% saturation, hardness was 250 mg ± 11 $CaCO_3$/l and pH 7.6 ± 0.2. Ammonia, nitrate and nitrite levels were kept below toxic concentrations (<0.1 mg/l).

5.3.2 Copper exposure and sampling procedures.

Before exposure, lava stones and charcoal were removed from the filter to prevent Cu adsorption. Cu was added manually at the start of the experiment as a copper nitrate solution ($Cu(NO_3)_2 \cdot 2H_2O$, Merck, Darmstadt, Germany) to the exposure aquaria. Fish were exposed to the Flemish standard of surface water (50 µg/l). The desired concentration was maintained by using a peristaltic pomp (Watson Marlow 505 S) connected to a stock solution of Cu, for the duration of the entire experiment. Cu concentrations were measured using graphite furnace atomic absorption spectroscopy (Varian, model Spectra AA-800).

Eight fish of each fish species and each condition (control and exposed) were sampled after 3 days. Fish were quickly netted, anaesthetized in a buffered MS222 solution (100mg/l Sigma Chemical, St. Louis, MO, USA) weighted and measured. Gill tissue was dissected on ice, rinsed and flash frozen in liquid nitrogen upon storage in -80°C for further analysis. To measure accumulated Cu, a fraction was weighed, dried and digested with 69% HNO_3 and, prior to digestion in a technical microwave oven, 30 % of H_2O_2 was added (Merck, Darmstadt, Germany). Cu concentrations were measured using ICP-MS (Varian Ultra Mass 700, Victoria, Australia). Actual recovery rates were 98.2 ± 2.6%. Analytical accuracy was achieved by the use of blanks containing Milli-Q water and solutions used for digestion and certified reference material "number 185R Bovine liver" of the Community Bureau of Reference (European Union, Brussels, Belgium) during all the destruction protocols. Generally, the concentrations of the blanks were below detection limits and recovery rates of the standards were 98.5 ± 4.2%.

5.3.3 Proteomics.

5.3.3.1 Protein extraction

Gill tissue from controls and exposed fish were homogenized in liquid nitrogen. The homogenate was transferred into a bullet tube and homogenized a second time in a small volume of TRIzol® (Invitrogen, Rockville, Maryland, USA. Cat. No. 15596-018) with a hand mixer (Xenox, Art. 68 518). More TRIzol® was added and the homogenate was left for 5 minutes at room temperature. After addition of 200 µl chloroform, a 15 second shake and 3 minutes at room temperature, the homogenate was centrifuged for 15 minutes at 11,000 rpm. This centrifugation step resulted in 3 distinct layers. The upper layer containing RNA was removed. The middle and bottom layer, containing DNA and proteins were submitted to a DNA precipitation using ethanol (VWR international, Radnor, PA, USA). Proteins were left in the supernatant after the DNA precipitation. Proteins were washed with isopropanol and 0.3 M guanidine HCl. The resulting protein pellet was dissolved in a resolubilization buffer (8 M urea, 2 M thiourea and 4 % chaps). For 2D-Dige analysis, protein samples were purified by a clean-up kit (GE Healthcare, Waukesha, WI, USA) and brought to pH 8-9 with 50 mM NaOH. Protein pellets were pooled in groups containing 3, 3 and 2 biological replicates, resulting in 3 replicates of each condition and fish species. Afterwards, subsamples of the

protein samples for both proteomics techniques were used to determine protein concentrations (RC DC protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

5.3.3.2 2D-Dige

Labeling of proteins with Cy Dyes

Cy3 and Cy5 of the Cy Dye Dige fluorochromes (GE Healthcare, Waukesha, WI, USA) were used to minimally label control and exposed gill protein extracts following the manufacturer's recommended protocol. Minimal labeling results in low-stoichiometry labeling of the terminal amine group of lysine side-chains. This ensures that quantification is performed using protein molecules that have been labeled once (Lilley and Dupree, 2006). Both control and exposed samples were labeled with Cy3 as well as Cy5 to exclude variation in labeling efficiency or dye effects. Additionally, an internal standard (for normalization), of a pool of equal amounts of control and exposed samples, was labeled with a third Cy Dye, Cy2. 50 µg proteins were labeled with 200 pmol Cy Dye for 30 minutes on ice, in the dark. The labeling was quenched by addition of 10 mM lysine.

Two-dimensional gel electrophoresis

A control sample, exposed sample and internal standard were combined, so that the final protein concentration prior to iso-electric focusing (IEF) was 150 µg. The volume was adjusted to an end volume of 450 µl with rehydration buffer (8 M urea, 2 M thiourea, 0.2% DTT, 0.5% IPG buffer, 0.5% bromophenol blue (GE Healthcare, Waukesha, WI, USA)) and the combined protein samples were loaded into strip holders (rehydratation loading). IEF was performed on an Ettan IPGphor 3 IEF system (GE Healthcare, Waukesha, WI, USA). Immobiline strips (24 cm, pH 4-7) were placed onto the samples. Afterwards, cover fluid was applied. The electrophoresis conditions were as follows: 12 h at 30 V, a desalting step for 2h at 150V, 500 Volt hours (Vh) at 500 V, 800 h at 1000 V, 16500 Vh at 10000 V and finally at 34700 Vh at 10000 V until 53800 Vh were reached. Focused strips were reduced (10 mg/ml dithiotreitol (DTT)) and alkalized (40 mg/ml iodoacetamide) in equilibration buffer (6 M urea, 30% glycerol and 2% SDS) at room temperature, each step lasting 15 min. Afterwards, equilibrated strips were placed on top of a 12.5 % SDS-PAGE gel (240 x 200 x 1 mm) and overlaid with a 0.5 % agarose sealing solution. For each fish species, gels were used which were prepared at the same time, to minimize intergel variability. The second dimension was performed with an Ettan DALT six electrophoresis unit (GE Healthcare, Waukesha, WI,

USA) in 1% SDS running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS) and started with a prerun at 2.5 W/gel, 600 V, 400 mA for 30 min. The following definite run at 100 watt, 600 V and 400 mA was run until the bromophenol blue front (added to the 0.5% agarose sealing solution) was approximately 5 mm off the bottom of the gel. A Typhoon Variable Mode Imager 9400 (GE Healthcare, Waukesha, WI, USA) was used to scan the gels. Cy3, Cy5 and Cy2 images were scanned at 100µm pixel resolution, using 532, 633 and 488 nm wavelength lasers respectively.

Image analysis

Image analysis was performed using Decodon Delta 2D software (DECODON GmbH, Greifswald, Germany). For each fish species, 3 gels (with a control, exposed and internal standard) were loaded into the program, resulting in 9 images. On these gel images spots were detected and Cy2 was used as internal standard. After the alignment of spot positions (gel warping) a fused image, of the multiple images of the Cy Dyes and the different gels, was formed (Luhn et al., 2003). On this gel, spot detection was performed and as a result, a consensus spot pattern was subsequently transferred to the original images for spot quantitation. Direct comparisons of spot volumes are made between the different Cy Dyes and the internal standard and these ratios are normalized. Using MultiExperiment Viewer (Artistic License v2.0), Student's t-tests were applied to the spot intensities to find proteins that showed significant differences in expression levels, meanwhile, attention was paid to exclude dye specific results (corrected for multiple testing). Typically, univariate statistical analysis has been used in the analysis of data from multi-gel experiments to identify differential expression between sample types by looking for significant changes in spot volume (Lilley and Dupree, 2006).

Mass spectrometry and protein identification

The gels were stained with colloidal Coomassie brilliant blue to facilitate excision of the differentially expressed proteins. Picked protein spots were then washed three times with 95% acetonitrile, one time with 50 mM NH_4HCO_3 and finally once more with 95% acetonitrile to destain and dehydrate the protein spots. When the picked protein spots were dry, they were incubated for 30 min at 4 °C with a 20 µl trypsin solution (25 ng/µl trypsin in 50 mM NH_4HCO_3, 10 % CH_3CN). Afterwards, 10 µl of substitute buffer (50 mM NH_4HCO_3, 10 % CH_3CN) was added, followed by an incubation of 4 hours at 37°C. At the end of this incubation period, supernatant was collected. An efficient peptide extraction was performed

using 30 µl 50 % CH_3CN/ 5% formic acid. Both supernatant and extract were combined and dried in a SpeedVac Concentrator 5301 (Eppendorf, Hamburg, Germany). Each sample was desalted using µ C18 ZipTips (Millipore, Bedford, MA) and spotted on Opti-TOF target plates (dried droplet method). To acquire peptide mass fingerprints (PMF) and peptide fragment fingerprinting (PFF), samples were analyzed using a MALDI-TOF/TOF (4800 Plus MALDI-TOF/TOF, Applied Biosystems, California, USA). Autolytic fragments of trypsin and fibrinopeptide B (*m/z* 1570.677) were used to internally calibrate all PMF spectra. These spectra and peptide sequence spectra of each sample were processed using GPS-Explorer 3.5, (Applied Biosystems, California, USA). The peak lists were searched against the full NCBInr database (NCBInr 20090710 (9283978 sequences; 3180197137 residues, Swiss-Prot) for peptide based homology identification (Mascot 2.1, Matrix Science). A peptide mass tolerance of ± 30 ppm, a fragment mass tolerance of ± 0.2 Da and a maximum of 1 missed cleavage was used together with the Mowse score (Mascot) to positively identify protein homologies. Results with a relative score exceeding 95% probability ($p<0.05$) were retained.

5.3.3.3 iTRAQ

Labeling proteins with iTRAQ reagents

For each fish species, 50 µg of each control and exposed replicate was used for iTRAQ labeling. Lobind Eppendorf tubes were used to ensure high recovery rates of proteins. 20 µl dissolution buffer, 1 µl denaturant and 2 µl of reducing agent (Multiplex buffer kit, Applied Biosystems) were added to each sample. Afterwards, all samples were incubated for 1 hour at 60 °C. Thereafter 1 µl Cysteine blocking reagent (Multiplex buffer kit, Applied Biosystems, California, USA) was added and samples were left at room temperature for 10 min. Prior to addition of 1.5 µg/µl trypsin (Promega, mass spectrometry grade trypsin, 1 µg/µl trypsin stock in 50 mM glacial acetic acid), samples were diluted with dissolution buffer, to minimize confounding effects of urea. Trypsin digestion was carried out overnight at 37°C. Samples were subsequently dried in a SpeedVac Concentrator 5301 (Eppendorf, Hamburg, Germany) to obtain volumes less than 33 µl (necessary to obtain good labeling conditions). The iTRAQ labels were prepared as described in the manufacturer's guidelines (Applied Biosystems, California, USA). Before labeling, the pH of the labels was adjusted to < 7.5 with dissolution buffer, while maintaining a 60 % isopropanol end concentration. Labels 113, 114 and 115 were added to control samples, labels 116, 117 and 118 to exposed samples and label 119 to

an internal standard. The latter consisted of a pool of both control and exposed samples. An incubation of 2 hours at room temperature was followed by an addition of 50 µl MQ to quench the labeling reaction and incubation for 30 min at room temperature. Finally, samples were combined in 1 Lobind Eppendorf tube for each fish species and partially dried using a SpeedVac Concentrator 5301 (Eppendorf, Hamburg, Germany) for further analysis.

Peptide separation

In the first dimension, each of the 3 iTRAQ-labeled samples was separated by strong cation exchange (SCX) using a Waters™ 600S controller, a Waters™ 626 pump and a Waters™ 996 PDA (Waters Corporation, Milford, MA, USA). Samples were loaded onto a polysulfoethyl-aspartamide SCX column with a 2.1mm internal diameter and a length of 200mm, packed with 5µm particles (Poly LC, Columbia, USA). Using 3 different solvents (A: 10mM KH_2PO_4 + 20% ACN at pH 2,7 / B: 10mM KH_2PO_4 + 650mM KCl + 20% ACN at pH 2,7 / D: 10mM KH_2PO_4 + 650mM KCl +20% ACN at pH 5,1) the following gradient was applied at a flow rate of 200µL/min: 100% A for 5 min; 7.5-30% B in 45 min; switch to 30% D; 30-100% D in 15 min; 100% D for 5 min and finally 100% A for 15 min. Fractions were collected from 5 to 80 min with an interval of 5 min. Peptide concentrations of the different fractions were determined based on the area under the curve (AUC). The first and the last fractions were pooled (due to low peptide concentration), resulting in a total of 10 SCX fractions per sample set. Fractions were dried by lyophilization and resuspended in reverse phase mobile phase (3% ACN + 0.1% formic acid) to a final concentration of 10µg/100µL.

In the second dimension, resuspended fractions were separated by reverse phase C_{18} (RP-C_{18}) using an Agilent 1100 micro-capillary HPLC system (Agilent Technologies, Waldbronn, Germany). Of each SCX fraction 10µg (100µL) was loaded on a Zorbax 300SB-C18 guard column (0.3 mm x 5 mm; 5µm particles) serially connected with a Zorbax 300SB-C18 analytical column with a 0,3mm internal diameter and a 150mm length packed with 3,5µm particles (Agilent Technologies). Used solvents were 0.1% formic acid in water (A) and 0.1% formic acid in 90% ACN (B). Columns were equilibrated with 3% solvent B. The following gradient was applied at a flow rate of 6µL/min: 5-55% B in 56.7 min; ramp to 90% over 3.3 min; 90% for 10 min and back to 3% B at the end of the run. From 5 till 51.7 min of the chromatographic run, 350 spots (800 nl/spot) for each fraction were spotted on an Opti-TOF MALDI-target (28 columns x 25 rows; 8 sec interval; 2 runs per target) (Applied Biosystems, Inc.). Afterwards each spot was covered with matrix using an external syringe pump at a flow

rate of 6 µL/min (800 nl/spot). The matrix consisted out of 3 mg/ml α-cyanohydroxy cinnamic acid in 70% ACN; internal calibrant: 63 pmol/ml human fibrinopeptide B.

Mass spectrometry and protein identification

Spots were analyzed using a MALDI-TOF/TOF (AB4800 Proteomics analyzer, Applied Biosystems). MS-analysis (reflectron mode; laser intensity: 3800; 25 x 20 laser shots per spot; mass-range 700-3000Da) was performed first, after which precursors were selected with a signal-to-noise (S/N) ratio above or equal to 50. MS/MS-analysis was performed on these selected precursors. A maximum of 50 unique precursors per spot were selected for fragmentation, starting from the precursors with the lowest S/N-ratio. These precursors were ionized (laser intensity: 4800; 25 x 20 laser shots per spot) and fragmented in a collision cell (1 kV collision) with air. Fibrinopeptide B (m/z 1570,677) was used as internal calibrant to calibrate all MS spectra. All MS/MS spectra of each sample were processed using GPS-Explorer 3.5 (Applied Biosystems) and screened against the full NCBInr database (NCBInr 20090710; 9283978 sequences; 3180197137 residues) using Mascot (Matrix Science, London, UK; version Mascot 2.1.03). Trypsin was selected as digestion enzyme with a maximum of 1 missed cleavage. Further a fragment ion mass tolerance of 0.2 Da and a parent ion tolerance of 200 ppm were selected. As fixed modifications iTRAQ-8plex of lysine and N-terminus and methyl methanethiosulfonate of cysteine were selected, while iTRAQ-8plex of tyrosine and oxidation of methionine were set as variable modifications.

Data analysis and relative quantification was performed using Scaffold Q3+ (version 3.00.03, Proteome Software Inc., Portland, OR). Protein identifications were accepted if they could be established at > 90% probability as specified by the Protein Prophet algorithm. The relative expression of identified proteins was based on the ratio of the reporter ions of the peptides. For each experiment, all values were normalized by using the internal standard (label 119).

5.3.4 Statistical analysis.

For 2D-Dige analysis, Decodon Delta 2D software (DECODON GmbH, Greifswald, Germany). Student's t-tests were used to find proteins that showed significant differences in expression levels, meanwhile, attention was paid to exclude dye specific results (corrected for multiple testing). The process of identification of significant different proteins is explained in 2.3.2.4. For iTRAQ analysis, all MS/MS spectra were processed using MASCOT and Scaffold 3 software (see 2.3.3.3).

5.3.5 WGCNA co-expression networks.

Network approaches provide a means to bridge the gap between individual genes or proteins and complex traits in one organism. The Weighted Gene Co-Expression Network Analysis (WGCNA) (Langfelder and Horvath, 2008) for R calculates proteomics co-expression networks and constructs protein sets from the observed expression data using unsupervised clustering.

WGCNA is used to calculate clusters of highly correlated proteins (called modules), for summarizing such clusters using the module eigengene or an intramodular hub gene, for relating modules to each other and to external sample traits such as the energy budget and metal accumulation data (using eigengene network methodology) and for calculating module membership measures. Protein sets are constructed from the protein expression data by using unsupervised clustering (Langfelder and Horvath, 2008). In case of a significant correlation between a certain module and the exposure concentration, the module was selected as being biologically relevant. Using this approach protein modules were linked to physiological and biochemical traits, revealing both direct interactions within a single module and indirect interactions between modules and physiological or biochemical parameters on different levels of biological organization. WGCNA alleviates the multiple testing problems inherent in large-scale datasets, as found in proteomics data analysis. Instead of relating thousands of proteins to a trait, WGCNA relates only a few modules. Because the modules may correspond to significantly affected processes, focusing the analysis on these modules amounts to a biologically motivated data reduction scheme (Horvath et al., 2006; Langfelder and Horvath, 2008; Presson et al., 2008). A significant correlation between a measured parameter and the protein modules was used to select the top 25% proteins for further analysis, based on the module membership (MM) and the gene significance (GS). The MM of a protein is a measure of the correlation of the protein expression profile with the module eigengene. The GS of a protein is defined as the correlation of the expression profile with an external trait. For a more detailed description of this software package, we refer to Langfelder and Horvath (2008).

5.4 Results and discussion.

The accumulation of Cu in gill tissue after exposure to 50µg/l Cu for 3 days was significantly increased compared to control values in all species. Rainbow trout gill Cu levels increased to 8.34 ± 0.61 µg/g dry weight (control value 5.72 ± 0.48 µg/g dry weight, $p<0.0049$), common carp gill Cu levels to 13.27 ± 1.59 µg/g dry weight (control value 6.83 ± 0.30 µg/g dry weight, $p<0.003$) and gibel carp gill Cu levels to 13.76 ± 0.81 µg/g dry weight (control value 9.52 ± 0.23 µg/g dry weight, $p<0.0001$).

5.4.1 2D-Dige.

5.4.1.1 2DE protein pattern

Fig. 21-23 illustrate typical 2D gels of gills of respectively rainbow trout, common carp and gibel carp. Table 4 presents the identification of the proteins with their significance indicated with the arrows in fig 21-23. The arrows designate the protein spots that were significantly different between controls and exposed and were identified as well.

For rainbow trout, 39 proteins out of 542 protein spots were significantly different between control and exposed gills. Of these 39 proteins, 16 were up regulated of which 1 (spot number 4) was successfully identified, 23 down regulated of which 3 (spot number 1, 2 and 3) were identified. In common carp, 43 proteins of 506 protein spots were significantly different of which 19 proteins showed a higher expression and 14 a lower one. Here, 3 up regulated (spot number 5, 6 and 7) and 7 down regulated (spot number 8, 9, 10, 11, 12, 13 and 14) proteins were successfully identified. Finally, in gibel carp, 40 proteins of 482 protein spots differed significantly between controls and exposed gills. 18 proteins of these 40 were down regulated and 22 of these proteins were up regulated. All 4 identified proteins of gibel carp's gill tissue were up regulated (spot number 15, 16, 17 and 18). In all gels, a comparable number of proteins were detected and similar numbers of these proteins were considered significant in the three fish species. However, the successful identification of the proteins was restrained, since rainbow trout, common and gibel carp are at present not fully annotated. The difficulty in discussing the results of the proteomic changes of three different fish species lies in the limited and different number of identified proteins for each fish species. At this moment, 5173 proteins of *Oncorhynchus* are identified, from which 303 are reviewed (UniProtKB/Swiss-Prot) and 4870 (UniProtKB/TrEMBL) are not. Common carp and gibel carp have even less

proteins identified (resp. for *Cyprinus* 109 are reviewed (UniProtKB/Swiss-Prot) and 1500 (UniProtKB/TrEMBL) are not and for *Carassius* 101 are reviewed (UniProtKB/Swiss-Prot) and 1307 (UniProtKB/TrEMBL) are not.

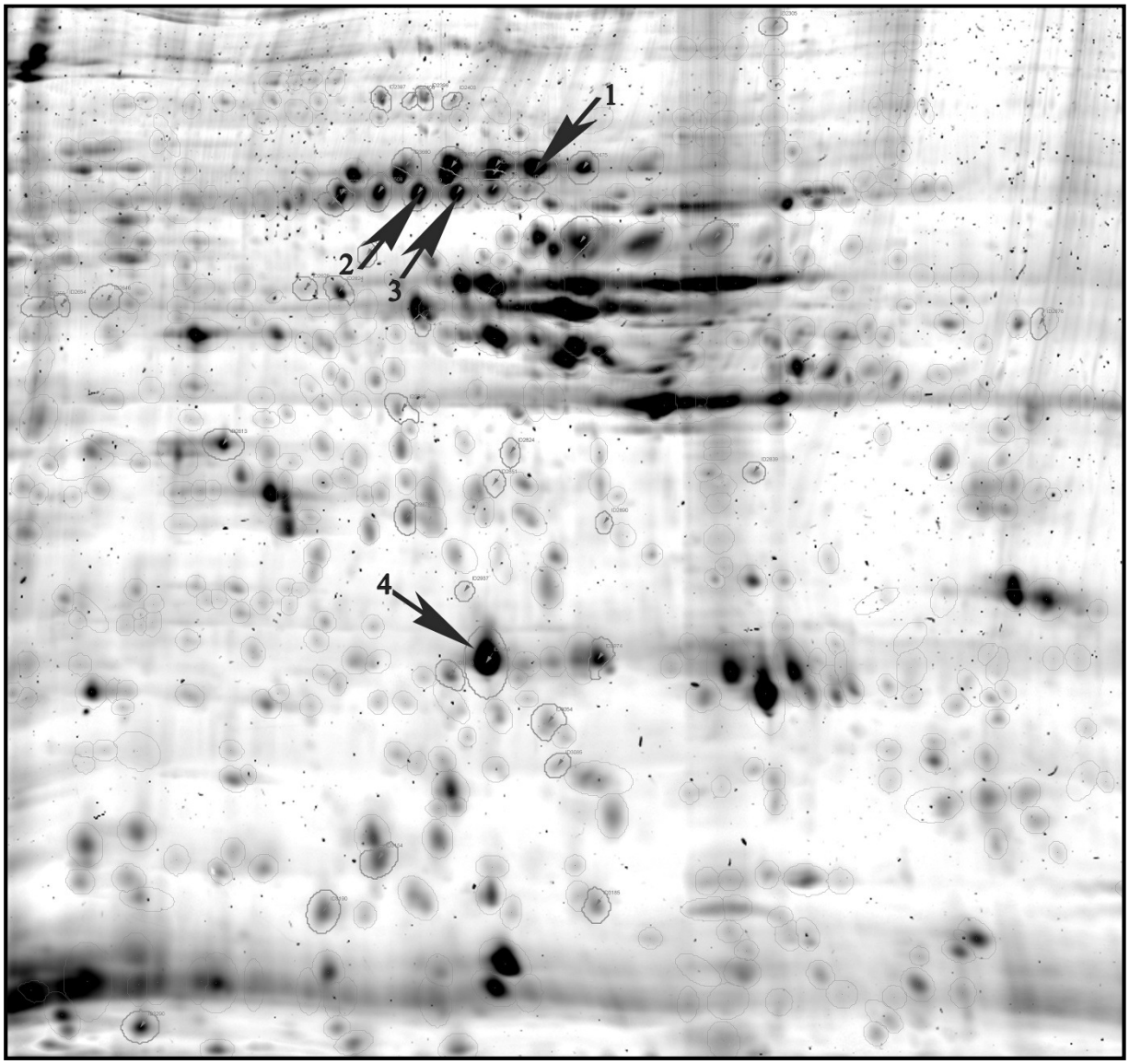

Fig. 21. 2D-Dige gel of rainbow trout. 542 spots were detected of which 39 proteins were significantly ($p<0.05$) different between control and exposed gills. Of these 39 proteins, 4 spots could be identified and are indicated by arrows 1(transferrin), 2 & 3 (serum albumin) and 4 (carbonic anhydrase).

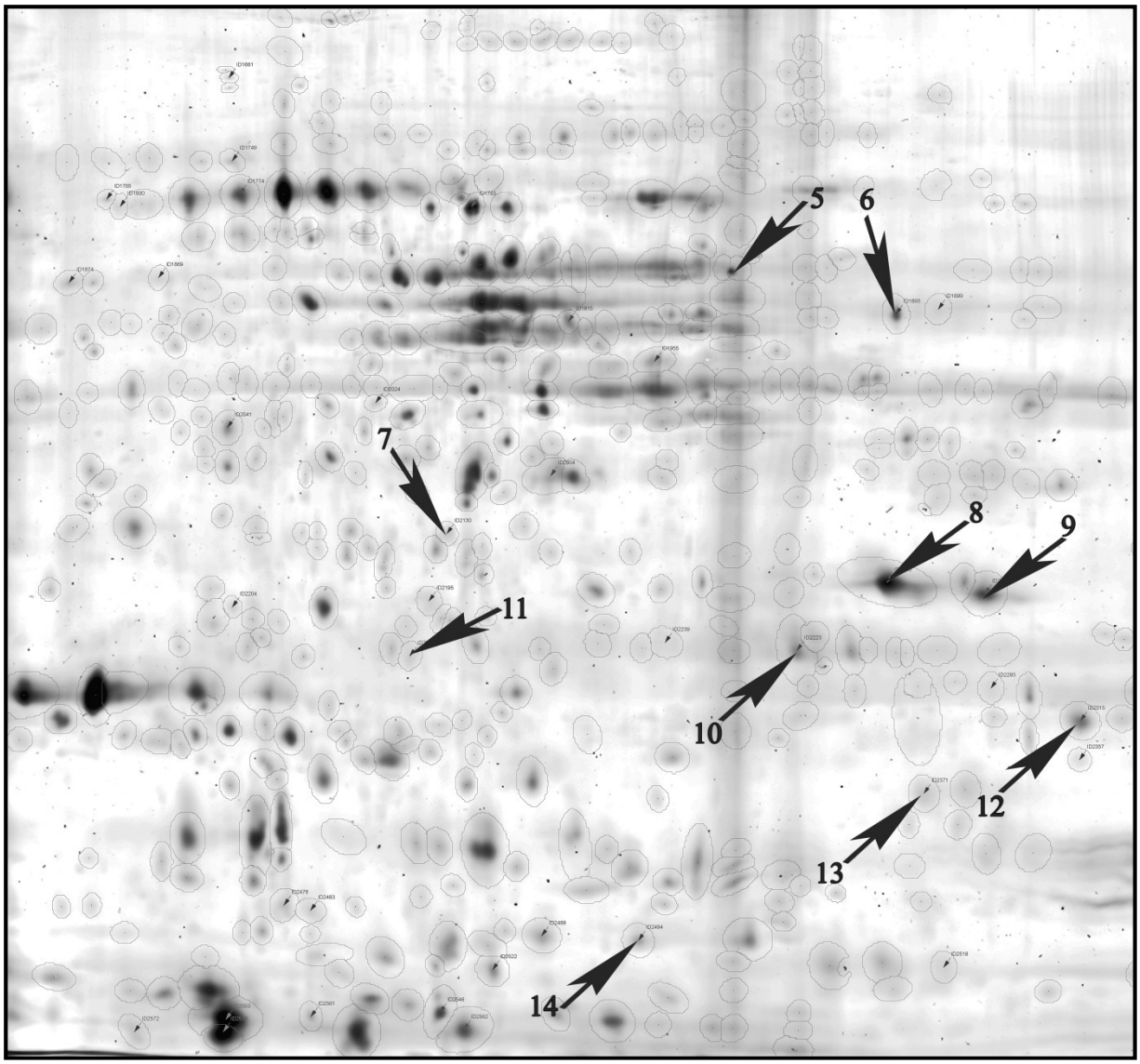

Fig. 22. 2D-Dige gel of common carp. 506 spots were detected of which 43 proteins were significantly ($p<0.05$) different between control and exposed gills. Of these 43 proteins, 10 spots could be identified and are indicated by arrows 5 (keratin 8), 6 (mitochondrial ATPsyntase beta subunit), 7 (F-actin-capping protein subunit beta), 8 (tropomyosin 3 isoform 2), 9 (tropomyosin alpha 4-chain), 10 (Rho-GDP dissociation inhibitor 2), 11 (proteasome subunit alpha type 6), 12 (translationally controlled tumor protein), 13 (myosin regulatory light chain, smooth muscle isoform) and 14 (type II keratin subunit protein).

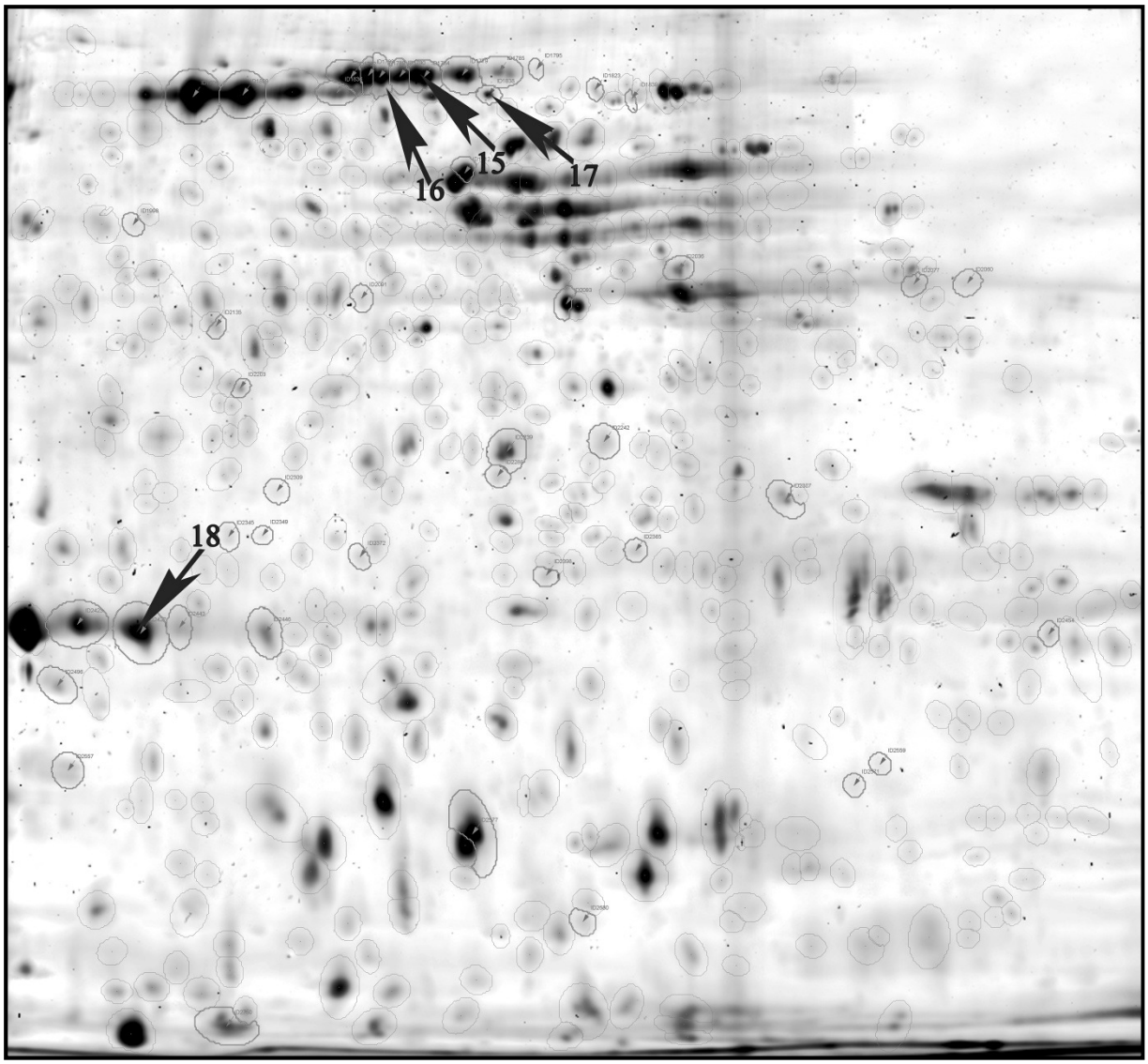

Fig. 23. 2D-Dige gel of gibel carp. 482 spots were detected of which 40 proteins were significantly ($p<0.05$) different between control and exposed gills. Of these 40 proteins, 4 spots could be identified and are indicated by arrows 15 & 16 (transferrin precursor), 17 (heat shock protein 17, mitochondrial precursor) and 18 (apolipoprotein A-I).

Species	Spot Nr.	Protein Name	Accession Nr.	MW (Kda)	Protein Score	P value	↓↑
Rainbow trout	1	transferrin [Oncorhynchus mykiss]	gi|218931236	76.92	966.00	0.001	↓
	2	serum albumin [Oncorhynchus mykiss]	gi|95931876	19.34	90.70	0.001	↓
	3	serum albumin [Oncorhynchus mykiss]	gi|95931876	19.34	78.70	0.001	↓
	4	carbonic anhydrase 1 [Oncorhynchus mykiss]	gi|218931238	28.55	785.00	0.05	↑
Common carp	5	keratin 8 [Danio rerio]	gi|29335502	55.57	509.00	0.05	↑
	6	mitochondrial ATP synthase beta subunit [Xenopus laevis]	gi|148223359	56.40	1100.00	0.05	↑
	7	F-actin-capping protein subunit beta [Salmo salar]	gi|209155424	30.79	361.00	0.05	↑
	8	tropomyosin 3 isoform 2 [Danio rerio]	gi|41393141	28.83	532.00	0.001	↓
	9	Tropomyosin alpha-4 chain [Salmo salar]	gi|213515262	28.60	256.00	0.001	↓
	10	Rho GDP-dissociation inhibitor 2 [Osmerus mordax]	gi|225708226	22.60	286.00	0.05	↓
	11	Proteasome subunit alpha type-6 [Salmo salar]	gi|221219958	27.78	105.00	0.001	↓
	12	translationally-controlled tumor protein [Cyprinus carpio]	gi|84569880	19.15	264.00	0.001	↓
	13	Myosin regulatory light chain 2, smooth muscle isoform [Oncorhynchus mykiss]	gi|238231434	19.84	244.00	0.05	↓
	14	type II keratin subunit protein	gi|386854	52.93	82.20	0.05	↓
Gibel carp	15	transferrin precursor [Carassius auratus gibelio]	gi|15290519	75.94	683.00	0.001	↑
	16	transferrin precursor [Carassius auratus gibelio]	gi|15290519	75.95	523.00	0.001	↑
	17	heat shock protein 70, mitochondrial precursor [Salmo salar]	gi|223648538	73.92	175.00	0.05	↑
	18	apolipoprotein A-I [Cyprinus carpio]	gi|13445027	20.80	77.80	0.05	↑

Table 4. Spot identification 2D-Dige. Protein identifications of designate proteins in fig 1-3 with accession number, Molecular Weight (MW), proteins score, an indication of the increase of decrease of its expression and p value.

5.4.1.2 Proteomic changes

In rainbow trout gills exposed to 50µg/l Cu for 3 days, the expressions of transferrin (spot number 1) and serum albumin (spot number 2 and 3) were inhibited whereas the expression of carbonic anhydrase (CA) (spot number 4) were increased (Table 4). In common carp, we found more identified proteins of which keratin 8, mitochondrial ATP synthase β subunit and F actin capping protein subunit β showed higher expressions (spot number 4, 5, 6) and tropomyosin 3 isoform 2, tropomyosin α 4 chain, rho GDP dissociation inhibitor 2, proteasome subunit α type 6, translationally controlled tumor protein, myosin regulatory light chain 2 smooth muscle isoform and type 22 keratin subunit protein (spot number 7-14) were down regulated (Table 4). Gills of Cu exposed gibel carp showed increased expressions of transferrin precursor (spot number 15 and 16), heat shock protein 70 mitochondrial precursor (Hsp70) (spot number 17) and apolipoprotein A-I (ApoAI) (spot number 18) (Table 4).

Interestingly, the down regulation of transferrin in the Cu sensitive rainbow trout, contrasts the increase in transferrin precursors in the more Cu resistant gibel carp. Transferrin is an iron transporter, mainly synthesized in the liver, which tightly regulates the concentration of iron (Neves et al., 2009). The binding of iron on transferrin renders iron unavailable for use in pathogen growth and unavailable for catalysis of superoxide radical formation via Fenton reactions (Lambert et al., 2005). As a common metalloprotein, transferrin is capable of binding other metals as well (Welch, 1992). For instance, Spears and Vincent (1997) and Zhu et al. (2006) showed that transferrin was able to bind Cu and cadmium, although not as tightly as iron, and is therefore able to play a role in the detoxification of these metals (De Smet et al., 2001). An increased transferrin concentration enhances the ability in capturing excess Cu ions and moreover may help to regulate the free or labile Cu concentration in the cell. The later is thought to be responsible for the oxidative stress effect caused by Cu exposure (Arabi and Allaeddini, 2005). Downregulation of this protein thus reduces binding capacity and increases risk of oxidative (and other) damage.

Despite the lower transferrin expressions in rainbow trout gills exposed to Cu, this fish species showed an increased CA expression. As shown in previous research, CA in gills is likely to be inhibited by waterborne Cu exposure, partly prohibiting Cl^- and Na^+ uptake at the gill (Blanchard and Grosell, 2006; Perry et al., 2003). Indeed, as illustrated in Eyckmans et al. (2010), rainbow trout's Cl^- concentration in plasma was decreased from the beginning of the exposure. It might be that the inhibited CA in rainbow trout gills triggered an increased CA

expression, visible after 3 days. In contrast, gibel and common carp showed decreased Cl^- concentrations in plasma only after 3 days of exposure and changes in CA expressions in their gills were probably not yet visible at the investigated time point.

Since the procedure of the dissection was consistent for all samples, gills were rinsed and biological replicates were pooled (see Materials and Methods), the slight differences in amount of blood present in the gill during dissection are most likely to be inconsequential. Therefore, the decreased expressions of serum albumin in gills of rainbow trout are effectively caused by the Cu exposure. Protein patterns, albeit in blood serum, of *O. niloticus* exposed to Cu showed comparable reduced albumin levels (Sharaf-Eldeen and Abdel-Hamid, 2002). Albumin, an important protein for i.a. maintaining osmotic pressure (Lepkovsky, 1930), can bind Cu and eventually transport the excess metal to the liver (Aaseth and Norseth, 1986). Again, this implies a reduced metal binding capacity in trout gills, with possible deleterious effects as a consequence.

Most of the identified proteins of the intermediate sensitive common carp were related to the cytoskeleton. For instance keratins, which are intermediary filament proteins who form specific heterodimers, are found in almost all differentiated eukaryote cells (Keyvanshokooh and Vaziri, 2008; Lu et al., 2011; Plowman, 2007) and showed changes in expressions. Myosin regulatory light chain (smooth muscle isoform) is used in cell motility and its expression was as well altered during Cu exposure in common carp gill tissue. Furthermore, it has been suggested that the actin cytoskeleton is an early target of reactive oxygen species toxicity (ROS) (Chen and Chan, 2009; England et al., 2004; Fagotti et al., 1996; Salas-Leiton et al., 2009) which coincides with effects of Cu exposure (a know oxidative stressor) and its resulting changes in expressions of F-actin capping protein, Tropmyosin and Rho GDP dissociation inhibitor. F-actin capping proteins bind to the fast growing ends of actin filaments, blocking exchange of subunits at these end, where tropomyosins bind actin as well, forming a molecular barrier by covering up active sites of actin (Cooper and Sept, 2008). Tropomyosins control different functions in collaboration with actin-binding proteins and regulate Ca^{2+} linked contraction as well (O'Neill et al., 2008; Salas-Leiton et al., 2009). There are different isoforms of tropomyosin that interact with actin which may have contrasting responses to Cu (Chen and Chan, 2009). Previous research has pointed out the possibility of oxidative stress related post translational modifications of tropomyosin caused by Cu exposure (Grøsvik et al., 2006). In this study, tropomyosin 3 and 4 expressions were down

regulated. Furthermore, members of the Rho family of small guanosine triphosphatases are regulators of actin cytoskeleton, and switch by cycling between active GTP bound and inactive GDP bound forms. The Rho GDP dissociation inhibitor regulates the GTP/GDP reaction by inhibiting the dissociation of GDP and subsequent the binding of GTP (Hall, 1998; Maddala et al., 2008). Furthermore, De Boeck et al. (2007) showed especially for common carp, cell swelling and hypertrophic lamellae in the first days of a Cu exposure. In contrast, rainbow trout showed little structural gill damage and in gibel carp, the onset of the occurring gill damage was only after 3 days of exposure (after the time point investigated in this study). According to Hoffmann et al. (2009), a rapid and extensive reorganization of the cytoskeleton after osmotic volume perturbations can occur in all sorts of cells which might be reflected here in the gills of common carp.

The remaining identified proteins appeared to be proteasome subunit α type 6, translationally controlled tumor protein and mitochondrial ATP synthase subunit b. The proteasome is a major cellular protease important for protein turnover and cell survival (Zhang et al., 2007) whereas translationally controlled tumor protein is thought to have functions involved in calcium binding, microtubule stabilization and a variety of other functions (Kim et al, 2000; Venugopal, 2005). Interestingly, the translationally controlled tumor protein can interact with Na^+/K^+- ATPase a subunit. Na^+/K^+- ATPase is a target of Cu exposure (Eyckmans et al., 2010) and is regulated by a wide range of signals including stress responses caused by heavy metals (Venugopal, 2005).

Besides the earlier mentioned up regulated expression of transferrin precursors, gills of gibel carp exposed to Cu showed elevated expressions of ApoAI and Hsp70 precursor. ApoAI is a highly expressed protein with a primary role in lipid metabolism (Cham, 1977) and, as Smith et al. (2005) noticed, it can influence gill branchial epithelial integrity. ApoAI determines trans-cellular permeability and could therefore be important in maintaining an intact cell membrane permeability since Cu is known to change this feature (Craig et al., 2007). Additionally, Hsp70, which plays a role in cell protection and repair when faced with acute stressors, is as well important for allowing the cell to cope with changes in the protein machinery (Airaksinen et al., 2003; Boone and Vijayan, 2002). An increase in expression can therefore be helpful in surviving excess metal exposure. Boone and Vijayan (2002) illustrated an increase in Hsp70 in rainbow trout hepatocytes when exposed to high Cu concentrations

for 24 hours. Although not many proteins were identified, those who were, indicate gibel carp's ability to minimize the negative effects resulting from Cu exposure.

As a result of the scarcity in protein identifications of the 2D-Dige experiment, performing extra research via iTRAQ became a necessity, as indicated in the introduction.

5.4.2 iTRAQ.

5.4.2.1 iTRAQ protein pattern

All MS/MS spectra were processed using MASCOT and Scaffold 3 software. For rainbow trout, 5221 queries in NCBI database (20090710) resulted in 1240 protein hits in MASCOT and 118 positively identified proteins with Scaffold. iTRAQ analysis of common carp gill proteome showed 5764 queries in NCBI database (20090710) which resulted in 937 protein hits in MASCOT and 42 positively identified proteins with Scaffold. Finally, for gibel carp, 5438 queries in NCBI database (20090710) resulted in 358 protein hits in MASCOT and 43 proteins positively identified in Scaffold. The number of queries exceeds the number of proteins detected in 2D-Dige to a great extent.

5.4.2.2 Proteomic changes

Of the three studied fish species, the most proteins were identified in rainbow trout and of these identified proteins, 41 proteins were significantly different from control gill tissue. Only a small majority (22) of these proteins showed down regulated expressions, while the other 19 proteins displayed up regulated expressions. Furthermore, 7 out of these 41 significantly different expressed proteins were not categorized. When annotated according to the GO classification system (<www.geneontology.org>), it appeared that 17 proteins (Table 5) were "translation and transcription" related whereas the other proteins were mostly divided between the "response to oxidative stress", "transport", "ATP metabolic process", "cytoskeleton and cell structure", "protein metabolism", "immune response", "unknown" groups. Although the changes in the translation and transcription group are very important, the significantly different proteins in the other groups draw the attention. For instance, Myeloperoxidase (MPO) precursor and myeloid specific peroxidase (a homologue of MPO) (response to oxidative stress) are both less expressed in Cu exposed gills of rainbow trout. MPO is in humans a well characterized enzyme located in azurophilic granules of neutrophils, which uses hydrogen peroxide to oxidize several substrates but has no well defined function

in fish (Castro et al., 2008; Loria et al., 2008). The increased oxidative stress during Cu exposure might have caused the reduced MPO expressions, possibly as a measure to reduce the production of additional ROS. Interestingly, two ATP synthesis proton coupled transport proteins displayed lower expression as well. This could indicate a decreased mitochondrial activity (Garcia et al., 2010).

Hemopexin, a serum glycoprotein, is known to function as a free radical scavenger, hereby protecting tissues against ROS. It is also important in iron homeostasis and has many other functions (Delange and Langois, 2001; Hirayama et al., 2004; Smith et al., 2007). In hypoxic goby Gillichthys mirabilis, Gracey et al. (2001) noticed increased levels of hemopexin-like protein mRNA. However, in this study, its expression was inhibited in Cu exposed gills of rainbow trout, possibly reflecting the onset of anemia (Singh et al., 2008) and again leading to a reduction in metal binding properties. Moreover, Smith et al. (2009) suggested a pivotal role for copper in iron homeostasis since Cu is required as a transition element for heme-hemopexin function in the hemopexin system. In the same GO classification, expression of stress induced phosphoprotein 1, or often named Hsp70/Hsp90 organizing protein (Hop), was up regulated. Besides being a chaperone during assembly of Hsp90, this protein regulates the activity of both Hsp70 and Hsp 90 and is thought to be overexpressed in exposure to stress (Lewis et al., 2010). Recently, chaperones are suspected to play a role not only in protein folding and assembly, but as well in protein degradation (Arndt et al., 2007). In rainbow trout gills exposed to Cu, three more proteins with significantly different expressions were related to protein metabolism. The HLYD family type I secretion membrane fusion protein, involved in protein secretion, displayed an enhanced expression while the hypothetical protein HPAG1_0260, involved in proteolysis as a metalloendopeptidase, showed a decreased expression. Furthermore, FK506 binding protein 1A expression was decreased as well. Members of this last family are associated with FK506 (Somarelli and Herrera, 2007), an immunosuppressant. In relation to the previous association, MHC class II β and Complement factor H1 expressions were respectively up and down regulated, indicating changes in the immune response of Cu exposed rainbow trout. More changes were found in the cytoskeleton and cell structure group, were keratin and procollagen expressions were down regulated. In the carbohydrate and lipid metabolism on the other hand, the up regulation of α–N-acetylgalactosaminidase expression and the decreased expression of glycerol-3-phosphate dehydrogenase indicate an increased energy demand. α–N-acetylgalactosaminidase is required for the breakdown of glycolipids, providing a source for energy production, whereas

glycerol-3-phospate dehydrogenase's inhibition leads to a shift towards more pyruvate production. Higher pyruvate concentrations increase the input in the citrus acid cycle or glycolysis, and thus provides an increased energy production. This enhanced energy production and above mentioned protein anabolism can be an indication of a mechanism to minimize the adverse stress effects in rainbow trout. In the iTRAQ analysis of rainbow trout gill tissue, the decrease of serum albumin in Cu exposed gills was as well present, comparable to the 2D-Dige analysis. In this study, Phenylalanyl-tRNA synthetases, which are enzymes that are very important during protein biosynthesis (Bullard et al., 1999), and the increased expression of the transcription factor protein, these transcription and translation related proteins indicate an enhanced protein production, possibly to ensure the best oxygen supply possible via the damaged gill tissue. Moreover, it could also be a reaction in response to the augmented expressions of nephrosin precursor and pancreatic caroboxypeptidase A1, which are both proteases albeit with different targets (Hung et al., 1997; Lyons et al., 2010).

In contrast, expression of preprotein translocase subunit secA was decreased in Cu exposed gills of common carp, resulting in reduced preprotein substrate transport (Economou, 1998) (table 6). Beside this protein, the hypothetical protein AFO388, with a function in the biosynthetic process, was in the gills of common carp the only other significantly different protein identified compared to controls that was down regulated. Next, there were 5 proteins with unknown functions and 3 more which indicated an enhanced energy consumption: FrdA (electron transport chain), triosephosphate isomerase (glycolytic pathway) and F0F1 ATP synthase subunit β (ATP production). Interestingly, ATP synthase appears to be one of the major mitochondrial proteins gluthionylated during times of oxidative stress (Garcia et al., 2010).

As in the 2D-Dige analysis, protein identifications of gibel carp samples were limited and only 7 out of 43 proteins of Cu exposed gills showed significantly different expressions compared to control samples (Table 7). These results pointed out that the hemoglobin complex was targeted, with an enhanced expression of hemoglobin subunit β and a slight decreased expression of hemoglobin subunit α. As previously mentioned, the dissection procedure was consistent for all groups and fish species so that the slight differences in amount of blood present in the gill during dissection are most likely to be inconsequential. Therefore, the changes in hemoglobin might be an effect of Cu exposure. In rainbow trout, researchers have found increased hemoglobin concentrations after 3 days of exposure to ~0.5

μM Cu which returned to normal levels afterwards (Dethloff et al., 1999). Often, these increased hemoglobin concentrations in Cu exposed fish are related to an increased hematocrit due to osmotic imbalances (Laurén and McDonald, 1987; Handy, 2003). However, in the neotropical pacu, Sampaio et al., (2008) noticed no differences in hemoglobin concentration when these fishes were exposed to 0.4 μM Cu for 24 hours. The role of the hemoglobin complex in fish Cu toxicity is as such not completely described. Although, for example in sheep, oxidative injury to hemoglobin can result in decreased concentrations of hemoglobin and ultimately death (Van Niekerk et al., 1994) which points out the possible importance of hemoglobin in Cu toxicity.

Most of the other significantly different and identified proteins in gills of gibel carp showed decreased expressions; Ubiquitin/actin fusion protein 2, inner membrane translocator, arginine/serine-rich splicing factor and an unknown predicted sc:d0375 protein. Ubiquitin/actin fusion proteins possibly play a more indirect role in cytoskeleton regulation and are related to the actin family (Archibald et al., 2003), whereas the inner membrane translocator provides the plasma membrane a means of efflux or influx of a diverse range of molecules (Webb and Lithgow, 2010). Further, arginine/serine-rich splicing factors are thought to play a role in the regulation of gene expression at the level of RNA processing (Fu, 1995). Finally, there was one other protein up regulated; ApoAI. Interestingly, 2D-Dige analysis showed a similar result which indicates the significance of ApoAI in maintaining intact cell membranes during stress situations, as discussed above.

Rainbow trout				
Go classification	Protein	Accession Number	P value	↓↑
ATP metabolic process	ATP synthase subunit d, mitochondrial [Salmo salar]	**B5DGF3**	0.0458	↓
ATP metabolic process	mitochondrial ATP synthase H+ transporting F1 complex delta subunit [Oncorhynchus m.f.]	**B0FFJ5**	0.0069	↓
carbohydrate metabolic proces	Alpha-N-acetylgalactosaminidase [Salmo salar]	**C0HAA7**	0.0232	↑
Cytoskeleton and cell structure	Keratin, type I cytoskeletal 18 [Osmerus mordax]	**C1BM11**	0.0015	↓
Cytoskeleton and cell structure	type I procollagen alpha 1 chain [Raja kenojei]	**Q4W6W6**	0.0386	↓
Cytoskeleton and cell structure	RecName: Full=Keratin, type I cytoskeletal 18	**Q7ZTS4**	0.0154	↓
glycerol-3-phospate dehydrogenase activity	glycerol-3-phosphate dehydrogenase 1 GlpD1 [Mycobacterium abscessus ATCC 19977]	**B1MH75**	< 0.0001	↓
immune system	Mhc class II beta [Oncorhynchus kisutch]	**Q31477**	0.0197	↑
immune system	complement factor H1 protein [Oncorhynchus masou formosanus]	**B0FFJ7**	0.0066	↓
Metal (ion) binding	hemopexin-like protein [Oncorhynchus mykiss]	**P79825**	0.0033	↓
Metal (ion) binding	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing) [Salmo salar]	**B5DG46**	0.0006	↑
protein metabolism	FK506-binding protein 1A [Salmo salar]	**B9ELK2**	0.0041	↓
protein metabolism	HlyD family type I secretion membrane fusion protein [Delftia acidovorans SPH-1]	**A9BV54**	0.0246	↑
protein metabolism	hypothetical protein HPAG1_0260 [Helicobacter pylori HPAG1]	**Q1CUP5**	< 0.0001	↓
Response to oxidative stress	Myeloperoxidase precursor [Salmo salar]	**C0PU42**	0.0050	↓
Response to oxidative stress	myeloid-specific peroxidase [Danio rerio]	**Q90WB4**	0.0311	↓
Translation and transcription	PREDICTED: similar to NME1-NME2 protein [Pan troglodytes]	**Q3T0Q4**	0.0302	↑
Translation and transcription	histone H3 [Strombus luhuanus]	**Q06SP4**	0.0430	↑
Translation and transcription	putative ribosomal protein L7 protein [Oncorhynchus mykiss]	**Q90YH2**	0.0199	↑
Translation and transcription	ribosomal protein S26 [Anopheles gambiae]	**Q9GT45**	< 0.0001	↑
Translation and transcription	PREDICTED: 40S ribosomal protein S24-like isoform 2 [Macaca mulatta]	**Q9W6X9**	0.0017	↑
Translation and transcription	large subunit ribosomal protein 30 [Koerneria sp. RS1982]	**A6YMS9**	0.0004	↑
Translation and transcription	ribosomal protein L6 [Oncorhynchus masou formosanus]	**A9Z0M5**	0.0104	↑
Translation and transcription	expressed hypothetical protein [Trichoplax adhaerens]	**B5DGD9**	0.0080	↑
Translation and transcription	putative ribosomal protein S7 [Sipunculus nudus]	**A8UA03**	0.0026	↑
Translation and transcription	KH domain-containing, RNA-binding, signal transduction-associated protein 1 [Mus M.]	**Q60749**	0.0142	↑

Translation and transcription	SNF2-related protein [Geobacter sp. M21]	**C6E6G1**	< 0.0001	↑
Translation and transcription	histone 2A family member ZA [Danio rerio]	**Q0Z946**	0.0369	↓
Translation and transcription	Heterogeneous nuclear ribonucleoprotein A0 [Salmo salar]	**B5X9N1**	0.0204	↓
Translation and transcription	PREDICTED: small nuclear ribonucleoprotein Sm D2-like [Macaca mulatta]	**B0CMA0**	0.0224	↓
Translation and transcription	40S ribosomal protein S14 [Psetta maxima]	**A0EZU3**	0.0222	↓
Translation and transcription	ribosomal protein S19 [Solea senegalensis]	**A2Q0T5**	0.0459	↓
Translation and transcription	Cold-inducible RNA-binding protein [Salmo salar]	**B5RI95**	0.0278	↓
Transport	serum albumin 1 precursor [Salmo salar]	**P21848**	0.0037	↓
Unknown	PREDICTED: similar to PABP3 isoform 5 [Pan troglodytes]	**not found**	0.0037	↑
Unknown	arsenite-resistance protein [Cricetulus griseus]	**Q60435**	0.0001	↑
Unknown	Neuroblast differentiation-associated protein AHNAK [Salmo salar]	**C0PU74**	0.0408	↑
Unknown	SAPK substrate protein 1 [Osmerus mordax]	**C1BM87**	0.0007	↓
Unknown	predicted protein [Laccaria bicolor S238N-H82]	**not found**	0.0023	↓
Unknown	hypothetical protein SSE37_03890 [Sagittula stellata E-37]	**not found**	< 0.0001	↓
Unknown	hypothetical protein PROVRETT_00358 [Providencia rettgeri DSM 1131]	**not found**	0.0494	↓

Table 5. Significantly different and identified proteins of gill rainbow trout with GO classification, Accession number, p value and an indication of the increase of decrease of its expression.

Common Carp				
Go classification	Protein	Accession Number	P value	↓↑
ATP metabolic process	F0F1 ATP synthase subunit beta [Sphingopyxis alaskensis RB2256]	**Q1GQS5**	0.0377	↑
cofactor biosynthetic process	hypothetical protein AF0388 [Archaeoglobus fulgidus DSM 4304]	**O29859**	0.0317	↓
Cytoskeleton and cell structure	keratin [Carassius auratus]	**Q07426**	0.0249	↑
Cytoskeleton and cell structure	Krt5 protein [Danio rerio]	**Q6P3K5**	0.0416	↑
Cytoskeleton and cell structure	PREDICTED: keratin 6A [Pan troglodytes]	**not found**	0.0248	↑
electron transport chain	FrdA [Helicobacter pylori]	**O06913**	0.0041	↑
Glycolythic pathway	triosephosphate isomerase [Cucumis sativus]	**A1BQP5**	0.0007	↑
protein metabolism	nephrosin precursor [Cyprinus carpio]	**O42326**	0.0067	↑
protein metabolism	pancreatic carboxypeptidase A1 precursor copy 2 [Tetraodon nigroviridis]	**Q6PHV5**	0.0144	↑
protein metabolism	preprotein translocase subunit SecA [Gramella forsetii KT0803]	**A0LYR8**	< 0.0001	↓
Translation and transcription	ribosomal protein S9 [Solea senegalensis]	**A2Q0S4**	0.0324	↑
Translation and transcription	cold inducible RNA binding protein [Danio rerio]	**Q566W6**	0.0141	↑
Translation and transcription	ribosomal protein L8 [Lateolabrax japonicus]	**A5JVQ0**	0.0205	↑
Translation and transcription	histone H3a [Pterophyllum scalare]	**Q5GAX6**	0.0263	↑
Translation and transcription	phenylalanyl-tRNA synthetase beta subunit [Rubrobacter xylanophilus DSM 9941]	**Q1AWF8**	0.0169	↑
Translation and transcription	Ribosomal protein L18 [Salmo salar]	**Q98SG3**	0.0133	↑
Translation and transcription	transcription factor protein [Ciona intestinalis]	**Q4H3H8**	0.0414	↓
Unknown	predicted protein [Physcomitrella patens subsp. patens]	**not found**	0.0453	↑
Unknown	hypothetical protein DESPIG_02731 [Desulfovibrio piger ATCC 29098]	**not found**	0.0424	↑
Unknown	ATP-dependent chaperone ClpB [marine gamma proteobacterium HTCC2148]	**not found**	0.0137	↑
Unknown	PREDICTED: 60S ribosomal protein L10-like isoform 1 [Macaca mulatta]	**not found**	0.0351	↑

Table 6. Significantly different and identified proteins of gill common carp trout with GO classification, Accession number, p value and an indication of the increase of decrease of its expression.

Gibel Carp				
Go classification	Protein	Accession Number	P value	↓↑
Cytoskeleton and cell structure	ubiquitin/actin fusion protein 2 [Bigelowiella natans]	**Q9GSP9**	0.0059	↓
Cytoskeleton and cell structure	inner-membrane translocator [Polaromonas naphthalenivorans CJ2]	**A1VNI7**	0.0348	↓
lipid metabolism	apolipoprotein A-I [Cyprinus carpio]	**Q98SI3**	0.0033	↑
Translation and transcription	Arginine/serine-rich splicing factor, putative, expressed [Oryza sativa]	**Q10KD9**	0.0468	↓
Transport	RecName: Full=Hemoglobin subunit beta	**P02139**	0.0008	↑
Transport	alpha-globin [Cyprinus carpio]	**O13137**	0.0105	↓
Unknown	PREDICTED: sc:d0375 [Danio rerio]	**F1QY29**	0.0367	↓

Table 7. Significantly different and identified proteins of gill gibel carp with GO classification, Accession number, p value and an indication of the increase of decrease of its expression.

5.4.2.3 WGCNA Co-expression networks: rainbow trout case study

Since rainbow trout's iTRAQ analysis provided enough identified proteins, we analyzed this dataset, together with all previously measured parameters (Eyckmans et al., 2010; 2011, unpublished data). A substantial amount of data points is needed for the software package to be able to cluster efficiently in different modules. If not enough data points are available, WGCNA will generated just one module. In this analysis, four protein modules were generated: brown, yellow, blue and turquoise. Each module contains highly interconnected proteins, based on their expression profiles. These modules were then linked to the introduced biological traits, for instance Cu concentration, or determined enzyme activity.
Interestingly, the turquoise module (98 proteins were associated within this module) appeared to be significantly related to the Cu concentration in the gills of Cu exposed rainbow trout. The majority of these proteins (33) were correlated to Transcription and translation (Fig. 24). Histones and especially ribosomes were vastly present; however some splicing factors and other nucleosome binding proteins were also represented in this module. For example, putative stathmin-like 3, which is a phosphoprotein that uses microtubule depolymerization to regulate cell cycle progression (Lewis and Keller, 2009; Ozon et al., 1997), was also differentially expressed at the genome level in fathead minnow larvae when exposed to Cu in an early life stage.

Seventeen of the proteins in this module were classified in the cell structure and cytoplasm group of GO annotations. Like in the univariate statistics, collagen and keratin were prominent proteins, however, tropomyosin, profilin, thymosin, calponin and actin related protein 2/3 complex (Arp 2/3) were added to the list. Once more, the oxidative stress effect of Cu on actin drew attention. When stimulated, actin can polymerize rapidly by means of actin monomer-binding proteins, of which profilin and thymosin are examples of (Yu et al., 1993). Profilin plays an important role not only in filament assembly (Dominguez, 2009), but also in cytokinesis, neuronal differentiation, synaptic plasticity and regulation of membrane trafficking and nuclear transport (Birbach, 2008; Yarmola and Bubb, 2009). Whereas thymosins are primarily actin monomer sequestering proteins and show higher affinity towards actin (Yu et al., 1993).

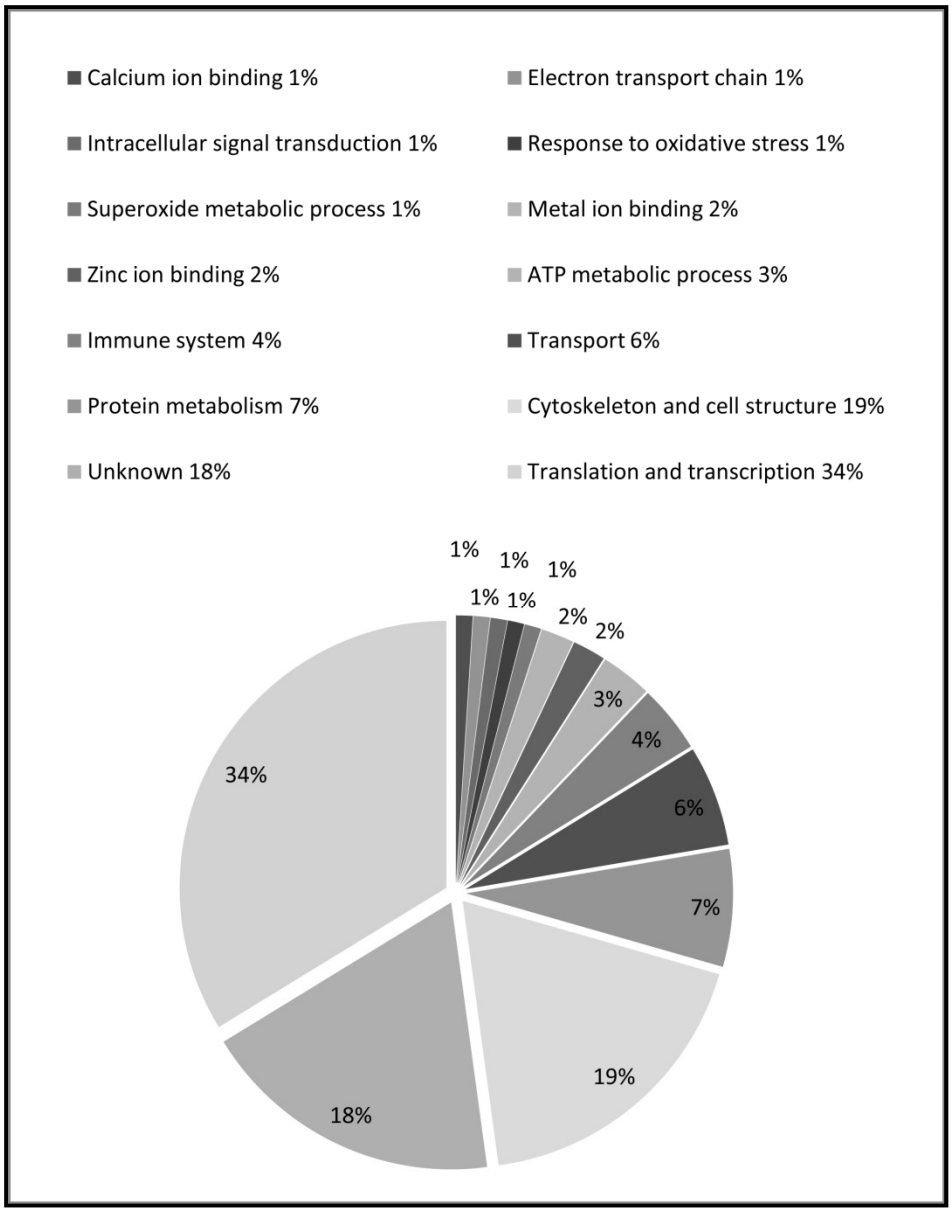

Fig. 24. GO classification WGCNA results rainbow trout of the turquoise module.

Transgelin, member of the calponin family of actin binding proteins, is one of the earliest markers of smooth muscle cell differentiation and is also involved in cancer suppression (Assinder et al., 2009). Another actin related protein is calponin. Calponins regulate actomyosin contraction and fulfills the role of actin filament stabilizing molecule (Rozenblum and Gimona, 2008). Finally, Arp 2/3 is one of the prominent orchestrators of actin filament nucleation (Chesarone and Good, 2009; Rottner et al., 2010). All these changes indicate a challenged actin cytoskeleton during increased oxidative stress caused by Cu exposure or a disturbed osmotic cell volume (Hoffmann et al., 2009).

Furthermore, thrombin is a coagulation system serine proteinase, important in hemostasis and is capable of inducing cell shape changes and gap formation in the monolayer by reorganization of the actin microfilament system (Vouret-Craviari et al., 1998). Moreover, Stenina et al. (2000) have shown a thrombin specific activation of a Y box protein and it is intriguing to see that there was also a Y-box protein present in this turquoise module.

In the GO classification system thrombin is categorized in the blood coagulation and proteolysis group. Complement factor D and H were also annotated as being proteolysis associated. Together with goose-type lysozyme, β-2-Microglobulin and H-2 class II histocompatibility antigen, A-B alpha chain precursor, which are important in specific recognition of antigens (Pérez-Casanova et al., 2010), the complement factors are involved in the immune system as well. The complement system consists of more than 30 proteins, all with a specific role in a cascade-like activation process. Both factor D and H are implicated in the alternative pathway of complement activation, as complement factor D is the rate-limiting enzyme and Factor H is essential for control of this pathway (Ferreira et al., 2010; Gonzalez et al., 2007). Kurtz et al. (2006) were able to link MHC based specific immunity to oxidative stress in three-spined sticklebacks exposed to parasites. As in this analysis, rising oxidative stress in Cu exposed rainbow trout gills, elicited an immune related response. Moreover, g-type fish lysozymes have antibacterial capacities and are therefore important in the destruction of Gram positive and negative bacteria (Callewaert and Michiels, 2010).

Several other proteins were linked to the protein metabolism: 2 peptidyl prolyl isomerase A, peptidyl prolyl isomerase B, FK506 binding protein 1A, HLYD family type 1 secretion membrane fusion protein, predicted protein Laccaria bicolor S238N-H82 and calpastatin long. The first four proteins were as well significantly different in the univariate statistics. The petidyl-prolyl isomerases are, similar to FK506 binding protein, proteins that catalyze cis-trans isomerisation of peptide bonds and have roles in protein folding (Shaw, 2002; Wang and Etzkorn, 2006).Where the predicted protein Laccaria bicolor S238N-H82 is involved in intracellular protein transport, calpastatin Long functions as an endogenous calpains specific inhibitor. Calpains are intracellular proteases (Chéret et al., 2007) which are capable of mobilizing hepatic proteins during starvation (Salem et al., 2007). The changes in protein metabolism can reflect an increased protein demand due to protein utilization as an energy source or due to an attempt to restore occurring damage and replacing conformationally Cu changed proteins.

The Cu exposure resulted also in changed expressions of iron homeostasis and blood function related genes including α and β globins, small heme-containing O_2 binding proteins and serum albumin. Wawrowski et al. (2011) observed similar changes in the Japanese flounder during acute and chronic hypoxia. Other transport proteins, such as Amino ABC transporter permease protein and ATP synthase transport proteins and metal binding proteins such as hemopexin like protein, Hop, cysteine and glycine rich protein, similar to cystein rich intestinal protein and matrix GLA protein, were also co expressed with changed Cu concentrations in the gills of rainbow trout. The ABC transporters have many functions of which transport of a wide range of substrates and xenobiotic compounds across biological membranes is one, which can indicate a protective effect (Popovic et al., 2010; Sturm et al., 2009). Related to the ATP metabolic processes, is adenylate kinase, which catalyzes a transfer of phosphate from ADP to ATP of AMP (Solaroli et al., 2009). Of the metal binding proteins, hemopexin like protein and Hsp70 were discussed previously, whereas the cystein containing proteins are related to zinc ion binding and the matrix GLA protein is essential in tissue calcification through calcium binding properties (Conceiçãio et al., 2008).

The occurrence of oxidative stress related proteins in the co-expression profiles of the turquoise module should be expected since Cu is a potent pro-oxidant (Falfushynska et al., 2011). Indeed, as was also noticed in the univariate analysis, the myeloperoxidase precursor was a part of the turquoise module. Furthermore, cytochrome c, a component of mitochondrial electron transport, was connected to oxidative stress, as described in Kim and Kang (2006). Although Cu is as well used in this protein, elevated Cu levels are known to lead to increased ROS production (Grosell and Wood, 2002). As such, it is interesting to have the enzyme superoxide dismutase (SOD) present in the turquoise model, linked to Cu concentration in gill tissue. SOD, a ROS-scavenging enzyme, showed indeed increased activity in rainbow trout gill tissue after 3 days of exposure to Cu (Eyckmans et al., 2011) and presents a start of the defense mechanism in rainbow trout.

5.5 Conclusion.

As a result of the proteome analysis, some characteristic responses were obvious in the studied fish species. Gibel carp showed increased transferrin, ApoAI and Hsp70 expressions in the 2D-Dige analysis, which all aided in the diminishing of adverse Cu reactions. Moreover, in the iTRAQ analysis ApoAI expression was found significantly different in exposed gills as well, which indicates a possible greater role in the Cu resistantness of gibel carp than previously thought. Changes in the proteome of common carp were primarily mediated by oxidative stress related alteration of the actin cytoskeleton (Chen and Chan, 2009) according to the 2D-Dige analysis, whereas an increased protein production and energy consumption were focal points in the iTRAQ analysis. For rainbow trout, 2D-Dige analysis indicated that the decreased expressions of transferrin and albumin in gills of the sensitive rainbow trout play a part in the negative effects caused by Cu exposure reducing its metal handling capacities. As these are interesting results on its own, it became clear for the three fish species studied that the possibilities of iTRAQ analysis were broader, especially when used with not completely annotated species. Furthermore, since more data were gathered in the iTRAQ analysis, WGCNA analysis, which is an ultimate tool for unraveling biologically relevant and important networks involved in (in this case) Cu toxicity, became possible for rainbow trout. Similar to common carp, an increased energy demand and protein production was noticeable in Cu exposed gills of rainbow trout. Additionally, Cu's adverse effects on the actin cytoskeleton were visible, while superoxide dismutase expressions were related to Cu concentrations in gill tissue as well. The appearance of superoxide dismutase in the WGCNA analysis confirmed the earlier observed importance of superoxide dismutase in rainbow trout's anti-oxidant mechanism since the activity of superoxide dismutase was as well increased at three days of exposure in gills of rainbow trout (Eyckmans et al., 2011).

The different results obtained by 2D-DIGE and the gel-free iTRAQ analyses highlight the complementary nature of these techniques which have distinct physicochemical properties that favor identification of different proteins. Nevertheless, as observed in Alvarez et al. (2009) as well, both proteins identified in both datasets were generally involved in similar biological processes (Briolant et al., 2010; Chen et al., 2009). Finally, the outcome of the different proteomics techniques demonstrates the eminent potential and usefulness of iTRAQ analysis compared to 2D-Dige.

5.6 Acknowledgements.

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Chapter 6

Subcellular differences in handling Cu excess in three freshwater fish species contributes greatly to their differences in sensitivity to Cu toxicity.

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6.1 Abstract.

Since changes in metal distribution among tissues and subcellular fractions can provide insights in metal toxicity and tolerance, we investigated this partitioning of Cu in gill and liver tissue of rainbow trout, common carp and gibel carp. These fish species are known to differ in their sensitivity to Cu exposure with gibel carp being the most tolerant and rainbow trout the most sensitive. After an exposure to 50µg/l (0.79 µM) Cu during 24 hours, 3 days, 1 week and 1 month, gills and liver of control and exposed fish were submitted to a differential centrifugation procedure. Interestingly, there was a difference in accumulated Cu in the three fish species, even in control fishes. Where liver of rainbow trout showed extremely high Cu concentrations under control conditions, the amount of Cu accumulated in their gills was much less compared to common and gibel carp. At the subcellular level, the gills of rainbow trout appeared to distribute the additional Cu exclusively in the biologically active metal pool (BAM; contains heat denaturable fraction, organelles fraction and nuclei cell debris fraction). The same could be seen in gill tissue of common carp, although the percentage of Cu in the BAM of common carp was lower compared to rainbow trout. Gill tissue of gibel carp accumulated more Cu in the biologically inactive metal pool (BIM compared to BAM; contains Heat stable fraction and metal rich granules fraction). The liver of rainbow trout seemed much more adequate in handling the excess Cu (compared to its gills), since the storage of Cu in the BIM increased. Furthermore, the high % of Cu in the metal rich granule fraction and heat stable fraction in liver of common carp and especially gibel carp after exposure and the better Cu handling in gill tissue, pointed out the ability of the carp species to minimize the disadvantages related to Cu stress. The differences in Cu distribution at the subcellular level of gills and liver of these fish species strongly reflects their capacity to handle Cu excess and is one of the greatest contributors to their differences in sensitivity to Cu toxicity.

Keywords: Carassius auratus gibelio, copper, Cyprinus carpio, Oncorhynchus mykiss, subcellular distribution.

6.2 Introduction.

Nowadays, effects of contaminants are explored at various levels of biological organization, ranging from cellular processes via physiology of individuals, to populations and ecosystems (Giguère et al., 2006a). At the first level of this organization, a variety of subcellular strategies are present, for instance to regulate the intracellular sequestration of copper (Cu) and other essential metals (Mason and Jenkins, 1995). Cu concentrations are meticulously controlled by binding to inducible metal binding proteins, such as metallothioneins, incorporation in lysosomes and membrane-bound vesicles (Giguère et al., 2006b) and by partitioning excess metal to metal rich granules (Wallace et al., 2003). In fish, the ability to withstand toxic metal concentrations might be associated with these detoxifying systems (Klerks and Bartholomew, 1991; Wallace et al., 2003) since metals sequestered or stored are not bioavailable to the metal sensitive cellular fractions such as organelles and enzymes (Hogstrand et al., 1991; Kraemer et al., 2005; Rainbow, 2002). Rainbow (2002) indicated that the onset of toxic effects is highly dependent of the concentration of metals in the biologically available form.

To investigate these subcellular compartments (metal rich granules (GR), nuclei cell debris (CD), organelles (OR), heat sensitive fraction (HSF; which also contains metallothionein (MT)) and heat denaturable fraction (HDF; which contains enzymes)), the subcellular fractionation technique according to Wallace et al., (1998) is widely used (Steen-Redeker et al., 2007; Kraemer et al., 2005). In yellow perch, major proportions of hepatic Cu were found in the heat stable fraction (Giguère et al., 2006b) whereas Kamunde and MacPhail (2008) studied juvenile rainbow trout exposed to 40µg/l Cu and noticed that the minority of the hepatocellular Cu was associated with the metal detoxified pool (HSF and GR; comparable to biologically inactive metal pool (BIM) of the BIM-BAM metal accumulation toxicity model (Steen-Redeker and Blust, 2004)) and the vast majority with a metabolically active pool (organelles, nuclei cell debris, HDF; comparable to biologically active metal pool (BAM) (Steen-Redeker and Blust, 2004)). This indicates that different fish species handle Cu differently, and that this is reflected at the subcellular level. When a metal concentration in BAM exceeds a certain threshold value, toxicity can occur. Therefore, the kinetics of internal compartmentalization into biologically active and less active pools is key to understand exposures toxicity relationships (Adams et al., 2010).

The concentration of Cu in the aquatic environment lies naturally below 0.5-1 µg/l (Subathra and Karuppasamy, 2008), however, as a result of industrial use in electric appliances, mining

activities and its use as an algaecide and herbicide (Eisler, 1998; Schjolden et al., 2007), this concentration is often exceeded considerably. The criterion maximum concentration of dissolved Cu recommended by the Environmental Protection Agency (USEPA 2007) for freshwater fish is 2.337µg/l. In Flanders (Belgium), a concentration of 50µg/l is set as the standard for surface water (total Cu concentration, not dissolved). In this study, the latter was used to expose rainbow trout (*Oncorhynchus mykiss)*, common carp (*Cyprinus carpio)* and gibel carp (*Carassius auratus gibelio)*. Previous studies revealed many differences in toxicity and it's adverse effects between these three fish species, such as differences in accumulation patterns, oxidative stress response, MT induction, ion regulation and swimming performance (De Boeck et al,, 2003, 2004, 2006; Eyckmans et al., 2010, 2011). The subcellular partitioning of copper over time could provide an opportunity to understand the mechanisms of toxicity and detoxification at the cellular level in these species (Kraemer et al., 2005). Fish gills serve as an important target for waterborne metals and represent a major uptake route of metals (Tao et al., 2006) whereas liver is known to regulate circulating Cu concentrations and Cu excretion (Grosell et al., 1996; Handy, 2003). Therefore, these tissues were analyzed within this study.

With this study, we want to evaluate the dynamics of the subcellular accumulation patterns in three different fish species under a similar scenario of sublethal copper exposure. The aim of the analysis of copper concentrations in subcellular compartments is to assess whether gill and liver cells of fish with a different sensitivity to copper exposure show intracellular differences in accumulation and handling of copper and whether this can help explain their differences in sensitivity to metal exposure.

6.3 Materials and Methods.

6.3.1 Experimental setup.

Rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibelio*) were purchased from a commercial fish farm (Luc and Patrick Bijnens, Zonhoven) in Belgium. Prior to Cu exposure, fish were able to acclimatize to lab conditions (200l aquaria with filtered tap water, 8 fish in each aquarium) at the University of Antwerp. The acclimatization period lasted for at least a month and was carried out in the same conditions as the experiments afterwards: a water temperature of 17 ± 1 °C, pH of 7.6 ± 0.2, oxygen concentration well above 90% saturation, hardness 250 ± 11 mg $CaCO_3$/l, 0.64 ±

0.32 mg DOC/l and ammonia, nitrate and nitrite levels below toxic concentrations (<0.1 mg/l). To ensure good water quality, a calibrated flow through system was used which renewed the total volume of the aquaria each 7 hours and an open trickling filter with filter wadding, activated charcoal and lava stones was used additionally. During acclimatization and exposure, fish were fed *ad libitum* once a day.

Each fish species was exposed to the Flemish standard for surface water (50 µg/l) for 24 hours, 3 days, 1 week and 1 month. Additionally, at each time point samples from the control groups were taken (N=6 fish for each group, species and time point). At the start of the experiment, lava stones and charcoal were removed from the filter and Cu was added subsequently as a copper nitrate solution ($Cu(NO_3)_2 \cdot 2H_2O$, Merck, Darmstadt, Germany). Once the aquaria were predosed properly, the chosen concentration was maintained by using a peristaltic pomp (Watson Marlow 505S) connected to a stock of the previous mentioned Cu solution. The total Cu concentration was checked daily and measured with graphite furnace atomic absorption spectroscopy (Varian Model 220 Z, Mulgrave, Australia) under manufacturer recommended conditions.
Fish were weighed and measured after anesthesia (buffered MS222 solution, 100mg/l Sigma Chemical, St. Louis, MO, USA) and prior to dissection. Using Li-heparinised 1 ml syringes, a blood sample was collected from the caudal blood vessel and centrifuged for 2 min in Li-heparinised 1.5 ml bullet tubes. The resulting plasma was transferred to a cryovial and, together with cryovials of dissected gill and liver, flash frozen in liquid nitrogen. All samples were stored at -80°C for further analysis.

6.3.2 Cu analysis in plasma.

Plasma samples were analyzed for their Cu content. These samples were acidified with 69% HNO_3 and prior to digestion in a technical microwave oven, 30 % of H_2O_2 was added (Merck, Darmstadt, Germany). After digestion, samples were analyzed with an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Varian Ultra Mass 700, Victoria, Australia).

6.3.3 Differential centrifugation and Cu analysis in subcellular fractions.

The subcellular fractionation was performed using the slightly adapted protocol of Wallace et al. (1998) and Kamunde and MacPhail (2008). Briefly, 3-5 volumes of a 20 mM Tris base (+ 2 mM β mercaptoethanol, 0.2 mM PMSF, pH 7.6) buffer was added to > 300 mg of tissue and

homogenized for 30 seconds with a 2 ml Potter Elvehjem homogenizer (VWR, Cat. 432-5032). Two thirds of the sample was used for further centrifugation, the remainder of the sample was used for measuring metal bioaccumulation and is referred to as whole gill/liver fraction. The first centrifugation step comprised of 15 min at 4°C 1450*g* (Eppendorf Centrifuge 5804 R, Hamburg, Germany). The supernatant was ultracentrifuged for 1h at 4°C 100,000*g* (Sorval Discovery^{TM} 90 Ultra speed centrifuge, Newton, CT). The resulting pellet contained the organelles fraction (OR). The supernatant was recentrifuged for 10 min 50,000*g* (Sorval Discovery^{TM} 90 Ultra speed centrifuge, Newton, CT) after a heating step of 15 min at 80°C. This step separated the heat denaturable proteins (HDF) (pellet) with the heat stable proteins (HSF) (supernatant). The pellet collected from the first centrifugation step was washed with 1 ml of 1N NaOH and incubated at 80°C for 30 min. A next centrifugation step of 10 min at 5,000*g*, collected the nuclei-cell debris (CD) (supernatant) and the metal rich granules (GR) (pellet). All fractions were weighed, dried and digested with 69% HNO_3 and, prior to digestion in a technical microwave oven, 30 % of H_2O_2 was added (Merck, Darmstadt, Germany). Cu concentrations were measured using ICP-MS (Varian Ultra Mass 700, Victoria, Australia). Subcellular Cu concentrations are expressed in µg/g dry weight. Recovery of Cu from the fraction was calculated by: (Sum of Cu in each fraction x 100%) / Cu in the whole gill/liver. Actual recovery rates were 98.2 ± 2.6%. Analytical accuracy was achieved by the use of blanks containing Milli-Q water and solutions used for digestion and certified reference material "number 185R Bovine liver" of the Community Bureau of Reference (European Union, Brussels, Belgium) during all the destruction protocols. Generally, the concentrations of the blanks were below detection limits and recovery rates of the standards were 98.5 ± 4.2%.

6.3.4 Statistics.

Data are presented as mean ± SEM, the number of experimental fish/samples used is indicated with N. All data passed a Kolmogorov-Smirnov test and each compared data set had similar standard deviations (method of Bartlett). Statistical analysis of the differences between control and Cu exposed fish (for each fish species separately) was performed by one way analysis of variance (ANOVA) followed by Tukey HSD post hoc test. Data were considered significant if $p<0.05$. All tests were performed using Graphad Instat. In control fish, no significant differences were observed over time. Because of the limited change in controls and for clarity of the graphs, graphs show pooled control data for each species.

6.4 Results.

Measured total [Cu] in each exposure was on average 98.4 ± 4.7 % of the nominal [Cu] during the entire experiment. No mortality was observed during the 1 month exposure period.

6.4.1 Total Cu concentration in plasma, liver and gill.

During the sub lethal exposure to 50µg/l Cu, plasma [Cu] was not highly affected in either fish species (Fig.25). We did notice a transient increase after 3 days in rainbow trout and after 1 week in common carp. Interestingly, control [Cu] in plasma of gibel carp was about 3.5 times higher than control [Cu] in rainbow trout, whereas control [Cu] plasma in common carp was 1.5 times higher than rainbow trout and 2.4 times lower than gibel carp. A similar pattern was seen in control gill tissue (Fig. 25), where [Cu] in gibel carp was about 1.5 times higher than in rainbow trout and 1.4 times higher than in common carp. Furthermore, common carp gill [Cu] appeared to be slightly higher than in rainbow trout. In contrast, control [Cu] in liver of rainbow trout (Fig. 25) was excessively high compared to common and gibel carp. The [Cu] concentration was 6 times more elevated than in common carp and even 30 times higher than in gibel carp. Common carp [Cu] in liver was 5 times higher compared to gibel carp.

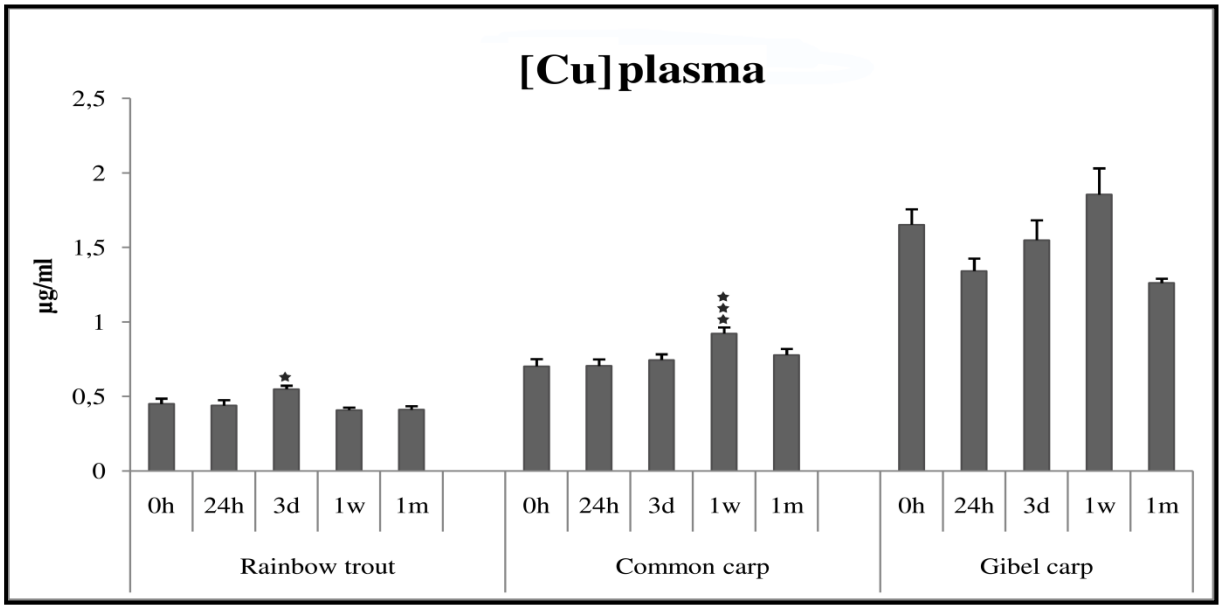

Fig.25. [Cu] in plasma of rainbow trout, common carp and gibel carp after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$ and ***: $p<0.001$).

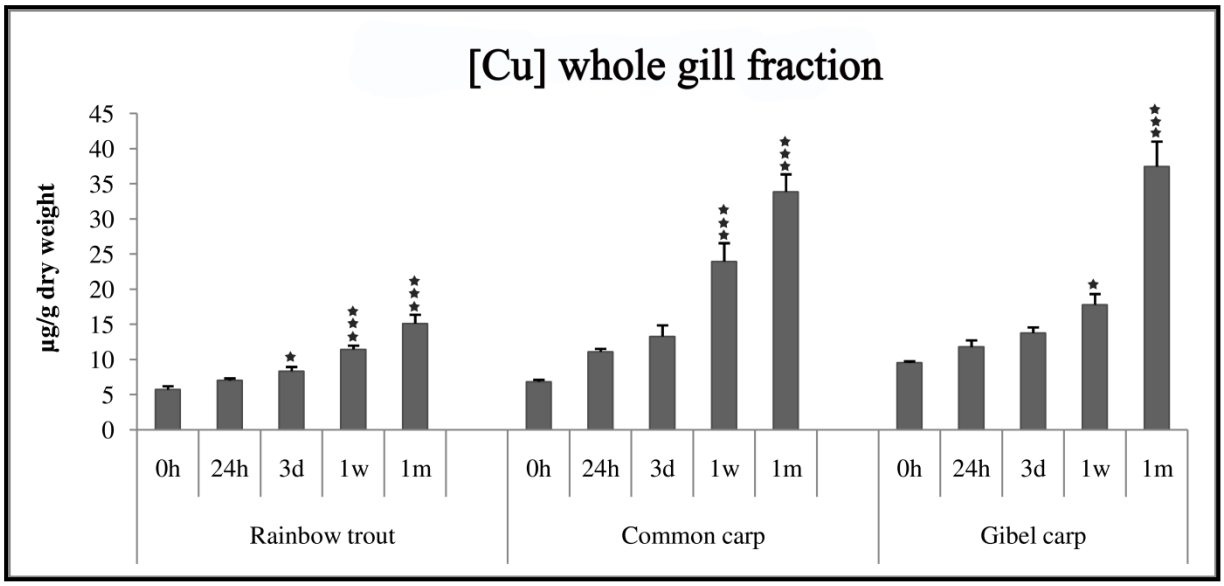

Fig.26. [Cu] in whole gill fraction of rainbow trout, common carp and gibel carp gill tissue after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$ and ***: $p<0.001$).

Liver [Cu] was raised in rainbow trout after 24 hours and 1 month, whereas common carp whole liver fraction was enhanced after 1 week and 1 month. Finally, gibel carp showed higher Cu concentrations in its whole liver fraction after 1 month. [Cu] in gill tissue of rainbow trout did increase stepwise, and this was significant from 3 days until the end of the exposure. Cu accumulation in common and gibel carp gill tissue was significant after 1 week and increased further thereafter.

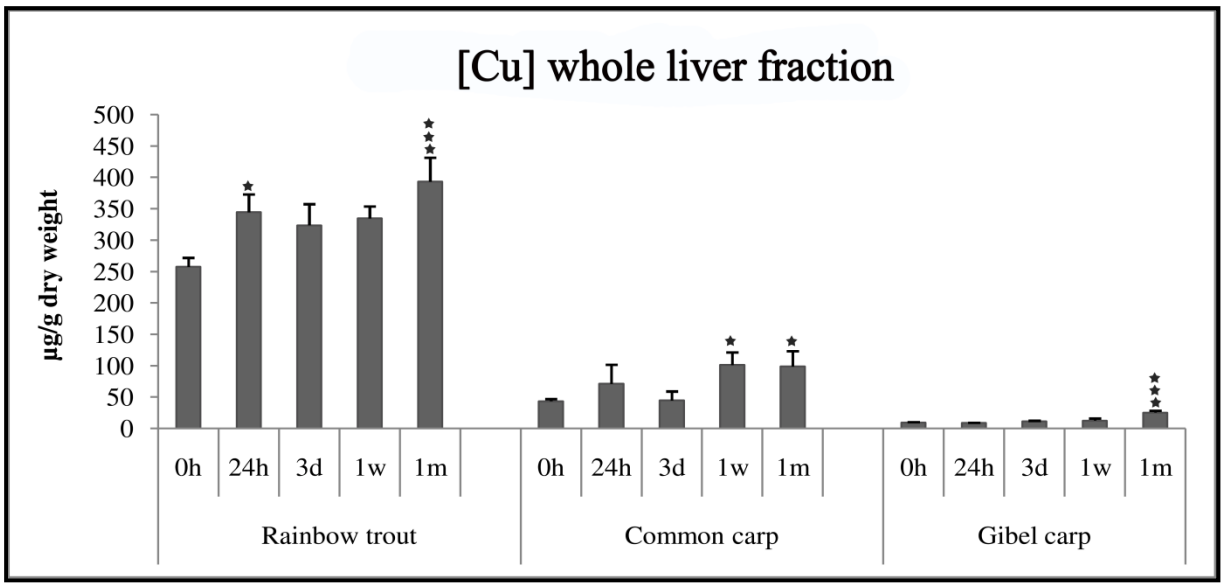

Fig.27. [Cu] in whole liver fraction of rainbow trout, common carp and gibel carp liver tissue after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$).

6.4.2 Subcellular Cu concentration in gill tissue.

The concentration of Cu in the subcellular fractions of gill of rainbow trout is presented in fig. 28. In the nuclei-cell debris fraction, the [Cu] increased with time from 0.50 µg/g dry weight at the start of the experiment, to significantly higher concentrations after 1 week (0.78 µg/g dry weight) and 1 month (1.20 µg/g dry weight) of exposure. Furthermore, a remarkable enhanced Cu accumulation occurred in the heat denaturable fraction, as well after 1 week and 1 month of exposure, from 1.70 to 6.55 and 8.61 µg/g dry weight, respectively. Cu concentration in the other subcellular fractions remained stable, however slight increases, although not statistically significant, were seen in the organelles fraction after 1 month, and in the heat stable fraction after 1 week and 1 month of exposure.

A different outcome was observed in the two carp species, were all subcellular fractions showed higher Cu accumulations at the end of the exposure period (Fig. 29 and 30). The Cu concentration in the nuclei-cell debris fraction of common carp was raised from 0.74 to 2.40 and 3.70 µg/g dry weight and the heat sensitive fraction from 2.64 to 4.46 and 7.06 µg/g dry weight after 1 week and 1 month, respectively. One month of exposure resulted in a 10 fold increase of [Cu] in the metal rich granules fraction (0. 40 to 4.00 µg/g dry weight) and a 5.5 fold increase of the heat denaturable fraction (2.31 to 11.63 µg/g dry weight). The latter fraction peaked as well in the beginning of the exposure (24 hours; 7.20 µg/g dry weight). The augmentation of [Cu] in the organelles fraction already started at 3 days and lasted until the end of the exposure (control values 1.60 to 5.00 µg/g dry weight).

In gibel carp, [Cu] was about 3 times more elevated after 1 month in the nuclei-cell debris fraction (from 0.83 to 2.41 µg/g dry weight), the metal rich granules fraction (from 0.36 to 1.00 µg/g dry weight) and the organelles fraction (from 2.18 to 6.98 µg/g dry weight). The heat denaturable fraction of gibel carp already showed an enhanced Cu accumulation after 24 hours (3.21 µg/g dry weight), returned to control concentrations (1.23 µg/g dry weight) thereafter and increased at the end of the exposure once more (to 4.70 µg/g dry weight). The Cu concentration in the heat stable fraction jumped up after 3 days of exposure (from 4.10 to 11.65 µg/g dry weight) and accumulation continued during the first week (11.90 µg/g dry weight) up to 1 month (19.15 µg/g dry weight) of exposure.

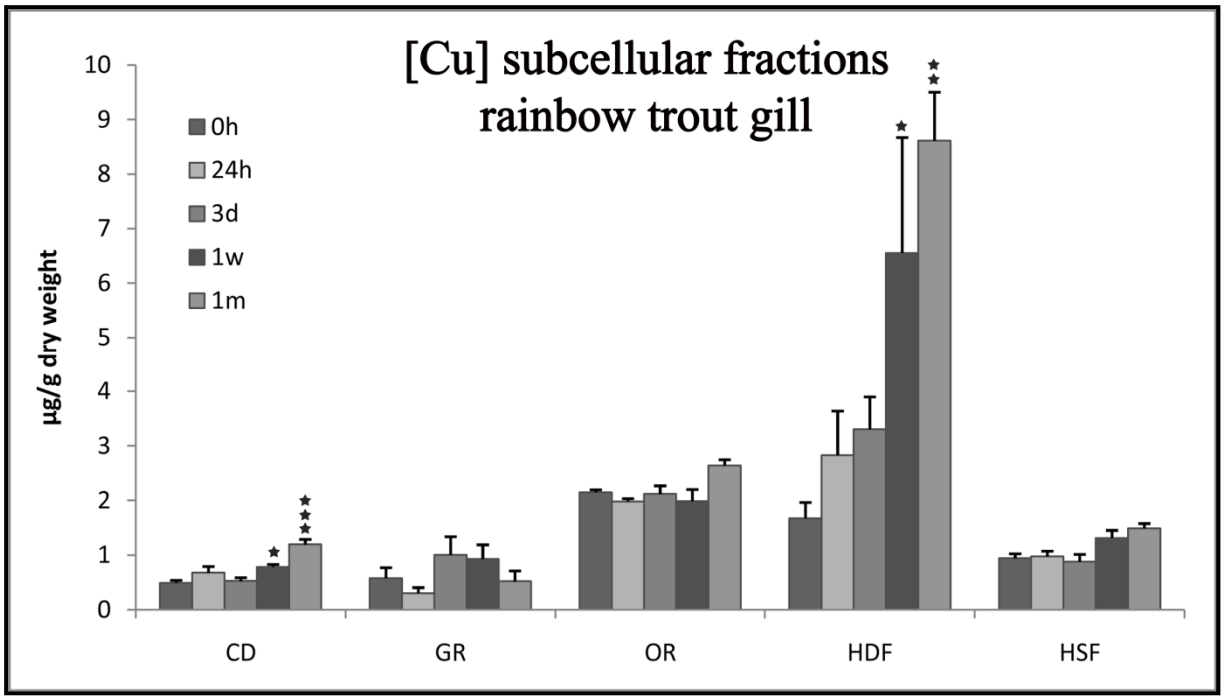

Fig.28. [Cu] in subcellular fractions of rainbow trout gill tissue after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

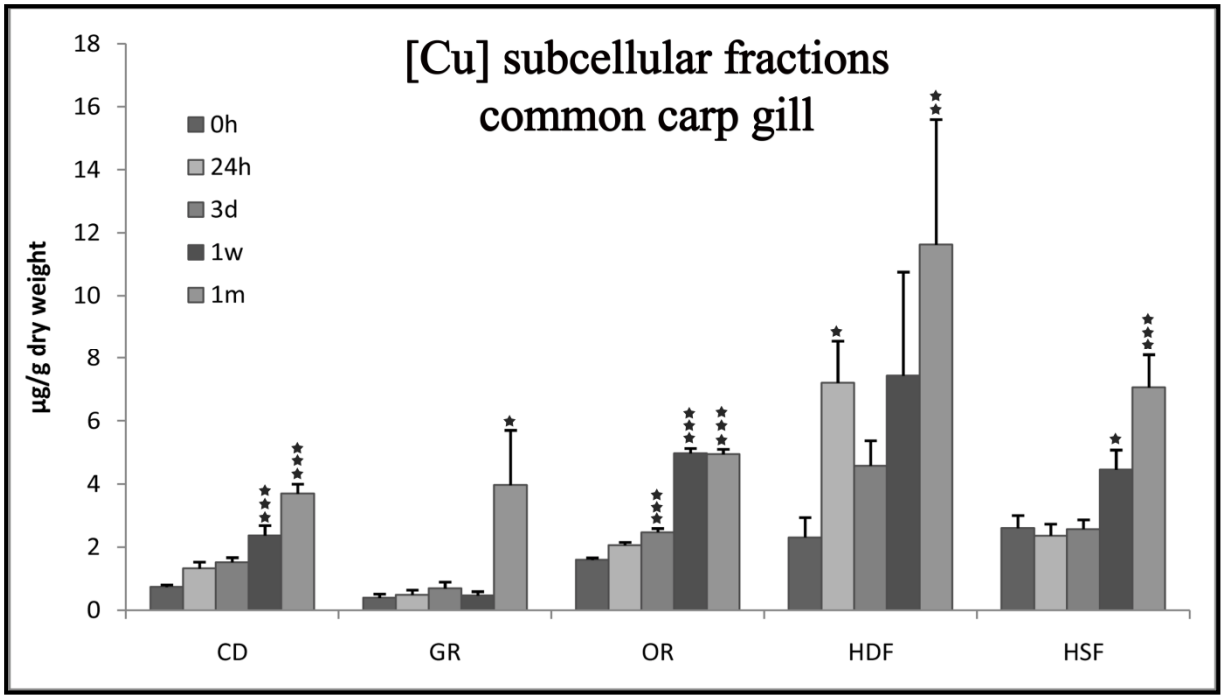

Fig.29. [Cu] in subcellular fractions of common carp gill tissue after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

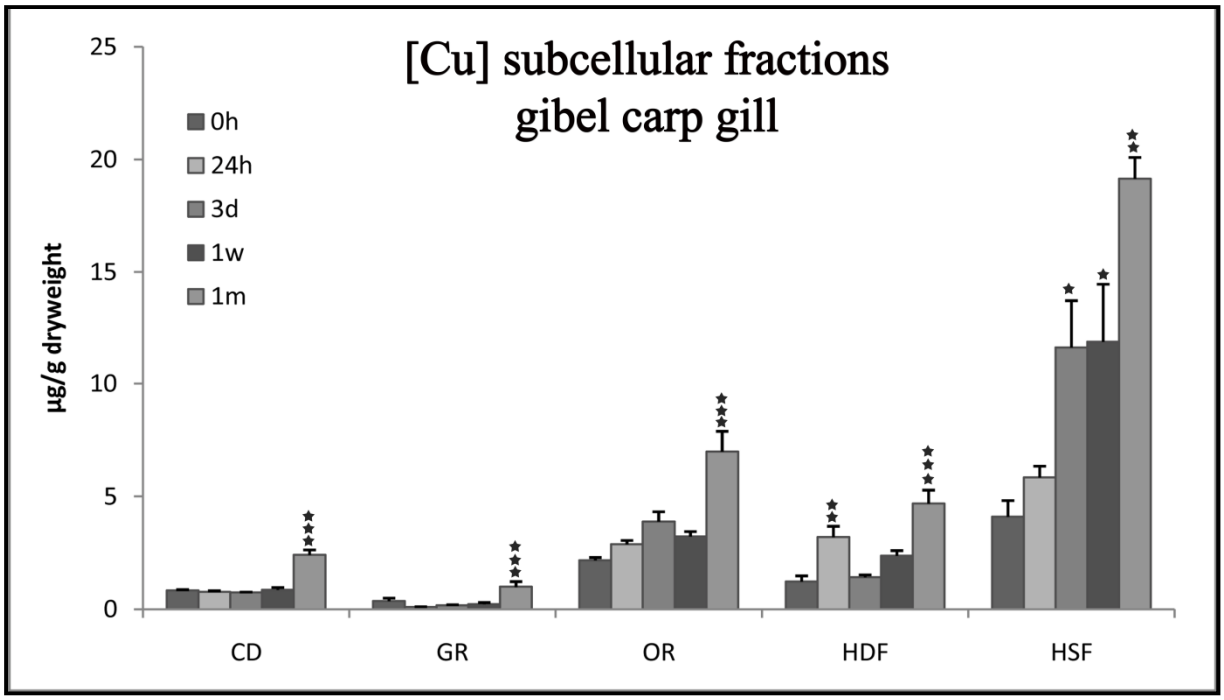

Fig.30. [Cu] in subcellular fractions of gibel carp gill tissue after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

6.4.3 Subcellular Cu concentration in liver tissue.

The increase of the whole liver [Cu] in rainbow trout after 24 hours (Fig. 27) was related to an increase of the nuclei-cell debris fraction (from 8.75 to 23.65 µg/g dry weight) and the metal rich granules fraction (from 6.75 to 31.67 µg/g dry weight) at the same time point (Fig. 31). There were no significant changes in the organelles and heat denaturable fraction of rainbow trout, however, the [Cu] of the heat stable fraction steadily increased with significantly higher levels after 1 month of exposure (from 76.94 to 182.05 µg/g dry weight).

Although the whole liver [Cu] in common carp (Fig. 27) did not show a statistically increased Cu concentration at 24 hours of exposure, the organelles (from 9.22 to 21.58 µg/ g dry weight) and heat denaturable fraction (from 1.77 to 5.13 µg/g dry weight) had accumulated higher concentrations than the corresponding controls at this time point (Fig. 32). The latter fraction still showed remnants of this increase 2 days later, at 3 days of exposure, were the concentration was 3.85 µg/g dry weight. The higher Cu concentration in the whole liver fraction after 1 week was related to a higher [Cu] in the organelles fraction (22.36 µg/g dry weight), whereas the increased whole liver concentration after 1 month was related to an increase of the metal rich granules fraction (from 5.25 µg/ g dry weight to 24.34 µg/g dry weight).

The whole liver fraction of gibel carp (Fig. 27) demonstrated an increase in accumulation of Cu only after 1 month of exposure, which was translated in a steep raise of the heat sensitive fraction at the same time period (from 3.69 to 7.61 µg/g dry weight) (Fig. 33). The remaining subcellular fractions did not accumulate significant amounts of Cu in the liver of gibel carp.

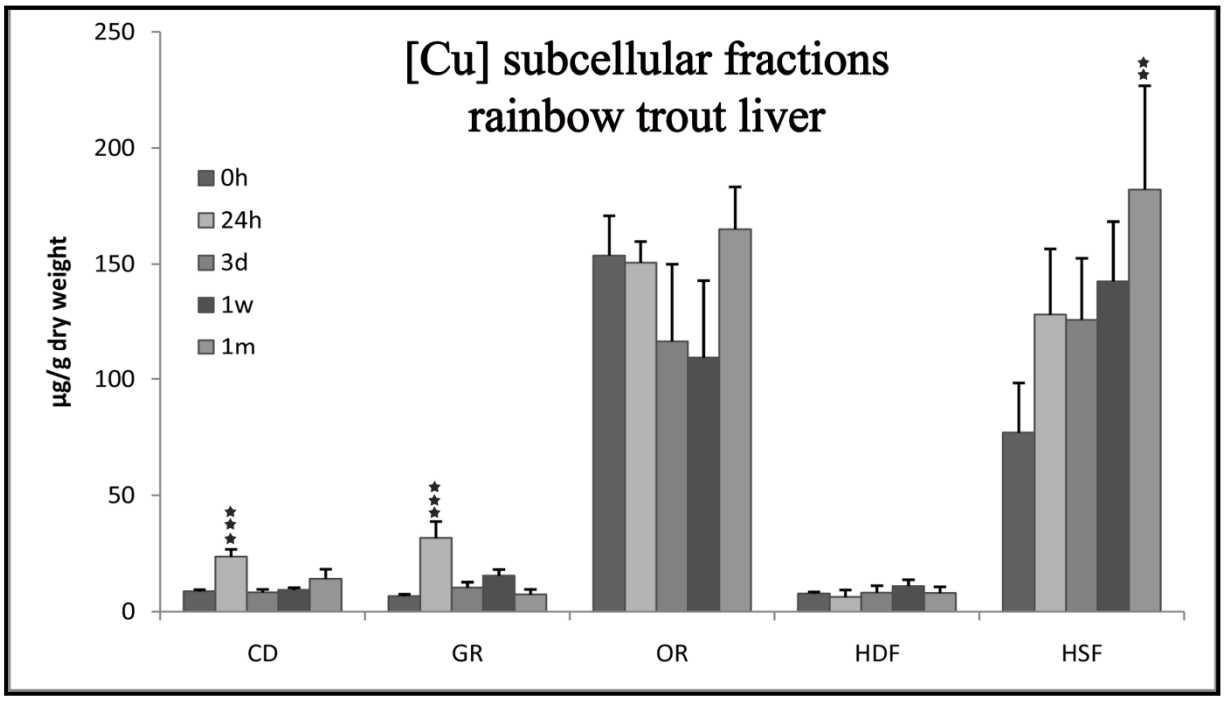

Fig.31. [Cu] in subcellular fractions of rainbow trout liver tissue after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

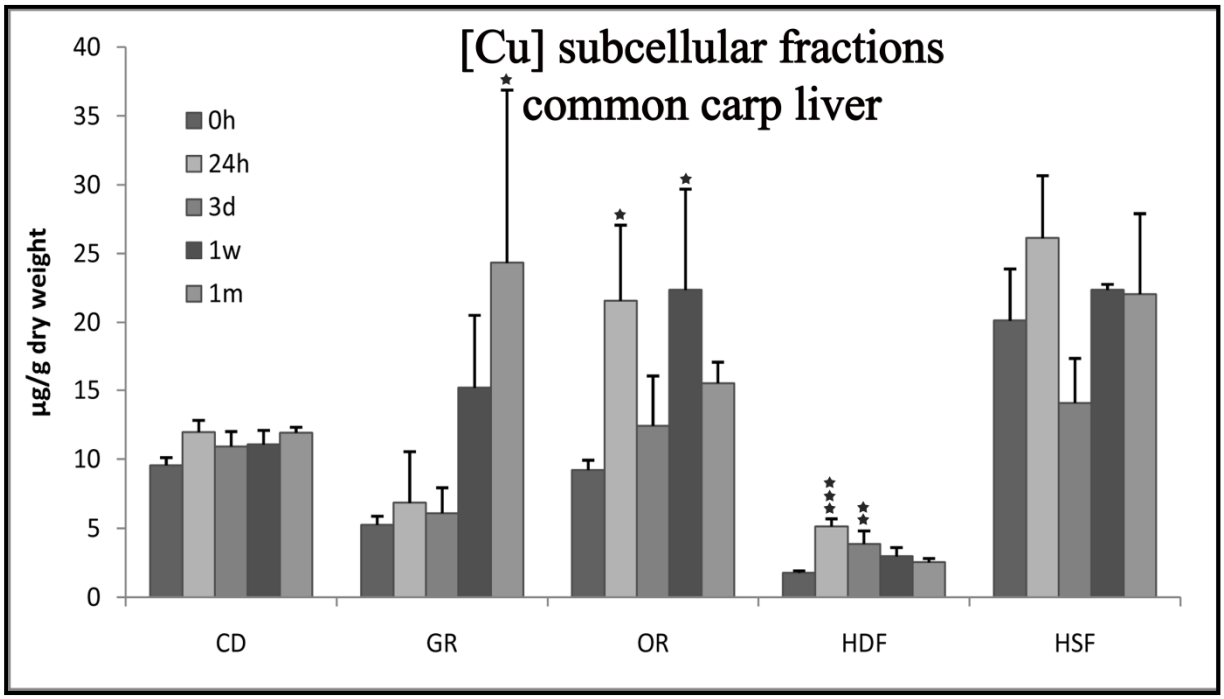

Fig.32. [Cu] in subcellular fractions of common carp liver tissue after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

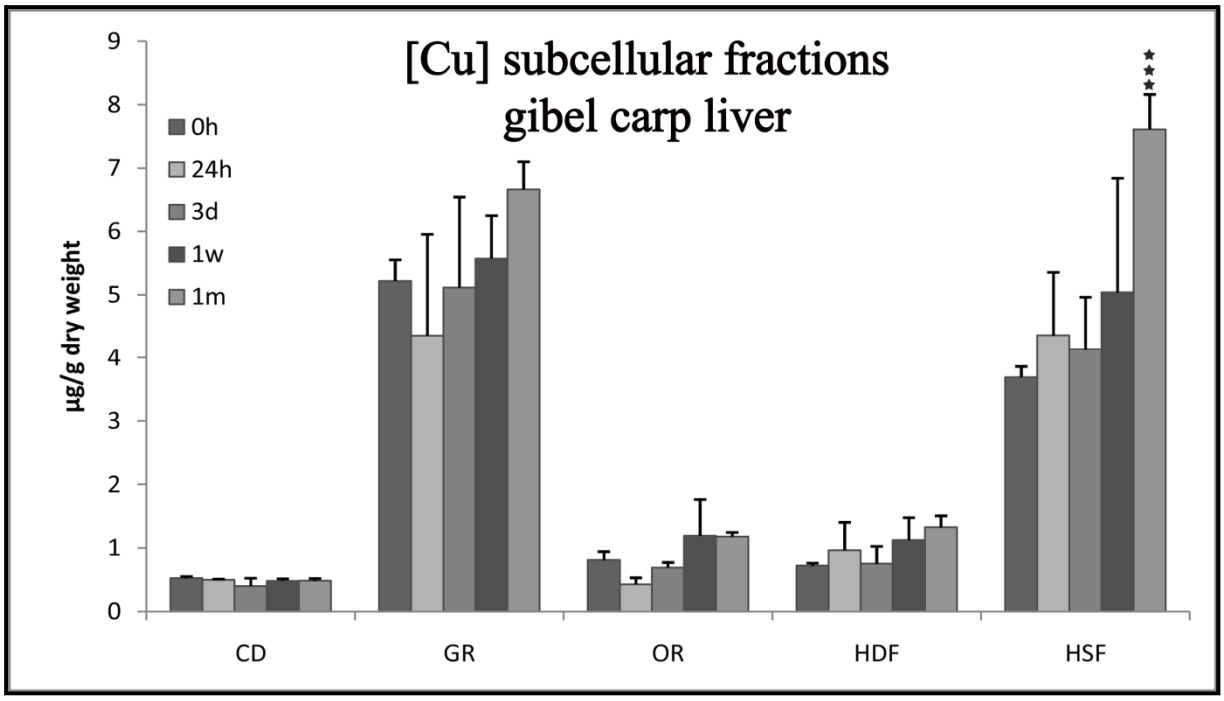

Fig.33. [Cu] in subcellular fractions of gibel carp liver tissue after 24h, 3 days, 1 week and 1 month to either control conditions (0h) or 50µg/l Cu. '*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

6.4.4 Distribution of Cu between Biologically active metal pool/ biologically inactive metal pool.

In Table 8, the distribution of Cu between the biologically active metal pool (BAM) (nuclei-cell debris, organelles and heat denaturable fraction) and the biologically inactive metal pool (BIM) (metal rich granules and heat stable fraction) is illustrated. In rainbow trout, distributions in gill and liver were comparable; both tissues had around 25-30% [Cu] in the BIM and 70-75% in the BAM. However, during Cu exposure, the tissues reacted differently to the opposed Cu stress. The accumulated Cu in gill tissue was primarily found in the BAM, whereas in liver, most of the excess Cu was rendered biologically inactive through binding with the BIM.

Cu in common carp gill tissue was situated mostly in the BAM as well, although the difference between the two pools was less pronounced (around 40% in BIM, 60% in BAM) than in rainbow trout. This was also shown in liver, where Cu was almost equally divided between the BIM (55%) and BAM (45%). Where the distribution of Cu in liver tissue did not change tremendously, common carp's gill tissue accumulated Cu in the BAM, since the % of

Cu increased during the first week (to 72-78%). At the end of the exposure period, distributions returned to normal levels.

Of all three fish species, gibel carp displayed the highest % BIM in gill and in liver. In gill, Cu was equally present in BIM (51%) and BAM (49%), whereas in liver, BIM comprised the largest part of stored Cu (80%) and was as such able to maintain this distribution during Cu exposure. Gill tissue BIM increased after 3 days to 1 week of exposure, however, after 1 month, the distributions were once more comparable to control conditions.

		Gill		Liver	
		%BIM	%BAM	%BIM	%BAM
Rainbow trout	0h	26.05 ± 0.98	73.95 ± 1.23	32.95± 1.47	67.05 ± 2.32
	24h	18.95 ± 2.70*	81.06 ± 1.95*	46.97 ± 2.64	53.03 ± 1.04
	3d	24.09 ± 3.11	75.91 ± 2.56	50.59 ± 2.49*	49.41 ± 3.46*
	1w	19.37 ± 0.88*	80.63 ± 0.79*	54.94 ± 2.11*	45.06 ± 1.09*
	1m	13.92 ± 2.92*	86.08 ± 3.09*	50.31 ± 1.01*	49.69 ± 2.69*
Common carp	0h	39.23 ± 1.86	60.77 ± 2.68	55.24 ± 2.70	44.75 ± 1.53
	24h	21.21 ± 1.05*	78.79 ± 3.84*	52.63 ± 1.11	47.37 ± 0.933
	3d	27.57 ± 1.28*	72.42 ± 2.72*	42.58 ± 1.44*	57.42 ± 1.61*
	1w	25.01 ± 1.16*	74.99 ± 2.91*	50.77 ± 1.12	49.23 ± 1.82
	1m	35.24 ± 3.04	64.76 ± 2.12	58.45 ± 2.05	41.55 ± 0.37
Gibel carp	0h	51.29 ± 1.88	48.70 ±2.54	81.21 ± 2.30	18.78 ± 0.93
	24h	46.41 ± 2.27	53.59 ± 2.91	82.15 ± 1.21	17.85 ± 2.59
	3d	66.12 ± 3.43*	33.88 ± 1.67*	83.35 ± 2.59	16.65 ± 1.84
	1w	65.18 ± 2.02*	34.82 ± 3.47*	79.11 ± 1.95	20.89 ± 1.14
	1m	58.85 ± 1.35	41.15 ± 1.98	82.66 ± 1.34	17.34 ± 0.93

Table 8. [Cu] distribution between the biologically active metal pool (BAM) and the biologically inactive metal pool (BIM) in gill tissue of rainbow trout, common carp and gibel carp. ('*' indicates a significant difference between exposed (N=6) and control fishes (N=12), *: $p<0.05$).

6.5 Discussion.

6.5.1 Total Cu concentration in plasma, liver and gill.

Environmental monitoring would benefit from a clear correlation between metal concentrations in the environment and tissue concentrations in a biomarker species. However, as illustrated by Blanchard and Grosell (2005) and De Boeck et al. (2004), there is lack of a dose-response relationship for dietary or waterborne Cu exposure and its intestinal, branchial or hepatic accumulation (Blanchard et al., 2009). In case of the gills, mucus production and surface related processes are apparently of great importance for metal accumulation in this organ (De Boeck et al., 2004; Tao et al., 2006). These characteristics may partially be responsible for the differences in Cu concentrations in gill tissue of the studied fish species in this work, even before onset of exposure. Gill tissue of rainbow trout appeared to have 15-40% less Cu present compared to the carp species. After the start of the exposure, concentrations increased stepwise in gill tissue of all fish species studied albeit more Cu accumulated in gills of common and gibel carp compared to rainbow trout. A 10 days exposure to 1µM Cu of De Boeck et al. (2004) presented increased Cu concentrations in gill tissue of rainbow trout, analogous to our results. Nonetheless, a study conducted a year earlier by the same researchers (De Boeck et al., 2003) revealed no significant changes in rainbow trout gill tissue after 7 days of exposure to the same Cu concentration (these experiments were conducted in similar conditions as the experiments of this study). As proposed by these authors, the relatively low copper binding capacity of rainbow trout gills might be reflected in a lower Cu uptake and accumulation (Laurén and McDonald, 1987). At the end of the one month exposures in this experiment, rainbow trout accumulated 3 times more Cu than control conditions in their gills, whereas the carp species accumulated 4-5 times more Cu. As for rainbow trout, Kamunde and MacPhail (2008) noticed as well a tripled Cu accumulation in the gill within 3 weeks. Elevated Cu concentrations in gill tissues have been related to a spectrum of negative effects, such as oxidative stress, DNA damage and a disrupted ion and osmotic homeostasis (Grosell et al., 2002; Handy, 2003; Wood, 2001). Another process indicative of Cu toxicity would be a raise in plasma Cu concentration, since these levels are tightly regulated (Kamunde and Wood, 2004). Although in this study, the effects on plasma were limited. In contrast, a doubling of the amount of Cu in liver tissue of control fishes was seen in common carp and triple in gibel carp. The liver is known to accumulate large amounts of excess Cu during chronically dietary or waterborne Cu exposure (Blanchard et al., 2009;

Hogstrand and Haux, 1991). In normal conditions, the liver is a principal site for metal storage in fresh water fish (Subathra and Karuppasamy, 2008) and it may also play a role in the homeostasis and detoxification of Cu (Hogstrand and Haux 1991; Kamunde et al., 2002b; Olsson et al., 1998). In contrast to the lower levels of Cu in gill and plasma, rainbow trout liver demonstrated 6 (common carp) to 30 (gibel carp) times more stored Cu in control conditions, which is remarkable. Such high Cu levels in liver tissue might be a common trait of salmonids, as this was also mentioned in a study of Julshamn et al. (1988). Despite the huge amounts of Cu present in control liver tissue of rainbow trout, this fish species accumulated ~90µg/g Cu/dry weight extra during exposure, which is a lot compared to the accumulation of ~40µg/g dry weight and ~18µg/g Cu/dry weight of respectively common and gibel carp's liver tissue during the same exposure conditions. On the other hand, ~8µg/g Cu/dry weight was stored in gill tissue of rainbow trout at the end of the exposure, whereas the carp species showed ~20.1 µg/g Cu/dry weight (common carp) to ~25.9 µg/g Cu/dry weight (gibel carp) of additional Cu accumulation. This highlights once more the relatively low Cu binding capacity of rainbow trout gills.

6.5.2 Subcellular Cu concentration in gill and liver tissue.

Differences in uptake kinetics, exposure pathways and influences of environmental conditions are factors responsible for the large differences in the bioaccumulation of metals among species (Wallace and Luoma, 2003). Furthermore, internal storage and detoxification events at the cellular level (these include metal binding proteins such as metallothionein, lysosomes, granules and membrane-bound vesicles) are of great importance to imminent toxicity (Anan et al., 2002; Mason and Jenkins, 1995; Roesijadi, 1980; Vijver et al., 2004; Wallace et al., 2003). As indicated in the introduction, this internal speciation can be studied by the use of differential centrifugation (Giguère et al., 2006a; Kraemer et al., 2006; Wallace et al., 1998). The resulting subcellular fractions can be divided into biologically active and biologically inactive metal pools, although due to the operationally defined fractions, some overlap (caused by breakage of particles or leakage of soluble constituents) is inevitable (Giguère et al., 2006b; Kamunde and MacPhail, 2008; Rainbow, 2002). Therefore, mitochondria, endoplasmatic reticulum and lysosomes were not separated and were collected in one fraction, the organelles fraction. Cu in this fraction is likely to cause toxicity when it's mostly bound to microsomes. These organelles contain fragmented endoplasmatic reticulum and are responsible for protein synthesis and transport. On the other hand, when bound to lysosomes,

it could reveal storage for eventual excretion/elimination (Bonneris et al., 2005; Fowler et al., 1987; Soto et al., 2002) and as such, detoxification. During the sub lethal exposure to Cu, only carp species had an increased Cu concentration in their gill organelles fraction, with common carp being the most pronounced. Furthermore, the organelles fraction of common carp liver was elevated in the beginning of the exposure and after 1 week. The associated Cu concentration in the whole liver fraction after 24 hours was not significantly different from control values. However, an increase in Cu concentration in the HDF was noticed. This fraction is assumed to contain denatured enzymes and other non-enzymatic proteins, such as albumin (Giguère et al., 2006b). When Cu concentration is higher in this fraction compared to control values, it could reflect conflicting binding of Cu to potential metal sensitive/biologically active sites (Giguère et al., 2006b), inhibiting proper enzyme function. Apart from common carp liver, the liver of the other 2 fish species studied showed no increase in the HDF. In contrast, gill tissue HDF was affected in all three fish species; in common and gibel carp, HDF Cu concentration was higher after 24 hours and 1 month, whereas in rainbow trout gill tissue, this raise was noticeable after 1 week and was again observed after 1 month. The relative amount of Cu in the HDF compared to the total amount of Cu accumulated in the three fish species differed quite remarkably as well. Where the HDF of rainbow trout gill tissue comprised 30% in control gills, its proportion of the total accumulated Cu rose from the start of the experiment to the end to 60% of the total Cu accumulated. While in the carp species, the Cu partitioned in the HDF fraction increased in the beginning (from 34% to 65% after 24 hours), but decreased thereafter (34%).

Beside HDF, the CD fraction showed elevated Cu concentrations at the same time points in gill tissue of rainbow trout and common carp. In gibel carp, there was only a difference with control values after 1 month of exposure. Rainbow trout's liver tissue showed solely an increase in CD concentration and this already after 24 hours. In rainbow trout, it seems that accumulated Cu is directed quickly to liver tissue, where it is assigned to the CD fraction and the GR fraction, but only during the first days of exposure. In the other tissues (liver and gill of common carp, gill of gibel carp), the latter fraction wasn't affected until the end of the exposure. Kamunde and MacPhail (2008) noticed no change in the GR fraction in liver during a 21 days Cu exposure of rainbow trout. However, as illustrated in this study with an increased Cu concentration in this liver fraction after 24 hours of exposure, a role for these granules in acute Cu exposure conditions is still plausible. These insoluble metal rich granules (Bonneris et al., 2005; George, 1983) and metallothioneins (Roesijadi, 1992) are among the

most studied components involved in the sequestration of metals and both are part of certain metal detoxification pathways (Klerks and Bartholomew, 1991; Wallace et al., 2003; Wallace and Lopez, 1997). For instance, in invertebrates, the sequestration of excessive amounts of metals with metal rich granules is one of the most important mechanisms to protect themselves against metal toxicity (Rainbow, 2002; Wallace et al., 2003). Metallothioneins (MT) and cytosolic cysteine rich metal binding proteins effectively buffer labile metal ion levels in the intracellular environment (Bonneris et al., 2005).

These MT's and glutathione (GSH) are heat stable proteins and form, together with other heat stable proteins, the remaining cytosolic fraction; the HSF (Giguère et al., 2006b). MT's are thought to be the major contributor to this fraction, although for rainbow trout gill this could be different since there are high amounts of GSH present in their gill tissue (Eyckmans et al., 2011). No induction of the HSF was seen in rainbow trout gill, in contrast to liver, where after 1 month of exposure, the HSF was increased. This result was consistent with a previous study of Kamunde and MacPhail (2008) who exposed rainbow trout to 40 µg/l Cu for 21 days and observed an increase in the Cu concentration of the HSF as well. De Boeck et al. (2003), studied MT induction in rainbow trout, common carp and gibel carp exposed to 1 µM Cu for 7 days. They noticed no difference in MT concentration in liver and gill tissue of rainbow trout, and stipulated that salmonid liver MT was not as easily induced by Cu as MT in cyprinid fish species. The increases of HSF in the carp species in this study were indeed more pronounced compared to rainbow trout. Especially gibel carp reacted swiftly in gill tissue, which correlated with the results of De Boeck et al. (2003) who saw a fast and elaborate defensive response in gibel carp gill tissue. This quick binding of excess Cu to MT could prevent toxic effects to occur and could be an important factor in the higher tolerance of gibel carp to Cu exposure. Other fish species, such as white suckers and perch, have also shown to increase the amount of metal in the HSF fraction (Hogstrand et al., 1991; Klaverkamp and Duncan, 1987). As well as the sequestration capacity of MT's, these proteins may protect against the elicited oxidative stress (Paris-Palacios and Biagianti-Risbourg, 2006; Review Viarengo et al., 2000). As seen in previous research (Eyckmans et al., 2011; Lloyd and Phillips, 1999), Cu is indeed a strong oxidative stressor in fish species. In this aspect, gibel carp was also able to induce their anti-oxidant defenses much sooner compared to common carp and rainbow trout, aiding in minimizing the toxic effects of excess Cu exposure (Eyckmans et al., 2011).

6.5.3 Distribution of Cu between biologically active metal pool/ biologically inactive metal pool.

Cu has various roles in the different subcellular fractions; the organelles fraction, nuclei-cell debris fraction and heat denaturable fraction contain Cu that is biologically active metal (BAM). In contrast, the Cu in the metal rich granule fraction and heat stable fraction is supposed to be biologically inactive metal (BIM) (Steen-Redeker and Blust, 2004) since Cu is sequestered and therefore not able to cause structural changes to vulnerable proteins, membranes and DNA. The more Cu is distributed in the BIM, the better the tissue can address the toxic effects of excess Cu exposure. Furthermore, increased tolerance has been related to the detoxification capacities of MT and GR. The opposite is true for a large pool of Cu in BAM, which could indicate an elevated risk of toxicity (Rainbow, 2002).

Even in control conditions, the Cu distribution in BAM/BIM was very different for the studied fish species. The most striking difference was the high % of Cu in BAM in rainbow trout gill and liver tissue, the most sensitive fish species in this study concerning Cu toxicity (De Boeck et al., 2004). This high % of Cu in the BAM of the liver of rainbow trout is not only 25-50% higher than in the carp species, the concentration of the accumulated amount of Cu in the liver is as well remarkably higher, which may contribute to increased availability. In the study of Kamunde and MacPhail (2008) a concurrent significant accumulation of Cu in BAM and BIM of rainbow trout liver was observed. Other studies showed similar results (Giguère et al., 2006b) and the suggestion has been made that Cu-binding sites are universal in liver tissue. In this experiment, only 3 subcellular fractions of rainbow trout liver had significantly accumulated Cu during this 1 month exposure. Nonetheless, the Cu distribution between BAM (~70%) and BIM (~30%) in control liver tissue was similar but the distribution changed (~50% in each pool) after 3 days, which lasted for the entire experiment. Although the organelles fraction (BAM) accounted for 60% of the total Cu in control liver, it did not accumulate a great amount of the excess Cu during exposure. The accumulated Cu was distributed in the HSF fraction of the biologically inactive metal pool, which went from a ~30% Cu concentration in control liver tissue to ~50% after the 1 month exposure. Chronic subcellular Cu handling in rainbow trout liver was probably characterized by MT sequestering or GSH binding and only little by accumulation in the biologically active metal pool.
In gill tissue of rainbow trout, control Cu distributions were comparable with liver tissue (BAM ~75%, BIM ~25%), however already early in the experiment, this distribution shifted

towards an increased BAM (~85%) and decreased BIM (~15%). The increased Cu accumulation in the BAM was caused by an enhanced partitioning of Cu in the HDF while no additional accumulation occurred in the HSF. So, in contrast with rainbow trout liver tissue, subcellular Cu handling in gills was defined by accumulation in the biologically active metal pool. Researchers studied subcellular Cd distribution in rainbow trout as well (Kamunde, 2009) and in this case, found a similar gill BAM increase while BIM decreased.

As the Cu concentration in common carp gill increased, concentrations of this metal were increased in all sub-cellular fractions, at the end of the exposure period. This contraindicates the spillover concept of metal toxicity which states that the excess of metal ions will leak into the BAM only when the detoxification capacity is exceeded (Kamunde and MacPhail, 2008; Rainbow, 2002; Vijver et al., 2004). BIM was responsible for ~40% of the accumulated Cu in gills of control common carp. This contribution decreased in the first week to ~20% but was brought to ~40% again at the end of the exposure. The accumulated Cu was mainly found in the HDF fraction, with an increased BAM as consequence (~75-80%) and thus showing similar subcellular Cu handling as rainbow trout gill.
In common carp's liver tissue, the Cu concentration in both pools increased to an equal level (~55% BIM and ~45% BAM) and Cu manifested itself in the GR, OR and the HDF. Hence, no mechanism was in place to prohibit Cu to accumulate in the BAM. The same was true for liver tissue of gibel carp, where the relative proportions were unchanged as well, although the original distribution favored Cu accumulation in the BIM (~80%) rather than the almost equal partitioning as in common carp. Subsequently, the subcellular handling portrayed more protection towards the BAM from the start. The vast amount of Cu present in the liver of gibel carp was constituted to the GR (47%) and HSF fraction (33%) and although the latter had a smaller contribution to the total, it accumulated (up to 44% of the total Cu at the end of the exposure) more Cu compared to GR. Interesting was the high % of Cu stored in the GR fraction, since this has been suggested to be a long-term storage of essential metals (Wallace et al., 2003). A number of invertebrates are known to deposit large amounts of detoxified metal in the form of granules (Rainbow, 2002; Wallace et al., 2003). Regardless of this high storage of Cu in the GR fraction of gibel carp liver, the actual amount of Cu accumulated in liver during the exposure was modest. As such, the biological available amount of Cu was minimal in gibel carp liver. In contrast to the Cu distribution in liver of gibel carp, the partitioning in gill did show equal accumulation in BIM and BAM (~50%) in an unexposed

situation. In the first week however, the Cu accumulated in BIM increased to ~ 65%, through an increased HSF. The increased HSF was therefore able to minimize the first toxic effects of Cu in gill tissue. Just as in common carp's gill tissue, it decreased again to control situations at the end of the exposure period.

As the most sensitive of the three studied fish species, rainbow trout gills are known to show toxic effects in the first days of Cu exposure, despite the relatively low Cu accumulation in this organ. Immediately, gill Na^+/K^+- ATPases are blocked and $[Na^+]$, $[Cl^+]$ and $[Ca^{2+}]$ in rainbow trout plasma are decreased as a result of the Cu exposure (Eyckmans et al., 2010). This sodium loss can ultimately lead to cardiac arrest and death (Evans et al., 1999). The % of Cu in the BAM of rainbow trout gills comprised the vast majority of the total Cu present in gills. Therefore, it is possible that these gills were not able to anticipate the changes in Cu concentration and consequently that the increased % of Cu in the BAM caused enzyme inhibition and increased oxidative stress. After 3 days of exposure, the superoxide dismutase in gill tissue of rainbow trout showed an increased activity to cope with the oxidative stress, where common and gibel carp did not show enhanced activities in neither of the studied anti-oxidant enzymes when exposed to 50µg/l Cu (Eyckmans et al., 2011). Moreover, the $[Na^+]$, $[Cl^+]$ and $[Ca^{2+}]$ in plasma of common and gibel carp were only decreased after a week of exposure and did not return to control concentrations after one month as was seen in rainbow trout. Surprisingly, no respiratory distress occurred in rainbow trout (De Boeck et al., 2006, 2007) compared to gibel and common carp who lowered their metabolic rate and were respiratory more challenged under Cu exposure. The accumulated Cu in gills of the carp species was higher compared to rainbow trout, however since the carp species brought their Cu levels in BAM and BIM in gill tissue at control levels after 1 month of exposure, they were more capable of surviving the toxic effects. On the other hand, rainbow trout liver does not seem to experience numerous negative effects of Cu exposure. The high concentration of Cu present in control fishes suggests a more flexible mechanism in this organ. The additional Cu that was accumulated during exposure was deposited mostly in the HSF fraction, thus in the BIM, rendering the Cu biologically unavailable. Common carp control livers displayed an equal distribution of Cu between the BAM and BIM together with an equal distribution between those pools when Cu concentration was increased. Gibel carp liver is actually even better equipped in handling excess Cu, since ~80% of the Cu in control fishes was already in the BIM and this distribution was kept constant during the Cu exposure to 50µg/l. The additional Cu was mainly added to the HSF and OR fraction, however, the total amount of

accumulated Cu in gibel carp liver was reasonably low. As proposed in De Boeck et al. (2003), the kidney of this fish species is probably responsible for this feature, since they are believed to clear Cu efficiently. These facets of gibel carp physiology are great contributors to its tolerance to Cu exposure.

6.6 Conclusion.

A striking difference between the studied fish species was the high Cu concentration in rainbow trout control liver and its low Cu concentration in control gills, compared to the carp species. The high Cu concentration in their liver is a perfect example of the theory that not only the total amount of Cu burden is important in causing toxicity, but the subcellular distribution is a key component in this matter. As Taylor et al. (2000) described, the elevated Cu burden of exposed fishes were not measures of effect, though were measures of chronic Cu exposure. This was also illustrated by the relatively low amounts of accumulated Cu in rainbow trout gill tissue compared to the numerous toxic effects present in this organ (previous research De Boeck et al., 2004; Eyckmans et al., 2010; 2011), showcasing this fish species high sensitivity to Cu.

Not surprisingly, Cu was found in all liver and gill subcellular compartments, which emphasizes the essential nature of this metal. When the amount of Cu accumulated in a metal sensitive fraction increases out of proportion, toxicity can occur easily. Therefore, it is fascinating that the HDF in rainbow trout gill tissue was used to store most of the additional Cu that was acquired during exposure in proportion to the total amount of Cu that was accumulated at a specific time point. As such, the enzymes in this fraction were accessible for Cu binding, rendering them highly vulnerable, which could lead to toxic effects in gill tissue. In contrast, the carp species were able to minimize the additional storage of Cu in HDF in the first week of exposure and thereafter partitioned the Cu to the biologically inactive metal pool (if looked at the % of Cu compared in the BIM to the total Cu accumulated at that time point). This was mainly the case for gill tissue of both carp species.

The liver of rainbow trout seemed much more adequate in handling the excess Cu compared to gill tissue, since the storage of Cu in the biologically inactive metal pool increased, with a proportional raise of the accumulated Cu in HSF from ~30 to ~50%. This suggests that the gills are more susceptible for Cu toxicity relative to liver, as was also noticed by Kamunde (2009) for Cd toxicity in rainbow trout gill and liver. In all three fish species studied here,

increasing trends of either GR or HSF were seen in liver tissue, suggesting that the detoxification systems were not saturated. The high % of Cu in GR and HSF of the carp species, and especially gibel carp, reflected their ability in handling Cu excess in a protective manner, which could imply a biological advantage. The differences in Cu distribution at the subcellular level of gills and liver of the studied fish species strongly reflects their capacity to handle Cu excess and explains ultimately their differences in sensitivity to Cu toxicity.

6.7 Acknowledgements.

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Chapter 7

Conclusion

Environmental awareness concerning toxicants has increased during the last few decades. After all, the quality of the environment is genuinely important in maintaining healthy ecosystems and protecting life as we know it today. Due to their extensive use, metals, that used to exist in traces in the environment, represent a substantial fraction of the pollutants released in air, soil and water. To prevent the situation of escalating any further, federal and state governments have instituted environmental regulations to protect the quality of surface and ground water from heavy metal pollutants and to protect the aquatic organisms. As exposed organisms try to cope with pollution, they develop species-specific defense mechanisms, which are rarely fully apprehended. This doctoral thesis aimed at revealing different players and protective mechanisms in the Cu resistant gibel carp, the intermediate common carp and the sensitive rainbow trout by the means of studying the involved hormone regulation, oxidative stress reactions, dynamics of specific metal binding protein, chaperones and transporters and subcellular distribution.

Investigating the relationships between plasma ions, plasma T3/T4, gill Na^+/K^+-ATPase activity and gill mRNA concentrations of PRL receptors and THR β gave us an insight in how these fish species manipulated their ionoregulatory important hormones and ion osmoregulatory processes when introduced to Cu (Chapter 4). As previous research indicated (Grosell et al., 2002; Pyle and Wood, 2008), the rate of the diffusive ion loss from extracellular fluids to the surrounding dilute environment played a crucial role in survival, more so than the total loss of ions. The fact that rainbow trout showed extensive decreases in $[Na^+]$, $[Cl^-]$ as well as $[Ca^{2+}]$ in the very first moments and days of exposure to 50µg/l Cu whereas the decline in plasma ions in carp species only started after 3 days, can be very substantial for the fish species ability to endure metal stress. Interestingly, the induced osmoregulatory disturbances in rainbow trout were compensated and returned to control levels after one month of exposure. Furthermore, this was also observed in the only surviving cannulated rainbow trout of a De Boeck et al. (2007) study on respiratory stress and Cu exposure. Common and gibel carp on the other hand did not regain normal ion concentrations in plasma by the end of the experiment although they were able to withstand higher Cu concentrations than rainbow trout. Ion leakage through damaged membranes, inhibition of carbonic anhydrase, Na^+/H^+ exchangers, Na^+ channels and Na^+/K^+-ATPase activity in gill tissue are known contributors to the decreased ion concentrations (Blanchard and Grosell,

2006; Kurilenko et al., 2002; Laurén and McDonald, 1985). Cu rapidly interfered with the Na^+/K^+-ATPase activity in this study since rainbow trout and gibel carp showed blocked Na^+/K^+-ATPase activity at 12 hours of exposure and for gibel carp activity was disturbed even up to one week of exposure to 150µg/l Cu. With compensatory mechanisms, such as stimulation of Na^+/K^+-ATPase synthesis and enhanced chloride cell turnover, elicited by an increased prolactin concentration, fish can try to repair the occurring damage. Rainbow trout, although used to transitions from fresh to salt water where prolactin is very important, showed little effects on prolactin receptor (PRLR) mRNA in gill tissue. The concentration of PRLR mRNA in carp species however, showed instead of the expected increase in mRNA, decreases at several time points during Cu exposure. Comparable results were found for thyroid hormone receptor β (THRβ) mRNA expressions in gill and [T3] in plasma of gibel carp, whereas in common carp and rainbow trout fewer or no effects were seen. These results could indicate that gibel carp depressed its metabolism as a protective response to the Cu exposure. Remarkably, all fish species showed an extensive peak in [T4] concentration after 1 hour of exposure to Cu, which lasted a week for rainbow trout exposed to 50µg/l. This steep rise could cause an initial augmentation in metabolism, in order to handle the first stress of the metal exposure, apparent in all three fish species.

Furthermore, depression of the metabolism by hypothyroidism as seen in gibel carp could lead to a decreased oxidant production and could hereby provide an extra protection mechanism of the organism to prevent oxidative damage by the excess Cu ions (Alturfan et al., 2007).

Since a well performing anti-oxidant defense system is essential for any organism to protect itself against the adverse effects of Cu exposure, superoxide dismutase activity, catalase activity, glutathione reductase activity and reduced glutathione and ascorbate concentrations in gill tissue after Cu exposure were determined. As such, we could assess if differences in anti-oxidant defenses in the gills of these fish contributed to the differences in sensitivity to Cu exposure (Chapter 5). Even without the opposed Cu exposures, striking differences between the studied fish species became apparent. Rainbow trout showed double amounts of reduced [GSH] and increased glutathione reductase (GR) activities in gill tissue compared to the cyprinids. On the other hand, gibel carp had more than twice the level of reduced [Asc] in their gills. Both features offer a certain asset in dealing with the increasing oxidative stress during Cu exposure, mostly by scavenging free Cu ions.

It has been demonstrated by De Boeck et al. (2004) that the most Cu sensitive species, the rainbow trout, slowly accumulates Cu in their gill tissue. Instead, most of the Cu was accumulated in liver tissue. Since the increase in Cu concentration in the gills was restrained, this might be a good explanation of the limited and delayed response of the investigated anti-oxidant enzymes in gill tissue. Furthermore, the high levels of GSH and GR present in rainbow trout gills possibly reflects the use of this molecule and enzyme in the first line of defense in reaction to oxidative stress. Moreover, it has been confirmed by De Boeck et al. (2003) that rainbow trout was unable to induce an increase in metallothionein, which are thought to be very important as a first response to increased metal concentrations, following a Cu exposure. Similar, no metallothionein response could be detected in the gills of common carp exposed to Cu. However, there were indications of GSH being important as a first defense response as well. Additionally, De Boeck et al. (2004) pointed out that Cu accumulated very quickly in gills of common carp. As such, it was very interesting to see that the anti-oxidant enzymes studied reacted immediately in the beginning of the exposure as well, albeit only when exposed to 65µg/l and not when exposed to 50µg/l Cu. Common carp exposed to 50 µg/l Cu did not seem to suffer much from oxidative stress since no anti-oxidant enzymes were induced or inhibited. The same was true for gill tissue of gibel carp exposed to 50 µg/l Cu. On the other hand, gibel carp exposed to 150 µg/l Cu showed the most efficient response in anti-oxidant enzymes. This response, combined with the high Asc levels and the established good correlation between gill metallothionein induction and Cu accumulation (De Boeck et al., 2003), give the gibel carp the most tools to minimize the damage caused by Cu elicited oxidative stress.

Intracellular differences in protein expressions of metallothionein or other transporters, carriers and metal binding proteins, might as well be key components in adequately diminishing negative effects opposed by Cu exposure. By studying the proteome of gills of the three fish species, we tried to evaluate the dynamics of these protein patterns and determine the resulting added value in the defense mechanisme to Cu (Chapter 6). During the 2D-Dige and iTRAQ analysis it became apparent that the succesful identification of the proteins was restricted due to the limited number of identified proteins concerning the studied fish species. As such, the study can only give a first indication of possible effects of the Cu exposure. Although this is a draw back, it is also inherent to proteomics and it does not diminish the valuability of the current results. The WGCNA software package used to cluster highly correlated expression profiles of

proteins (or genes) proved to be very helpful in approaching the large scale dataset and offered a tool to link biological traits or parameters with groups of correlated proteins.

The sensitive rainbow trout showed some signs of increased oxidative stress, energy production and decreased metal binding capacities. This decrease in metal binding capacities was especially highlighted in the 2D-Dige analysis with a decreased expression of transferrin and albumin in the gills. On the other hand, an increased carbonic anhydrase expression might be a defense response to counteract the inhibition of this enzyme during Cu exposure (Perry et al., 2003). The iTRAQ analysis and subsequent WGCNA analysis revealed some additional assets such as an increased Hsp70/Hsp90 organizing protein, a decreased meyeloperoxidase expression and a correlation between Cu concentration and superoxide dismutase activity (Castro et al., 2008; Lewis et al., 2010). Other observed effects were situated in the immune response, protein metabolism and the oxidative stress related alteration of the actin cytoskeleton (Chen and Chan, 2009). The latter group of differentially expressed proteins was highly represented in the effects of Cu on the proteome of common carp gills as well. In this fish species, a number of proteins related to transcription and translation, increased protein production and energy consumption were present in the proteome analysis, indicating an enhanced use of energy and proteins to give a first start in the recovery from the toxic effects caused by Cu. The decreases of translationally controlled tumor protein and cold inducible RNA binding protein were very interesting since both are thought to be related to effects caused by metal stress (De Leeuw et al., 2007; Venugopal et al., 2005). Furthermore, the translationally controlled tumor protein is associated with Na^+/K^+-ATPase of which the activity is mostly inhibited by excess Cu as well (Eyckmans et al., 2010; Venugopal et al., 2005). Of all three fish species studied, gibel carp's proteome is the least annotated. However, proteins that were positively identified indicated overall an increased protection to adverse Cu effects. Transferrin increase ensurres an enhanced metal binding property (Welch, 1992), Apolipoprotein A I protects cell membrane integrity (Smith et al., 2005) and Heat shock protein 70 aids in repair and protection of the cell (Boone and Vijayan, 2002). The location of the accumulated Cu in the cell is therefore also very important. As such, we wanted to evaluate the dynamics of the subcellular accumulation patterns in three different fish species under a similar scenario of sublethal copper exposure.

The aim of the analysis of copper concentrations in subcellular compartments was to assess whether gill and liver cells of fish with a different sensitivity to copper exposure show

intracellular differences in accumulation and handling of copper and whether this could increase the insights in their differences in sensitivity to metal exposure (Chapter 7). And indeed, this study proved to be the most promising in terms of understanding the occuring differences between the three studied fish species. Previous research already drew attention to the low Cu accumulation in gill compared to high Cu accumulation and control concentrations in rainbow trout liver (De Boeck et al., 2004). Compared to common and gibel carp, rainbow trout had 15 to 40% less Cu in their control gills and 60 to 300% more Cu in their control liver. Especially for liver, these differences are immens. During the exposure to Cu, gill tissue accumulation increased stepwise in all three fish species albeit more Cu was found in gills of the carp species (~20.1 µg/g Cu/dry weight for common carp and ~25.9 µg/g Cu/dry weight for gibel carp) compared to gills of rainbow trout (~8.0 µg/g Cu/dry weight), highlighting the relatively low Cu binding capacity of rainbow trout gills. Likewise, the vast capacity of Cu accumulation in rainbow trout liver was also confirmed since an additional ~90µg/g Cu/dry weight was added to control concentrations of ~250µg/g Cu/dry weight. This is significantly higher compared to the ~40µg/g dry weight and ~18µg/g Cu/dry weight of respectively common and gibel carp's liver tissue added to the control Cu concentrations of ~43µg/g Cu/dry weight and ~10 µg/g Cu/dry weight during similar Cu exposures. Besides these differences in total accumulated Cu concentrations, possibly more importantly is the partitioning of Cu within the cell. As Steen-Redeker and Blust (2004) proposed, the proportion of metal stored in the biologically active metal compartment (BAM) (which in this study comprises the organelles fraction, nuclei-cell debris fraction and heat denaturable fraction) is more susceptible of toxic effects caused by increased metal concentrations. In contrast, the biologically inactive metal compartment (BIM) (which in this study comprises the heat stable fraction and the metal rich granule fraction) sequesters excess metals and renders it unavailable for adverse effects (Rainbow, 2002).

Interestingly, the most sensitive species, rainbow trout, showed a high accumulation of ~70% of Cu in BAM in both investigated tissues even in control conditions. Moreover, the gill tissue distributed the additional Cu in BAM (increase to ~85%) through deposits in the heat denaturable fraction (more available for adverse interactions) (comprised 60% Cu in gill compared to 30% in control conditions) before restoring control distributions. Furthermore, liver tissue was able to use the heat stable fraction to accumulate the excess Cu (more protection). Although the organelles fraction (BAM) accounted for 60% of the total Cu in control liver, it did not accumulate a great amount of the excess Cu during exposure, which is

remarkable and may indicate a good developed partitioning system in liver tissue of rainbow trout. The opposite was true for liver of common carp, were no mechanism was in place to prohibit Cu of accumulation in the BAM since Cu was deposited in the organelles fraction, heat denaturable fraction and metal rich granule fraction. Moreover, in gill tissue of common carp, all subcellular fractions showed increased Cu concentrations at the end of the exposure period with a main distribution in the heat denaturable fraction resulting in an increased BAM %. Most surprisingly, the resistant gibel carp was able to control Cu distribution in both tissues by increasing the partitioning of Cu in the heat stable fraction in gills (with a return to control conditions at the end of the exposure) and in the same fraction as well as in the metal rich granule fraction in liver. The original distribution in control conditions of liver of gibel carp with an immense ~80% of Cu stored in BIM and the persistence of this distribution during Cu exposure implicates a great advantage for gibel carp. Moreover, the biological available amount of Cu was minimal in the liver of gibel carp, protecting essential enzymes, proteins and DNA.

In summary, some advantages and disadvantages of each fish species physiology while handling Cu excess will be discussed, beginning with the most sensitive fish species studied. Rainbow trout is a more vulnerable species with a high need for clean streams with abundant oxygen concentrations compared to the more robust cyprinid species, which can withstand a greater array of environmental conditions. However, as a migrating species, rainbow trout are thought to be able to handle changes in ion concentrations more adequately compared to the cyprinids. And indeed, once rainbow trout survived the initial shock phase i.e. losing vast amounts of plasma ions in the first days of Cu exposure combined with an inhibited Na^+/K^+-ATPase activity in the gill, they restored their ion homeostasis quickly to control values. An increased expression profile of carbonic anhydrase, as noticed in the proteomic analysis, could be an additional advantage in the restoring process. However, the losses of ions in the first hours of exposure can also result in cardiac arrest if the amount of ions lost was too high to overcome.

Beneficiary for rainbow trout is the slow Cu accumulation in gill and its relatively fast transfer of absorbed Cu to the liver. Compared to the gills, the liver appeared better equipped for handling the excess Cu, since the liver demonstrated very high Cu concentrations in control conditions and stored the additional Cu in the biologically inactive metal

compartment, preventing the interaction with enzymes, proteins and DNA. In gill, most of the Cu was present in the biologically active metal compartment where the excess Cu was deposited during Cu exposure as well, rendering the gill cells susceptible for toxic effects. As such, the quick distribution of absorbed Cu to the liver is an advantage for rainbow trout. Furthermore, De Boeck et al. (2004, 2006 and 2007) noticed relatively little gill damage in a week exposure to Cu correlating to the lack of respiratory distress. Nevertheless, the Cu concentration in gill tissue increases when exposure is prolonged and after a few days, superoxide dismutase activity was enhanced to cope with the oxidative stress. Interestingly, the expression profile of superoxide dismutase was as well increased at the same time point when the activity of this enzyme was enhanced. However, it is likely that the high reduced glutathione concentrations and glutathione reductase activity in gill are the defense mechanisms of choice for rainbow trout, more so than other anti-oxidant enzymes. This hypothesis is supported by the observation that metallothionein was not induced in Cu exposed gills of rainbow trout which makes the reduced glutathione even more important in handling metal toxicity (De Boeck et al., 2003). Despite several interesting adaptations to increased metal concentrations, the rainbow trout remains 3 to 7 times (De Boeck et al., 2004) more sensitive to Cu than common and gibel carp and is just not flexible enough to handle excessive increases of Cu.

Common carp and especially gibel carp can withstand much higher Cu exposures. These cyprinid species display similar reactions to increased metals with gibel carp reacting more thoroughly in almost each aspect. When common and gibel carp were exposed to 50µg/l, the concentration where rainbow trout demonstrated an array of effects, almost no changes in the observed parameters were seen. However, exposure to their 10%LC50 value (for common carp 65µg/l and for gibel carp 150µg/l Cu) elicited different results. As mentioned, rainbow trout are very vulnerable for ion losses in the beginning of a toxic metal situation. Common and gibel carp did loose ions as well, however this became apparent only after a week of Cu exposure. No recovery of ion levels in plasma was seen when the exposure ended, suggesting a changed and reestablished ion homeostasis for the cyprinids. Where rainbow trout was efficient in distributing excess Cu to the liver, the cyprinids accumulated more Cu in their gills. However, for these fish species, it did not seem to be a real disadvantage (despite an increased Cu distribution in the BAM of common carp gill tissue in the beginning of the exposure) since they were capable to restore the distribution of Cu in the BIM and BAM to control levels after 1 month of exposure. Especially for gibel carp, were the percentage of Cu

in the BIM was exceptionally high in gill tissue, as well as in liver tissue, the amount of free or labile Cu was maintained at minimum levels. The clear correlation of increasing Cu concentrations in gill with increasing metallothionein induction (De Boeck et al., 2003) adds to the tight regulation of Cu in gibel carp gill tissue. Moreover, where the proteomics results point in a direction of increased use of proteins and energy in common carp, the few identified proteins in gibel carp all were capable of decreasing negative effects by increased binding to excess metal (increased transferrin) and increased membrane and cell protection (increased apolipoprotein A I and heat shock protein 70). Furthermore, both cyprinid species induced the activity of the studied anti-oxidant enzymes in the beginning of Cu exposure, albeit gibel carp outdid common carp by reacting even more swiftly. Perhaps this difference can be related to the observed difference in the pattern of gill damage (De Boeck et al., 2007), where common carp showed a first shock in the beginning of the exposure, followed by repair and recovery. In contrast, gills of gibel carp experienced less gill damage up to three days of exposure and only thereafter loose epithelia and gill damage increased. Moreover, gibel carp possibly attempts to reduce ion loss and decrease Cu uptake by embedding their secondary lamellae in an interlamellar cell mass (Sollid et al., 2003). In both cyprinid species respiratory challenges during the first days of exposure were also more apparent compared to rainbow trout (De Boeck et al., 2007), possibly likewise linked to the changes in gill structure. Additionally, gibel carp, and to a lesser extend common carp, can lower their metabolic rate to further decrease Cu uptake via the gills and restrain the production of reactive oxygen species. Besides these features, gibel carp can benefit from high ascorbate concentrations in their gills to help in decreasing free or labile Cu concentrations and can profit from an efficient Cu clearance by the kidney (De Boeck et al., 2003). All these facets of gibel carp's physiology contribute to its tolerance to Cu and reflects their efficient response to increased concentrations of this metal.

To conclude, the results clearly provide an insight in the sensitiveness of rainbow trout, the intermediarity of common carp and the resistantness of gibel carp in handling excessive Cu concentrations during waterborne Cu exposure.

Chapter 8

Nederlandstalige samenvatting

Ondanks de recente verbeteringen van de Vlaamse oppervlaktewateren, voldoet 85% nog steeds niet aan de vooropgestelde richtlijnen. Door hun wijdverspreide gebruik blijven metalen een groot deel uitmaken van het probleem rond watervervuiling. Sommige van deze metalen zijn essentiële sporenelementen voor bepaalde cellulaire processen maar kunnen toxisch worden wanneer de concentratie een zekere drempelwaarde overschrijdt. Koper is bijvoorbeeld een metaal met deze eigenschap. Dit roodachtige metaal komt voor in de aardkorst en wordt al meer dan 7000 jaar gebruikt door de mensheid, eerst voornamelijk in wapens en juwelen, later, bij het ontstaan van de mijnindustrie, ook in elektrische draden, metalen pijpen, als pesticide in de agricultuur, enz. Het feit dat metalen niet biologisch afbreekbaar zijn maar juist persistent, is betreurenswaardig omdat de concentratie in het ecosysteem dan sneller wordt opgebouwd. Bodem, sediment, water en organische materialen in stedelijke en industriële gebieden zijn gekend voor hun hogere metaalconcentraties. De mate van toxiciteit wordt vooreerst bepaald door de biobeschikbaarheid van het metaal. Wanneer de biobeschikbaarheid laag is, heeft het metaal complexen gevormd met anorganische of organische materie waardoor het moeilijker wordt opgenomen in organismen. Wanneer het metaal niet gebonden is en in een vrije vorm voorkomt, is de biobeschikbaarheid groot en kan het snel worden opgenomen. Voor koper wordt er aangenomen dat Cu^{2+} de meest toxische vorm is, gevolgd door $CuOH^{-}$ en $Cu(OH)_2$. Wanneer zoetwater vissen in contact komen met toxische koper concentraties, vertoont het kieuwweefsel acute oedeem en loskomen van het epitheelweefsel. Dit wordt veroorzaakt door een osmotische influx door (onder andere) de inhibitie van het enzym Na^{+}/K^{+}-ATPase in de kieuwcellen en door de vervanging van Ca^{2+} door Cu^{2+} in de membranen, wat een accumulatie van ionen in de kieuwcellen en een verlies van ionen aan de omgeving teweegbrengt. Dit kan uiteindelijk leiden tot een cardiovasculair falen en sterfte. Tijdens een minder acute maar meer chronische blootstelling kunnen vele fysiologische processen, waaronder de ademhaling, het metabolisme, de immuniteit, de endocriene functies, de zwemsnelheid, de groei en de reproductie aangetast worden. Koper wordt voornamelijk in de kieuw, lever, darm en nieren van zoetwater vissen opgeslagen. Omdat de kieuwen een groot contactoppervlakte met het omgevende water hebben en onmisbaar zijn in de ion regulatie, wordt dit weefsel als erg gevoelig beschouwd, zeker omdat het weefsel als eerste in contact komt met de hoge metaalconcentraties. Ook tijdens chronische blootstellingen zijn de tekortkomingen op het vlak van ion regulatie de eerst merkbare effecten. Bovendien bezit koper pro-oxidant eigenschappen door zijn chemische speciatie, waardoor reactieve zuurstof radicalen gevormd

worden die enzymen inhiberen en schade toebrengen aan lipiden, eiwitten en DNA. Uit vergelijkende fysiologische studies is gebleken dat blootgestelde organismen soort specifieke verdedigingsmechanismen hebben ontwikkeld die, bij de ene soort al beter dan de andere, toelaten om met verhoogde metaal concentraties om te gaan. Doch de onderliggende verschillen die aan de basis liggen van de reacties op stressoren, zijn vaak nog onbegrepen. Deze doctoraatsthesis had dan ook als doel om dit verder te bestuderen in 3 vissoorten waarvan reeds voordien opgemerkt was door De Boeck et al. (2004) dat ze verschilden in gevoeligheid aan koper; de gevoelige regenboogforel (*Oncorhynchus mykiss*), de intermediaire karper (*Cyprinus carpio*) en de resistentere giebel (*Carassius auratus gibelio*). In deze drie vissoorten (waarvan de karper en de giebel beiden karperachtigen zijn) werd er gekeken naar ion regulatie, oxidatieve stress reacties, expressie patronen van het kieuw proteoom en de intracellulaire distributie van koper.

De experimenten werden uitgevoerd in een klimaatskamer (17°C) aan de Universiteit van Antwerpen, België. De vissen werden in groene 200l aquaria gehouden voorzien van gefilterd drinkwater en ad libitum gevoed. De waterkwaliteit (zuurstofsaturatie >90%, pH 7.6 ± 0.2, ammonium, nitraat en nitriet <0.1 mg/l) alsook de totale concentratie metaal in het water, werden doorheen de acclimatisatie en de blootstelling op vaste tijdstippen nagekeken. De vissoorten werden telkens onderworpen aan 2 verschillende sub letale koper concentraties. In een eerste reeks experimenten werkten we met de Vlaamse norm voor oppervlaktewater (50µg/l Cu). Door het blootstellen aan eenzelfde concentratie was het mogelijk om verschillen in gevoeligheid waar te nemen. In een tweede reeks experimenten gebruikten we een concentratie die 10 maal lager was dan de 96u LC50 waarde van de soort. Zo kunnen we een idee krijgen van de verschillen die optreden wanneer de vissen eenzelfde belasting van koperblootstelling ondergaan (regenboogforel: 20µg/l Cu, karper: 65µg/l Cu en giebel: 150µg/l Cu; uit De Boeck et al., 2004). Tijdens elke blootstelling werden de vissen intensief gevolgd met staalnames (per 8 voor elke conditie, tijdspunt en vissoort) na 1u, 12u, 24u, 3 dagen, 1 week en 1 maand.

Het onderzoeken van de relatie tussen plasma ionen, plasma T3/T4, kieuw Na^+/K^+-ATPase activiteit en kieuw mRNA concentraties van prolactine receptoren en schildklierhormoon receptor β, bezorgde ons een inzicht in de manier waarop de bestudeerde vissoorten hun ion regulatieve hormonen en ion osmo-regulatieve processen manipuleerden wanneer ze in contact kwamen met toxische koper concentraties (Hoofdstuk 3). Zoals eerder onderzoek aantoonde is de snelheid waarmee plasma ionen door diffusie naar de omliggende omgeving

verloren gaan erg belangrijk voor het overleven, belangrijker dan de totale hoeveelheid ionen die verloren is gegaan tijdens de hele blootstelling. Het feit dat de regenboogforel een duidelijke daling in natrium, chloor en calcium vertoonde vanaf het begin van de blootstelling en de karperachtigen (karper en giebel) pas na 3 dagen, is zeer nadelig voor de regenboogforel. Boeiend is wel dat de osmo-regulatieve verstoring in de regenboogforel relatief snel weer gecompenseerd werd tot controle waarden. Bij de karper en de giebel werden de plasma ion controle concentraties daarentegen niet meer bereikt tijdens de duur van de blootstelling ondanks hun hogere weerstand tegen toxische koper concentraties. De daling in plasma ionen kan worden veroorzaakt door beschadigde membranen, inhibitie van carboanhydrases, Na^+/H^+ uitwisselaars, Na^+ kanalen en Na^+/K^+-ATPase activiteit in kieuw weefsel. In dit onderzoek interfereerde koper met de werking van Na^+/K^+-ATPase in regenboogforel en giebel na 12 uur blootstelling. Voor de giebel duurde deze vermindering van activiteit zelfs tot een week na de start van de blootstelling aan 150µg/l Cu. Om deze schade te herstellen kan een stimulatie van de Na^+/K^+-ATPase synthese plaatsvinden, alsook een verhoogde chloride cel aanmaak. Dit zijn effecten die kunnen veroorzaakt worden door een gestegen circulerende prolactine concentratie. Ondanks het feit dat de regenboogforel gewend is aan overgangen tussen zoet en zout water en dus aan fluctuerende ionen concentraties, waar prolactine noodzakelijk voor is, werden er weinig veranderingen waargenomen in de mRNA concentraties van de bestudeerde prolactine receptor in kieuwweefsel van regenboogforel. De concentratie van deze receptor in de karperachtigen was echter niet gestegen maar gedaald op verschillende tijdspunten van de koper blootstelling. Vergelijkbare resultaten werden opgemerkt voor de mRNA expressies van de schildklierhormoon receptor β in kieuw en de schildklierhormoon T3 concentratie in plasma van de giebel. De karper en de regenboogforel vertoonden echter minder of geen veranderingen in de schildklierhormoon gerelateerde parameters. Het feit dat alle vissoorten een grote piek in T4 concentraties vertoonden na het eerste uur van de blootstelling (en voor regenboogforel blootgesteld aan 50µg/l duurde deze verhoging tot een week na blootstelling) is op zijn minst opmerkelijk te noemen en kan een indicatie zijn van een eerste algemene stressreactie en daarmee gepaard gaande metabole stimulatie. Door de verminderde mRNA expressies en sterk gedaalde schildklierhormoon T3 concentraties in de giebel, wordt het duidelijk dat deze vissoort mogelijk zijn metabolisme onderdrukt als reactiemechanisme op de toxische koper concentraties. Door dit te doen, wordt het ademhalingsritme onderdrukt en komt er bijgevolg minder koper langs de kieuwen. Verder zorgt een onderdrukking van het

metabolisme en de bijhorende hypothyreoidie ook voor een vermindering in reactieve zuurstof radicalen wat een extra bescherming geeft door een verminderde pro-oxidant productie. Een goed functionerend anti-oxidant verdedigingssysteem is dan ook onontbeerlijk voor elk organisme om zichzelf te beschermen tegen de reactieve zuurstof radicalen die ontstaan tijdens de normale werking van het metabolisme. Wanneer de concentratie van deze radicalen erg stijgt, door bijvoorbeeld de negatieve effecten van koper, wordt de efficiëntie van dit verdedigingssysteem dan ook erg belangrijk. Daarom werden de activiteiten van superoxide dismutase, catalase en glutathion reductase samen met de concentraties van het gereduceerde glutathion en ascorbaat in koper blootgesteld kieuwweefsel bepaald. Zodoende konden we nagaan of de diversiteit in dit anti-oxidant verdedigingssysteem een bijdrage leverde tot de verschillen in gevoeligheid voor koper (Hoofdstuk 4). Merkwaardig genoeg werden er reeds contrasten waargenomen in controle situaties. De regenboogforel vertoonde dubbel zoveel gereduceerde glutathion en glutathion reductase in vergelijking met de kieuwen van de karperachtigen. Daartegenover demonstreerde de giebel meer dan een verdubbeling van gereduceerde ascorbaat concentraties in hun kieuwweefsel in vergelijking met de andere vissoorten. Deze verhoogde concentraties van glutathion en ascorbaat bezorgen de beide vissoorten een voordeel door de grotere capaciteit om vrije koper ionen onschadelijk te maken tijdens de toegenomen oxidatieve stress veroorzaakt door de koper blootstelling. De Boeck et al. (2004) toonden aan dat de meeste gevoelige soort, de regenboogforel, relatief traag koper accumuleerde in de kieuwen. In plaats daarvan accumuleerde de opgenomen koper voornamelijk in de lever. De veranderingen in activiteiten van de onderzochte anti-oxidant enzymen konden ook pas later of helemaal niet worden waargenomen, waarschijnlijk omdat de stijging in koper concentratie in de kieuwen eerder beperkt was gebleven in het begin van de blootstelling. Overigens reflecteren de hoge concentraties glutathion en glutathion reductase in de kieuwen van de regenboogforel mogelijks een belangrijke functie van deze molecule en enzym in de eerste verdediging tegen de oxidatieve stress. Het werd immers ook bevestigd door De Boeck et al. (2003) dat de regenboogforel niet in staat was om een verhoogde productie van metallothionein (normaal de metaalcomplexer bij uitstek) te weeg te brengen als reactie op verhoogde koper concentraties. In de kieuwen van de karper werden in hetzelfde onderzoek ook geen gestegen metallothionein concentraties gevonden. Maar, ook in deze vissoort lijkt glutathion een belangrijke molecule in de verdediging tegen oxidatieve stress te zijn. Bovendien werd door de Boeck et al. (2004) opgemerkt dat koper erg snel accumuleert in de kieuwen van de karper. Het was dan ook erg interessant om te zien dat de

anti-oxidant enzymen die bestudeerd werden onmiddellijk bij het begin van de blootstelling aan 65µg/l koper verhoogde activiteiten vertoonden. De karpers die werden blootgesteld aan 50µg/l demonstreerden weinig tot geen veranderingen in deze activiteiten. Hetzelfde kan gezegd worden voor de giebel blootgesteld aan 50µg/l koper. Wanneer de giebel dan werd blootgesteld aan 150µg/l koper werd overigens wel de meest efficiënte reactie van de anti-oxidant enzymen waargenomen. Deze snelle reactie gecombineerd met de hoge ascorbaat concentraties en de goede correlatie tussen de kieuw metallothionein inductie en koper accumulatie (De Boeck et al., 2003), geeft de giebel de meeste mogelijkheden om de schade veroorzaakt door de koper gerelateerde oxidatieve stress te minimaliseren.

Intracellulaire verschillen in expressies van metallothionein of andere transporters en metaal bindende eiwitten kunnen ook belangrijke componenten zijn om de negatieve effecten van koper blootstelling adequaat het hoofd te kunnen bieden. Door het proteoom van de kieuwen van de drie vissoorten te bestuderen, trachtten we de dynamiek van deze eiwitten patronen te evalueren en te bepalen of er belangrijke positieve voordelen aanwezig zijn in het verdedigingsmechanisme tegen koper (Hoofdstuk 5). Tijdens de 2D-Dige en iTRAQ analyse werd het duidelijk dat deze technieken elk een favoriete subset van eiwitten identificeren en dat de succesvolle identificatie van de eiwitten beperkt werd door het gelimiteerde aantal gekende eiwitten in de databanken van de bestudeerde vissoorten. Als gevolg geeft deze studie slechts een eerste indicatie van mogelijke effecten veroorzaakt door koper blootstelling. Desondanks dat dit voor een kleinere dataset zorgde, is dit ook inherent aan proteoom onderzoek en doet dit niets af aan de significantie van de bekomen resultaten. Het WGCNA software pakket dat gebruikt werd om sterk gecorreleerde expressie profielen van eiwitten te correleren, bleek erg bruikbaar in het behandelen van relatief grotere datasets en gaf de mogelijkheid om een link te vinden tussen biologische gemeten parameters en groepen sterk gecorreleerde eiwitten.

De gevoelige regenboogforel vertoonde enkele tekenen van verhoogde oxidatieve stress, verhoogde energie productie en verminderde metaal bindende eigenschappen. Deze daling in metaal bindende eigenschappen werd vooral uitgelicht in de 2D-Dige analyse met een verminderde expressie van transferrine en albumine in de kieuwen. Aan de andere kant werd tijdens deze analyse ook een verhoging van de carboanhydrases opgemerkt welke een reactie zou kunnen zijn om de inhibitie van dit enzym tijdens koper blootstelling tegen te gaan. De iTRAQ analyse en bijhorende WGCNA analyse onthulde enkele bijkomende voordelen zoals

een verhoogde Hsp70/Hsp90 organiserend eiwit, een verminderde myeloperoxidase expressie en een correlatie tussen de koper concentratie in het kieuwweefsel en de superoxide dismutase activiteit. Ook eiwitten in verband met de immuniteit, het eiwit metabolisme en de oxidatieve stress evenals gerelateerde veranderingen van het actine cytoskelet konden worden opgemerkt. In de kieuwen van karper omvatten de veranderingen in het actine cytoskelet de grootste groep van beïnvloedde eiwitten. In deze vissoort bleken eiwitten gerelateerd aan transcriptie en translatie, verhoogde eiwit productie en verhoogd energie verbruik ook erg belangrijk in de proteoom analyse. Dit gestegen gebruik van energie en eiwitten kan een eerste start geven aan het herstel van de toxische effecten veroorzaakt door koper. De dalingen in het translationeel gecontroleerd tumor eiwit en het koude geïnduceerde RNA bindende eiwit bleken erg interessant omdat beide eiwitten door onderzoekers verbonden werden met metaal gerelateerde stress situaties. Bovendien werd het translationeel gecontroleerd tumor eiwit ook geassocieerd met Na^+/K^+-ATPase, waarvan geweten is dat de activiteit verandert bij blootstelling aan koper. Van de drie bestudeerde vissoorten is het proteoom van de kieuwen van de giebel het minst geannoteerd. Niettegenstaande bleken de geïdentificeerde eiwitten te duiden op een verhoogde bescherming tegen koper toxiciteit. Een stijging in de transferrine expressie verzekert een verhoogde metaal bindende eigenschap, de stijging in Apolipoeiwit A-I zorgt dan weer voor bescherming van de cel membraan integriteit en de toegenome expressie van het "heat shock protein 70" helpt in het herstel en bescherming van de cel. De locatie van de geaccumuleerde koper in de cel is daarom ook erg belangrijk dus werd er in de volgende studie gefocused op de dynamiek van de subcellulaire accumulatie patronen in de drie vissoorten tijdens eenzelfde blootstelling aan 50µg/l koper. Het doel van deze analyse was dan ook om na te gaan of verschillen in subcellulaire partitie van koper in kieuw en lever cellen van vissen met verschillende gevoeligheden aan koper belangrijke contrasten naar voor brengen die geassocieerd kunnen worden met hun mate aan gevoeligheid aan metaalblootstelling (Hoofdstuk 6).

En inderdaad, deze veelbelovende studie zorgde voor een groter inzicht in de verschillen tussen de drie vissoorten. Voorafgaand onderzoek vestigde al de aandacht op de lage koper accumulatie in kieuwen van de regenboogforel in vergelijking met de hoge deposities van koper in controle levers van dezelfde vissoort (De Boeck et al., 2004). Vergeleken met de karper en de giebel was er in de kieuwen van de regenboogforel 15 tot 40% minder koper en 60 tot 300% meer koper aanwezig in de lever van de regenboogforel in controle situaties. Vooral voor de lever zijn deze verschillen echt opmerkelijk. Tijdens de bloostelling aan koper steeg de

geaccumuleerde koper concentratie in de kieuwen van de drie vissoorten in gradaties alhoewel meer koper werd teruggevonden in de karperachtigen (~20.1 µg/g Cu/drooggewicht voor de karper en ~25.9 µg/g Cu/drooggewicht voor de giebel) in vergelijking met de kieuwen van de regenboogforel (~8.0 µg/g Cu/drooggewicht). Dit onderstreepte nogmaals de relatief lage koper bindende capaciteit van de kieuwen van de regenboogforel. De grote capaciteit van de lever van de regenboogforel om koper op te slagen werd ook bevestigd nl. ~90µg/g Cu/drooggewicht werd extra geaccumuleerd boven op een controle concentratie van ~250µg/g Cu/ drooggewicht. In vergelijking, ~40µg/g drooggewicht en ~18µg/g Cu/drooggewicht van respectievelijk de lever van de karper en de giebel werden toegevoegd aan controle concentraties van ~43µg/g Cu/drooggewicht en ~10 µg/g Cu/drooggewicht tijdens blootstelling aan dezelfde concentratie koper. Nog belangrijker dan deze verschillen in totale geaccumuleerde koper concentraties, is de verdeling van de opgestapelde koper in de cel. Zoals Steen-Redeker en Blust (2004) voorstelden is het metaal dat wordt opgeslagen in biologische actieve metaal compartimenten (BAM) (wat in deze studie bestond uit de organellen, de kern-cel debris en hitte denatureerbare fracties) toegankelijker voor het onstaan van schadelijke effecten door de verhoogde metaalconcentraties. Aan de andere kant is metaal opgeslagen in de biologische inactieve metaal compartimenten (BIM) (wat in deze studie bestond uit de hitte stabiele fractie en de metaal rijke granule fractie) gedetoxificeerd door complexvorming en dus niet meer beschikbaar voor het uitlokken van schadelijke effecten.

Boeiend was dan ook het feit dat de meest gevoelige soort, de regenboogforel, zelfs in controle situaties meer koper in de BAM vertoonden van kieuw en lever, dan de karperachtigen. Wanneer de regenboogforel werd blootgesteld aan koper, werd de additionele koper in de kieuw opgeslagen in de BAM (een verhoging van een 70 naar 85% van de totale koper concentratie in de kieuw) en dit door distributie van koper in de hitte denatureerbare fractie die vooral bestaat uit gevoelige enzymen en eiwitten. Deze fractie bevatte tijdens de blootstelling dubbel zoveel koper in vergelijking met een controle situatie in kieuw, maar was tegen het einde van de blootstelling weer hersteld. In de lever van de regenboogforel werd dan het teveel aan koper opgeslagen in de hitte stabiele fractie (metaal bindende eiwitten) wat zorgde voor meer bescherming in dit weefsel. Ondanks dat de organellen fractie voor 60% van de totale koper concentratie in de lever instond, accumuleerde het niet veel extra koper tijdens de blootstelling, wat een voordeel is van de koper distributie processen in de lever. Het tegenovergestelde gebeurde in de lever van de karper, waar ogenschijnlijk geen mechanisme aanwezig was om te verhinderen dat koper in de BAM accumuleerde. Extra koper werd dan

ook in de organellen fractie, de hitte denatureerbare fractie en de metaal rijke granule fractie gedeponeerd. In de kieuwen van de karper accumuleerde de koper zelfs in alle fracties tegen het einde van de blootstelling met de hitte denatureerbare fractie als belangrijkste afzetplaats. De giebel echter kon in beide weefsels de distributie van koper onder controle houden door de toewijzing van koper aan de hitte stabiele fractie in kieuwen en dezelfde fractie alsook de metaal rijke granule fractie in lever. De oorspronkelijke distributie tussen de BIM en de BAM in controle condities van de lever toonden al aan dat de BIM rond de 80% van de totale koper concentraties bevatte. Tijdens de blootstelling werd deze distributie behouden wat erg belangrijk is om de schadelijke effecten van koper te kunnen voorkomen. Meer nog, de biologische beschikbare hoeveelheid koper was minimaal in de lever van de giebel, wat het organisme nog meer beschermd voor interacties van koper met essentiële enzymen, eiwitten en DNA.

Om te besluiten worden nog eens de belangrijkste voordelen en nadelen van elke vissoort bij het verwerken van een koper blootstelling besproken. De meest gevoelige vissoort, de regenboogforel, is op zich al een kwetsbare soort die nood heeft aan zuivere stromen met overvloedige zuurstofconcentraties, in vergelijking met de meer robuuste karperachtigen, die een grotere waaier aan omgevingscondities kunnen weerstaan. Hoe dan ook, als migrerende soort wordt de regenboogforel geacht om veranderingen in ion concentraties adequaat en efficiënt het hoofd te kunnen bieden. En inderdaad, nadat de regenboogforel de eerste schok fase kon overleven (dus het verlies van grote hoeveelheden ionen gecombineerd met een geblokkeerde activiteit van Na^+/K^+-ATPase) was het in staat om de ion homeostase sneller te herstellen en controle concentraties te herwinnen. Het verhoogde expressie profiel van carboanhydrase, zoals de proteoomanalyse demonstreerde, kan ook aanzien worden als een extra voordeel in het herstellen van de ion homeostase. Dit herstel kan ook alleen maar plaatsvinden als de hoeveelheid ionen die in de eerste uren van de blootstelling verloren zijn gegaan de drempelwaarde voor het ontstaan van een hartstilstand niet hebben overschreden.

De trage koper accumulatie in de kieuw en de relatief snelle transfer van opgenomen koper naar de lever van de regenboogforel werkt ook in hun voordeel. De lever bleek dan ook nog eens erg goed uitgerust om de additionele koper te verwerken en op te slagen in de biologische inactieve metaal compartimenten, wat verhinderde dat de vrije koper ionen konden reageren met enzymen, eiwitten en DNA. In kieuwweefsel van regenboogforel bleek dat de meeste koper aanwezig was in de biologisch actieve metaal compartimenten waar ook

de additionele koper werd opgeslagen tijdens koper blootstelling. Dit maakt de kieuw dus extra vatbaar voor de schadelijke effecten gerelateerd aan koper blootstelling. Zodoende is de snelle distributie van opgenomen koper van de kieuw naar de lever een belangrijk voordeel voor de regenboogforel. Verder werd door De Boeck et al. (2004, 2006 en 2007) ook opgemerkt dat de kieuwschade in een week blootstelling aan koper relatief klein was en dat er ook weinig sprake was van ademhalingsmoeilijkheden. Desondanks verhoogde de koper concentratie in kieuwweefsel na een aantal dagen en waren de superoxide dismutase enzymen actiever (wat ook terug gezien werd in een verhoogd expressie profiel van deze enzymen tijdens de proteoomanalyse) om met de extra oxidatieve stress om te kunnen gaan. Het is waarschijnlijk dat ook de hoge concentraties gereduceerd glutathion en bijhorend enzyme erg belangrijk zijn om de oxidatieve stress te kunnen voorkomen, zeker omdat er geen inductie van metallothionein en eerder een daling in metaalbindende eiwitten (zoals albumine en transferrine) in kieuwweefsel werd gezien. Alhoewel de regenboogforel enkele adaptaties vertoont om met verhoogde koper concentraties om te gaan, blijft deze vissoort toch 3 tot 7 keer gevoeliger aan koper in vergelijking met de karper en de giebel. Hierbij wordt duidelijk dat de regenboogforel niet flexibel genoeg is om te overleven tijden blootstelling aan excessieve hoeveelheden koper.

De karper en vooral de giebel kunnen vele hogere koper concentraties trotseren. Deze karperachtigen vertonen vaak gelijkaardige reacties op de gestegen metaalconcentratie waarbij de giebel steeds nóg uitgebreider of sneller reageert in de bestudeerde aspecten. Wanneer beide vissoorten werden blootgesteld aan 50µg/l Cu werden er weinig veranderingen in de fysiologie van deze vissen opgemerkt. Maar indien ze te maken kregen met de blootstelling aan 10% of hun 96 uur LC50 waarde (voor karper 65µg/l Cu en voor giebel 150µg/l Cu) veranderde dit snel. Zoals eerder vermeld is de regenboogforel erg kwetsbaar voor het verlies van ionen in het begin van een schadelijke metaal blootstelling. Karper en giebel hadden ook te maken met ionen verlies, maar pas na een week van blootstelling aan koper. In tegenstelling tot de regenboogforel werd de controle situatie niet meer geëvenaard maar werd er veeleer een nieuwe ion homeostase ingesteld, welke het mogelijk maakt voor deze vissoorten om te overleven. De regenboogforel was dan ook erg efficiënt in het distribueren van koper naar de lever terwijl bij de karperachtigen bleek dat ze meer koper accumuleerden in hun kieuwen. Desondanks zorgt dit niet voor onoverkomelijke problemen omdat beide karperachtigen de oorspronkelijke verdeling van koper tussen de BIM en de BAM van de kieuw na een maand blootstelling weer hersteld hadden. Vooral in de

giebel, waar het percentage koper in de BIM uitzonderlijk hoog bleek te zijn in de kieuw, maar ook in de lever, waardoor de hoeveelheid labiele of vrije koper concentraties steeds tot een minimum beperkt konden worden, bleek erg goed met de verhoogde koper concentraties om te kunnen gaan. De duidelijke correlatie tussen stijgende koper concentraties in kieuw en gelijktijdige verhogingen van metallothionein inductie in de kieuw zorgt nog meer voor een strikte regulatie van koper in de kieuwen van de giebel. En waar de proteoom analyse voor de karper in de richting van verhoogde eiwitten en energie verbruik wees, waren de geïdentificeerde eiwitten van de giebel telkens betrokken bij het afremmen van schadelijke effecten door de bindingscapaciteit te verhogen (gestegen transferrine concentratie) en de membranen en de cellen meer te beschermen (verhoogde apolipoprotein A-I en heat shock protein 70). Bovendien vermeerderden beide karperachtigen, en dan vooral de giebel, de activiteit van de bestudeerde anti-oxidant enzymen in het begin van de koperblootstelling. Dit verschil met de regenboogforel zou geassocieerd kunnen zijn met het verschil in kieuwschade bij beide vissoorten tijdens deze blootstelling (De Boeck et al., 2007). De kieuwen van karper vertoonden namelijk een eerste schokfase in het begin, gevolgd door herstel. De kieuwen van de giebel daarentegen leken in het begin van de blootstelling weinig schade te ondervinden maar na 3 dagen blootstelling werd toenemende kieuwschade vastgesteld. Bij beide karperachtigen was de verstoring van de ademhaling dan ook veel groter dan bij de regenboogforel. Daarbij kan de giebel, en in mindere mate ook de karper, de metabole snelheid verminderen zodat de koper opname via de kieuw wordt verminderd en zodat de productie van reactieve zuurstof radicalen wordt beperkt. Naast deze uitzonderlijk eigenschappen kan de giebel genieten van hoge ascorbaat concentraties in de kieuw, om nog eens extra de vrije of labiele koper concentraties te doen dalen, en van een zeer efficiënte verwijdering van koper via de nier (De Boeck et al., 2003). Deze waaier aan positieve facetten van de fysiologie van de giebel weerspiegelt de tolerantiemogelijkheden wat koper betreft en geeft de efficiënte reacties van de giebel weer wanneer ze worden uitgedaagd met toxische concentraties metaal in het milieu. Zodanig kan er dus geconcludeerd worden dat de resultaten een goed inzicht verschaffen in de onderliggende redenen van de gevoeligheid van de regenboogforel, de intermediaire gevoeligheid van de karper en de resistentie van de giebel aan koper.

Chapter 9

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