2012

An integrated approach to assess impact of environmental stress in carp, Cyprinus carpio L.: Biochemical, genotoxic, histopathological and individual level effects

Sanaa A. Mustafa

PHD

An integrated approach to assess impact of environmental stress in carp, Cyprinus carpio L.: Biochemical, genotoxic, histopathological and individual level effects

Mustafa, Sanaa A.

Award date:
2012

Awarding institution:
University of Plymouth

Dedicated to my beloved father, Abdul Aziz Nasoof

Copyright Statement

An integrated approach to assess impact of environmental stress in carp, *Cyprinus carpio* L.: Biochemical, genotoxic, histopathological and individual level effects

By

SANAA ABDULAZIZ MUSTAFA

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biomedical and Biological Sciences
Faculty of Science

May 2012

An integrated approach to assess impact of environmental stress in carp, *Cyprinus carpio* L.: Biochemical, genotoxic, histopathological and individual level effects

SANAA A. MUSTAFA

Abstract

Studies were undertaken to determine toxicological effects in a model species, *Cyprinus carpio* L. following hypoxic exposure either alone or in combination with representative heavy metal (i.e. copper; Cu) via a dietary route, at different levels of biological organisation (viz. biochemical, histological and individual level effects). Initially, the validation study of biological responses using a range of concentrations of dietary Cu as a relevant environmental contaminant was carried out (Chapter 3). The results showed a range of biological responses in exposed fish including significant genotoxic response as determined by induction of DNA strand breaks (i.e. the Comet assay) with bacterial enzymes Fpg and Endo-III (for detection of oxidative DNA damage) and reduction in growth rate suggesting the robustness of selected biomarkers. Subsequently, this approach was used initially to determine the biological responses following chronic hypoxic and hyperoxic exposure (Chapter 4). The results suggested that both hypoxic and hyperoxic conditions lead to a range of comparable biological responses. Following relative evaluation of chronic hypoxic and hyperoxic exposures, experiments were carried out to elucidate potential interactive effect of hypoxia in combination with dietary Cu (Chapter 5). The combined exposure of hypoxia and Cu induced a significantly higher level of DNA damage suggesting that DNA damage in fish can serve as a sensitive biomarker for changes in water quality as well as presence of genotoxic chemicals. The final sets of experiment were carried out to determine the biological responses in *C. carpio* following exposure to chronic hypoxic stress and subsequent recovery in normoxic condition for 7 days. Real-time PCR (qPCR) technology was used to examine the hypoxia inducible Factor-1 α (HIF-1α) gene expression pattern (Chapter 6). The results suggested that the expression levels of HIF-1α in response to hypoxia were significantly higher compared to normoxic controls, a high level of oxidative DNA damage under hypoxia and re-exposure to normoxic condition (i.e. recovery period). This will shed lights for development of adaptive response in higher vertebrates, which could also have significant clinical implications in human health.

Table of Contents

Figures List

CHAPTER 1.

CHAPTER 2.

CHAPTER 3.

CHAPTER 4.

CHAPTER 5.

CHAPTER 6.

CHAPTER 7.

Tables List

CHAPTER 1.

CHAPTER 2.

CHAPTER 3.

CHAPTER 4.

CHAPTER 5.

CHAPTER 6.

Plates list

Acknowledgements

Foremost, I would like to express my sincere gratitude to my supervisor Professor Awadhesh Jha for his unreserved help, motivation, enthusiasm and immense knowledge from the beginning up to the completion of this study. I must also acknowledge my second supervisor Professor Simon Davies for his encouragement, suggestions and for providing me all the facilities in the aquarium to complete my experiments.

Besides my supervisors, I would like to thank the following people for their time and support: Dr. John Moody for guidance in statistical treatment of the data, technical colleagues, especially William Vevers, Andy Artfield and Angela Harrop for their support in various ways. Thanks are also due to Dr. Andy Fisher who helped me with analytical techniques (i.e. ICP-OES). Colleagues in electron microscopy unit, Glenn Harper and Peter Bond helped me for TEM work. Appreciation also goes out to Peter Russel and Binjamin Eynon for helping me during the 'exposure period' to set up the gas cylinders and to maintain the fish stock. A special thank also goes to Nutrition lab, particularly to Liz Preston for helping me in the analysis of the carcases composition. Mark Rawling is thanked for standing with me in the lab during my last experiment to do the NBT test. I cannot forget to express appreciation to my friend Sahar Karieb for teaching me PCR techniques. I also thank colleagues in Cefas, Weymouth for their training during the histopathology workshop. I would also like to extend my thanks to all my colleagues in the School of Biomedical and Biological Sciences, who answered my request for help. Further, I must acknowledge the Ministry of the Higher Education and Scientific Research, Republic of Iraq for the financial support.

My special thanks go to my best friend Sherain Al-Subiai for supporting me to overcome the hard times during my study, and for standing with me in sampling and assays for all the experiments. Last but not the least, I would have not finished this study without the support of my Dad, Sisters (Sumaya and Dalia) and Brothers (Saad and Raad) who have always been there for me whenever I needed them, their never ending encouragement and love kept me going. I hope I have made you proud!

Platform presentations

Determination of impact of aflatoxins on fish health: toxicological and histopathological approach. University of Plymouth, Plymouth, UK, April 2008.

Linking DNA damage with ecotoxicological parameters in the changing environment. University of Plymouth, Plymouth, UK, Nov 2010.

Poster presentations (please refer to Appendix II)

An integrated approach to determine effects of copper in bivalve mollusc, *Mytilus edulis*. Plymouth Marine Sciences Partnership Symposium: Marine Science for a Changing World, Plymouth, UK, April 2009.

An integrated approach to determine oxidative stress in mirror carp *Cyprinus carpio*. 15th International Symposium on Pollutant in Marine Organisms. Bordeaux, France, May 2009.

Linking DNA damage with specific growth rate: differentiating nutritional and ecotoxicological impact of dietary copper in fish. Annual Meeting of the European Environmental Mutagen Society (EEMS), Oslo, Norway Sept 2010.

Diploma supplement (please refer to Appendix III)

Word count of main body of thesis: 60,094

Signed ----------

Date ----------

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. This study was financed with the aid of Ministry of the Higher Education and Scientific Research/Iraq. Relevant scientific seminars and conferences were attended at which work was presented and papers have been prepared for publication:

Publications (please refer to Appendix I [*for the abstract only; Figure (Text/chart/diagram etc.) has been removed due to Copyright restrictions]*

Ferguson, R.M.W., Merrifield, D.L., Harper, G.M., Rawling, M.D., **Mustafa, S.A.,** Picchietti, S., Balcázar, J.L.Davies, S.J. 2010. The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). *Journal of Applied Microbiology,* 109, 851-862.

Al-Subiai, S.N., Moody, A.J., **Mustafa, S.A.** Jha, A.N. 2011. A multiple biomarker approach to investigate the effects of copper on the marine bivalve mollusc, *Mytilus edulis*. *Ecotoxicology and Environmental Safety,* 7**4,** 1913-1920.

Merrifield, D., Harper, G., **Mustafa, S.,** Carnevali, O., Picchietti, S.Davies, S. 2011. Effect of dietary alginic acid on juvenile tilapia (*Oreochromis niloticus*) intestinal microbial balance, intestinal histology and growth performance. *Cell and Tissue Research*, 344, 1-12.

Mustafa, S.A., Al-Subiai, S.N., Davies, S.J. Jha, A.N. 2011. Hypoxia-induced oxidative DNA damage links with higher level biological effects including specific growth rate in common carp, *Cyprinus carpio* L. *Ecotoxicology,* 20**,** 1455–1466.

Mustafa, S.A., Davies, S.J.Jha, A.N. 2012. Determination of hypoxia and dietary copper mediated sub-lethal toxicity in carp, *Cyprinus carpio*, at different levels of biological organisation. *Chemosphere*, 87, 413-422.

Harper, G.M., Saoud, I.P., Emery, M., **Mustafa, S.A.,** Rawling, M.D., Eynon, B.S.J., Davies, S.J., Merrifield, D.L.Monfor, M. 2012. In Press. An ex vivo approach to studying the interactions of probiotic *Pediococcus acidilactici* and Vibrio (*Listonella*) anguillarum in the anterior intestine of rainbow trout *Oncorhynchus mykiss*. *Journal of Aquaculture Research & Development,* doi.org/10.4172/2155-9546.S1-004.

Abbreviations

%	Percent
µl	Microliter
µg	Microgram
µm	Micrometer
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AFB_1	Aflatoxin B_1
Cu	Copper
DO	Dissolved oxygen
DMSO	dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleotide triphosphate sodium
DOC	dissolved organic carbon
DPBS	Dulbecco's Phosphate-Buffered Saline
D.W	Dry weight
EDTA	Ethylenediaminetatra acetic acid
Endo-III	Endonucleases-III
Fpg	Formamidopyrimidine glycosylase
GPx	Glutathione peroxidise
GSH	Glutathione (reduced)
GST	Glutathione–S-transferase
Hb	Haemoglobin
Hct	Haematocrit
H & E	Haematoxylin and eosin stain
H_2O_2	Hydrogen peroxide
HEPES	*N-2*-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HIF-1α	Hypoxia Inducible factor-1α
H_2SO_4	Sulphuric acid
ICP-OES	Inductivity coupled plasma-optical emission spectrometer
IMS	Industrial methylated spirit
LMPA	Low melting point agarose
LSD	Less significant difference
MDA	melanodialaldehyde
MFO	mixed function oxygenase
MTC	maximum tolerance concentration
NADH	reduced β nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferase
NBT	nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information
NMP	Normal melting point agarose
OD	Optic Density
OECD	Organisation of Economic Cooperation and Development
P	Statistical probability
PCV	Pack cell volume
P-450	Cytochrome P-450
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biophenyls
PMA	phorbol myristate acetate
PBDEs	Polybrominted diphenyl ethers
PUFA	Polyunsaturated fatty acids

RBC	Red blood cell
ROS	Reactive Oxygen Species
SCGE	Single Cell Gel Electrophoresis
SGR	Specific Growth Rate
S.E	Standard error
TEM	Transmission electron microscopy
TBA	Tris buffered saline
TBARS	thiobarbituric Acid Reactive Substances
WBC	White blood cell
ww	wet weight
w/v	weight/volume

.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

1.1 Environmental pollution

The increasing pollution of aquatic ecosystems with thousands of anthropogenic and natural chemicals is becoming the major environmental threat facing human and environmental health (Schwarzenbach et al., 2006). In addition to anthropogenic chemicals and radionuclides, in recent years, significant attention has been paid to the problems of environment contamination by pathogenic microbes and organisms (bacteria, viruses, and parasites), harmful algal blooms, increased ultraviolet (UV) radiation, and nutrient enhancement or deprivation (Fleming et al., 2006). The major classes of toxic compounds of concern for aquatic ecosystems and in particular for fish are heavy metals, polycyclic aromatic hydrocarbons (PAHs), chlorine, cyanides, polychlorinated biphenyls (PCBs), petroleum products and domestic and industrial effluents, which might contain a range of different contaminants with potential detrimental impact on the health of humans and the biota (Walker et al., 2001).

The contaminants in the natural environment however occur as complex mixture in all probable combinations (Jha, 2004). Most of the knowledge and understanding of the potential effects of pollutants however is based upon the effects of single compound assessed in the laboratory. Much less is known about the impacts of the complex environmental mixtures. Many of these contaminants, as a part of complex mixture, could occur at very low concentrations often below the detection level by available analytical techniques. However, potential synergistic or even additive interactive toxic effects can make such mixtures hazardous (Bickham et

al., 2000; Dixon et al., 2002; Novák et al., 2008; Schwarzenbach et al., 2006). For instance, Brian et al., (2005) showed that when five estrogenic compounds are mixed in concentrations all below levels at which their individual effects can be detected, their cumulative effect on fish was detrimental. Also exposure of aquatic organisms to environmental contamination often results in genotoxic insult, either via direct genotoxicity, or through the induction of cellular or oxidative stress (Klobučar et al., 2010). Furthermore, the carcinogenic and mutagenic compounds are the most dangerous as their effects may exert a damage beyond that of individual and could be active through several generations (Dixon et al., 2002).

In addition, chronic, low level exposure of pollutants have detrimental effects on reproduction, behavior, resistance against diseases and other physiological problems such as immunosuppression (Kime, 1999). As a consequence, all of these pollutants have implications with human and animal activities. This could include direct interaction from toxic chemicals in drinking water and indirectly via the food chain (e.g. by consuming contaminated fish or seafood) (Li et al., 2006). The assessment of a wide range of xenobiotically- induced variations in cellular or biochemical components, processes, structures or functions (biomarkers) in sentinel organisms (bioindicators) is very important to detect the potential toxicity of pollutants. Considering, the importance of DNA in maintaining the homeostasis of all organisms and in the transfer of information to the next generation, an assessment of the integrity of DNA is essential when detecting pollution related stress in aquatic organisms (Bickham and Smolen, 1994; Klobučar et al., 2010). In order to sustain healthy life on this planet, detection of the effects of contaminants in aquatic ecosystems is an essential task requiring improved analytical and

modeling biomarkers to probe (toward) the bioavailability, distribution, and biological impacts of single compounds and of chemical mixtures (Schwarzenbach et al., 2006).

1.2 Types of pollutants

1.2.1 Organic pollutants

Persistent organic pollutants (POPs) represent a threat to aquatic ecosystem because they are resistant to environmental degradation via biological, chemical, and photolytic mechanisms and also have ability to accumulate in sediments and bioaccumulate in human and animal adipose tissue and biomagnify in food chain. This group covers a wide range of products including: polyhalogenated, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biophenyls (PCBs), and polybrominted diphenyl ethers (PBDEs). (Livingstone, 1998). Also, pseudo persistent xenobiotics (e.g. pharmaceuticals) have recently been recognized as an important class of organic pollutants due to their physical–chemical properties, which allow their persistence and bioaccumulation in the environment. Due to their high prescription rate, they are however continuously supplied to the aquatic ecosystem provoking harmful effects in aquatic environments in concentration down to a few nanograms per litter (Barceló and Petrovic, 2007; Trovó et al., 2008). Many of these contaminants are highly bioactive, most are polar, many are optically active and all (when present in the environment) usually occur at no more than trace concentrations such as pharmaceuticals which are relatively readily degradable (e.g. ibuprofen, diclofenac) (Trovó et al., 2008).

Aquatic biota including fish species are frequently exposed to these pollutants during lifespan via direct contact to sediment as embryo and/or by consumption of contaminated invertebrates as food. In addition, the uptake of these contaminants by aquatic organisms could also occur via the suspended particulate matter and food sources. A proportion of these potentially toxic substances enter the aquatic environment where they are readily taken up into the tissue of fish and other biota causing adverse systemic disorders including: reproductive impairment, damage to the nervous system and disruption to the immune system (Li et al., 2006). More recently, these compounds, in the environment are known or suspected pro-carcinogens that require metabolic activation to exert their genotoxicity (Perk, 2006). The fate of the organic pollutants in an organism can comprise four phases: absorption, distribution, metabolism and excretion (i.e. ADME). Absorption of organic contaminants is dependent on the molecular properties of the compound (e.g. lipophilicity, polarity, size) and on the characteristics of the organism. In fish, the main absorption mechanism of the organic compound is the passive diffusion through the gills and skin. Distribution of organic compounds through tissues via circulatory system follows the same principles as absorption (Fig. 1.1). The toxicity of the compound depends on its degree of uptake (distribution) and metabolism (Walker et al., 2001), whereas the importance of biotransformation (metabolism) will be explained in detail as described in section 1.3. Foreign chemicals in teleost fish are primarily excreted via urine and bile. Xenobiotics and their metabolites excreted via urine are filtered in the glomerulus and/or secreted by tubular transport within the kidney. For many organic pollutants, the extent to which conjugates are formed is extremely important in determining the rate of excretion of

the pollutant. This is because most conjugates (glycosides, sulfates, amino acid conjugates, mercapturic acids) are organic anions which are readily water-soluble and are rapidly excreted by fish (and probably higher invertebrates) by a combination of glomerular filtration and tubular transport (Livingstone, 1998).

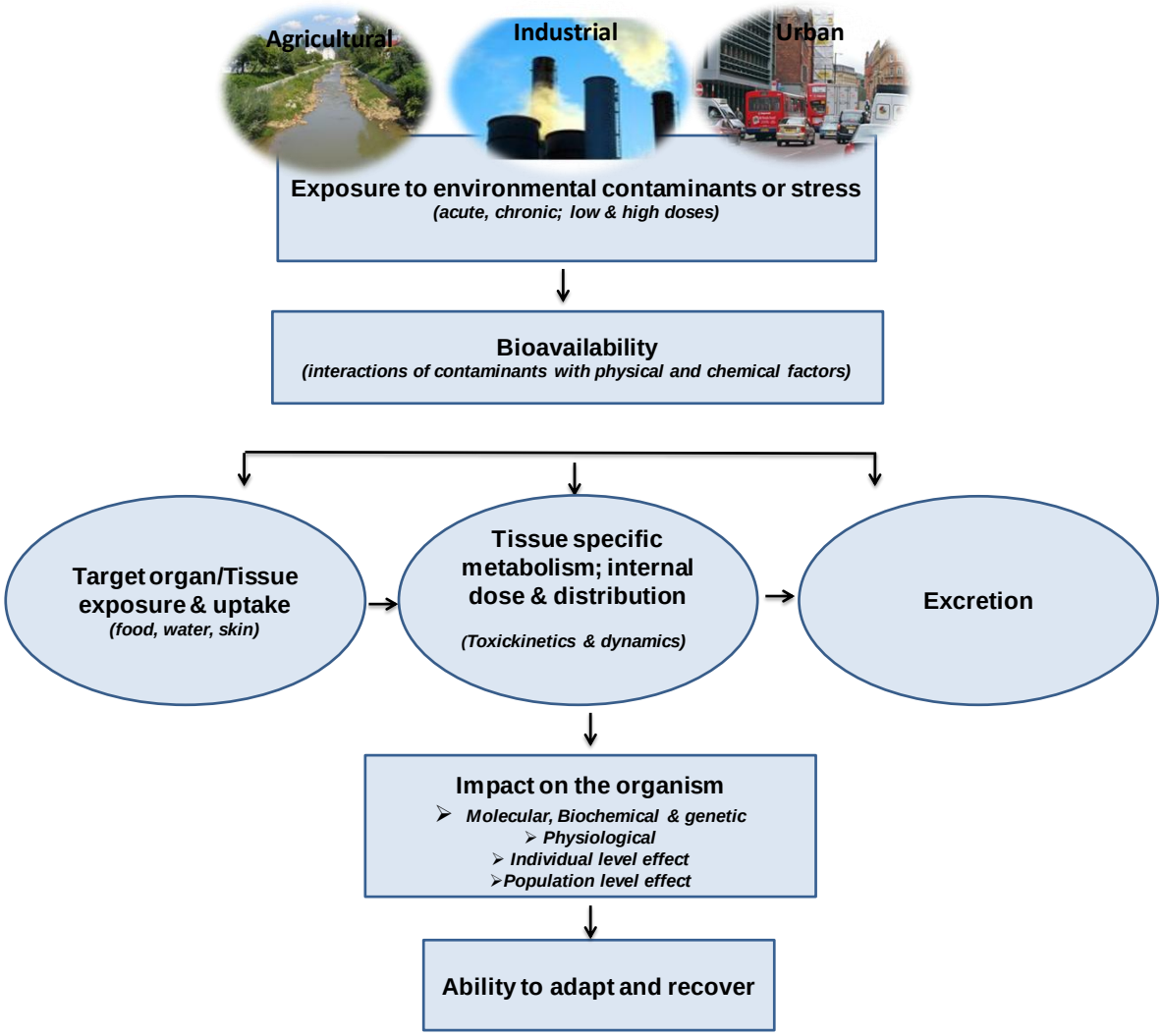


Fig. 1.1 Interaction of contaminants with environmental and biological system leading to responses at different levels of biological organisation.

1.2.2 Heavy metal

1.2.2.1 Bioavailability of metals in water and the pathways of their uptake by fish

There are three possible pathways by which heavy metals enter into the body of fish: The skin (body surface), gills, and the alimentary tract (Dallinger et al., 1987). The routes of metals entering into aquatic organisms depend on specific features of water chemistry, sediments and on biological factors of the organisms. Among the biological characteristics affecting metal bioavailability, species specific differences such as feeding behavior (Baudin and Fritsch, 1989; Phillips and Rainbow, 1989; Van Campenhout et al., 2009; Van Hassel et al., 1980; Veltman et al., 2008) and habitat preferences can play an important role (Hendricks, 1994; Van Hassel et al., 1980). Moreover, these basic features are modified by physiological factors (bioaccumulation levels and the binding ability) in an animal. In addition, the bioavailability of metals could be modified by water quality criteria like salinity, temperature and pH (Angehrn-bettinazzia et al., 1989; Wang, 1987).

Little is known about the entry of metals or other contaminants in fish via the skin. There are some indicators that mucous secretion may prevent heavy metals from entering fish body. However, body surface of fish is more or less impermeable to harmful agents in the aquatic environment (Eddy and Fraser, 1982; Tao et al., 2000). Gills represent a highly specialized and exposed part of the body surface and also play an important pathway for uptake of essential and non-essential metal ions from the water (Ayse Bahar et al., 2010; Fenwick and So, 1974). For example, after exposure of fish to soluble hexavalent chromium or cadmium, these metals are found in the gills (Ayse Bahar et al., 2010; Kim and Kang, 2004). Many studies

have been shown that cadmium is taken up by perfused gill in aquatic organisms (Part and Svanberg, 1981; Pedersen and Bjerregaard, 2000), a marked uptake occurring immediately after exposure (Dang and Wang, 2009; de Conto Cinier et al., 1999). From gills, the observed metals are distributed throughout the whole body and accumulate to specific tissues. Heavy metals have been shown to evoke severe changes in gill morphology. Hence, the gill represents an important site for the soluble fractions of heavy metals in the aquatic organisms. (Arellano et al., 1999; Evans, 1987; Martinez et al., 2004).

Uptake of particulate metal fractions by aquatic organisms occurs, if at all, from contaminated suspended matter, sediments, and organisms such as invertebrates which act as food sources, the only possible route being the alimentary tract. In many aquatic systems pollution has lead to metal contamination of the food sources (including organisms) for various fish species (Dallinger et al., 1987; Mudre and Ney, 1986; Spehar et al., 1978; Van Hassel et al., 1980; Wang and Rainbow, 2008).

1.2.2.2 The relative importance of food as a source of heavy metals

Metal-containing food represents a much more highly contaminated source than water, in which the levels are lower, even at highly contaminated sites. Food or diets therefore could be an important and effective source of metal contamination in aquatic organisms. Though, most of the work supporting this observation is based on the laboratory experiments (Amiard and Amiard-Triquet, 1979; Dallinger et al., 1987; Hamdy and Prabhu, 1979) some studies have also suggested very

high level of accumulation of metals in invertebrates which serve as food source for fish (Rainbow et al., 2007).

Aquatic organisms of higher trophic levels will have a diet enriched by these metals and may bioaccumulate, biomgnificate or biotransfer certain heavy metals to levels high enough to bring about harmful effects, especially by transferring the heavy metals up-the food chain (Dallinger et al., 1987). Generally, the uptake of heavy metals from food depends on various factors. Some studies indicate that significant absorption of a metal by fish takes place only, if the metal content in the food exceeds a minimum threshold concentration (Chan et al., 2003). Furthermore, expenditure rate, feeding frequency (Li et al., 2009), diet quality (Woodward et al., 1994) and interactions with water borne metals may modify food related metal accumulation in fish (Dallinger et al., 1987). The extent of metal from water or food is reflected in increased levels in different organs. For instance, after contamination with copper or cadmium, high levels of metal are accumulated in liver and kidney (Kim and Kang, 2004; Malik et al., 2010), as these organs are targets for final accumulation of different heavy metals. The concentrations in both organs seem to be independent of the pathway of uptake. However, the contributions of water and food to metal uptake in fish are reflected by high levels in gills and gut tissues, respectively. For example, for mercury, a harmful metal for which most studies have involved the organic or methylated metal, Lock (1975) reported that in *Salmo gairdneri* the uptake rate of methyl mercury from water was higher compared to food. The percentage uptake of the metal was however higher from the food (5-10 times higher). Table 1.1 summarises data comparing the levels of various tissues contaminated following both aqueous and food or dietary route. Soluble metal

fractions may accumulate preferentially via gills whereas particulate metal fractions via digestive tract (Dallinger and Kautzky, 1985). In fishes uptake of metal via water, the higher levels are generally found in gills than gut tissues. In contrast, fishes accumulating heavy metals via food, the higher concentration is in the gut and liver tissues compared to other tissues. Therefore, it appears that both gills and gut tissues are important pathways for metal uptake in fish.

Table 1.1 Comparison of metals levels in different tissues of fish following different routes of uptake (i.e. food and water). Metals, fish species, tissues are specified. The levels in organs are ranked in decreasing order.

Heavy metal	Species	Tissue ranked by concentration	Reference
A: accumulation by food			
Cu	*Sebastes schlegeli*	liver > intestine > kidney > gill	(Kim and Kang, 2004)
Cd	*Terapon jarbua*	liver > digestive gland > gill	(Dang and Wang, 2009)
Cd	*Pseudosciaena crocea* R	kidney > liver > gill > muscle	(Li et al., 2009)
Cu	*Cyprinus carpio* L.	liver > Intestine > kidney > gill	(Hashemi et al., 2007)
Cd & Cu	*Salmo salar*	liver > Intestine > kidney > gill	(Lundebye et al., 1999)
Hg	*Cichlasoma facetem*	gut > liver > muscle > head	(Hamdy and Prabhu, 1979)
B: accumulation by water			
Cu	*Platichthys flesus*	gill > liver > kidney > plasma	(Stagg and Shuttleworth, 1982)
Cu & Pb	*Tilapia zillii*	gill > kidney > liver	(Ay et al., 1999)
Cd	*Cyprinus carpio* L.	kidney > liver > muscle	(de Conto Cinier et al., 1999)
Cd	*Pleuronectes platessa*	gill > kidney > liver > gut	(Pentreath, 1977)
Co-60	*Cyprinus carpio* L.	gill > digestive tract	(Amiard and Amiard-Triquet, 1979)
Cr	*Oreochromis niloticus*	liver > gill > muscle	(Nuray et al., 2010)

1.2.2.3 Modifying factors affecting heavy metals toxicity

As mentioned earlier, aquatic organisms living in contaminated environments tend to accumulate heavy metals in their organs. Generally, metal distribution in various tissues are related to their uptake, concentration, time of exposure, environmental factors (pH, water temperature, hardness, salinity, dissolved oxygen) and biological factors (feeding habits, fish age), resulting in structural lesions and impairment of functions (Jezierska and Witeska, 2001; Jezierska and Witeska, 2006). Different metals however show various affinities to fish tissues. Most of them accumulate mainly in liver, kidney and gills. Fish muscles, comparing to the other tissues, usually contain the lowest levels of metals (Dural et al., 2007; Karadede and Ünlü, 2000). Metal accumulation in fish tissues usually follow the grading: Fe > Zn >Pb >Cu > Cd > Hg. Zn concentration may be very high over 300 μg g^{-1} d.w. Maximum levels of Cu and Pb are very lower and usually do not exceed 10 μg g^{-1} d.w. Cd and Hg accumulated in fish tissues in very low amount and is usually below 1 μg g^{-1} d.w. (Jezierska and Witeska, 2006).

The environmental conditions affect uptake and accumulation of metals in fish. Douben (1989) found that the rate of uptake and elimination of Cd by *Neomacheilus barbatulus* L. increased with water temperature on metal absorption than on elimination. According to Köck et al. (1996), Cd and Pb levels in *Salvelinus alpinus* kidney and liver indicate that higher uptake rate of both metals occur at high temperature during summer. The authors explained that this probably was due to increased metabolic rate.

Many studies indicate that water acidification affect metal uptake and accumulation. Çogun and KargIn, (2004) found that the accumulation of the Cu increased at lower pH. Also, data generated from different lakes indicate that the levels of Pb and Cd but not Zn are significantly increased in fish from acidified lakes (Grieb et al., 1990; Haines and Brumbaugh, 1994; Wiener et al., 1990). The authors explained that water acidification affects metals accumulation by direct way via damage to the epithelia which become more permeable to metals, and in addition, competitive uptake of H^+ ions could inhibit metal absorption or by indirect route by changing the solubility of the metals rates in aquatic organisms.

Water hardness or alkalinity (particularly Ca concentration) significantly affects metals uptake of metals across gill epithelium. According to Comhaire et al., (1994) the effect of Ca concentrations on Co^{+2} uptake involve direct interactions with system involved in the translocation of Co^{+2} across the gill epithelium of the common carp. Data obtained by Baldisserotto et al. (2004) showed that enrichment of water with Ca^{+2} protect against both, dietary and waterborne uptake of cadmium. Pagenkopf (1983) reported that calcium might compete with other metals for binding sites in the gill surface.

In common with hardness, salinity reduces the uptake and accumulation of metals by fish. Bugenyi and Lutalo (1990) showed that high concentrations of mineral salts in the water have the ability to reduce the effectiveness of copper ionic activity through adsorption, precipitation, and ionic interference. The high concentration of organic compounds in the water also complex and chelates Cu^{2+} ion. With dissolved oxygen (DO), it has been found that as DO decreases, toxicity of a given concentration of Cu increases. This may be partly due to increased ventilation,

which in turn increases with the amount of Cu presented to the absorptive gill surfaces (Llyod, 1961). Such relationship has been reviewed in details in Chapter 5 (section 5.1).

Intrinsic factors also affect metals uptake and accumulation. Different species of fish from the same water body may accumulate differing amounts of metals. Interspecies differences in metal accumulation could be related to living (i.e. physiology) and feeding habits. Kidwell et al. (1995) reported that predatory fish species accumulated more Hg but the benthivores contained more Cd and Zn. Higher levels of Hg in predatory fishes compared to non-predatory one was also reported by Voigt (2007). The data obtained by Yilmaz (2003) suggested that Cu, Ni, Cr and Pb levels were higher in benthic fish.

The affinity of different metals to fish organs may differ. In general, accumulation of essential metals such as Cu, Zn, Fe, Mn or Co is organ specific, even at low environmental levels. For instance, Cu shows high affinity to the liver (Kim and Kang, 2004), while Zn shows distinct affinity to the gonads (Yilmaz et al., 2010). The gills are directly in contact with water and in general, concentration of metals in gills reflects their concentration in water where the fish lives, whereas the concentration in liver represent storage of metals in the water (Roméo et al., 1999).

Furthermore, the accumulation of metals in fish tissues is time dependent. Generally in the initial stage of exposure metal is accumulated at high level, and then the rate stabilizes when equilibrium of metal uptake and elimination rate is attained. Many data indicate that dynamics of metals concentrations in various organs during exposure and depuration may be different. At the beginning of waterborne exposure metals levels in the gills rapidly increase, and then usually

decrease. Whereas, at the end of exposure, metals are rapidly removed from gill (Hollis et al., 2001). In case of dietary exposure, metals levels in the gills and digestive tract are generally increased much lower and usually reach lower rates, while in the liver remain high until the end of the exposure (Ausseil et al., 2002; Kim and Kang, 2004).

1.2.2.4 The importance of aquatic food chains in metal accumulation and toxicity in fish

The transfer of the heavy metals through food chains remains an important issue in metal assimilation by fish. Most heavy metals are effective at very low concentrations, however the extent to which the food chain effect in fish could be influenced by ecological factors, two possible factors should be regarded: The first kind of influence is related to degree of contamination of the food supply which is reflected significantly by high concentrations of heavy metals in sediment, benthic animals and macrophytes than by elevated concentrations in water (Clearwater et al., 2000; Dallinger et al., 1987; Di Giulio and Scanlon, 1985). This model of distribution may occur with both low and high concentrations of contamination (Dallinger and Kautzky, 1985), depending on their habitat preferences or specialized food requirements. For example, bottom dwelling fish species accumulate heavy metals due to association with metal containing sediments (Ney and Van Hassel, 1983). Therefore, ingestion of sediments or sediments dwelling invertebrates may present an important source of metal uptake by fishes (Czarnezki, 1985; Prosi, 1989; Vogt and Quinitio, 1994).

The secondary factor might be related to reduction in species diversity. Numerous reports indicate that heavy metal contamination may lead to elimination of

susceptible species (Roch et al., 1985). As a result, the food chain is shortened and predictor fish are forced to feed more on only one kind of metal tolerant organisms as food (Boyle et al., 2011).

Metal tolerance of food organisms is based on two opposite effects: detoxification and accumulation of metals. Among metal detoxifying food organisms aquatic isopods, snails and sludge worms have ability for storing large amount of heavy metal (Bryan and Langston, 1992). At the same time, these organisms possess effective detoxification mechanisms by which the metals combine to metal binding proteins or stored in cellular structures like vacuoles and lysosomes (Brown, 1976; Maltby et al., 1987). In highly polluted (metals) aquatic environment, the opposite effect might be seen in which metal tolerant phytoplankton species may favor the growth and are characterized by a decreased uptake of heavy metal per unit of biomass (Gächter and Geiger, 1979). Therefore, the tolerance is achieved by the exclusion of heavy metals. This process may play an effective role in ecosystem by decreasing the bioavailability of metals for organisms belonging to higher trophic levels.

1.2.2.5 Mode of action of heavy metal contamination

Many industrial and agricultural activities have contributed to the pollution of fresh water system with various heavy metals, thereby bringing harmful effects on both aquatic biota and human health (Yang et al., 2009). This is particularly in less developed countries, compared to most developed countries where emissions have declined over the last 100 years (Jarup, 2003). Most of the heavy metal ions

induce toxicity via the formation of coordination complexes and clusters in the animal cells (Albinas et al., 2005). Low level of heavy metals may exhibit a chronic stress which may not kill the individual fish but cause reduction for the growth rate (Vosylienė et al., 2003), thus reducing their ability to compete for food and habitat. These metals cannot be destroyed via biological degradation and have the ability to bioaccumulate in the environment causing adverse effects to the aquatic environment and consequently to the human via aquatic products as a sources of food (Kalay et al., 1999). Thus, humans can be at great risk through contamination of the food chain (Cheraghalia et al., 2010).

The main threats to human health from heavy metals are associated with exposure to lead, cadmium, mercury, chromium, nickel, copper, and arsenic which are considered the most important heavy metals that may cause health risks from consumption of contaminated foods. The effects of heavy metal toxicity studies confirm that heavy metals can directly influence behavior by impairing mental and neurological function, influencing neurotransmitter production and utilization, and changing several metabolic pathways. In biological systems toxic metals can induce impairment and dysfunction including the blood and cardiovascular, detoxification pathways (i.e. colon, liver, kidneys, skin), endocrine (hormonal), energy production pathways, enzymatic, gastrointestinal, immune, nervous (peripheral and central), reproductive, and urinary as reviewed by Mudgal et al., (2010).

Mercury (Hg) is considered to be a global pollutant because it is the predominant form of atmospheric Hg and highly toxic metal for living organisms (Li et al., 2001;

Schroeder and Munthe, 1998). Even at very low concentration, Hg could be converted to methylmercury (Me-Hg) and accumulate in the food chain, posing a potential hazard to humans' health. The concern of Hg pollution arises from the health effects caused by Me-Hg through the consumption of fish (Clarkson, 1993). Poisoning by methyl mercury compounds have been observed in large-scale outbreaks in Japan, China, Iraq and various parts of the world (Horvat et al., 2003; Li et al., 2009). The profound capacity of soft acid (acceptor) CH_3Hg^+ to bind soft ligands explains the high toxicity of methyl mercury compounds than Hg (Pelletier and Larocque, 1987; Rabenstein, 1978).

Cadmium (Cd) causes various toxic effects in the body. It can react with polythiol groups of cellular macromolecules such as lipids, glycogen and amino acids. Cd bioaccumulated in tissues can replace the essential element zinc present in the enzymes carboxypeptidase (Price and Morel, 1990) and metallothionein (Jensen et al., 1996). The metal causes oxidative damage by alteration of mitochondrial activity and to other biomolecules including DNA.

In the environment, Chromium (Cr) exists mainly in the trivalent and hexavalent states, the latter being the predominant species in natural water. Cr in combination with nickel as trace metals function as potential health hazard that causes maldisfunction in gastrointestinal, hepatic and neurological activities. Hexavalent chromium generates reactive oxygen species (ROS), which increase risk for cellular and hepatic DNA damage, enhance intracellular oxidised states, and decrease cell viability with necrosis and programmed cell death (apoptosis) (Bagchi et al., 2002).

Nickel (Ni) salts considerably increase the level of lipid peroxidation and simultaneously decrease glutathione level and glutathione peroxidase activity in the liver (Das et al., 2001).

Lead (Pb) exposure produce adverse effect on the central nervous system as it is extremely toxic to most of the aquatic organisms (Jarup, 2003). The absorption of relatively small amounts of lead over a long period of time in the human body can lead to the malfunctioning of the organs (Admus et al., 2007). Children are mainly sensitive to lead exposure due to high gastrointestinal uptake and the permeable blood–brain barrier (WHO, 1995). Recent studies have indicated that lead could lead to neurotoxic effects at lower levels of exposure than previously anticipated (Jarop, 2003).

Copper is an essential trace element to most aquatic organisms at very low concentrations (5-20 μg g^{-1}) by humans, other mammals, fish and shellfish for carbohydrate metabolism and the functioning of more than 30 enzymes. It is also required for the formation of haemoglobin and haemocyanin, the oxygen-transporting pigments in the blood of vertebrates and shellfish respectively. However, copper concentrations that exceed 20 μg g^{-1} can be toxic, as explained by Bradl (2005) and Wright and Welbourn (2002). The use of copper to control algae, fungi and molluscs demonstrates that it is highly toxic to aquatic organisms. The toxicity of copper depends on many water characteristics. Increased amounts of natural organic matter, carbonate, and higher pH levels may reduce the toxic effect of copper. The presence of dissolved organic carbon (DOC) in the water column provides some protection from the effects of copper on the gills because copper forms complexes with DOC and will therefore be less bioavailable. Copper

toxicity also depends on the organism considered. Some aquatic organisms are more susceptible to copper than others. The concentration required for killing 50% (LC_{50}) of the marine mussel *Mylitus edulis* in 4, 10, 14, and 30 days is 200 to 300, 90, 15, and 2 mg l^{-1} total copper respectively (Luoma and Carter, 1991).

The effects of copper on aquatic organisms can be directly or indirectly lethal. In fishes, the gill surfaces low affinity for metal allows greater entry of the metal to the intracellular compartment. Once there, more complex binding sites are present. Binding to these ligands causes one or more of the following toxic mechanisms: (a) blocking of the essential biological functional groups of biomolecules; (b) displacing the essential metal ion in molecules; or (c) modifying the active conformation of biomolecules. These mechanisms may account for the specific inhibition of ion transport from ionic copper (Cu^{+2}) exposure (Luoma and Rainbow, 2005). Gills become frayed and lose their ability to regulate transport of salts such as NaCl and KCl into and out of fish. These salts are important for the normal functioning of the cardiovascular and nervous systems. When the salt balance is disrupted between the body of a copper-exposed fish and the surrounding water the death of the fish can result (Luoma and Rainbow, 2005). .

Therefore, the presence of heavy metal at high concentrations in water or sediment does not involve direct toxicological risk to fish, especially in the absence of significant bioaccumulation. It is known that bioaccumulation is to a large extent mediated by biotic and biotic factors that influence metal uptake, due to the harmful effects of metals on aquatic ecosystems. In addition, metal interactions in the intestines of fish may affect the assimilation of essential and non-essential metals with possible toxicological consequences for fish (Boyle et al., 2011). It is

necessary to monitor their bioaccumulation in key species, because this will give an indication of the temporal and spatial extent of the process, as well as an assessment of the potential impact on organism's health (Zhou et al., 2008). Therefore, measures should be taken in order to minimize the risk of adverse health effects.

1.2.2.6 Mechanism of acute and chronic copper toxicity in fish

Cu is generally toxic to aquatic organisms particularly to fish, when ambient concentrations exceed physiological thresholds. The mechanisms of toxicity have been well elucidated for teleost fishes in the literature. In general, Cu exposure disturbs physiological functions in fish (Clear water et al., 2000). It accumulates in tissues and predominantly interferes with ionregulatory homeostasis both by decreasing branchial Na^+/K^+ adenosine triphosphate (ATPase) activity and by causing gill damage. Cu also affects energy metabolism, reduces swimming capacity and induces a corticosteroid stress response at lethal and sublethal concentrations (De Boeck et al., 2001). In addition, molecular data obtained indicated that intestinal Cu concentrations and ^{110m}Ag metal interactions in the intestines of fish may affect the assimilation of essential and non-essential metals with possible toxicological consequences for fish (Boyle et al., 2011).

The mechanism of acute Cu toxicity to fish can be easily explained by direct target organ effects of Cu which results from the combined effects of a reduction in sodium (Na^+) influx and an increase in Na^+ efflux. The main target organ for waterborne exposure is the gill epithelium, which suffers an acute oedema and epithelial lifting during exposure (Taylor et al., 1999). This oedema perhaps

originates by Cu^{2+}- dependent inhibition of the branchial $Na^{+/}K^{+}$-ATPase (Handy, 2003). Reduced Na^{+} influx is thought to be associated with non-competitive binding of Cu ions to the basolateral Na^{+}-pump, Na^{+}/K^{+}-ATPase, resulting in lower Na^{+} uptake rates into the blood (Nadella et al., 2007; Pelgrom et al., 1995; Li et al., 2009). The result of this net loss of Na^{+} is a solute accumulation in the epithelial cells resulting osmotic influx of water into the cells. This initial disturbance is then followed by a general loss of ionoregulatory control by the gill, efflux of electrolytes from the blood over the gill epithelium, resulting cardiovascular failure and fish death (Pilgaard et al., 1994; Nussey et al., 1995). A moderate hypoxia due damaging gill could also contribute to the latter stages of toxicity (Sellers et al., 1975).

Chronic sub-lethal exposure to Cu causes a series of cellular and physiological changes in fish that enable the animal to survive. Cu is also an endocrine disrupting metal in the aquatic ecosystem, and has a number of normal neuro-endocrine roles in vertebrates (reviewed by Handy, 3003). Similar to other heavy metals, Cu could be accumulated in fish tissues not only from the aqueous phase but also via the dietary route. Waterborne Cu accumulates in numerous tissues during chronic exposure including the gill, liver and kidney and to a lesser amount in the muscle (Grosell and Wood, 2002). Whilst these target tissues are broadly the same as acute exposures, during chronic exposure fish have more time to down-regulate Cu uptake across the gills and re-distribute newly acquired Cu to the liver for excretion to minimise toxic effects (Grosell and Wood, 2002). As in mammals, the liver is the major homeostatic organ for controlling excretion and circulating Cu concentrations, and also biliary Cu excretion is elevated in situations of elevated

uptake (Grosell and Wood 2002; Kamunde et al., 2002). Whole body Cu status in fish is also a function of body size (as in humans, Linder, 1991). Several studies have shown that adult fish are able to regulate tissue Cu levels to lower concentrations than smaller juvenile fish of the same species (e.g. intestine, Handy et al., 2002; muscle, Grosell et al., 1996). Therefore, both the temporal change of Cu distribution and excretion, and apparent body-mass dependence of these events suggest a well regulated physiological function in fish.

1.3 Biotransformation reactions and enzymes

All animal cells possess a suite of biotransformation enzymes, usually present in highest levels in the liver. In fish, the activity of these enzymes may be induced or inhibited upon exposure to contaminants (Bucheli and Kent, 1995). In general term, the major function of the biotransformation enzymes is the conversion of lipophilic xenobiotics to more hydrophilic, water- soluble excretable metabolites, to reduce half-life of the contaminants and to reduce the effect of exposure as well as to avoid the accumulation in the process of biotransformation (Gatlin and Wilson, 1986). Three major phases of enzymes involved in xenobiotic biotransformation are distinguished as: phase I, phase II and phase III enzymes (Livingstone and Pipe, 1992). A summary of metabolic reactions is given in Table 1.2. The phase I of metabolism, adding reactive functional groups, includes oxidation, reduction or hydrolysis. For the most of the xenobiotic compounds the phase I reactions are catalyzed by microsomal monoxygenase (MO) enzymes, also known as the mixed-function oxidase (MFO) system (i.e. cytochrome P450 [CYP450], cytochrome b_5 [CYb_5], and NADPH cytochrome P450 reductase (Livingstone, 1998). The most important enzyme system catalyzing phase I metabolic reactions for environmental

contamination in fish is the induction of CYP450 1A (CYP1A) (Bucheli and Kent, 1995; Goksøyr and Förlin, 1992). This enzyme system is also involved in the oxidative metabolism of xenobiotics in the liver (Stegeman, 1985). Several studies have established an increase in the hepatic CYP1A protein levels in different species of fish after exposure to organic trace contaminants. Particularly, PAHs, PCBs, PCDDs and PCDFs caused a significant or a higher increase (500% of control) in CYP1A content (Hahn et al., 1998; Klemz et al., 2010; Miller et al., 2004).

The phase II of metabolism involves a conjugation of the xenobiotic parent compound or its metabolites with an endogenous ligand. Conjugations are addition reactions in which large and often polar chemical groups or compounds such as sugars, glutathione and amino acids are covalently added to xenobiotic compounds thus facilitating transport and produce water- soluble molecules which is generally non-toxic, more easily excreted via biliary and renal route (Livingstone, 1998). In mammals and fish, the organic anions formed by phase II reaction are frequently substrates for facilitated renal tubular transport and are therefore rapidly excreted in urine by a combination of glomerular filtration and tubular transport. Thus, depending on structure and the extent of phase one I biotransformation of a particular organic pollutant, the rate of excretion will be influenced by the extent to which the pollutant is conjugated. Pollutants that are rapidly excreted show no lasting toxicity (James, 1987). Phase III of metabolism includes catalysis by enzymes of the xenobiotic compounds which are also active in phase I and/or phase II reactions in which the xenobiotic conjugates may be further hydrolyzed or metabolized (Livingstone and Pipe, 1992).

Table 1.2 The principle reactions in the biotransformation of xenobiotics.

	Reaction	Enzyme	Substrates
Phase I	• Oxidation • Reduction • Hydration • Hydrolysis	• CYP* and MFO* • NADPH*-CYP reductase • Epoxide hydrolase • Esterases and amidases	• Amins and many sulphur compounds • Quinones • Epoxides • Estrase & amides
Phase II	• Glucuronide conjugation • Amino acid conjugation • Glutathione conjugation • Sulphation	• UDP-glucuronosyltransferase • Amino acid N-acyltransferase • Glutathione –S- transferase • Sulphotransferases	• Phenol, alcohols, amins & amide • Phenol, alcohols, amins, amide & thiols • Epoxide, haloakannes, nitroalkanes & alkenes • Phenol, alcohols, amins and thiols
Phase III	• Oxidation • Hydrolysis • Deamination	• MFO • Peptidases • Transaminases	• Thioethers • Glutathion conjugates • Amino acid conjugates

* CYP = Cytochrome P-450, MFO = mixed function oxygenase system, NADPH = Nicotinamide adenine dinucleotide phosphate.

1.4 Stress in aquaculture

The term stress has been widely used and redefined by many biologists. Jha (2004) defined stress as: *"situations where the fitness of individuals (or populations) is reduced because of changed environmental conditions".* Natural environmental stress factors that fish may encounter include change in season, temperature and salinity. Social stress (i.e. crowding and hierarchy) is also considered in the literature (Iwama, 1997). Furthermore, hypoxic areas, or 'dead zones' which are also becoming an increasingly worldwide problem could have serious consequences on ecosystem functioning (Diaz and Rosenberg, 2008). As described earlier, anthropogenically induced stress factors include xenobiotics including metal contaminants.

From an ecological or aquaculture point of view, severe cases of stress my result in high rates of mortality, sub lethal stress may result in disturbance in physiological and behavioural functions, immunosuppression and decreased disease resistance, decreased growth rates and adverse effects on health (Yin et al., 1995). Hence, the management of the stress including its dietary modulation is critical in running and maintaining a successful aquaculture system.

1.5 Hypoxia and hyperoxia Induce oxidative stress in fish

The oxygen demands of fish mainly depends on species, activity and fish size, and fish have to adapt to fluctuations in environmental factors, such as temperature, salinity and water quality, that affect oxygen concentration. Sensitivity to oxygen is very dependent on species. Thus, lethal concentration of oxygen in water varies from 0.5 to 3 mg l^{-1} depending on species and was estimated around 2-3 mg l^{-1} for salmonids (Landman et al., 2005). Hypoxic as

well as hyperoxic conditions are common in polluted and naturally eutrophic waters. Moreover, a rapid change from hypoxia to hyperoxia can occur within only a few hours as the result of photosynthetic oxygen production. Chronic hypoxic conditions prevailing in so called 'dead zones' in different parts of the world, which is linked to anthropogenic activities, often leads to mass mortality of sensitive biota and could lead to overall reduction in biodiversity (Diaz and Rosenberg, 2008). Elevated oxygen concentration serves as an inductor for oxidative stress in aquatic animals and may lead to DNA damage (Halliwell and Aruoma, 1991). Also, hypoxic conditions can lead to cell death and apoptotic DNA disintegration (Gorokhova et al., 2012).

In fact, any kind of physiological stress response includes modification of ROS concentrations, which in certain states result in the development of oxidative stress. Oxygen availability is among the factors that critically affect cellular ROS levels and respectively induce oxidative stress (Lushchak and Bagnyukova, 2006; Nikinmaa, 2002). It is generally established that reduced environmental oxygen concentration (termed hypoxia) or its full absence (termed anoxia) reduces ROS level, but normoxic recovery sharply increases ROS level resulting in oxidative stress (Halliwell and Gutteridge, 1999). Studies on anoxia/hypoxia effects on the common carp *Cyprinus carpio* generally fit the above. Hyperoxia clearly results in a transient oxidative stress in common carp tissues (Lushchak et al., 2005).

1.6 Reactive oxygen species (ROS) and production of oxidative stress

Reactive oxygen species (ROS) are constantly generated as undesirable bi-products of normal metabolic pathways and also by some specific sites under certain cellular control. At the same time, ROS are degraded through several

processes. Two different mechanisms; generation, degradation of ROS, generally under delicate cellular control are very low ($<10^{-8}$ M) steady state ROS levels are controlled (Halliwell and Gutteridge, 2007). However, under some circumstances, the dynamic equilibrium can be disturbed leading to enhanced ROS level and damage to cellular constituents which is called "oxidative stress". Hence, oxidative stress is caused by an imbalance between the generation of intra- and extracellular ROS and the ability of the antioxidants to scavenge them (Livingstone, 2003; Lushchak, 2011).

ROS containing one or more unpaired electrons, e.g., superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\bullet}$), and non-radical species such as hydrogen peroxide (H_2O_2), as well as reactive nitrogen species such as nitric oxide ($NO^{\cdot}$), nitric dioxide ($NO_2^{\cdot}$), and peroxynitrite ($ONOO^-$) (Livingstone, 2003). The generation of the different ROS can be inter-related, producing ultimately the potential damaging OH^-Thus $O_2^{\cdot-}$ can be reduced to H_2O_2 via dismutation. H_2O_2 and $O_2^{\cdot-}$ can react together to produce extremely reactive hydroxyl anion and hydroxyl radical via metal catalysed Harber Weiss reaction (Fig. 1.2). This leads to peroxidation of lipids, proteins, damage to cell structure (DNA, RNA) and apoptosis and programmed cell death (Di Giulio et al., 1989).

The main endogenous site of ROS generation, in living organisms (usually over 90%) is produced by electron transport chain in mitochondrial, endoplasmic reticulum, plasmatic and nuclear membranes, and photosynthetic system (Puddu et al., 2008). Additionally, minor ROS amounts are produced by various enzymatic oxidase reactions such as cytochrome P450 reductase, lipoxygenases, and xanthine oxidase (Chance et al., 1979). Many environmental pollutants are essentially exogenous sources of ROS in

biological systems. In particular, polycyclic aromatic hydrocarbons, organochlorine, polychlorinated biphenyl, heavy metals (including Cu and Fe) and other chemical toxic pollutants are capable of inducing oxidative stress in aquatic animals (Lackner, 1998). In addition to pollutants, ROS are linked to a variety of environmental factors such as ultraviolet radiation, which plays an important role in the mechanistic aspects of oxidative stress (Valavanidis et al., 2006). Thus, oxidative stress is a pathological xenobiotic-induced mechanism related to overproduction of ROS in tissues, and is considered to be an essential factor in aging processes, DNA damage, and is closely associated with aging and a number of diseases including cancer, cardiovascular diseases, diabetes and diabetic complications in humans (Agarwal et al., 2008).

Recently contaminant- stimulated ROS formation and resultant oxidative damage has been considered to be a significant mechanism of toxicity in aquatic organisms exposed to pollution. Oxidative stress can also be produced from other sources, including: hypoxia, hyperoxia and the use of the ozonisation in aquaculture (Livingstone, 2003; Luschack, 2011). Dirmeier et al. (2002) also reported that oxidative stress results from hypoxic conditions. Moreover, high oxygen solubility at cold-water temperatures is considered to be responsible for elevated levels of ROS and antioxidant concentrations in polar marine invertebrates and fish (Abele and Puntarulo, 2004). In addition, several investigators have observed ROS production after exposure to hyperoxic event, and have investigated that these ROS act as upstream signaling molecules that induce oxidative damage (Buccellato et al., 2004).

Aniagu et al. (2006) demonstrated that polluted waterways and acute extreme exercise could result in DNA damage associated with oxidative stress. Little is

known of the relationships between the oxidative stress, disease and fitness in aquatic organisms. Functional and pathological alterations have been seen with exposure to pollutants, including altered in intracellular Ca^{+2} concentrations in *O. mykiss* with exposure to lindane. At the whole organism level, reduced growth rates were seen in several fish species with exposure to Fe, PCBs and binary mixtures of dichloraniline and lindane (Livingstone, 2001, 2003).

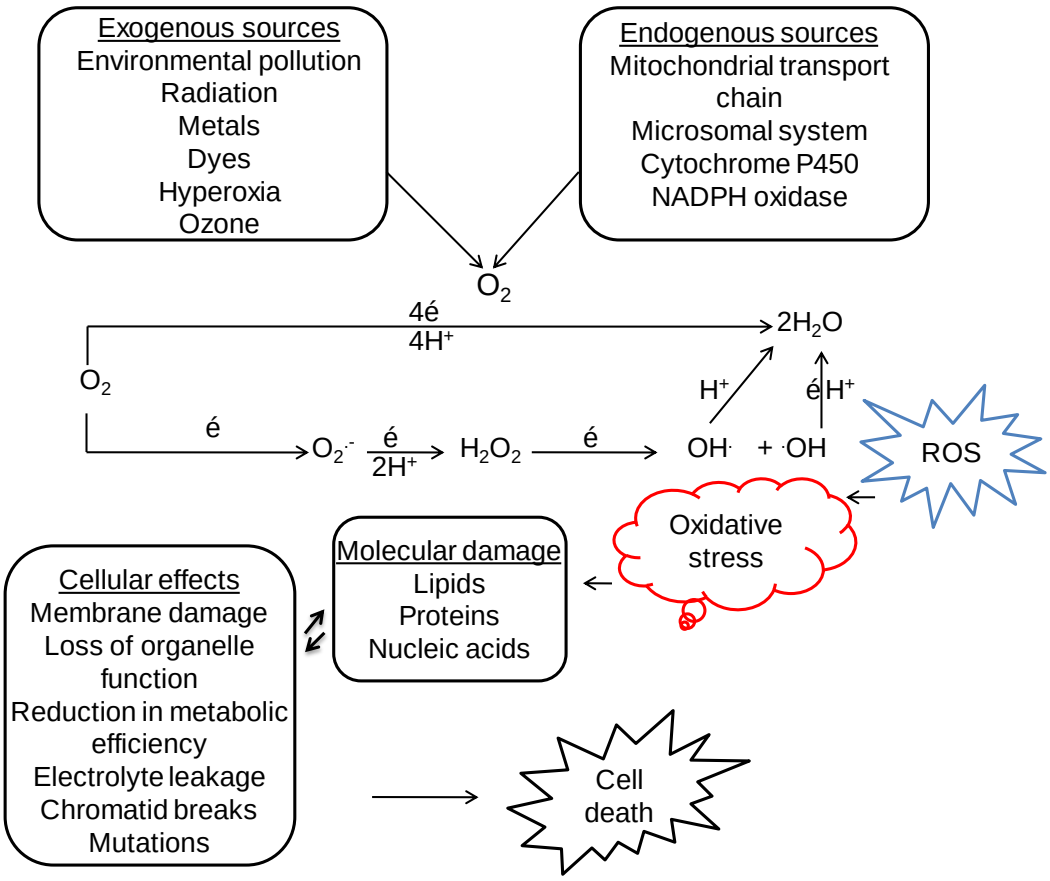


Fig. 1.2 Different ways of oxygen reduction in biological systems with cellular generation of reactive oxygen species (ROS) and their interrelationships with the sequence of major cellular effects.

1.7 Biomarkers

Numerous definitions have been specified for the term 'biomarker', which is generally used in a broad sense to involve almost any measurement reflecting a relation between a biological system and a potential threat, which could be, physical, chemical or biological (Decaprio, 1997). A 'biomarker' is defined by (McCarthy and Shugart, 1990) *"measurements of body fluid, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response"*

Peakall and Walker, (1994) redefined the term 'biomarker' *"as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes) which can be related to exposure to or toxic effects of environmental chemicals"* In the past 25 years, several biomarkers have been developed with the objective to apply them to evaluate the impact of pollution on organisms. More recently, the Water Framework Directive (WFD) of the European Union adopted monitoring programs required to allow accidental pollution assessment for all water bodies by 2015 (Sanchez and Porcher, 2009). Hence, biomarkers have the potential to play vital role in environmental assessment because they can provide information on the impact of contaminants rather than mere quantification of the levels present, as well as, can detect the early biological events, biochemical or physiological change resulting from given exposure that can predict the onset of adverse health effects (Peakall and Walker, 1994).

The biomarkers include several ranges of measurements to assess the level of exposure to toxicants and also the consequences for the organisms. Organisms may elicit responses at all levels of biological organisation from molecular level to the individual level (Decaprio, 1997). Certain biomarkers can be used in *vitro*,

as well as in *vivo,* some of them are specific to mammals or other organisms, whereas others are applicable to all organisms (Timbrell, 1998). According to WHO, (1995) ecotoxicological biomarkers can be classified into three categories (see next sections).

1.7.1 Biomarkers of exposure

These biomarkers are including the detection and assessment of an exogenous compound or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism. Biomarkers of exposure are divided into three classes of biomarkers:

- Biomarkers of **internal dose**: these markers indicate exposure to a particular compound takes place by measuring the compound or its metabolites in body fluids. Measuring the specific metabolites such as mercapturic acids or the measurement the of GSH conjugation are used as a biomarkers for internal dose but need prior information about the structure of the compound.
- Biomarkers of **effective dose**: these biomarkers indicate that the exposure to a particular compound has resulted in the compound or its metabolites, reaching a toxicological significant target. Due to differences in the rate and route of metabolism, DNA or protein adducts which could be quantified and can potentially considered to be a biomarker of exposure to chemical carcinogen such as aflatoxin B_1 (AFB_1), which may be present in the diet, and may lead to mutation and hence development of cancer (Groopman et al., 1994). Another biomarker is urinary 8-hydroxy-2-deoxygunanosine, which is considered to be a biomarker of oxidative damage (Li et al., 2005).

1.7.2 Biomarkers of response or effect

These biomarkers range from simple biomarkers (e.g. measuring the body weight and population changes) to the complex biomarkers (e.g. detection of specific isoenzymes by using immunochemical test, chromosomal aberrations, histopathological lesions etc.). Generally, biomarkers can be classified into invasive biomarkers which include. Invasive markers in tissues cover an array of pathological methods, including gross pathology and histopathology (using either light or electron microscopy) and biochemical changes. Non-invasive biomarkers extensively used in the aquatic organisms, which is based on physical parameters in order to assess body function in health and disease when no break in the skin is created and there is no contact with the mucosa, such as body weight, micronucleus induction in the blood cells (Van Campenhout et al., 2004).

1.7.3 Biomarkers of susceptibility

These biomarkers deal with ability of an organism to respond to the challenge of exposure to a specific xenobiotic compound, including genetic factors, DNA repair capacities, immune response and changes in receptors which alter the susceptibility of an organism to that exposure. The activities of enzyme systems such as glutathione–S-transferase (GST), cytocrome P-450 and N-acetyltransferase (NAT) represent a better biomarker of susceptibility markers.

1.8 Applications of biomarkers

1.8.1 Lipid peroxidation

Oxidative stress can damage any biological molecules; indeed, proteins and DNA are more significant target of injury than lipids, whereas lipid peroxidation often occurs late in the injury processes. Lipid peroxidation is the most

commonly used approach in free radical research field since many species especially aquatic organisms; contain high amounts of lipids with polyunsaturated fatty acids residues, a substrate for oxidation. Lipid peroxidation usually begins with the abstraction of a hydrogen atom from an unsaturated fatty acid. Polyunsaturated fatty acids (PUFA) (especially arachidonate) and those incorporated into lipids are readily attacked by free radicals due to its double bonds between carbon atoms. By contrast, both monounsaturated and saturated fatty acids are more resistant to free-radical attacks. Biological membranes are often rich in unsaturated fatty acids and bathed in an oxygen-rich, metal-containing fluid. Therefore, it is not surprising that membranelipids are susceptible to peroxidative attack. The occurrence of lipid peroxidation in biological membranes causes impairment of membrane functioning, changes in fluidity, inactivation of membrane –bound receptors and enzymes, and increased nonspecific permeability to ions such as Ca^{+2}. For example, deformation of red blood cells after exposure to peroxides causes them to become leaky to K^{+} ions (Halliwell and Chirico, 1993).

One of the most commonly applied assays for measuring lipid peroxidation is the thiobarbituric acid reactive substances (TBARS) test. The TBARS method is based on the measuring end products of lipid peroxidation primarily melanodialaldehyde (MDA) formed in peroxiding lipid systems under acidic condition, it involves reaction of MDA (or malonaldehyde–type products including unsaturated carbonyls) with TBA to yield a colored compound that is measured spectrophotometrically (Gutteridge, 1986).

1.8.2 DNA strand breaks (as a genotoxic biomarker)

Significance of DNA strand breaks has been highlighted by Jha (2004). This could be easily measured by single cell gel electrophoresis or the Comet assay, which is commonly used for evaluating impact of genotoxicants in the environment (Collins, 2009; Frenzilli et al., 2008; Jha, 2008; Mitchelmore and Chipman, 1998). Ostling and Johanson (1984) were the first to develop a new sensitive technique of microgel electrophoresis for measuring DNA damage. This technique was later modified by Singh et al (1988) to become the alkaline Comet assay for detecting DNA damage in single cells via both *in vivo* and *in vitro* exposures. Since its initial development 28 years ago, Comet assay has become most popular test in aquatic toxicology to investigate the exposure to environmental contaminants (Ohe et al., 2004), with wide range of applications in genotoxicity, molecular epidemiology and human health risk (Collins et al., 1997; Jha, 2008). In addition it has been used to study apoptosis as well as to detect physical stress agents like sunlight, radioactivity and nutritional toxicology (Olive, 1999; Roser et al., 2001).

Several studies on fish recognized the Comet assay as one of the most sensitive methods available for measuring DNA strand breaks in contrast to other biomarkers widely used in genetic ecotoxicology, such as the micronucleus test and sister chromatid exchanges (Buschini et al., 2004; Cavas and Konen, 2007; Kim and Hyun, 2006). The main advantages of the Comet assay are: flexibility, ease in application, low costs, only a small number of sample cells are required, and it is capable of measuring DNA strand damage and repair at single cell level following genotoxic exposure (Lee and Steinert, 2003; Mitchelmore and Chipman, 1998).

The overall principle of this assay is that DNA strand breaks create fragments or supercolid DNA-loops that, when embedded in an agarose gel, migrate in an electric field. In 1999 an expert panel at an International Workshop on Genotoxicity Test Procedures (IWGTP), held in Washington, identified guidelines and methodological steps for this assay under *in vivo* and *in vitro* conditions. The methodological of this assay include slide preparations with agarose gel embedded with single cells, lyses of cell membranes alkaline unwinding, electrophoresis (pH >13), neutralization, staining and cell scoring (visual scoring) under fluorescence microscope. There are also some modifications in the standard protocol of the alkaline version of the Comet assay enabling the detection of specific classes of DNA damage. One of these alternative procedures have been used to detect oxidative base damage, using lesion-specific repair enzymes from *Escherichia coli* to convert the oxidative damage into single strand breaks (Collins et al., 1997; Collins, 2004; Collins, 2009; Collins et al., 1993). The formamido pyrimidine glycosylase (Fpg) protein is recommended for the detection of oxidised purines, in particular, 8-oxoguanine (Albertini et al., 2000; Tice et al., 2000) and endonuclease III (Endo-III) is recommended for the detection of oxidised pyrimidines (Albertini et al., 2000).

Various measurements are used to assess the DNA damage but the most common parameters are the tail extent moment, the olive tail moment (OTM), and the percentage of DNA in the tail (%tail DNA) (Kumaravel and Jha, 2006). The shape, size, and amount of DNA within the comet, plays vital roles in the determination the level of damage (Kumaravel et al., 2009). Among all the

parameters provided by the software, Tail DNA (%) is considered to be most reliable (Kumararvel and Jha, 2006).

1.8.3 Histopathologic biomarkers

Histopathological alterations in fish tissues are biomarkers that signal effects resulting from exposure to environmental stressors. This category of biomarkers has the advantage of allowing one to examine specific target tissues and cells as they affected under both *in vivo* and *in vitro* conditions (Bernet et al., 1999). In addition, for field assessment, histopathology is the most rapid tool of detecting adverse sub- lethal and chronic impacts of exposure in different organs and tissues including individual finfish or shellfish (Admas et al., 1989). During the last two decades, a variety of histological changes in fish and mussels have been used as indicators for pollution monitoring, and many of these have been adopted in major national monitoring programmes which are designed to assess the effects of environmental pollution on histopathological features in fish (Admas et al., 1989; Au, 2004).

Furthermore, histopathological analysis provides a possibility of allowing investigators to directly examine the specific tissues and cells affected by exposure to environmental pollutants (Bernet et al., 1999). Therefore, histopathological biomarkers are higher level responses occurring in some toxic conditions following chemical and cellular interaction and often signify prior metabolism and macromolecular binding (Au, 2004). Most toxicants which are potentially genotoxic require metabolic activation to an ultimate from that binds covalently, forming DNA adducts. If the adduct persists and is not repaired, subsequent changes could result in an acute toxicity (cell death) or could possibly abnormal growth and tumour formation (Hinton et al., 1992). Similarly,

exposure to environmental chemicals can increase or decrease hepatic enzyme activities, resulting to an increase in cellular detoxification mechanisms, and leading to cellular toxicity and death, which is subsequently detected as a tissue necrosis or apoptosis (Au, 2004). Histopathological biomarkers also may reflect prior contaminant induced reduction in host defences. For example, xenobiotic exposure may reduce immune response, leading to infections disease, perhaps neoplasia or death (Hinton et al., 1992).

Biomarker responses may include all levels of biological organisation with an integrator of biochemical and physiologic changes. The changes may be noted in the distribution of molecules, such as glycoproteins on cell surfaces, organelle number, volume, morphology or distribution; cell number, or distribution; and relative weight (Hinton et al., 1992). Histopathological changes and increase in size have been reported in the liver, kidney and gills of many fish as a result of exposure to different toxicants (Camargo and Martinez, 2007; Figueiredo-Fernandes et al., 2007; Handy et al., 2002; Mohamed, 2009). Several pathological alterations have been reported in the kidney of *Cyprinus carpio* exposed to sewage (Kakuta and Murachi, 1997), *Lates calcarifer* exposed to cadmium (Thophon et al., 2003), *Channa punctatus* exposed to zinc (Gupta and Neera, 2006), *Solea senegalensis* exposed to copper (Arellano et al., 1999). Following chronic exposure of rainbow trout to high concentrations of naphthenic acids, gill and liver cells showed a reduction in cell membrane integrity, mitochondrial activity and lysosomal function (Nero et al., 2006). Active fish with high oxygen requirements (hyperoxic condition) show larger gill surface areas than slow moving benthic fish (De Jager and Dekkers, 1974). Moreover, species known to live and tolerate hypoxic events have also been reported to

have increased gill surface area (Matey et al., 2008; Saroglia et al., 2002; Timmerman and Chapman, 2004; Wells et al., 1989).

1.9 Fish as a model organism to assess the impact of environmental contaminants

Fish models have attracted wide interest among non-mammalian species in toxicological studies. This is particularly for the pollutants which are likely to exert their effects on environment. Two common fish species, zebrafish (*Danio rerio*) medaka (*Oryziaslatipes*) and have attracted wide attention as an experimental model for laboratory-based experimental studies. Fish models have also been developed for diseases such as neurodegenerative disease, diabetes and muscular dystrophy in addition to determining the molecular mechanisms of mutagenesis and carcinogenesis following exposure to environmental contaminants (Teather and Parrott, 2006). For instance, zebrafish (*Danio rerio*) have been used as a prime model for the cancer studies for many years (Raisuddin and Seong Lee, 2008). Furthermore, physiological processes have also been elucidated in fish to study aging process (Gerhard, 2007). Such studies are not only enhancing our knowledge of disease mechanisms but also for the development of drugs for a range of diseases.

Furthermore, fish are considered as common model species to evaluate the health of aquatic ecosystems because pollutants build up in the food chain and are responsible for adverse effects including death in the aquatic systems (Farkas et al., 2002). For example, endocrine disrupting chemicals (EDCs) have been regarded as a major environmental concern (Hecker and Hollert, 2011; Jenssen, 2006). EDCs could not only disrupt the natural population but also the human health. Moreover, EDCs may potentially induce genetic damage and can modify carcinogenic responses of chemicals (Jha, 2004; Jha et al., 2000a,

2000b; Taylor and Harrison, 1999). However, there is limited information available pertaining to potential likely effects of their exposure on environmental carcinogenicity although many EDCs are linked with development of cancer (Jha et al., 2000a). Additionally, there is no specific experimental model to assess the impact of potential of EDCs on aquatic species. In this regard, fish could represent a suitable model in its natural habitats since they are exposed to a wide range of toxic compounds and a considerable proportion of these compounds are known carcinogens (Zhou et al., 2009).

In contrast to the potential nutritional benefits of dietary intake, an issue of concern related with frequent fish consumption is the risk derived from exposure to chemical pollutants accumulated in fish and shellfish (Dallinger et al., 1987; Handy, 2003). Until recently, methylmercury and polychlorinated biphenyls (PCBs) were the chemical contaminants to which great attention was paid (Mozaffarian and Rimm, 2006). However, a number of recent studies have shown that fish can also be a potential cause of human exposure to other known toxic contaminants such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) and polycyclic aromatic hydrocarbons (PAHs), or pollutants such as polybrominateddiphenyl ethers (PBDEs), polychlorinated diphenyl ethers (PCDEs), and polychlorinated naphthalenes (PCNs) (Albert et al., 1998; Hu et al., 2002; Mozaffarian and Rimm, 2006). Consumption of fish contaminated with environmental pollutants are known to pose threat to human health (Tomasallo et al., 2010; Urban et al., 2009). Therefore, fish can be used as a prime model for assessing the impacts of various environmental pollutants on human and environmental health.

1.9.1 Cyprinus carpio as a model species for assessing the toxicological potential of environmental contaminants

Common carp (*Cyprinus carpio* Linnaeus, 1758.) have been largely employed in ecotoxicology assessment, as they can serve as bioindicators of environmental contamination (Alinnor, 2005; Dabrowski et al., 2004). A number of unique features have contributed for its attraction as a prime model in our study, such as rapid growth, easy maintenance in the aquarium. Also, carp can tolerate 4 weeks exposure to pollutants with minimal stress handling (Oikari, 2006; van der Oost et al., 1998). Other reasons attributed to common carp include high ability to endure at low oxygen levels, pollutants and turbidity compared to most native fish (Koehn, 2004). They also have species-specific biochemical strategies to allow long-term survival at low oxygen levels (Hochachka and Lutz, 2001). Therefore it has become one of the most popular model organisms for studying responses to various environmental pollutants (van der Oost et al., 1998). Moreover, common carp have previously been successfully used for assessing the quality of the aquatic ecosystems and also the genotoxicity studies. Given so many attributes mentioned above, this has also been recommended as an Organisation of Economic Cooperation and Development (OECD) test species (Klobučar et al., 2010). In contrast to some of the model species (e.g. zebra fish, 3 spined stickleback, *Gasterosteus aculeatus*; European flounder, *Platichthys flesus*), not enough progress has been made to characterise the genome of this important fish species. It is hoped that characterisation of the genome of this species will further enhance its usefulness in toxicological work.

1.10 Hypothesis

Overall, the thesis aimed to probe the following hypotheses:

(a) Elevated dietary copper induce responses at different levels of biological organisation in a representative carp species, *Cyprinus carpio*. (*Chapter 3*).

(b) Chronic hypoxia (i.e. low level of dissolved oxygen) and hyperoxia (i.e. elevated level of dissolved oxygen) induce responses at different levels of biological organisation (i.e. biochemical, DNA, histopathological and growth effect at individual level). These stresses, manifest in varieties of way and could be correlated. (*Chapter 4*).

(c) Chronic hypoxia modifies the responses of dietary copper exposure at different levels of biological organisation (i.e. biochemical, oxidative DNA damage, histopathological and growth effect at individual level). (*Chapter 5*).

(d) Chronic hypoxia and transition back to normoxia induce biological stresses or responses (including oxidative stress) and modify expression of hypoxia related gene in *C. carpio* L. (*Chapter 6*).

1.11 Aims and objectives

The overall aim of the thesis is to assess the biological responses at different levels of organisation (i.e. biochemical, DNA, tissue and individual level responses) caused by exposure to hypoxic condition either alone or in combination with dietary copper in *Cyprinus carpio* L. The study used carp fish, *C. carpio* L. as a model organism which is also recommended as a regulatory fish species, and used the copper as a representative toxic metal. Copper was chosen and tested on fish at different levels because it is widely used to control pathogens in fish culture ponds (Carvalho and Fernandes, 2006) and its

concentration in the aquatic environment is likely to increase due to anthropogenic activities (Al-Subiai et al., 2011).

The specific objectives of this research were:

(i) Assess the biological responses following chronic exposure (i.e. 10 weeks) to different concentrations of dietary copper (250, 500 and 1000 mg kg^{-1}) in *C. carpio*. Another objective was to detect the highly toxic level of Cu on fish (see *Chapter 3* for details).

(ii) Determine the biological responses at different levels of organisation in *C. carpio* following long term exposure to both hypoxic and hyperoxic (1.8, 12.3 mg l^{-1} respectively) compared to normoxic condition. Also the study aimed to assess the correlations between DNA damage with other biomarkers and specific growth rate (SGR) of the fish (see *Chapter 4* for details).

(iii) Investigate whether the impact of hypoxia either alone or in combination with dietary copper will be greater than hypoxia or Cu alone. Also to assess the effects of this combination could enhance the sublethal toxicity at different level of biological functions (see Chapter 5 for details).

(iv) Determine whether tissue specific accumulation of Cu is influenced by Cu and oxygen level (*Chapter 5*).

(v) Measure the hypoxia inducible factor (HIF-1α) gene expression following chronic exposure (i.e. 21 days) to hypoxia and after recovery in normoxia for 7 days in addition to assess the oxidative DNA damage, lipid peroxidation and gill morphology (see *Chapter 6* for details).

CHAPTER 2

GENERAL MATERIALS & METHODS

GENERAL METHODOLIGIES

2.1 Overview

A range of procedures and analytical techniques were fundamental to the experimental design to probe the hypothesis undertaken in the present study. Other unique methods to particular trials (including diet formulation) are described in the relevant experimental chapters. Unless otherwise stated, all materials, chemicals and reagents were purchased from Sigma-Aldrich Ltd. (Poole, Dorset, UK) and Fisher Scientific Ltd. (Loughborough, Leicestershire, UK). All experimental work involving fish was conducted under the UK Home Office project licence (PPL 20/2644) and personal licence P/L 30/8250 in accordance with the Animals (Scientific Procedures) Act 1986.

2.2 Rearing facilities and maintenance of water quality

All experimental trials were conducted within a freshwater recirculation system (RS), number 'D', at the Plymouth University's Aquaculture and Fish Nutrition Research Aquarium (see Plate 2.1). Recirculation systems are widely used in fisheries research to increase fish production. Recirculation system's filters are used in a way that clean water can be recycled. These have several other advantages including reduced water requirements and space, often in close proximity to markets, and with a high degree of environmental control which is particulary advantageous to researchers. In order to provide an appropriate environment for the rearing of fish, water quality parameters in a RS were effectively managed to keep within certain limits for conservation of natural resources.

Plate 2.1 Recirculation system 'D' located at Plymouth University. White arrow shows the direction of water over flow to experimental tanks from the drum filter through the biological filter (not visible).

The facility used was a closed RS with a total water volume of~2250 l. 12 experimental 80 L fibreglass tanks, each received water at rate of ~600 l h^{-1}. An automated 12 h dark / light system was maintained throughout all experimental trials. The biology and function of the RS used in the present study is common to many RS. The description of the RS is as follows: an activated carbon filter (Commandomatic TCF, Waterco Ltd., Sittingbourne, Kent, UK) removed chlorine and organic compounds from the incoming water. As a by-product of the breakdown of proteins, fish excrete ammonia ions (NH_3) through the gills, which is very toxic to fish. Ammonium (NH_4^+) is relatively nontoxic to fish and is present in rates relative to water pH. NH_3 is removed from re-circulated water by a submerged biological filter of nitrifying bacteria in two stage processes: the biological oxidation of NH_3 to relatively less toxic nitrite (NO_2^-) by the *Nitrosmonas bacteria* which is subsequently converted to nitrate by the

Nitrobater species. Nitrogenous compound were monitored on a weekly basis using an AQ2- Automated Discrete Analyser (Seal Analytical Ltd. Sussex, UK). The following levels of nitrogenous compound were considered acceptable: ammonia (unionized) <0.1mg l^{-1}, nitrate <50mg l^{-1} and nitrite <1.0 mg l^{-1}. If required nitrogenous compounds were controlled by a partial water exchange.

Saturation oxygen needs to be maintained above 80% and was maintained by a side supply of compressed air (compressor; Rietschle, UK) delivered via air stones and a perforated pipe to each tank and sump water respectively. Temperature was maintained at 23 ± 1°C with a thermostatically controlled heater (Optipac R407C, PSA, Saint Barthelemy,D' Anjou, France). The pH of the water system was maintained between 7-8 using alkaline buffer sodium bicarbonate ($NaHCO_3$) as necessary. Oxygen saturation, temperature and pH were monitored daily with an electronic meter (Hach HQ40d- Multi Parameter, Loveland, USA).

2.3. Collection and maintenance of experimental fish (Cyprinus carpio L.)

Mirror carp (*Cyprinus carpio* L.) obtained from Bowlake fish farm carp hatcheries (Hampshire, UK) were used for all experimental trials. Fish were transported directly from the hatchery to the aquarium facility in a 1000 l tank supplied with pure oxygen (BOC, UK); transport time was ~4 h. Depending on the requirements, fish were procured in different months of the year, throughout the study. Fish were gradually acclimated to the temperature of the aquarium facility over a period of 2 h. All fish were checked on arrival, monitored daily and fed a standard commercial diet of 2% body weight (Ewos, Micro 20 p, Ewos Ltd., Westfield, Bathgate West Lothian, UK). A period of at least three weeks was given until fish were randomly allocated into experimental tanks prior to

experimental trials. Anaesthetisation of fish was carried out ethically according to Home Office procedures with tricainemethanesulphonate (MS-222; Pharmaq, Ltd. Fordingbridge, Hampshire, UK) at a dose rate of (100 mg l^{-1}).

2.3.1 External features of the carp

Mirror carp is a large, soft-finned freshwater fish which has a more rounded shape. Mirror carp can be recognised with a linear line of scales down each side of the fish (linear mirror) and also with a full set of large random scales all over the body (fully scaled). Carp have a thick leathery appearance and two barbells on each side of the mouth representing a distinguished marking for the carp. The carp's body is robust, deep and thick, and arched toward a lengthy dorsal fin, with nearly 20 soft rays (Plate 2.2A).

Dorsal fin extends well along the back, and the fin edge is high in the front and straight in the back. The caudal fin is forked and is articulated on a series of flat plates. The first dorsal and anal fin spines are serrated. The pectoral fins are suspended immediately behind the opercular region of the skull on the lower sides of the body, which act as the main braking fins; they achieved this by placing the fins out to provide a large surface area to the water. The pelvic or ventral fins are paired act as hydrofoils and suspended on the lower side approximately mid body (Plate 2.2A).

Lateral line is a sense organ in aquatic organisms (mainly fish) used to detect movement and vibration in the surrounding environment. The basic sensory unit of the lateral line system is the neuromas, which is a bundle of sensory and supporting cells, located superficially on the skin or under the skin in fluid filled canals on the head and body. The mucus layer covers the entire external area

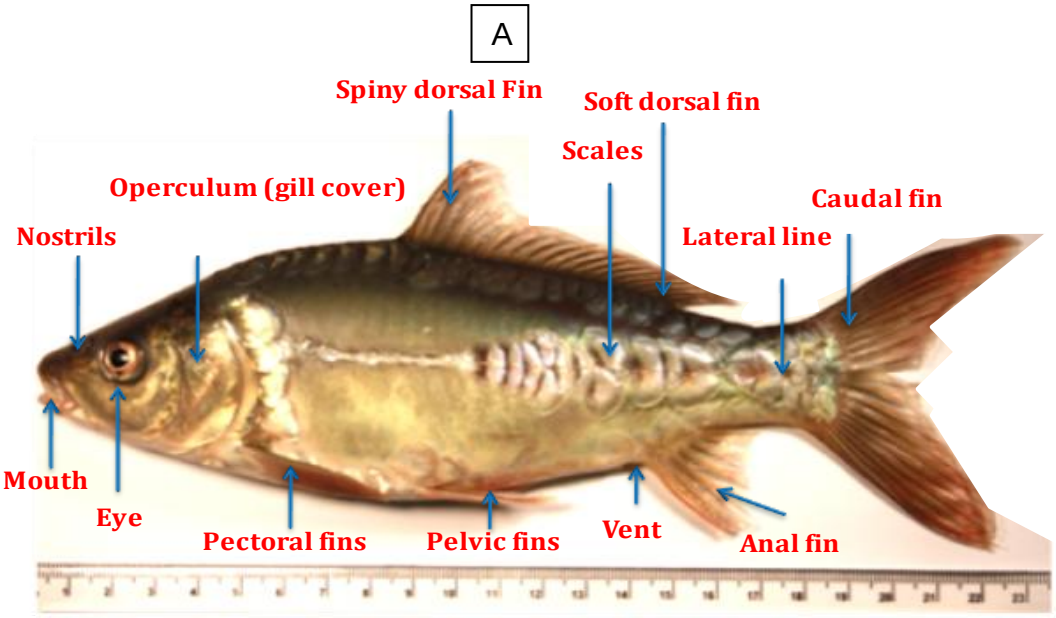
of the fish. The mucus layer provides protection from bacteria and fungus and also gives the fish its slippery feel (Plate 2.2A).

2.3.2 Internal anatomy of the carp

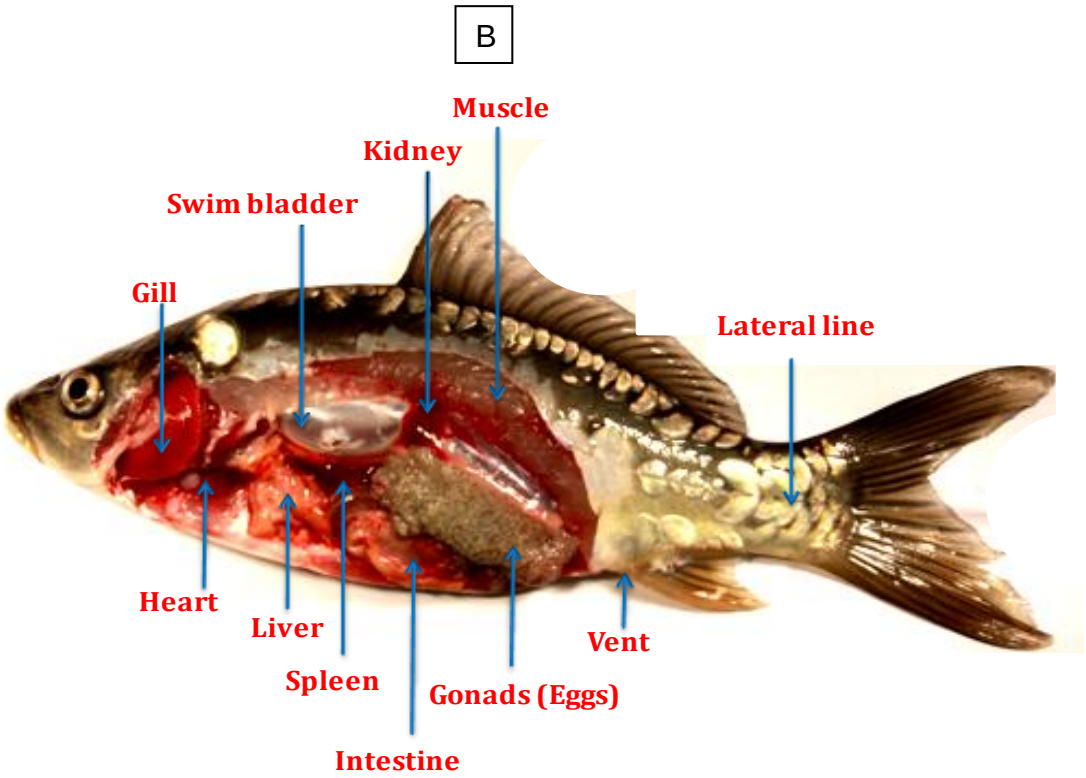
With respect to the internal anatomy of the carp, eyes are just forward of the gills, which can see in two directions at the same time, to either side of the body as well as above or below on each side. The nostrils are just forward and slightly above the eyes, used purely for scent. The gills of carp consist of two sets of holobranches arranged in each side in four pairs of the buccal cavity. Each holobranch is composed of two hemi-branches arising from the posterior edge of the bronchial arch or gill arch in such a way that the free ends diverge and touch those of the adjacent holobranches, an additional primal gill hemi-arch is also present (Plate 2.3). Gills have similar function to the lungs of mammals, not only the primary site for respiration but are also the principle and often exclusive site for osmoregulation, acid base balance and metabolism of circulating hormones and xenobiotics (Roberts, 2001). The swim bladder, located below the backbone consists of 2 different size chambers (Plate 2.2B). Fish adjust their position by inflating or deflating these chambers. This changes the density relative to the surrounding water. In conjunction with the auditory system, it controls the orientation, and the level at which fish swim (Roberts 2001).

The liver is large, multi-lobed, fairly prominent, reddish brown in colour, lying in the abdominal cavity and vasculature drains into the sinus venous. The primary functions of hepatic tissue are metabolism and filter the blood draining the intestine before it enters the general systemic circulation. The liver plays a prominent role in detoxification process. It also produces bile which is used in the digestion/absorption of fat. The gall bladder is located next to the liver which

plays an important role in fat digestion through excretion of the bile. The internal sex organs of the males and females are the testes and ovaries respectively. In both the male and female, these organs are located below the swim bladder. Eggs and sperm exit the body via the gonopores which are located in front of the urinary opening. The gonopores are connected by the gonoduct. The kidney is also extremely important in regulating water and salt concentrations within the body. The head kidney (or pronephros) is a major antibody producing organ as well as antigen trapping organ. Head kidney has been also recognised in fish as possessing endocrine, haemopiotic, excretory and lymphatic tissues (Roberts, 2001). The urinary bladder plays an important role in osmoregulation as the salt content in the carp body is higher than the surrounding water where it lives. The carp fish is continually taking in water trying to equalize salt concentration. The waste products (faeces and urine) of the carp digestive system are excluded via the anal pore (Plate 2.2B).



External Features



Internal Anatomy

Plate 2.2 The external and internal views of the mirror carp *Cyprinus carpio* L. (A) showing external features; (B) the internal view showing position of the major internal organs.

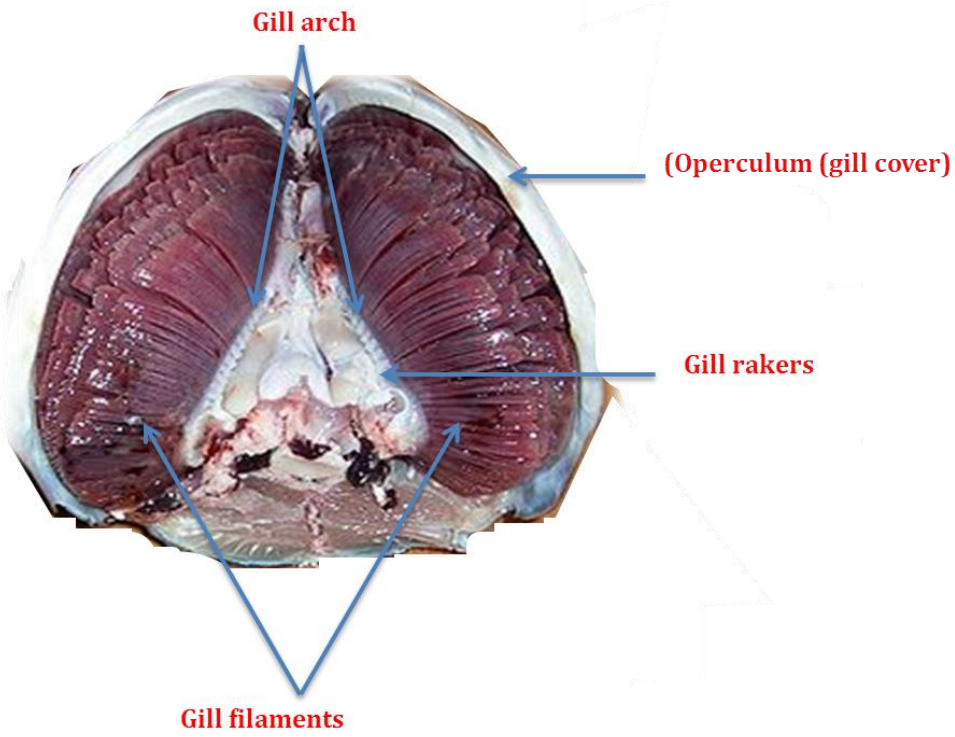


Plate. 2.3 Transverse section of fish gill showing the gill arch, with rakers and primary lamellae

2.4 Gene expression analysis using reverse transcription Polymerase Chain Reaction quantitative (Q-PCR) and Real-time polymerase chain reaction (RT-PCR)

Major steps involved in the gene expression analysis have been outlined in the figure 2.1. Each of these steps has been further elaborated in the following sections (i.e. 2.4.1-2.4.5).

2.4.1 RNA Extraction

In addition to HIF-1α as the target gene, ß-actin was considered as a house keeping gene for internal control, which was selected on the basis of the previous studies carried out under hypoxia using this gene (Terova et al., 2008; Ton et al., 2003). Liver tissues dissected from fish (n=6) and stored at -80°C until RNA extraction. Liver samples from individual fish were used for gene expression analysis. Total RNA of samples was extracted using GenElute mammalian Mammalian Total RNA Miniprep Kit (Sigma Aldrich, UK). Sample of liver tissue (0.02 g) was homogenised in Ice using sonicator (Misonix, Microson XL., 20 levels) in eppendrof tubes containing 350 µl of lysing solution [5 µl 2-mercaptoethanol plus 500 µl lysis buffer] on level 4-5 for 10 sec to confirm full break of the tissue/cells in order to improve the quality of extracted RNA. Following this step, samples were immediately stored on ice while other samples from the same batch were being processed. The tissue lysate were centrifuged for 2 min at max speed (Micro Centaur, MES, >8,000X g), supernatant containing the RNA was gently transferred to filtration tube and centrifuged for 2 min. 400 µl of 70% ethanol was added to the throw-flow liquid, and transferred to the binding tube and spin for 15 sec at < 13,000, g, the eluted flow-through was discarded and the sample washed with wash solution 1 and 2 as summarised in Fig. 2.2.

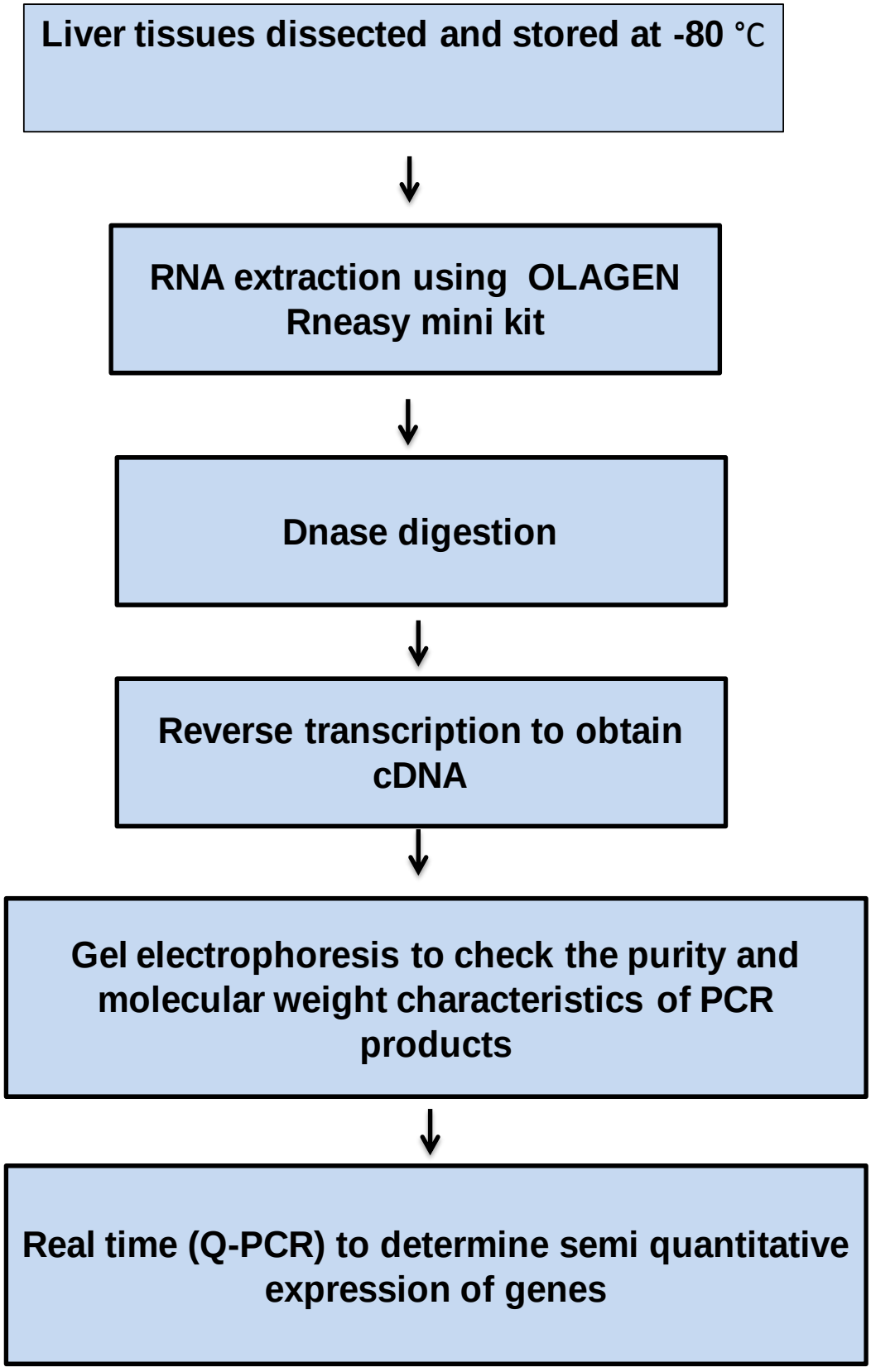


Fig. 2.1 Major experimental steps to determine relative expression of HIF-1α and β-Actin genes in the liver samples of *C. carpio.*

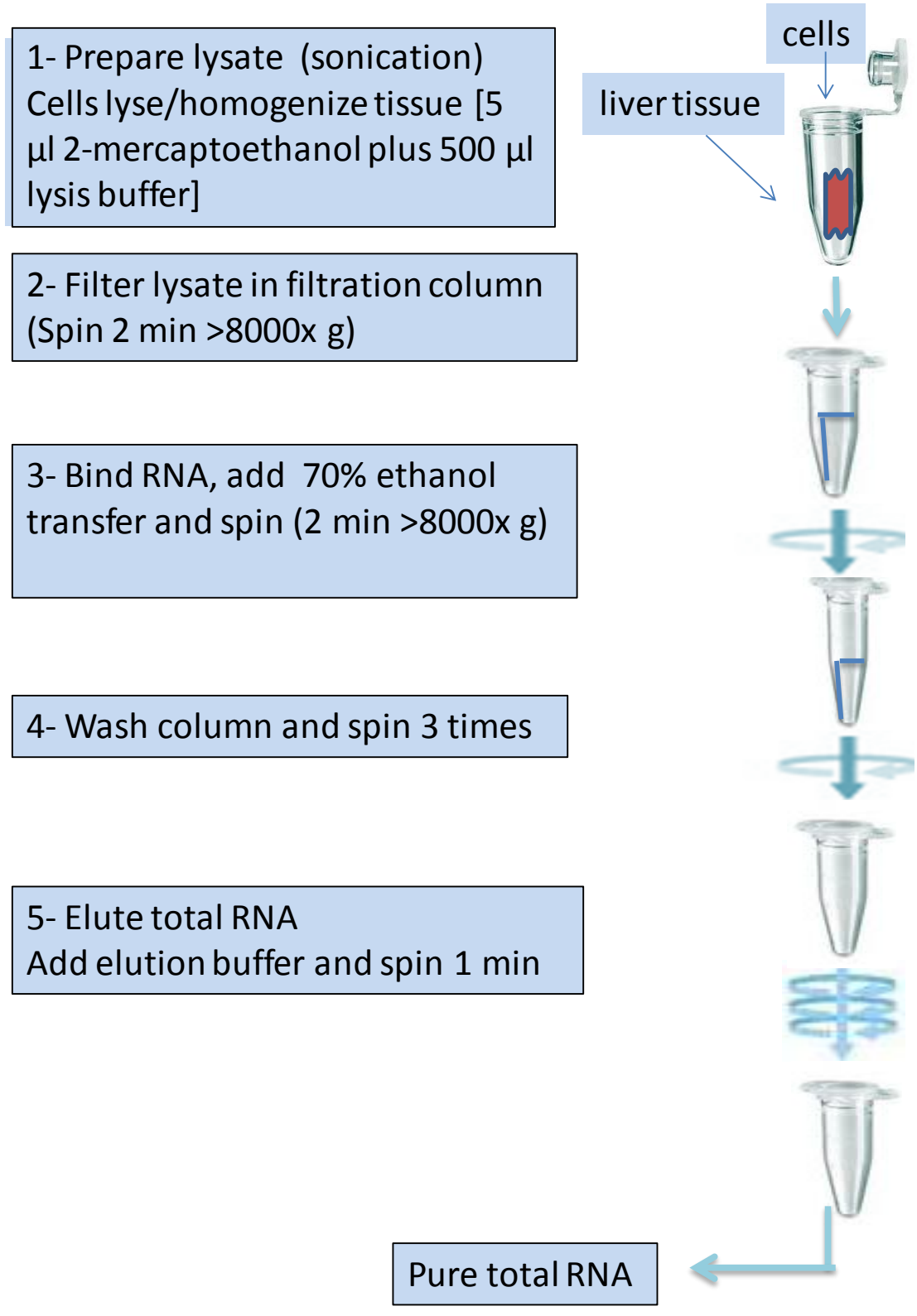


Fig. 2.2 Flowchart to explain major steps involved in RNA extraction using RNeasy kit.

2.4.2 DNA digestion

To avoid the contamination with genomic DNA, DNA digestion method was used to digest any potential DNA contamination present in the RNA using DNase I column enzyme (Sigma Aldrich, UK) for 15 min for DNA digestion. Samples were washed with wash solution and elute using elute solution. RNA quantity and purity was measured using NanoDrop spectrometer (ND-1000) by measuring the absorbance of the RNA samples at 260 nm. The ratio of RNA:DNA and RNA: Protein was in the range of 2.10-2.23 and above 1.5 respectively (Fig. 2.3). RNA samples were stored in a freezer at -80 °C until required.

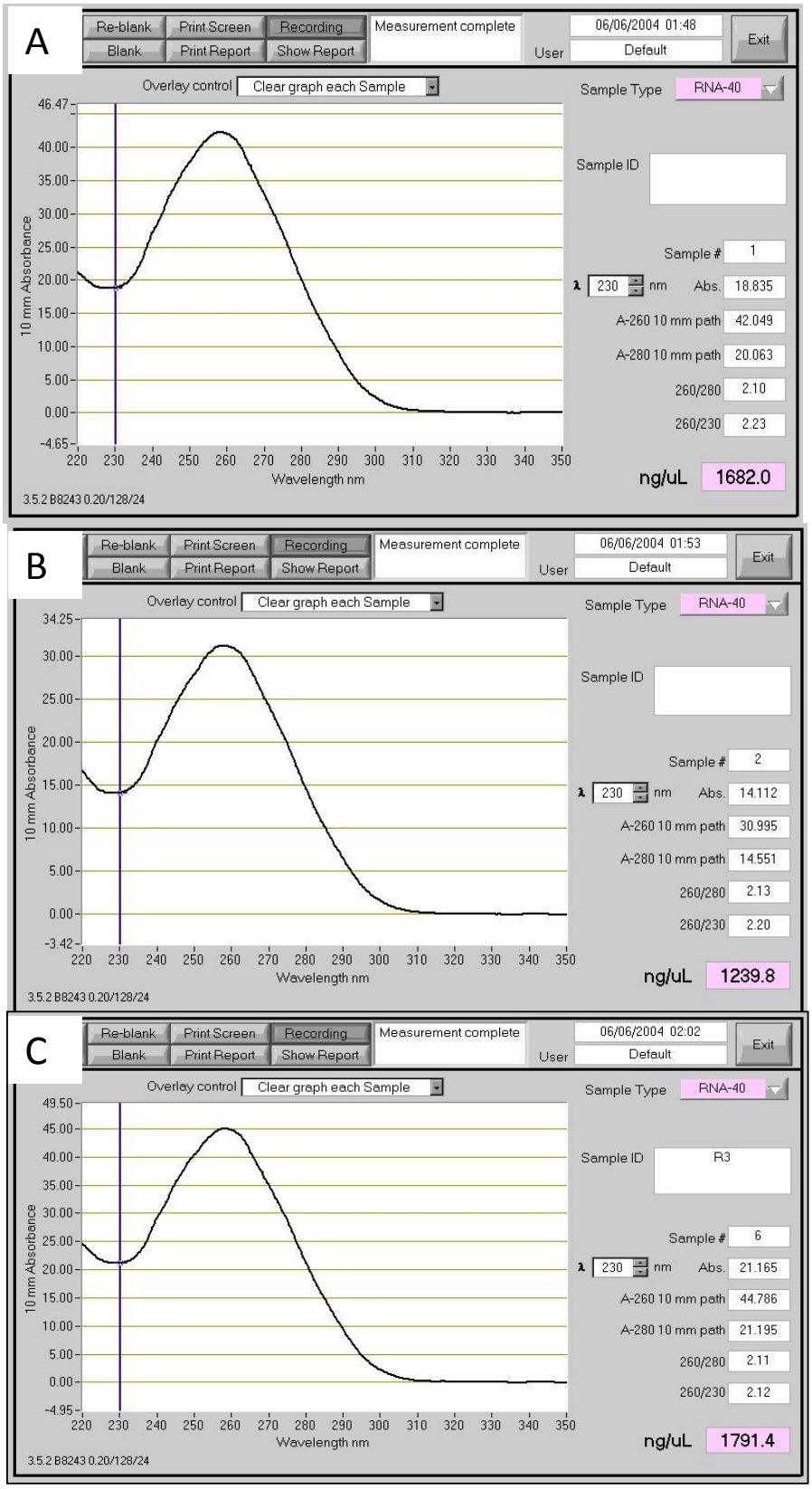


Fig. 2.3 RNA purity and concentrations data from liver samples of *C. carpio.* Highlighted number is RNA concentration in 2 µl of the sample measured by NanoDrop spectrophotometer.

2.4.3 Reverse transcription to obtain complementary DNA (cDNA)

To convert the total RNA samples to cDNA material, the reverse transcriptase method was used. Briefly, cDNA was synthesised using 1µg of total RNA incubated with reverse transcriptase DNTPs mix (Deoxynucleotide triphosphate sodium) along with random hexamers. Furthermore, 1.6 ml of molecular water was added to 1 unit vial to make a final concentration of 1-5 µM for each sample as a master mix. The mixture (master mix; Table 2.1) was briefly centrifuged < 2,000 for 5 sec to gently collect all components to the bottom of the tube and to prevent it from sticking to the wall of the eppendorf. The mixture was incubated (Gene Amp PCR system 9700) at 70 °C for 10 min following which the tubes were immediately removed and placed on ice for 5 min. 10 µl of master mix was added to each previously incubated sample to obtain a final volume 20 µl. The reaction was incubated at room temperature for 10 min to ensure the elongation of random primers. The reaction was incubated at 21 °C for 10 min, 37 °C for 50 min, 94 °C for 5 min to denature the Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase, and then preserved at 4 °C for a month.

Primers were designed using the mRNA sequences, which was obtained from a primer blast in the NCBI (National Centre for Biotechnology Information; Table 2.2). The criteria for primer included the GC ratio, temperature and product length. The method development and optimisation took several weeks and many primers were used to obtain specific carp PCR product for cDNA.

Table 2.1 Preparation of CDNA Master Mix A and Master Mix B.

Chemical name	Volume	Master mix type
10 mM dNTPs	1	**A**
Random Hexamers	1	
Total volume	2	
10x M-MLV Reverse Trascriptase Buffer	2	**B**
M-MLV Reverse Trascriptase	1	
Molecular water	7	
Total volume	10	

Table 2.2 Primers used for PCR reactions for the target genes. The length of bases for the genes ranged between 90 and 170 bases.

Primer / Gene	Sequence 5'-3'	Product size	Accession No.
β-Actin	Forward: 5'TCGCTTAGGCCTTGCTCTTCAAACA-3' Reverse: 5'- GGCTGTCGCGTGCACATTGC-3'	92	M24113
(HIF-1α)	Forward: 5'- CCGTGTGCAGGAGCGCAGTG-3' Reverse: 5'- TCCAGAGTGTGGCGGCTAAGGA-3'	153	EU144225

HIF-1α =hypoxia inducible factor 1α

2.4.4 Gel electrophoresis to check the purity of PCR products

In order to check the purity and molecular weight characteristics of PCR product, agarose gel electrophoresis was run. The DNA was produced by the ordinary PCR machine (Gene, Amp, PCR system 9700, Applied biosystem, UK) and was separated by gel electrophoresis in order to confirm the size, location and quality of the PCR specific product for specific primers. The electrophoresis tank was prepared using a standard method (Sambrook et al., 1989). Gel was prepared by mixing 70 ml of Ix TAE (Tris Acitate EDTA) with 0.70 g agarose to make 1% gel for electrophoresis, and was microwaved for 2 min on medium/high power. Subsequently, 2.5 µl of the Orange G dye (1/10 sample volume) was added to each sample. To calibrate the gel, 25 µl of 1 kb ladder was prepared as follows; 21.5 µl of molecular water, 2.5 µl of Orange G dye and I µl of ladder (100bp, Promega). The gel was left to warm, then was poured into an electrophoresis tank (Pharmacia Bioteech, GNA 100, 8X10.5 cm) and was left to solidify, then the electrophoresis tank was filled with I x TAE buffer, 25 µl of each sample was loaded into the wells starting with the ladder. The electrophoresis tank then was covered and connected to the power on 40 volts for 1 h. DNA stained using SYBR safe and then visualized and photographs were taken of the gel using UVItec Limited, England.

2.4.5 Real-time quantitative polymerase chain reaction (Q-PCR)

Real-time- quantitative Polymerase Chain Reactions (Q-PCR) experiments were carried out to compare semi-quantitative analysis of the gene expression in the experimental relative to the reference (control) group. The Syber Green PCR Master Mix was prepared for one reaction (Table 2.3). Negative controls and samples for target and housekeeping genes were used in triplicate. Each

sample was run three times by RT-PCR protocol to confirm the results from 9 fish treatment^{-1}.

The thermal protocol for PCR reactions were: 3 min incubation at 95 °C, followed by 40 reaction cycles: 15 s at 95 °C, 30 s at 60 °C, 20 s at 72 °C where the fluorescent amplification signal was read (Dondero et al., 2006). Melting curves for PCR products were adjusted between 60-90 °C. The data were analysed based on the differences between the reference (control) and the treatment groups using a comparative Ct analyses, using the following equation:

$\Delta\Delta C_t = \Delta C_{t\ sample} - \Delta C_{t\ reference\ control}$

Amount of target (RQ) = $2^{-(\Delta\Delta Ct)}$

where Ct is threshold cycle.

Table 2.3 Reagents used to prepare sybergreen PCR Master Mix for thermocycling reactions for Q-PCR (Dondero et al., 2006).

Chemical / reagent	Volume (µl)
SYBR® Green JumpStar *Taq* ReadyMix	12.5
reference Dye	0.25
Forward Primer (10 µM)	0.5
Reverse Primer (10 µM)	0.5
Template cDNA	2.0
Molecular water	9.5
Total volume	25.25

2.5 Nitroblue tetrazolium (NBT) reduction assay

Respiratory burst activity of different blood cells (mainly neutrophils and monocytes) were quantified using the reduction of nitroblue tetrazolium (NBT) to formazon as a measure of superoxide anion (O_2^-) production. This assay was carried out as per the method described by Kumar et al., (2005). Briefly, the blood samples were collected by piercing the caudal peduncle in a test tube containing 2.5% EDTA as anticoagulant. Fifty µl of blood was placed into the wells of 'U' bottom microtitre plates (three replicate wells were used sample^{-1}) and incubated at 22 °C for 1 h to facilitate adhesion of cells. Following this step, the supernatant was removed and the adhered wells were washed three times in PBS. After washing, 50 µl of 0.2% NBT+1 µl ml^{-1} of phorbol myristate acetate (PMA) were added and resulting solution was incubated for a further h at 22 °C. The cells were then fixed with 100% methanol for 2–3 min and again washed (3x) with 70% methanol. The plates were then air dried. Sixty µl 2 M potassium hydroxide and 70 µl dimethyl sulphoxide were added into each well to dissolve the formazon blue precipitate formed. The optical density (OD) of the turquoise blue solution was then read in a plate reader at 540 nm.

2.6 Determination of lipid peroxidation using thiobarbituric acid-reactive substances (TBARS) assay

Determination of thiobarbituric acid-reactive substances (TBARS) assay was used to determine lipid peroxidation as described by Camejo et al., (1998) with slight modifications. Briefly, liver samples (from individual fish) were weighed and homogenized (1:10, wet tissue weight/buffer volume) using a Potter-Elvjeham glass homogenizer in a buffer of 20 mM Tris-HCl (pH 7.6), containing 1 mM dipotasium EDTA, 0.15 M potassium chloride, 0.5 M sucrose and 1 mM dithiotheritol. Homogenates were centrifuged (4 °C at 10,500 xg for 10 min) and the supernatant was transferred to a polypropylene microcentrifuge tubes. Forty µl of homogenate were added to 10 µl of 1 mmol l^{-1} butylatedhydroxytoluene (2,6-Di-O- tert-butyl-4-methylphenol or BHT) to labelled eppendorf to avoid undesired further oxidation of samples. 1,1,3,3-tetraethoxypropane (TEP) was used as the standard in the range of 0-100 nmol ml^{-1} dissolved in phosphate buffer pH 7.5 (as shown in Fig. 2.4). Following this step, 140 µl of 0.1 M phosphate buffer (pH 7.5) was added to bring the volume to 190 µl, subsequently 50 µl of 50% (w/v) trichloroacetic acid was added to each tube (Al-Sabti and Metcalfe, 1995). The reactions were allowed to continue by adding 75 µl of 1.3% (w/v) thiobarbituric acid (TBA) dissolved in 0.3% (w/v) NaOH to each well, followed by incubation at 60 °C for 60 min. Supernatants were centrifuged (10,000 xg for 2 min at 4 °C) and pipetted out 200 µl of the supernatant was transferred into each well, and then OD was measured spectrophotometrically at 530 nm. Absorbances were expressed as TBARS nmol gww^{-1}.

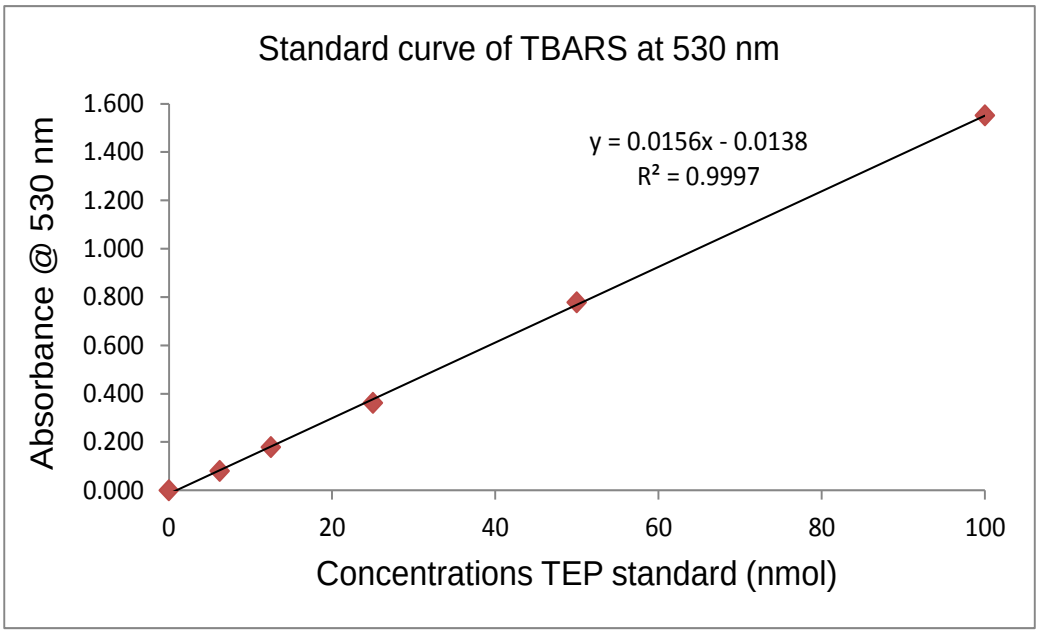


Fig. 2.4 Standard curve for lipid peroxidation assay. Data from three independent experiments expressed as means ± S.E; n=3.

2.7 Determination of DNA strand breaks using Single Cell Gel Electrophoresis (SCGE) or the Comet assay

2.7.1 Assessment of cell viability with trypan blue

Prior to determination of DNA strand breaks using single cell gel electrophoresis (SCGE) or the Comet assay, erythrocyte viability was assessed through trypan blue dye exclusion assay. This test relies on membrane integrity to distinguish between viable or non-viable erythrocytes. Briefly, 500 µl of 0.4% trypan blue dye was added to 500 µl of erythrocyte and mixed. Using a haemocytometer, percentage viability was calculated as living cells/total cells counted. Any cells that exhibited uptake of the blue stain were considered as non-vital. Only samples with >90% viability were considered for determination of DNA strand breaks using the Comet assay (Tice et al., 2000).

2.7.2 Single Cell Gel Electrophoresis (SCGE) -'Comet assay'

The Comet assay protocol was constructed using guidelines outlined by Tice et al., (2000). In brief, peripheral blood sample was collected from a caudal vein using a 1 ml syringe with a 25 gauge needle (illustrated in Plate 2.4). Depending upon the fish size, up to 300 µl blood sample was collected from each fish (300 µl per 30g fish weight). To prevent blood clotting, syringes were flushed twice with a heparin (Porcine Intestinal Mucosa) solution diluted with physiological saline containing 1000 units ml^{-1}. The basic steps of the Comet assay are summarised in Fig. 2.5.

2.7.2.1 Slide preparation

Frosted slides were prepared by coating with molten NMP agarose [1% in phosphate buffer saline (PBS)] and left at 30°C for 15 min until set. Whole blood samples from fish were diluted 1 in 5000 with Ca and Mg free physiological saline solution [Dulbecco's Phosphate-Buffered Saline (DPBS)] (Gibco,

Invitrogen, Paisley, UK), to give a cell count of approximately $2.5x10^5$ cells ml^{-1}. Cells were then transferred into 24 microcentrifuge tubes which contained DPBS and centrifuged 2,500 xg for 2 min. The resulting pellet was resuspended in 170µl low melting agarose (LMP) and added to the prepared slides to give two replicates of 85 µl at either end of the slide. Cover slips or glasses were placed over each drop and gels were allowed to set at 4°C for approximately 5-10 min.

2.7.2.2 Lysis

Coverslips were gently removed and slides were immersed for 1 h in the dark at 4°C in the lysis solution [2.5 mM NaCl, 100 mM Na_2EDTA, 10 mM tris-BASE, 1% n-lauroylsarcosinate, 1% Triton X-100, 10% DMSO and adjusted with NaOH to pH 10] to remove cellular proteins.

2.7.2.3 Alkali (pH<13) unwinding

DNA was left to unwind in the electrophoresis chamber (Pharmacia Biotech GNA200) at 4°C in freshly prepared alkaline electrophoresis buffer [1 mM Na_2EDTA, 300 Mm NaOH, pH 12.3]. Slides were placed side by side in the electrophoresis chamber with labelled ends towards anode. Unwinding of DNA was optimised by investigating various times- 10, 20, 30 and 40 min (Section 2.7.3).

2.7.2.4 Electrophoresis

Electrophoresis was performed at 25 V, 300 mA at 4°C in the dark for 20 min. Both unwinding and electrophoresis times were optimised in the first part of the study using different unwinding and electrophoresis times (see section 2.4.3).

2.7.2.5 Neutralisation and staining

Embedded cells were gently immersed in neutralization buffer [0.4 M Tris-BASE, pH 7.5] for 5 min and this step was repeated three times followed by rinsing with distilled water for 10 min. Slides were then allowed to air dry for 24 h. Finally, to visualise Comets, 40 µl (2 µg ml-1 stock) Ethidium bromide (in a fume cupboard, gloves worn) stain was applied to each gel and coverslips added.

2.7.2.6 Comet visualisation and scoring

Scoring was achieved using a fluorescence microscope (Leica, DMR) and Komet 5.0 image analysis software (Kinetic Imaging Ltd., Liverpool, UK) was used to score 100 cells for each slide (50 randomly selected cells per gel from each exposed individual fish). Slides were independently coded by given a numbers and scored without knowledge of the code. Initially, the software provided several parameters but tail DNA (%) and Olive tail moment (OTM) were considered to be the important parameters (Kumaravel and Jha, 2006).

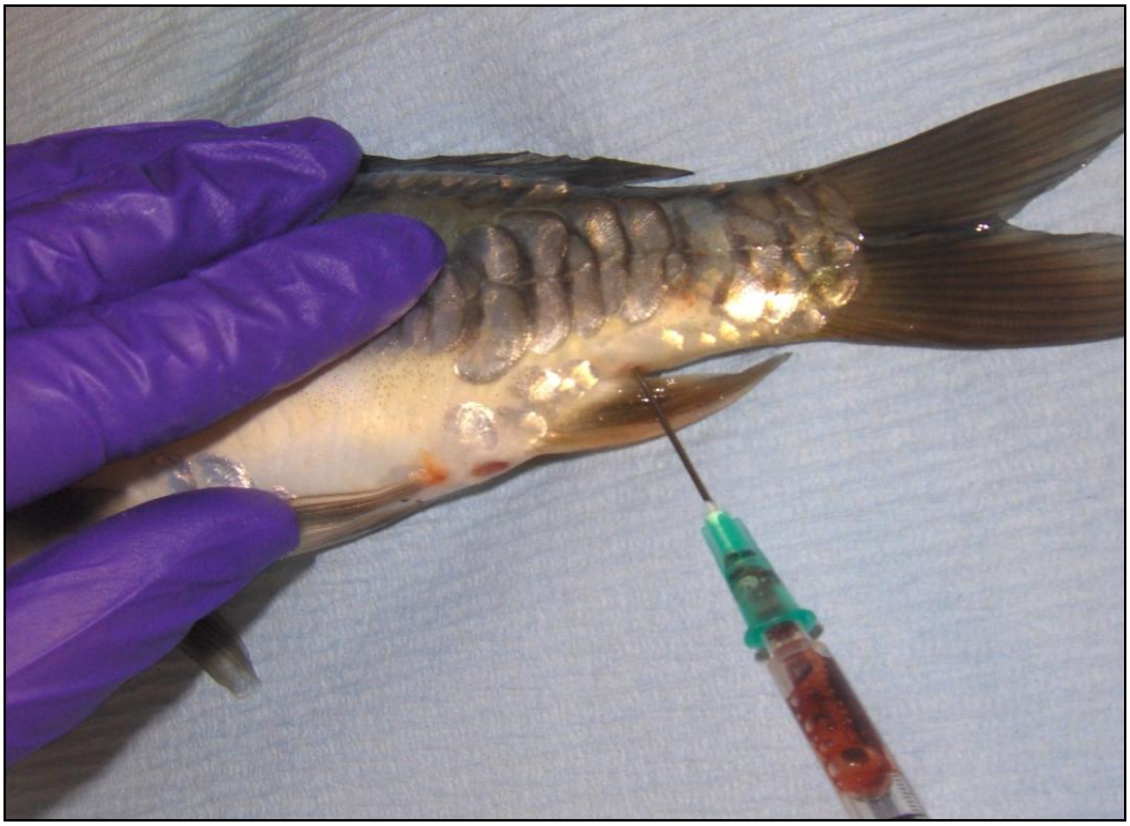


Plate 2.4 Photograph illustrating collection of blood samples (300 µl) from the caudal vessel of *Cyprinus carpio* L.

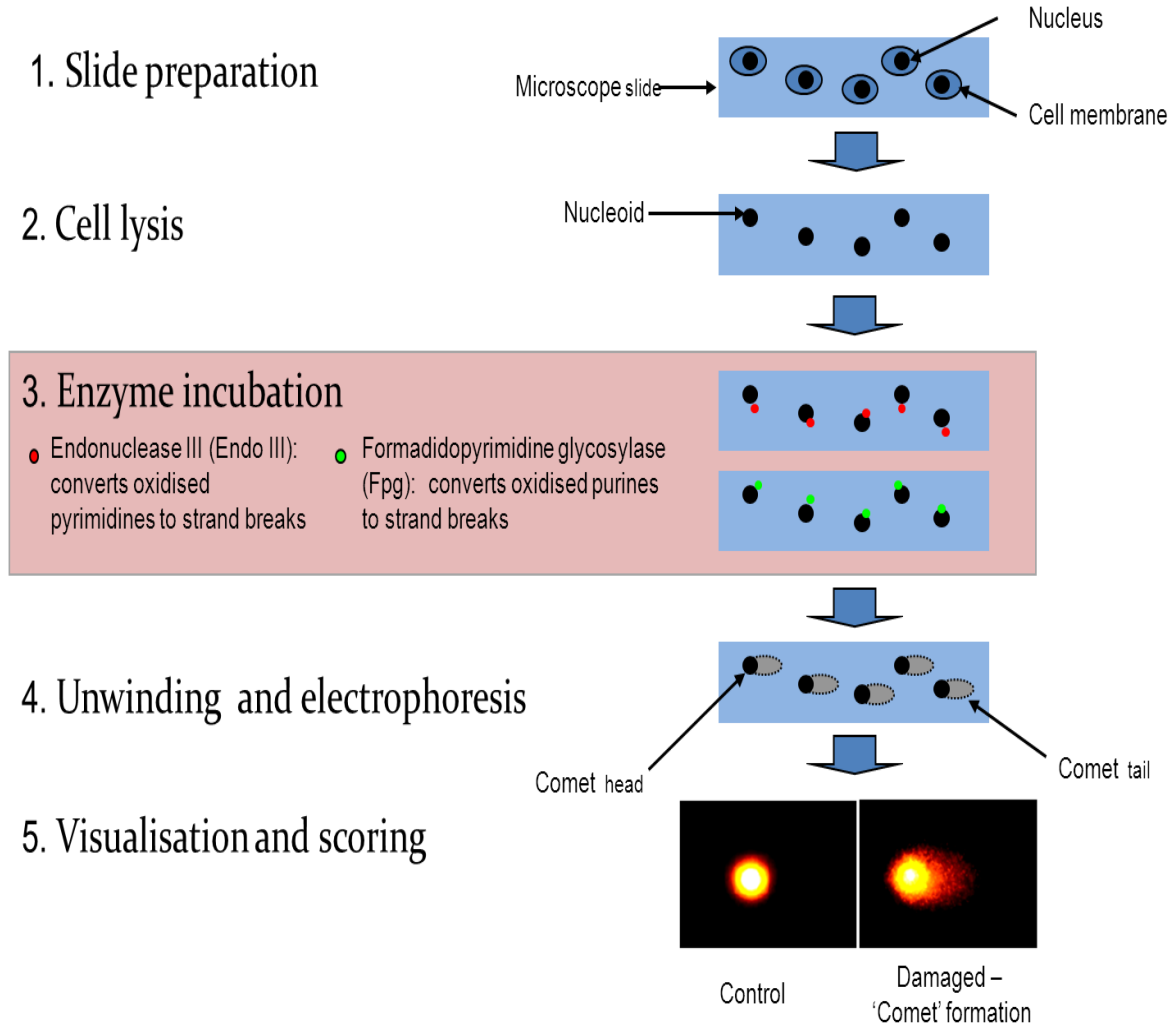


Fig. 2.5 The critical steps involved in the modified Comet assay (adapted from Tice et al., 2000)

2.7.3 Comet assay optimisation using hydrogen peroxide (H_2O_2)

The aim of the Comet assay optimisation was to establish the optimal unwinding and electrophoresis times for the erythrocytes which gives the desired level of DNA damage (i.e. low background levels). Hydrogen peroxide (H_2O_2) was used as a reference chemical (positive control) at an exposure concentration 100 µM (10 min). This concentration was selected based on previous studies using H_2O_2 as a reference agent (Cheung et al., 2006; Tice et al., 2000). Time intervals investigated were 10, 20, 30 and 40 min. for both unwinding and electrophoresis. Slides were then prepared as per the standard Comet assay protocol described in section 2.7.2.

2.7.4. Validation of the Comet assay under in vitro conditions using hydrogen peroxide (H_2O_2)

Once unwinding and electrophoresis times had been optimised, the Comet assay was validated using H_2O_2. This was conducted by *in vitro* exposure of fish erythrocytes to a range of H_2O_2 concentrations (Tice et al., 2000). After collection, blood samples were centrifuged at (500 g for 5 min) the supernatant was removed and replaced with 200 µl H_2O_2 solution (diluted with DPBS) obtaining final nominal concentrations of 0, 1, 10, 50, 100, 200 µM H_2O_2 respectively. The concentrations of H_2O_2 were based on an earlier study by Tice et al. (2000). The samples were then incubated for 10 min at 4°C in the dark. Following the incubation, cells were washed with DPBS to remove any H_2O_2 residues. Slides were then prepared as described in section 2.7.2.

2.7.5. Modified Comet assay for the detection of oxidised DNA bases

This modified Comet assay for the detection of oxidised DNA bases (i.e. purines and pyrimidines) is essentially identical to the conventional protocol (but modified with the addition of bacterial enzymes: (i.e. Formamidopyrimidine DNA glycolyase (Fpg) and Endonucleases III (Endo-III) to treat the slides after lysis step to allow the enzymes to recognise the oxidised bases and convert them into DNA strand breaks, thus increasing the sensitivity of the assay and allowing indirect assessment of oxidized purines (the majority of which are 8-oxodG recognised by Fpg) and pyrimidine bases. Enzymes as well as protocol for the storage and use of the enzymes were kindly provided by Professor Andrew Collins, Department of Nutrition, University of Oslo, Norway as described by Reeves et al. (2008). Briefly, slides were prepared as described in 2.7.2.except, following cell lysis, the slides were washed three times with enzyme reaction buffer (4 mM HEPES, 0.01 mM KCl, 0.05 mM Na_2EDTA, and 0.02 $mgml^{-1}$ bovine serum albumin at pH 8). Fpg and Endo-III enzymes (1 unit of enzyme diluted in 50 µL of buffer per gel) were added to each duplicate gel. After enzyme addition, covered with coverslips and incubated in a humid chamber at 37°C for 45 min. Following incubation, the slides were processed identically undergoing the standard protocol which included steps of unwinding, electrophoresis and neutralization. Comet scores were compared to reference slides (buffer incubation with no enzymes).

2.8 Determination of haematological parameters

2.8.1 Determination of haemoglobin content

Determination of hemoglobin (Hb) level was performed using the cyanmethaemoglobin method (Dacie and Lewis, 1995). Briefly, the Drabkin's reagent consists of 1.0 g sodium bicarbonate, 50 mg potassium cyanide, 200 mg potassium ferricynide made to a 1l solution using distilled water and stored in borosilicate glass bottle for later use. Assay was performed in test tubes where 20 µl of freshly collected blood was mixed with 5 ml of diluents. Solution was inverted several times before being allowed to incubate at room temperature (25 ^{o}C) for 10 min. Absorbance was measured using a spectrophotometer (Thermo spectronic, Helious Epsilon, USA) at 540 nm. A stock solution of cyanmethaemoglobin was used as a reference or standard which was diluted into several concentrations using the diluents. Absorbance values generated from the serial dilution was used to calculate a standard curve for the unknown samples reading.

2.8.2 Determination of total erythrocyte and leukocyte counts

Both total erythrocyte and leukocyte counts were accomplished by diluting 20 µl of freshly collected whole blood sample with 0.98 ml of Dacie's fluid (10ml of 40% formaldehyde, 31.3 g trisodium citrate, 1.0 g brilliant crystal blue dissolve in1 l of distilled water and filtered through 0.45 µm syringe filter) mixed gently to disperse the cells. Counts were performed on an improved Neubauer haemocytometer (Fig. 2.6). Total erythrocytes were carried out in the central counting chamber; containing 25 squares with subdivision of further 16 squares. Counting was performed in 5 squares (the central one and the 4 square in the corner) from the divided 25 squares, (volume counted per square = 0.2 × 0.2 × 0.1=0.004 mm^3). Erythrocyte counts are expressed as 10^6 cells mm^3. White cell

(i.e. leukocytes) counts were performed on the same sample in a similar way by counting 5 of the large areas (volume = 1 × 1× 0.1 mm = 0.1 mm^3). White cell counts are expressed as 10^3 mm^3. All counts included cells that touch the right and top side of the boundary lines of each square. Both erythrocyte and leukocyte counts were performed in duplicate per a sample. Calculation is determined according to the following equation:

$$\text{Cell count (x } 10^6 \text{ cells/mm}^3) = ((\text{average cell count / volume of square (mm}^3)) \times \text{dilution factor})) / 1{,}000{,}000.$$

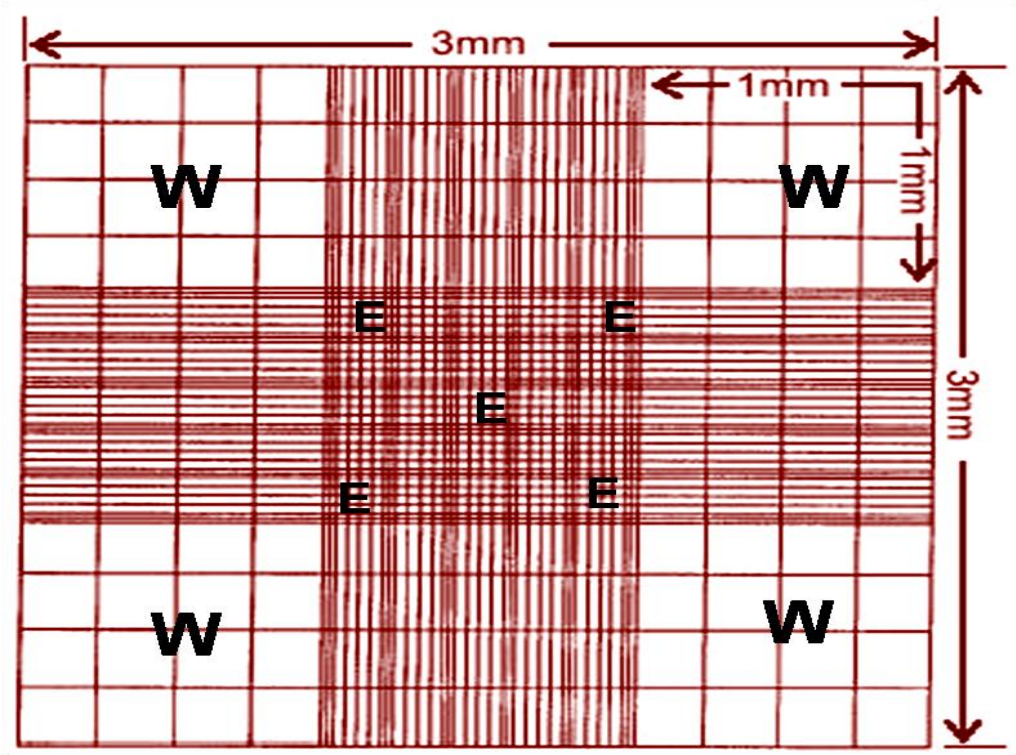


Fig. 2.6 Positions of erythrocytes and leucocytes for counting on Neubauer haemocytometer. W= white blood cell (leukocyte), E = erythrocyte (Dacie and Lewis, 1995).

2.8.3 Determination of haematocrit value (HCT)

Haematocrit (packed cell volume) of whole blood was determined in duplicate as described by (Klontz, 1997). Whole blood was collected in 300 µl heparinised microhaematocrit tubes and subsequently separated using a Centurion haematocrit centrifuge (10,500 g for 5 min). Hct value was determined as the total percentage packed cell volume using Hawksley reader (Hawksley, Sussex, UK).

2.8.4 Determination of differential leucocyte counts

Blood smears were made by dropping 4 µl of fresh whole blood onto a glass slide, the end of the second slide (‘‘spreader slide’’) was placed against the surface of the slide with the blood drop, at an angle of 45°. By drawing the ‘‘spreader slide’’ up against the drop of blood, it spread across the end of the slide by capillary attraction and filled the angle between the two slides. The ‘‘spreader slide’’ was then pushed back along the other slide (Dacie and Lewis, 1995). The prepared smears were left to dry at room temperature for an hour prior to being fixed in pure methanol for 5 min. Cells were stained using 20% Giemsa stain (pH 7.0) for 10 min and washed with three changes of distilled water. When thoroughly dried, slides were mounted with coverslips (glasses) using DPX mountant.

Counting was accomplished by observing the slides under the light microscope (Olympus Vanox-T microscope) at a final magnification of x1000. To prevent potential errors arising from uneven distribution of leucocytes, the slide was divided into four segments and 50 leucocytes per segments were counted. Leucocytes were counted in a parallel row beginning from the outside edge of the slide to the inside. The 200 leucocytes counted per slide were classified according to their general form, identified and recorded in a table as a specific

cell type (for example a lymphocyte or monocyte) by dividing the sum of each type of leukocyte by two, the percentage of each cell was obtained. Classification of the leucocytes was based on the classification system used by Groff and Zinkl (1999) for common carp (Fig. 2.7). Photographs of selected slides were also taken using a digital camera (Olympus camedia C-2020 Z) at a total magnification of x 1000 (zoom on the camera was x2.5).

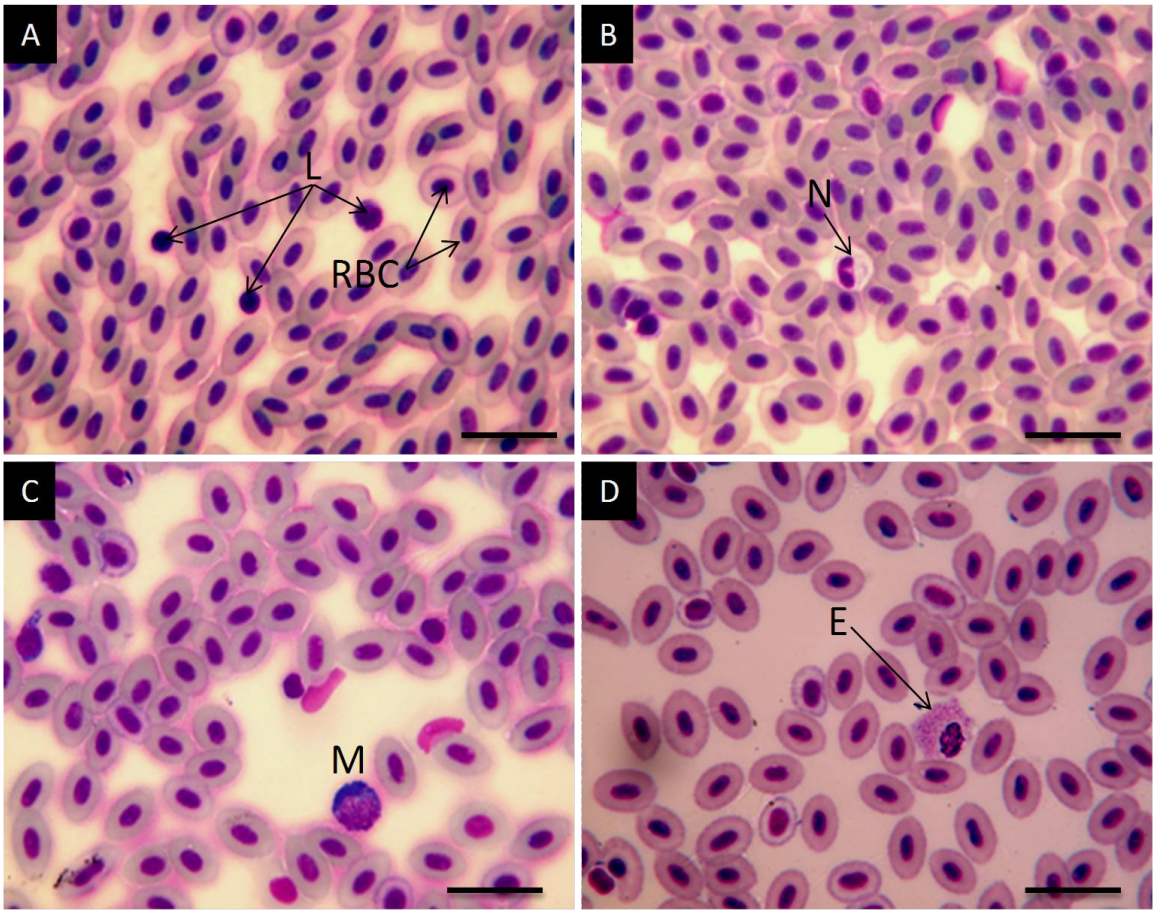


Fig. 2.7 Blood cells of *Cyprinus carpio* L. A: Red blood cells (RBC) and lymphocytes (L) B: Neutrophil (N) with blue granular cytoplasm; C: Monocytes (M) with large oval nucleus; D: Eosinophil (E) with pink granular cytoplasm: Giemsa stain; Scale bars: 25µm.

2.8.5 Plasma collection

Prior to plasma collection, the remaining blood per fish was immediately centrifuged at 10,500 xg for 10 min; collected supernatant was subjected to a further 1 min spinning at 10,500 xg. The blood plasma samples were stored at -80^{o}C until they were needed for analysis.

2.9 Determination of Cu accumulation in the tissues

The Cu concentration in the tissues was measured according to Federici et al., (2007). Fish were removed from their tanks and dissected out (6 fish treatment^{-1}). Briefly about 0.05 g of fresh tissue (i.e. gill, liver, intestine and kidney) were washed with Milli-Q water and placed on clean individual slides and dried to constant weight in an oven at 70°C for 24 h. All glassware were acid-washed (5% Aristar HNO_3 for at least 2 h) to ensure that minimal contamination occurs, and then triple rinsed in deionised water. In a fume cupboard, each piece of dried tissue was placed in 20 ml polythene screw-top digestion vials (scintillation vials, Simport, Canada) and 1 ml of concentrated Aristar HNO_3 was added. Samples were digested at 70°C for 2 h in water bath. Once digestion was completed (no brown fumes stopped evolving), the tubes were allowed to cool, then diluted with 4 ml of Milli-Q water. Tissue digest aliquots were stored at room temperature. Cu concentrations were measured by using Inductivity Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Varian, Yarnton, UK. 725-ES). The software used with ICP was ICP Expert II Vision 1.1. Cu standards were also prepared in order to calibrate the instrument before metal analysis. The standard solutions were made by using 1000 mg l^{-1} Cu stock solution, diluted by 2% nitric acid. The Cu standards used were 0, 0.5, 1, 4 µg l^{-1} the wavelength chosen for Cu measurements was 327.39 nm. Cu concentrations in the tissues were expressed as µg g^{-1} dry wt (dw). Calculation

to determine Cu concentrations in the tissues was carried out as per the following equation:

$$\boldsymbol{Cu\ concentration}\ (\mu \boldsymbol{g/g}) = \frac{\boldsymbol{volume}\ (\boldsymbol{ml})}{\boldsymbol{sample\ wt.}\ (\boldsymbol{g})} \times \boldsymbol{digest\ conc.}\ (\boldsymbol{mg/l})$$

2.10 Histopathological studies

Histological examinations were carried out at the end of each exposure as described by Myers et al. (1998). Fish were removed from their tanks and dissected out (6 fish treatment^{-1}), and whole gill, liver, intestine were carefully collected. Tissue samples were immediately fixed in 10% formaldehyde solution (ratio of the tissue to the fixative solution was 1:100; 100 ml of 40% formaldehyde made to 1 l with distilled water, adjusted to pH 7.4) for 48 h . The preserved tissues were then dehydrated via a series of alcohols to remove excess water (70% for 24 h, 90% industrial methylated sprit, IMS, for 2h and 100% IMS for 2h), cleared in three changes of Xylene (1h for each change) to remove alcohol and to make the tissues ready for paraffin infiltration. Tissues were then transferred to the paraffin oven (58-60°C) for 60-120 min to ensure the tissue was completely permeated with paraffin and manually set in wax blocks, which were then left to harden for 4 h., Transverse sections (thickness of 5-7 µm) were cut and stained with Haematoxylin and Eosin (Mayer's H and E), following standard method with some modifications of staining time to achieve the good results. Staining time was adjusted as follows: slides were cleared in Xylene 3 times for 2 min each. Then dehydrated in 2 changes of absolute alcohol; 90, 70, 50% for 2 min each. Sections were then stained with in Haematoxylin for 40 min, and then followed by good wash with tap water. The slides were then blued in alkaline $LiCO_3$ (5 rinses), differentiated with acid

alcohol (2 rinses) followed by good wash with distilled water finally stained with Eosin for 1 min. Following staining, a good wash with distilled water was performed. Dehydration with descending alcohol series for 2 min each was applied. Clearing in 3 changes of Xylene for 2 min each was done (Plate 2.5). The slides were then covered with clean coverslips, making sure that there were no air bubbles trapped underneath them. Slides were examined by light microscopy using an Olympus Vanox-T microscope and photographed using a digital camera (Olympus camedia C-2020 Z) at total magnifications of x 100 and x 400 (zoom on the camera was x2.5).

Detailed descriptions of pathology were done for the experiments according to Bernet et al., (1999). For the gill tissues, histological features were recognized, measured when appropriate and counted in relation to the number of lamellae. Only those secondary lamellae that were complete, from base to tip, were considered for analysis. For each experiment, treated/ exposed and untreated/ control, 6 fish were sampled for each treatment and 100 secondary lamellae were counted. A quantitative assessment of lesions in a histopathological investigation was done through practicable statistics (ANOVA).

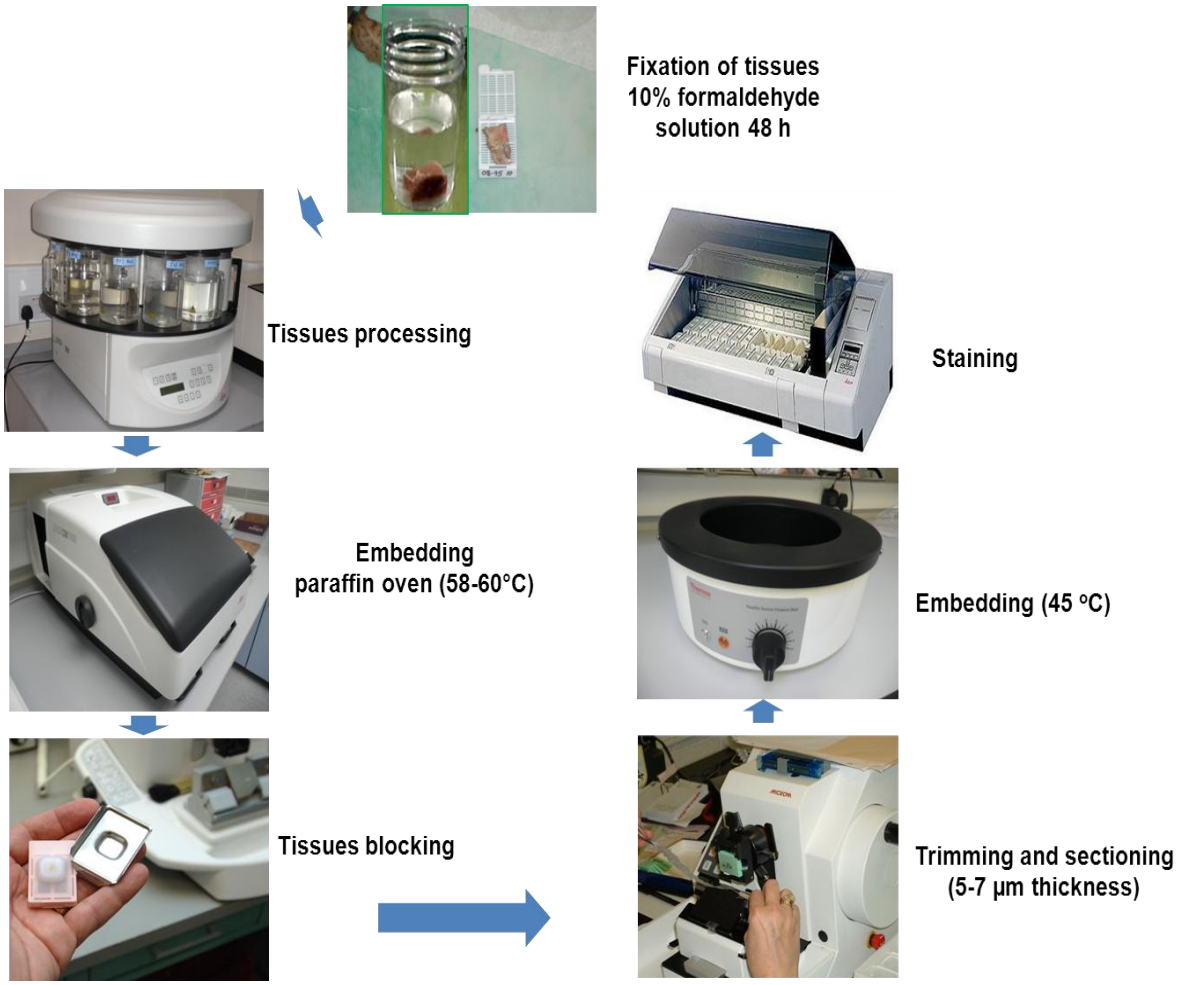


Plate 2.5 The major steps for the sectioning of tissues for histopathological studies using different instruments.

2.11 Ultrastructural analysis using transmission electron microscopy (TEM)

For ultrastructural studies, tissue samples were prepared as described by Au et al., (1999). Tissues (i.e. liver, gill or intestine) were cut into 1 mm^3 cubes and immediately fixed (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, made up with filtered freshwater). Tissue samples were fixed overnight at 4°C, followed by washing [in buffer, 1:1 of buffer: DI and then DI, 10 min each step] before post fixing in 1% aqueous osmium tetroxide for 2 h to provide contrast to the images. Samples were rinsed thoroughly with sodium cacodylate buffer (2 times at 15 min) followed by dehydration schedule through a graded ethanol series, then infiltrated gradually in Spur's resin for several days and finally the resin is polymerised in a small capsule. Before embedding, semi-thin sections (0.5 mm) were cut with an ultramicrotome, collected on grids, positively stained with methylene blue/azur II and prepared for orientating the tissue for ultrathin sectioning. Ultrathin sections for TEM were stained in 2% uranyl acetate, followed by 1% lead citrate for 15 min for each stain, to produce high contrast stain for cellular and tissue components, and examined under a transmission electron microscope (JEOL, TEM-1200EX II) at 120 kV, and imaged using soft Imaging system, Mega View 3 (Plate 2.6).

Plate 2.6 JEOL, TEM-1200EX II used for TEM analysis.

2.12 Feeding and weighing the fish

All fish in each experimental tank were weighed at *t* = 0 and fed relative to 3 % biomass day^{-1} in three equal rations three times daily at ~ 09:00,13:00 and 18:00 h. Throughout experiments/ trials, fish were reweighed every week and within this period feed input was adjusted daily based on a predicted feed conversion ratio value (FCR) (Cech et al., 1984).

2.13 Growth and nutritional performance

Growth performance and feed utilisation was assessed by specific growth rate (SGR) and feed conversion ratio (FCR). Calculations were conducted according to Cech et al., (1984) as per the following equations:

$$\boldsymbol{SGR}\ (\%) = \frac{\boldsymbol{Ln\ final\ wt(g) - Ln\ initial\ wt(g)}}{\boldsymbol{Days\ fed - 1}} \times \mathbf{100}$$

$$\boldsymbol{FCR} = \frac{\boldsymbol{diet\ feed(g)}}{\boldsymbol{weight\ gain(g)}}$$

2.14 Proximate analysis of diets and carcass

The proximate composition of the carcass and diets were subjected to analysis for the determination of moisture, ash, lipid, and protein levels. All diets were ground by use of a house hold blender and analysed on a wet weight basis. Analysis was conducted in triplicate according to Baker and Davies, (1996) as described in the following sub sections.

2.14.1 Moisture

Diets (in duplicate) and carcasses (whole with peritoneal cavity and viscera) were weighed and dried at 105°C with a fan assisted oven (Genlab Ltd., UK) until a constant weight was achieved. Percentage moisture was determined by following equation:

$$\boldsymbol{Moisture}\ (\%) = \frac{(\boldsymbol{wet\ wt.}\ (\boldsymbol{g}) - \boldsymbol{dry\ wt.}\ (\boldsymbol{g}))}{\boldsymbol{wet\ wt.}\ (\boldsymbol{g})} \times \mathbf{100}$$

2.14.2 Ash

Ash (total mineral or inorganic content) was determined in duplicate by adding a known sample weight (~ 500 mg) to a pre- weighed ceramic crucible. The crucibles and samples were then incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550°C for 12 h. After cooling in a dehumidification chamber, percentage ash was determined from the sample residue using the following formulae:

$$\boldsymbol{Ash}(\%) = \frac{(\boldsymbol{sample\ residue}\ (\boldsymbol{g}) - \boldsymbol{crucible\ wt.}\ (\boldsymbol{g}))}{\boldsymbol{initial\ sample\ wt.}\ (\boldsymbol{g})} \times \mathbf{100}$$

2.14.3 Lipid

Lipid content was determined in duplicate using the Soxhelt extraction method. Diets were weighed (~ 3 g) and placed into cellulose thimble lightly plugged with cotton wool and inserted into the condensers (raised into the 'rinsing' position) of a SoxTec^{Tm}extraction system (Tecator Systems, Högnäs, Sweden; model 1043 and service 1046). Pre- weighed cups containing 40 ml of petroleum ether were clamped into the condensers and extraction levers moved to the boiling position for 30 min, after which extraction levers were set to the 'rinsing' position for 45 min. The cups containing extracted lipid were then transferred to a fume cupboard, cooled for 30 min and weighed. Total lipid content was determined as per the following formulae:

$$\textbf{\textit{Total lipid}}\ (\%) = \frac{(\textbf{\textit{cup}}\ (\textbf{\textit{inct. Lipid}})\ \textbf{\textit{wt.}}\ (\textbf{\textit{g}}) - \textbf{\textit{cup wt.}}\ (\textbf{\textit{g}})}{\textbf{\textit{initial sample wt.}}\ (\textbf{\textit{g}})} \times \mathbf{100}$$

2.14.4 Crude protein

Determination of the total crude protein in diets and fish samples was achieved in triplicate by the Kjeldhal method, which measures protein from the total nitrogen content of the samples. Total nitrogen is multiplied by a factor of 6.25 to calculate apparent protein content. In brief, ~ 100 mg of sample was weighed directly into a micro kjeldhal tube along with one catalyst tablet (3 g K_2SO_4, 105 mg $CuSO_4$ and 105 mg TiO_2; BDH Ttd., Poole, UK) and 10 ml concentrated sulphuric acid (H_2SO_4) (Sp. Gr. 1.84, BDH Ltd. Poole, UK). Digestion was performed with a Gerhardt Kjelda Thermo digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) with the following schedule; 100°C for 30 min, 225°C for 45 min (one hour if samples had particularly high lipid content) and 380°C for 60 min. Once digestion was completed and following a cooling period, the samples were distilled using a Vodapest 40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany), the distillate was neutralised with concentrated H_2SO_4 and from the titration value crude protein determined using the following formulae:

$$\textbf{\textit{Crude protein}}\ (\%) = \frac{((\textbf{\textit{ST}} - \textbf{\textit{BT}})\ \textbf{\textit{X}}\ \mathbf{0.1}\ \textbf{\textit{X}}\ \mathbf{14}\ \textbf{\textit{X}}\ \mathbf{6.25})}{\textbf{\textit{SW}}} \times \mathbf{100}$$

Where; 0.1 is the molarity of the acid; 14 is the relative atomic mass of nitrogen (N); 6.25 is the constant relationship between N and animal protein; ST is the sample titre (ml); BT is the blank titre (ml) and SW is the initial sample weight.

2.15 Statistical analysis

Statistical analysis was performed using Statgraphics v5.1 software (StatSoft, USA). All data were presented as mean ± standard error (S.E.) and normality tests are used to determine whether a data set is well-modeled by a normal distribution or not, or to compute how likely an underlying random variable is to be normally distributed and analysed using one way analysis of variance (ANOVA) or Kurskal Wallis test, followed by multiple range tests. For the Comet assay data were analysed using multifactor analysis of variance (two way ANOVA) followed by Turkey's multiple comparison test. Only P values < 0.05 were considered significant. Any correlations between variables were determined using the Pearson's correlation coefficient.

2.16 Results

2.16.1 Comet assay optimisation

The results for 10, 20, 30 or 40 min. electrophoresis on either 10, 20, 30 or 40 min. unwinding for the considered endpoint (i.e. tail DNA %) has been presented in Fig 2.8 (A&B) and Fig 2.9 (C&D). The results are shown for the negative and positive controls. Overall, there was no significant difference between the unwinding times for the different electrophoresis times or between the electrophoresis times for different unwinding times (ANOVA, $P > 0.05$; n=3). Therefore, 20 min unwinding time and 20 min electrophoresis time, giving a background of about 15% (Fig. 2.8B) was chosen for the duration of the study.

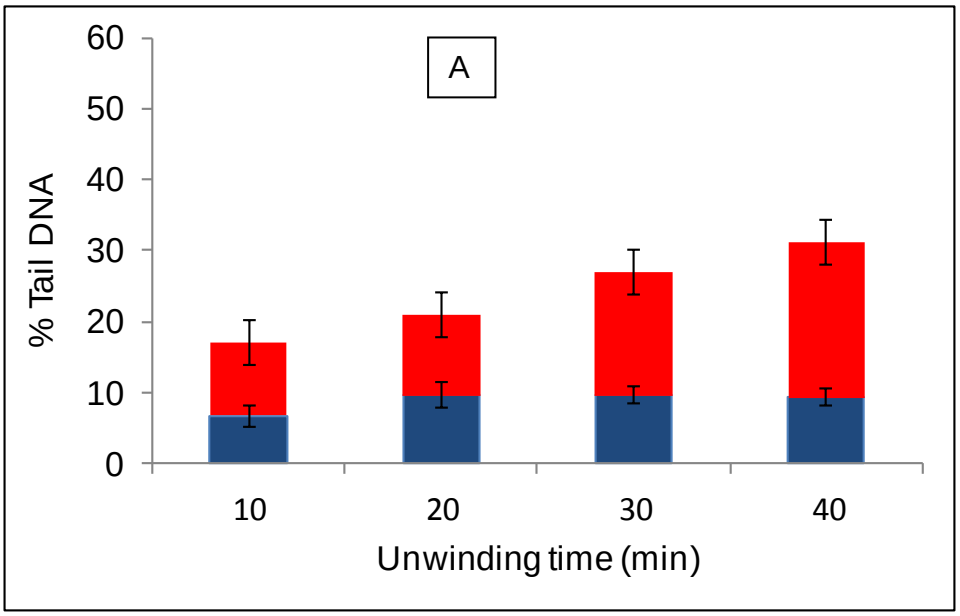
2.16.2 Validation of the Comet assay under in vitro conditions using hydrogen peroxide (H_2O_2)

Validation experiment was conducted with H_2O_2 exposure to erythrocytes. Results for % tail DNA demonstrated concentration-dependent increase in DNA damage in erythrocytes (Fig. 2.10). There were significant differences between H_2O_2 concentrations (10-200 µM) in comparison with control (Kurskal-Wallis, $P< 0.03$). Moreover, multiple range tests indicated a significant difference between H_2O_2 concentrations in both % tail DNA and Olive tail moment (Fig. 2.11 A&B). Thus, Comet assay using erythrocytes of the carp proved to be a sensitive technique for the detection of DNA damage.

2.16.3. Validation of the Comet assay under in vitro conditions using hydrogen peroxide (H_2O_2) to determine oxidative DNA damage (modified Comet assay protocol)

Another validation experiment was conducted using H_2O_2 as reference genotoxic agents to optimize modified Comet assay. Two different enzymes were used: Fpg and Endo-III. The result showed a concentration- dependent increase for the induction of DNA damage. The induction of DNA damage following enzyme treatments was ranked as follow:

Fpg > Endo-III > buffer (Two way ANOVA, $P < 0.05$), (Fig. 2.12 A&B).



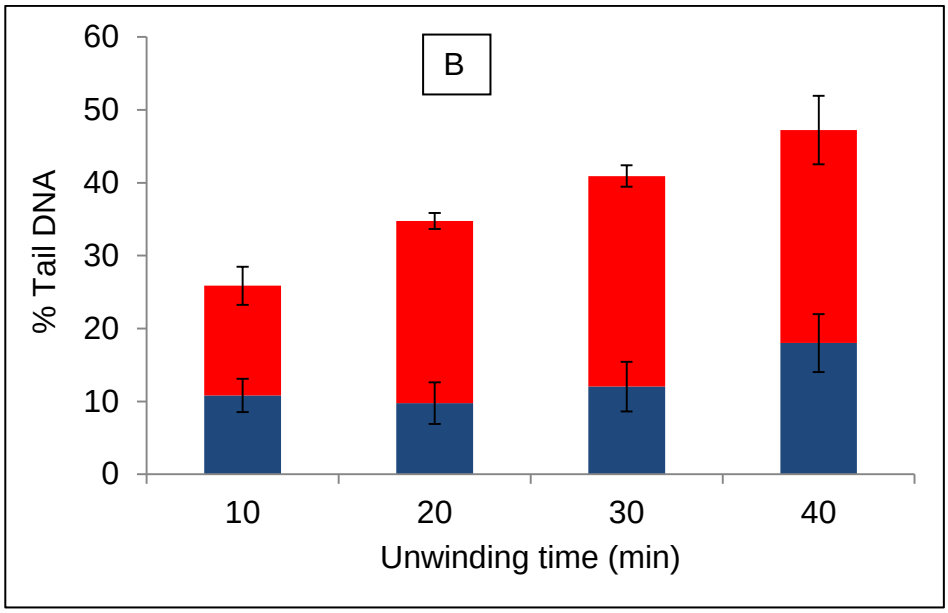
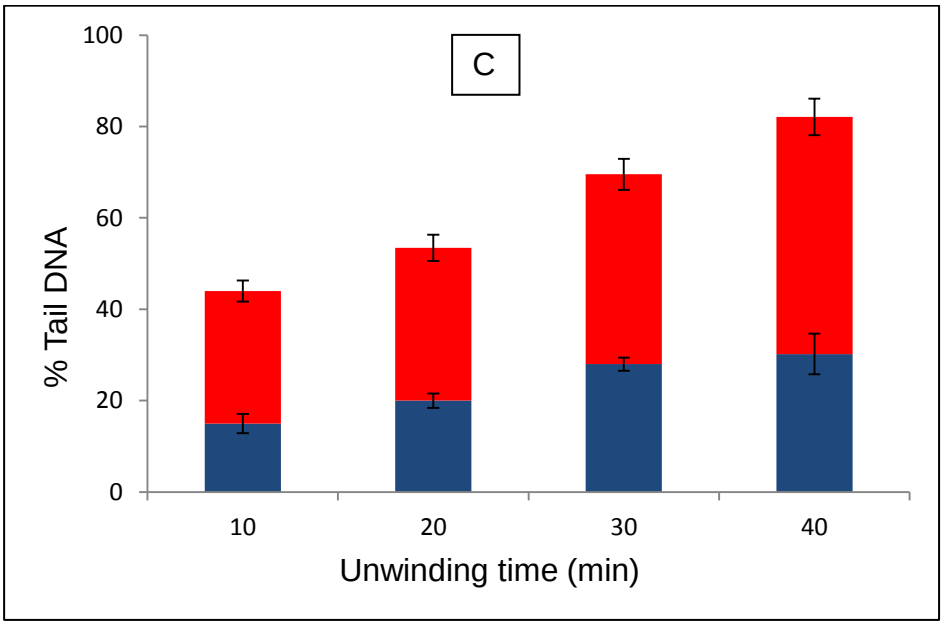


Fig. 2.8 The effect of unwinding time (A&B) negative control (blue) and positive control-100 µM H_2O_2 (red). (A) 10 min electrophoresis (B) 20 min electrophoresis; n=3.



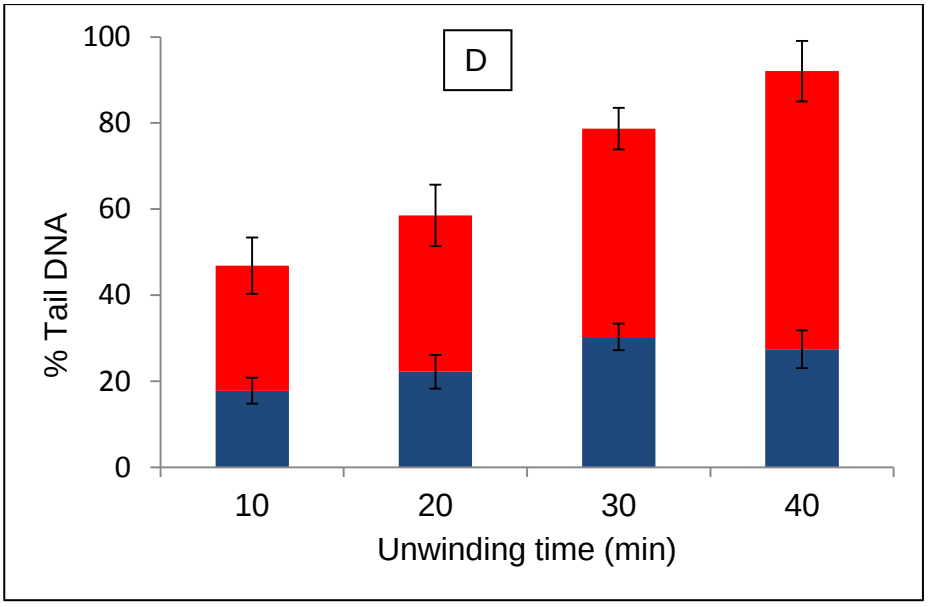


Fig. 2.9 The effect of unwinding time (C&D) negative control (blue) and positive control-100 µM H_2O_2 (red). (C) 30 min electrophoresis (D) 40 min electrophoresis; n=3.

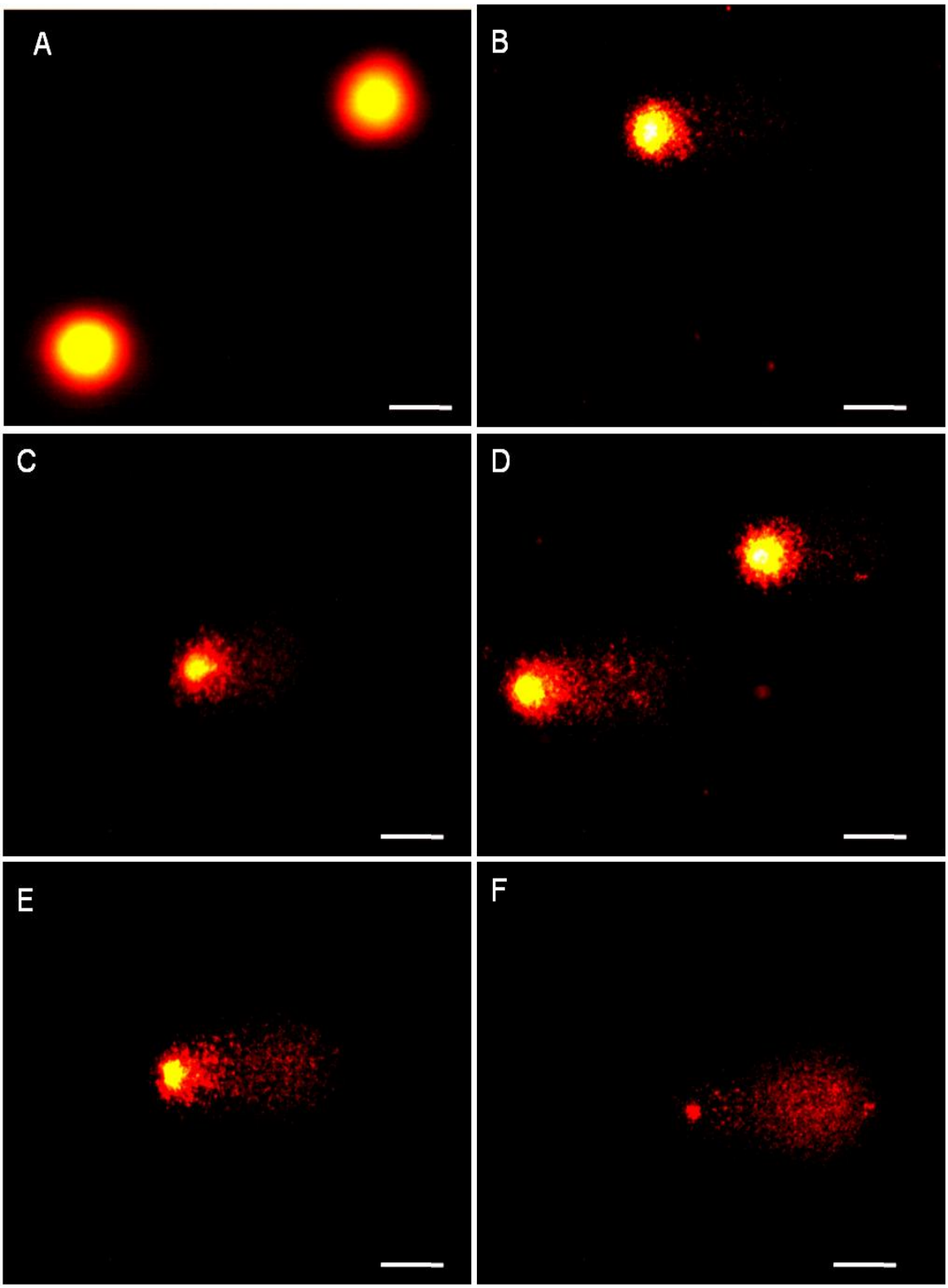
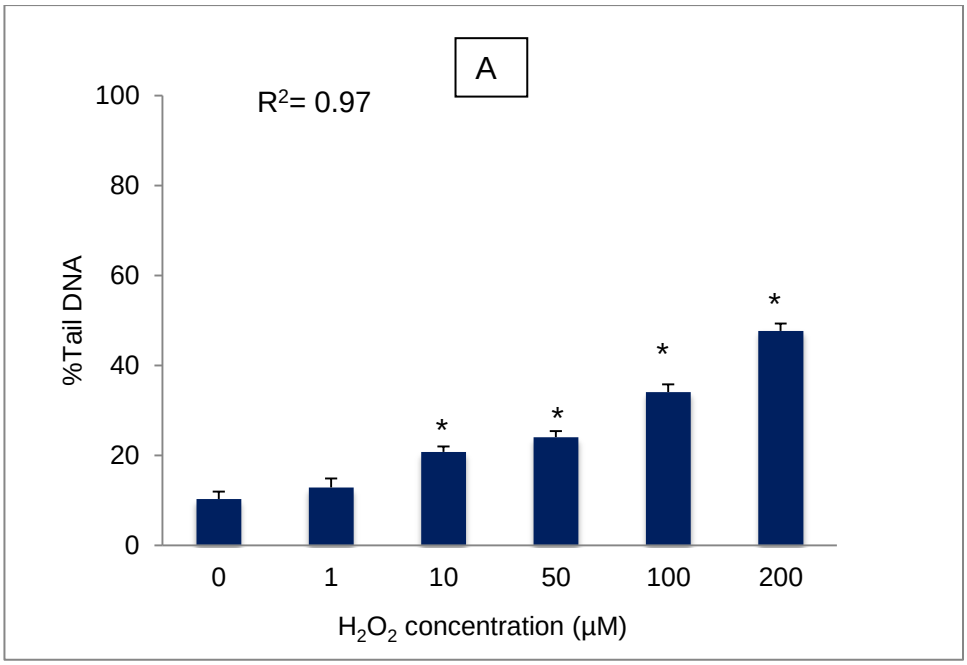


Fig. 2.10 Representative comet images of *C. carpio* L. erythrocytes following exposure to H_2O_2 for 10 min. for different unwinding and electrophoresis times. These comets illustrate the visual scoring classification; class 0 (A), class 1 (B), class 2 (C&D), class 3 (E) and class 4 (F). Cells are stained with Ethidium bromide. Total magnification = 400x. Scale bars 50 µm.



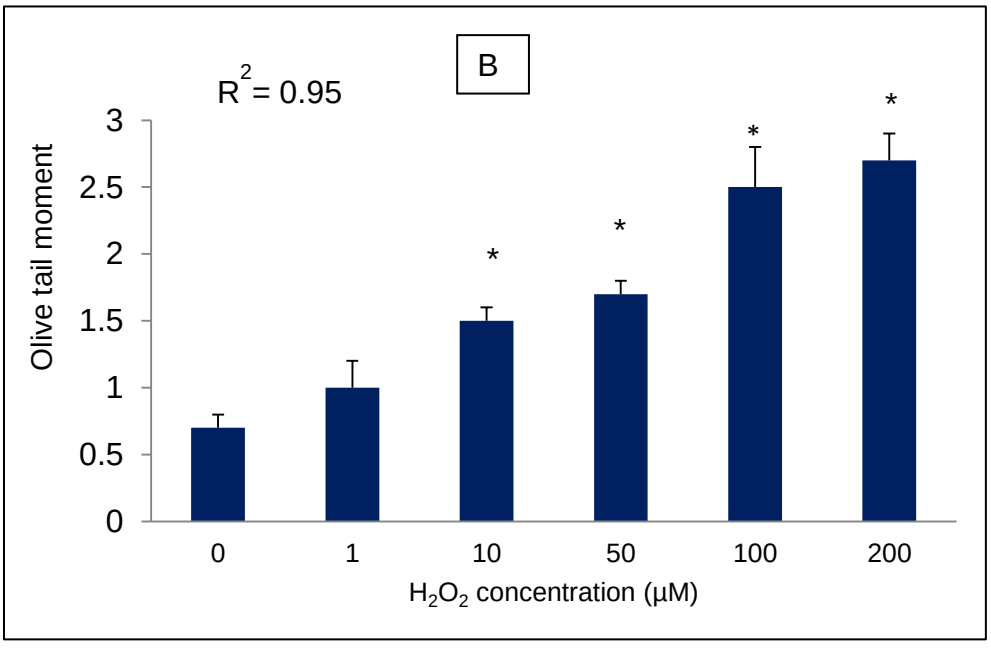
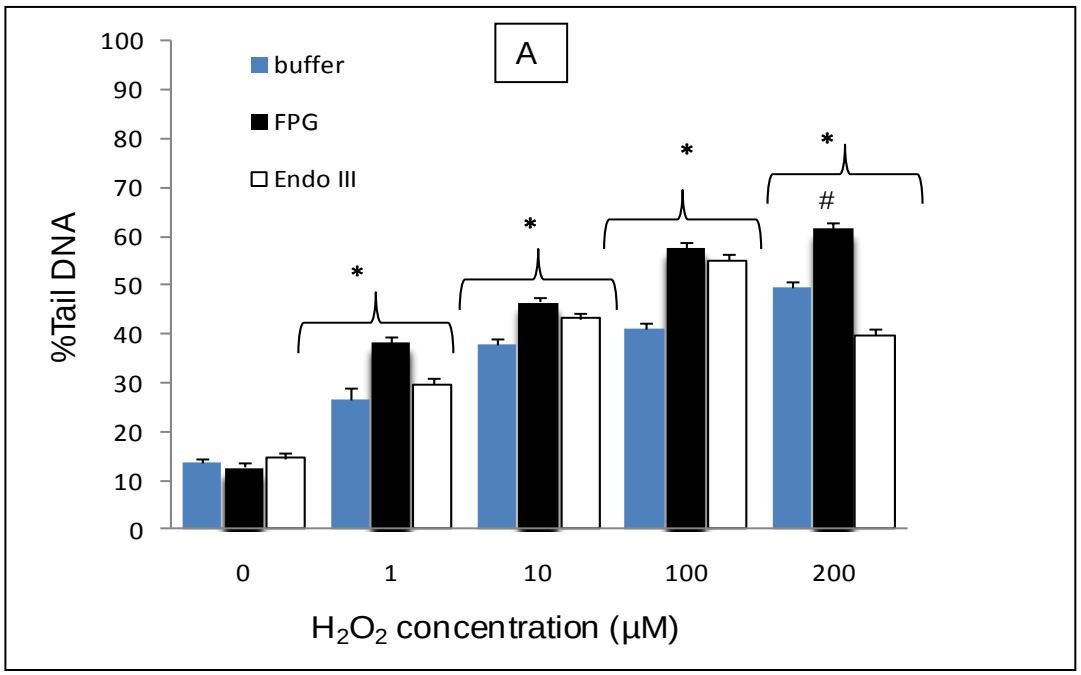


Fig. 2.11 DNA damage expressed as (A) % tail DNA (B) Olive tail moment in *C. carpio* erythrocytes following 10 min *in vitro* incubation with different concentrations of H_2O_2 (1, 10, 50, 100, 200 µM). Data are means ± S.E (*) indicates significant differences from control, using Kurskal Wallis test. (n = 3).



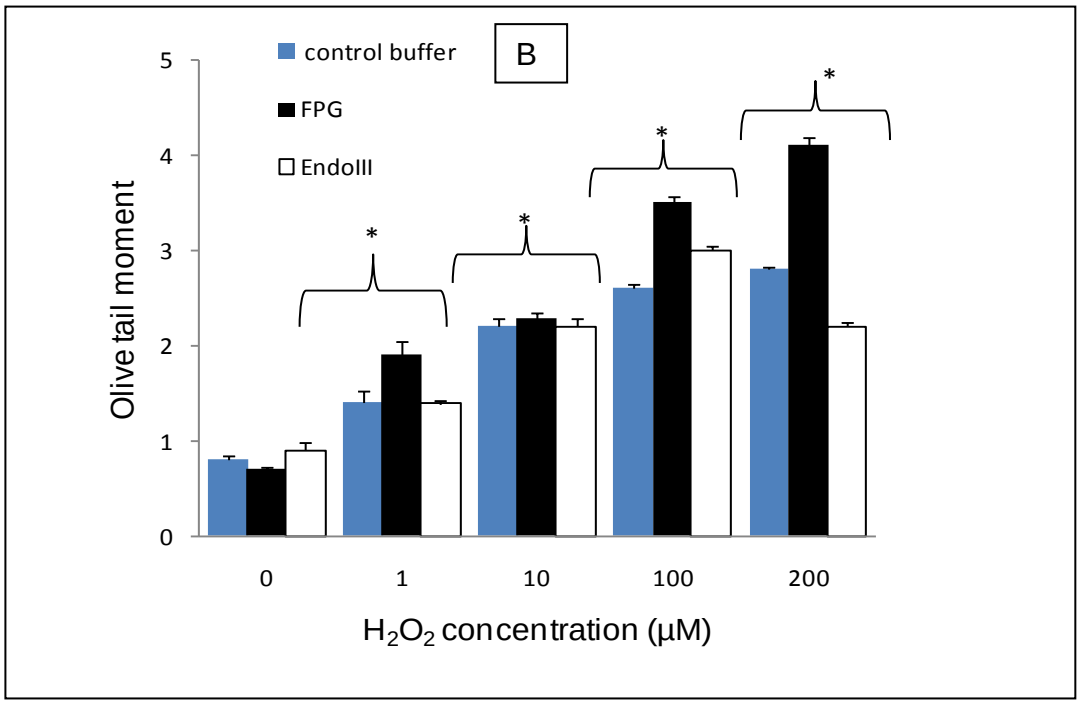


Fig. 2.12 DNA damage expressed (A) as %tail DNA (B) Olive tail moment in *C. carpio* erythrocytes following 10 min *in vitro* incubation with different concentrations of H_2O_2 (1, 10, 100, 200 µM) using bacterial enzymes (Fpg and Endo-III). Data are mean ± S.E. (*) indicate significant differences from control; (n=3).

CHAPTER 3

ELEVATED DIETARY COPPER INDUCES BIOCHEMICAL, GENETIC AND HISTOPATHOLOGICAL CHANGES IN CARP, *CYPRINUS CARPIO* L.

Results from this chapter have been presented at the 40th EEMS annual meeting, Oslo, Norway, Sep 2010 (poster presentation) and in Marine Institute Conference, Plymouth, UK, Dec 2010.

Hypotheses: (a) Elevated concentrations of dietary Cu induce responses at different levels of biological organisation

Abstract

Copper (Cu) is an essential element for all organisms including fish. Conversely, in line with other heavy metals, excessive amounts of this metal could be detrimental to normal physiological functions manifested primarily by impaired feed efficiency, reduced growth and tissue damage at both histological and molecular levels. Consequently we adopted an integrated approach to evaluate the impact of dietary borne copper on mirror carp, *C. carpio,* determining varying levels of biological responses, including specific growth rate (SGR), feed conversion ratio (FCR) and particularly DNA stability. Fish (mean weight 8.23 ± 0.14) were exposed chronically for 10 weeks to dietary Cu concentrations of 12 (control) -1000 mg kg $d.w^{-1}$. Several endpoints at different levels of biological organisations were evaluated. These included oxidative DNA damage (using Comet assay in combination with Fpg and Endo-III enzymes), haematological and histopathological parameters (including ultrastructural changes) in selected organs. Cu accumulation in different organs was also determined at the end of this period.

Oxidative damage in the DNA (determined in erythrocytes) showed a significantly higher level of damage at elevated Cu concentrations compared to control groups, suggesting that high dietary Cu exposure induces oxidative DNA damage. There was no significant effect of exposure on haematological parameters except for haemoglobin concentration. The Cu levels in liver were significantly higher in fish fed with 1000 mg kg $d.w^{-1}$. The order of Cu accumulation in tissues was liver > intestine > gill > bone which are dose response. Highest concentration (1000 mg kg $d.w^{-1}$), showed hepatocellular

coagulative necrosis with multifocal areas of lipid vacuolation of hepatocytes. There was a marked inverse relationship between specific growth rate and dietary Cu concentration for carp. These findings indicate complex stress responses occurring at different levels of biological organisation with oxidative DNA damage being precipitated at the higher organisational level. In common with human studies, this approach could be adopted to determine the impact of dietary factors on general health of individuals and populations. The ecological impact on fish biology is obvious given that growth, maturation and reproductive success ultimately reflects the Darwinian fitness of the organisms and ultimate survivability. Exposure to specific contaminants may have profound implications too for the global aquaculture industry dependent on quality feed ingredients and traceability of the food chain.

3.1 Introduction

Cu is an essential trace element required in small concentrations for all organisms including fish. The optimal requirement of copper in diet as determined for several fish range from 3 to 5 mg Cu kg $d.w^{-1}$ (Watanabe et al., 1997). Conversely, at high concentrations this metal is considered to be a hazardous inorganic contaminant to aquatic organisms (Pedder and Maly, 1985). Cu from natural and various anthropogenic sources constantly influx the aquatic environment where they create threats to fish health due to their toxicity, long persistence, bioaccumulation and biomagnification in the food chain (Zhou et al., 2008).

Lanno et al. (1985) have reported that dietary uptake to copper is considerably less toxic relative to waterborne exposure. This is attributable to the mucosal layer in the gut which act as a great barrier to toxic metals (Handy, 1992), although Cu ultimately absorbed via the gut. Thus, the bioavailability of its much lower in contaminated feed compared to the equivalent quantity offered in dissolved form (Miller et al., 1993b). Whatever the uptake, Cu accumulated mainly in liver, intestine, gill and kidney. Thus, the bioaccumulation model of metals in fish can be utilized as effective biomarkers of environmental metal pollution (Larsson et al., 1985). Furthermore, tissue specific accumulation has been proposed as a key biomarker to assess the effect of the chronic exposure of metals in aquatic organisms (Kim and Kang, 2004).

Growth is a good quantitative biomarker for measuring the long-term toxicological investigations and also provides an index of physiological status and performance (Rijnsdorp, 1990). Furthermore, histopathological and ultrastructural studies are also useful tools to assess chronically exposed environment (Arellano et al., 1999).

Since blood variables respond quickly to sub chronic effects of pollutants than other commonly measured endpoints, they have been widely used for the description of healthy fish and for predicting systematic relationships and the physiological status of fish. Moreover, detection of oxidative DNA damage is also sensitive and reliable biomarker to measure the impaired cellular process. But, few studies are available on the effects of Cu on oxidative DNA damage particularly through dietary route.

Whereas toxic effects in fish exposed to highest waterborne levels of copper are well known, relatively few studies have been addressed the dietary metal toxicity, regardless of diet being an important pathway of contamination in wild fish (Dallinger and Kautzky, 1985). Also, there are still many gaps in the knowledge of Cu toxicity in fish, and which organ respond to elevated levels is essential. Therefore, this study aimed to test the following hypotheses:

1. Elevated concentrations of dietary Cu (250, 500 and 1000 mg Cu kg $d.w^{-1}$) can induce oxidative DNA damage in *C. carpio*.

2. Elevated concentrations of dietary Cu will have an adverse influence on the growth rate of *C. carpio*.

We adopt a holistic approach and achieve the following objectives:

a. Determine damage to the DNA (using modified Comet assay as a biomarker of DNA damage)

b. Determination the potential influences on haematological parameters (erythrocytes and leukocytes count, haematocrit value and haemoglobin concentration).

c. Determine the effects of dietary Cu exposure on tissue specific accumulation (liver, intestine, gill and bone) in carp.

d. Examine the effects of dietary Cu exposure on histopathological and ultrastructural aspects in the major tissues and organs in carp.

e. Determine the specific growth rate and food conversion ratio of the *C. carpio* exposed to elevated concentrations of dietary Cu.

3.2 Diet formulation

A ten-week feeding trial was initiated with four dietary treatments based on herring meal and lysamine pea protein as the source of protein, corn starch and viten were added as the carbohydrate source, vegetable oil provided lipids, and molasses was added as binder. In addition, mineral premix formulated to supply the recommended requirements for carp (Table 3.1). Cu- supplemented diets were prepared using the same formulation of control diet except that 1, 2 and 4 g kg^{-1} of corn starch was omitted to compensate for the mass of Cu sulphate ($CuSO_4.5H_2O$) were supplemented with 0, 250, 500 and 1000 mg Cu kg^{-1} dry weight (subsequently termed as mg Cu kg^{-1} in the text). These concentrations of Cu were selected on the basis of previous studies carried out in our laboratory conditions in rainbow trout following exposure to dietary Cu (500 mg Cu kg^{-1}) for 3 months (Handy et al., 1999). In addition, the concentrations used in the study were in the range of other studies in our and other laboratory conditions where different fish species have been exposed to dietary Cu for physiological and toxicological studies (for review see Handy, 2003). In the absence of any mortality for these reports, the concentration range was justified especially for evaluation of genetic damage which is determined below the maximum

tolerated concentration or dose (MTC or MTD) for which mortality is considered to be final arbitrator (Canty et al., 2009; Jha, 2008).

The copper sulphate was dissolved in 300 ml deionised water and mixed well with other feed ingredients. The resulting paste was extruded through a Hobart food mixer (model A-120, 3 mm extrusion plate) and dried at 40°C for 96 h. Pellets were stored at - 20°C to prevent lipid peroxidation until use. The control diet was similarly treated except that no Cu was added to the diet. The Cu content of diets was confirmed by atomic absorption spectrophotometer (ICP-OES, Varian 725-ES ICP- optical emission spectrometer). Final Cu concentrations in the experimental diets were 11± 0.2, 241 ± 0.6, 481 ± 1.4 and 993 ± 1.3 mg Cu kg^{-1} respectively, n=3 in the control and Cu supplemented diets. Proximate analyses of the diets indicated in Table 3.1.

Table 3.1Dietary ingredients, proximate composition of diet.

	Cu concentrations (mg kg d.w^{-1})			
Ingredient (g kg^{-1})	Control	250	500	1000
Corn starch[1]	390.99	389.99	388.99	386.99
Herring meal (LT92)[2]	350.00	350.00	350.00	350.00
Lysamine pea protein[3]	140.01	140.01	140.01	140.01
Viten[4]	50.00	50.00	50.00	50.00
Vegetable oil[5]	30.00	30.00	30.00	30.00
Vitamin mineral premix[6]	20.00	20.00	20.00	20.00
Molasses	10.00	10.00	10.00	10.00
Proximate analysis (%) n=3				
Crude protein	38.6 ± 1.24	38.09 ± 0.32	38.07± 0.60	38.30 ± 1.20
Lipid	13.2 ± 0.31	13.05 ± 1.65	13.09 ± 1.54	12.98 ± 0.02
Ash	14.92± 2.04	15.00 ± 0.41	16.25 ± 0.43	15.07 ± 0.93
Moisture	34.81± 1.32	35.13± 0.62	34.11 ± 0.25	34.04 ± 1.05

[1] Sigma-Aldrich Ltd, UK.
[2]Herring meal LT92 - United Fish Products Ltd., Aberdeen, UK.
[3] Roquette Frêres, France.
[4] Sigma-Aldrich Ltd, UK.
[5] Sunflower oil.
[6] Premier nutrition vitamin/mineral premix: 121 g kg^{-1} calcium, Vit A 1.0 µg kg^{-1}, Vit D3 0.1 µg kg^{-1}, Vit E (as alpha tocopherol acetate) 7.0 g kg^{-1}, Cu (as cupric sulphate) 250 mg kg^{-1}, Magnesium 15.6 g kg^{-1}, Phosphorous 5.2 g kg^{-1}.

3.3 Experimental design

Mirror carp *C. carpio* weighing about 5.39 ± 0.14 g were obtained from Hampshire carp hatcheries (Bowlake fish farm, UK) and acclimated for a period of 3 weeks, during this time fish were fed standard commercial diet of 2% body weight (Ewos, Micro 20 p, Ewos Ltd., Westfield, Bathgate, West Lothian. UK). Post acclimation, 120 fish were selected for dietary exposure (average weight, 8.2 ± 0.1 g), were equally distributed into twelve 80-l fibreglass tanks, with 10 fish $tank^{-1}$ (four triplicate groups). Each tank was provided with 98% of re-circulated-aerated fresh water at a flow rate of 600 l h^{-1}. The physicochemical characteristics water quality was presented in Table 3.2. A 12-h light/12-h dark photoperiod was maintained throughout the trial duration. Weekly water changes, the self-cleaning design of the system and the routine cleaning weekly were carried out to ensure that food and faecal materials were cleared from each tank. Prior to experiment, all fish were fed the control basal diet with no added Cu for one week to acclimatise them to the experimental diet. At the start of the experiment all fish were fed the diet three times daily at a rate of 3% of average body mass (equal rations at 9.00, 13.00 and 17.00 h) for 10 weeks, three tanks remained on the basal diet (no added Cu) containing 11 mg Cu kg^{1}, while the others were fed a high-Cu diet containing 250, 500 and 1000 mg kg^{1} added as $CuSO_4.5H_2O$. Throughout experiments/trials, fish were reweighed every week and within this period feed input was adjusted daily based on a predicted feed conversion ratio value (FCR). Daily feed was corrected on a weekly basis following batch weighing after a 24 h starvation period (see 2.12). SGR and FCR were determined (see section 2.13). Total mortality during the experiment was 0%.

Table 3.2 Average data of physical-chemical parameters of water used in the dietary Cu exposure experiment.

Water quality parameters	Values*
pH	7.3 ± 0.3
Temperature (°C)	24 ± 0.05
Dissolved oxygen (mg l^{-1})	7.1 ± 0.02
Ammonia (mg l^{-1})	0.002 ± 0.01
Nitrite (mg l^{-1})	0.02 ± 0.3
Nitrate (mg l^{-1})	24.30 ± 0.06
Copper (mg l^{-1})	3.04 ± 0.6

* Values are means ± SE.

3.4 Biological sampling and analysis

After 10 weeks of trial, fish were not fed the day before the sampling times in order to empty the gut and to facilitate dissection. Fish were netted (2 fish $tank^{-1}$, 6 $treatment^{-1}$) and euthanized in a buffered solution of methane sulphonate (MS-222; 100 mg l^{-1} water for 10 min). Blood samples were obtained from the caudal vessel using a 25 gauge needle and 1 ml heparinized syringe immediately collected into test tubes for determination of oxidative DNA damage using modified Comet assay (see 2.7.5). Also for analysis of percentage haematocrit (2.8.3), measurement of haemoglobin concentration (see 2.5.1) and total leucocyte and red blood cell counts (2.8.2). Major tissues of interest (liver, intestine, and kidney) were dissected out, and fixed in 10% formaldehyde solution for histopathological studies (see 2.10). Another portion of liver tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for ultrastructural studies by TEM (see 2.11). A further two fish per tank (6 fish $treatment^{-1}$) were sampled for copper analysis, and the following tissues were sampled: liver, intestine, gill and bone (see 2.9; Cu analysis). For analytical procedures (lipid, protein and ash) 2 fish per tank (6 fish $treatment^{-1}$) were dried, and percentage moisture also determined from initial and final weight (see 2.14 and subsections).

3.5 Statistical analysis

Statistical analysis was performed using Statgraphics v5.1 software (StatSoft, USA). All results are expressed as mean ± Standard error (S.E.). A way one ANOVA was used to test the differences between the treatments on the parameters measured at the end of the experiment. For the modified Comet assay (with the use of enzymes) data presented as average median ± S.E. was analysed using multifactor analysis of variance (two way ANOVA). Significant ANOVA was followed by a multiple comparison test. Level of significance was taken as $P < 0.05$. General linear model was used to determine the differences for the growth rate between weeks using one of the appropriate statistical methods (LSD). The numbers of measured for each parameter is specified in the table captions.

3.6 Results

3.6.1 Determination of oxidative DNA damage

In the present study after 10 weeks of exposure to different concentrations of dietary Cu, no loss of cell viability was observed in any of the treatments (cell viability in the trypan blue exclusion dye, > 90% in all cases). Oxidative DNA damage was relatively low in control group and in low dietary Cu group (i.e., 250 mg kg^{-1}) compared to both medium and high dietary exposure groups. Oxidative DNA strand breaks increased significantly ($P < 0.00005$) on dietary exposure groups at high concentration (1000 mg Cu kg^{-1}) compared to control and 250 mg Cu kg^{-1} by approximately 200% and 150% respectively. Also, DNA damage at 500 mg kg $d.w^{-1}$ was significantly increased compared to control and 250 mg Cu kg^{-1} groups by approx. 130% and 85% respectively. In addition, there was a significant difference between all dietary exposures groups. However, the enzymes (i.e. Fpg and Endo-III) and enzymes/Cu interaction was not significant (Fig. 3.1). These results suggesting that oxidative DNA damage depended upon the dietary Cu concentration.

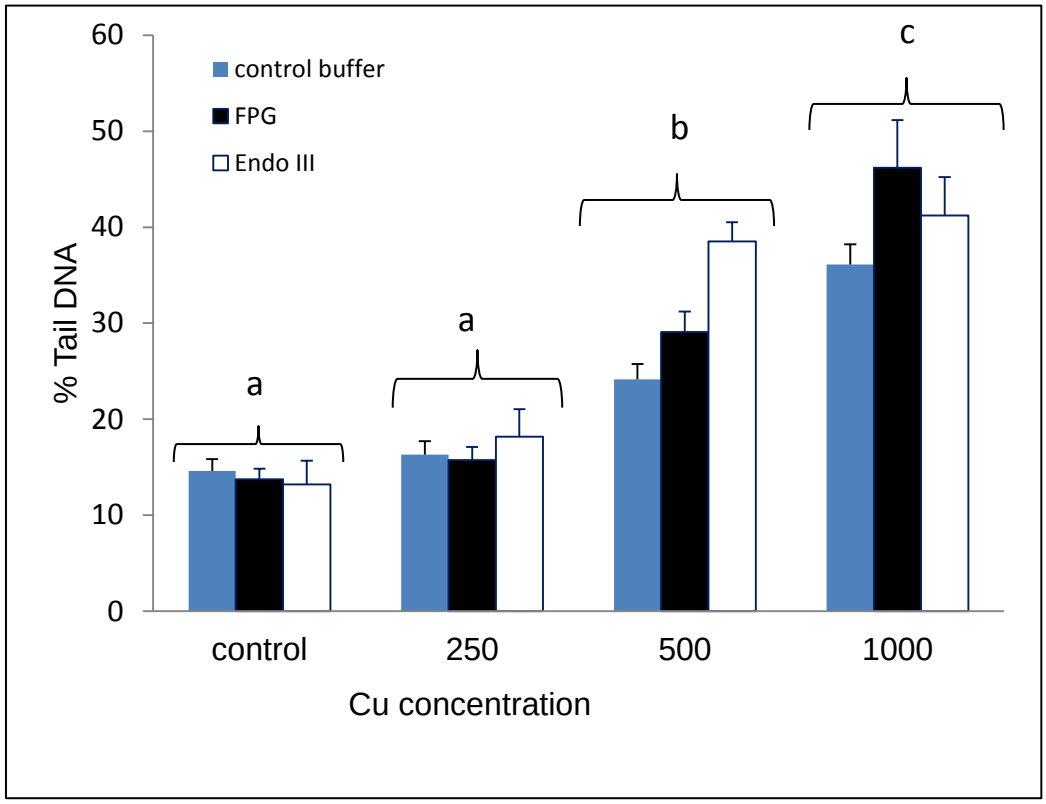


Fig. 3.1 Induction of DNA single strands breaks (represented as percentage tail DNA) in *C. carpio* erythrocytes following 10 weeks exposure to dietary Cu concentrations (250, 500, 1000 mg kg d.w^{-1}). Values are mean ± S.E. Different letters indicate significant different at $P < 0.05$, corresponding 95.0% confident intervals (n = 6).

3.6.2 Haematological parameters

Haematological variables in the control and all dietary exposure groups are summarised in Table 3.3. For in haematocrit (Hct%) values there were no significant differences observed in all dietary Cu groups compared to control group, as well as no significant differences were observed between all dietary Cu treatments. The results upon red blood cell count (RBC) are indicated no significant differences in dietary Cu groups at 250, 500 mg kg^{-1}; compared to control group fed basal diet. Only the group fed the highest Cu concentration (1000 mg kg^{-1}) had a significant decrease by approximately 30% compared to control group and also was significant decrease compared to both Cu dietary groups at 250, 500 mg Cu kg^{-1} respectively (ANOVA, P = 0.046). For white blood cell count (WBC) there were no significant differences observed in all dietary exposure groups compared to control group. Haemoglobin concentration showed significant decreased in dietary exposure groups at 1000, 500 mg kg^{-1} by approximately 25% compared to control group and low dietary Cu group (250 mg kg^{-1}) (ANOVA, P = 0.028).

Table 3.3 Effect of 10 weeks exposure to dietary Cu on haematological parameters of *C. carpio.*

variables	Cu concentrations (mg kg d.w^{-1})			
	Control	250	500	1000
Hct (%)	31.25 ± 0.73^{a}	30.63 ± 0.80^{a}	28.58 ± 1.63^{a}	29.22 ± 1.62^{a}
RBC (cells x $10^{6}\mu l^{-1}$)	1.77 ± 0.06^{a}	1.42 ± 0.30^{a}	1.47 ± 0.18^{a}	1.21 ± 0.09^{b}
WBC (cells x $10^{3}\mu l^{-1}$)	13.09 ± 0.04^{a}	14.36 ± 0.06^{a}	13.18 ± 1.43^{a}	14.01 ± 0.97^{a}
Hb (g dl^{-1})	9.34 ± 0.08^{a}	9.68 ± 0.03^{a}	6.89 ± 0.02^{b}	7.03 ± 0.06^{b}

Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significantly different at $P < 0.05$; (n=6).

3.6.3 Histopathological studies

Liver, intestine and kidney were examined at the end of the feeding trial. For the kidney, all tubules, glomeruli and other elements of the nephrons appeared normal, in both control and in all dietary Cu groups with no evidence of oedema, necrosis (Fig. 3.2 A&B). Intestine also showed normal structure, with no evidence of necrosis, oedema, haemorrhage or excessive epithelial sloughing in control and all dietary groups (Fig. 3.2 C&D) and there were no significant differences in the length and width of the villi in all treatment groups. The liver histology from control and exposed fish is briefly illustrated in Fig. 3.3. In control groups, sections of liver hepatocytes exhibited a typical architecture with no lesions in the hepatocytes. The hepatocytes present a homogenous cytoplasm around centrally or sub central located spherical nucleus (Fig. 3.3 A). The hepatic tissues of dietary Cu exposures exhibited histopathological changes after 10 weeks exposure to 500 and 1000 mg kg^{-1}, the cells lost its normal size and structure especially at 500 and 1000 mg kg^{-1}. At 500 mg Cu kg^{-1} diet hepatic parenchyma cells 83.3% of fish showed a typical perivascular

infiltrateration of mononuclear inflammatory cells (monocytes/macrophages and lymphocytes), with subendothelial fibroblastic nuclei on the back side of the vein (Fig. 3.3 B). At high Cu concentration (1000 mg Cu kg^{-1}), 67% of fish exhibits some hepatocellular coagulation necrosis and intravascular fibrin deposition with also a typical perivascular infiltrate of mononuclear inflammatory cells (monocytes/macrophages and lymphocytes) (Fig.3.3 C). Additionally, 66% of fish exhibited large, multifocal moderate to severe areas of fatty change/lipid vacuolation of hepatocytes. (Fig.3.3 D). For the low dietary Cu exposure (250 mg Cu kg $d.w^{-1}$) the hepatic parenchyma cells did not show any histopathological alterations.

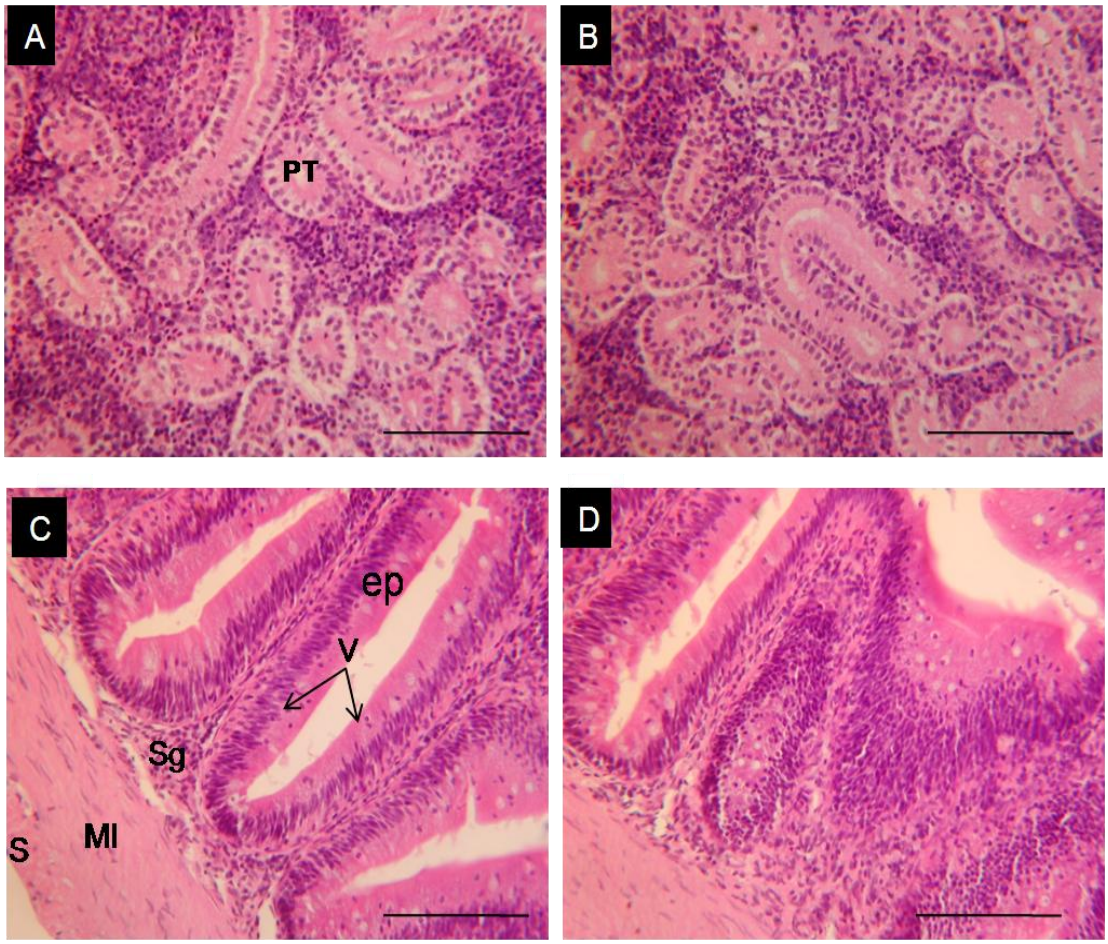


Fig. 3.2 Light micrographs of sections through kidney and intestine of *C. carpio* showing histological structures of control and dietary Cu exposures stained with H&E at 5-7 µm thickness. (A) kidney control (B) 1000 mg Cu kg^{-1} (C) intestine control (D) 1000 mg Cu kg-1 dw. PT = proximal tubule; S= Serous membrane; Ml= Muscularis longitudinal; Sg= Stratum granulosum; V= Villi; ep= epithelium. Scale bars: 100 µm.

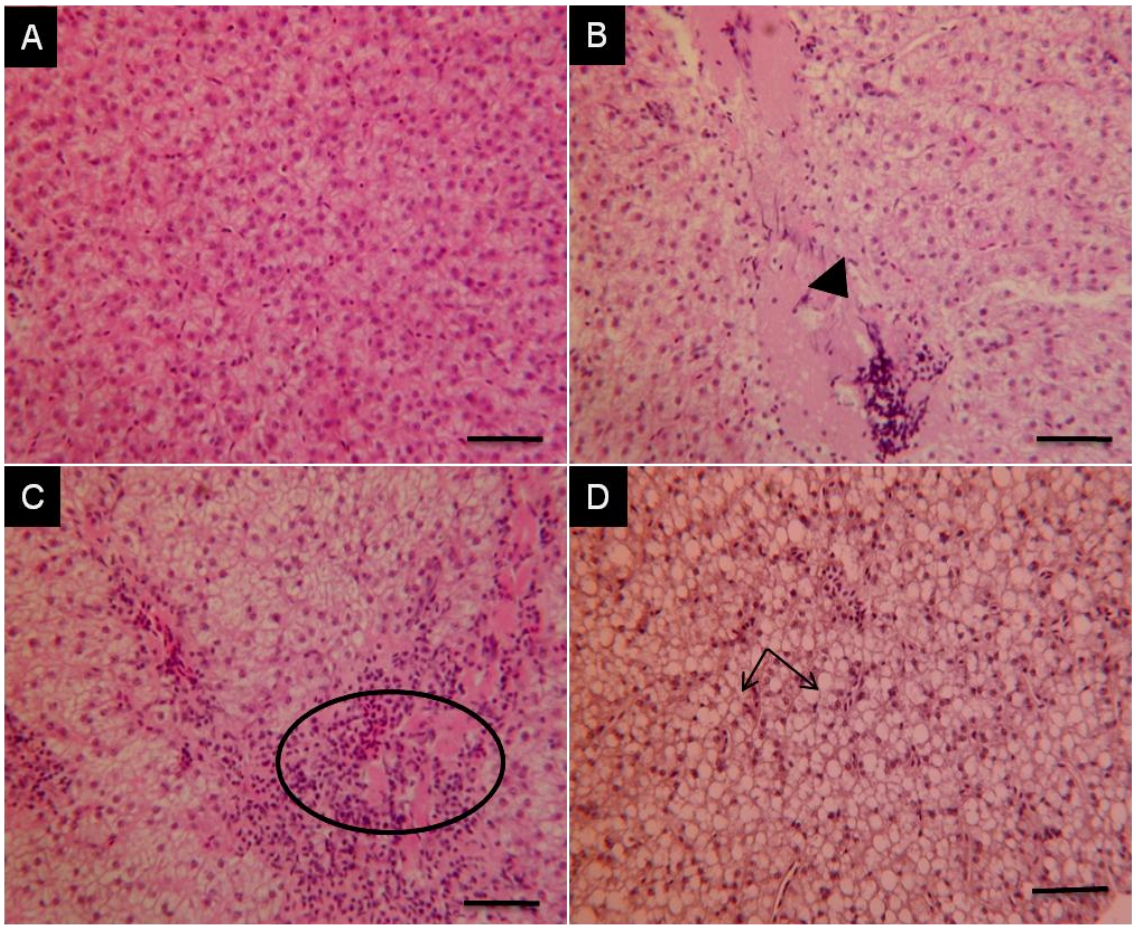


Fig. 3.3 Light micrograph sections showing histological structures through liver of *C. carpio* L. from control and dietary Cu exposures stained with H&E at 5-7 µm thickness. (A) control liver showing normal histology of hepatocytes (white arrow); (B) 500 mg Cu kg^{-1} diet subendothelial fibroblastic nuclei with aggregations of mononuclear inflammatory cells (arrowhead); (C&D) 1000 mg Cu kg $d.w^{-1}$ showing hepatocellular coagulative necrosis and infiltrateration of mononuclear inflammatory cells (black circle) and multifocal moderate to severe areas of fatty change/lipid vacuolation of hepatocytes (black arrow). Scale bars: 50 µm.

3.6.4 Ultrastructural study

In control group, sections of liver hepatocytes exhibited a typical architecture with no pathological changes (Fig. 3.4 A). Hepatocellular size was between 9 and 14 µm. The rounded hepatocelluar nuclei (2.0 ± 1.0 µm) displayed an electron–lucent euchromatin. The nucleus was surrounded by two to three cistern of rough endoplasmic reticulum and the smooth endoplasmic reticulum was poorly developed. A clear Golgi complex was not observed. A large number of mitochondria were diffused though the cytoplasm. The few numbers of roundish lysosomes of a very dark homogenous matrix was also observed. The hepatocytes contained few lipid droplets (vacuoles).

The hepatocytes of dietary Cu exposures revealed that the number of lipid droplet (homogeneous semi-electron dense vacuole) had increased significantly at 1000 mg kg $d.w^{-1}$ and 500 mg kg $d.w^{-1}$ dietary Cu exposures compared to control group (Fig. 3.4 B&C; 17.8 ± 0.99 $hepatocyte^{-1}$ and 11.3 ± 1.68 $hepatocyte^{-1}$) respectively (ANOVA, P = 0.016). These fat vacuoles were different in size (4.1 ± 2.9 µm) and were larger compared to control group. Also 1000 mg Cu kg $d.w^{-1}$ group showed an area of necrosis in some of the hepatocytes (Fig. 3.4 D).

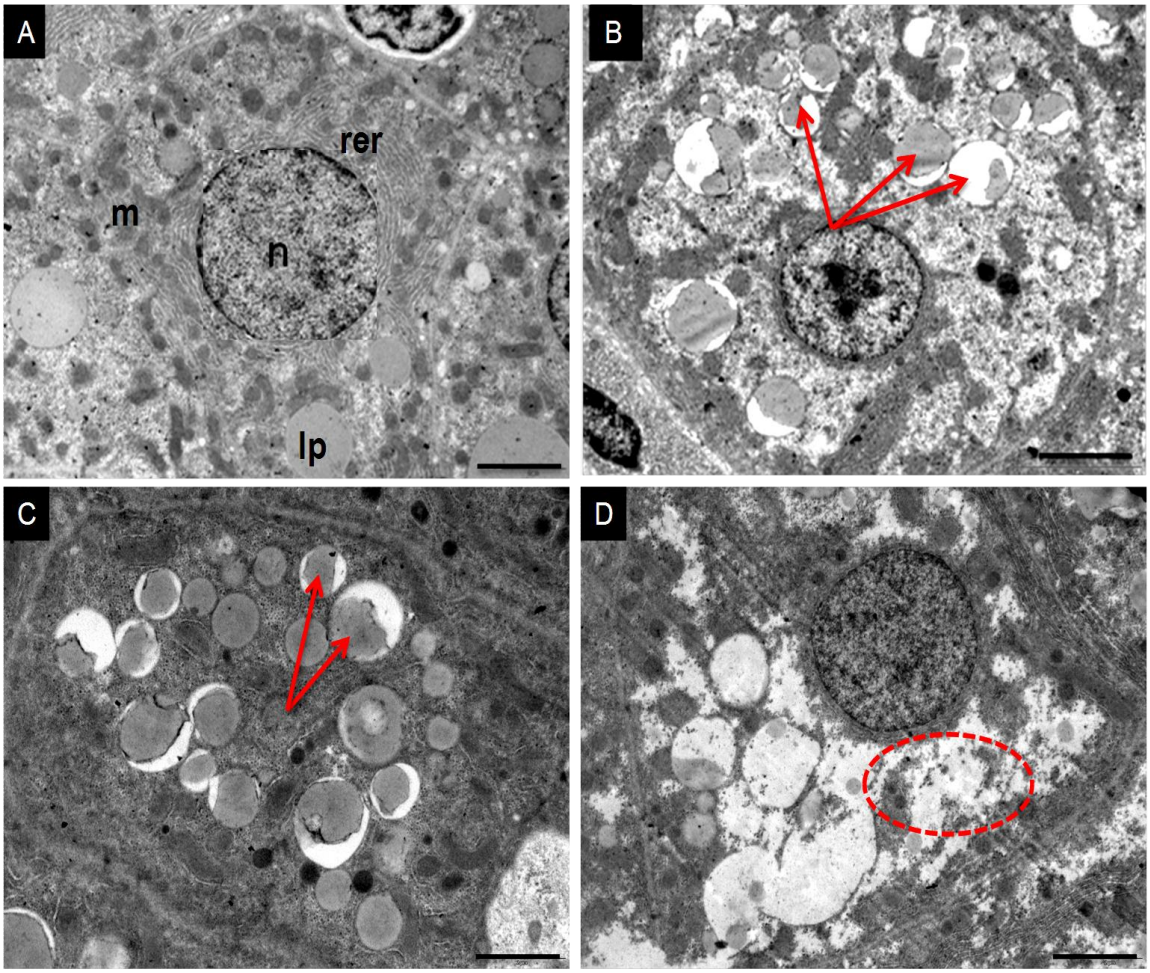


Fig. 3.4 Transmission electron microscopy images: (A) control liver; nucleus (n), rough endoplasmic reticulum (rer) nucleolus (nu), mitochondria (m), rough endoplasmic reticulum (rer), lysosomes (l) (B) 500 mg Cu kg $d.w^{-1}$ (C&D)1000 mg Cu kg $d.w^{-1}$, the number of lipid droplets (lp) increased (red arrow); necrosis (red circle). Scale bars: 2µm.

3.6.5 Cu analysis

Cu accumulation in the liver, intestine, gill and bone of *C. carpio*, are shown in Table 3.4. After 10 weeks of Cu dietary exposure, it was found that Cu accumulated in tissues was concentration dependent, and the order of Cu accumulation in tissues were liver > intestine > gill > bone. The highest Cu concentration was registered in the liver at 1000 mg Cu kg^{-1} (60 and 1.8 fold increase, respectively) compared to the 250 mg Cu kg^{-1} and control groups (Kruskal-Wallis, P = 0.022). On the other hand, Cu levels in the intestine were lower in comparison to liver. Also, Cu levels in the intestine was significantly higher at 1000 mg Cu kg^{-1} and 500 mg Cu kg^{-1} dietary exposure treatments compared to control and to 250 mg Cu kg^{-1} groups (14.7 and 11 fold, respectively). In addition, the level of Cu in intestine was higher at 500 mg Cu kg^{-1} over in those exposed to control and to 250 mg Cu kg^{-1} dietary exposure groups (10 and 8 fold, respectively) (ANOVA, P = 0.003). Cu concentration in gill was showed much lower in comparison to liver and intestine in all dietary exposure groups. There was increase of Cu levels in gill at 500 1000 mg Cu kg^{-1} (6 and 8 fold increase respectively) compared to control group. Also there was a significant difference between the 500 and 1000 mg Cu kg^{-1} (Kruskal-Wallis, P = 0.018). Cu accumulated in bone in lesser extent when compared to other tissues and also there was no significant difference between all dietary Cu groups.

Table 3.4 Accumulation of copper (µg Cu g^{-1}) by tissues of *C. carpio* fed diets containing different levels of Cu mg kg $d.w^{-1}$ for 10 weeks.

Tissue	Cu concentrations (mg kg $d.w^{-1}$)			
	Control	250	500	1000
Liver	12.55 ± 4.04^{a}	339.97 ± 184.22^{b}	679.40 ± 155.21^{c}	737.03 ± 162.02^{c}
Intestine	22.73 ± 2.64^{a}	29.74 ± 2.76^{a}	243.72 ± 33.17^{b}	336.11 ± 92.68^{b}
Gill	3.93 ± 0.66^{a}	7.25 ± 0.63^{a}	19.61 ± 1.05^{b}	24.80 ± 4.01^{c}
Bone	2.45 ± 0.69^{a}	3.23 ± 0.63^{a}	3.63 ± 0.35^{a}	5.76 ± 3.02^{a}

Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significant difference at $P < 0.05$; (n=6).

3.6.6 Growth and nutritional performance

Initial body weight of mirror carp from all replications of control and experimental groups varied from 82 - 82.9 g and there were no statistical differences at the starting time of the trial ($P < 0.05$). All the data for growth and nutritional performance are summarized in Table 3.5.

No fish died during the experimental period. After 10 weeks exposure to dietary Cu, there was a significant inverse relationship between the weight gain and the Cu concentrations (Fig. 3.5). The SGR of exposed *C. carpio* L. to dietary Cu was 1.6%, 1.6%, 1.5% at 250, 500, 1000 mg Cu kg^{-1} respectively, which was significantly lower compared to control group, and also there was a significant difference between high dietary exposure at 1000 mg Cu kg^{-1} and 500, 250 mg Cu kg^{-1} (ANOVA, $P = 0.006$). On the other hand, food conversion ratio (FCR) of dietary Cu groups was significantly different compared to control group and also a significant different between 1000 mg Cu kg^{-1} and 250, 500 mg Cu kg^{-1} (ANOVA, $P = 0.004$). These results, indicating that the highest level of dietary

Cu caused a gradual decline in growth rate of the Cu-fed groups relative to the control fed basal diet (Fig 3.4).

Mean Weight of the control and Cu dietary groups for 10 weeks are presented in table 3.6. The growth rate of *C. carpio* for all treatments showed ascending trend from week 0 till the end of the trial (Fig. 3.6). Different concentrations of Cu revealed lower growth rate than control which started from week 5 till week 10. Dramatically, decrease for all treatments appeared from week 9 and 10 (LSD=3.1). The proximate composition of the carcass was similar for all the treatments and control group, with a trend for the high Cu concentration (1000 mg Cu kg^{-1}) to have a higher lipid, and lower ash content. However, these differences were not statistically significant (Table 3.5).

Table 3.5 The effect of dietary Cu exposure on nutrition and growth [a] of *C. carpio.*

Variable	**Cu concentrations mg kg d.w^{-1}**			
	Control	250	500	1000
Initial mean weight (g)	08.23 ± 0.03^{a}	08.30 ± 0.05^{a}	8.26 ± 0.02^{a}	08.25 ± 0.02^{a}
Final mean weight (g)	30.05 ± 1.05^{a}	26.50 ± 0.8^{ab}	26.05 ± 0.75^{b}	23.5 ± 0.01^{b}
Weight gain (g)	21.82 ± 1.08^{a}	18.21 ± 0.75^{ab}	17.79 ± 0.77^{b}	15.25 ± 0.12^{b}
SGR (% g^{-1})	01.80 ± 0.02^{a}	01.69 ± 0.03^{b}	1.68 ± 0.04^{b}	01.53 ± 0.01^{c}
FCR	01.39 ± 0.01^{a}	01.56 ± 0.01^{b}	1.55 ± 0.03^{b}	01.70 ± 0.05^{c}
Proximate carcass composition[b]				
Moisture (%)	70.20 ± 1.31^{a}	70.51 ± 0.83^{a}	71.60 ± 0.05^{a}	71.53 ± 0.26^{a}
Crude protein (%)	54.32 ± 0.40^{a}	52.25 ± 0.87^{a}	54 .23 ± 1.10^{a}	52.91 ± 0.52^{a}
Lipid (%)	30.11 ± 0.34^{a}	30.40 ± 1.08^{a}	31.92 ± 2.72^{a}	33.41 ± 1.20^{a}
Ash (%)	08.51 ± 2.02^{a}	09.01 ± 1.98^{a}	09.9.32 ± 4.8^{a}	07.65 ± 3.82^{a}

[a]Values are means ± S.E. values within a row with different superscript letters are significantly different as determined by ANOVA at $P < 0.05$; (n= 6).
[b]Values are for fish at the end of the experiment expressed as percentage of dry matter. Proximate carcass composition of initial fish for moisture, protein, lipid and ash respectively were (means ± S.E. n=6): 75.08±0.03; 52.80±3.19; 31.62±0.01; 5.18±0.5.

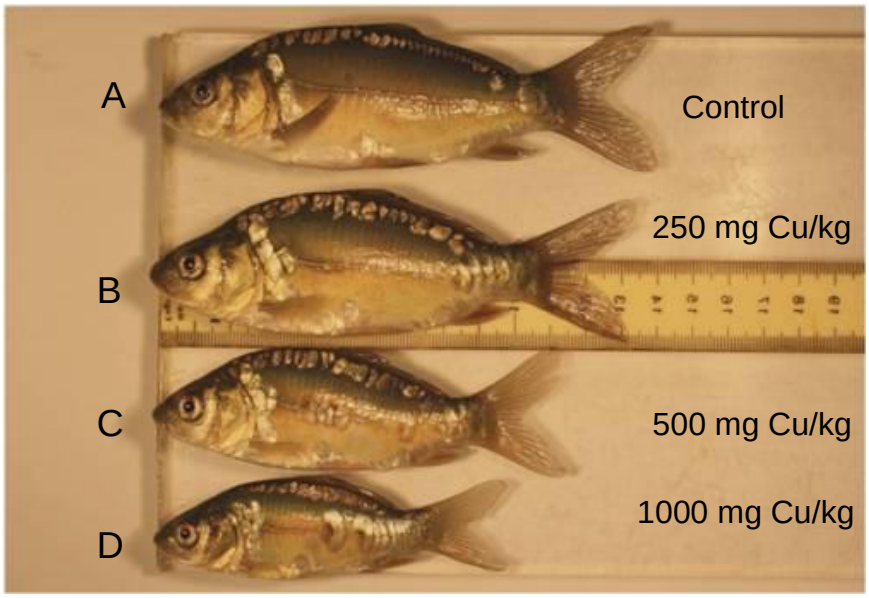


Fig. 3.5 Mirror carp *C. carpio* (A) fed basal diet without adding Cu. B, C & D fed Cu diets at 250, 500 and 1000 (mg kg d.w^{-1}) respectively. Weight gain from control group was higher than this from experimental groups.

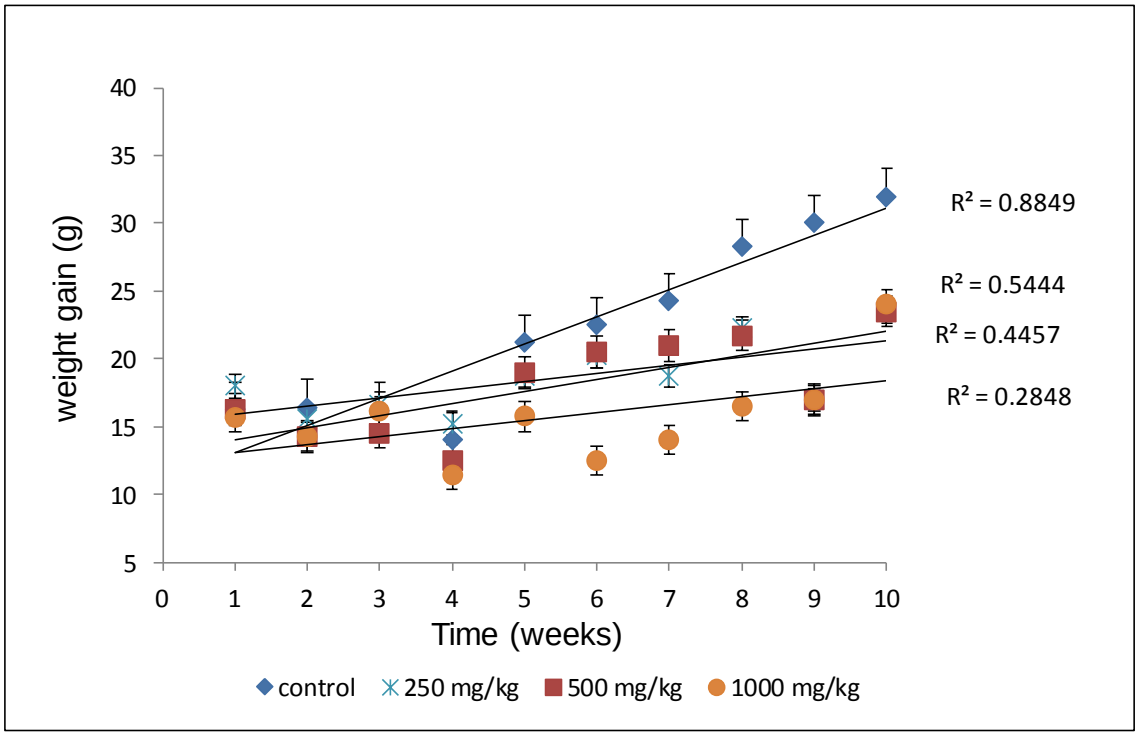


Fig. 3.6 Mean of weight gain of *C. carpio* exposed to elevated dietary Cu concentrations for 10 weeks.

Table 3.6 Mean weight of the control and Cu dietary groups for 10 weeks (n=3).

Cu concentrations mg kg d.w^{-1}	Weight (Mean (g) ± S.E.) per week									
	1	2	3	4	5	6	7	8	9	10
Control	9.86±0.34	10.90±0.37	12.52±0.57	13.92±0.52	16.05±0.65	18.3±0.8	20.7±0.9	23.2±1.0	27.15±1.3	30.05±1.05
250	10.1±0.27	11.26±0.25	12.92±0.22	14.45±0.45	16.32±0.42	18.35±0.5	20.2±1.0	22.4±1.1	24.14±0.9	26.5±0.8
500	9.89±0.29	11.02±0.27	12.47±0.27	13.72±0.37	15.62±0.52	17.7±0.47	19.8±0.3	22±0.6	23.07±0.6	26.05±0.75
1000	9.82±0.12	10.75±0.16	12.37±0.17	13.52±0.32	15.1±0.7	16.35±0.5	17.7±0.4	19.4±0.7	21.1±0.8	23.5±0.1

LSD=3.1; Less significant difference.

3.7 Discussion

The data obtained from the Comet assay; showed that the high Cu supplemented groups exert a significant genotoxic action after 10 weeks of exposure. The analysis of the data, moreover, suggests that the genotoxicity is based on the concentration of the Cu: both 500, 1000 mg Cu kg^{-1} showed increasing DNA strand breakage in mirror carp (Fig. 3.1). While there is some evidence of Cu genotoxicity and carcinogenicity on aquatic ecosystem including fish, However, its genotoxic potential is poorly understood in fish (Atienzar et al., 2001). Increasing evidence indicates that multifactorial mechanisms proposed to explain Cu genotoxicity one of the well-known mechanisms is though Cu initiated free radical generation that can damage biomolecules, including unsaturated lipids and DNA (Becker et al., 2009; Yourtee et al., 1992). The alternative mechanism for Cu genotoxicity is that Cu ions can directly interact with specific sequences of nucleotides in DNA leading to an inactivation of proteins involved in DNA replication, transcription and repair mechanisms (Hartwig, 1995; Prá et al., 2008).

Cu was previously reported to induce DNA damage but such an effect was only observed in European eel *Anguilla anguilla* exposed for 24 h to Cu: 1 or 2.5 µM) with and without pre exposure to B-naphthoflavone, gill showed DNA integrity loss in all exposure conditions (Ahmad et al., 2008). The data obtained from the effect of 0.002 µM of Cu^{+2} on DNA erythrocytes from the teleost gilthead sea bream *Sparus aurata* and the bivalve mollusk *Scapharca inaequivalvis* showed that the *in vivo* treatment with 0.002 µM of Cu increased the susceptibility of DNA to be damaged, and all three comet parameters significantly increased (tail length, tail intensity, and tail moment) (Gabbianelli et al., 2003). Therefore, it is difficult to compare the DNA strand break measurements from the various

studies, because of various methods of measurement used to determine the extent of the DNA strand breaks after carrying Comet assay. However, the induction of DNA strands break in most of the studies measured by various methods was higher after exposure to standard well known DNA damaging chemicals.

Haematological parameters have been considered as valuable biomarker for assessing fish health (Singh and Srivastava, 2010; van der Oost et al., 2003). In the present study most haematological parameters analysed remained unaffected. The results are indicating that the chronic dietary exposure of Cu has no significant effects on haematocrit (%) and for the total and for white blood cell count except for red blood cell count which exhibited significant decrease at high Cu dietary exposure (1000 mg Cu kg^{-1}) compared to control group and to Cu dietary groups. These results are in agreement with the previous studies, Handy et al., (1999) reported no significant differences in haematocrit (%) and erythrocytes count in rainbow trout fed 500 mg kg^{-1} dietary Cu for 3 months. Furthermore, Gatlin and Wilson (1986) found that the haematocrit and RBC count were not affected in catfish exposed to 40 mg kg^{-1} dietary Cu. Heavy metal exposure is known to induce changes in haematological indices in fish reviewed by Heath, (1995). However, no significant change in total RBC (for 250 and 500 mg Cu kg^{-1}) and WBC count possibly the fish body tries to maintain this count with the limits of physiological standards using different physiological mechanisms. Significant decrease in red blood cell count and haemoglobin concentration at high concentration of dietary Cu (1000 mg Cu kg^{-1}) may indicate osmotic disturbance and changes in blood's O_2 carrying capacity and this developing anaemia (Gatlin and Wilson, 1986). But, long term waterborne exposure (0.2 mg l^{-1}) resulted significant decreased

in erythrocytes count and haemoglobin concentration in trout (Vosyliene, 1996). In addition, the difference between dietborne and waterborne, that waterborne metal exposure could associated in gill damage which may lead to physiological disturbances (Pelgrom et al., 1995).

After 10 weeks exposure to 250, 500 and 1000 mg Cu kg^{-1} liver showed a perivascular infiltration of mononuclear inflammatory cells (monocytes/macrophages and lymphocytes). These changes were more evident in fish exposed to high concentration (1000 mg kg^{-1}); and also there was a moderate to severe areas of fatty change/lipid vacuolation of hepatocytes (Fig. 3.3). These alterations may be attributed to toxic effects of Cu on hepatocytes, since the liver associated with detoxification and biotransformation of all types of contaminants and toxins, the accumulation of inflammatory cells may indicate the reaction of melanodialdehyde in oxidative stress developed by Cu exposure. The present results are in agreement with those observed by several authors who studied the effects of various contaminants on fish (Arellano et al., 1999; Shaw and Handy, 2006). Intestine did not show histological alterations (Fig. 3.2), this is consistent with few studies on temperate species such as rainbow trout (Kamunde et al., 2001; Shaw and Handy, 2006).

For ultrastructural studies in liver, there was a significant increase in the number of lipid droplet compared to control group (Fig. 3.4). These lipid droplets could possibly indicate an alteration in lipid metabolism or partial changes in their morphology. In *Solea senegalensis* exposed to Cu (100 µg Cu l^{-1}), Arellano et al. (1999) observed an increase in the number of lipid droplet, which were larger in size compared to control group.

The present results showed Cu accumulation in tissues was dependent on the Cu concentration. There was a significant Cu accumulation in the intestine at high concentration (1000 mg Cu kg^{-1}) in contrast to other Cu levels and control group (Table 3.4). Also, liver exhibited high level of dietary Cu accumulation, suggesting that dietary Cu transferred from intestine to the liver via a portal system, and also indicating a overloading of the Cu regulating capacity.

The intestine seems to have an important role in controlling the uptake of dietary Cu, as was indicated by the considerable increase in intestinal Cu concentrations in the Cu dietary groups. This agrees with Handy et al. (1999) who also observed the greatest increases of Cu concentration in the liver and intestine in rainbow trout exposed to 300 mg Cu kg^{-1} for 3 months. Similarly, Berntssen et al., (1999) found the large increases of the Cu accumulation in the intestine and liver in Atlantic salmon *Salmo salar* L. exposed to 700 mg Cu kg^{-1} for 4 weeks. While Lorentzen et al. (1998), found no increase in hepatic Cu content in Atlantic salmon exposed to dietary Cu concentration up to 100 mg Cu kg^{-1} for 12 weeks.

Generally, there are two major routes for uptake of metal in aquatic organisms. These include digestive tissue in case of metals in diet or sediment, and the gill, in the case of dissolved form (Lapointe and Couture, 2009). In this study, Cu content in the liver of the exposed fish was approximately 60, 55 and 32 fold greater compared to controls at 1000, 500, 250 mg Cu kg^{-1} respectively. This finding is in accordance with results of Miller et al., (1993b) reported that the higher Cu accumulation in liver of rainbow trout increased as the Cu concentration increased in the diet. Moreover, Kim and Kang (2004) found that

the Cu concentration in the liver of rockfish was approximately 51, 18 and 11-fold higher compared to control at 500, 250 and 125 mg Cu kg^{-1} respectively.

It can be concluded that the mirror carp liver is typically important for Cu storage in contrast with other tissues. The high accumulation of Cu in the liver clearly confirmed that liver is the vital organ for detoxification and excretion of Cu through the induction of metal binding proteins such as metalthioneins (Handy et al., 1999), which is a sequestering agent. The detoxification of the Cu may be by sequestration rather than elimination by excretion (Phillips and Rainbow, 1989). Therefore the high level of Cu in the liver may be an induction of the storage of sequestered products in it.

Interestingly, levels of Cu accumulation in the liver and intestine tissues were higher than levels of accumulation in the gill tissue. Its appear to be the digestive system is more active compared with activity in the gill tissue, so the accumulation of metals in the digestive system is more than is usual. In addition, since the gill tissue is in direct contact with water and osmoregulation, gill easily disposed of Cu. Similar pattern of Cu accumulation were also shown in another study carried out with rainbow trout (Gundogdu et al., 2009). Levels of Cu accumulation in the bone were lower than levels in the liver, intestine and gill. This suggesting that carp bone does not play a major role in Cu detoxification.

No mortalities were observed by elevated dietary Cu concentrations during the experiment. However, Cu dietary exposure resulted reduction of *C. carpio* growth rate and there was opposite relationship between growth and Cu concentrations. (Table 3.5.), and was reflected by a decrease in SGR of exposed mirror carp to dietary Cu. Several researchers have observed

reductions in growth rate during dietary Cu uptake in fish but others are not. Baker et al. (1998) found that 2400 mg Cu kg^{-1} diet produced depression in growth rate of Grey Mullet *Chelon labrosus* in 10 weeks. Lanno et al.(1985) reported a transient depression of growth rate in fresh water adapted rainbow trout *Onchorhyncus mykiss* when exposed to 664 mg Cu kg^{-1} for 8 weeks. Berntssen et al. (1999) also reported a significantly growth reduction in juvenile Atlantic salmon when exposed to $\geq$ 500 mg Cu kg^{-1} for 12 weeks. It is also similar to juvenile rockfish *Sebastes schlegeli* which revealed a depression on growth rate at (125, 250, 500 mg Cu kg $d.w^{-1}$) in 60 days (Kim and Kang, 2004).

A possible explanation for impairment of growth the physiological changes permitting metal detoxification and homeostasis cost energy and reduced growth caused by exposure to Cu has been attributed to metabolic costs associated with metal detoxification as suggested by Kim et al., (2006). Furthermore, toxicants that interact with energy yielding reactions indirectly inhibit the syntheses of RNA, DNA and protein. In the case of mirror carp, the intestinal morphology remained similar for both control and all exposure groups, the reduction in growth which reflected by a decrease in SGR, appear due to increased energy consumption for sustaining normal metabolism leaving less energy available for growth. Other explanation for decreased growth is perhaps explained by reduced feed intake during copper exposure and poor absorption of the major nutrients.

In conclusions, the data obtained from the Comet assay showed that the high Cu supplemented groups exert a significant genotoxic action after 10 weeks of exposure. The analysis of the data, moreover, suggests that the genotoxicity is based on the concentration of the Cu; both 500, 1000 mg kg^{-1} showed

increasing DNA damage in mirror carp. Therefore, this test is a promising tool for estimation of the relationship between DNA damage and the exposure of fish to genotoxic pollutants at the single cell level.

The results of this integrated study of the dietary Cu exposure affects only Hb concentration and RBC count at high Cu concentration (1000 mg Cu kg^{-1}) and did not affect the other parameters. Cu accumulation clearly reflected the level of dietary exposure. The liver is a more important storage tissue than other tissues, and the order of Cu accumulation in tissues was liver > intestine > gill > bone. Dietary Cu exposure resulted in reduction of mirror carp specific growth rate and had inverse relationship between growth and Cu concentration. These findings indicate complex stress responses occurring at different levels of biological organisation with oxidative DNA damage being precipitated at the higher Cu level. In addition, the results of this study indicate that maximum allowable Cu concentration in food of mirror carp should be below 250 mg kg^{-1}

CHAPTER 4

HYPOXIA-INDUCED OXIDATIVE DNA DAMAGE LINKS WITH HIGHER LEVEL BIOLOGICAL EFFECTS INCLUDING SPECIFIC GROWTH RATE IN CARP, *CYPRINUS CARPIO* L.

Results from this chapter have been presented at the 15th International Symposium on Pollutant in Marine Organisms, Bordeaux, France, May 2009 and at the Plymouth Marine Sciences Partnership Symposium 2009, Plymouth, UK, April 2009 The results have also been published in Ecotoxicology, 20, 1455-1466 (Mustafa et al., 2011).

Hypotheses: Both hypoxia and hyperoxia induce responses at different levels of biological organisation (i.e. DNA to individual) in a representative carp species.

Abstract

Both hypoxia and hyperoxia, albeit in different magnitude, are known stressors in the aquatic environment. Adopting an integrated approach, mirror carp (*Cyprinus carpio* L.), were exposed chronically (i.e. 30 days) to hypoxic (1.8 ± 1.1 mg O_2 l^{-1}) and hyperoxic (12.3 ± 0.5 mg O_2 l^{-1}) conditions and resultant biological responses or biomarkers were compared between these two treatments as well as with fish held under normoxic conditions (7.1 ± 1.04 mg O_2l^{-1}). The biomarkers determined included the activities of glutathione peroxidase (GPx), measurement of oxidative DNA damage (using modified Comet assay employing bacterial enzymes: Fpg and Endo-III), haematological parameters, histopathological and ultrastructural examination of liver and gills. Specific growth rate of the fish, as an important ecotoxicological parameter, was also determined over the exposure period. The study suggested that while the levels of hepatic GPx were unaffected, there was a significant difference in activity in the blood plasma under different exposure conditions. Interestingly, oxidative DNA damage was significantly higher in both hypoxic and hyperoxic compared to normoxic conditions, Fpg showing enhanced level of damage compared to the Endo-III treatment. The haematological parameters showed enhanced values under hypoxic conditions. Transmission electron microscopic (TEM) studies revealed damage to liver and gill tissues in both the extreme conditions. Interestingly specific growth rate of fish was significantly lowered in hypoxic compared to normoxic condition and this was found to be correlated with DNA damage. Taken together, these results indicate that prolonged

exposure to both hypoxic and hyperoxic conditions induce oxidative stress responses at both DNA and tissue levels, and hypoxia can result in compensatory changes in haematological and growth parameters which could influence Darwinian fitness of the biota with wider ecological implications.

4.1 Introduction

Chronic exposures to both hyperoxia and hypoxia could be damaging to aquatic organisms leading to suboptimal growth and biomass production (Wedemeyer, 1997). Although lack of dissolved oxygen (DO) or hypoxia (DO < 2.8 mg l^{-1}) could be a natural phenomenon caused by daily fluctuations in oxygen concentrations (Nikinmaa, 2002), chronic hypoxic conditions prevailing in so called 'dead zones' in different parts of the world, which is linked to anthropogenic activities, often leads to mass mortality of sensitive biota and could lead to overall reduction in biodiversity (Diaz and Rosenberg, 2008). With respect to hypoxia-induced biological responses, most of the mechanistic studies have been carried out using mammalian cells under *in vitro* conditions (Wu, 2002). Our understanding of molecular responses using *in vitro* models therefore requires further elucidation at whole organism level.

Fish as a group are considered to be prime models to study oxygen dependent processes as they demonstrate acclamatory or adaptive responses with respect to their requirements (Lushchak and Bagnyukova, 2006; Soitamo et al., 2001). Most of the studies carried out using fish involving hypoxic or hyperoxic exposures have however used only short term exposures (Wu, 2002). Where a chronic exposure has been performed, only a selected biochemical or physiological responses have been studied, which do not provide a holistic picture of the potential impact at the individual level. In this context, the significance of oxidative stress associated with both environmental contaminants and also in aquaculture related activities have been subject of scientific investigations (Livingstone, 2003; Lushchak, 2011). For example,

effects of hypoxia (0.40 mg O_2 l^{-1}) for 2, 6 or 10 h and subsequent normoxic recovery has shown induced oxidative stress and a compensatory changes of a range of antioxidant enzymes in different tissues of goby, *Perccottus glenii* (Lushchak and Bagnyukova, 2007). A 42 d exposure to common carp, *Cyprinus carpio* L. to 0.50 mg O_2 l^{-1} has shown to induce DNA damage (determined by TUNEL signal) in liver cells, especially during the first week of exposure. There was however no change in other cellular parameters (e.g. proliferation, number or size, caspase activity) including induction of DNA single strand breaks (Poon et al., 2007). Gene expression analyses in gonads of mature zebrafish (*Danio rerio*) maintained under normoxia (3 mg O_2 l^{-1}) and hypoxia (1 mg O_2 l^{-1}) following short (4 d) and long term (14 d) exposures showed differential expression of genes associated with initial adaptive response (e.g. metabolism of carbohydrate, proteins, nucleic acid) and a suite of genes belonging to different ontology categories related with lipid metabolism, steroid synthesis and immune response which could lead to reproductive impairment (Martinovic et al., 2009). Field and laboratory studies in different fish species have also demonstrated that hypoxic conditions could potentially lead to abnormal developments of reproductive systems (Thomas and Rahman, 2010; Wu, 2002).

A 5 h exposure of rainbow trout to varying degrees of oxygen saturation (3.30-21.10 mg l^{-l}), showed enhanced degree of DNA single strand breaks (as determined by the alkaline unwinding technique) under hypoxic and hyperoxic conditions compared to normoxic conditions (i.e. 11 mg O_2 l^{-l}). The highest rate of DNA breaks occurred when the fish were kept under hypoxic conditions (Liepelt et al., 1995). Apart from this study, where a single endpoint was studied, to our knowledge there have been no further investigations to compare

the biological responses of these two contrasting environmental conditions concurrently. In particular, comparison of levels of oxidative DNA damage is lacking. Whilst exposure to both hyperoxia and hypoxia have been shown to cause DNA damage and apoptosis in cells of diverse origin (Cacciuttolo et al., 1993; Gozal et al., 2005; Poon et al., 2007), evaluation of oxidative DNA damage and its potential knock-on effects at individual level has not been elucidated. This is particularly important given that oxidative stress (including oxidative DNA damage) has been implicated in a variety of pathophysiological conditions including impairment of reproductive success in humans and, could well correlate to ecotoxicological parameters affecting other species (Jha, 2008). Furthermore, although both hypoxic and hyperoxic conditions are commonly observed in the aquatic environment (van Raaij et al., 1994), to our knowledge, concurrent comparison of impact of hyperoxia and hypoxia at different levels of biological organisation, elucidating cause-effects coupling is lacking. This is particularly important for illuminating the mechanisms of biological responses, as considerable overlap or commonalities in molecular pathways could exist for these contrasting conditions (Lushchak and Bagnyukova, 2006).

In the backdrop of above information, overall aim of this study was to compare the biological or biomarker responses at different levels of organisation (i.e. DNA to individual) in a representative carp species, *Cyprinus carpio* L., following chronic exposure (i.e. 30 days) to both hypoxic and hyperoxic compared to normoxic condition. This species has been used for such studies by different workers e.g., (Lushchak et al., 2005; Poon et al., 2007). We also aimed to test the correlations between DNA damage with other biomarkers and specific growth rate (SGR) of the fish. A modified single cell gel electrophoresis

or the Comet assay was employed as a robust assay to determine oxidative DNA damage (Azqueta et al., 2009). Levels of antioxidant enzyme, glutathione peroxidase (GPx activity) in plasma and liver samples and, haematological parameters were also determined along with histopathological and ultrastructural studies in selected tissues. Finally, measurement of feed conversion rate (FCR) and specific growth rate (SGR) and its potential correlation with DNA damage were also determined.

4.2 Materials and methods

4.2.1 Fish and their maintenance

Cyprinus carpio L. (genetically male; ca. 30 g) were obtained from Hampshire carp hatcheries (Bowlake fish farm, UK) and were transported to the Aquarium. Fish were acclimated and grown to attain an average weight of 98.06 ± 0.39 g within 2 months in a re-circulated aerated fresh water at a rate of 600 l h^{-1}. Water temperature was maintained at 23.0-23.5 °C with an electric immersion heater; pH was between 7.2 and 8.0 and adjusted with $NaHCO_3$ as necessary. Dissolved oxygen (DO) concentration was maintained at 7.40 mg l^{-1} using air stones. All the water quality parameters were monitored and recorded daily using an electric meter (Hach HQ4d). Water was renewed weekly and nitrogenous compound were monitored weekly using a Hanch Lange DR 2800 and cuvettes for ammonia (Lange, LCK 304), nitrite (Lange, LCK 341) and nitrate (Lange, LCK 340) (Hach Lange Ltd., Salford, UK). The following levels of nitrogenous compounds were considered acceptable; ammonia (un-ionized) (< 0.10 mg l^{-1}), nitrite (< 1.0 mg l^{-1}), nitrate (< 50 mg l^{-1}). Mechanical filtration media was washed twice a week to maintain high filtration efficiency and reduce biological loading on the filter system.

4.2.2 Experimental design

Following the acclimation period, 36 fish (weighing 98.06 ± 0.39 g) were randomly distributed into six 80-l fiberglass tanks (duplicate tanks treatment^{-1}), normoxia (control group) at 7.1 ± 1.04 mg O_2 l^{-1}, hypoxic treatment group at 1.8 ± 1.1 mg O_2 l^{-1} [achieved using pumping nitrogen gas; $N_{2:}$ purity 99.99%], at specific water flow through the system ≈ 1 l min^{-1}) and a hyperoxic treatment group at 12. 3 ± 0.5 mg O_2 l^{-1} [O_2 injected: purity 99.95%] for 30 days (Plate 4.1). The concentration of oxygen in the water was measured three times daily by using an oxygen electrode (Oxy Guard, Handy Polaris, DK). Water temperature was maintained at 23.0-23.5 °C and pH was maintained at 7.07 ± 0.25. A 12 h light/12 h dark photoperiod was maintained through the exposure duration. The exposure start time began when the desired dissolved oxygen level was achieved, which was within 2 days after the initiation of nitrogen and oxygen pumping. During the experimentation period the fish in each treatment group were fed commercial feed pellets (Ewos, Micro 20 p, Ewos Ltd., Westfield, Bathgate, West Lothian. UK) at 2% biomass per day provided in equal rations at 09:00, 13:00 and 17:00 h. Daily feed was corrected weekly following batch weighing after a 36 h starvation period (see 2.12), SGR and FCR were determined (2.13).

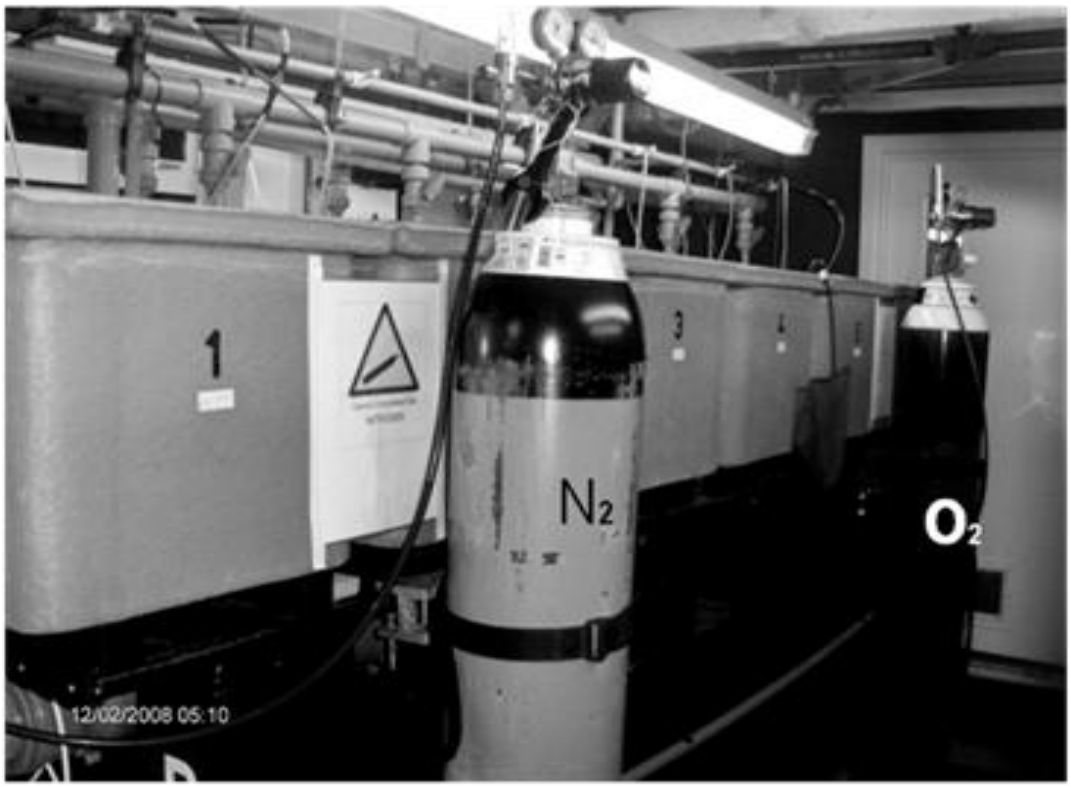


Plate 4.1 A view of the experimental set up using nitrogen and oxygen cylinders to maintain hypoxia and hyperoxia in the water tanks.

4.2.3 Analytical procedures

Fish were sampled at the end of experimental period 30 days (see 2.3), which by the nature of feeding schedule included one day of feed withdrawal prior to sampling. Fish were ethically anaesthetised with tricaine methanesulfonate (MS-222; 100 mg l^{-1} water for 10 min) (Pharmaq, Fordingbridge, UK). Whole blood was sampled from the caudal vein using a needle and syringe from 6 random individuals treatment^{-1} and kept under room temperature (22 ± 1°C) in heparin treated tubes until immediate analysis of percentage haematocrit (2.8.3), the measurement of haemoglobin concentration (2.8.1) and total leucocyte and red blood cell counts (2.8.2). Plasma removed and stored at – 80 °C (see 2.8.5) until analysis of glutathione peroxidase activity.

Blood samples were taken and stored on ice on heparin treated tubes for analysis by the single cell electrophoresis 'modified Comet assay' (2.7.5). The liver tissue excised and stored – 80 ^{o}C for enzyme analysis. Also, liver and gill tissues were dissected and immediately fixed in 10% formaldehyde solution for histopathological studies (see 2.10) another portions of these tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for ultrastructural studies (see 2.11).

4.2.4 Measurement of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was measured in plasma and liver as described by Lushchak et al., (2005) with slight modifications. Liver samples were weighed and homogenized (1:9 w/v) using a Potter- Elvjeham glass homogenizer in 100 mM Tris-HCl (pH 7.5), containing 2.5 mM dipotasium EDTA, 0.01% Triton X-100, and 2.5 mM sodium azide. Homogenates were

centrifuged (4°C for 20 min at 10,500 xg). The supernatant was transferred to a polypropylene microcentrifuge tube. Just prior to the GPx assay, a mixture containing 50 mM potassium HEPES buffer, (pH 7.5), 1 mM dipotasium EDTA, 0.21 mM NADPH, 1 Uml^{-1} glutathione reductase (Sigma G-3664 from *Saccharomyces cerevisiae*) and 1 mM GSH was prepared; 270 µl of this mixture and 50 µl of sample were mixed and the reaction was initiated by the addition of 5 µl of 12.4 mM H_2O_2. The decrease in absorbance was monitored for 60 sec in a microplate reader (Optimax, Molecular Devices, Sunnyvale, CA, USA) using 96 well plates. All measurements were carried out in triplicate. The assay temperature in each case was 20 ± 2.00°C. Activity was expressed as nmol min^{-1} ml^{-1} in plasma and nmol min^{-1} g^{-1}ww (wet weight) in liver.

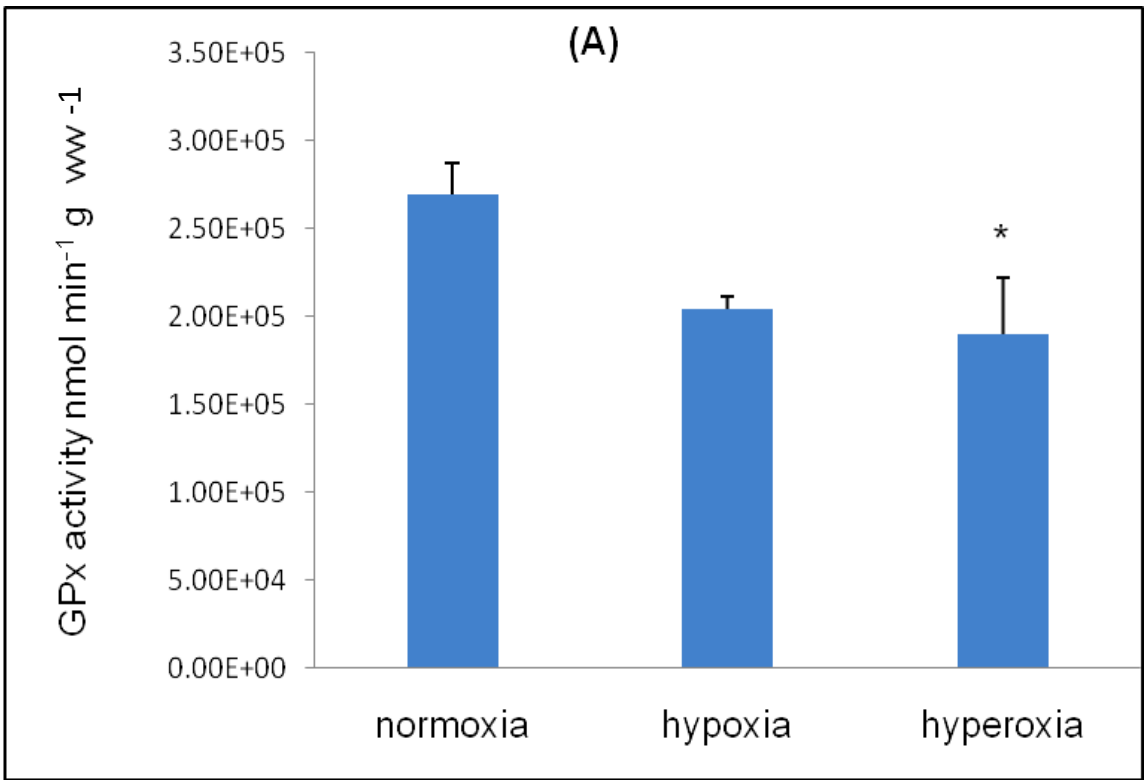
4.2.5 Statistical analysis

Statistical analysis was performed using Statgraphics v5.1 software (StatSoft, USA). All data were presented as mean ± standard error (S.E.), and analysed using a one way analysis of variance (ANOVA) or Kruskal Wallis test, followed by multiple range tests. For the modified Comet assay (with the use of enzymes) data presented as median ± S.E. were analysed using multifactor analysis of variance (ANOVA) followed by Turkey's multiple comparison test. P values < 0.05 were considered significant. Any correlations between variables were determined using Pearson's correlation coefficient.

4.3 Results

4.3.1 Determination of GPx activity

The liver GPx activity in hyperoxic reared fish was significantly decreased in comparison with normoxic group (ANOVA, $P = 0.04$). Also, the GPx activity in hypoxia decreased, but it was not significantly different from the normoxic group. Moreover, multiple range tests indicated that there was no significant difference between the hypoxic and hyperoxic group (Fig. 4.1 A). Over all, the GPx activity in the liver tissue of mirror carp seems to be unaffected by hypoxic condition for the exposure periods, (i.e. 30 days). In contrast to liver, a significant decrease in GPx activity was observed in the blood plasma in hypoxic group compared to normoxic group (Fig. 4.1 B). On the other hand, the hyperoxic group showed increased GPx activity compared with normoxic group and was highly significant indicating increased oxidative stress in this group. This significant difference was detected between hypoxic and the hyperoxic treatment groups (ANOVA, $P = 0.002$).



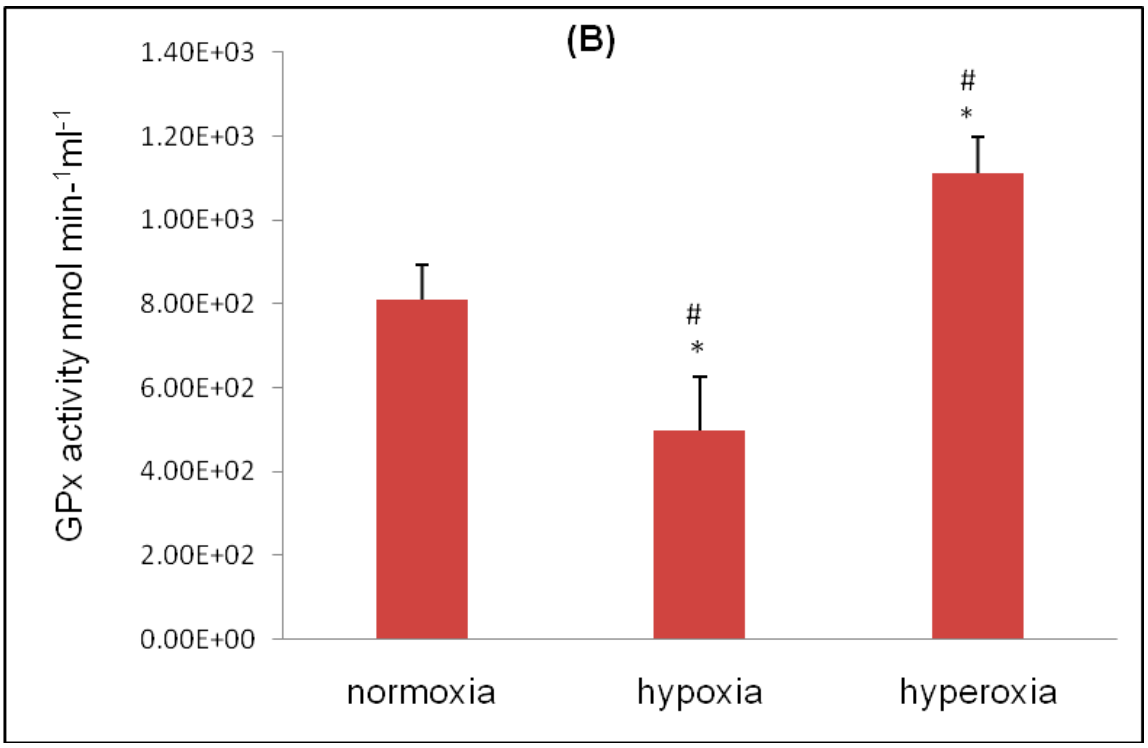


Fig. 4.1 Activity of GPx (A) in liver (B) in plasma, following 30 days of exposure to normoxia, hypoxia and hyperoxia. Values are mean ± S.E. * indicates significant differences from normoxic group; # indicates significant differences between the hypoxia and hyperoxia groups at $P < 0.05$; n=6.

4.3.2 Determination of oxidative DNA damage

Both hyperoxic and hypoxic conditions showed a strongly significant ($P < 0.00005$) increase for oxidative DNA damage compared to normoxic condition. Oxidative DNA damage was relatively low in normoxic fish compared to hyperoxic and hypoxic groups. Conversely, the highest degree of oxidative DNA strand breaks (i.e. %DNA in tail) was seen in the presence of Fpg enzyme which increased by about 25% in the both cases (hypoxic group and hyperoxic group) compared to normoxic condition. This enzyme showed a statistically significant different compared to buffer control and Endo-III treatments using two way ANOVA, P= 0.0001 (Fig. 4.2).

.

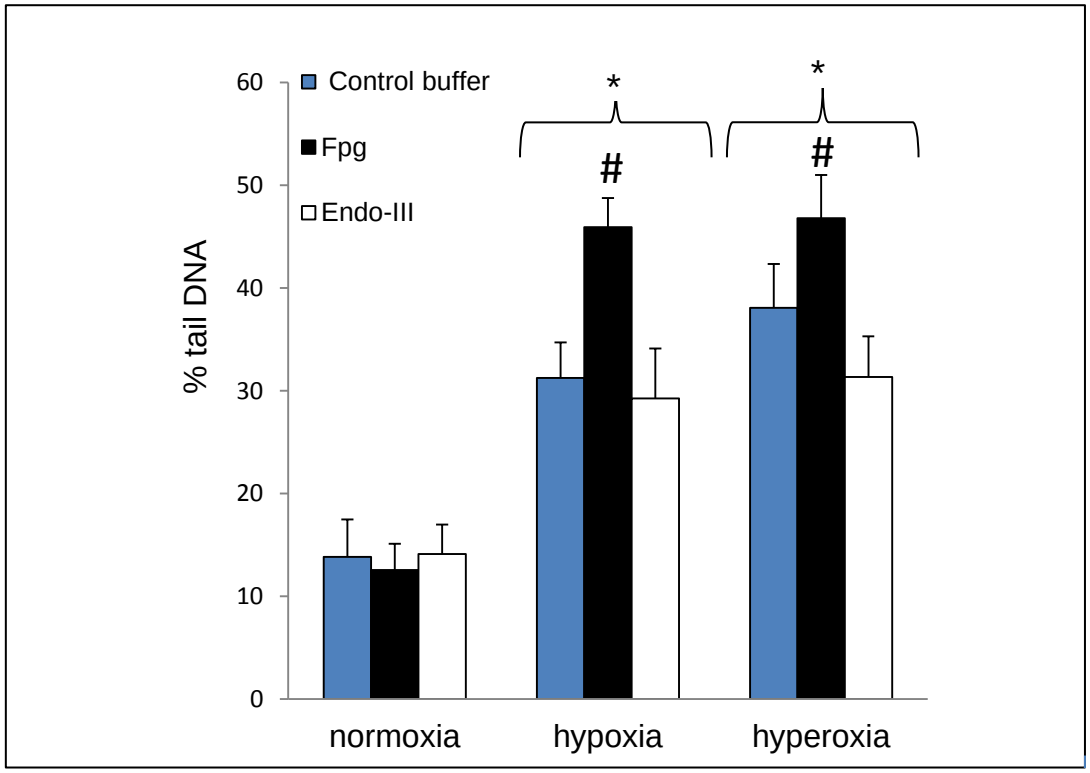


Fig. 4.2 Induction of DNA single strands breaks (represented as percentage tail DNA) in *C. carpio* erythrocytes following 30 days exposure to normoxia, hypoxia and hyperoxia (1.8-7.0-12.3 mg l^{-1} respectively). Values are average median ± S.E. * statistically significant different versus normoxia; # statistically significant different versus buffer and Endo-III at $P < 0.05$; n = 6.

4.3.3 Determination of haematological parameters

The results of the various indices for the hypoxic, hyperoxic and normoxic treatment groups are summarised in Table 4.1 Differences among groups were found for Hct value which increased in hypoxic group by about 25% than the normoxic group. Hct value in hypoxic group (38.00 ± 0.04) was highly significant from the normoxic group value (31.00 ± 0.03) and also from hyperoxic group value (30 ± 0.01) (ANOVA, P = 0.001). The highest Hb concentration was registered in hypoxic group (9.57 ± 0.66). This value was significantly higher by about 40% than value registered in normoxic group (6.76 ± 0.60) (ANOVA, P = 0.03). The Hb concentration in the hyperoxic group marginally increased (7.41 ± 0.48) from the normoxic group value, but was not significantly different from either control or hypoxic treatment values. On the other hand, RBC count increased ~90% in hypoxic group and ~ 50% in hyperoxic group compared to the normoxic group. RBC count was having highest value in hypoxic (3.42 ± 0.30) and hyperoxic (2.70 ± 0.08) groups, these values were significantly higher (Kruskal-Wallis, P = 0.0003) in comparison to the normoxic group value (1.82 ± 0.20). In addition, multiple range test detected significant differences between hypoxic and the hyperoxic treatment groups. No differences among the groups were found for total leukocyte counts.

Table 4.1 Haematological parameters in *Cyprinus carpio* exposed to normoxic, hypoxic and hyperoxic conditions for 30 days.

parameters	Normoxia	Hypoxia	Hyperoxia
Hct (%)	31.16 ± 1.24^{a}	38.00 ± 0.80^{b}	30.83 ± 1.44^{a}
Hb (g/dl)	06.76 ± 0.60^{a}	09.57 ± 0.66^{b}	07.41 ± 0.48^{ab}
RBC (cells x 10^6µl)	01.82 ± 0.20^{a}	03.42 ± 0.30^{b}	02.70 ± 0.08^{c}
WBC (cells x 10^3µl)	14.70 ± 0.43^{a}	14.32 ± 0.51^{a}	15.11 ± 0.93^{a}

Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significant difference at $P < 0.05$; (n=6).

4.3.4 Histopathological Studies

The liver sections of control (normoxic) group exhibited normal morphological structures with no abnormalities in the hepatocytes. It showed a homogenous cytoplasm around a centrally located spherical nucleus. Microscopic examination of hepatocytes and their nuclei from hypoxic group areas showed histopathological changes after 30 days of exposure compared to the normoxic group. Hepatocytes in 4 fish (out of 6) lost their normal boundaries. There were cellular and nuclear degeneration, cytoplasmic vacuolation in most regions of the liver sections. Livers on the hyperoxic treatment didnt show any histopathological changes under the light microscopy (Fig. 4.3 A-D).

Gill morphology in the control (normoxic) group had normal morphological structures in which lamellae were lined by squamous epithelium composed of non-differentiated cells. Gills from hypoxic condition showed several histological alterations including lifting of lamellar epithelium and enhanced presence of goblet cells on the lamellae compared to normoxic group. In addition, in some cases aneurysm resulted in the fusion of some secondary lamellae. These changes were significantly different compared to normoxic and hyperoxic group

(Table 4.2; ANOVA, $P < 0.05$). Gills from hyperoxic group showed lifting of the lamellar epithelium and curling of secondary lamellae. The extent of the damage was however not severe compared with hypoxic condition. Most of these changes were significantly different from the normoxic group (Fig. 4.4 A-D).

Table 4.2 Histopathological changes presented as a percentage in the gills of *C. carpio* L. exposed to normoxic, hypoxic and hyperoxic conditions for 30 days.

Lesion (%)	**Normoxia**	**Hypoxia**	**Hyperoxia**
Lifting epithelium	1.50 ± 0.71^{a}	36.66 ± 3.64^{b}	20.50 ± 3.43^{c}
Hyperplasia	00.00 ± 0.00^{a}	16.66 ± 3.60^{b}	1.83 ± 0.48^{a}
Necrosis	0.00 ± 0.00^{a}	2.16 ± 0.60^{b}	0.54 ± 0.22^{a}
Fusion	0.83 ± 0.40^{a}	13.33 ± 1.54^{b}	15.50 ± 2.96^{b}

Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significant difference at $P < 0.05$; (n=6).

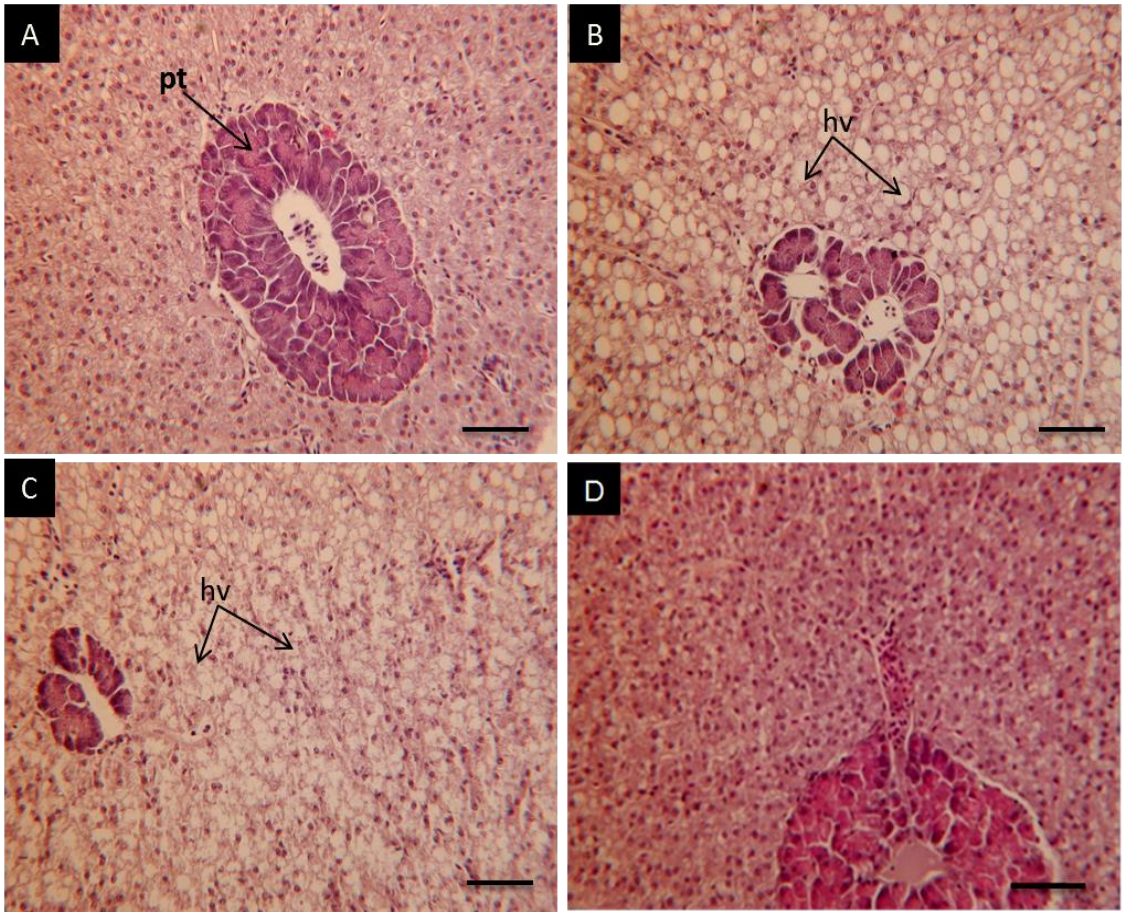


Fig 4.3 Light micrographs of sections through liver of *C. carpio* showing histological structures of normoxic, hypoxic and hyperoxic treatments stained with H&E at 5 µm thickness. A: normoxic liver; B&C: hypoxic liver; D: hyperoxic liver. (pt) pancreatic tissue, (hv) hydrobic vacuolation. Scale bars: 50 µm.

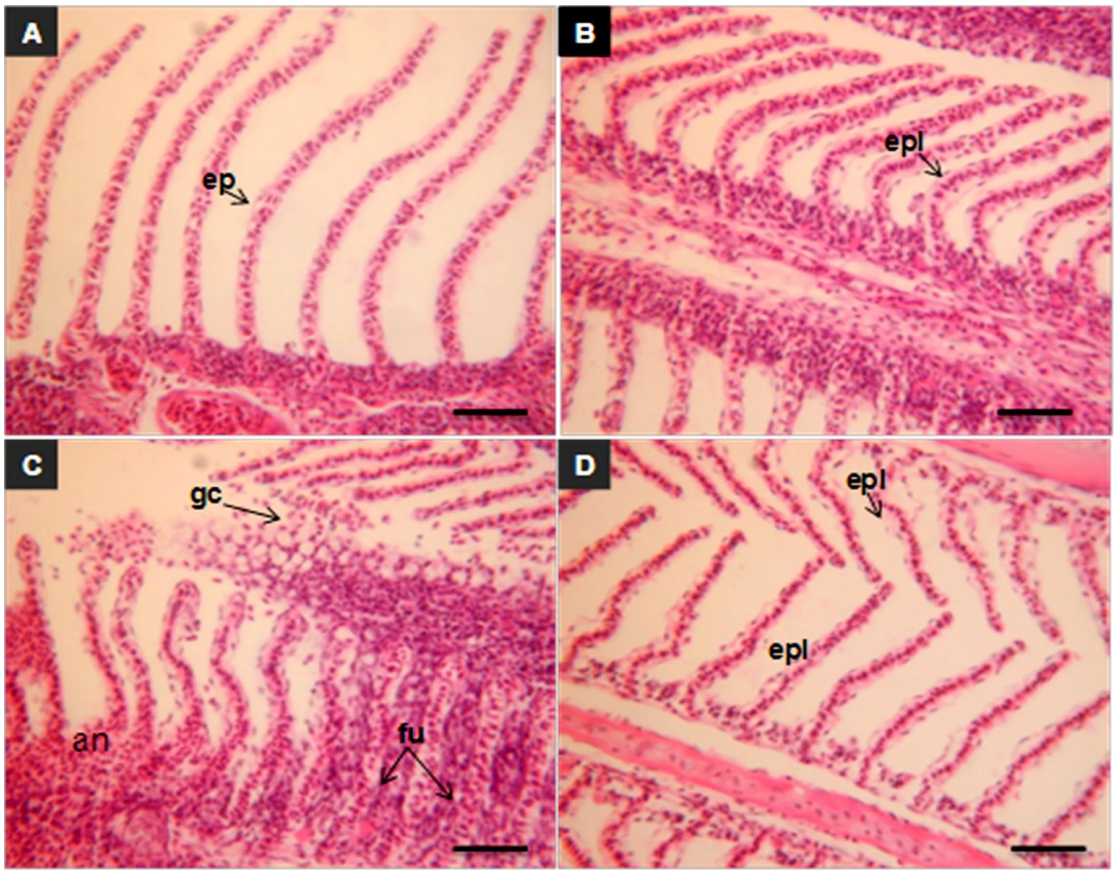


Fig. 4.4 Light micrographs of secondary lamellae from *C. carpio* showing histological structures of normoxic, hypoxic and hyperoxic treatments stained with H&E at 5 µm thickness. A: normoxic gill; B&C: hypoxic gill and D: hyperoxic gill. (ep) epithelial cell; (epl) epithelial cell lifting; goblet cell; (fu) fusion; (an) aneurysm. Scale bars: 50 µm.

4.3.5 Transmission electron microscopic (TEM) studies

For TEM studies, liver in normoxic condition showed normal organelles (e.g. cell membranes, nuclei, mitochondria, rough endoplasmic reticulum; Fig. 4.5 A&B), hypoxic specimens revealed significantly increased number of lipid droplets in the cells (homogeneous semi-electron dense vacuole) of varying sizes (17.3 µm ± 0.4) compared with normoxic and hyperoxic specimens (2.50 ± 0.61 and 6.20 ± 0.90) respectively (ANOVA, P = 0.03). In hypoxic condition, nucleus showed areas which were relatively clear and occupied by dense heterochromatin within nuclear envelope (Fig. 4.5 C&D). On the other hand, liver from hyperoxic treatment showed increased number of the mitochondria (23.40 ± 0.44) which was significantly different (ANOVA, P = 0.03) from normoxic and (8 ± 0.02) hypoxic conditions (11.30 ± 0.51) (Fig. 4.5 E&F). The nucleus of the hepatocytes also showed irregular shape occupied by dense heterochromatin in hyperoxic condition, which was not apparent while conducting light microscopic studies.

TEM images in secondary lamellae of normoxic gill exhibited normal organelles with epithelial and pillar cells (Fig. 4.6 A). Hypoxic specimens showed lifting of the epithelial cells. There was also a breakdown of pillar cells and disorganisation of blood spaces (Fig. 4.6 B&C). The secondary lamellae of hyperoxic gill also revealed separation of epithelial cells and intracellular oedema (Fig. 4.6 D).

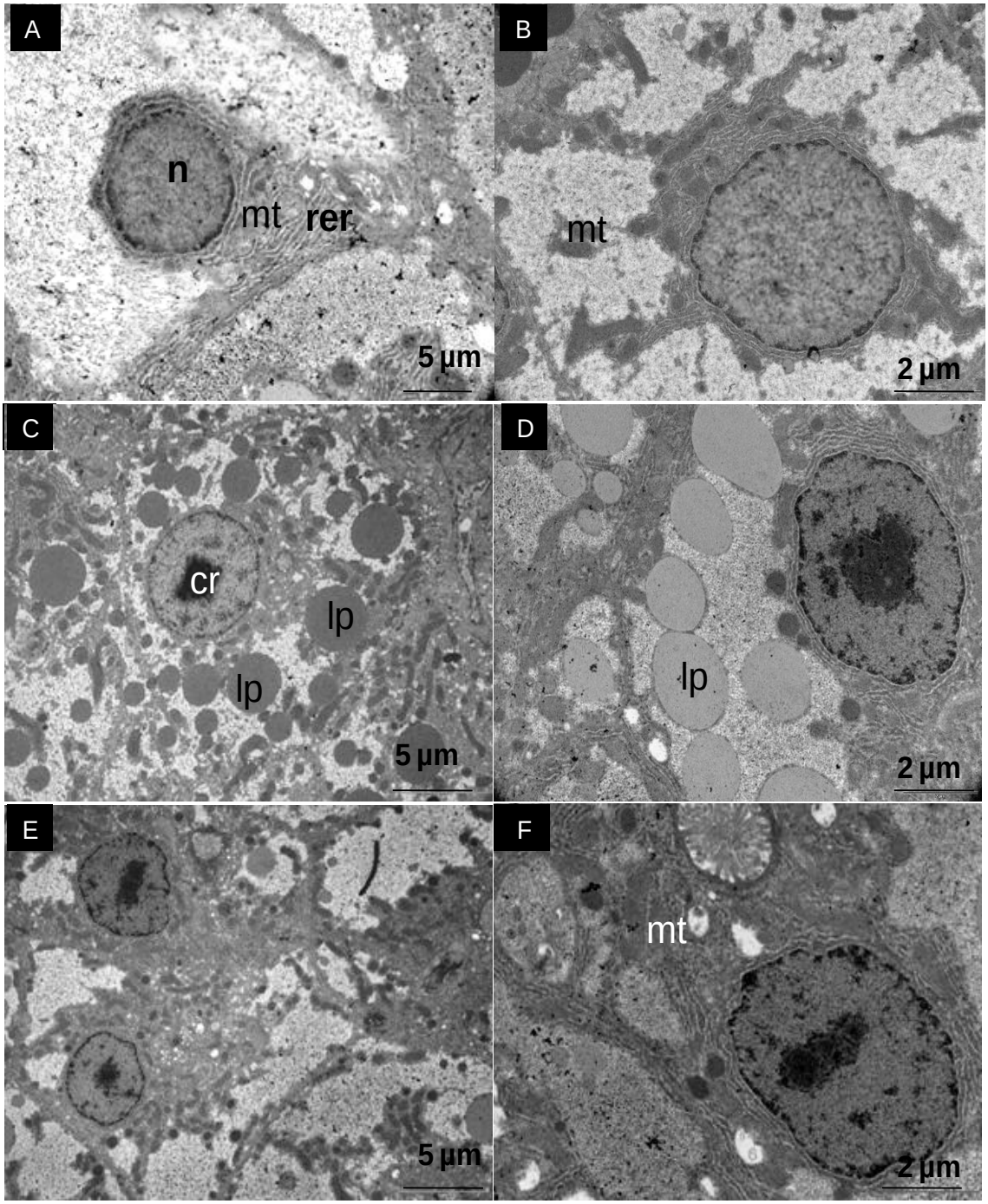


Fig. 4.5 Transmission electron microscopy images: A&B: normoxic liver; (n) nucleus, (nu) nucleolus, (mt) mitochondria, (rer) rough endoplasmic reticulum C&D: hypoxic liver; showing (lp) lipid droplets E&F: hyperoxic liver; the mitochondria are large and numerous.

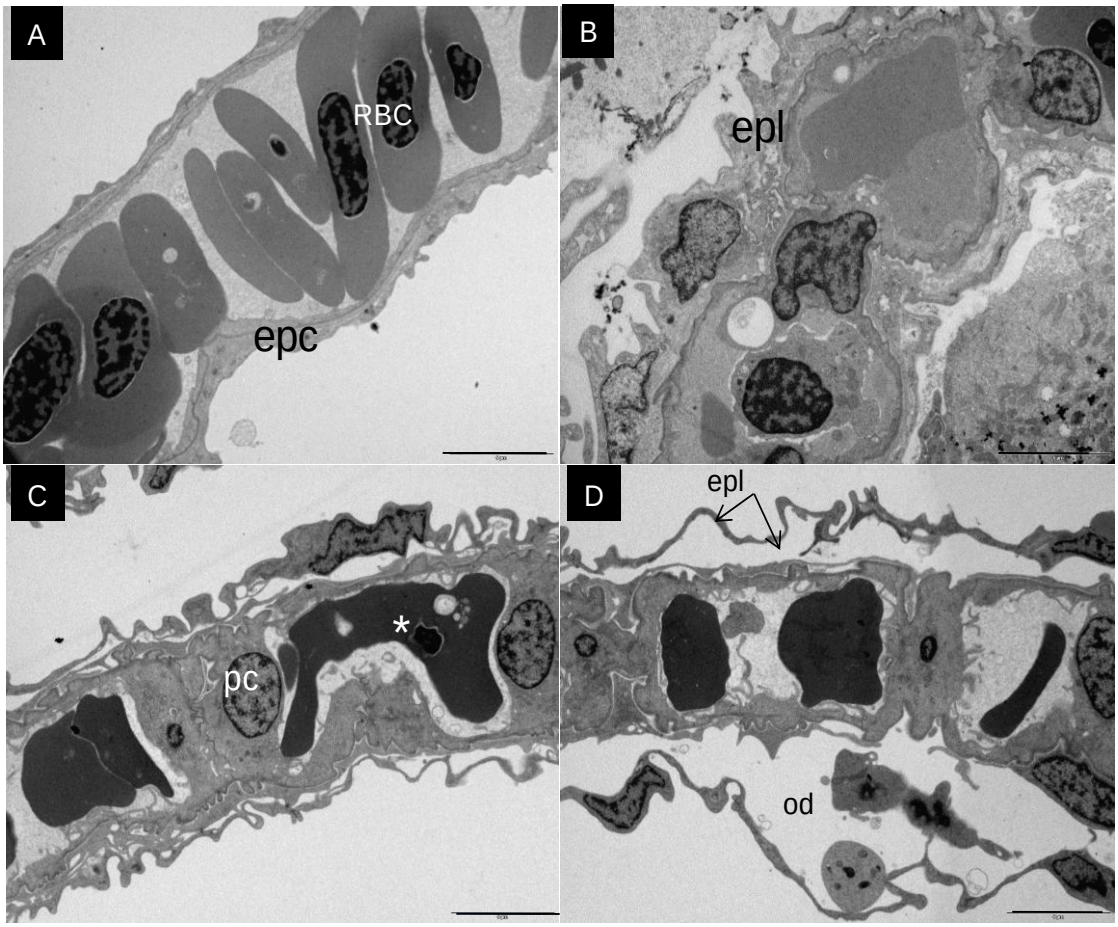


Fig. 4.6 Transmission electron microscopy images of the secondary lamellae from A: normoxic condition; B&C: hypoxic secondary lamellae and D: hyperoxic secondary lamellae.(IPCS) pillar cell; (RBC) red blood cell; (ep) epithelial cell; (epl) epithelial cell lifting; (od) oedema; white star disorganisation of the pillar cells. Scale bars: 5 µm.

4.3.6 Determination of growth performances

No mortalities occurred during the experimental period. Specific growth rate after 30 day of exposure (hypoxic and hyperoxic conditions) was significantly lower in the hypoxic group by approximately 30% compared to fish exposed to normoxic condition (ANOVA, $P = 0.03$). There were no significant differences for SGR between hypoxic and hyperoxic conditions (Table 4.3). This result suggests that the SGR was significantly affected by level of dissolved oxygen as evident by significant growth depression in the hypoxic group.

Table 4.3 Growth performance of carp exposed normoxic, hypoxic and hyperoxic conditions for 30 days.

Variable	**Normoxia**	**Hypoxia**	**Hyperoxia**
Initial weight (g)	98 .3 ± 1.73^{a}	97.3 ± 2.41^{a}	98 .6 ± 2.09^{a}
Final weight (g)	165.2 ± 4.61^{a}	139.8 ± 7.08^{b}	146.3 ± 4.24^{ab}
Weight gain (g)	66.7 ± 3.52^{a}	42.2 ± 6.43^{b}	47.7 ± 3.75^{ab}
SGR (% g^{-1})	1.73 ± 0.01^{a}	1.23 ± 0.10^{b}	1.30 ± 0.09^{ab}
FCR	1.01 ± 0.21^{a}	1.07 ± 0.11^{b}	1.02 ± 0.08^{ab}
Survival rate (%)	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00

Data are mean ± S.E. Groups with different alphabetic superscripts indicate significant difference at $P < 0.05$; (n=6).

4.4 Discussion

Several carp species (e.g. Crucian carp: *Carassius carassius;* gold fish: *Carassius auratus;* the mirror or common carp: *C. carpio)* are highly tolerant to hypoxic and even to anoxic conditions. This adaptive capability is critically important to allow them to occupy specific environmental niches where oxygen level can drop down to low concentrations (van den Thillart and van Waarde, 1985). Therefore, species that routinely tolerate hypoxic status need a defensive strategy to cope with oxygen limiting conditions. This strategy could include changes in tissue-specific activities of antioxidant enzymes (Lushchak and Bagnyukova, 2006; Lushchak et al., 2005; Lushchak et al., 2001). In this context, GPx activity has been used as indices of oxidative stress, since this is considered to be the most important enzyme providing protection against this stress (Livingstone, 2001; Valavanidis et al., 2006).

The results suggest that in the liver, hyperoxia induced a marked decrease in GPx activity compared to normoxic condition. Decreased activity of liver GPx in hyperoxic condition is possibly related with inactivation under enhanced ROS levels (Halliwell and Gutteridge, 1999). This decrease was not significantly different in hypoxic compared with normoxic group. Although not significant at the end of exposure period, decreased activity of this enzyme under hypoxic condition may reflect a general depression of metabolic activities that may affect both xenobiotic processing and protein synthesis (Lushchak et al., 2005). In contrast, significant increased GPx activity in blood plasma in the hyperoxic compared with normoxic and hypoxic groups indicates increased oxidative stress in this treatment group. It is possible that exposure to hyperoxia can stimulate ROS production which acts as upstream signalling molecules to

enhance oxidative stress. Such a response has previously been reported in rat exposed to hyperoxia (Buccellato et al., 2004). In fish, a previous study with rainbow trout has also demonstrated an elevation of GPx activity following exposure to ozonized water. This also increased the level of lipid peroxidation indicating that these fish were subjected to oxidative stress (Ritola et al., 2002). The decreased GPx activity in blood plasma in hypoxic compared with normoxic and hyperoxic groups probably indicates that the activity of this enzyme was not sufficient to deal with oxidative stress arising under hypoxic condition.

For the Comet assay, significant increase for DNA strand breaks observed under hyperoxic condition is not surprising. Hyperoxic condition is known to elevate the production of ROS leading to oxidative stress (Lushchak and Bagnyukova, 2006). In contrast, the observed increase for DNA damage under hypoxic condition compared to normoxic condition is more intricate to explain. Liepelt et al. (1995) reported that highest rate of DNA strand breaks in the gills occur when the rainbow trout were kept under hypoxic condition (3.3 mg O_2 l^{-1}; 5 h exposure) followed by a rapid increase of the oxygen concentration. It is however not clear why hypoxic condition could lead to oxidative damage. Given that under hypoxic, opposed to anoxic condition, some degree of molecular oxygen is still available, ROS level could increase due to reduction of mitochondrial electron transport chain and their leakage to residual oxygen molecules leading to oxidative stress (Dirmeier et al., 2002). It has also been speculated that under hypoxic condition, xanthine dehydrogenase can be converted into xanthine oxidase which produces ROS as products (Lushchak, 2011; Lushchak and Bagnyukova, 2007). Under hypoxic condition, limited proteolysis or oxidation could lead to production of efficient ROS producer (Lushchak, 2011). In addition, hypoxia may result in inactivation of carriers of

the electron transport chain and this process could increase the chance of electrons to 'escape' and reduce oxygen via one-electron mechanisms (Lushchak and Bagnyukova, 2007). Furthermore, it has been suggested that repair of DNA strand breaks under hypoxic condition is less effective compared to normoxic condition (Modig et al., 1974). It could therefore be assumed that spontaneous DNA strand breaks could accumulate under chronic hypoxic condition as a result of inefficient DNA repair capability. Our results show that both hypoxic and hyperoxic conditions have considerable impact on the level of DNA strand breaks in fish erythrocytes. These results are consistent with previous studies where fish erythrocytes have been shown to be sensitive for measuring the genotoxic effects either in laboratory or in field studies (Belpaeme et al., 1998; Buschini et al., 2004).

A significant increase in haemoglobin concentration in response to hypoxia compared with normoxic condition (approximately 40%) is consistent with a number of studies in fish chronically exposed to hypoxic condition (Greaney and Powers, 1978). However, carp have not been shown to demonstrate significant increases when exposed in a similar way (Jensen and Weber, 1985; Lykkeboe and Weber, 1978). This suggests that increasing haemoglobin concentration possibly improves preserving oxygen delivery during hypoxic challenge by protecting oxygen diffusion gradient from blood to tissues. Besides, there was a steep increase for the haematocrit value which was significantly different (approx. 25%) relative to normoxic and hyperoxic conditions, resulting possibly from a significant increase in number of red blood cells. This response has been observed in many marine and freshwater fish species (Muusze et al., 1998; Smit and Hattingh, 1978; Soldatov, 1996). The processes responsible for the increased numbers of RBCs in hypoxic condition however are not clear.

Perhaps, this increase is associated with both their release from blood storing organ (e.g. spleen) and the activation of the erythropoiesis in blood forming tissues concentrated in head kidney (Estebani et al., 1989). Both mammalian and fish studies have suggested that hypoxia inducible factor 1 (HIF-1α) is also responsible for activation of erythropoietin (EPO) gene transcription for enhancement of red blood cell production (Guillemin and Krasnow, 1997; Okino et al., 1998; Soitamo et al., 2001). The overall effect of differential gene expression have been suggested to lead to a series of biochemical and physiological responses, allowing the organisms to survive under hypoxic conditions with net decrease in metabolic rate and protein synthesis (Wu, 2002).

For histopathological and ultrastructural studies, the high accumulation of lipid droplets observed in hypoxic liver cells has been suggested to be associated with increased elongation of fatty acid and inhibition of lipolysis (van den Thillart et al., 2002). Increased synthesis or decreased catabolism and mobilization of lipid could account for lipid accumulation during hypoxia. Reduction in molecular available oxygen is known to decrease the intensity of oxidative phosphorylation occurring in mitochondria. This could lead to reduction in the formation of adenosine triphosphate (ATP) which is needed for lipolysis (van Raaij et al., 1994). Our results are in line with previous study by Poon et al., (2007) who reported increased lipid content in the liver after 42 days exposure of common carp to hypoxia. Furthermore, ultrastructural study of flat fish (*Plathichthys flesus* L.) collected from highly contaminated sites with organochlorines and heavy metals have been shown to contain increased lipid droplet (Kohler, 1990). For hyperoxic condition, liver didn't show any significant histopathological

changes, however, under the TEM; mitochondria were relatively larger in size and higher in number. This finding is consistent with observed increase in mitochondrial number per alveolar epithelial cells in hyperoxically exposed rat (Khazanov and Poborskii, 1991). This confirms that increased number of mitochondria is a generalised response to oxidative stress in both fish and mammals.

The gills showed alterations in hypoxic conditions such as epithelial lifting, aneurysms, increased numbers of goblet cells, besides fusion of some secondary lamellae. These could possibly be examples of defense mechanisms. Similar histological changes in gills were common in a channel catfish, *Ictalurus punctatus,* exposed to sublethal hypoxic condition (Camargo and Martinez, 2007). The epithelial lifting have also been reported in gills of sea bass *Dicentrarchus labrax* exposed for three months to hypoxic and hyperoxic conditions (Coutinho and Gokhale, 2000). These histological alterations are non-specific and common to different environmental stresses and a range of contaminants (Mallatt, 1985).

Our study confirms that mirror carp demonstrate high tolerance to long term hypoxia as no mortalities occurred over the exposure period. There was however significantly decreased SGR in fish exposed to hypoxic compared to normoxic condition. The lower SGR and growth reduction are in line with reported studies in other studies showing decline in fish growth under reduced or hypoxic environments (Chabot and Dutil, 1999; Dabrowski et al., 2004; Thetmeyer et al., 1999). As mentioned earlier, this probably results as a consequence of down regulation of maintenance energy required for optimal growth (Miller, 2005), which could also affect food intake (Glencross, 2009). In

this context, correlation between oxygen consumption and protein synthesis in fish cell lines has been reported (Smith and Houlihan, 1995). It is interesting to note that induction of oxidative DNA damage showed no correlation with GPx activities in liver under hyperoxic and hypoxic conditions (Fig. 4.7 A-C). Furthermore, SGR showed a significant negative correlation with oxidative DNA damage under hypoxic compared to hyperoxic and normoxic conditions (Fig. 4.7 D-F). Such chronic conditions in the natural environment or in aquaculture would have profound knock-on effects on the Darwinian fitness of the organisms.

In conclusion, our study suggests that compared to normoxia, both chronic hypoxia and hyperoxia induce oxidative DNA damage, oxidised purines showing higher levels of damage compared to pyrimidines in a carp species. Hypoxia also induced a significant increase in most haematological parameters along with ultrastructural changes in both liver and gills. Different exposure conditions also affected the specific growth rates of the fish, which was observed to correlate with oxidative DNA damage. Given the increasing number of ‘dead zones’ in different parts of the world, we need to better comprehend the hypoxic conditions on the biota either alone or in combination with other contaminants and environmental stressors. Additionally, the intensive aquaculture or experimental rearing of new fish within recirculation systems with high aeration and varying sensitivity to ROS could influence production efficiency and biological responses which will also warrant further investigations for this globally expanding industry.

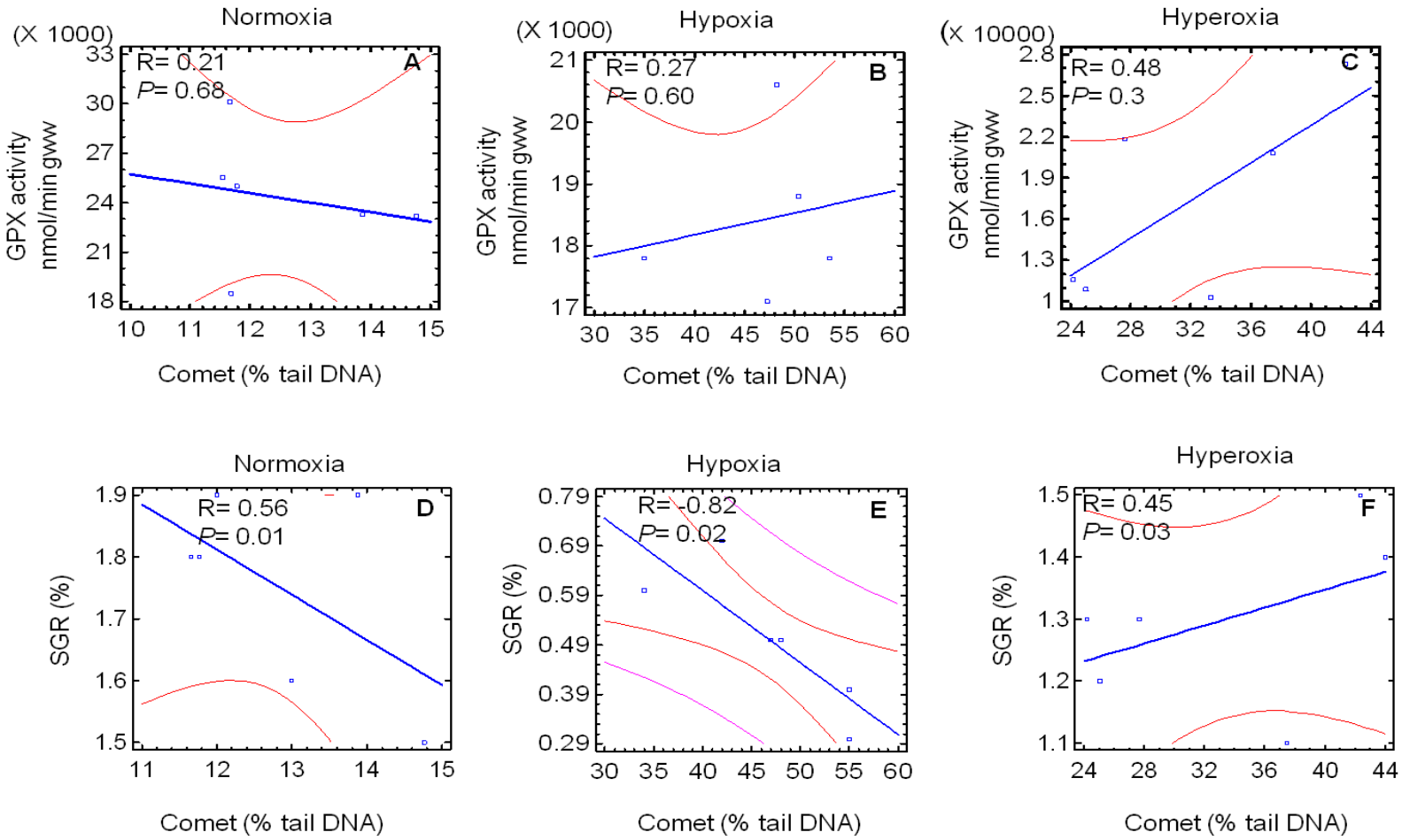


Fig. 4.7 Linear regression analysis illustrating correlations between the comet assay and the GPx activity, correlations between the comet assay and the SGR (D-F) in *C. carpio* following 30 days exposure to normoxia, hypoxia and hyperoxia. The solid line is a linear regression and the dashed lines represent 95% confidence limits.

CHAPTER 5

HYPOXIA AND DIETARY COPPER INTERACTS DIFFERENTIALLY TO INDUCE SUB LETHAL TOXICITY IN CARP, *CYPRINUS CARPIO* L. AT DIFFERENT LEVELS OF BIOLOGICAL ORGANISATION

Results from this Chapter have been published in Chemosphere, 87, 413-422 (Mustafa et al., 2012).

Hypotheses: An elevated dietary Cu level impairs or modifies the biological functions in a representative carp species exposed to chronic hypoxic condition.

Abstract

Hypoxic event, (i.e., temporal or chronic depletion of oxygen) frequently occurs in the natural environment. It has been suggested that accumulation and toxicity of micro pollutants during such occasions increases. However, few experimental studies are available on the toxicity of heavy metal (which also serve as micronutrient) under hypoxic condition. To elucidate this phenomenon, mirror carp *Cyprinus carpio* L. weighing 16.13-16.23 g were exposed chronically to dietary copper at concentration 250 and 500 mg kg^{-1} d.w. for 30 days under normoxic (8.25 mg O_2 l^{-1}) and hypoxic (3 mg O_2 l^{-1}) conditions. We studied the potential modifying effects at different levels of biological organisation, including specific growth rate (SGR), feed conversion ratio (FCR) and oxidative DNA damage (using comet assay in combination with Fpg and Endo-III enzymes), haematological and histopathological parameters (including ultrastructural changes) in selected organs. Cu accumulation in different organs was also determined at the end of this period.

This study showed that the combined action of dietary copper and hypoxia leads to increased DNA damage formation compared to the effects of the individual stressor. These results are consistent with a hypothesis that Cu in presence of hypoxia affects DNA integrity, causing increased oxidative DNA damage. Haematological parameters showed that the combined action of dietary Cu plus hypoxia result in increase red and white blood cells, haematocrit value, and increase in haemoglobin concentration. These changes suggest a compensatory response to respiratory surface reduction of gills (tissue damage and cell proliferation) in order to maintain oxygen demands from water to the tissues. The order of Cu accumulation in tissues was liver > intestine > kidney >

gill. Quantitative histology showed changes in gills in hypoxic group and in all dietary Cu groups under normoxic and hypoxic conditions. This included lifting and hyperplasia of the lamellar epithelium. Coagulative necrosis with multifocal areas lipid vacuolation of hepatocytes. Interestingly, SGR of fish fed with dietary Cu under normoxic hypoxic conditions reduced with elevating supplemental copper levels in diets. Overall, the results provide evidence for enhanced toxicological responses in fish following exposure to Cu either alone or in combination with hypoxic condition and lends support to the evolving viewpoint that many water quality guidelines should be revisited in terms of new ecotoxicological criteria.

5.1 Introduction

Mostly linked with anthropogenic activities, hypoxia or temporal depletion of oxygen is now considered to be amongst the most pressing and critical problems for the hydrosphere in the world. Although common in both freshwater and marine environments, its impact on densely populated coastal regions with intense aquaculture activities, creating so called 'dead zones' is of particular concern (Diaz and Rosenberg, 2008). Needless to mention, decreases in oxygen concentration has profound detrimental impact on the biological functions of the organism, oxidative stress being one of the mechanisms of production of these responses (Lushchak, 2011; Mustafa et al., 2011). It is also being realised that hypoxia could enhance the vulnerability to environmental chemicals in aquatic organisms by impairing the physiology and food/ contaminant uptake rate (Hattink et al., 2005). A strong relationship between ventilation rate and uptake of micropollutants has been suggested in aquatic ecosystems resulting in toxic impact when correlated with hypoxic conditions (Diaz and Rosenberg, 2008; Schiedek et al., 2007). This higher toxicity is explained by increased ventilation rate, causing a higher water flow over the gill epithelium, leading to severe physiological and behaviour responses in aquatic organisms (Sijm et al., 1994). In this context, aquatic ecosystems that undergo seasonal hypoxia can also be concurrently tainted with contaminants such as heavy metals (Diaz and Rosenberg, 2008). With rapid industrialisation and population growth, industrial effluents and domestic sewage containing diverse range and large quantities of potentially toxic metals are being discharged in the aquatic environment with long term consequences for the sustainability (Mohsen and Jaber, 2003; Rai, 2008). In addition, the presence of some metals has also been suggested to mimic the hypoxic action in the aquatic environment

(Kubrak et al., 2011). In common with other contaminants, the presence of heavy metals and variable oxygen availability may interact differentially to exert detrimental effects on aquatic ecosystems which need further elucidation to disentangle cause-effect coupling at different levels of biological organisation for hazarded risk assessment.

Whilst copper (Cu) is essential for normal physiological functioning, it is toxic at elevated concentrations for aquatic life (Carvalho and Fernandes, 2006). World production of Cu has increased in the last few decades and contamination by Cu has become increasingly prevalent in the aquatic environment (IPCS, 1993) which is likely to increase bearing in mind manufacture and disposal of wide varieties of Cu-based products including agrochemicals. In relatively unpolluted marine waters Cu concentrations are less than 5ppb, but may reach 3 ppm in heavily polluted areas (Parry and Pipe, 2004; Soegianto et al., 1999). Furthermore, the transfer of metals through food chains can be substantial to reach high concentrations in fish tissues and the dietary accumulation of Cu dominates the aqueous route (Dallinger et al., 1987). In the environment common carp typically browse on the bottom (i.e., sediment) could accumulate more contaminants. It has also been suggested that concentration of the Cu in natural food (invertebrates) could reach up to 3750 µg g^{-1} in contaminated areas (Rainbow, 2007). Furthermore, hypoxia under chronic condition on its own right is likely to target the gills inducing adverse physiological and morphological (i.e. histopathological, ultrastructural) effects in addition to imparting indirect effects through assimilated Cu via dietary intake. The effect of Cu on aquatic ecosystems is complex and depends not only on its concentration but also on physicochemical characteristics of the water (e.g. alkalinity, hardness, pH etc.) which affect its speciation and subsequently bioavailability (Rathore and

Khangarot, 2003; Tao et al., 2001; Yim et al., 2006). Other environmental parameters such as oxygen level, temperature, salinity and presence of other metals may also affect metal toxicity to aquatic life. The standard laboratory based toxicological studies for risk assessments of chemicals carried out under well-defined conditions fail to consider these potential interactive effects.

For metallic contaminants, the accumulation of Cu in the mud shrimp, *Corophium volutator* increased but was not significant under hypoxic condition (19% air saturation, Eriksson and Weeks, 1994). Pilgaard et al., (1994) showed that the uptake of Cu and zinc (Zn) was not enhanced under hypoxic events in rainbow trout, *Oncorhynchus mykiss*. Hattink et al., (2005) also showed that Zn uptake was not altered in carp, *Cyprinus carpio* L. under hypoxic conditions, despite an hyperventilation rate and a three-times enhanced toxicity under hypoxic compared to normoxic condition. Furthermore, using a toxicokinetic study, higher sensitivity of carp to cadmium (Cd) under hypoxic condition was observed, although hypoxic condition did not influence the uptake rate or the accumulation dynamics (Hattink et al., 2005).

Given that (a) only limited information exists with respect to potential interactive effects of hypoxia with other environmental factors and (b) most of the studies to date have only been carried out following short-term aqueous exposures to heavy metals or hypoxia, taking into account only a limited number of biological responses, we aimed to probe the hypothesis that elevated dietary Cu concentrations impairs or modifies the biological functions in a representative carp species exposed to chronic hypoxic stress. We also emphasised the importance of the food chain as a conduit for metal toxicity in association with environmental stressors. These effects are examined on specific biochemical

and histological parameters thereby, adapting an integrated approach to the whole organism.

5.2 Experimental design

Cyprinus carpio L. weighing 3.5-4 g, length 6-7 cm (n = 250) were obtained from the Hampshire carp hatcheries (Bowlake Fish Farm. UK). Fish were acclimated for 3-4 weeks to the experimental (~2250-L) re-circulating aquarium system, with a pump filtration system (filtered Plymouth city mains water, pH 7.2, dissolved oxygen 7.5-8.2 mg l^{-1}, temperature 22-23 °C). During acclimation fish were fed standard commercial diet of 2% body weight (Ewos, Micro 20 p, Ewos Ltd., Westfield, Bathgate, West Lothian. UK).

Following acclimation, 150 fish weighing 16.13-16.23 g were randomly distributed into 10 x 80-l fibreglass tanks, with 15 fish per tank. Each treatment was conducted in duplicate (two tanks per treatment). The treatments were:

Normoxia (control): two fish groups (15 fish per tank) were fed control diet (no added Cu) and kept under normoxic condition (8.25 mg O_2 l^{-1}); the oxygen level was maintain to air saturation (equivalent to 95%).

Normoxia plus copper: two groups were fed a high-Cu diet containing 500 mg kg^{-1} dry weight (subsequently termed as mg Cu kg^{-1} in the text) added as $CuSO_4$. $5H_2O$ (diets preparations as described in Chapter 3 section 3.2) in normoxic water.

Hypoxia: two groups were exposed to hypoxic condition ~3 mg O_2 l^{-1} (air saturation equivalent 35 %); oxygen levels were maintained by bubbling the tanks with nitrogen gas [N_2: purity 99.99%] and maintaining steady water flow through the system ~1 l min^{-1}).

Hypoxia plus copper: two groups were fed 250 mg Cu kg^{-1} and maintained in hypoxic conditions (~3 mg O_2 l^{-1}).

***Hypoxia plus copper*:** another two groups were fed 500 mg Cu kg^{-1} and maintained in hypoxic conditions (~3 mg O_2 l^{-1}).

The exposure time began when the desired dissolved oxygen level (3 ±0.41mg L^{-1}) was achieved (i.e. within 2 d after the initiation of nitrogen pumping), in line with our previous study (Mustafa et al., 2011). Oxygen concentration was monitored (three times day^{-1}) using a hand-held dissolved oxygen meter (Oxy Guard, Handy Polaris, DK), which did not vary significantly after the exposure period started. As the level of oxygen declined gradually, there were no mortalities either during the first two days or over the exposure period. The physico-chemical parameters of the water were presented in Table 5.1. A 12-h light/12-h dark photoperiod was maintained throughout the duration of the experiment was maintained throughout the duration of the experiment. Water was renewed at least weekly and the self-cleaning design of the system ensured that food and faecal material were cleared from each tank. Fish were fed 3 % biomass per day (equal rations at 09.00, 13.00 and 17.00 hours) for 30 days. Daily feed was corrected on a weekly basis following batch weighing after a 24 h starvation period (see 2.12). SGR and FCR were determined (see section 2.13).

5.3 Biological sampling and analysis

At the end of the trial (i.e. 30 days), fish were not fed day before the sampling times in order to empty the gut before dissection. Three fish per tank (n = 6) were netted and quickly anaesthetized in a buffered solution of methane sulfonate (MS-222; 100 mg l^{-1} water for 10 min). Fresh blood samples were

immediately collected from the caudal vessel using a 25 gauge needle and 1 ml heparinized syringe into eppendrof tubes for analysis by single cell electrophoresis 'modified Comet assay' (see 2.7.5) and for analysis of percentage haematocrit (2.8.3), measurement of haemoglobin concentration (see 2.8.1) and total leucocyte and red blood cell counts (2.8.2) and also blood smears were made for determination of differential leucocytes cell counts (see 2.8.4).

Samples of gill and liver tissue were immediately dissected out and fixed in 10% formaldehyde solution for histopathological studies (see 2.10) another portions of gill tissue were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for ultrastructural studies (see 2.11). A further three fish per tank (6 per treatment) were dissected out for Cu analysis, and the following tissue were sampled: liver, intestine, gill and kidney (see 2.9). For analytical procedures (lipid, protein and ash) three fish per tank (6 per treatment) were dried, and the percentage of the moisture also determined from initial and final weight (see 2.14 and subsections).

5.4 Statistical analysis

Statistical analysis was performed using Statgraphics v5.1 software (StatSoft, USA). All data were presented as mean ± standard error (S.E.) and analysed using one way analysis of variance (ANOVA) or Kruskal Wallis test, followed by multiple range tests. For the modified Comet assay (with the use of enzymes), data presented as median ± S.E. were analysed using multifactor analysis of variance (ANOVA) followed by a multiple comparison test. P values < 0.05 were considered significant.

Table 5.1 Average data of Physical-chemical parameters of water used in dietary Cu exposure plus normoxia and hypoxia experiment.

Water quality parameters	Normoxia	Hypoxia	500 mg Cu kg^{-1}/normoxia	250 mg Cu kg^{-1} /hypoxia	500 mg Cu kg^{-1}/hypoxia
Temperature °C	23.38 ± 0.18	22.40 ± 0.10	23.38 ± 0.18	22.54 ± 0.07	22.61 ± 0.07
O_2 mgl^{-1}	08.25 ± 0.02	03.05 ± 0.10	08.25 ± 0.02	03.13 ± 0.06	2.55 ± 0.08
O_2%	95.21 ± 0.81	40.15 ± 0.09	95.21 ± 0.81	36.42 ± 0.83	34.36 ± 1.03
pH	7.66 ± 0.07	7.66 ± 0.07	7.66 ± 0.07	07.66 ± 0.07	7.66 ± 0.07
Nitrogenous compound (mgl^{-1})					
Ammonia mgl^{-1}	0.01 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.07 ± 0.01	0.09 ± 0.02
Nitrite mgl^{-1}	0.01 ± 0.08	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.05 ± 0.02
Nitrate mgl^{-1}	25.43 ± 0.77	27 ± 0.58	25.43 ± 0.77	25.20 ± 1.22	26.73 ± 0.65
Copper mgl^{-1}	2.07 ± 0.06	2.80 ± 0.01	3.01 ± 0.01	2.86 ± 0.07	3.04 ± 0.07

Values are means ± SE;

5.5 Results

5.5.1 Determination of oxidative DNA damage

After the exposure period, no loss of cell viability (determined by trypan blue exclusion dye) was observed in any of the treatments (cell viability > 90% in all cases). Oxidative DNA damage was relatively low in normoxic compared to all treatment groups. Compared to normoxic condition, the level of oxidative DNA damage showed strong significant difference following exposure to dietary Cu level under normoxic condition (1.6-fold) as well as under hypoxic condition at both Cu levels (2.1 and 2.5-fold respectively; Fig. 5.1). In addition, there was a significant difference found between the dietary Cu groups at both levels plus hypoxia and the 500 mg Cu kg^{-1} for normoxic and also compared to hypoxic group (ANOVA, P = 0.0001). However, there was no statistically significant interaction (at 95% confidence level) between the two enzymes (i.e. Fpg and Endo-III) used and the Cu concentrations. These results suggest that oxidative DNA damage depended upon the dietary Cu concentration and oxygen levels.

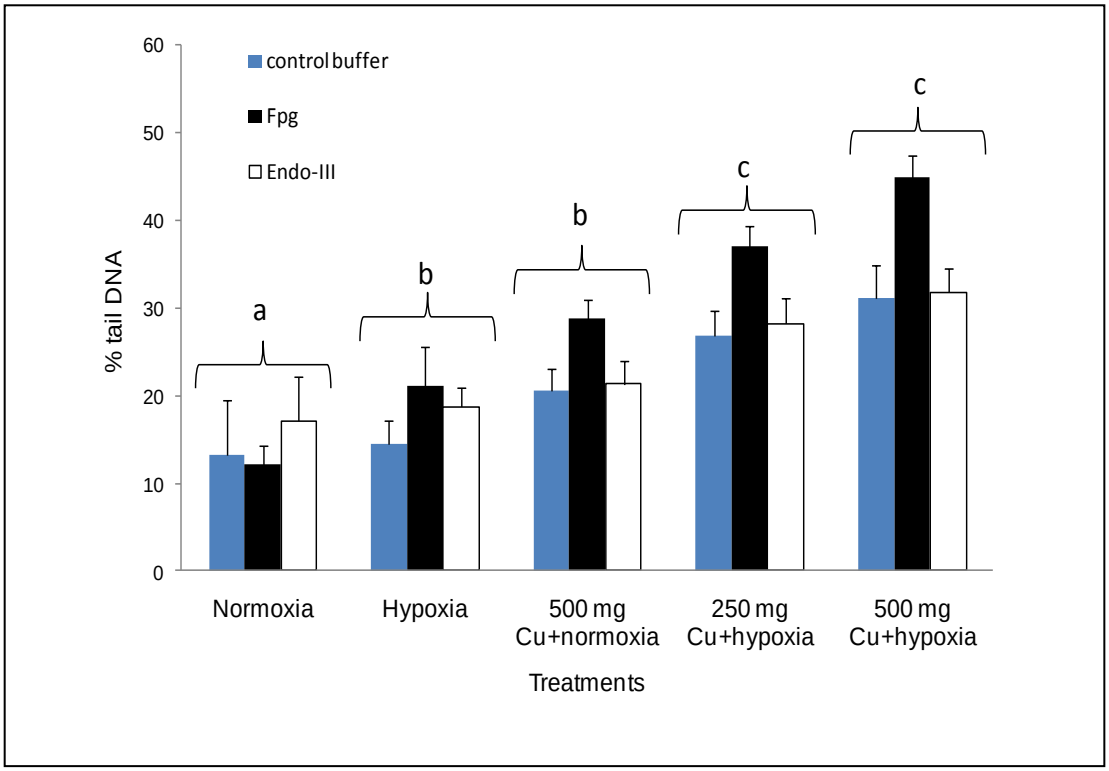


Fig 5.1 Induction of DNA single strands breaks (represented as percentage tail DNA) in *C. carpio* L. erythrocytes following 30 days exposure to dietary Cu concentrations (250, 500 mg kg^{-1}) under normoxic and hypoxic conditions. Values are average median ± S.E. Different letters indicate significant different at $P < 0.05$, corresponding 95.0% confident intervals (n = 6).

5.5.2 Haematological parameters

Results for haematological parameters are presented in Table 5.2. Following exposure to dietary Cu, Hct value increased significantly in the hypoxic (approx. 18%) compared to normoxic group. Also Hct values increased significantly at both Cu exposure levels under hypoxic condition (approx. 8%). In addition, there was a significant difference between both Cu levels under hypoxia and 500 mg Cu kg^{-1} for normoxia treatment (Kruskal-Wallis, P = 0.03). A slight increase at 500 mg Cu kg^{-1} for normoxia treatment was observed compared to only normoxia (control) but was not significant. On the other hand, the number of red blood cells (RBC) was increased (approx. 25%) significantly (ANOVA, P = 0.002) in hypoxic groups compared to normoxic group. Also, RBC count in hypoxic groups was significantly increased compared to 500 mg Cu kg^{-1} under normoxic condition. The significantly increased value for Hb value was observed in hypoxic group (approx. 30%) compared to normoxia and compared to both Cu levels under hypoxia (approx. 12%). Also, both Cu levels under hypoxia were significantly increased (approx. 20%) compared to normoxia (ANOVA, P = 0.01).

Interestingly, the number of white blood cells (WBC) was increased significantly at both Cu levels under hypoxic (approx. 8% and 15% respectively) and normoxic (approx. 8%) compared to both normoxic and hypoxic groups (ANOVA, P = 0.043). For differential leucocyte counts, four types of cells (viz., lymphocytes, neutrophiles, monocytes and eosinophiles) were observed but no basophiles were identified. Numerous types of lymphocytes were found. Significant increase in lymphocyte counts at both Cu levels under hypoxic (approx. 6% and 4% respectively) and normoxic (approx. 6%) compared to normoxic and also to hypoxic group were observed (ANOVA, P = 0.032). In

contrast, in the hypoxic group, frequency of lymphocyte marginally increased compared to normoxic (control) group but this increase was not deemed significant. Eosinophiles increased significantly at both Cu levels under hypoxia (appox. 76%) compared to both normoxic and hypoxic groups. Also fish fed a diet (500 mg Cu kg^{-1}) under normoxia showed significant increases for eosinophiles (approx. 80%) compared to normoxic and hypoxic groups (ANOVA, P = 0.041). Neutrophiles decreased significantly (appox. 40%) in all Cu treatments compared to normoxia (ANOVA, P = 0.03). Also, monocytes decreased significantly in all Cu treatments plus normoxia and hypoxia (approx. 17%, ANOVA, P = 0.031).

Table 5.2 Haematological parameters of *C. carpio* L. exposed to normoxic and hypoxic condition for 30 days either alone or fed with different levels of Cu containing diets (mg Cu kg dw-^{1}).

Parameter	Normoxia	Hypoxia	500 mg Cu kg^{-1}/normoxia	250 mg Cu kg^{-1} /hypoxia	500 mg Cu kg^{-1}/hypoxia
Hct (%)	32.8 ± 0.8^{a}	38.1 ± 2.3^{b}	33.5 ± 0.7^{a}	35.5 ± 1.0^{c}	35 ± 1.0^{c}
Hb (g dl^{-1})	8.6 ± 1.8^{a}	11.3 ± 0.7^{b}	8.8± 1.04^{a}	10.1 ± 1.60^{c}	10.7 ± 0.5^{c}
RBC (cells x 10^{6}µl)	1.5 ± 0.2^{a}	1.9 ± 0.4^{b}	1.4 ± 0.1^{a}	1.6 ± 0.20^{b}	1.5 ± 0.4^{b}
WBC (cells x 10^{3}µl)	13.0 ± 1.4^{a}	13.4 ± 1.1^{a}	14.05 ± 1.9^{b}	14.02± 1.10^{b}	14.9 ± 2.9^{c}
Differential leukocyte count (%)					
Lymphocytes (%)	79.0 ± 2.3^{a}	80.4 ± 4.9^{a}	84.1 ± 4.9^{b}	84.1 ± 2.4^{b}	82.1 ± 4.4^{b}
Neutrophiles (%)	7.2 ± 0.1^{a}	5.9 ± 0.2^{a}	4.1 ± 5.3^{b}	4.1 ± 1.1^{b}	4.2 ± 2.2^{b}
Monocytes (%)	12.3 ± 3.7^{a}	12.0 ± 3.3^{a}	9.07 ± 5.8^{b}	10.1 ± 2.2^{b}	9.1 ± 0.8^{b}
Eosinophiles (%)	1.5 ± 1.5^{a}	1.7 ± 1.5^{a}	3.17 ± 5.4^{b}	3.0 ± 1.2^{b}	3.03 ± 2.7^{b}

Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significant difference at $P < 0.05$; (n=6).

5.5.3 Histopathological study

The gill morphology in the control (normoxic) group showed typical structures in which lamellae were lined by epithelial cells (Fig. 5.2A). After 30 days exposure to dietary Cu under hypoxia and normoxia, identical lesions were observed in all hypoxic groups. However, the extent of the damage was not severe in gills from 500 mg Cu kg^{-1} under normoxic condition and also in gill tissue from the experimental hypoxic group (Fig. 5.2B-F).

Quantitative analysis showed fusion of the secondary lamellae and lifting up of the epithelium. Epithelial hyperplasia and necrosis in the primary and secondary lamellae were significantly higher at both Cu levels under hypoxic compared to the normoxic group. Furthermore, a significant difference at both Cu levels under hypoxic compared to only hypoxic group and also to dietary Cu under normoxic condition were observed. On the other hand, clubbing of the ends of the secondary lamellae, which was significantly higher at both Cu levels under hypoxic compared to hypoxic group and also to dietary Cu plus normoxic group (Table 5.3; ANOVA; $P < 0.05$). A large percentage of the lamellar mucous cells were actively discharging mucous. Shortening of the secondary lamellae (atrophy) was also observed in all experimental Cu groups but it was not significant compared to other groups. In addition, several histopathological alterations in vascular system were observed in the gills. Erythrocytes congestion was general in the marginal channel (telangiectasis) and also throughout the entire lamellae (aneurysm) (Fig. 5.2F) in fish exposed to 250 and 500 mg Cu kg^{-1} under hypoxic and also in only hypoxic condition.

The liver of the control group exhibited normal hepatocytes (e.g. centrally located spherical nucleus) with no abnormalities (Fig. 5.3A). The hepatic tissues showed pronounced pathological changes after 30 d exposure to hypoxia either

alone or with different levels of dietary Cu (Fig. 5.3B-D). The hypoxic group and all dietary Cu groups plus hypoxia and normoxia showed marked cytoplasmic vacuolation of multifocal moderate to severe areas of lipid vacuolation of hepatocytes. On the other hand, necrosis in hepatic tissue was evident in hypoxic group and in all dietary Cu groups. For the intestine, all the groups showed normal histological structures, with no evidence of necrosis, oedema, haemorrhage or excessive epithelial sloughing in any of exposed fish.

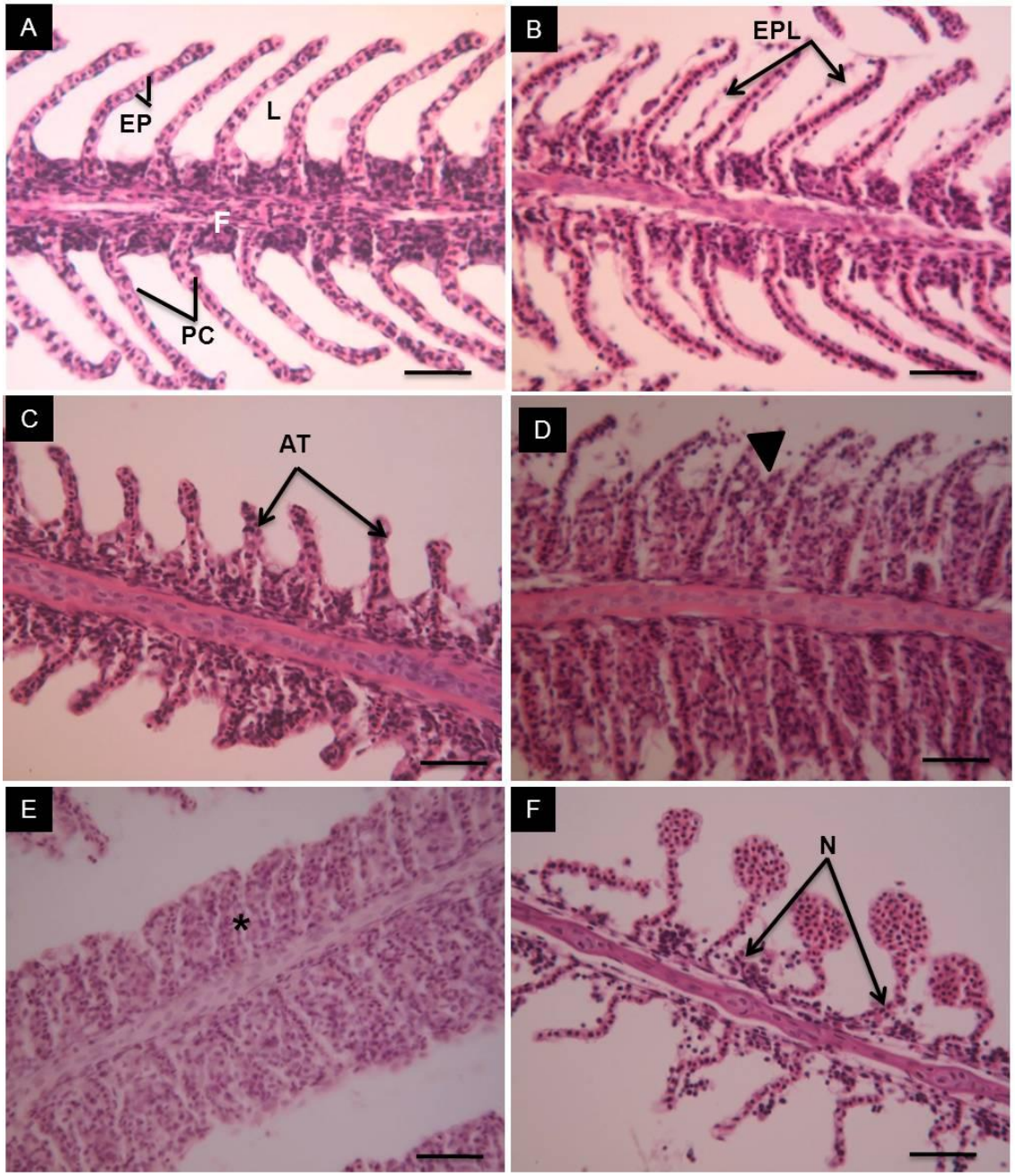


Fig. 5.2 Light micrograph showing histological structures of the gill of *C. carpio* L. from normoxia, hypoxia and dietary Cu exposures plus hypoxia stained with H&E at 5-8 µm thickness. (A) control gill showing the (F), filament; (L), secondary lamellae; , pillar cell; and (EP), epithelial cell (B) hypoxic gill showing (EPL) epithelial lifting; (C&D) 250 mg Cu kg^{-1} plus hypoxia demonstrating shortening of the secondary lamellae (black arrow) and hyperplasia of the epithelial cells (arrow head); (E&F) 500 mg Cu kg^{-1} plus hypoxia showing fusion of the secondary lamellae (white star); (AN), aneurysm; (N) necrotic cells occupying in the interlamellar space and secondary lamellae (black arrow). Scale bars: 50 µm.

Table 5.3 Histopathological changes presented as a percentage in the gills of *C. carpio* L. exposed to hypoxic condition and fed diets containing different levels of Cu mg kg^{-1} d.w plus hypoxia and normoxia for 30 days.

Lesion (%)	Normoxia	Hypoxia	500 mg Cu kg^{-1}/normoxia	250 mg Cu kg^{-1} /hypoxia	500 mg Cu kg^{-1}/hypoxia
Gill					
Hyperplasia	3.40 ± 1.44^{a}	23.00 ± 5.81^{b}	3.20 ± 1.07^{c}	31.4 ± 4.23^{d}	29.6 ± 7.26^{d}
Fusion	2.40 ± 1.17^{a}	15.00 ± 4.01^{b}	2.60 ± 2.60^{c}	21.2 ± 8.58^{d}	23.4 ± 7.72^{d}
Aneurism	0.00 ± 0.00^{a}	10.00 ± 0.71^{b}	0.00 ± 0.00^{a}	2.6 ± 2.14^{abc}	6.6 ± 5.88^{abc}
Club tips	9.06 ± 4.77^{a}	25.00 ± 5.07^{b}	3.80 ± 6.12^{a}	33.6 ± 8.09^{c}	34 ± 9.84^{c}
Lifting epithelium	0.00 ± 0.00^{a}	15.20 ± 8.87^{b}	0.00 ± 0.00^{c}	27.4 ± 3.64^{d}	42.4 ± 2.24^{d}
Atrophy	0.00 ± 0.00^{a}	00.00 ± 0.00^{a}	07.00 ± 3.92^{b}	09 ± 5.42^{b}	09.6 ± 2.77^{b}
Necrosis	0.00 ± 0.00^{a}	15.20 ± 4.47^{b}	2.20 ± 1.36^{c}	24 ± 0.18^{d}	29 ± 2.66^{d}

Data are mean ± S.E. Groups with different alphabetic superscripts indicate significantly different at $P < 0.05$; (n=6)

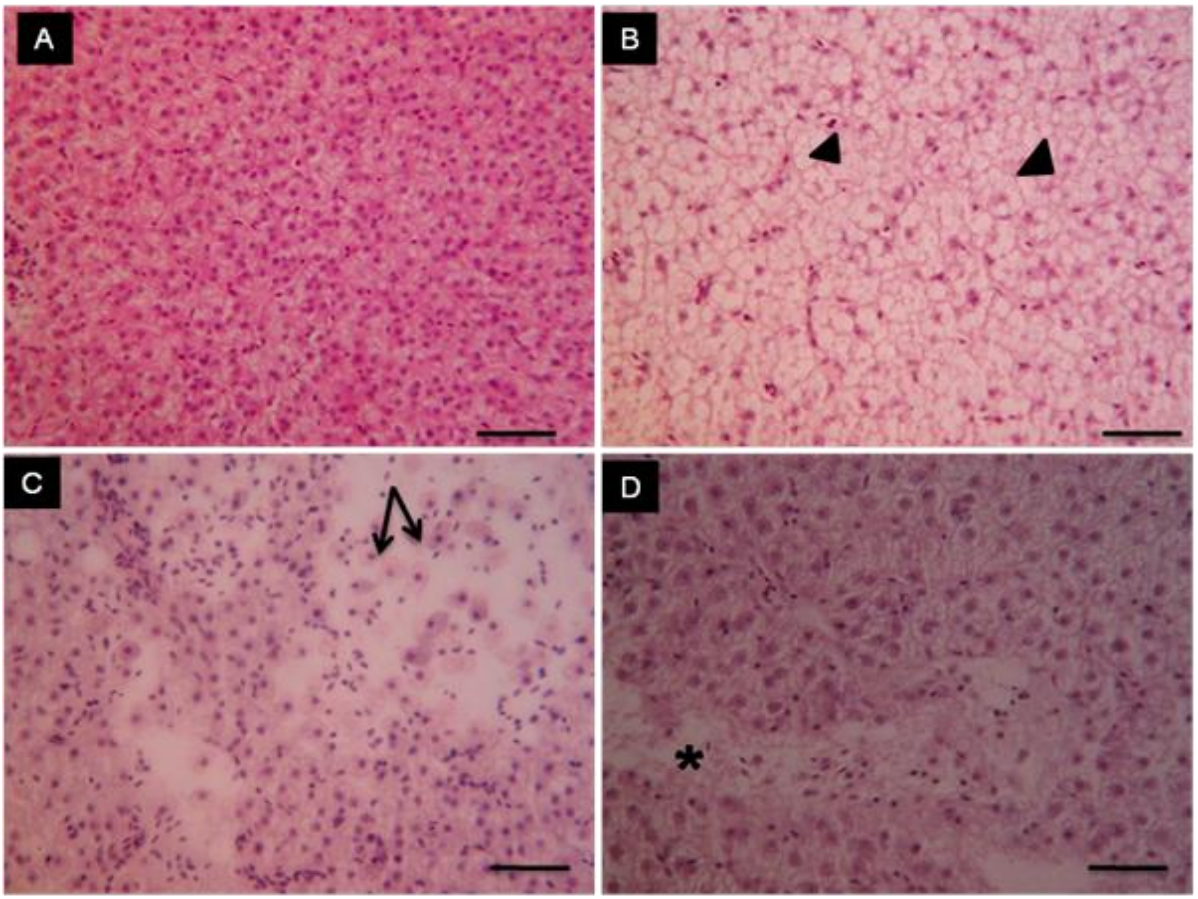


Fig. 5.3 Light micrograph sections showing histological structures through liver of *C. carpio* L. from normoxia, hypoxia and dietary Cu exposures plus hypoxia stained with H&E at 5-8 µm thickness. (A) control liver showing normal histology (B) hypoxic liver showing multifocal moderate to severe areas of lipid vacuolation of hepatocytes (arrow head) (C) 250 mg Cu kg^{-1} d.w./hypoxia showed hepatocellular coagulative necrosis and enlargement of the hepatocytes (black arrow) (D) 500 mg Cu kg^{-1} d.w./hypoxia showing hepatocellular necrosis (black star). Scale bars: 50 µm

5.5.4 Ultrastructural study

For the ultrastructural study, secondary lamellae under normoxic (control) condition exhibited typical regular structures with no lesions (i.e. ordered and parallel arrangement, coaxial system made up of wrapping epithelium and inner endothelium supported by pillar cells with central portion occupied by large nuclei; Fig. 5.4 A-D). Under hypoxic condition, secondary lamellae showed detachments of the epithelial cells and intracellular oedema. The epithelial cells contained swollen mitochondria with enlargement of numerous microvesicles under apical membrane (Fig. 5.4 E&F). Oedema and detachment of the cells were severe in 250 mg Cu plus hypoxia with up to 50% of detachment in one side of the secondary lamellae (Fig 5.4. 4G). Additionally, mitochondrion rich cells showed enlargement and increase in the number of the mitochondria and microvesicles (Fig. 5.4H). In 500 mg Cu kg^{-1} plus hypoxia treatment, approx. 40% of detachment of the epithelial cells was observed on both sides of secondary lamellae along with hyperplasia, breakdown of pillar cells and disorganization of blood cells (Fig. 5.4I&J). In addition, up to 70% of the mitochondrion rich cells exhibited swollen and damaged mitochondria along with irregular meshes of endoplasmic reticulum (Fig. 5.4 K&L).

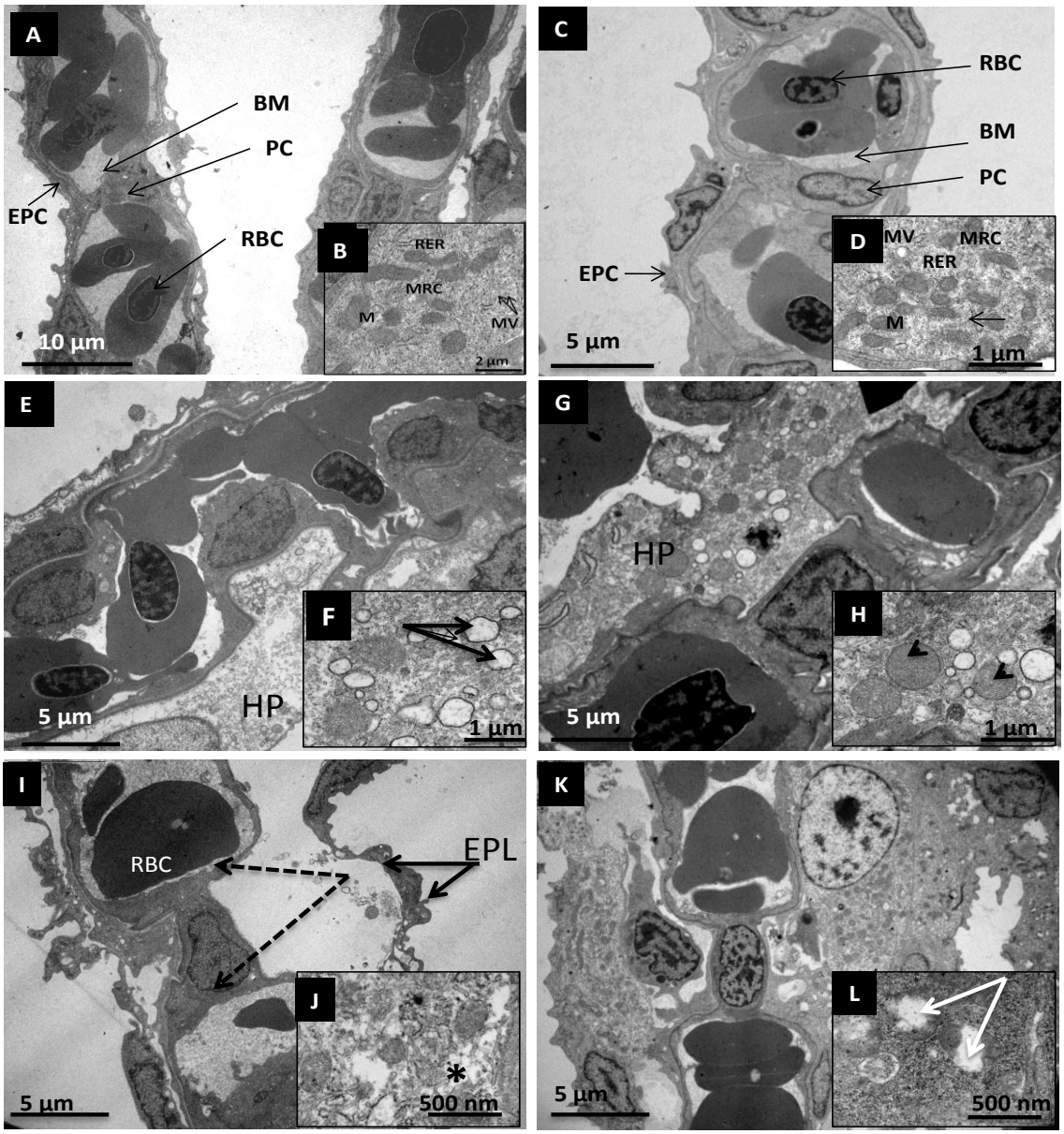


Fig. 5.4 Ultrastructural differences in gills viewed by TEM. (A-D) secondary lamellae of normoxic gill, (E&F) secondary lamellae exposed to hypoxia; (G&H) secondary lamellae exposed to 250 mg Cu kg^{-1} plus hypoxia; (I-L) secondary lamellae exposed to 500 mg Cu kg^{-1} plus hypoxia BM, basement membrane; PC, pillar cell; RBC, red blood cell; EPC, epithelial cell; MRC, mitochondrion rich cells; M, mitochondria; MV, microvesicles; RER, rough endoplasmic reticulum; HP, hyperplasia of the epithelial cells; EPL, epithelial lifting; black arrows indicates high concentration of numerous microvesicles under the apical membrane; arrow head indicates indicate swollen mitochondria; intermittent arrows, breakdown of pillar cells and disorganization of RBC; black star, irregular meshes of tubular reticulum; white arrow, indicate mitochondrial damage.

5.5.5 Copper accumulation

Cu accumulation in the liver, intestine, gill and kidney of *C. carpio*, are shown in Table 5.4. After 30 days of exposure to dietary Cu under hypoxic and normoxic conditions, the liver showed significantly increased of Cu accumulation at 500 mg Cu kg^{-1}d.w (under both hypoxic and normoxic conditions) compared to normoxia group (11 and 10 fold increase respectively), compared to 250 mg Cu kg^{-1}d.w/hypoxia (3.2 and 3 fold increase respectively), and also to hypoxia group (12 and 11 fold increase respectively (Kruskal-Wallis, P = 0.0003). There was no significant difference at 500 mg Cu kg^{-1} d.w under hypoxia and normoxia. In contrast, Cu levels in the intestine was significantly higher at both Cu levels 250 and 500 mg Cu kg^{-1}/hypoxia-exposed groups and also at 500 mg Cu kg^{-1}/normoxia over in those exposed to the normoxic condition and also to hypoxic condition. Interestingly, there was a significantly different between 500 mg Cu kg^{-1}/normoxia-exposed group and 500 mg Cu kg^{-1}/hypoxia-exposed group (Kruskal-Wallis, P = 0.0001). On the other hand, Cu concentration in the kidney was significantly increased at 500 mg Cu kg^{-1}under hypoxia and normoxia in comparison to normoxia group (4 and 7 fold increase respectively) and also to hypoxia group (5 and 10 fold increase respectively). Also there was a significant difference between 500 mg Cu kg^{-1}/normoxia-exposed group and 500 mg Cu kg^{-1}/hypoxia-exposed group (Kruskal-Wallis, P = 0.0003). In contrast, Cu accumulated in gill in lesser extent compared to other tissues and also there was no significant difference between all exposure groups under normoxic and hypoxic conditions.

Table 5.4 Accumulation of copper (µg Cu g^{-1}) by tissues and organs of *Cyprinus carpio* L. exposed to hypoxic condition and fed diets containing different levels of Cu mg kg^{-1} d.w. under normoxic and hypoxic conditions for 30 days.

Tissue	Normoxia	Hypoxia	500 mg Cu kg^{-1}/normoxia	250 mg Cu kg^{-1} /hypoxia	500 mg Cu kg^{-1}/hypoxia
Liver	66.98 ± 6.42^{a}	62.97 ± 7.38^{a}	688.16 ± 75.50^{b}	234.45 ± 36.36^{c}	763.94 ± 95.56^{b}
Intestine	14.09 ± 1.59^{a}	18.87 ± 2.64^{a}	590.91 ± 149.68^{b}	221.26 ± 50.21^{c}	326.35 ± 74.55^{d}
Gill	6.57 ± 1.00^{a}	6.34 ± 0.44^{a}	10.28 ± 0.54	7.88 ± 0.41^{a}	10.68 ± 0.67^{a}
Kidney	7.25 ± 0.76^{a}	5.81 ± 1.15^{a}	51.91 ± 11.16^{b}	16.32 ± 2.47^{c}	26.54 ± 3.50^{c}

Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significant difference at $P < 0.05$; (n=6)

5.5.6 Growth and nutritional performance

All the data for growth and nutritional performance are summarized in Table 5.5. Initial body weight of fish from all replications of control and experimental groups varied from 16.13 g to 16.26 g with no significant differences at the starting time of the trial. No mortalities occurred during the experimental period (i.e. 30 d). Whilst fish from all treatment groups gained weight over the exposure period, weight gain following dietary Cu plus hypoxia exposure was however reduced with elevating copper supplements. There was significant differences in final weight in the Cu exposed and in hypoxic compared to normoxic groups (ANOVA, P = 0.012) which had knock-on effects on SGR which was significantly lower in all treatment compared to normoxic group (ANOVA, P = 0.019). On the other hand, feed conversion ratio (FCR) of dietary Cu groups at 500 mg Cu kg^{-1} under both normoxic and hypoxic conditions was significantly different compared to normoxic and hypoxic groups. Also, FCR at 500 mg Cu kg^{-1} under normoxic was significantly different compared to 250 mg Cu kg^{-1} hypoxic condition (ANOVA, P = 0.007).

This result suggests that the SGR was significantly affected by the level of dissolved oxygen and Cu concentration as evident by significant growth reduction in hypoxic and in all dietary Cu groups. The proximate composition of the carcass was similar for all dietary Cu and normoxic groups, and there were no significant differences between the treatment groups compared to normoxic group (Table 5.5).

Table 5.5 The effect of dietary Cu exposure and hypoxia on nutrition and growth performances [a] of *C.*

Variables	Normoxia	Hypoxia	500 mg Cu kg^{-1}/normoxia	250 mg Cu kg^{-1} /hypoxia	500 mg Cu kg^{-1}/hypoxia
Initial mean weight (g)	16.26 ± 0.06[a]	16.13 ± 0.06[a]	16.23 ± 0.16[a]	16.26 ± 0.13[a]	16.23 ± 0.03[a]
Final mean weight (g)	31.56 ± 0.16[a]	28.60 ± 0.36[b]	25.67 ± 1.13[c]	28.70 ± 1.76[b]	26.68 ± 0.03[bc]
Weight gain (g)	15.30 ± 0.23[a]	12.47 ± 0.43[b]	9.44 ± 0.96[c]	12.44 ± 1.63[b]	10.45 ± 0.06[bc]
SGR (% g^{-1})	2.23 ± 0.03[a]	1.84 ± 0.09[bc]	1.59 ± 0.10[b]	1.95 ± 0.16[c]	1.72 ± 0.01[bc]
FCR	1.00 ± 0.02[a]	1.18 ± 0.02[a]	1.52 ± 0.08[b]	1.22 ± 0.09[c]	1.39 ± 0.0[cb]
Proximate carcass composition[b]					
Moisture (%)	72.39 ± 1.15	73.98 ± 0.80	72.95 ± 0.78	74.31 ± 0.84	74.21 ± 2.11
Crude protein (%)	51.51 ± 0.75	55.78 ± 0.55	56.63 ± 0.67	59.17 ± 0.84	56.01 ± 0.72
Lipid (%)	36.59 ± 0.66	33.48 ± 0.07	33.10 ± 1.17	32.46 ± 0.30	32.81 ± 0.23
Ash (%)	06.81 ± 0.46	07.31 ± 0.31	08.23 ± 0.005	07.85 ± 0.07	08.44 ± 0.18

[a]Values are means ± S.E. values within a row with different superscript letters are significantly different as determined by ANOVA at $P < 0.05$; (n= 6).
[b]Values are for fish at the end of the experiment expressed as percentage of dry matter. Proximate carcass composition of initial fish for moisture, protein, lipid and ash respectively were (means ± S.E. n=6): 73.84±0.34; 59.83±0.04; 26.26 ±0.31; 10.26±0.07. The values within the rows without superscripts did not differ significantly ($P > 0.05$).

5.6 Discussion

There is no doubt that exposure to high concentration of Cu can damage cells and tissues because Cu ions and its complexes can lead to increase the ROS followed by oxidative damage to biomolecules including unsaturated lipid, proteins and DNA (Becker et al., 2009; Yourtee et al., 1992). The alternative mechanism for Cu genotoxicity, Cu ions can directly interact with specific sequences of nucleotides in DNA leading to an inactivation of proteins involved in DNA replication, transcription and repair mechanisms (Hartwig, 1995; Prá et al., 2008). As well as, elevated oxygen concentration acts as an inductor for oxidative DNA damage in aquatic animals and may lead to DNA damage (Halliwell and Aruoma, 1991).

Whilst there have been a large number of studies pertaining to Cu-induced toxicity, there is paucity of information related to Cu-induced DNA damage in aquatic organisms, especially in fish either through aqueous or dietary exposure. Higher concentrations of Cu is known to induce damage at cellular and tissue levels either through generation of reactive oxygen species (ROS) or by direct interaction with biomolecules including enzymes and DNA (Prá et al., 2008). These results in *C. carpio* exhibiting highest DNA damage in Cu supplemented groups held under hypoxic and normoxic, and also under hypoxic conditions compared to normoxic group is in line with *in vitro* or *in vivo* studies showing Cu-induced DNA damage (Al-Subiai et al., 2011; Atienzar et al., 2001; Lloyd and Phillips, 1999; Prá et al., 2008). Hypoxia induced oxidative DNA damage also confirms with our previous study where it has been significantly correlated with the specific growth rate (SGR) and GPx activity in this fish species (Mustafa et al., 2011). Oxidative DNA damage in the present

investigation is directly related with Cu and oxygen levels. Moreover, induced DNA damage with stressors alone or as a result of interaction is largely due to oxidative stress, oxidised purines showing higher damage compared to pyrimidines. Needless to mention, induction of genetic damage has implications for both short- and long-term survival of the natural species which could also act as surrogates for human health (Jha, 2004, 2008). It is therefore not surprising that to maintain ecological quality of the hydrosphere attention is being focussed on those contaminants which are carcinogenic, mutagenic or show reproductive toxicity (Borja et al., 2004; Fuerhacker, 2009).

Changes in haematological parameters (e.g. increase in RBC and lymphocyte numbers, Hb values) suggest water quality as one of the most important factors which could have knock-on effects on the overall physiology of the organisms (Casillas and Smith, 1977). A significant increase in the number of white blood cells at different Cu levels under hypoxic and normoxic conditions indicates activation of the immune system either directly or from the oxidative stress (Nussey et al., 1995b). In common with mammals, the nuclein products released from the damaged tissues (e.g. gill, liver) could stimulate the production of leucocytes (Wepener et al., 1992a, 1992b). Similar increases have been reported when fish were exposed to different pollutants including heavy metals (Nussey et al., 1995a; Wepener et al., 1992a). The increase in the number of lymphocytes and eosinophiles at both Cu levels under hypoxia and normoxia also indicate some immune response occurring after the exposure to Cu (Ellis, 1981). The increases in haematocrit and Hb values, influencing the oxygen carrying capacity of blood is potentially an indication of polycythemia due to impaired osmoregulation and damage to gill cells (Wepener et al., 1992a, 1992b). During anaerobic respiration, lactic acid is released causing a

rise in the acidity of blood which in turn leads to swelling and increased number of RBC (Soivio et al., 1974). Fish also compensate for poor oxygen supply by increasing the demand for oxygen intake resulting in epithelial lifting of the lamellae and mucous deposition on gills. Additional stress could cause adrenergic stimulation of the erythropoietic organs (mainly the pronepheric kidney and spleen) to contract and release stored erythrocytes into circulating blood (Nilsson and Grove, 1974). Overall, changes in the haematological profile indicate an attempt by the fish to adapt in an environment with an increased requirement for oxygen leading to further knock-on effects on other body systems.

Exposure to Cu and hypoxia is known to exert a wide range of histopathological effects in fish (Arellano et al., 1999; Handy et al., 1999; Mallatt, 1985). The most noticeable histopathological alterations in the liver being increasing vacuolation associated with lipid accumulation, necrosis and cellular swelling. Increased hepatic lipid content could be attributed by either increased lipid deposition in excess of nutritional requirements or a failure to mobilize lipid stores under stressed conditions. Similar findings were also documented in Sleek Unicorn fish (*Naso hexacanthus*) exposed to heavy metals at contaminated sites in the Red sea areas (Montaser et al., 2010).

Following exposure to hypoxia either alone or in combination with dietary Cu, gills exhibited several histopathological changes which were significantly higher at combined exposures. These changes in histological features are however not exclusive to oxidative or oxygen related stresses. In fact some characters (e.g. lack of arrangement of the lamellae, the detachment of the epithelial layer from the basal lamina etc.) have been previously reported in other stressed

conditions such as exposure to pesticides, effluents from wastewater treatment plans and other environmental stresses in different fish species (Fanta et al., 2003; Szakolczai, 1997). With particular reference to exposure to metals, Skidmore and Tovell (1972) observed severe curling and oedema of the secondary lamellae, with the epithelium lifted away from the basement membrane in rainbow trout, *Oncorhynchus mykiss* exposed to Zn. Similar changes were observed in Senegales sole, *Sole senegalensis* subjected to sublethal concentrations of Cu for 7 d (Arellano et al., 1999). The changes observed in the gill tissue could impair respiratory functions (e.g. gas exchange) generating an internal hypoxia which may stimulate the RBC to be released from the erythropoietic tissues into the blood stream (Pilgaard et al., 1994). All these alterations may represent a defence mechanism to adapt in the changing environment (Arellano et al., 1999; Camargo and Martinez, 2007). In this integrated study, we have determined the potential effects at different levels of biological organisation as well as in different tissues and organs (i.e. liver, intestine, gill and kidney). It is interesting to note that although fish were exposed to dietary Cu levels and they did not show significant accumulation of this metal in gills, but they exhibited histopathological abnormalities in this organ. It is to be remembered that hypoxia also induces stress on the gills. Even though the fish were exposed to Cu through dietary route, it will cause stress after assimilation and absorption. Indeed it has been shown that dietary Cu exposure induces histopathological changes in gills, despite the fact that it does not get accumulated in significant amount in this organ (Handy, 2003; Handy et al., 1999), which is in line with our observation.

For the ultrastructural studies, whilst the secondary lamellae under normoxic condition showed typical regular structures, it displayed distinct morphological

changes compared to the hypoxic groups either alone or in combination with Cu. Deformation of erythrocytes was also noticed which could be due to the reduced vascular space. The restricted lumen of the vessels could also force the RBC to move into reduced space giving rise to cellular deformation. Nilsson et al., (1995) have suggested that this deformation may be an adaptation to increase oxygen uptake, since it can counteract the formation of a diffusion boundary layer around the erythrocytes resulting in mixing of intracellular haemoglobin molecules. Due to restricted passage, however, cells have to pass slowly via the lamella favouring oxygen uptake. The large ultrastructural changes observed in the gill could be due to a remodelling during hypoxia which is suggested to be a general characteristic associated with regulation of ion disturbances (Matey et al., 2008).

Cu accumulation in tissues was seen to be directly related to its levels, highest in the liver followed by intestine, kidney and gill. Higher accumulation in the liver compared to other tissues possibly related to its important role in the detoxification and excretion of toxicants (Kim and Kang, 2004; Roesijadi, 1992). In addition, accumulation of Cu in the liver was virtually similar at 500 mg kg^{-1} under hypoxic and normoxic conditions. A similar result has been reported in rainbow trout *Oncrohynchus mykiss* exposed to Cu (0.5 ppm) for 9 d in normoxic and hypoxic conditions (Pilgaard et al., 1994). For intestine, Cu accumulation at 500 mg Cu kg^{-1} under normoxia was significantly higher compared to hypoxia. The latter effect could be attributed to the suppression of the cellular metabolic rate in response to hypoxia (Hochachka, 1997). The intestine seems to have an important role in regulating the uptake of dietary Cu and higher accumulation of Cu in the intestine compared to liver and gill in rainbow trout has been reported (Handy et al., 1999). Lloyd (1961) has

proposed that higher toxicity of Cu in hypoxic condition may be associated with increased water coming in contact with gills as a result of hypoxia induced hyperventilation. This could however be only possible if sufficient quantity of Cu entering the interlamellar spaces of the gills is taken up as the water passes to the secondary lamellae. The Cu content of the kidney probably reflects the Cu level in the blood content of the organ and its sequestration by metallothionein.

Dietary Cu exposure either in hypoxic or in normoxic conditions resulted in reduction in SGR. This could be explained by reduced daily feed intake since the lower feed intake could reduce the amount of nutrients available for growth. Impairment of growth rate following dietary Cu exposure in fish has been conflicting. Whilst some workers have reported reduction in growth rate (Baker et al., 1998; Clearwater et al., 2000) others have not (Handy et al., 1999; Kamunde et al., 2001; Lanno et al., 1985). Also, significant growth reductions have been demonstrated in different fish species exposed to hypoxic condition including by us (Mustafa et al., 2011; Petersen and Pihl, 1995). A possible explanation for impairment of growth could be that physiological changes permitting metal detoxification and homeostasis are highly energy dependent (Kim and Hyun, 2006). Furthermore, toxicants that interfere indirectly with energy production inhibit the synthesis of biomolecules (i.e. nucleic acids and protein). It is therefore likely that upon prolonged exposure to hypoxia, in order to conserve energy expenditure by metabolic depression, fish may have to eventually enhance the limited supply of energy from anaerobic sources. As a result, the physiological and biochemical adjustments may ultimately manifest in growth reduction and feed utilization efficiency (Hochachka, 1997; West and Boutilier, 1998).

In conclusion, hypoxia either alone or in combination with dietary Cu induces sub-lethal responses at different levels of biological organisation in fish. Whilst Cu accumulated differentially in the tissues, hypoxia did not significantly influence this accumulation. Combined exposure of hypoxia and Cu induced a significantly higher level of oxidative DNA damage suggesting that DNA damage in fish can serve as a sensitive biomarker for changes in water quality as well as presence of genotoxic chemicals. The study further supports the notion that water quality should be regarded as one of the most important factors responsible for individual variations in genotoxic and other sub-lethal biological responses in aquatic biota inhabiting different ecological niches where contaminants either in hypoxic or in normoxic conditions could be present in all probable combinations.

CHAPTER 6

GENE EXPRESSION PROFILE, LIPID PEROXIDATION AND OXIDATIVE DNA DAMAGE IN CARP *CYPRINUS CARPIO* L. FOLLOWING EXPOSURE TO HYPOXIC AND SUBSEQUENT RECOVERY IN NORMOXIC CONDITIONS

Hypothesis: (a) hypoxia and transition back in normoxic condition induce oxidative stress and modify expression of hypoxia related genes in *C. carpio* L.

(b) hypoxia-induced oxidative stress are manifested in varieties of way and these responses could be interrelated

Abstract

In fish a complex set of physiological and biochemical strategies are employed to cope with environmental stress including hypoxic stress, underlying mechanism of which could be expression of certain key genes. In order to probe the hypotheses that hypoxia induces oxidative stress and this stress could be manifested in varieties of way, *C. carpio* were chronically exposed to hypoxic condition (1.8±0.6 mg l^{-1}) for 21 days and subsequently allowed to recover under normoxic condition for 7 days. At the end of these exposure periods (i.e. 21 days hypoxic and 7 days recovery under normoxic condition), maximising the use of biological samples, adopting an integrated approach at different level of biological organisation, several endpoints were evaluated. These included determination of (a) expression of hypoxia inducible factor 1 (HIF-1α) gene using RT-PCR in liver samples (b) oxidative DNA damage in erythrocytes (using modified Comet assay employing bacterial enzymes: Fpg and Endo-III), (c) lipid peroxidation in liver samples by measuring the MDA production using the 2-thiobarbituric acid (TBARS test) (d) respiratory burst (RB) activity of neutrophiles using reduction of nitroblue tetrazolium and (e) histopathological changes in gills. The results suggested that the expression levels of HIF-1α in response to hypoxia were significantly higher compared to concurrent normoxic controls, which reverted to control values within 7days exposure to normoxic condition *(P* < 0.05). Interestingly, the highest rate of oxidative DNA damage occurred when the fish were kept under hypoxic conditions followed by a rapid increase of the oxygen concentration (recovery period for 7 days) compared to

fish maintained under normoxic condition as concurrent controls. Hypoxic groups showed significantly increased values for TBARS levels (by ~ 2 fold) compared to both normoxic and recovery groups. RB activities increased under both hypoxic and recovery groups but there were no significant differences compared to normoxic control groups. Histopathological changes revealed damage in gill tissue under both hypoxia and recovery stages. Taken together, the results suggest that exposure to hypoxia resulted in the induction of oxidative damage at different levels of biological organisation. This also corroborated with adequate of activation of HIF-1α gene.

6.1 Introduction

Fresh water fish and, to some extent marine fish are continuously exposed to frequent episodes of environmental and physiological hypoxia. Such events are likely to produce elevated levels of reactive oxygen species (ROS) during or in recovery of physiological stress, capable of inducing damage to biological systems (Abele and Puntarulo, 2004). The direct effects of oxidative stress include peroxidative damage to important macromolecules (i.e. DNA, proteins and lipids). Indirectly, changes induced by reactive oxygen metabolites in cellular membranes and components can modify the metabolic process which could lead to changes in physiology and possibly pathological changes in the organisms (Miller et al., 1993a).

Generally, hypoxia even for short-term can be fatal or detrimental to humans and most mammals as they possess only little tolerance to anoxia and their tissues are normally debilitated by any prolonged lack of O_2 (van der Meer et al., 2005). Unlike mammals, fish have however evolved to survive on long exposures to hypoxia owing to a range of biochemical, physiological adaptations collectively known as the “hypoxia responses” (Iwama et al., 2005; Nikinmaa, 2002). Carp is one of the several fish species having the ability to tolerate hypoxic conditions ranging from hours to weeks (van den Thillart and van Waarde, 1985).

Most important alterations that help fish to cope under this condition includes decreased metabolic rate (van den Thillart and van Waarde, 1985), increased ventilation rate (Jensen et al., 1993), increased haematocrit and haemoglobin concentration (Mustafa et al., 2011). By reducing the energy consumption of their tissues using glycogen reserves and anaerobic ATP which is mainly produced from glycolysis (Hochachka, 1997). Thereby, fish respond to stress by

inducing physiological responses including endocrine, metabolic and cellular changes (Bonga, 1997). However, metabolic damage associated with hypoxia/anoxia arises not only from hypoxia, but can also occur during tissue reoxygenation (recovery).

The reintroduction of oxygen into hypoxic tissue results into rapid transient increase in ROS causing oxidative stress (Di Giulio et al., 1989; Halliwell and Aruoma, 1991; Lushchak et al., 2001). Hence, to successfully survive, hypoxia exposed organisms must not only maintain its viability under low levels of oxygen but also have effective mechanisms to prevent the oxidative stress during transition back from hypoxic to aerobic form.

At molecular level, *in vitro* studies have suggested that many genes inducible by hypoxia are regulated by a ubiquitous, highly conserved DNA-binding protein, hypoxia inducible factor 1α (HIF-1α) (Guillemin and Krasnow, 1997; Soitamo et al., 2001). HIF-1α receives signal from the molecular oxygen sensor through redox reaction and or phosphorylation, which in turn activates the transcription of a number of hypoxia-inducible genes. The overall effect of differential gene expression have been suggested to lead to a series of biochemical and physiological responses, allowing the organisms to survive under hypoxic conditions with net decrease in metabolic rate and protein synthesis (Wu, 2002).

On the other hand, although recent technological developments have made it possible to detect patterns of gene expression, only little published reports are available on tissue expression patterns of HIF-1α in fish exposed to hypoxia. In addition to the natural environment, the oxidative status and the consequences of the hypoxic event are very important to the aquaculture. In this study

therefore, we have taken the opportunity to adopt a holistic approach to determine the biological responses at different levels of biological organisation. In particular, we used reverse transcription and real- time quantitative PCR (i.e. RT-PCR and q-PCR) technology to examine the HIF-1α gene expression pattern in *C. carpio* under hypoxic condition and subsequent recovery period. In addition, we also investigated oxidative DNA damage, lipid peroxidation, respiratory burst activity (i.e. measurement of release of reactive oxygen species by immune cells) and histopathological changes in the gills of mirror carp, *C. carpio* L., chronically (i.e. 21 days) exposed to hypoxia (1.8 mg l^{-1}) and subsequently to recovery period under normoxic conditions for 7 days to verify if hypoxia and transition back from hypoxic to aerobic condition (normoxia) is capable of inducing any oxidative stress with concurrent modification in gene expression profile in this species

6.2 Materials and Methods

6.2.1 Fish and their maintenance

Mirror carp (*Cyprinus carpio* L.) were obtained from Hampshire carp hatcheries (Bowlake fish farm, UK), and kept in our aquarium at Plymouth University. Fish were stocked into tanks and allowed to acclimate for 30 days before starting the trial. The tanks were connected to a tap water recirculation system (~2250-L), with strictly controlled water conditions: temperature 24 ± 1 °C, pH 7 ± 0.5, total ammonia below 0.1 mg l^{-1}, nitrate below 22 mg l^{-1} and nitrite below 0.01 mg l^{-1}. Dissolved oxygen (DO) was maintained at over 95% of the saturation value by adding pure O_2 to the system. During the acclimation period, all fish were fed twice daily (8:00 a.m and 1:00 p.m) with a commercial diet (Ewos, Micro 20 p, Ewos Ltd., Westfield, Bathgate, West Lothian. UK) at a rate of 2% of average body mass.

6.2.2 Exposure to hypoxic condition and sampling strategy

After the acclimation period, 60 fish (weighing an average 30.2 g; length 14.4 ± 2 cm) were transferred into each of six experimental tanks (10 fish $tank^{-1}$) connected to the same recirculation system and allowed to acclimate for five days. Following this, first set of three tanks (control) were maintained under normoxic conditions (DO, 8.2± 0.5 mg l^{-1}, 95–100% saturation), the second set of three tanks were maintained under hypoxic condition (DO, 1.8±0.6 mg l^{-1}; 30% of saturation). The DO value for hypoxic period was chosen on the basis of our observations on hypoxia tolerance level of common carp and in accordance with experimental protocols used in previous studies using identical set up (Mustafa et al., 2011, 2012). Low level of hypoxia was achieved using pumping nitrogen gas (purity: 99.99%) at specific water flow through system ≈ 1 l min^{-1}, as described in earlier studies, which occurred within two days. This period did not led to significant built-up of excreta compared to controls, so that groups did not differ in more than ambient O_2 levels (pH 7.5–7.8, nitrite 0.01– 0.02 mg l^{-1}, and ammonia 0.061 ± 0.008 mg l^{-1}). The concentration of oxygen in the water was measured three times daily by using a hand-held dissolved oxygen meter (Oxy Guard, Handy Polaris, DK).

The exposure start time began when the desired dissolved oxygen levels were achieved, which was within 2 days after the initiation of nitrogen pumping. After 3 weeks of exposure to hypoxia, 3 fish replicate $tank^{-1}$ were randomly captured and immediately anaesthetized in a buffered solution of methane sulfonate (MS-222; 100 mg l^{-1} water for 10 min). Fresh blood samples were immediately collected from the caudal vessel using a 25 gauge needle and 1 ml heparinised syringe into eppendorf tubes for analysis by modified single cell electrophoresis “modified Comet assay” (Mustafa et al., 201, 2012) and NBT reduction test

(section 2.5). Fish were dissected; liver samples were isolated, frozen immediately in liquid N_2, and stored at -80 °C for gene expression (HIF-1α) (sections 2.4.1-2.4.5) and lipid peroxidation analyses (see section 2.6). Gill samples were immediately fixed in 10% formaldehyde solution for histopathological study (section 2.10).

Following exposure to hypoxic condition (i.e. 3 weeks), tanks were opened and the bubbling of nitrogen was replaced by bubbling of air; oxygen levels returned to normal values after about 30 min (fish kept under recovery for 7 days). After recovery period (i.e. 7 days), 3 fish from each tank (n=9) were sampled to carry out same analysis as described above.

6.3 Results

6.3.1 Gene expression analyses using quantitative real time PCR (qPCR)

The expression levels of HIF-1α in the liver sample from individual fish in response to hypoxic condition as estimated by quantitative real-time PCR (Q-PCR) are presented in Fig. 6.1. Fig. 6.2 provides qualitative expression of the two genes (i.e. marker β-actin and HIF-1α in agarose gel). These data demonstrate that longer exposure to hypoxic stress (21 days) induced up regulation of HIF-1α which was significantly higher compared to concurrent normal (normoxic) levels. Recovery period significantly decreased the HIF-1α levels which returned to control values within 7days of exposure to normoxic conditions (Kurskal- Wallis; $P < 0.000$).

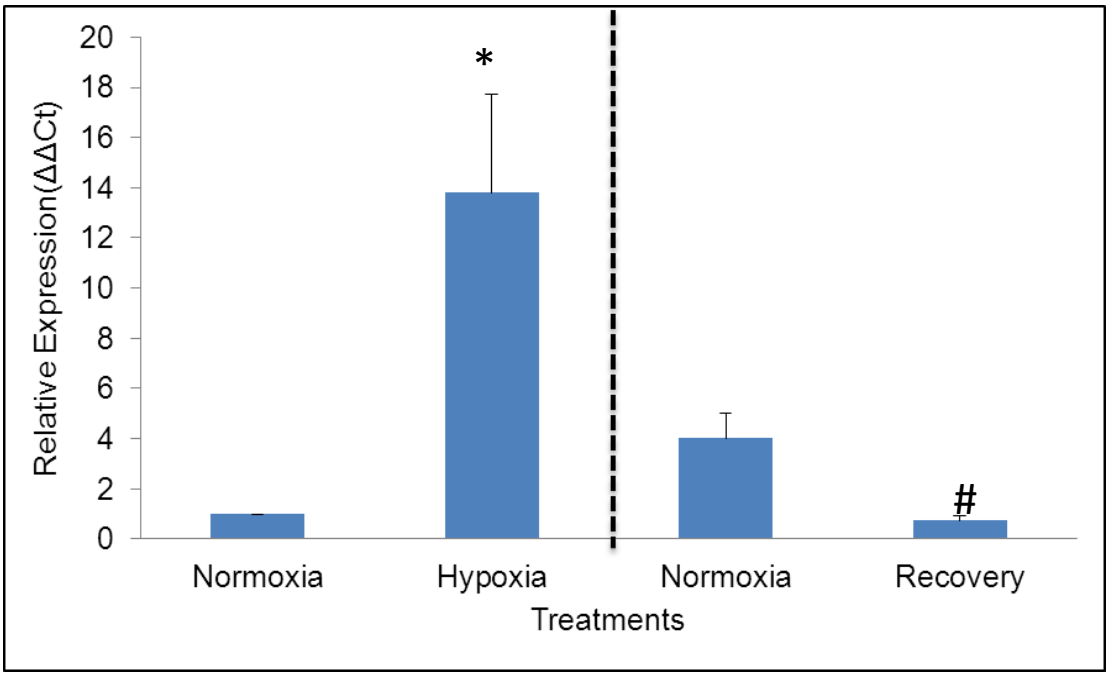


Fig. 6.1 Expression pattern of HIF-1α mRNA as determined by real-time quantitative PCR in *C. carpio* exposed to normoxia and hypoxia (DO, 1.8±0.6 mg l^{-1}, 31% of saturation) for 3 weeks and for 7days as recovery phase. Values are mean ± S.E; * indicates significant differences from normoxic groups; # indicates significant differences between hypoxia and recovery groups at $P< 0.001$; n = 6.

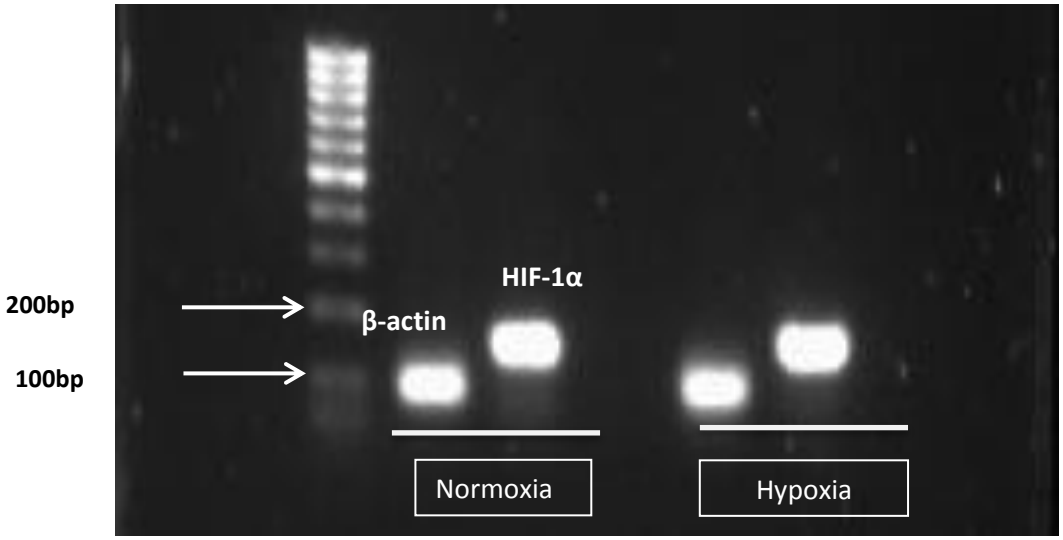


Fig. 6.2 Photographs of PCR products showing HIF-1α and β-Actin detected in nuclear extracts of liver from hypoxic and normoxic fish in 1.5% agarose.

6.3.2 Determination of oxidative DNA damage

Hypoxic condition showed highly significant ($P < 0.00001$) increase for oxidative DNA damage compared to normoxic condition. The highest rate of oxidative DNA damage occurred when the fish were kept under hypoxic conditions followed by a rapid increase of the oxygen concentration (recovery stage for 7days). Also there was no significant difference between the hypoxic group and the recovered group for oxidative DNA damage (Fig. 6.3). On the other hand, the highest degree of oxidative DNA strand breaks was found in the presence of Fpg enzyme in hypoxic condition and following recovery stage compared to the normoxic groups. This enzyme showed a statistically significant difference compared to both control buffer and Endo-III treatments using two ways ANOVA (Fig. 6.3).

6.3.3 Respiratory burst activity

The respiratory burst activity (NBT reduction) of neutrophils of *C. carpio* of the experimental groups is shown in Fig. 6.4. Higher respiratory burst activity was found in hypoxic (0.47 ± 0.03) and recovery groups (0.50 ± 0.02), but there were no significant differences compared to normoxic groups.

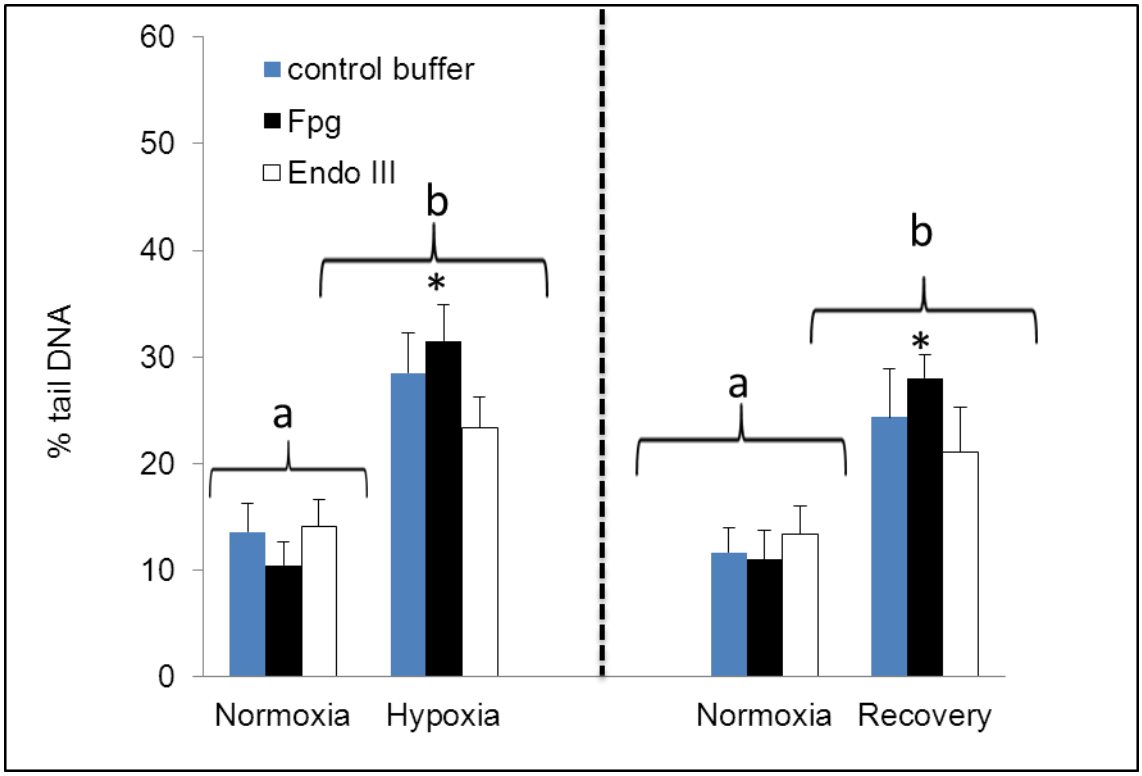


Fig. 6.3 Induction of DNA single strands breaks (represented as percentage tail DNA) in *C. carpio* erythrocytes following 3 weeks exposure to normoxia, hypoxia and following 7days recovery in normoxic condition. Values are average median ± S.E. Different alphabetic letters denote statistically significant different between groups *statistically significant different versus buffer and Endo-III at $P< 0.001$; n =9.

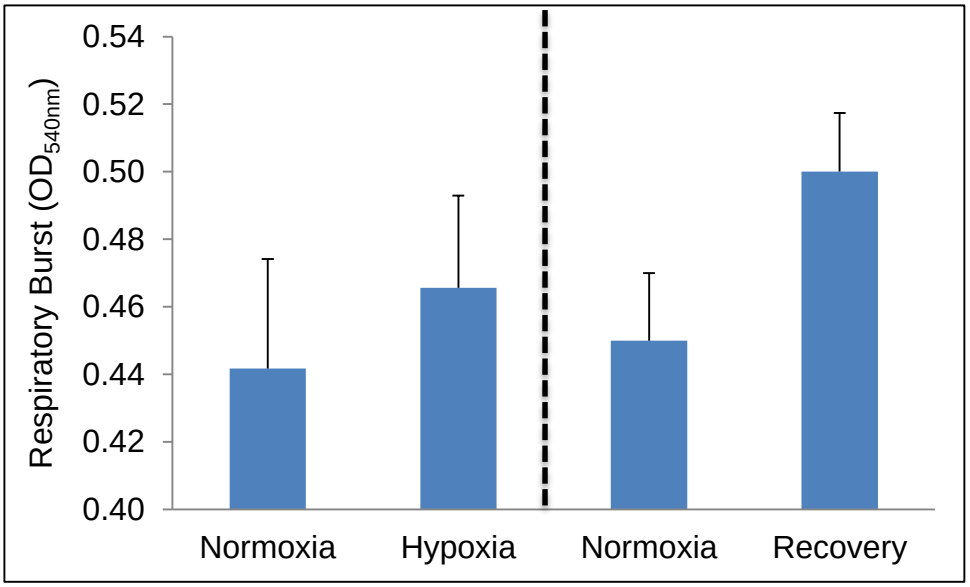


Fig. 6.4 Respiratory burst activity (NBT reduction) in *C. carpio* neutrophils following 3 weeks exposure to normoxia, hypoxia and for 7days recovery under normoxic condition. Values are mean ± S.E; n =9.

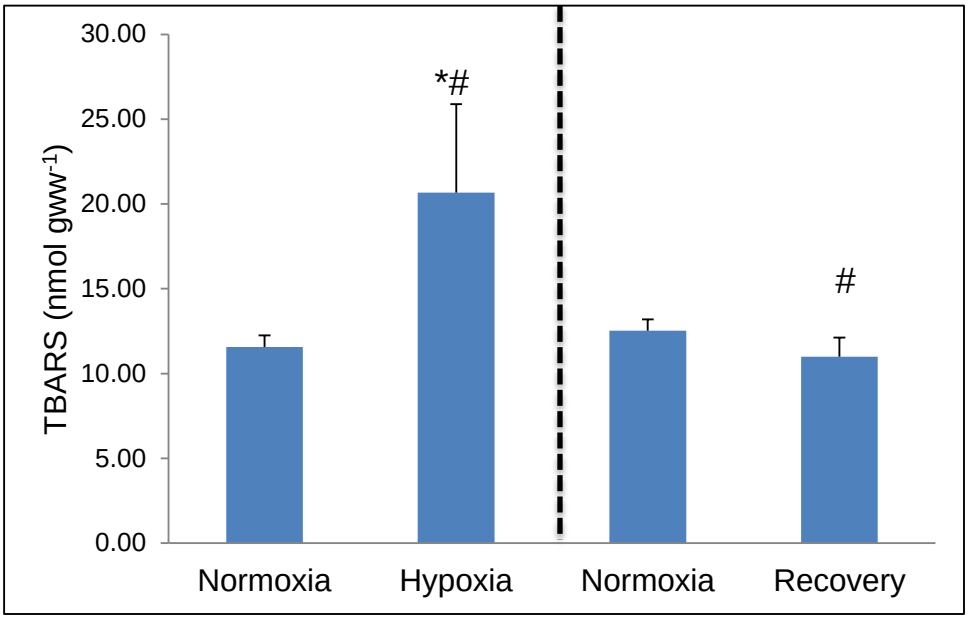


Fig. 6.5 Effect of hypoxia and recovery on levels of thiobarbituric acid reactive substances (TBARS) in the liver of *C. carpio* Values are mean ± S.E; * indicates significant differences from normoxic groups; # indicates significant differences between hypoxia and recovery groups at $P< 0.05$; n = 9.

6.3.4 Determination of lipid peroxidation (TBARS assay)

The levels of TBARS in carp liver from the experimental groups are shown in Fig. 6.5. Hypoxic groups showed significantly higher values for TBARS levels, approx. 2 fold increases compared to normoxic groups. Also, there was a significant difference between hypoxia and recovery groups (ANOVA, P= 0.013). In contrast, the level of TBARS decreased in recovered fish, which was similar to normoxic groups. There were no significant differences between the normoxic and recovery (under normoxic condition) groups.

6.3.5 Histopathological studies

The gill morphology of normoxic group exhibited the typical structure in which lamellae were lined by epithelial cells (Fig. 6.6 A). After 21 days exposure to hypoxia, gills showed several notable histological alterations including lifting of lamellar epithelium, epithelial hyperplasia and necrosis in primary and secondary lamellae (Fig. 6.6 B-C). The magnitude of these changes decreased following 7 days exposure to normoxia (recovery period). However the recovery was not complete within 7 days in normoxic condition and the histological alterations persisted in this group (Fig. 6.6 D). Quantitative analysis for 3 main parameters (viz. epithelial lifting, hyperplasia and necrosis) showed that most of these changes were significantly higher under hypoxic compared to the normoxic groups. Also, these changes were significantly higher in the recovery group especially the necrosis compared to the normoxic group suggesting that 7 days was not an adequate length of time for mirror carp to fully recover from necrosis after prolonged hypoxic stress (Table 6.1).

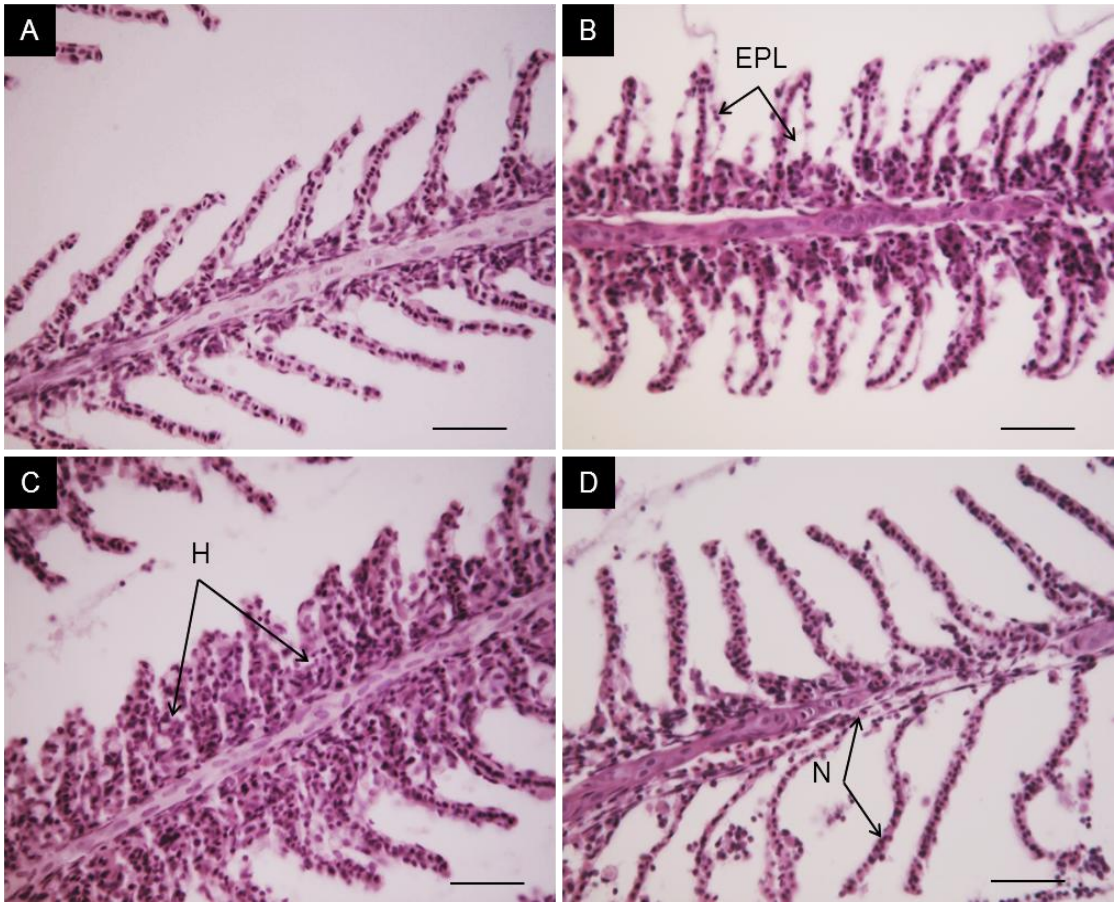


Fig. 6.6 General view of the *C. carpio* secondary lamellae of the gill viewed by light microscopy: *C. carpio* exposed to normoxia (control; A), (B&C) hypoxic gill exposed to 1.8 mg O_2 l^{-1} for 21 days showing (EPL) epithelial lifting; (H) hyperplasia, (D) recovery gill for 7 days showing (N) necrosis. H& E stain; Thickness 5-8 µm. Scale bars 50µm.

Table 6.7 Histopathological changes presented as a percentage in the gills of *C. carpio* following 3 weeks exposure to normoxia, hypoxia and for 7days to recovery (normoxia).

Lesion (%)	Normoxia	Hypoxia	Recovery after hypoxia
Epithelial lifting	2.0 ± 2.5^{a}	$43.\ 2 \pm 7.0^{b}$	$24.\ 08 \pm 5.4^{c}$
Hyperplasia	0.0 ± 0.0^{a}	40.4 ± 5.3^{b}	23 ± 3.7^{c}
Necrosis	0.0 ± 0.0^{a}	37.2 ± 5.3^{b}	32.53 ± 6.4^{b}

Data are mean ± S.E. Groups with different alphabetic superscripts with in rows indicate significantly different at $P < 0.05$.

6.4 Discussion

Several studies have been carried out to demonstrate the adaptive responses in fish exposed to hypoxic condition. These studies have selected a range of parameters at different level of biological organisation, from biochemical to individual level (Lushchak and Bagnyukova, 2007) Our previous investigations have shown that, in the common carp (*C. carpio*), which is especially suited to tolerate hypoxic condition; oxidative DNA damage haematological and histopathological changes in selected organs are induced (Mustafa et al., 2011; 2012). Other studies have also demonstrated histological abnormalities, a decline in growth rate, and feed utilisation performance under a reduced oxygen or hypoxic event (Chabot and Dutil, 1999; Dabrowski et al., 2004; Scott and Rogers, 1980). However, the molecular responses to hypoxia have not been investigated extensively in fish, even though these animals are prime models for such studies. In this respect, considerable attention has been paid to oxygen related effects, as the hypoxic condition are known to affect growth, food intake and the physiological status of fishes (Jobling, 1994; Mustafa et al., 2011). In contrast, in mammals, hypoxia-induced changes for the expression of a broad range of genes have been reported (Bruick, 2003) and extensive amount of information is available on HIF-1α mRNA transcript regulation (Beasley et al., 2002; Turner et al., 2002; Zhao et al., 2004; Zhong et al., 1998). In contrast, only limited numbers of studies on HIF-1α regulation have been reported in teleost fish (Rytkönen et al., 2007; Terova et al., 2008). These studies have adopted different approaches with differing exposure scenarios in different fish species to elucidate the expression of this key gene (i.e. HIF-1α) which plays an important role to facilitate adaptation in the adverse environmental condition

by controlling the induction of several genes involved in the process of glycolysis, erythropoiesis and angiogenesis (Wu, 2002, Soitamo et al., 2001).

To fill the gaps in our knowledge, in the present study, we utilized real-time PCR technique to determine the changes in HIF-1α mRNA expression levels in response to chronic (i.e. 21 days) hypoxic stress. This extended exposure period promoted a remarkable increase in the transcript levels of HIF-1α mRNA, which significantly exceeded (by 13-fold) compared to normoxic controls. The expression patterns were then rapidly reversed upon re-exposure to normoxic condition. These data suggest that HIF-1α is involved in the adaptation response to hypoxia in the common carp. Similarly, in another hypoxia-tolerant species, Atlantic croaker (*Micropogoniasundulatus*), the levels of expression of HIF-1α and HIF-2α mRNAs were found to be in the similar range after long-term (3 weeks) exposure to hypoxia (Rahman and Thomas, 2007). In hypoxic-sensitive species, sea bass *(Dicentrarchus labrax)*, rapid recovery in terms of HIF-1α gene expression was also seen after 24h acute hypoxic stress (Terova et al., 2008). Therefore, it seems that the extent to which hypoxically stressed fish display high levels of HIF-1α expression upon return to adequate oxygenation condition is not related to the exposure period of the hypoxic stress sustained by the animals. In this context, it is to be pointed out that HIF-1α gene is highly conserved showing 61% similarity between fish and mammals (humans and mouse; Soitamo, 2001). Indeed, the mechanism of stabilization and degradation of HIF-1α protein is likely to be the same in fish as in mammals (Soitamo et al., 2001). Although the exposure scenarios in fish and mammals are not comparable, in fish (Chinook, salmon and rainbow trout) stabilization (i.e. return to normal level of gene product after hypoxic exposure) occurred at much higher oxygen levels than in mammals which inherently are exposed to

low levels of oxygen for normal physiological activities. It is to be remembered that the gene expression analyses in the present study were carried out in the liver samples of fish. Mammalian studies have shown that a large number of factors such as life stage, fasting, sex, age, diet, circadian rhythm and liver lobe source can profoundly influence the expression pattern in the liver for a range of genes (Corton et al., 2012). Despite some variation in the levels of HIF-1α in different studies involving fish, it appears that expression of this gene provides a robust marker in response to hypoxic exposure.

For the Comet assay, the high level of oxidative DNA damage observed under hypoxic compared to normoxic condition is not surprising (Fig. 6.3), which is in line with our previous studies (Mustafa et al., 2011, 2012). Several mechanisms have been proposed for the increased levels of DNA strand breaks under hypoxic condition (Lushchak, 2011). It is suggested that under hypoxia the sites of electron-transport chains are more reduced due to limited oxygen availability. Therefore, there are more electrons to escape from the chains and join oxygen molecules. The second mechanism could be related with operation of xanthine reductase/xanthine oxidase system. Under hypoxic conditions the first enzyme can be theoretically converted to the second via limited proteolysis or oxidation and be converted as an effective ROS producer.

Also, following hypoxic exposure, recovered fish in normoxic condition showed high level of oxidative DNA damage after 7d of re-oxygenation. This level was significantly higher compared to concurrent normoxic control group. Lushchak et al. (2011) have suggested that during hypoxia, the tissues of aerobic aquatic organisms, such as fish, are depleted of ATP, which is converted to adenosine and xanthine. After oxygenation, the accumulated xanthine is converted to uric acid by the enzyme xanthine oxidase. As a by-product of this reaction, several

ROS are produced such as superoxide anion radical [$\cdot O_2$], which are responsible for the induction of oxidative stress, leading to DNA strand breaks, lipid peroxidation and other types of cellular damage.

A significant increase for TBARS levels in response to hypoxic compared to normoxic conditions (by approx. 2 fold) was found. Similarly, a significant increase for TBARS levels in response to hypoxia in *C. carpio* and *Carassius auratus* has been reported (Luschack et al., 2001, 2005). As mentioned above, that ROS level could increase since the reduction of mitochondrial electron transport chain and their subsequent leakage to join molecular oxygen resulting oxidative stress. The TBARS in hypoxic recovered fish was significantly decreased compared to hypoxic condition which might be a compensatory consequence of the maintenance. It is to be pointed out that malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation which is mutagenic and carcinogenic.

A general mechanism that supports survival under hypoxic condition in the organisms is depression of metabolic rate (Storey and Storey, 2004) by maintaining high levels of carbohydrates reserves in the tissues for anaerobic energy supply. However, the problem that these adaptive species face a new risk when oxygen is reintroduced during the hypoxic to aerobic move (recovery); this is called the ‘oxygen paradox’. During oxygen deficiency cells become highly reduced (Lushchak, 2011) and during re-oxygenation, reactive reductive equivalents can reduce molecular oxygen via a one electron mechanism resulting in the generation of high levels of ROS–superoxide anion, hydrogen peroxide and hydroxyl radical. Overproduction of these ROS leads oxidative stress (Lushchak, 2011).

The respiratory burst activity of blood cells (e.g. phagocytes) measured by reduction of nitroblue tetrazolium (NBT) did not show any significant decrease in the activity, either in the hypoxic condition or after 7d of recovery period. These findings suggested the levels of production of ROS could not reach a threshold which could induce respiratory burst activity in the target cells, although erythrocytes showed enhanced levels of oxidative DNA damage. It appears therefore that the mechanism of production of this biological response is different from those responses (e.g. DNA damage, lipid peroxidation and histological changes) which showed positive response for these exposure scenarios. In addition, perhaps increased sample size could have enhanced the sensitivity of this assay under the experimental conditions.

For the histopathological changes in the gills, hypoxic conditions showed significant morphological changes, even if these features are not limited to oxygen stress (Mallatt, 1985). Changes such as epithelial hyperplasia, detachment of the epithelial layer from the basal lamina have been previously reported following exposure to other environmental stress (Fanta et al., 2003). This appears to be a paradoxical response to a reduced level of oxygen availability (Camargo and Martinez, 2007). In fact, 7 days was not an adequate length of time for mirror carp to fully recover from histological damage after prolonged hypoxic stress (Table 6.1).

In conclusion, a high level of oxidative DNA damage was seen under hypoxia and re-exposure to normoxia. Also, high TBARS levels in response to hypoxia was observed, which reversed after exposure to normoxia. An increased level of lipid peroxidation was significantly correlated with oxidative DNA damage. We also isolated and characterized HIF-1α in *C. carpio* in response to hypoxia. The expression patterns for this gene in liver samples reversed upon recovery

to normoxia. Future studies are necessary before HIF-1α can be reliably used as a biomarker for hypoxia and to completely elucidate the underlying mechanism of HIF-activation in fish responded to hypoxia. This will open new opportunities for development of this adaptive response in vertebrates, which could have significant clinical implications in human health.

CHAPTER 7

GENERAL DISCUSSION

7.1 Discussion

The study carried out in this project focused on evaluating the biological or biomarker responses at different levels of organisation (i.e. DNA to individual level) following chronic exposure to hypoxic condition either alone or in combination with a representative metal i.e. Cu in a fresh water fish species, *Cyprinus carpio*. Hypoxic conditions in the water and levels of dietary Cu (500 and 1000 mg kg $d.w^{-1}$) showed significant impact on the amount of DNA single strand breaks (DSSB) reflecting high level of oxidative DNA damage. These findings proved the usefulness of the measurement of DNA breaks as determined by the Comet assay in fish as a useful indicator for genotoxicity (*Chapter 3-6*). Also, determination of glutathione peroxidase (GPx) activity in liver and blood plasma complemented the results for the Comet assay (*Chapter 4*). The haematological variables proved that fish have the efficiency to produce compensatory changes which possibly improves preserving oxygen delivery during hypoxic challenge (*Chapter 3&5*). Histopathology and ultrastructural changes in liver (lipid vacuolation, necrosis) and gill (e.g., epithelial lifting, hyperplasia, fusion) are good indicators of what cells and tissues can do to react with metal toxicity and hypoxic challenge (*Chapter 4-6*). Growth rate reduction as a result of exposure to hypoxic event is likely to be a reflection of the fish adaptation to the stressors under prolonged hypoxia to conserve energy expenditure by metabolic depression (*Chapter 4*). Also, growth reduction was observed in fish exposed to high levels of dietary Cu, which could be attributed to metabolic costs associated with metal detoxification (*Chapter 3&5*). Overall, in this study, I determined a range of sub-lethal biological effects using biochemical, genetic, haematological, histopathological and physiological effects. Based on these observations, it could be concluded that the toxic

effects of hypoxia and dietary Cu could manifest their responses in a varieties of way at different levels. The relative sensitivities of these responses have been further elaborated later in this chapter (section 7.1.3). Schematic outlines of the thesis with main the results for each experiment are summarised in Fig. 7.1.

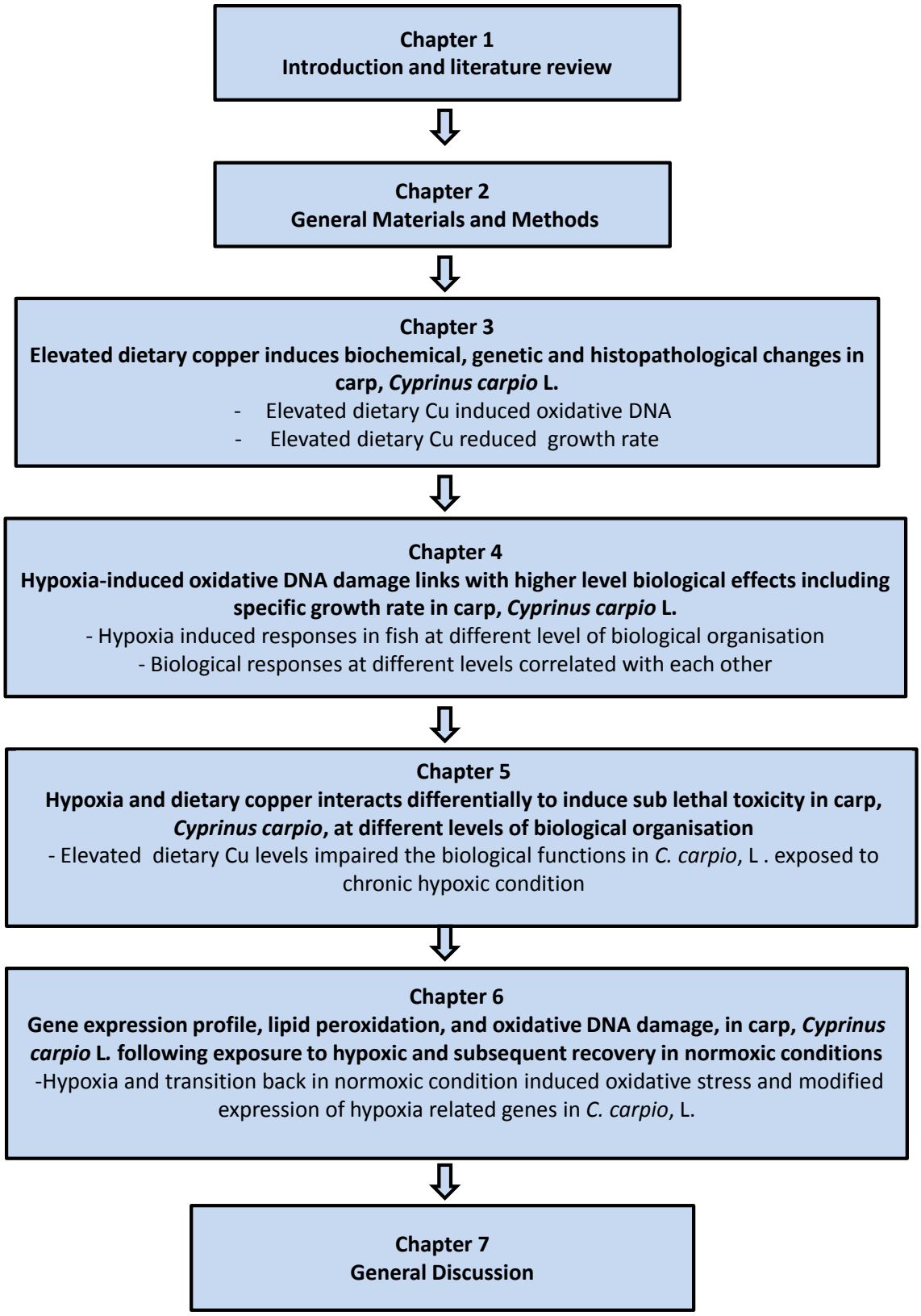


Fig. 7.1 A schematic outline of the thesis and research hypothesis tested for each experiment.

7.1.1 Stress induced by water quality parameters and chemicals

The physical or abiotic environment consists of many factors that can function as stressors either alone or in combination. These include chemical stressors such as temperature, oxygen, salinity, pH and ultraviolet (UV) light, as well as anthropogenic factors (viz. heavy metals, organic contaminants), physical stressors (e.g. handling, capture) and perceived stressors (i.e. stress response as a result of predators) (Barton, 2002; Cossins and Crawford, 2005). These can all affect aquatic ecosystems via two ways: either by debilitation and finally death (resulting tolerant or resistance effects) or by sublethal level effects affecting physiological processes which could result in toxic effects (Cossins and Crawford, 2005). Physical, chemical and perceived stressors can all induce non-specific responses in fish, which could be reflected by adaptation to enable the fish to cope with the disturbance and maintain its homeostatic status (Barton, 2002). If the stressor persists for long time (chronic), the fish is not capable of maintaining homeostasis, then the responses themselves may become detrimental threatening the fish health (Barton, 2002). Fish display a wide variation in their physiological responses to stress, which is classified as (a) primary response: which involves endocrine changes such as measurable levels of circulating catecholamines and corticosteroids, and (b) secondary responses: which include changes in features related to metabolism, hydromineral balance, cardiovascular, respiratory and immune functions (see Fig. 7.2). This integrated study further supports the observation that a range of biological responses could be induced in the fish following exposure to environmental contaminants and stressors. Whilst range of parameters for the studies, due to logistics and technical limitations, it was not possible to use

other parameters especially the molecular (e.g. microarrays, metabolomics etc.), physiological and immunotoxic responses to get a more holistic picture of the impact of these stressors.

Furthermore, ecosystems are contaminated with complex mixture rather than a single agent. Several interactions can be characterised when organisms are exposed to combinations of contaminants depending on the direction of the combined effect (i.e., additive, synergistic, antagonistic or protective). Additive effects might occur when the toxicity of the mixture is equal to the amount of the toxicities of the individual components. Synergistic effects arise when exposure to two pollutants results in a response that is much greater than the sum of individual effects. Antagonistic effects result when exposure to two or more pollutants occurs, with the total response less than the sum of individual effects (Preston et al., 2000). Synergism between two environmental pollutants might occur via a variety of mechanisms. Firstly, the two pollutants could act at the same or different stages in the same mechanistic pathway; secondly, the presence of one might affect ability to mode the action of the other; and thirdly, the presence of one might influence the dose of the other. There are the possibilities that the presence of synergism could be dose or concentration-dependent, that the same combined exposure might be synergistic for one effect and not for others. Toxicities of combinations of pollutants were greater than predicted from addition of individual toxicities (Mauderly and Samet, 2009). Authorities generally enforce regulations that assume that acceptable concentrations for pollutants can be treated independently, even when they are present in mixtures. However, serious consequences may result when such assumptions are incorrect (Preston et al., 2000). Therefore, the evaluation of

synergies and antagonisms among pollutants, including their dose–response relationships, is a necessary foundation for progressing toward a multipollutant water quality controlling measures. Also, toxicity assessment of the impact of combined environmental pollutant exposure on health outcome requires application of sensitive, rapid, inexpensive, and reliable ecotoxicity biomarkers. In this study whilst increased, modest biological responses were observed with combined exposures of hypoxia and different levels of dietary Cu, the interactive effects were neither additive nor synergistic at the levels of Cu used. Enhanced levels of hypoxia and dietary Cu in future studies could throw some light on the possible interactive effects of these two factors in observed biological effects at different levels of biological organisation.

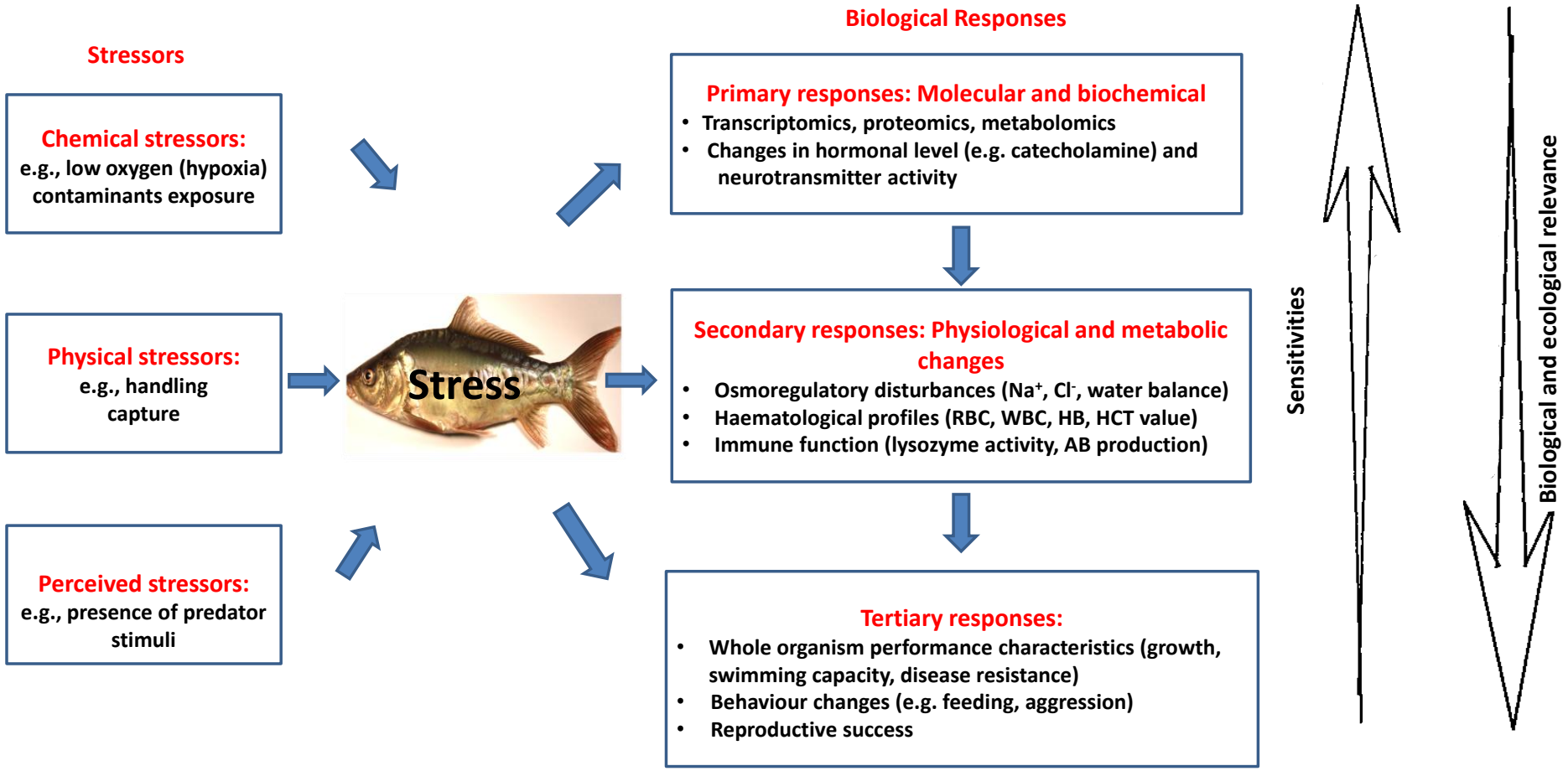


Fig. 7.2 Physical, chemical and other perceived stressors and their biological and ecological relevance (modified from Barton, 2002, Moore et **al.,** 2004).

7.1.2 Molecular methods in environmental monitoring

Over the last decade, the extensive developments of our understanding of interaction of contaminants with biomolecules (i.e. DNA, protein and lipid) at biochemical and molecular levels and subsequent developments in our understanding of how genes and genomes are organised and work, have introduced several new areas of research. In addition to mechanistic toxicity, expression of gene transcripts (i.e. transcriptomics), proteins (i.e. proteomics) and small cellular molecules or metabolites (i.e. metabolomics) have significantly contributed to our understanding of the way environmental agents could induce adverse effects in the biota (Fent and Sumpter, 2011; Hines et al., 2007; Hines et al., 2010). In common with human health arena, the importance of these technologies in aquatic toxicology facilitates to analyse how the toxicants interacts with and integrates from the environment to produce both stress and adaptive responses at molecular and individual levels.

Technologies such as real time quantitative PCR (i.e. RT-PCR) is considered to be the most sensitive and reliable method for detection of gene expression level in terms of low amount of mRNA (Dondero et al., 2006). This direct method allows analysis of known or suspected expressional changes based on the mode of action of the toxicants. However, a limitation of this tool is that only a small set of genes can be analysed. In contrast, microarray technology is a holistic or global approach. This approach analyse thousands of genes (or almost the entire genome) at the same time and at a specific levels. This approach is considered to be superior as the toxicity of toxicants includes mostly multiple modes of actions and a cascade of gene interactions and

pathways rather than the changes in transcript expression of only a few genes. The concept of microarray technology is simple: thousands of copies of cDNA/oligonucleotides probes are immobilised onto a solid substrate. RNA samples to be studied are reverse-transcribed into fluorescent/radioactive-labelled cDNA which is then applied to the microarray in a solution phase. But, the disadvantages of this method are transcript expression levels of some genes (in response to a toxicant) are depended on time and concentration. Thus, important time points may be missed. Also, many genes are not annotated and their functions remain unknown. In recent years, some of these technologies have been used to determine the hypoxia induced changes at the gene or whole genome levels (McElroy et al., 2012; Rahman and Thomas, 2007; Ton et al., 2003). It is however important to link these changes at gene or genome expression level at individual or population levels (Jha, 2004; Jha, 2008; Moore et al., 2004). Another new technologies assessing a large number of mRNA (transcriptomics), proteins (proteomics), or small molecules (metabolomics) in correlation with bioinformatic tools have the potential to determine the protein and/or metabolite changes revealing the mode of action of chemicals and its potential impact. They could enhance our understanding of the mechanism of the mode of action of toxicants as well as predict changes through comparison the chemicals sharing the same mode of action.

Despite the progress made with these techniques (toxicogenomics), as mentioned above, to date only very studies have been conducted that correlates gene expression pattern with biochemical, physiological and histological responses at higher levels of biological organisation. Additionally, the use of these techniques in aquatic toxicology remain in its early (research)

stages, data cannot yet be applied to environmental risk assessment or for regulatory purposes until more studies is conducted to establish the validity of the data within physiological and toxicological contexts (Ankley et al., 2006). Thus, a correlation between the molecular (transcriptomics) and biochemical responses to adverse changes in physiology, growth, development, reproduction, and survival is important to enhance our understanding of adverse effects of chemicals. Limited study carried out in this project to link the gene expression data with higher level effects goes some way towards achieving these goals. Also, epigenetic mechanisms seem to allow an organism to respond to the environment through changes in gene expression "meaning interpretation of the genotype during development to give the phynotype". Therefore, epigenetic processes are essential for development and differentiation which represents an exciting area of future research (Anway and Skinner, 2006). Further studies must be performed to establish robust correlation between gene expression changes and responses measured at the cellular or individual levels using different contaminants or stresses to move the science forward. Also, studies should be conducted to better define background or baseline data (gene expression, protein or metabolic profiles (Fent and Sumpter, 2011).

7.1.3 Sensitivity and specificity of the biomarkers

Choosing the right samples and organs for the biological end points is an important feature in establishing biomarkers as robust tools in environmental risk assessment (ERA). Given that toxicity of a chemical is a tissue or cell specific phenomenon (Jha, 2004; 2008), assessing the right end point in the

wrong tissue might lead to no results and also to misinterpretation of the data. The useful application of physiological and biochemical diagnostic biomarkers in aquatic toxicology could therefore be categorized on three parts. Firstly, these assays can serve as sensitive, rapid, and sublethal indicators of the potential effects of the toxic contaminants on survival, reproduction and/or growth of the aquatic species. Secondly, the sensitive nature of the biological systems to determine presence and quantity of selected chemicals can be used for “true or specific bioassays” (e.g. endocrine disruption, neurotoxicity, immunotoxicity, genotoxicity etc.). Thirdly, physiological–biochemical methods can be used to elucidate the mechanism of action of toxic chemicals in aquatic organisms. These endpoints (biomarkers) have given some emphasis in this study.

This study has shown that quantitative real time PCR analysis is an appropriate method to study the responses to hypoxic condition. We identified the expression of HIF-1α gene in the liver samples of the common carp. However, it remains to be determined which of these changes are tissues specific. Expression of this gene in other tissues or organs (e.g. gill) would have provided relative importance for the selection of the tissues or organs. One of the potential endpoints as an indicator of early warning is oxidative DNA damage using modified Comet assay employed with bacterial enzymes (Fpg and Endo-III). As DNA damage is efficiently repaired by cellular enzymes, its measurement gives a snapshot view of the level of oxidative stress. This study, demonstrating the loss of DNA integrity can serve as an ideal and sensitive biomarker in common with human or mammalian studies (Azqueta et al., 2009). Also, our results indicated an increase in the level of DNA strand breaks under

hypoxic and hyperoxic conditions and with increasing Cu concentration (*Chapter 3-6*). Therefore, Comet assay is considered to be a sensitive and reproducible technique for investigating effects of environmental stress on cells. The inclusion of lesion specific enzymes has improved the sensitivity and specificity of the assay, which detects broad classes of oxidized bases (pyrimidines and purines by digestion with Endo-III and Fpg, respectively). The premutagenic 7-hydro-8-oxo-2o-deoxyguanosine lesion probably is one of the most important lesions detected by the Fpg protein, which has been linked with development of malignancy (Collins, 2004; Collins, 2009). Although, malignancy in natural biota might not be so important in ecological terms as they have reproductive surplus, genotoxic effects however can impact other systems in the body leading to reduced Darwinian fitness (Dixon et al., 2002; Jha, 2004; Jha, 2008; Wurgler and Kramers, 1992).

Despite Comet assay being used as a preferred tool for measuring most kinds of DNA damage at individual cell level, due to lack of a standardised protocol it has been difficult to compare results from different investigators. Differences in assay conditions and various methods of measurement used to determine the extent of the DNA strand breaks make the comparison hard. The earliest studies employing this assay used DNA tail lengths, while more current studies used image analysis softwares to calculate DNA tail moments or percentage of DNA in the tail. The % tail DNA has the advantage that it can be 'standardised' over studies, while tail length and moment, although constant within a study, may not be comparable across studies. Hence, there is an increasing emphasis on the use of the % tail DNA as the preferred metric or the primary end point

(Kumaravel and Jha, 2006). Measurement of oxidative DNA damage, as carried out in this study, is considered to be an important parameter, which has implications for many pathophysiological conditions (Jha, 2008). Whilst this technique is widely used in mammalian or human studies (Collins et al., 1997), there is lack of studies in aquatic organisms measuring oxidative damage (Valavanidis et al., 2006). Moreover, whatever little information related to oxidative damage in fish is available; they are mostly confined to measurements of lipid peroxidation (Lushchak, 2011). This study therefore contributes to only limited amount of information present in the literature measuring oxidative DNA damage in fish or shellfish (Aniagu et al., 2006; Emmanouil et al., 2007). These results is consistent with the mammalian or in vitro studies showing higher sensitivity for purine bases (Reeves et al., 2008).

Lipid peroxidation was measured in this study using TBARS assay. This assay measures various malonic dialdehyde (MDA) that are end products of long chain reactions in the peroxidation processes. TBA also reacts with many types of compounds, such as different aldehydes, amino acids, and carbohydrates. Therefore, it is not appropriate to refer to MDA measurement, but rather to TBA-reactive substances (TBARS), taking into account the low specificity of this assay. Although the assay is not specific it still could estimate the generation of ROS which lead to oxidative stress and DNA damage (Marnett, 1999). This study is in line with other studies showing lipid peroxidation as a result of exposures to hypoxia (Luschak, 2011) and dietary Cu. Interestingly, there was a significant correlation between oxidative DNA damage and lipid peroxidation (*Chapter 6*). This is an area where the relative sensitivity of different

biomolecules (i.e. DNA, protein and lipid) for oxidative damage could be further explored.

Haematological variables widely used for the detection of physiopathological alterations in different conditions of stress (Nussey et al., 1995b). Blood parameters such as changes in haemoglobin, haematocrit, number of erythrocytes and white blood cells are indicators of toxicity with a wide potential for application in environmental monitoring and toxicity studies in aquatic animals since the blood is known to exhibit pathological changes before the onset of any external symptoms of toxicity (Blaxhall and Daisley, 1973). Thereby, our study demonstrates the suitability of haematological variables as sensitive biomarkers in monitoring the physiological status of fish and as indicators of the health for the aquatic environment which appears to have direct correlation with the Darwinian fitness and the immediate adaptation in the stressed condition.

Histopathological and ultrastructural analysis and quantification has been suggested as tool to assess chronically exposed environment and a robust biomonitoring tool in environment risk assessment (Hinton et al., 1992). Histopathological responses in this stud*y* detected changes when there was little or no effect in other biochemical parameters. Also, ultrastructural studies have proved to be a useful tool for investigating the cellular changes, as well as providing complementation to histopathological studies for many features in different tissues. Fish gills are more sensitive to water-borne environmental stress. Any damage in their structure can interfere with respiration. Therefore this study had the aim of using *C. carpio* gill structure as biomarkers to study

the morphological alterations which could occur in branchial tissue after exposure to hypoxic condition. The liver is also an important internal target organ for stress related studies. It is an important detoxifying organ and the site of accumulation for many metals (essential and non-essential). It also contain of many enzymes including metabolic enzymes that could serve as biomarkers (Kim and Kang, 2004). This organ showed severe histopathological changes as a result of both the stresses in this study. To our knowledge, this study adds to very limited information in the literature pertaining to hypoxia induced ultrastructural and histopathological changes in a representative fish species and it should serve as a benchmark for future such studies.

Growth rate of fish is also a good quantitative parameter in measuring the amount of energy available to an organism for various functions, (Rijnsdorp, 1990). Furthermore, measurement of the growth can often be used to provide an index of physiological status and performance. Theoretically, in a contaminated environment an organism would use energy to cope with the toxic stress and hence has less energy available for growth, therefore it is very useful to record the morphmetrics of the fish (Storey and Storey, 2004). To our knowledge, no studies have been carried out to measure SGR in fish following exposure to hypoxia. This evident reduction in SGR was as a result of chronic rather than acute exposure, which most of the earlier studies have performed. Interestingly, in this study the levels of DNA strand breaks significantly correlated with the SGR under hypoxic condition (Chapter 4), which suggesting a possible “cause and effect” relationship between the two endpoints (Fig. 7.3). This suggests that DNA strand breaks could be considered as an ecologically

relevant assay as it directly links with the growth of the organism. In future, links with reproductive success of the organisms would be an important parameter to evaluate. This could be accrued out be measuring DNA damage in the sperms of the fish species.

We have chosen biomarkers that are promising and relate to stressor response and physiological status. A good biomonitoring programme will use a suite of biomarkers (Handy et al., 2002). Additionally, a useful biomarker should, among others, be ecologically related (i.e. able to show effects at higher level of biological organisation), permitting identification/predictions to be made on ecological risk in the receiving environment, and have an obvious correlation between an observable biomarker response (mainly in the case of an exposure biomarker) and a significant biological effect (Lam, 2009).

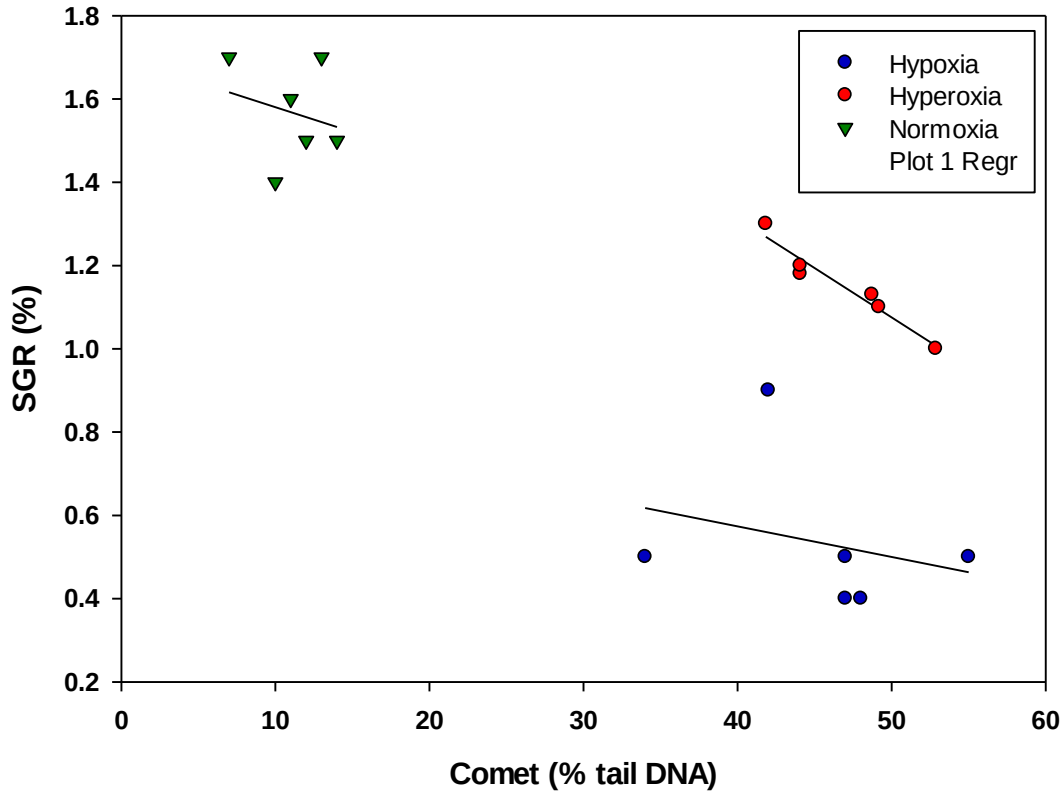


Fig 7.3 Relationship between % tail DNA damage and % SGR under normoxic, hypoxic and hyperoxic condition in *C. carpio.* Each point represents one fish, n=6.

7.1.4 Future work

The present study serves as a spring board to stimulate further studies in the field. There are many areas where the present study could be further elaborated and extended. At molecular and biochemical level, it will be very useful to obtain an assessment of tissue- (and species-specific) gene expression changes and to define the role of individual genes involved in the adaptation following hypoxic exposure (e.g. genes involved in haematopoiesis process, oxygen supply etc.). This will lead to a better understanding of the adaptive responses to hypoxia and results might have significant implications in human health arena (e.g. treatment of malignancy). The genetic and individual susceptibility and potential transgenerational effects of environmental contaminants have not been elucidated for environmental chemicals or complex mixtures, particularly in the aquatic environment and this could be considered novel aspect for future studies. Such studies could be conducted using molecular based investigations to identify the potential mechanisms (e.g. identification of the mutational changes, epigenetic effects etc.) through which tolerance is transmitted. It is however difficult to target specific genes for such studies. In mammalian studies, it has been shown that embryonic exposure of certain chemicals (e.g. endocrine disrupting agents) to rats can lead to decreased sperm quality and increased incidence of sterility. These effects were inherited to male offsprings through three subsequent generations (Anway and Skinner, 2006). In this context, DNA methylation plays an important role in the expression of genes. It is however known that DNA methylation is only important for the somatic lineages and has no role in embryonic lineages (including the germ line). Furthermore, among vertebrates, genomic imprinting is found only in mammals.

Numerous hypotheses have suggested an essential function to imprinting because of the unique development and physiology exhibited by mammals (Reik et al., 2001). It is therefore not surprising that no work pertaining to role of methylation on transgenerational effects has been carried out in fish or other vertebrates (except mammals as mentioned above). Therefore the role of methylation for transgenerational effects in fish is yet to be established.

Challenges for future study also include determination of further suitable molecular biomarkers (e.g. metabolomics) which could serve as excellent indicators of exposures and to elucidate complex metabolic pathways (Hines et al., 2010). Additionally, establishment of strong links among the end points in fish and development (and refinement) of methods that can be used for regulatory purposes need further attention. In this context, it could be pointed out that the Comet assay, in the study is considered for *in vivo* mammalian regulatory toxicological studies (Burlinson et al., 2007; Jha, 2008). It is to be remembered that the aquatic environment and the species rarely encounter only one chemical or stressor at a time. Multiple endpoints assessed in a single experiment and evaluated together may therefore offer stronger evidence for the level of hazard present in an aquatic system. Information obtained through integrated studies using simultaneous applications of numerous biomarkers on various aquatic organisms (i.e. multiple biomarkers and multiple species) will provide a holistic assessment of toxicological impact of environmental pollutants. This could have significant clinical implications for human health as it goes without saying that ‘our’ health depends on the health of the ‘environment’

which includes not only the physical environment but the extant biota on this planet.

References

Abele, D., Puntarulo, S., 2004. Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 138, 405-415.

Admas, S.M., Shepard, K.L., Greeley, M.S., Jemenez, B.D., Ryon, M.G., Shugart, L.R., McCarthy, J.F., 1989. The use of bioindicators for assessing the effects of pollutants stress on fish. Marine Environmental Research 28, 459-464.

Admus, M.A.O.B., Audu, T.O.K., Anyata, B.U., 2007. Removal of lead ion from industrial wastewaters by activated carbon prepared from periwinkle shells (*Typanotonus fuscatus*). Turkish Journal of Engineering and Environmental Sciences 31, 251-263.

Agarwal, A., Cocuzza, M., Abdelrazik, H., Sharma, R., K,, 2008. Oxidative stress measurement in patients with male or female factor infertility, in: Lewin, I.P.a.G. (Ed.), Handbook of Chemiluminescent Methods in Oxidative Stress Assessment. Kerala, India, pp. 195-218.

Ahmad, I., Maria, V.L., Oliveira, M., Pacheco, M., Santos, M.A., 2008. Modulatory role of copper on B-naphthoflavone-induced DNA damage in European eel (*Anguilla anguilla* L.). Ecotoxicology and Environmental Safety 71, 806-812.

Al-Sabti, K., Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. Mutation Research/Genetic Toxicology 343, 121-135.

Al-Subiai, S.N., Moody, A.J., Mustafa, S.A., Jha, A.N., 2011. A multiple biomarker approach to investigate the effects of copper on the marine bivalve mollusc, *Mytilus edulis*. Ecotoxicology and Environmental Safety 74, 1913-1920.

Albert, C.M., Hennekens, C.H., O'Donnell, C.J., Ajani, U.A., Carey, V.J., Willett, W.C., Ruskin, J.N., Manson, J.E., 1998. Fish consumption and risk of sudden cardiac death. The Journal of the American Medical Association 279, 23-28.

Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E., Tice, R., Waters, M.D., Aitio, A., 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. Mutation Research 463, 111-172.

Albinas, L., Loreta, L., Dalia, P., Jûratë, R., Algirdas, M., Rimantas, V., Igoris, P., 2005. Effect of copper, zinc and lead acetates on microorganisms in soil. Ecological 1, 61-69.

Alinnor, I.J., 2005. Assessment of elemental contaminants in water and fish samples from Aba river. Environmental Monitoring and Assessment 102, 15-25.

Amiard, J.C., Amiard-Triquet, C., 1979. Distribution of cobalt 60 in a mollusc, a crustacean and a freshwater teleost: Variations as a function of the source of pollution and during elimination. Environmental Pollution 20, 199-213.

Angehrn-bettinazzia, C., Thonia, L., Hertza, J., 1989. An attempt to evaluate some factors affecting the heavy metal accumulation in a forest stand International Journal of Environmental Analytical Chemistry 35, 69 - 79.

Aniagu, S.O., Day, N., Chipman, J.K., Taylor, E.W., Butler, P.J., Winter, M.J., 2006. Does exhaustive exercise result in oxidative stress and associated DNA damage in the chub (*Leuciscus cephalus*)? Environmental and Molecular Mutagenesis 47, 616-623.

Ankley, G.T., Daston, G.P., J., D.S., 2006. Toxicogenomics in regulatory ecotoxicology. Environmental Sciences Technology 40, 4055–4065.

Anway, M.D., Skinner, M.K., 2006. Epigenetic Transgenerational Actions of Endocrine Disruptors. Endocrinology 147, s43-s49.

Arellano, J.M., Storch, V., Sarasquete, C., 1999. Histological changes and copper accumulation in liver and gills of the *Senegales Sole, Solea senegalensis*. Ecotoxicology and Environmental Safety 44, 62-72.

Atienzar, F.A., Cheung, V.V., Jha, A.N., Depledge, M.H., 2001. Fitness parameters and DNA effects are sensitive indicators of copper-induced toxicity in *Daphnia magna*. Toxicological Sciences 59, 241-250.

Au, D.W.T., 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Marine Pollution Bulletin 48, 817-834.

Au, D.W.T., Wu, R.S.S., S., Z.B., Lam, P.K.S., 1999. Relationship between ultrastructural changes and EROD activities in liver of fish exposed to Benzo[a]pyrene. Environmental Pollution 104, 235-247.

Ausseil, O., Adam, C., Garnier-Laplace, J., Baudin, J.-P., Casellas, C., Porcher, J.-M., 2002. Influence of metal (Cd and Zn) waterborne exposure on radionuclide (134Cs, 110mAg, and 57Co) bioaccumulation by rainbow trout (*Oncorhynchus mykiss*): A field and laboratory study. Environmental Toxicology and Chemistry 21, 619-625.

Ay, Ö., Kalay, M., Tamer, L., Canli, M., 1999. Copper and lead accumulation in tissues of a freshwater fish *Tilapia zillii* and its effects on the branchial Na,K-ATPase activity. Bulletin of Environmental Contamination and Toxicology 62, 160-168.

Ayse Bahar, Y., Cemal, T., Tahsin, T., 2010. Uptake and distribution of hexavalent chromium in tissues (gill, skin and muscle) of a freshwater fish, Tilapia, *Oreochromis aureus*. Journal of Environmental Chemistry and Ecotoxicology 2, 28-33.

Azqueta, A., Shaposhnikov, S., Collins, A.R., 2009. DNA oxidation: Investigating its key role in environmental mutagenesis with the comet assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 674, 101-108.

Bagchi, D., Stohs, S.J., Downs, B.W., Bagchi, M., Preuss, H.G., 2002. Cytotoxicity and oxidative mechanisms of different forms of chromium. Toxicology 180, 5-22.

Baker, R.T.M., Davies, S.J., 1996. Changes in tissue &-tocopherol status and degree of lipid peroxidation with varying &-tocopheryl acetate inclusion in diets for the African catfish. Aquaculture Nutrition 2, 71-79.

Baker, R.T.M., Handy, R.D., Davies, S.J., Snook, J.C., 1998. Chronic dietary exposure to copper affects growth, tissue lipid peroxidation, and metal composition of the grey mullet, Chelon labrosus. Marine Environmental Research 45, 357-365.

Baldisserotto, B., Kamunde, C., Matsuo, A., Wood, C.M., 2004. A protective effect of dietary calcium against acute waterborne cadmium uptake in rainbow trout. Aquatic Toxicology 67, 57-73.

Barceló, D., Petrovic, M., 2007. Pharmaceuticals and personal care products (PPCPs) in the environment. Analytical and Bioanalytical Chemistry 387, 1141-1142.
Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids Integrative and Comparative Biology 42, 517-525.
Baudin, J.P., Fritsch, A.F., 1989. Relative contributions of food and water in the accumulation of ^{60}Co by a freshwater fish. Water Research 23, 817-823.
Beasley, N.J.P., Leek, R., Alam, M., Turley, H., Cox, G.J., Gatter, K., Millard, P., Fuggle, S., Harris, A.L., 2002. Hypoxia-inducible factors HIF-1α and HIF-2α in head and neck cancer. Cancer Research 62, 2493-2497.
Becker, T.W., Krieger, G., Witte, I., 2009. DNA single and double strand breaks induced by aliphatic and aromatic aldehydes in combination with copper (II). Free Radical Research 24, 325-332.
Belpaeme, K., Cooreman, K., Kirsch-Volders, M., 1998. Development and validation of the in vivo alkaline comet assay for detecting genomic damage in marine flatfish. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 415, 167-184.
Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. Journal of Fish Diseases 22, 25-34.
Berntssen, M.H.G., Hylland, K., Wendelaar Bonga, S.E., Maage, A., 1999. Toxic levels of dietary copper in Atlantic salmon (*Salmo salar* L.) parr. Aquatic Toxicology 46, 87-99.
Bickham, J.W., Smolen, M.J., 1994. Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environmental Health Perspectives 102, 25-28.
Bickham, J.W., Sandhu, S., Hebert, P.D.N., Chikhi, L., Athwal, R., 2000. Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutation Research/Reviews in Mutation Research 463, 33-51.
Blaxhall, P.C., Daisley, K.W., 1973. Routine haematological methods for use with fish blood. Journal of Fish Biology 5, 771-781.
Bonga, S.E.W., 1997. The stress response in fish. Physiological Reviews 77, 591-625.
Borja, A., Valencia, V., Franco, J., Muxika, I., Bald, J., Belzunce, M.J., 2004. The water framework directive: water alone, or in association 4 with sediment and biota, in determining quality standards? Marine Pollution Bulletin 49, 8-11.
Boyle, D., Hogstrand, C., Bury, N.R., 2011. Physiological response to a metal-contaminated invertebrate diet in zebrafish: Importance of metal speciation and regulation of metal transport pathways. Aquatic Toxicology 105, 21-28.
Bradl, H., 2005. Heavy metals in the environment: origin, interaction and remediation (interface science and technology). Elsevier/Academic Press, London.
Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonf, A., Marcomini, A., Sumpter, J.P., 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environmental Health Perspectives 113, 721-728.

Brown, B.E., 1976. Observations on the tolerance of the isopod *Asellus meridianus* Rac. to copper and lead. Water Research 10, 555-559.
Bruick, R.K., 2003. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. Genes and Development 17, 2614-2623.
Bryan, G.W., Langston, W.J., 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. Environmental Pollution 76, 89-131.
Buccellato, L.J., Tso, M., Akinci, O.I., Chandel, N.S., Budinger, G.R.S., 2004. Reactive oxygen species are required for hyperoxia-induced bax activation and cell death in alveolar epithelial cells. Journal of Biological Chemistry 279, 6753-6760.
Bucheli, T.D., Kent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems Environmental Science and Technology 25, 201-268.
Bugenyi, F., Lutalo-Bosa, A., 1990. Likely effects of salinity on acute copper toxicity to the fisheries of the lake George-Edward basin. Hydrobiologia 208, 39-44.
Burlinson, B., Tice, R.R., Speit, G., Agurell, E., Brendler-Schwaab, S.Y., Collins, A.R., Escobar, P., Honma, M., Kumaravel, T.S., Nakajima, M., Sasaki, Y.F., Thybaud, V., Uno, Y., Vasquez, M., Hartmann, A., 2007. Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 627, 31-35.
Buschini, A., Martino, A., Gustavino, B., Monfrinotti, M., Poli, P., Rossi, C., Santoro, M., Dörr, A.J.M., Rizzoni, M., 2004. Comet assay and micronucleus test in circulating erythrocytes of *Cyprinus carpio* specimens exposed in situ to lake waters treated with disinfectants for potabilization. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 557, 119-129.
Cacciuttolo, M.A., Trinh, L., Lumpkin, J.A., Rao, G., 1993. Hyperoxia induces DNA damage in mammalian cells. Free radical Biology and Medicine 14, 267-276.
Camargo, M.M.P., Martinez, C.B.R., 2007. Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. Neotropical Ichthyology 5, 327-336.
Camejo, G., Wallin, B., Enojarvi, M., 1998. Analysis of oxidation and antioxidants using microtiter plates. In: Jikai, L. (Ed.), Free radical and antioxidant protocols. (Methods in molecular Biology. NJ: Hummana Press Inc, Totawa, pp. 377-386.
Canty, M.N., Hutchinson, T.H., Brown, R.J., Jones, M.B., Jha, A.N., 2009. Linking genotoxic responses with cytotoxic and behavioural or physiological consequences: Differential sensitivity of echinoderms (*Asterias rubens*) and marine molluscs (*Mytilus edulis*). Aquatic Toxicology 94, 68-76.
Carvalho, C.S., Fernandes, M.N., 2006. Effect of temperature on copper toxicity and hematological responses in the neotropical fish *Prochilodus scrofa* at low and high pH. Aquaculture 251, 109-117.
Casillas, E., Smith, L.S., 1977. Effect of stress on blood coagulation and haematology in rainbow trout (*Salmo gairdneri)*. Journal of Fish Biology 10, 481-491.

Cavas, T., Konen, S., 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis, 263-268.
Cech, J.J., Mitchell, S.J., Wragg, T.E., 1984. Comparative growth of juvenile white sturgeon and striped bass: Effects of temperature and hypoxia. Estuaries and Coasts 7, 12-18.
Chabot, D., Dutil, J.D., 1999. Reduced growth of Atlantic cod in non-lethal hypoxic conditions. Journal of Fish Biology 55, 472-491.
Chan, S.M., Wang, W.-X., Ni, I.H., 2003. The uptake of Cd, Cr, and Zn by the macroalga and *Enteromorpha crinita* and subsequent transfer to the marine herbivorous rabbitfish and *Siganus canaliculatus*. Archives of Environmental Contamination and Toxicology 44, 298-306.
Chance, B., Sies, H., Boveris, A., 1979. Hydroperoxide metabolism in mammalian organs. Physiological Reviews 59, 527-605.
Cheraghalia, A.M., Kobarfardb, F., Noroldin, F., 2010. Heavy metals contamination of table salt consumed in Iran. Iranian Journal of Pharmaceutical Research 9, 129-132.
Cheung, V.V., Depledge, M.H., Jha, A.N., 2006. An evaluation of the relative sensitivity of two marine bivalve mollusc species using the Comet assay. Marine Environmental Research 62, S301-S305.
Clarkson, T.W., 1993. Mercury: Major issues in environmental health. Environmental Health Perspectives 100, 31-38.
Clearwater, S.J., Baskin, S.J., Wood, C.M., McDonald, D.G., 2000. Gastrointestinal uptake and distribution of copper in rainbow trout. Journal of Experimental Biology 203, 2455-2466.
Çogun, H.Y., KargIn, F., 2004. Effects of pH on the mortality and accumulation of copper in tissues of *Oreochromis niloticus*. Chemosphere 55, 277-282.
Collins, A., Mária, D., Michael, F., Martina, S., Helena, P., Susan, D., Laurence, F., Mihalis, P., Katarína, R., Nicholas, V., 1997. Comet assay in human biomonitoring studies: Reliability, validation and applications. Environmental and Molecular Mutagenesis 30, 139-146.
Collins, A.R., Duthie, S.J., Dobson, V.L., 1993. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis 14, 1733-1735.
Collins, A.R., 2004. The Comet assay for DNA damage and repair: Principles, applications, and limitations. Molecular Biotechnology 26, 249-261.
Collins, A.R., 2009. Investigating oxidative DNA damage and its repair using the comet assay. Mutation Research/Reviews in Mutation Research 681, 24-32.
Comhaire, S., Blust, R., Van Ginneken, L., Vanderborght, O.L.J., 1994. Cobalt uptake across the gills of the common carp, *Cyprinus carpio*, as a function of calcium concentration in the water of acclimation and exposure. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 109, 63-76.
Corton, J.C., Bushel, P.R., Fostel, J., O'Lone, R.B., 2012. Sources of variance in baseline gene expression in the rodent liver. Mutation Research/Genetic Toxicology and Environmental Mutagenesis.
Cossins, A.R., Crawford, D.L., 2005. Fish as models for environmental genomics. Nature Reviews Genetics 6, 324-333.
Coutinho, C., Gokhale, K.S., 2000. Selected oxidative enzymes and histopathological changes in the gills of *Cyprinus carpio* and

Oreochromis mossambicus cultured in secondary sewage effluent. Water Research 34, 2997-3004.
Czarnezki, J.M., 1985. Accumulation of lead in fish from missouri streams impacted by lead mining. Bulletin of Environmental Contamination and Toxicology 34, 736-745.
Dabrowski, K., Lee, K.J., Guz, L., Verlhac, V., Gabaudan, J., 2004. Effects of dietary ascorbic acid on oxygen stress (hypoxia or hyperoxia), growth and tissue vitamin concentrations in juvenile rainbow trout (*Oncorhynchus mykiss*). Aquaculture 233, 383-392.
Dacie, J.V., Lewis, S.M., 1995. Practical haematology, 8th ed. Churchill Livingstone, Edinburgh ; New York, p. 609.
Dallinger, R., Kautzky, H., 1985. The importance of contaminated food for the uptake of heavy metals by rainbow trout (*Salmo gairdner*i): A field study. Oecologia 67, 82-89.
Dallinger, R., Prosi, F., Segner, H., Back, H., 1987. Contaminated food and uptake of heavy metals by fish: a review and a proposal for further research. Oecologia 73, 91-98.
Dang, F., Wang, W.X., 2009. Assessment of tissue-specific accumulation and effects of cadmium in a marine fish fed contaminated commercially produced diet. Aquatic Toxicology 95, 248-255.
Das, K.K., Das, S.N., Gupta, S.D., 2001. Influence of ascorbic acid against nickel-induced hepatic lipid peroxidation in rats. Journal of Basic and Clinical Physiology and Pharmacology 12, 187-196.
De Boeck, G., Vlaeminck, A., Balm, P.H.M., Lock, R.A.C., De Wachter, B., Blust, R., 2001. Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term copper exposure: Interactions between Cu^{2+} and plasma cortisol elevation. Environmental toxicology and Chemistry 20, 374-381.
de Conto Cinier, C., Petit-Ramel, M., Faure, R., Garin, D., Bouvet, Y., 1999. Kinetics of cadmium accumulation and elimination in carp *Cyprinus carpio* tissues. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 122, 345-352.
De Jager, S., Dekkers, W.J., 1974. Relations between gill structure and activity in fish. Netherlands Journal of Zoology 25, 276-308.
Decaprio, A.P., 1997. Biomarkers: coming of age for environmental health and risk assessment. Environmental Science and Technology 31, 1837-1848.
Di Giulio, R.T., Scanlon, P.F., 1985. Heavy metals in aquatic plants, clams, and sediments from the Chesapeake Bay, U.S.A. implications for waterfowl. The Science of The Total Environment 41, 259-274.
Di Giulio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W., Jewell, C.S., 1989. Biochemical responses in aquatic animals: a review of determinats of oxidative stress. Environmental Toxicology and Chemistry 8, 1103-1123.
Diaz, R.J., Rosenberg, R., 2008. Spreading dead zones and consequences for marine ecosystems. Science 321, 926-929.
Dirmeier, R., O'Brien, K.M., Engle, M., Dodd, A., Spears, E., Poyton, R.O., 2002. Exposure of yeast cells to anoxia induces transient oxidative stress: Implications for the induction of hypoxic genes. Journal of Biological Chemistry, 34773-34784.
Dixon, D.R., Pruski, A.M., Dixon, L.R.J., Jha, A.N., 2002. Marine invertebrate eco-genotoxicology: a methodological overview. Mutagenesis 17, 495-507.

Dondero, F., Dagnino, A., Jonsson, H., Caprì, F., Gastaldi, L., Viarengo, A., 2006. Assessing the occurrence of a stress syndrome in mussels (*Mytilus edulis*) using a combined biomarker/gene expression approach. Aquatic Toxicology 78, 13-24.

Douben, P.E.T., 1989. Uptake and elimination of waterborne cadmium by the fish *Noemacheilus barbatulus* L. (stone loach). Archives of Environmental Contamination and Toxicology 18, 576-586.

Dural, M., Göksu, M.Z.L., Özak, A.A., 2007. Investigation of heavy metal levels in economically important fish species captured from the Tuzla lagoon. Food Chemistry 102, 415-421.

Eddy, F.B., Fraser, J.E., 1982. Sialic acid and mucus production in rainbow trout (*Salmo gairdneri* Richardson) in response to zinc and seawater. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 73, 357-359.

Ellis, A.E., 1981. Stress and the modulation of defence mechanisms in fish. In: Pickering, A.D. (Ed.), Stress and fish. Academic Press, London, pp. 147-169.

Emmanouil, C., Sheehan, T.M.T., Chipman, J.K., 2007. Macromolecule oxidation and DNA repair in mussel (*Mytilus edulis* L.) gill following exposure to Cd and Cr(VI). Aquatic Toxicology 82, 27-35.

Eriksson, S.P., Weeks, J.M., 1994. Effects of copper and hypoxia on two populations of the benthic amphipod Corophium volutator (Pallas). Aquatic Toxicology 29, 73-81.

Estebani, M.A., Meeguer, J., Garcia, A.A., Aglleiro, B., 1989. Erythropoiesis and thrombopoiesis in the head-kidney of the sea bass (*Dicentrarchus labrax* L.): an ultrastructural study. Archives of Histology and Cytology 52, 407-419.

Evans, D.H., 1987. The fish gill: site of action and model for toxic effects of environmental pollutants Environmental Health Perspectives 71, 47-58.

Fanta, E., Rios, F.S.A., Romão, S., Vianna, A.C.C., Freiberger, S., 2003. Histopathology of the fish *Corydoras paleatus* contaminated with sublethal levels of organophosphorus in water and food. Ecotoxicology and Environmental Safety 54, 119-130.

Farkas, A., Salánki, J., Specziár, A., 2002. Relation between growth and the heavy metal concentration in organs of bream *Abramis brama* L. populating Lake Balaton. Archives of Environmental Contamination and Toxicology 43, 236-243.

Federici, G., Shaw, B.J., Handy, R.D., 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. Aquatic Toxicology 84, 415-430.

Fent, K., Sumpter, J.P., 2011. Progress and promises in toxicogenomics in aquatic toxicology: Is technical innovation driving scientific innovation? Aquatic Toxicology 105, 25-39.

Fenwick, J.C., So, Y.P., 1974. A perfusion study of the effect of stanniectomy on the net influx of calcium 45 across an isolated eel gill. Journal of Experimental Zoology 188, 125–131.

Figueiredo-Fernandes, A., Ferreira-Cardoso, J.V., Garcia-Santos, S., Monteiro, S.M., Carrola, J., Matos, P., Fontaínhas-Fernandes, A., 2007. Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. Pesquisa Veterinária Brasileira 27, 103-109.

Fleming, L.E., Broad, K., Clement, A., Dewailly, E., Elmir, S., Knap, A., Pomponi, S.A., Smith, S., Solo Gabriele, H., Walsh, P., 2006. Oceans and human health: Emerging public health risks in the marine environment. Marine Pollution Bulletin 53, 545-560.

Frenzilli, G., Nigro, M., Lyons, B.P., 2008. The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutation Research 681, 80-92.

Fuerhacker, M., 2009. EU water framework directive and stockholm onvention. Environmental Science and Pollution Research 16, 92-97.

Gabbianelli, R., Lupidi, G., Villarini, M., Falcioni, G., 2003. DNA damage induced by copper on erythrocytes of gilthead sea bream *Sparus aurata* and mollusk *Scapharca inaequivalvis*. Archives of Environmental Contamination and Toxicology 45, 350-356.

Gächter, R., Geiger, W., 1979. An experimental heavy metal pollution study: Behaviour of heavy metals in an aquatic food chain. Aquatic Sciences - Research Across Boundaries 41, 277-290.

Gatlin, D.M., Wilson, R.P., 1986. Dietary copper requirement of fingerling channel catfish Aquaculture 54, 277-285.

Gerhard, G.S., 2007. Small laboratory fish as models for aging research. Ageing Research Reviews 6, 64-72.

Glencross, B.D., 2009. Reduced water oxygen levels affect maximal feed intake, but not protein or energy utilization efficiency of rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition 15, 1-8.

Goksøyr, A., Förlin, L., 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. Aquatic Toxicology 22, 287-311.

Gorokhova, E., Löf, M., Reutgard, M., Lindström, M., Sundelin, B., 2012. Exposure to contaminants exacerbates oxidative stress in amphipod *Monoporeia affinis* subjected to fluctuating hypoxia. Aquatic Toxicology In Press, doi: 10.1016/j. aquatox. 2012.01.022.

Gozal, E., Sachleben, L.R., Jr., Rane, M.J., Vega, C., Gozal, D., 2005. Mild sustained and intermittent hypoxia induce apoptosis in PC-12 cells via different mechanisms. American Journal of Physiology- Cell Physiology 288, C535-542.

Greaney, S.G., Powers, A.D., 1978. Allosteric modifiers of fish hemoglobins: *In vitro* and *in vivo* studies of the effect of ambient oxygen and pH on erythrocyte ATP concentrations. Journal of Experimental Zoology 203, 339-349.

Grieb, T.M., Bowie, G.L., Driscoll, C.T., Gloss, S.P., Schofield, C.L., Porcella, D.B., 1990. Factors affecting mercury accumulation in fish in the upper michigan peninsula. Environmental Toxicology and Chemistry 9, 919-930.

Groff, J.M., Zinkl, J.G., 1999. Hematology and clinical chemistry of cyprinid fish. Common carp and goldfish. The veterinary clinics of North America Exotic animal practice 3, 741-776.

Groopman, J.D., Wogan, G.N., Roebuck, B.D., Kensler, T.W., 1994. Molecular biomarkers for aflatoxins and their application to human cancer prevention. Cancer Research 54, 1907-1911.

Grosell, M.H., Boe¨tius, I., Hansen, J.M., Rosenkilde, P., 1996. Influence of pre-exposure to sub-lethal levels of copper on ^{64}Cu uptake and distribution among tissues of the European eel (*Anguilla anguilla*). Comparative Biochemistry and Physiology Part C 114, 229–235.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. Journal of Experimental Biology 205, 1179-1188.
Guillemin, K., Krasnow, M.A., 1997. The hypoxic response: huffing and HIFing. Cell 89, 9-12.
Gundogdu, A., Haramantepe, F., Dogan, G., Karsli, Z., Yagan Asci, M., 2009. Effects of dietborne copper on accumulation in the tissues and organs, growth and feed utilization of rainbow trout (*Oncorhynchus mykiss,* walbaum, 1792) juvenile. Journal of Animal and Veterinary Advances 8, 2495-2502.
Gupta, P., Neera, S., 2006. Effects of sub-lethal concentrations of zinc on histological changes and bioaccumulation of zinc by kidney of fish *Channa punctatus* (Bloch). Journal of Environmental Biology 27, 211-215.
Gutteridge, J.M., 1986. Aspects to consider when detecting and measuring lipid peroxidation. Free Radical Research Communications 1, 173-184.
Hahn, M.E., Woodin, B.R., Stegeman, J.J., Tillitt, D.E., 1998. Aryl hydrocarbon receptor function in early vertebrates: Inducibility of cytochrome P450 1A in agnathan and elasmobranch fish. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 120, 67-75.
Haines, T.A., Brumbaugh, W.G., 1994. Metal concentration in the gill, gastrointestinal tract, and carcass of white suckers (*Catostomus commersoni*) in relation to lake acidity. Water, Air and Soil Pollution 73, 265-274.
Halliwell, B., Aruoma, O.I., 1991. DNA damage by oxygen-derived species Its mechanism and measurement in mammalian systems. FEBS Letters 281, 9-19.
Halliwell, B., Chirico, S., 1993. Lipid peroxidation: its mechanism, measurement, and significance. The American Journal of Clinical Nutrition 57, 715S-724S.
Halliwell, B., Gutteridge, J.M.C., 1999. Free radicals in biology and medicine, 3rd ed. Clarendon Press; Oxford University Press, Oxford New York.
Halliwell, B., Gutteridge, J.M.C., 2007. Free radicals in biology and medicine, 4th ed. Oxford University Press, Oxford; New York.
Hamdy, M.K., Prabhu, N.V., 1979. Behavior of mercury in biosystems III. Biotransference of mercury through food chains. Bulletin of Environmental Contamination and Toxicology 21, 170-178.
Handy, R.D., 1992. The assessment of episodic metal pollution. I. Uses and limitations of tissue contaminant analysis in rainbow trout (*Oncorhynchus mykiss*) after short waterborne exposure to cadmium or copper. Archives of Environmental Contamination and Toxicology, 74-81.
Handy, R.D., Sims, D.W., Giles, A., Campbell, H.A., Musonda, M.M., 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. Aquatic Toxicology 47, 23-41.
Handy, R.D., Runnalls, T., Russell, P.M., 2002. Histopathologic biomarkers in three spined sticklebacks, *Gasterosteus aculeatus*, from several rivers in Southern England that meet the freshwater fisheries directive. Ecotoxicology 11, 467-479.

Handy, R.D., 2003. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 135, 25-38.
Hartwig, A., 1995. Current aspects in metal genotoxicity. BioMetals 8, 3-11.
Hashemi, S., Blust, R., de Boeck, G., 2007. The effect of food rations on tissue-specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio*. Environmental Toxicology and Chemistry 26, 1507-1511.
Hattink, J., Boeck, G.D., Blust, R., 2005. The toxicokinetics of cadmium in carp under normoxic and hypoxic conditions. Aquatic Toxicology 75, 1-15.
Heath, A.G., 1995. Water pollution and fish physiology. CRC Press, Boca Raton
Hecker, M., Hollert, H., 2011. Endocrine disruptor screening: regulatory perspectives and needs. Envirnmental Sciences Europe 23, 1-15.
Hendricks, J.D., 1994. Carcinogenicity of aflatoxins in nonmammalian organisms. In: Eaton, D. L., Groopman, J. D. (Eds.), Toxicology of aflatoxins: human health, veterinary, and agricultural significance. . Academic press, San Diego pp 6-8.
Hines, A., Oladiran, G.S., Bignell, J.P., Stentiford, G.D., Viant, M.R., 2007. Direct sampling of organisms from the field and knowledge of their phenotype: Key recommendations for environmental metabolomics. Environmental Science and Technology 41, 3375-3381.
Hines, A., Staff, F.J., Widdows, J., Compton, R.M., Falciani, F., Viant, M.R., 2010. Discovery of metabolic signatures for predicting whole organism toxicology. Toxicological Sciences 115, 369-378.
Hinton, D.E., Baumann, P.C., Gardner, G.R., Hawkins, W.E., Hendricks, J.D., Murchlano, R.A., Okihiro, M.S., 1992. Histopathologic biomarkers. In: Huggett, R.J., Kimerle, R.A., Mechrle, J.P.M., Bergman, H.L. (Eds.), Biomedical, physiological, and histological markers of anthropogenic stress. Lewis Publisher, Boca Raton, pp. p156-159.
Hochachka, P.W., 1997. Oxygen-A key regulatory metabolite in metabolic defense against hypoxia. American Zoologist 37, 595-603.
Hochachka, P.W., Lutz, P.L., 2001. Mechanism, origin, and evolution of anoxia tolerance in animals. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 130, 435-459.
Hollis, L., Hogstrand, C., Wood, C.M., 2001. Tissue-specific cadmium accumulation, metallothionein induction, and tissue zinc and copper levels during chronic sublethal cadmium exposure in juvenile rainbow trout. Archives of Environmental Contamination and Toxicology 41, 468-474.
Horvat, M., Nolde, N., Fajon, V., Jereb, V., Logar, M., Lojen, S., Jacimovic, R., Falnoga, I., Liya, Q., Faganeli, J., Drobne, D., 2003. Total mercury, methylmercury and selenium in mercury polluted areas in the province Guizhou, China. The Science of The Total Environment 304, 231-256.
Hu, F.B., Bronner, L., Willett, W.C., Stampfer, M.J., Rexrode, K.M., Albert, C.M., Hunter, D., Manson, J.E., 2002. Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. The Journal of the American Medical Association 287, 1815-1821.
IPCS, International Programme on Chemical Safety, 1993. Biomarkers and risk assessment: concepts and principles. Environmental Health Criteria. World Health Organization, Geneva.

Iwama, G.K., 1997. Fish stress and health in aquaculture. Cambridge University Press, Cambridge [England] ; New York.
Iwama, G.K., Afonso, L.O.B., Vijayan, M.M., 2005. Stress in fish. In: Evans, D.H., Claiborne, J.B. (Eds.), The physiology of fishes, 3rd ed. CRC Press, Florida, pp. 319-342.
Jarup, L., 2003. Hazards of heavy metal contamination. British Medical Bulletin 68, 167-182.
Jensen, F.B., Weber, R.E., 1985. Kinetics of the acclimational responses of tench to combined hypoxia and hypercapnia. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 156, 197-203.
Jensen, F.B., Nikinmaa, M., Weber, R.E., 1993. Environmental, perturbations of oxygen transport in teleosts fishes: causes, consequences and compensations. In: Rankin, J.D., Jensen, F.B. (Eds.), Fish ecophysiology. Chapman and Hall, London, pp. 161–179.
Jensen, L.T., Howard, W.R., Strain, J.J., Winge, D.R., Culotta, V.C., 1996. Enhanced effectiveness of copper Ion buffering by CUP1 metallothionein compared with CRS5 metallothionein in *Saccharomyces cerevisiae*. Journal of Biological Chemistry 271, 18514-18519.
Jenssen, B.M., 2006. Endocrine-disrupting chemicals and climate change: a worst-case combination for arctic marine mammals and seabirds? Environmental and Health Perspective 114, 76-80.
Jezierska, B., Witeska, M., 2001. Metal toxicity to fish. Reviews in Fish Biology and Fisheries 11, 279-279.
Jezierska, B., Witeska, M., 2006. The metal uptake and accumulation in fish living in polluted waters. In: Twardowska, I., Allen, H.E., Häggblom, M.M., Stefaniak, S. (Eds.), Soil and Water Pollution Monitoring, Protection and Remediation. Springer Netherlands, pp. 107-114.
Jha, A.N., Cheung, V.V., Foulkes, M.E., Hill, S.J., Depledge, M.H., 2000a. Detection of genotoxins in the marine environment: adoption and evaluation of an integrated approach using the embryo–larval stages of the marine mussel, *Mytilus edulis*. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 464, 213-228.
Jha, A.N., Hagger, J.A., Hill, S.J., 2000b. Tributyltin induces cytogenetic damage in the early life stages of the marine mussel, *Mytilus edulis*. Environmental and Molecular Mutagenesis 35, 343-350.
Jha, A.N., 2004. Genotoxicological studies in aquatic organisms: an overview. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 552, 1-17.
Jha, A.N., 2008. Ecotoxicological applications and significance of the comet assay. Mutagenesis 23, 207-221.
Jobling, M., 1994. Fish Bioenergetics. Chapman & Hall, London.
Kakuta, I., Murachi, S., 1997. Physiological response of carp, *Cyprinus carpio*, exposed to raw sewage containing fish processing wastewater. Environmental Toxicology and Water Quality 12, 1-9.
Kalay, M., Ay, Ö., Canli, M., 1999. Heavy metal concentrations in fish tissues from the northeast Mediterranean sea. Bulletin of Environmental Contamination and Toxicology 63, 673-681.
Kamunde, C.N., Grosell, M., Lott, J.N., Wood, C.M., 2001. Copper metabolism and gut morphology in rainbow trout (*Oncorhynchus mykiss*) during chronic sublethal dietary copper exposure. Canadian Journal of Fisheries and Aquatic Sciences 58, 293-305.

Kamunde, C., Grosell, M., Higgs, D., Wood, C.M., 2002. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. Journal of Experimental Biology 205, 279-290.
Karadede, H., Ünlü, E., 2000. Concentrations of some heavy metals in water, sediment and fish species from the Atatürk Dam Lake (Euphrates), Turkey. Chemosphere 41, 1371-1376.
Khazanov, V.A., Poborskii, A.N., 1991. Respiration of rat brain mitochondria during hyperoxia and normoxia. Bulletin of Experimental Biology and Medicine 112, 1258-1261.
Kidwell, J., Phillips, L., Birchard, G., 1995. Comparative analyses of contaminant levels in bottom feeding and predatory fish using the national contaminant biomonitoring program data. Bulletin of Environmental Contamination and Toxicology 54, 919-923.
Kim, I.Y., Hyun, C.-K., 2006. Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms. Ecotoxicology and Environmental Safety 64, 288-297.
Kim, S.G., Kang, J.C., 2004. Effect of dietary copper exposure on accumulation, growth and hematological parameters of the juvenile rockfish, *Sebastes schlegeli*. Marine Environmental Research 58, 65-82.
Kim, S.G., Eom, K.H., Kim, S.S., Jin, H.G., Kang, J.C., 2006. Kinetics of Cd accumulation and elimination in tissues of juvenile rockfish (*Sebastes schlegeli*) exposed to dietary Cd. Marine Environmental Research 62, 327-340.
Kime, D.E., 1999. A strategy for assessing the effects of xenobiotics on fish reproduction. The Science of The Total Environment 225, 3-11.
Klemz, C., Salvo, L.M., Bastos Neto, J.d.C., Bainy, A.C.D., Assis, H.C.d.S.d., 2010. Cytochrome P450 detection in liver of the catfish *Ancistrus multispinis* (*Osteichthyes, Loricariidae*). Brazilian Archives of Biology and Technology 53, 361-368.
Klobučar, G., Štambuk, A., Pavlica, M., Sertić Perić, M., Kutuzović Hackenberger, B., Hylland, K., 2010. Genotoxicity monitoring of freshwater environments using caged carp (*Cyprinus carpio*). Ecotoxicology 19, 77-84.
Klontz, G.W., 1997. Fish haematology. In: Stolen, J.S., Fletcher, T.C., Rowley, A.F., Zelikoff, J.T., Kaattari, S.L., Smith, S.A. (Eds), Techniques In Fish Immunology-3 (2^{nd}), SOS Puplications, NJ, USA., 1st ed, pp. 258, 245
Köck, G., Triendl, M., Hofer, R., 1996. Seasonal patterns of metal accumulation in Arctic char (*Salvelinus alpinus*) from an oligotrophic Alpine lake related to temperature. Candian Journal of Fisheries and Aquatic Sciences 53, 780–786.
Koehn, J.D., 2004. Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. Freshwater Biology 49, 882-894.
Kohler, A., 1990. Identification of contaminant-induced cellular and subcellular lesions in the liver of flounder (*Platichthys flesus* L.) caught at differently polluted estuaries. Aquatic Toxicology 16, 271-293
Kubrak, O.I., Husak, V.V., Rovenko, B.M., Storey, J.M., Storey, K.B., Lushchak, V.I., 2011. Cobalt-induced oxidative stress in brain, liver and kidney of goldfish *Carassius auratus*. Chemosphere 85, 983-989.
Kumar, S., Sahu, N.P., Pal, A.K., Choudurry, D., Yengkokpam, S., Mukherjee, S.C., 2005. Effect of dietary carbohydrate on haematology, respiratory

burst activity and histological changes in *L. rohita juveniles*. Fish and Shellfish Immunology 19, 331-344.
Kumaravel, T., Jha, A.N., 2006. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 605, 7-16.
Kumaravel, T., Vilhar, B., Faux, S., Jha, A., 2009. Comet Assay measurements: a perspective. Cell Biology and Toxicology 25, 53-64.
Lackner, R., 1998. Oxidative stress in fish by environmental pollutants. In: Braubeck, T., Hilton, D. E. and Streit, B., Editord, 1998, Fish Ecotoxicology, Birkhause Veriag, Basel, pp203-224.
Lam, P.K.S., 2009. Use of biomarkers in environmental monitoring. Ocean and Coastal Management 52, 348-354.
Landman, M.J., Van Den Heuvel, M.R., Ling, N., 2005. Relative sensitivities of common freshwater fish and invertebrates to acute hypoxia. New Zealand Journal of Marine and Freshwater Research 39, 1061-1067.
Lanno, R.P., Slinger, S.J., Hilton, J.W., 1985. Maximum tolerable and toxicity levels of dietary copper in rainbow trout (*Salmo gairdneri* Richardson) Aquaculture Production and Management 49, 257-268.
Lapointe, D., Couture, P., 2009. Influence of the route of exposure on the accumulation and subcellular distribution of nickel and thallium in Juvenile Fathead Minnows (*Pimephales promelas*). Archives of Environmental Contamination and Toxicology 57, 571-580.
Larsson, Å., Haux, C., Sjöbeck, M.-L., 1985. Fish physiology and metal pollution: Results and experiences from laboratory and field studies. Ecotoxicology and Environmental Safety 9, 250-281.
Lee, R.F., Steinert, S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutation Research/Reviews in Mutation Research 544, 43-64.
Li, X., Poon, C.-s., Liu, P.S., 2001. Heavy metal contamination of urban soils and street dusts in Hong Kong. Applied Geochemistry 16, 1361-1368
Li, C.-S., Wu, K.-Y., Chang-Chien, G.-P., Chou, C.-C., 2005. Analysis of Oxidative DNA damage 8-Hydroxy-2‘-deoxyguanosine as a biomarker of exposures to persistent pollutants for marine mammals. Environmental Science and Technology 39, 2455-2460.
Li, Q.Q., Loganath, A., Chong, Y.S., Tan, J., Obbard, J.P., 2006. Persistent organic pollutants and adverse health effects in humans. Journal of Toxicology and Environmental Health, Part A 69, 1987 - 2005.
Li, H., Mai, K., Ai, Q., Zhang, C., Zhang, L., 2009. Effects of dietary squid viscera meal on growth and cadmium accumulation in tissues of large yellow croaker, *Pseudosciaena crocea* R. Frontiers of Agriculture in China 3, 78-83.
Liepelt, A., Karbe, L., Westendorf, J., 1995. Induction of DNA strand breaks in rainbow trout *Oncorhynchus mykiss* under hypoxic and hyperoxic conditions. Aquatic Toxicology 33, 177-181.
Linder, M.C., 1991. Biochemistry of Copper. Plenum Press, New York.
Livingstone, D.R., Pipe, R.K., 1992. The mussel Mytilus: ecology, physiology, genetics and culture. Developments in aquaculture and fisheries science. Elsevier, Amsterdam.
Livingstone, D.R., 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by

invertebrates and fish. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 120, 43-49.
Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidativedamage in aquatic organisms. Marine Pollution Bulletin 42, 656-666.
Livingstone, D.R., 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Revue de Médecine Vétérinaire 6, 427-430.
Lloyd, D.R., Phillips, D.H., 1999. Oxidative DNA damage mediated by copper(II), iron(II) and nickel(II) Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 424, 23-36.
Llyod, R., 1961. Effect of dissolved oxygen concentrations on the toxicity of several poisons to rainbow trout (*Salmo gairdnerii* Richardson). Journal of Experimental Biology 38, 447-455.
Lock, R.A.C., 1975. Uptake of methylmercury by aquatic organisms from water and food. In: Sublethal effects of toxic chemicals on aquatic animals. In: Kroeman, J.H., Striks, J.J.T.W.H. (Eds.), Sublethal effects of toxic chemicals on aquatic animals, Elsevier, Amsterdam, pp. 61-79
Lorentzen, Maage, Julshamn, 1998. Supplementing copper to a fish meal based diet fed to Atlantic salmon parr affects liver copper and selenium concentrations. Aquaculture Nutrition 4, 67-72.
Lundebye, A.K., Berntssen, M.H.G., Bonga, S.E.W., Maage, A., 1999. Biochemical and physiological responses in atlantic salmon (*Salmo salar*) following dietary exposure to copper and cadmium. Marine Pollution Bulletin 39, 137-144.
Luoma, S. N., Carter, J.L., 1991. Effects of Trace Metals on Aquatic Benthos, Metal. Ecotoxicology, Concepts and Applications, In: Ecotoxicity: Concepts and Applications. Advances in Trace Substances Research Series. Lewis Publishers, Inc., Chelsea, Michigan, pp. 261-300.
Luoma, S.N., Rainbow, P.S., 2005. Why Is metal bioaccumulation so variable? biodynamics as a unifying concept. Environmental Science and Technology 39, 1921-1931.
Lushchak, V.I., Lushchak, L.P., Mota, A.A., Hermes-Lima, M., 2001. Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. American Journal of Physiology- Regulatory, Integrative and Comparative Physiology 280, R100-107.
Lushchak, V.I., Bagnyukova, T.V., Lushchak, O.V., Storey, J.M., Storey, K.B., 2005. Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. The International Journal of Biochemistry and Cell Biology 37, 1319-1330.
Lushchak, V.I., Bagnyukova, T.V., 2006. Effects of different environmental oxygen levels on free radical processes in fish. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 144, 283-289.
Lushchak, V.I., Bagnyukova, T.V., 2007. Hypoxia induces oxidative stress in tissues of a goby, the rotan *Perccottus glenii*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 148, 390-397.
Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101, 13-30.

Lykkeboe, G., Weber, R.E., 1978. Changes in the respiratory properties of the blood in the carp,*Cyprinus carpio*, induced by diurnal variation in ambient oxygen tension. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 128, 117-125.
Malik, N., Biswas, A., Qureshi, T., Borana, K., Virha, R., 2010. Bioaccumulation of heavy metals in fish tissues of a freshwater lake of Bhopal. Environmental Monitoring and Assessment 160, 267-276.
Mallatt, J., 1985. Fish gill structural changes induced by toxicants and otherirritants: a statistical review. Canadian Journal of Fisheries and Aquatic Sciences 42, 630–648.
Maltby, L., Snart, J.O.H., Calow, P., 1987. Acute toxicity tests on the freshwater isopod, Asellus aquaticus using $FeSO_4$. $7H_2O$, with special reference to techniques and the possibility of intraspecific variation. Environmental Pollution 43, 271-279.
Marnett, L.J., 1999. Lipid peroxidation—DNA damage by malondialdehyde. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 424, 83-95.
Martinez, C.B.R., Nagae, M.Y., Zaia, C.T.B.V., Zaia, D.A.M., 2004. Acute morphological and physiological effects of lead in the neotropical fish *Prochilodus lineatus*. Brazilian Journal of Biology 64, 797-807.
Martinovic, D., Villeneuve, D.L., Kahl, M.D., Blake, L.S., Brodin, J.D., Ankley, G.T., 2009. Hypoxia alters gene expression in the gonads of zebrafish (*Danio rerio*). Aquatic Toxicology 95, 258-272.
Matey, V., Richards, J.G., Wang, Y., Wood, C.M., Rogers, J., Davies, R., Murray, B.W., Chen, X.-Q., Du, J., Brauner, C.J., 2008. The effect of hypoxia on gill morphology and ionoregulatory status in the Lake Qinghai scaleless carp, *Gymnocypris przewalskii*. Journal of Experimental Biology 211, 1063-1074.
Mauderly, J.L., Samet, J.M., 2009. Is there evidence for synergy among air pollutants in causing health effects? Environmental Health Perspectives 11, 1-6.
McCarthy, J.F., Shugart, L.R., 1990. Biomarkers of environmental contamination, Liwes puplishers, Boca Ratan, Florida.
McElroy, A., Clark, C., Duffy, T., Cheng, B., Gondek, J., Fast, M., Cooper, K., White, L., 2012. Interactions between hypoxia and sewage-derived contaminants on gene expression in fish embryos. Aquatic Toxicology 108, 60-69.
Miller, A.T., 2005. The role of oxygen in metabolic regulation. Helgoland Marine Research 14, 392-406.
Miller, J.K., Brzezinska-Slebodzinska, E., Madsen, F.C., 1993a. Oxidative stress, antioxidants, and animal function. Journal of Dairy Science 76, 2812-2823.
Miller, P.A., Lanno, R.P., McMaster, M.E., Dixon, D.G., 1993b. Relative contributions of dietary and waterborne copper to tissue copper burdens and waterborne-copper tolerance in rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Fisheries and Aquatic Sciences 50, 1683-1689.
Miller, K.A., Addison, R.F., Bandiera, S.M., 2004. Hepatic CYP1A levels and EROD activity in English sole: biomonitoring of marine contaminants in Vancouver Harbour. Marine Environmental Research 57, 37-54.
Mitchelmore, C.L., Chipman, J.K., 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental

monitoring. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 399, 135-147.
Modig, H.G., Edgren, M., Re´ve´sz, L., 1974. Dual effect of oxygen on the induction and repair of single-strand breaks in the DNA of x-irradiated mammalian cells. International Journal of Radiation Biology 26, 341-353.
Mohamed, F.A.S., 2009. Histopathological studies on *Tilapia zillii* and *Solea vulgaris* from Lake Qarun, Egypt. World Journal of Fish and Marine Sciences 1, 29-39.
Mohsen, M.S., Jaber, J.O., 2003. Potential of industrial wastewater reuse. Desalination 152, 281-289.
Montaser, M., Mahfouz, M.E., El-Shazly, S.A.M., 2010. Toxicity of heavy metals on fish at Jeddah coast KSA: Metallothionein expressionas a biomarker and histopathological study on liver and gills. World Journal of Fish and Marine Sciences 2, 174-185.
Moore, M.N., Depledge, M.H., Readman, J.W., Leonard, D.R.P., 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 552, 247-268.
Mozaffarian, D., Rimm, E.B., 2006. Fish intake, contaminants, and human health. The Journal of the American Medical Association 296, 1885-1899.
Mudgal, V., Madaan, N., Mudgal, A., Singh, R.B., Mishra, S., 2010. Effect of toxic metals on human health. The Open Nutraceuticals Journal 3.
Mudre, J.M., Ney, J.J., 1986. Patterns of accumulation of heavy metals in the sediment of roadside streams. Archives of Environmental Contamination and Toxicology 15, 489-493.
Mustafa, S.A., Al-Subiai, S.N., Davies, S.J., Jha, A.N., 2011. Hypoxia-induced oxidative DNA damage links with higher level biological effects including specific growth rate in common carp, *Cyprinus carpio* L. Ecotoxicology 20, 1455–1466.
Mustafa, S.A., Davies, S.J., Jha, A.N., 2012. Determination of hypoxia and dietary copper mediated sub-lethal toxicity in carp, *Cyprinus carpio*, at different levels of biological organisation. Chemosphere 87, 413-420.
Muusze, B., Marcon, J., van den Thillart, G., Almeida-Val, V., 1998. Hypoxia tolerance of Amazon fish: Respirometry and energy metabolism of the cichlid *Astronotus Ocellatus*. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 120, 151-156.
Myers, M.S., Johnson, L.L., Hom, T., Collier, T.K., Stein, J.E., Varanasi, U., 1998. Toxicopathic hepatic lesions in subadult English sole (*Pleuronectes vetuls*) from Puget Sound, Washington, USA: Relationships with other biomarkers of contaminant exposure. Marine Environmental Research 45, 47-67.
Nadella, S., Grosell, M., Wood, C., 2007. Mechanisms of dietary Cu uptake in freshwater rainbow trout: evidence for Na-assisted Cu transport and a specific metal carrier in the intestine. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 177, 433-446.
Nero, V., Farwell, A., Lee, L.E.J., Van Meer, T., MacKinnon, M.D., Dixon, D.G., 2006. The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. Ecotoxicology and Environmental Safety 65, 252-264.

Ney, J.J., Van Hassel, J.H., 1983. Sources of variability in accumulation of heavy metals by fishes in a roadside stream. Archives of Environmental Contamination and Toxicology 12, 701-706.
Nikinmaa, M., 2002. Oxygen-dependent cellular functions--why fishes and their aquatic environment are a prime choice of study. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 133, 1-16.
Nilsson, G.E., Lofman, C.O., Block, M., 1995. Extensive erythrocyte deformation in fish gills observed by *in vivo* microscopy: apparent adaptations for enhancing oxygen uptake. Journal of Experimental Biology 198, 1151-1156.
Nilsson, S., Grove, D.J., 1974. Adrenergic and cholinergic innervation of the spleen of the cod: *Gadus morhua*. European Journal of Pharmacology 28, 135-143.
Novák, J., Benísek, M., Hilscherová, K., 2008. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. Environment International 34, 898-913.
Nuray, C., Bedii, C., Cahit, E., Ozcan, A., Celal, G., 2010. Accumulation of chromium in liver, gill and muscle tissues of *Orechromis niloticus* Journal of Animal and Veterinary Advances 9, 1958-1960.
Nussey, G., Van Vuren, J.H.J., Du Preez, H.H., 1995a. Effect of copper on the differential white blood cell counts of the Mozambique tilapia (*Oreochromis mossambicus*). Comparative Biochemistry and Physiology Part C: Comparative Pharmacology and Toxicology 111, 381-388.
Nussey, G., Van Vuren, J.H.J., du Preez, H.H., 1995b. Effect of copper on the haematology and osmoregulation of the Mozambique tilapia, *Oreochromis mossambicus* (Cichlidae). Comparative Biochemistry and Physiology Part C: Comparative Pharmacology and Toxicology 111, 369-380.
Ohe, T., Watanabe, T., Wakabayashi, K., 2004. Mutagens in surface waters: a review. Mutation Research/Reviews in Mutation Research 567, 109-149.
Oikari, A., 2006. Caging techniques for field exposures of fish to chemical contaminants. Aquatic Toxicology 78, 370-381.
Okino, S.T., Chichester, C.H., Whitlock, J.P., 1998. Hypoxia-inducible mammalian gene expression analyzed *in vivo* at a TATA-driven promoter and at an initiator-driven promoter. Journal of Biological Chemistry 273, 23837-23843.
Olive, P.L., 1999. DNA damage and repair in individual cells: applications of the comet assay in radiobiology. International Journal of Radiation Biology 75, 395-405.
Ostling, O., Johanson, K.J., 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochemical and Biophysical Research Communications 123, 291-298.
Pagenkopf, G.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environmental Science and Technology 17, 342-347.
Parry, H.E., Pipe, R.K., 2004. Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). Aquatic Toxicology 69, 311-325.
Part, P., Svanberg, O., 1981. Uptake of cadmium in perfused rainbow trout (*Salmo gairdneri*) gills. Canadian Journal of Fisheries and Aquatic Sciences 38, 917-923.

Peakall, D.B., Walker, C.H., 1994. The role of biomarkers in environmental assessment (3). Vertebrates. Ecotoxicology 3, 173-179.
Pedder, S.C.J., Maly, E.J., 1985. The effect of lethal copper solutions on the behavior of rainbow trout,*Salmo gairdneri*. Archives of Environmental Contamination and Toxicology 14, 501-507.
Pedersen, T.V., Bjerregaard, P., 2000. Cadmium influx and efflux across perfused gills of the shore crab, *Carcinus maenas*. Aquatic Toxicology 48, 223-231.
Pelgrom, S.M.G.J., Lock, R.A.C., Balm, P.H.M., Bonga, S.E.W., 1995. Integrated physiological response of tilapia, (*Oreochromis mossambicus*), to sublethal copper exposure. Aquatic Toxicology 32, 303-320.
Pelletier, E., Larocque, R., 1987. Bioaccumulation of mercury in starfish from contaminated mussels. Marine Pollution Bulletin 18, 482-485.
Pentreath, R.J., 1977. The accumulation of cadmium by the plaice, *Pleuronectes platessa* L. and the thornback ray, *Raja clavata* L. Journal of Experimental Marine Biology and Ecology 30, 223-232.
Perk, M.v.d., 2006. Soil and water contamination : from molecular to catchment scale. Taylor & Francis, London; New York pp 45-49.
Petersen, J.K., Pihl, L., 1995. Responses to hypoxia of plaice, *Pleuronectes platessa*, and dab, *Limanda limanda*, in the south-east Kattegat: distribution and growth. Environmental Biology of Fishes 43, 311-321.
Phillips, D.J.H., Rainbow, P.S., 1989. Strategies of trace metal sequestration in aquatic organisms. Marine Environmental Research 28, 207-210.
Pilgaard, L., Malte, H., Jensen, F.B., 1994. Physiological effects and tissue accumulation of copper inwet-water rainbow trout *Oncorhynchus mykiss* under normoxic and hypoxic conditions. Aquatic Toxicology 29, 197-212.
Poon, W.L., Hung, C.Y., Nakano, K., Randall, D.J., 2007. An *in vivo* study of common carp (*Cyprinus carpio* L.) liver during prolonged hypoxia. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 2, 295-302.
Prá, D., Franke, S., Giulian, R., Yoneama, M., Dias, J., Erdtmann, B., Henriques, J., 2008. Genotoxicity and mutagenicity of iron and copper in mice. BioMetals 21, 289-297.
Preston, S., Coad, N., Townend, J., Killham, K., Paton, G.I., 2000. Biosensing the acute toxicity of metal interactions: Are they additive, synergistic, or antagonistic? Environmental Toxicology and Chemistry 19, 775-780.
Price, N.M., Morel, F.M.M., 1990. Cadmium and cobalt substitution for zinc in a marine diatom. Nature 344, 658-660.
Prosi, F., 1989. Factors controlling biological availability and toxic effects of lead in aquatic organisms. The Science of The Total Environment 79, 157-169.
Puddu, P., Puddu, G.M., Cravero, E., Rosati, M., Muscari, A., 2008. The molecular sources of reactive oxygen species in hypertension. Blood Pressure 17, 70-77.
Rabenstein, D.L., 1978. The aqueous solution chemistry of methylmercury and its complexes. Accounts of Chemical Research 11, 100-107.
Rahman, M.S., Thomas, P., 2007. Molecular cloning, characterization and expression of two hypoxia-inducible factor alpha subunits, HIF-1α and HIF-2α, in a hypoxia-tolerant marine teleost, Atlantic croaker(*Micropogonias undulatus*). Gene 396, 273-282.

Rai, P.K., 2008. Heavy metal pollution in aquatic ecosystems and its phytoremediation using wetland plants: an ecosustainable approach. International Journal of Phytoremediation 10, 133 - 160.
Rainbow, P.S., 2007. Trace metal bioaccumulation: Models, metabolic availability and toxicity. Environment International 33, 576-582.
Raisuddin, S., Seong Lee, J., 2008. Fish models in impact assessment of carcinogenic potential of environmental chemical pollutants: an appraisal of hermaphroditic mangrove killifish *Kryptolebias marmoratus*. Interdisciplinary Studies on Environmental Chemistry—Biological Responses to Chemical Pollutants 1, 7-15.
Rathore, R.S., Khangarot, B.S., 2003. Effects of water hardness and metal concentration on a freshwater *Tubifex tubifex* Muller. Water, Air and Soil Pollution 142, 341-356.
Reeves, J.F., Davies, S.J., Dodd, N.J.F., Jha, A.N., 2008. Hydroxyl radicals (OH) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 640, 113-122.
Reik, W., W., D., Walter, J., 2001. Epigenetic reprogramming in mammalian development. Science 293, 1089-1093.
Rijnsdorp, A.D., 1990. The mechanism of energy allocation over reproduction and somatic growth in female North Sea plaice, *Pleuronectes platessa* L. Netherlands Journal of Sea Research 25, 279-289.
Ritola, O., Livingstone, D.R., Peters, L.D., Lindström-Seppä, P., 2002. Antioxidant processes are affected in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to ozone and oxygen-supersaturated water. Aquaculture 210, 1-19.
Roberts, R.J., 2001. The anatomy and physiology of teleosts. In: Eliss, A.E., Roberts, R.J. (Eds.), Fish pathology. USA, Philedaphia, pp. 12-55.
Roch, M., Nordin, R.N., Austin, A., McKean, C.J.P., Deniseger, J., Kathman, R.D., McCarter, J.A., Clark, M.J.R., 1985. The effects of heavy metal contamination on the aquatic biota of Buttle lake and the Campbell river drainage (Canada). Archives of Environmental Contamination and Toxicology 14, 347-362.
Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquatic Toxicology 22, 81-113.
Roméo, M., Siau, Y., Sidoumou, Z., Gnassia-Barelli, M., 1999. Heavy metal distribution in different fish species from the Mauritania coast. The Science of The Total Environment 232, 169-175.
Roser, S., Pool-Zobel, B.-L., Rechkemmer, G., 2001. Contribution of apoptosis to responses in the comet assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 497, 169-175.
Rytkönen, K.T., Vuori, K.A.M., Primmer, C.R., Nikinmaa, M., 2007. Comparison of hypoxia-inducible factor-1 alpha in hypoxia-sensitive and hypoxia-tolerant fish species. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 2, 177-186.
Sambrook, J., Fritcsh, E.F., Maniatis, T., 1989. Molecular cloing: A laboratory manual. Gold spring Harbour Labrotary Press, New York, p 3-19.
Sanchez, W., Porcher, J.-M., 2009. Fish biomarkers for environmental monitoring within the Water Framework Directive of the European Union. Trends in Analytical Chemistry 28, 150-158.

Saroglia, M., Terova, G., De Stradis, A., Caputo, A., 2002. Morphometric adaptations of sea bass gills to different dissolved oxygen partial pressures. Journal of Fish Biology 60, 1423-1430.
Schiedek, D., Sundelin, B., Readman, J.W., Macdonald, R.W., 2007. Interactions between climate change and contaminants. Marine Pollution Bulletin 54, 1845-1856.
Schroeder, W.H., Munthe, J., 1998. Atmospheric mercury. An overview. Atmospheric Environment 32, 809-822.
Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants inaquatic systems. Science 313, 1072-1077.
Scott, A.L., Rogers, W.A., 1980. Histological effects of prolonged sublethal hypoxia on channel catfish *Ictalurus punctatus* (Rafinesque). Journal of Fish Diseases 3, 305-316.
Sellers, C.M., Heath, A.G., Bass, M.L., 1975. The effect of sublethal concentrations of copper and zinc on ventilator activity, blood oxygen and pH in rainbow trout (*Salmo gairdneri*). Water Research. 9, 401–408.
Shaw, B.J., Handy, R.D., 2006. Dietary copper exposure and recovery in Nile tilapia, *Oreochromis niloticus*. Aquatic Toxicology 76, 111-121.
Sijm, D.T.H.M., Verberne, M.E., Pärt, P., Opperhuizen, A., 1994. Experimentally determined blood and water flow limitations for uptake of hydrophobic compounds using perfused gills of rainbow trout (*Oncorhynchus mykiss*): allometric applications. Aquatic Toxicology 30, 325-341.
Singh, N., Srivastava, A., 2010. Haematological parameters as bioindicators of insecticide exposure in teleosts. Ecotoxicology 19, 838-854.
Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175, 184-191.
Skidmore, J.F., Tovell, P.W.A., 1972. Toxic effects of zinc sulphate on the gills of rainbow trout. Water Research 6, 217-228.
Smit, G.L., Hattingh, J., 1978. The effect of respiratory stress on carp haemoglobin. Comparative Biochemistry and Physiology Part A: Physiology 59, 369-374.
Smith, R.W., Houlihan, D.F., 1995. Protein synthesis and oxygen consumption in fish cells. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 165, 93-101.
Soegianto, A., Charmantier, D.M., Trilles, J.P., Charmantier, G., 1999. .Impact of copper on the structure of gills and epipodites of the shrimp *Penaeus japonicus* (Decapoda). Journal of Crustacean Biology 19, 209-223.
Soitamo, A.J., Råbergh, C.M.I., Gassmann, M., Sistonen, L., Nikinmaa, M., 2001. Characterization of a hypoxia-inducible factor (HIF-1α) from rainbow trout. Journal of Biological Chemistry 276, 19699-19705.
Soivio, A., Nikinmaa, M., 1981. The swelling of erythrocytes in relation to oxygen affinity of the blood of the rainbow trout , *Salmo gairdneri*. In: Pickering, A.D. (Ed.), Stress and fish. Academic Press, London ; New York, p. 367.
Soivio, A., Westman, K., Nyholm, K., 1974. Changes in haematocrit values in blood samples treated with and without oxygen: a comparative study with four salmonid species. Journal of Fish Biology 6, 763-769.
Soldatov, A.A., 1996. The effect of hypoxia on red blood cells of flounder: a morphologic and autoradiographic study. Journal of Fish Biology 48, 321-328.

Spehar, R.L., Anderson, R.L., Fiandt, J.T., 1978. Toxicity and bioaccumulation of cadmium and lead in aquatic invertebrates. Environmental Pollution 15, 195-208.
Stagg, R.M., Shuttleworth, T.J., 1982. The accumulation of copper in *Platichthys flesus* L. and its effects on plasma electrolyte concentrations. Journal of Fish Biology 20, 491-500.
Stegeman, J.J., 1985. Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic. Marine Biology 89, 21-30.
Storey, K.B., Storey, J.M., 2004. Metabolic rate depression in animals: transcriptional and translational controls. Biological Reviews 79, 207-233.
Szakolczai, J., 1997. Histopathological changes induced by environmental stress in common carp, Japanese coloured carp, European eel, and African catfish. Acta Veterinaria Hungarica 45, 1-10.
Tao, S., Li, H., Liu, C., Lam, K.C., 2000. Fish uptake of inorganic and mucus complexes of lead. Ecotoxicology and Environmental Safety 46, 174-180.
Tao, S., Wen, Y., Long, A., Dawson, R., Cao, J., Xu, F., 2001. Simulation of acid-base condition and copper speciation in the fish gill microenvironment. Computers and Chemistry 25, 215-222.
Taylor, M.R., Harrison, P.T.C., 1999. Ecological effects of endocrine disruption: Current evidence and research priorities. Chemosphere 39, 1237-1248.
Teather, K., Parrott, J., 2006. Assessing the chemical sensitivity of freshwater fish commonly used in toxicological studies. Water Quality Research Journal of Canada 41, 100-105.
Terova, G., Rimoldi, S., Corà, S., Bernardini, G., Gornati, R., Saroglia, M., 2008. Acute and chronic hypoxia affects HIF-1α mRNA levels in sea bass *(Dicentrarchus labrax)*. Aquaculture 279, 150-159.
Thetmeyer, H., Waller, U., Black, K.D., Inselmann, S., Rosenthal, H., 1999. Growth of European sea bass (*Dicentrarchus labrax* L.) under hypoxic and oscillating oxygen conditions. Aquaculture 174, 355-367.
Thomas, P., Rahman, M.S., 2010. Region-wide impairment of Atlantic croaker testicular development and sperm production in the northern Gulf of Mexico hypoxic dead zone. Marine Environmental Research 69, 59-62.
Thophon, S., Kruatrachue, M., Upatham, E.S., Pokethitiyook, P., Sahaphong, S., Jaritkhuan, S., 2003. Histopathological alterations of white seabass, *(Lates calcarifer*), in acute and subchronic cadmium exposure. Environmental Pollution 121, 307-320.
Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/Comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environmental and Molecular Mutagenesis 35, 206-221.
Timbrell, J.A., 1998. Biomarkers in toxicology. Toxicology 129, 1-12.
Timmerman, C.M., Chapman, L.J., 2004. Hypoxia and interdemic variation in *Poecilia latipinna*. Journal of Fish Biology 65, 635-650.
Tomasallo, C., Anderson, H., Haughwout, M., Imm, P., Knobeloch, L., 2010. Mortality among frequent consumers of Great Lakes sport fish. Environmental Research 110, 62-69.
Ton, C., Stamatiou, D., Liew, C.-C., 2003. Gene expression profile of zebrafish exposed to hypoxia during development. Physiological Genomics 13, 97-106.
Trovó, A.G., Melo, S.A.S., Nogueira, R.F.P., 2008. Photodegradation of the pharmaceuticals amoxicillin, bezafibrate and paracetamol by the photo-

Fenton process. Application to sewage treatment plant effluent. Journal of Photochemistry and Photobiology A: Chemistry 198, 215-220.
Turner, K.J., Moore, J.W., Jones, A., Taylor, C.F., Cuthbert-Heavens, D., Han, C., Leek, R.D., Gatter, K.C., Maxwell, P.H., Ratcliffe, P.J., Cranston, D., Harris, A.L., 2002. Expression of hypoxia-inducible factors in human renal cancer. Cancer Research 62, 2957-2961.
Urban, J.D., Tachovsky, J.A., Haws, L.C., Wikoff Staskal, D., Harris, M.A., 2009. Assessment of human health risks posed by consumption of fish from the Lower Passaic River, New Jersey. Science of The Total Environment 408, 209-224.
Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety 64, 178-189.
Van Campenhout, K., Bervoets, L., Redeker, E.S., Blust, R., 2009. A kinetic model for the relative contribution of waterborne and dietary cadmium and zinc in the common carp (*Cyprinus carpio*). Environmental toxicology and Chemistry 28, 209-219.
Van Campenhout, K., Infante, H.G., Adams, F., Blust, R., 2004. Induction and binding of Cd, Cu, and Zn to metallothionein in carp (*Cyprinus carpio*) using HPLC-ICP-TOFMS. Toxicological Sciences 80, 276-287.
van den Thillart, G., van Waarde, A., 1985. Teleosts in hypoxia: Aspects of anaerobic metabolism. Molecular Physiology 8, 393-409.
van den Thillart, G., Vianen, G., Zaagsma, J., 2002. Adrenergic regulation of lipid mobilization in fishes; a possible role in hypoxia survival. Fish Physiology and Biochemistry 27, 189-204.
van der Meer, D.L.M., van den Thillart, G.E.E.J.M., Witte, F., de Bakker, M.A.G., Besser, J., Richardson, M.K., Spaink, H.P., Leito, J.T.D., Bagowski, C.P., 2005. Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 289, R1512-R1519.
van der Oost, R., Lopes, S.C.C., Komen, H., Satumalay, K., van den Bos, R., Heida, H., Vermeulen, N.P.E., 1998. Assessment of environmental quality and inland water pollution using biomarker responses in caged carp (*Cyprinus carpio*): Use of a bioactivation:detoxication ratio as a Biotransformation Index (BTI). Marine Environmental Research 46, 315-319.
van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13, 57-149.
Van Hassel, J.H., Ney, J.J., Garling, D.L., 1980. Heavy metals in a stream ecosystem at sites near highways. Transactions of the American Fisheries Society 109, 636-643.
van Raaij, M.T.M., Breukel, B.-J., van den Thillart, G.E.E.J.M., Addink, A.D.F., 1994. Lipid metabolism of goldfish, (*Carassius auratus* L.) during normoxia and anoxia. Indications for fatty acid chain elongation. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 107, 75-84.
Veltman, K., Huijbregts, M.A.J., Kolck, M.v., Wang, W.-X., Hendriks, A.J., 2008. Metal bioaccumulation in aquatic species: Quantification of uptake and elimination rate constants using physicochemical properties of metals

and physiological characteristics of species. Environmental Science & Technology 42, 852-858.
Vogt, G., Quinitio, E.T., 1994. Accumulation and excretion of metal granules in the prawn, *Penaeus monodon*, exposed to water-borne copper, lead, iron and calcium. Aquatic Toxicology 28, 223-241.
Voigt, H.-R., 2007. Heavy metal (Hg, Cd, Zn) concentrations and condition of eelpout (*Zoarces viviparus* L.), around Baltic sea. Polish Journal of Environmental Studies 16, 909-917.
Vosylienė, M., Kazlauskienė, N., Svecevičius, G., 2003. Effect of a heavy metal model mixture on biological parameters of rainbow trout *Oncorhynchus mykiss*. Environmental Science and Pollution Research 10, 103-107.
Vosyliene, M.Z., 1996. The effect of long term exposure to copper on physiological parametersof rainbow trout (*Onchorhynchus mykiss*). Acta Zoologica Lituanica 1, 3-6.
Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B., 2001. Major classes of pollutant. In: Principles of ecotoxicology, 2nd ed. Taylor and Francis, London, New York, pp 3-6.
Wang, W.-X., Rainbow, P.S., 2008. Comparative approaches to understand metal bioaccumulation in aquatic animals. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 148, 315-323.
Wang, W., 1987. Factors affecting metal toxicity to (and accumulation by) aquatic organisms. Overview. Environment International 13, 437-457.
Watanabe, T., Kiron, V., Satoh, S., 1997. Trace minerals in fish nutrition. Aquaculture 151, 185-207.
Wedemeyer, G.A., 1997. Effects of rearing conditions on the healthand physiological quality of fish in intensive culture. In: Iwama.
Wells, R.M.G., Grigg, G.C., Beard, L.A., Summers, G., 1989. Hypoxic responses in a fish from a stable environment: Blood oxygen transport in the antarctic fish *Pagothenia borchgrevinki*. Journal of Experimental Biology 141, 97-111.
Wepener, V., Van Vuren, J.H.J., Du Preez, H.H., 1992a. The effect of hexavalent chromium at different pH values on the haematology of *Tilapia sparrmanii* (Cichlidae). Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 101, 375-381.
Wepener, V., Vuren, J.H., Preez, H.H., 1992b. Effect of manganese and iron at a neutral and acidic pH on the hematology of the banded tilapia (*Tilapia sparrmanii)*. Bulletin of Environmental Contamination and Toxicology 49, 613-619.
West, T.G., Boutilier, R.G., 1998. Metabolic suppression in anoxic frog muscle. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 168, 273-280.
WHO, 1995. Lead. Environmental Health Criteria, vol. 165. Geneva: World Health Organization. CHF 43. (CHF 30.10 in developing countries) ISBN 92-4-157165-9.
Wiener, J.G., Martini, R.E., Sheffy, T.B., Glass, G.E., 1990. Factors influencing mercury concentrations in Walleyes in northern Wisconsin lakes. Transactions of the American Fisheries Society 119, 862-870.
Woodward, D.F., Brumbaugh, W.G., DeLonay, A.J., Little, E.E., Smith, C.E., 1994. Effects of rainbow trout fry of a metals-contaminated diet of benthic

invertebrates from the Clark Fork River, Montana. Transactions of the American Fisheries Society 123, 51-62.
Wright, D.A., Pamela, W., 2002. Environmental Toxicology. Cambridge University Press, Cambridge, UK.
Wu, R.S.S., 2002. Hypoxia: from molecular responses to ecosystem responses. Marine Pollution Bulletin 45, 35-45.
Wurgler, F.E., Kramers, P.G.N., 1992. Environmental effects of genotoxins (eco-genotoxicology). Mutagenesis 7, 321-327.
Yang, P., Mao, R., Shao, H., Gao, Y., 2009. An investigation on the distribution of eight hazardous heavy metals in the suburban farmland of China. Journal of Hazardous Materials 167, 1246-1251.
Yilmaz, A.B., 2003. Levels of heavy metals (Fe, Cu, Ni, Cr, Pb, and Zn) in tissue of *Mugil cephalus* and *Trachurus mediterraneus* from Iskenderun Bay, Turkey. Environmental Research 92, 277-281.
Yilmaz, A.B., Sangün, M.K., YaglIoglu, D., Turan, C., 2010. Metals (major, essential to non-essential) composition of the different tissues of three demersal fish species from Iskenderun Bay, Turkey. Food Chemistry 123, 410-415.
Yim, J.H., Kim, K.W., Kim, S.D., 2006. Effect of hardness on acute toxicity of metal mixtures using *Daphnia magna*: Prediction of acid mine drainage toxicity. Journal of Hazardous Materials 138, 16-21.
Yin, Z., Lam, T.J., Sin, Y.M., 1995. The effects of crowding stress on the non-specific immuneresponse in fancy carp (*Cyprinus carpio* L.). Fish & Shellfish Immunology 5, 519-529.
Yourtee, D.M., Elkins, L.L., Nalvarte, E.L., Smith, R.E., 1992. Amplification of doxorubicin mutagenicity by cupric ion. Toxicology and Applied Pharmacology 116, 57-65.
Zhao, T.B., Ning, H.X., Zhu, S.S., Sun, P., Xu, S.X., Chang, Z.J., Zhao, X.Q., 2004. Cloning of hypoxia-inducible factor 1α cDNA from a high hypoxia tolerant mammal—plateau pika (*Ochotona curzoniae*). Biochemical and Biophysical Research Communications 316, 565-572.
Zhong, H., Agani, F., Baccala, A.A., Laughner, E., Rioseco-Camacho, N., Isaacs, W.B., Simons, J.W., Semenza, G.L., 1998. Increased expression of hypoxia inducible factor-1α in rat and human prostate cancer. Cancer Research 58, 5280-5284.
Zhou, J., Zhu, X.-s., Cai, Z.-h., City, S., 2009. Endocrine disruptors: an overview and discussion on issues surrounding their impact on marine animals. Environmental Research 2, 7-17.
Zhou, Q., Zhang, J., Fu, J., Shi, J., Jiang, G., 2008. Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. Analytica Chimica Acta 606, 135-150.

Appendix I

Published papers

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*)

R.M.W. Ferguson[1], D.L. Merrifield[1], G.M. Harper[2], M.D. Rawling[1], S. Mustafa[1], S. Picchietti[3], J.L. Balcázar[4] and S.J. Davies[1]

1 Aquaculture and Fish Nutrition Research Group, School of Marine Science and Engineering, Marine Institute, University of Plymouth, Plymouth, UK
2 Electron Microscopy Centre, University of Plymouth, Plymouth, UK
3 Department Environmental Sciences, Tuscia University, Viterbo, Italy
4 Catalan Institute for Water Research, Science and Technology Park of the University of Girona, Girona, Spain

Keywords
fish, health, histology, PCR-DGGE, probiotic.

Correspondence
Daniel L. Merrifield, Aquaculture and Fish Nutrition Research Group, School of Marine Science and Engineering, Marine Institute, University of Plymouth, Plymouth, UK.
E-mail: daniel.merrifield@plymouth.ac.uk

2010/0098: received 19 January 2010, revised 18 February 2010 and accepted 22 February 2010

doi:10.1111/j.1365-2672.2010.04713.x

Abstract

Aim: To assess *Pediococcus acidilactici* as a dietary supplement for on-growing red tilapia (*Oreochromis niloticus*).

Methods and Results: Tilapia were fed either a control diet or control diet supplemented with *Ped. acidilactici* at 10^7 CFU g^{-1} for 32 days. *Ped. acidilactici* colonized the intestinal tract and significantly affected the intestinal microbial communities. PCR-DGGE revealed direct antagonism of gastric *Ped. acidilactici* with an endogenous uncultured bacterium during a period of reverting to nonsupplemented feeding. Light microscopy revealed that gut integrity and leucocyte levels were unaffected by *Ped. acidilactici*; however, blood leucocyte levels and serum lysozyme activity were elevated after 14-days' feeding. No significant improvements in growth performance were observed at the end of the trial (day 32), but survival was significantly higher in the probiotic group.

Conclusions: The study demonstrates that oral supplementation of *Ped. acidilactici* modulates intestinal bacterial communities in on-growing red tilapia and also stimulates some aspects of the nonspecific immune response.

Significance and Impact of the study: To our knowledge this is the first study assessing the effects of probiotics on the gut microbiota of tilapia using culture-independent methods. Such methods are crucial to understand the mechanisms which underpin and mediate host benefits.

Introduction

Over the past decade, new initiatives towards the assessment and appraisal of probiotic applications have demonstrated a range of benefits in fish (Carnevali *et al.* 2004, 2006; Panigrahi *et al.* 2004, 2005, 2007; Picchietti *et al.* 2007, 2009) including tilapia *Oreochromis niloticus* (Lara-Flores *et al.* 2003; El-Haroun *et al.* 2006; Pirarat *et al.* 2006; Shelby *et al.* 2006; Taoka *et al.* 2006; Aly *et al.* 2008a,b; Wang *et al.* 2008). These studies provide a basis of the effects on health parameters and growth performance. However, our understanding of the mechanisms involved in mediating these responses is limited because many studies have lacked investigations of the gut microbiota or have employed culture-dependent methods. We must have a clear understanding on the effects of probiotics on the indigenous gut microbiota to begin to understand the underlying mechanisms which result in host benefits.

Pediococcus acidilactici, a lactic acid bacteria (LAB), has recently been reported to alleviate vertebral column compression syndrome, elevate blood leucocyte levels,

Ecotoxicology and Environmental Safety 74 (2011) 1913–1920

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

A multiple biomarker approach to investigate the effects of copper on the marine bivalve mollusc, *Mytilus edulis*

Sherain N. Al-Subiai, A. John Moody, Sanaa A. Mustafa, Awadhesh N. Jha *

Ecotoxicology Research and Innovation Centre, School of Biomedical and Biological Sciences, University of Plymouth, Plymouth, PL4 8AA, UK

ARTICLE INFO

Article history:
Received 15 November 2010
Received in revised form
4 July 2011
Accepted 17 July 2011
Available online 17 August 2011

Keywords:
Copper
Genotoxicity
Total glutathione
Histopathology
Clearance rate
Biomarkers
Bivalve mollusc

ABSTRACT

While copper (Cu) is considered to be an essential trace element for many organisms, overexposure to this metal can induce a wide spectrum of effects including DNA damage. Given that Cu is a highly relevant contaminant in the marine environment, we aimed to evaluate the induction of DNA strand breaks (using the comet assay) in haemocytes and concurrently also determined biological responses at higher levels of biological organisation in bivalve molluscs, *Mytilus edulis*, following exposure for 5 days to a range of environmentally realistic levels of Cu (18–56 $\mu g\,l^{-1}$). Prior to evaluation of genetic damage, the maximum tolerated concentration (MTC) was also determined, which was found to be ($100\,\mu g\,l^{-1}$) above which complete mortality over the exposure period was observed. In addition to DNA damage, levels of glutathione in adductor muscle extracts, histopathological examination of various organs (*viz.*, adductor muscle, gills and digestive glands) and clearance rates as a physiological measure at individual level were also determined. Furthermore, tissue-specific accumulation and levels of Cu in water samples were also determined using ICP-MS. There was a strong concentration-dependant induction for DNA damage and total glutathione levels increased by 1.8-fold at 56 $\mu g\,l^{-1}$ Cu. Histological examination of the organs showed qualitatively distinct abnormalities. Clearance rate also showed a significant decrease compared to controls even at the lowest concentration (i.e. 18 $\mu g\,l^{-1}$; $P=0.003$). Cu levels in adductor muscle ($P=0.012$), digestive gland ($P=0.008$) and gills ($P=0.002$) were significantly higher than in the control. The multi-biomarker approach used here suggests that in some cases clear relationships exist between genotoxic and higher level effects, which could be adopted as an integrated tool to evaluate different short and long-term toxic effects of pollutants.

1. Introduction

The marine environment has long been subjected to contamination by heavy metals (e.g. As, Cd, Cr, Co, Cu, Pb, Hg and Ni), either of natural origin, such as volcanic activity, windblown dust or decaying vegetation, or from anthropogenic sources related to industrial processes and the use of antifouling paints (Rainbow, 2002). World production of copper (Cu) has increased in the last few decades and contamination by Cu has become increasingly prevalent in marine environments (IPCS, 1998), and this is likely to increase in coming decades. In relatively unpolluted coastal waters Cu concentrations are less than 5 ppb (Soegianto et al., 1999) but can reach 3 ppm in heavily polluted areas (Parry and Pipe, 2004). The increasing Cu concentrations in marine ecosystems are therefore a potential threat to living organisms. Although Cu is essential for normal functioning within organisms (e.g. being a cofactor in many enzymes), it can be toxic if present in high levels or if organisms are exposed chronically to low levels in the environment (Gaetke and Chow, 2003).

Long-term exposure of organisms to Cu is typically associated with impairment of feeding mechanisms (Nicholson, 2003), growth rates and reproduction (Fitzpatrick et al., 2008) and increased susceptibility to disease and to development of histopathological abnormalities (Zorita et al., 2006). Cu toxicity can result from its binding to DNA (Lloyd and Phillips, 1999) and proteins involved in DNA replication, transcription and repair, leading to impairment of these processes (Nor, 1987). However, Cu toxicity seems mostly to be a consequence of oxidative stress (Gaetke and Chow, 2003). Cu can directly induce oxidative stress by catalysing hydroxyl radical ($OH^{\bullet}$) production in a Fenton-like reaction leading potentially to damage to every class of biological molecule including DNA (Becker et al., 1996; Bremner, 1998; Gaetke and Chow, 2003). It is also possible that it can cause oxidative stress indirectly by binding to reduced glutathione (Friedman et al., 1989), thereby depleting a key antioxidant.

While there is relatively good understanding of Cu toxicity and bioaccumulation in aquatic organisms from earlier studies, to our knowledge there has been no integrated study where effects at

* Corresponding author. Fax: +44 1752 584605.
E-mail address: a.jha@plymouth.ac.uk (A.N. Jha).

0147-6513/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2011.07.012

Cell Tissue Res (2011) 344:135–146
DOI 10.1007/s00441-010-1125-y

REGULAR ARTICLE

Effect of dietary alginic acid on juvenile tilapia (*Oreochromis niloticus*) intestinal microbial balance, intestinal histology and growth performance

Daniel L. Merrifield · Glenn M. Harper · Sanaa Mustafa · Oliana Carnevali · Simona Picchietti · Simon J. Davies

Received: 13 September 2010 / Accepted: 17 December 2010 / Published online: 22 January 2011

Abstract The aim of the present study was to assess the effect of a commercial alginic acid source (Ergosan) on tilapia *Oreochromis niloticus* intestinal microbial balance, intestinal morphology, and growth parameters. Fish were fed a basal control diet or the basal diet plus a source of alginic acid (5 gkg^{-1} Ergosan; Schering-Plough Aquaculture, UK) for 9 weeks. At the end of the trial, light and electron microscopy demonstrated that the morphology of the intestinal tract at the gross and ultra-structural level was not affected by dietary alginic acid inclusion. Both groups of fish displayed healthy, normal morphology with no signs of disease, cell or tissue damage. Intestinal epithelial leucocyte infiltration was not affected by dietary alginic acid. Molecular bacterial profiles derived from PCR-DGGE illustrated highly similar microbial communities (both within the lumen and associated with the intestinal mucosa) in the respective treatment groups. Microbial ecological parameters (e.g. species diversity and richness) also remained unaffected. Although not significant, trends towards elevated survival and body protein content were observed in the alginic acid-fed fish. These results are suggestive that alginic acid does not adversely impact the indigenous gastrointestinal microbial balance and subsequently does not impact upon the epithelial brush border integrity. Validation of non-detrimental impacts of immunostimulatory products on gastric microbiota and epithelial integrity should be pursued in future studies as maintaining microbial balance and epithelial integrity is essential for proper gut functionality.

Keywords Gut microbiota · DGGE · Alginic acid · Ultra histology · Intestine

D. L. Merrifield (✉) · S. Mustafa · S. J. Davies
Aquaculture and Fish Nutrition Research Group,
School of Biomedical and Biological Sciences,
The University of Plymouth,
Plymouth, Devon, UK PL4 8AA
e-mail: daniel.merrifield@plymouth.ac.uk

G. M. Harper
Electron Microscopy Centre, University of Plymouth,
Plymouth, UK

O. Carnevali
Department of Marine Sciences,
Polytechnic University of Marche,
Ancona, Italy

S. Picchietti
Department Environmental Sciences, Tuscia University,
Viterbo, Italy

Introduction

The use of preventive approaches to combat disease, essential for further development of sustainable aquaculture practices, is becoming increasingly important. Promising among these preventative approaches are immunostimulants. Immunostimulants are compounds able to stimulate the immune response by promoting phagocytic cell function, increasing their bactericidal activity and/or non-specific cytotoxic cells and antibody production (Sakai 1999). Although treatment and prevention of infectious diseases are the most common reasons to use immunomodulators, amelioration of stress-induced immunosupression and improved general animal well being are also reported benefits (Blecha 2001; Vollostad et al. 2006).

A range of different substances are known to act as immunostimulants but few are suitable for use in aquacul-

Ecotoxicology (2011) 20:1455–1466
DOI 10.1007/s10646-011-0702-5

Hypoxia-induced oxidative DNA damage links with higher level biological effects including specific growth rate in common carp, *Cyprinus carpio* L.

Sanaa A. Mustafa · Sherain N. Al-Subiai · Simon J. Davies · Awadhesh N. Jha

Accepted: 7 May 2011 / Published online: 8 June 2011

Abstract Both hypoxia and hyperoxia, albeit in different magnitude, are known stressors in the aquatic environment. Adopting an integrated approach, mirror carp (*Cyprinus carpio* L.), were exposed chronically (i.e. 30 days) to hypoxic (1.8 ± 1.1 mg O_2 l^{-1}) and hyperoxic (12.3 ± 0.5 mg O_2 l^{-1}) conditions and resultant biological responses or biomarkers were compared between these two treatments as well as with fish held under normoxic conditions (7.1 ± 1.04 mg O_2 l^{-1}). The biomarkers determined included the activities of glutathione peroxidase (GPx), measurement of oxidative DNA damage (using modified Comet assay employing bacterial enzymes: Fpg and Endo-III), haematological parameters, histopathological and ultrastructural examination of liver and gills. Specific growth rate (SGR) of the fish, as an important ecotoxicological parameter was also determined over the exposure period. The study suggested that while the levels of hepatic GPx were unaffected, there was a significant difference in activity in the blood plasma under different exposure conditions; the hyperoxic group showed increased GPx activity by approximately 37% compared to normoxic group and the hypoxic group showed a decrease by approximately 38% than the normoxic group. Interestingly, oxidative DNA damage was significantly higher in both hypoxic and hyperoxic by approximately 25% compared to normoxic conditions, Fpg showing enhanced level of damage compared to the Endo-III treatment ($P < 0.001$). The haematological parameters showed enhanced values under hypoxic conditions. Transmission electron microscopic (TEM) studies revealed damage to liver and gill tissues for both the treatments. Interestingly, SGR of fish was significantly lowered in hypoxic by approx. 30% compared to normoxic condition and this was found to be correlated with DNA damage ($R = -0.82$; $P = 0.02$). Taken together, these results indicate that prolonged exposure to both hypoxic and hyperoxic conditions induce oxidative stress responses at both DNA and tissue levels, and hypoxia can result in compensatory changes in haematological and growth parameters which could influence Darwinian fitness of the biota with wider ecological implications.

Keywords Hypoxia · Hyperoxia · Oxidative DNA damage · Histopathology · Ultrastructural changes · Specific growth rate · Carp fish

S. A. Mustafa · S. N. Al-Subiai · S. J. Davies · A. N. Jha (✉)
School of Biomedical & Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK
e-mail: a.jha@plymouth.ac.uk

Introduction

Chronic exposures to both hyperoxia and hypoxia could be damaging to aquatic organisms leading to suboptimal growth and biomass production (Wedemeyer 1997). Although lack of dissolved oxygen (DO) or hypoxia ($DO < 2.8$ mg l^{-1}) could be a natural phenomenon caused by daily fluctuations in oxygen concentrations (Nikinmaa 2002), chronic hypoxic conditions prevailing in so called 'dead zones' in different parts of the world, which is linked to anthropogenic activities, often leads to mass mortality of sensitive biota and could lead to overall reduction in biodiversity (Diaz and Rosenberg 2008). With respect to hypoxia-induced biological responses, most of the mechanistic studies have been carried out using mammalian cells under in vitro conditions (Wu 2002). Our understanding of molecular responses using in vitro models therefore requires further elucidation at whole organism level.

Chemosphere 87 (2012) 413–422

Contents lists available at SciVerse ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Determination of hypoxia and dietary copper mediated sub-lethal toxicity in carp, *Cyprinus carpio*, at different levels of biological organisation

Sanaa A. Mustafa, Simon J. Davies, Awadhesh N. Jha *

School of Biomedical & Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK

ARTICLE INFO

Article history:
Received 29 September 2011
Received in revised form 8 December 2011
Accepted 13 December 2011
Available online 10 January 2012

Keywords:
Hypoxia
Dietary copper
Oxidative DNA damage
Histopathology
Ultrastructural changes
Cyprinus carpio

ABSTRACT

Hypoxic events frequently occur in the aquatic environment in association with micro pollutants, including heavy metals. Only a few studies are however available on the uptake and biological responses of heavy metals under hypoxic conditions. To elucidate the phenomenon, mirror carp *Cyprinus carpio* L. (16.13–16.22 g) were exposed chronically to dietary copper (Cu; 250 and 500 mg kg dry wt.$^{-1}$) for 30 d under normoxic (8.25 mg O_2 L^{-1}) and hypoxic (~3 mg O_2 L^{-1}) conditions and adopting an integrated approach, sub-lethal biomarker responses were determined at different levels of biological organisation. Level of oxidative DNA damage (as determined by modified Comet assay) showed strong significant difference following exposure to dietary Cu level under normoxic (1.6-fold) as well as under hypoxic condition at both Cu levels (2.1 and 2.5-folds respectively). Significant difference was also observed for haematological parameters (i.e. increased red and white blood cells, haematocrit value and haemoglobin concentration). Quantitative histology revealed alterations in tissues (i.e. liver and gills) for hypoxic and all dietary Cu treatment groups under both normoxic and hypoxic conditions suggesting a compensatory response to these organs ($p < 0.05$). The order of Cu accumulation in tissues (as determined by ICP-OES) was liver > intestine > kidney > gill. Interestingly, SGR under both normoxic and hypoxic conditions reduced with elevating Cu levels ($p = 0.019$). Overall, the results provide evidence for enhanced toxicological responses in fish following exposure to Cu either alone or in combination with hypoxic condition and lends support to the evolving viewpoint that many water quality guidelines should be revisited in terms of new ecotoxicological criteria.

1. Introduction

Mostly linked with anthropogenic activities, hypoxia or temporal depletion of oxygen is now considered to be amongst the most pressing and critical problems for the hydrosphere in the world. Although common in both freshwater and marine environments, its impact on densely populated coastal regions with intense aquaculture activities, creating so called 'dead zones' is of particular concern (Diaz and Rosenberg, 2008). Needless to mention, decreases in oxygen concentration has profound detrimental impact on the biological functions of the organism, oxidative stress being one of the mechanisms of production of these responses (Lushchak, 2011; Mustafa et al., 2011). It is also being realised that hypoxia could enhance the vulnerability to environmental chemicals in aquatic organisms by impairing the physiology and food/contaminant uptake rate (Hattink et al., 2005). In this context, aquatic ecosystems that undergo seasonal hypoxia can also be concurrently tainted with contaminants such as heavy metals (Diaz and Rosenberg, 2008). With rapid industrialisation and population growth, industrial effluents and domestic sewage containing diverse range and large quantities of potentially toxic metals are being discharged in the aquatic environment with long term consequences for the sustainability (Mohsen and Jaber, 2003; Jha, 2004, 2008; Rai, 2008). In addition, the presence of some metals has also been suggested to mimic the hypoxic action in the aquatic environment (Kubrak et al., 2011). In common with other contaminants, the presence of heavy metals and variable oxygen availability may interact differentially to exert detrimental effects on aquatic ecosystems which need further elucidation to disentangle cause-effect coupling at different levels of biological organisation for hazard and risk assessment.

Whilst copper (Cu) is essential for normal physiological functioning, it is toxic at elevated concentrations for aquatic life (Carvalho and Fernandes, 2006). World production of Cu has increased in the last few decades and contamination by Cu has become increasingly prevalent in the aquatic environment (IPCS, 1998) which is likely to increase bearing in mind manufacture and disposal of wide varieties of Cu-based products including agrochemicals. In relatively unpolluted coastal waters Cu concentrations are less than 5 ppb, but may reach 3 ppm in heavily polluted areas (Soegianto et al., 1999; Parry and Pipe, 2004). Furthermore, the transfer of metals through

* Corresponding author. Tel.: +44 1752 584633; fax: +44 1752 584605.
E-mail address: a.jha@plymouth.ac.uk (A.N. Jha).

0045-6535/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2011.12.037

http://www.omicsonline.org/jardhome.php

ISSN: 2155-9546

Journal of Aquaculture Research & Development

Open Access

Aquaculture plays a vital role in the economical betterment of a country. Aquaculture also termed as aqua farming is the cultivation of plants or animals such as fish, shellfish and seaweed, in natural or controlled marine or freshwater environments.

Journal of Aquaculture Research & Development is an International, open access journal which publishes hypothesis-driven papers on applied or scientific research relevant to freshwater aquaculture, brackish water aquaculture and marine aquaculture.

Journal of Aquaculture Research & Development - Open Access using online manuscript submission, review and tracking systems of Editorial Manager® for quality and quick review processing. Submit your manuscript at http://www.editorialmanager.com/environsci/

Editors & Editorial Board

OMICS Publishing Group

5716 Corsa Ave., Suite 110, Westlake, Los Angeles, CA 91362-7354, USA, Phone: +1- 650-268-9744, Fax: +1-650-618-1414, Toll free: +1-800-216-6499

Aquaculture
Research & Development

Harper et al. J Aquac Res Development 2011, S1
http://dx.doi.org/10.4172/2155-9546.S1-004

Research Article Open Access

An ex vivo approach to studying the interactions of probiotic *Pediococcus acidilactici* and *Vibrio* (*Listonella*) anguillarum in the anterior intestine of rainbow trout *Oncorhynchus mykiss*

Glenn M. Harper[1], Imad P. Saoud[2], Matthew Emery[3], Sanaa Mustafa[3], Mark Rawling[3], Ben Eynon[3], Simon J. Davies[3] and Daniel L. Merrifield[3]*, Matthias Monfort

[1]*Electron Microscopy Centre, The University of Plymouth*
[2]*American University of Beirut, Beirut, Lebanon*
[3]*The School of Biomedical and Biological Sciences, The University of Plymouth, Plymouth, UK*

Abstract

The aim of the present study was to observe the antagonistic relationship of the probiotic *Pediococcus acidilactici* and the pathogen *Vibrio* (*Listonella*) *anguillarum* in the intestine of rainbow trout (*Oncorhynchus mykiss*) by using the ex vivo intestinal sac method. Rainbow trout (240-250g) were fed either a control diet (commercial diet: 43% protein, 20% lipid) or a probiotic diet (control diet supplemented with *P. acidilactici* [Bactocell] at log 7 CFU g^{-1}) for two weeks. The anterior intestine was then isolated for an ex vivo challenge study and intestinal sacs formed by shutting one end of the intestinal portion. The sacs were filled with PBS solutions containing either no bacteria (as a control), the probiotic (*P. acidilactici*), the pathogen *Vibrio anguillarum*, or probiotic+pathogen and incubated for one hour. At the end of the exposure bacterial levels in the lumen were determined by culture based approaches and colonisation of the mucosa assessed with PCR-DGGE and electron microscopy (EM). Intestinal morphology (observation of gross morphological damage, ultrstructural differences and the quantification of goblet cells and intra epithelial lecuocyte numbers) and bacterial infection/translocation was assessed using light microscopy (LM) and EM.

Results revealed that *V. anguillarum* caused extensive histological damage to the gut but *P. acidilactici* did not. No signs of translocation of either the probiotic or pathogen were observed. Microbiological analyses indicated that P. acidilactici was able to outcompete V. anguillarum in the rainbow trout intestine and also to populate or colonise the mucosa. Additionally, elevated leucocyte levels and goblet cells in the epithelium of P. acidilactici fed fish, and intestines exposed to *P. acidilactici*, suggests that *P. acidilactici* might have potential use in controlling vibriosis. In vivo disease-challenge studies are warranted to ascertain if *V. anguillarum* infections can be controlled in rainbow trout using dietary applications of *P. acidilactici*.

Keywords: Fish, Vibriosis, Disease, Health, Microbiology, Bactocell

Introduction

Evidence suggests that the gastrointestinal (GI) tract of fish is a major portal of entry for a range of fish pathogens including *Vibrio* (*Listonella*) *anguillarum*, the causative agent of vibriosis [1,2]. Recent studies have sought to use probiotics to fortify the intestinal microbiome of fish and enhance the defensive barrier against such diseases [3,4]. Such studies have shown a great deal of success and several probiotic strains have been demonstrated to elevate disease resistance of trout to pathogenic bacteria [3,5-7]. The mechanisms which mediate these benefits are thought to result from host immunostimulation and competitive exclusion. Such conclusions are based on empirical evidence but information regarding probiotic-pathogen antagonism in fish studies are primarily in vitro based, with evidence that probiotics are able to outcompete or antagonise (via the production of bacteriocins, organic acids, siderophores, competition for adhesion sites etc) pathogens on agar plates, broth cultures or in adhesion assays. However, such information does not fully represent probiotic vs. pathogen interactions in situ (within the fish GI tract) where localised responses such as gut associated lymphoid tissues (GALT) and antimicrobial properties of intestinal mucus are likely to be an important factor. Therefore, we aim to further the present knowledge by observing the antagonistic relationship of probiotics and pathogens in the intestine of fish by using the ex vivo intestinal sac method [8,9]. Due to previous evidence suggesting a positive role as a probiotic for fish [10-12], *Pediococcus acidilactici* was used as the probiotic and because of pathogenicity and industrial relevance, *V. anguillarum* was used as the pathogen in the present study.

Materials and Methods

Experimental fish and diets

Female rainbow trout were acquired from a local fish farm (Hatchlands Fisheries, Greyshoot Lane, Rattery, South Brent, UK) and transported to the Aquaculture and Fish Nutrition Research facility at The Univeristy of Plymouth, UK. Fish were offered a commercial trout diet (Skretting, Skretting, 43% protein, 20% lipid) *ad libitum* for four weeks. After grading, batches of 20 fish (240-250g) were randomly distributed into six 150 L fibreglass tanks, each provided with 98% re-circulated aerated freshwater at a rate of 150 L hr^{-1}. Tanks were randomly divided among two treatments with three replicate tanks per treatment. Fish in treatment-1 were offered a control diet whilst fish in treatment-2 were offered the same diet supplemented with *P.*

***Corresponding author:** Daniel L. Merrifield, The School of Biomedical and Biological Sciences, The University of Plymouth, Plymouth, UK. Tel: +44 -0- 1752 584888; E-mail: daniel.merrifield@plymouth.ac.uk

Received July 04, 2011; **Accepted** September 01, 2011; **Published** September 10, 2011

Citation: Harper GM, Monfort M, Saoud IP, Emery M, Mustafa S, et al. (2011) An ex vivo approach to studying the interactions of probiotic *Pediococcus acidilactici* and *Vibrio* (*Listonella*) *anguillarum* in the anterior intestine of rainbow trout *Oncorhynchus mykiss*. J Aquac Res Development S1:004. doi:10.4172/2155-9546.S1-004

Appendix II

Poster presentation

Dead zones, hypoxia and fish health: an integrated study to determine impact of oxidative stress in fish

Sanaa A. Mustafa, Sherain N. Al-Subiai, Simon J. Davies and Awadhesh N. Jha

School of Biological Sciences, University of Plymouth, Plymouth, PL4 8AA

Background

❖ Oxidative stress may lead to pathophysiological conditions and could severely affect health and fitness of humans and natural organisms[1]

❖ *Hypoxia* and *hyperoxia* are potential environmental stressors in the aquatic environment, particularly in intensive aquaculture[2]

❖ No scientific studies have evaluated the impact of different levels of oxygen concurrently at different levels of biological organisation

Aims & objectives

❖ Investigate the effects of chronic *hypoxia* and *hyperoxia* on a model fish species, *Cyprinus carpio* (mirror carp) at different levels of biological organisation (viz. biochemical, DNA and tissue levels)

❖ Compare the degree of biological responses under different exposure conditions

❖ Link and correlate the responses with 'fitness' and growth of carp

Experimental design & methodologies

❖ 36 carp (118 ± 2.4 g) distributed and acclimatised in 6 tanks in duplicate (i.e. 6 fish tank^{-1} in duplicate)

❖ Fish exposed to three oxygen levels: 7.1 ± 0.4 mg l^{-1} *(normoxia)*; 1.8 ± 0.1 mg l^{-1} (*hypoxia*- by lowering the dissolved oxygen level using nitrogen gas; purity 99.99%), and 12.3 ± 0.2 mg l^{-1} (*hyperoxia*- by oxygen enrichment: purity 99.95%) for 30 days (Figure.1)

❖ Following 30 days exposure period:

- Blood samples collected to study the induction of DNA strand breaks applying modified comet assay using two bacterial enzymes: *Formamidopyrimidine DNA glycosylase* (FPG) and *Endonuclease III* (Endo III) to determine oxidative DNA damage in the erythrocytes[3]
- Glutathione peroxidise (GPx), an anti- oxidative enzyme activity measured in liver and plasma[2]
- Ultrastructural changes in the tissues (e.g. liver, gill) using TEM[4]

Figure1. Experimental setup.

Results

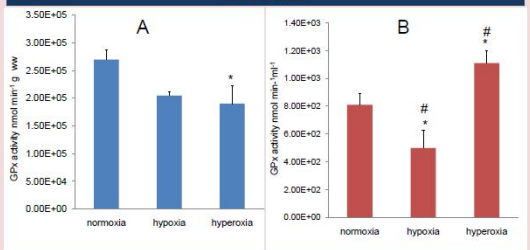


Figure 2. Activity of GPx (A) in liver (B) in plasma, following 30 days of exposure to *normoxia* , *hypoxia* and *hyperoxia*. Values are mean ± S.E. (*) significant differences from *normoxic* group (#) indicates significant differences between groups.

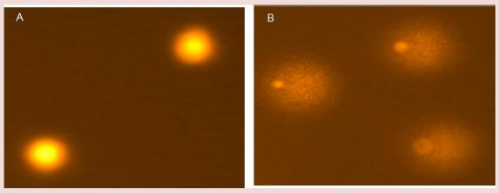


Figure 3. Comet response in erythrocytes of carp: (A) normal cell (B) damaged cell.

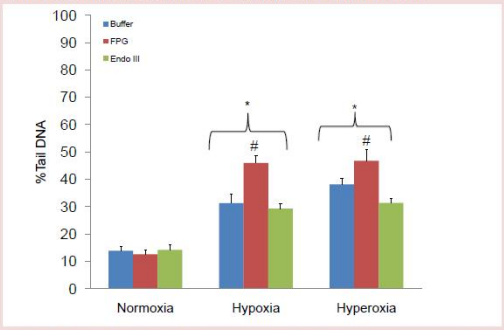


Figure 4. DNA single strand break in mirror carp erythrocytes following 30 days exposure to *normoxia*, *hypoxia* and *hyperoxia*. Values are mean ± SE. n = 6, (*) statistically significant different versus *normoxia*, (#) statistically significant different versus buffer and EndoIII.

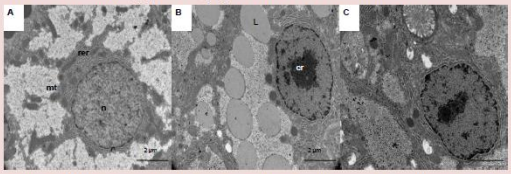


Figure 5. Transmission electron microscopy (TEM) images of liver: (A) *normoxic:* nucleus (n), mitochondria (mt), rough endoplasmic reticulum (rer) (B) *hypoxic:* increased number of lipid droplets (L) and condensed chromatin (cr) (C) *hyperoxic:* large and increased number of mitochondria and condensed chromatin.

Conclusions

❖ Compared to *normoxia*, both *hypoxia* and *hyperoxia* induced DNA damage; oxidised purines showing higher levels of damage compared to pyrimidines; no significant difference observed between oxidised pyrimidine and controls for *hypoxic* and *hyperoxic* conditions

❖ GPx activity significantly decreased in the *hyperoxic* liver compared to the *normoxic* conditions; In the plasma compared to controls, the levels decreased in *hypoxic* conditions and increased in *hyperoxic* condition, indicating some compensatory response mechanism

❖ TEM studies revealed ultrastructural changes in different tissues (i.e. liver and gill)

❖ Different exposure conditions also affected the specific growth rate (SGR) of the fish

References

1. Barker GF *et al.* (2006) *Am. J Respiratory Cell and Molec. Biol. 35, 277-288.*

2. Lushchak V &, Bagnyukova TV (2006) *Comp. Biochem and Physiol. Part B:* 144, 283-289.

3. Frenzilli G. *et al.* (2004) *Mutation Res.* 552, 187-195.

4. Au DWT *et al.* (1999) *Environ. Pollu.* 104, 235-247.


AN INTEGRATED APPROACH TO DETERMINE EFFECTS OF COPPER IN BIVALVE MOLLUSC, *Mytilus edulis*

Sherain N. Al-Subiai, A. John Moody, Sanaa A. Mustafa and Awadhesh N. Jha

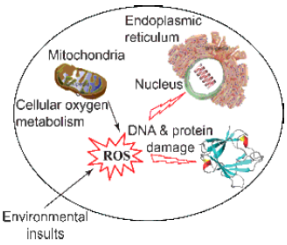
School of Biological Sciences, University of Plymouth, Plymouth, PL4 8AA, UK

Background

➢ Copper (Cu) is an essential trace element for growth and metabolism

➢ Overexposure to Cu could induce production of reactive oxygen species (ROS) thereby causing oxidative damage, resulting in patho-physiological conditions

➢ Environmental levels of Cu range from 0.03 - 100 µg l^{-1} in water and 3.6 – 280 mg kg^{-1} in sediment



Aims

➢ To adopt an integrated approach to evaluate the impact of Cu at different levels of biological organisation in the marine mussel, *Mytilus edulis*

➢ To correlate the levels of exposure with biological responses or biomarkers

Methods

Mussels were collected from a reference site (Cornwall, UK), allowed to acclimatize (2 wks) and then exposed to Cu (Bolognesi *et al.* ,1999), as shown in Figure 1

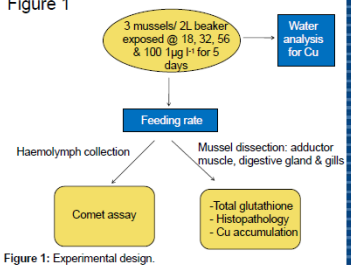


Figure 1: Experimental design.

Results

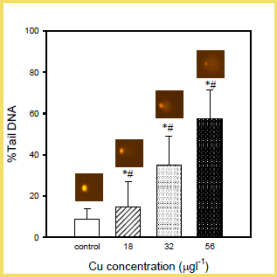


Figure 2: DNA strand breaks, in *Mytilus edulis* haemocytes. The values are mean ± S.E. (*) indicates significant difference from the control. (#) indicates a significant difference between treatments.

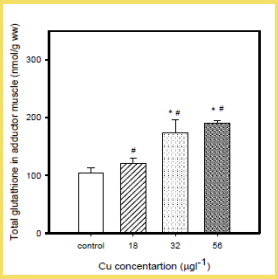


Figure 3: Levels of total glutathione content . The values are mean ± S.E. (*) indicates significant difference from the control. (#) indicates a significant difference between treatments.

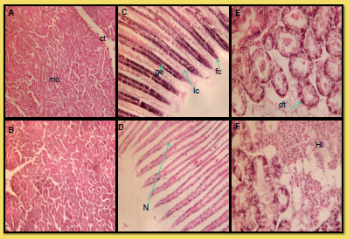


Figure 4: Light micrographs of sections through adductor muscle, gills & digestive gland of *M. edulis* showing histological structure of control and treated mussels stained with H & E at 5-8 µm thickness. (A, C & E) control; (B, D & F) exposed to Cu. mc = myocyte cell; ct = connective tissue; gf = gill filaments; fc = frontal cilia; lc = lateral cilia; ge = gill epithelium; dt = digestive tubules; HI = haemocyte infiltration; N = necrosis.

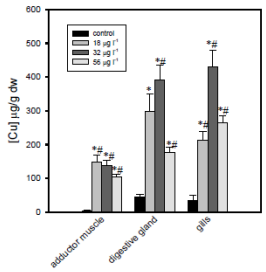


Figure 5: Copper concentrations in tissues. Data are mean ± S.E., n = 12 mussel per treatment. (*) indicate significant difference from the control, (#) indicates a significant difference between treatment.

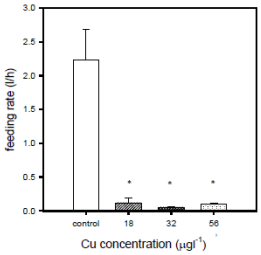


Figure 6: Feeding rates of *M. edulis* (n = 12/exposure). The values are mean ± S.E. (*) indicates significant difference from the control.

Conclusions

➢ Environmentally relevant Cu exposure capable of inducing DNA damage & antioxidant defence

➢ Damage at lower levels precipitate at higher levels of biological organisation

References

Bignell *et al.* (2008) *Aquatic Biol.* 2: 1-15.
Bolognesi *et al.* (1999) *Environ Mol Mutagen.* 33: 287-292.
Jha *et al.* (2005) *Mutat Res.* 588: 47-57.
Owen & Belcher (1965) *Biochem. J.* 94: 705-711.

Acknowledgements

Financial support: Kuwait Institute for Scientific Research

Linking DNA damage with specific growth rate: differentiating nutritional and ecotoxicological impact of dietary copper in fish

Sanaa A. Mustafa, Simon J. Davies and Awadhesh N. Jha

School of Biomedical & Biological Sciences, University of Plymouth, Plymouth, PL4 8AA

Introduction

❖ Copper (Cu) is an essential trace element required in small concentrations for all organisms including fish[1]

❖ Cu can have adverse toxicological impacts on fish health due to its extreme persistence, high toxicity, affinity to build up in the food chain, bioaccumulation and its high availability via many and various anthropogenic sources[2]

❖ Cu also has effects on fitness parameters and DNA integrity at elevated concentrations[3]

Aims & Objectives

❖ To investigate the effects of chronic exposure to dietary Cu on a model fish species, *Cyprinus carpio* (mirror carp) at different levels of biological organisation (i.e. DNA, tissue or whole organism level)

❖ Compare the degree of biological responses including the specific growth rate (SGR) under different dietary Cu concentrations to discriminate nutritional & ecotoxicological response

Experimental Design & Methodologies

❖ 120 carp (8.23 ± 0.14 g) distributed and acclimatised in 12 tanks in triplicate (i.e. 10 fish tank^{-1} in triplicate)

❖ Fish exposed chronically for 10 weeks to three levels of dietary copper : 250 (low); 500 (medium) & 1000 mg Cu kg^{-1} d.w. (high)

❖ Following 10 weeks exposure period:

- Blood samples collected to analyse the induction of DNA strand breaks applying modified comet assay using two bacterial enzymes: *Formamidopyrimidine DNA glycosylase* (FPG) and *Endonuclease III* (Endo III) to determine oxidative DNA damage in the erythrocytes[4]

- Cu analysis (ICP-OES) in different tissues (liver, intestine, gill and bone)[5]

- Histopathological & ultrastructural changes in the tissues (e.g. liver, intestine) using TEM[6]

Results

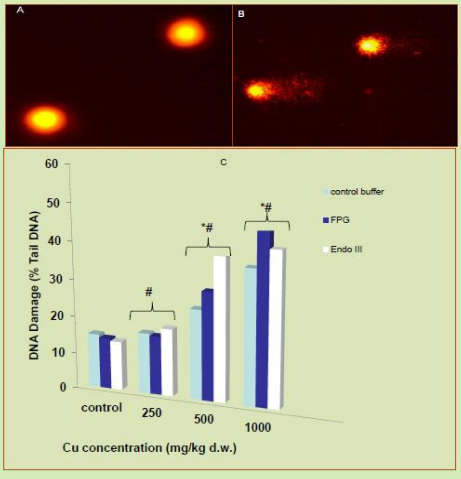


Fig. 1: (A) Comet response in erythrocytes of carp: normal cell (B) damaged cell (C) DNA single strand breaks in mirror carp erythrocytes following 10 weeks exposure to dietary copper. Values are means ± S.E. n = 6, (*) significantly different versus control, (#) significant difference between groups (P<0.05, ANOVA).

Table1. Accumulation of copper (µg Cu g^{-1}) in organs of *Cyprinus carpio* fed diets containing different levels of Cu mg kg^{-1} over 10 weeks. Data are mean ± S.E. Groups with different alphabetic superscripts indicate significant difference at P < 0.05; (n=6).

Tissue	Cu concentrations (mg kg^{-1} dw)			
	Control	250	500	1000
Liver	201.55 ± 9.04^{a}	339.99 ± 184.22^{a}	679.40 ± 155.21^{ab}	737.03 ± 162.02^{b}
Intestine	22.73 ± 2.64^{a}	71.64 ± 25.73^{ab}	212.12 ± 40.73^{b}	336.11 ± 92.68^{b}
Gill	3.93 ± 0.66^{a}	7.25 ± 0.63^{a}	19.61 ± 1.05^{a}	87.68 ± 56.30^{b}
Bone	2.45 ± 0.69^{a}	3.23 ± 0.63^{a}	3.63 ± 0.35^{a}	5.76 ± 3.02^{a}

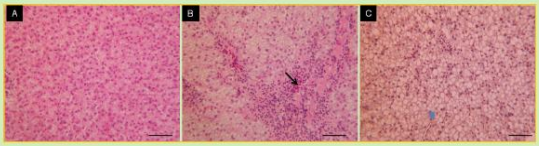


Fig. 2: Light micrograph sections showing histological structures through liver from control and dietary Cu exposure fish stained with H&E at 5-8 µm thickness. (A) control liver showing normal histology, ((B &C) 1000 mg Cu kg^{-1} d.w. showed hepatocellular coagulative necrosis & infiltrateration of mononuclear inflammatory cells (black arrow),& lipid vacuolation of hepatocytes (blue arrow). Scale bar: 50 µm.

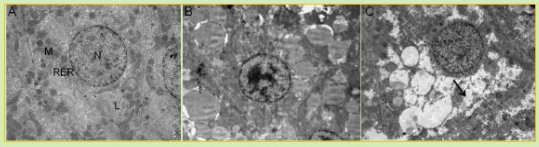


Fig. 3: Transmission electron microscopy images: (A) *control* liver nucleus (N), mitochondria (M), rough endoplasmic reticulum (RER), lipid droplet (L) (B&C) 1000 mg Cu kg^{-1} d.w. showing increased number of lipid droplets &coagulative necrosis (black arrow). Scale bar: 2 µm.

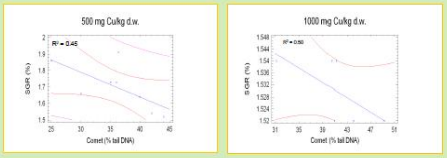


Fig. 4: Linear regression analysis illustrating correlations for, DNA damage and SGR in *Cyprinus carpio* following 10 weeks exposure to dietary Cu The solid line is a linear regression and the dashed lines represent 95% confidence limits.

Conclusions

❖Compared to control both dietary Cu exposures (medium and high) induced DNA damage; significant difference observed between dietary exposure groups

❖ The order of Cu accumulation in tissues: liver > intestine > gill > bone

❖ Histopathological and TEM studies revealed changes in liver

❖Cu supplemented groups also affected the growth of the fish

❖DNA damage linked with SGR

References

1. Watanabe T *et al.* (1997) *Aquaculture.* 151,185-207.
2. Zhou Q *et al.* (2008) *Analytica Chimica Acta.* 606, 135-150.
3. Atienzar FA *et al.* (2001) *Environ. Toxi.* 59, 241-250.
4. Reeves JF *et al.* (2008) *Mutation. Res.* 640, 113-122.
5. Handy RD *et al.* (2000) *J. Exp. Biol.* 203, 2365-2377.
6. Au DWT *et al.* (1999) *Environ. Pollu.* 104, 235-247.

Appendix III

Diploma supplement

Diploma Supplement

The University of Plymouth

DIPLOMA SUPPLEMENT

INFORMATION IDENTIFYING THE HOLDER OF THE QUALIFICATION

Surname
Mustafa

First Name(s)
Sanaa

Date of Birth
01.10.1969

Student Reference Number
10133033

INFORMATION IDENTIFYING THE QUALIFICATION

Name of Qualification and title conferred
PhD

Title of thesis
An integrated approach to assess impact of environmental stress in carp, *Cyprinus carpio* L.: Biochemical, genotoxic, histopathological and individual level effects

Names of Supervisors
Awadhesh Jha

Name(s) of collaborating institution(s)
University of Plymouth

Language of thesis
English

INFORMATION ON THE LEVEL OF THE QUALIFICATION

Level of the Qualification
PhD

Mode of study (full time/part time/mixed mode)
Full time

Official length of the programme
4 years

Completion date
12.03.2012

FURTHER INFORMATION

Professional Status

Official Stamp of the Graduate School

Diploma supplement Appendix

RESEARCH SKILLS

Subject Specific Skills Training completed

Course Title	Module Code	Date Attended	Performance Pass/Dist or %	Credit rating and level
Postgraduate Research Skills and method in biology	BioL 5124	Oct. 2007-Feb. 2008	61.3	20
Advanced techniques in aquatic biology	Bio 5101	Oct. 2007-Jan. 2008	71.80	10
Academic English for Research Student		Sep. 2007-Apr. 2008		
English Summer/Winter program For Research Students	ELC004	Jun 2008-Sep. 2008		A

Verified by Director of Studies

Name …….. Signature …… Date ……

Generic Skills Training completed

Course Title	Joint Research Councils Skills Statement category	Date Attended	Location of Course
Home office licence-1-3 fish module-licence gained for fish species		14-15th April 2008	Plymouth Marine Laboratory
General Teaching Associates Course (GTA)		6-10 July 2009	University of Plymouth
Histopathology workshop		10-14 May 2010	(CEFAS)/ Weymouth

Verified by Graduate School Skills Development Officer

Name …….. Signature …… Date ……

Other Skills Training completed (e.g UKGRAD School; English/Foreign Language) and/or conferences/meetings attended

Title	Location	Organisers	Date Attended
Getting started with Microsoft office 2007	Babbage building	IT Training and Documentation Team	10th Dec. 2007
Excel 2007 - further features	Babbage building	IT Training and Documentation Team	1rd Feb. 2008
Your words or other peoples? Plagiarism	Babbage building	Ms Pollyanna Magne	13th may 2008
Endnote Bibliographic For Beginnings	Babbage building	Professor Peter Brooks	30th may 2008
Endnote Bibliographic Referencing - Advanced	Babbage building	Professor Peter Brooks	13th Aug. 2008
preparing to transfer	Roland Levinsky building	Professor Mick Fuller	5th Nov.2008
Microsoft excel 2007Introduction to essential features	Babbage building	Jacqui Hunter	20th Nov. 2008
Introduction to E Resources	Babbage building	Library academic support	18th Nov. 2008
Stress management skills session	Roland Levinsky building	Sarah Kearns	23Oct. 2009
Preparing for the Viva	Rolle building	Professor Mick Fuller	5th Mar. 2009
Marine Institute Research Centre showcase day	Roll building		13th Mar. 2010
GenStatistics	Scott Building	Paul Hewson	25 October 2010
Brixham Environmental laboratory Aston Zeneca	Roland Levinsky Building Room 210		18th Sep. 2008
EndNote Users Clinic	Library Room 114		17 March 2011
Training for Metabolomics (workshop)	University of Birmingham, UK		27-31 Oct. 2011

Verified by Director of Studies

Name …….. Signature …… Date ……

RESEARCH OUTPUTS

Seminar/Conference/Performance presentations

Title of Paper or Performance	Title of Meeting & location	Poster or Oral paper	Date	Published* or not published
An integrated approach to determine effects of copper in bivalve mollusc, *Mytilus edulis*.	Plymouth Marine Sciences Partnership Symposium: Marine Science for a Changing World, Plymouth, UK	Poster	April 2009	Published (Abstract)
An integrated approach to determine oxidative stress in mirror carp *Cyprinus carpio*.	15th International Symposium on Pollutant in Marine Organisms. Bordeaux, France,	Poster	May 2009	Published (Abstract)
Linking DNA damage with specific growth rate: differentiating nutritional and ecotoxicological impact of dietary copper in fish	Annual Meeting of the European Environmental Mutagen Society (EEMS), Oslo, Norway	Poster	Sept 2010	Published (Abstract)
The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*)	Journal of Applied Microbiology	Paper	109, 851-862. 2010	Published
A multiple biomarker approach to investigate the effects of copper on the marine bivalve mollusc, *Mytilus edulis*.	Ecotoxicology and Environmental Safety	Paper	74**,** 1913-1920. 2011	Published
Effect of dietary alginic acid on juvenile tilapia (*Oreochromis niloticus*) intestinal microbial balance, intestinal histology and growth performance	Cell and Tissue Research	Paper	344, 1-12. 2011	Published
Hypoxia-induced oxidative DNA damage links with higher level biological effects including specific growth rate in common carp, *Cyprinus carpio* L	Ecotoxicology	Paper	20**,** 1455–1466,2011	Published
Determination of hypoxia and dietary copper mediated sub-lethal toxicity in carp, *Cyprinus carpio*, at different levels of biological organisation	Chemosphere	Paper	87, 413-422, 2012.	Published

An ex vivo approach to studying the interactions of probiotic *Pediococcus acidilactici* and Vibrio (*Listonella*) anguillarum in the anterior intestine of rainbow trout *Oncorhynchus mykiss*	Journal of Aquaculture Research & Development	Paper	doi.org/10.4172/2155-9546.S1-004.	Published

Verified by Director of Studies

Name …….. Signature …… Date ……

PUBLISHED REFEREED/EDITED PUBLICATIONS

* Give the full bibliographic reference (including Journal ISSN No., DOI (Digital Object Identifier) and Web address if appropriate)

Verified by Director of Studies

Name …….. Signature …… Date ……

Official Stamp of the Graduate School