NTNU
Norwegian University of
Science and Technology

Uptake, organ distribution and physiological effects of an anti-diabetic II drug (metformin) in Atlantic salmon (Salmo salar)

Typhaine Le Doujet

Biotechnology
Submission date: June 2016
Supervisor: Augustine Arukwe, IBI

Norwegian University of Science and Technology
Department of Biology

Preface and acknowledgments

This master thesis was accomplished at the the Norwegian University of Science and Technology (NTNU) at Department of Biology from 2014 to 2016. The author wishes to acknowledge and thank the Department of Biology and Biotechnology at the Norwegian University of Science and Technology for providing me with the opportunity to gain knowledge and to experience the Norwegian lifestyle.

First and foremost, I would like to extend my deepest gratitude to my supervisor Professor Augustine Arukwe, for his guidance, continuous encouragement, discussion capability and support during the last year, and of course for providing me with very interesting industrial cases that will shape my thinking for the rest of my career. I am especially thankful that he welcomed me in his group so that I can perform my research.

I also wish to thank friends, fellow students especially Mohammed and Nhung, colleagues and professionals within NTNU laboratories for their support and SeaLab especially senior engineer Randi Røsbak for her help during the fish experiment and her patience when providing me with training. I am also very thankful to Elvira Mennillo, PhD exchange from Italy for her support in laboratory with the enzymatic assay, and our mutual help when needed. And for her support in general. Also thanks to Professor Monika from Germany for assisting with the analysis. My special thanks go to my family for their support.

Trondheim, June, 2016

Typhaine Le Doujet

Abstract

Pharmaceutical and personal care products (PPCPs) are recognized as emerging contaminants due to their high occurrence and persistence in the environment. Metformin is an anti-diabetic II drug and one of the most prescribed PPCPs and found in relatively high concentration in the environment. Upon metformin ingestion by diabetic people, the drug is not metabolized and is excreted as non-modified molecule in the urines to reach the treatment facilities where it is unfortunately not well degraded. Metformin can reach the aquatic environment where aquatic organism might absorb the drug and probably result in its way back up to the food chain. Some studies has showed relatively high amount of metformin in sludge and waste water treatment plants. Therefore, it is important to perform health risk assessment on such xenobiotic and for that it is necessary to have good knowledge about PPCPs chemical structure, uptake and translocation, and physiological effect in aquatic species. However, there is lack of data concerning fish and particularly with metformin. In this study, the uptake, organ distribution and physiological effects of metformin in Juvenile Atlantic salmon (*Salmo Salar*) were investigated. Four group containing respectively five Juvenile Atlantic salmon were exposed to different concentrations of metformin (0, 5, 50 or 500µg/L) during 3, 7 and 10 days. Chemical analysis was performed in order to determine uptake, distribution in organ of the drug. Gene expression was performed with Real-time RT-PCR in order to analyse the transcript level of various genes. First, StAR and P450scc genes, involved in the steroidogenesis were investigated in the brain. Other genes including those responsible for phase I and II biotransformation (cyp1a1, cyp3a, GST), multi-xenobiotic transporters (P-glycoprotein and multi-R), organic anion transporters (OATs) and protection against oxidative stress was also studied. In addition, biochemical analysis was

performed in order to determine the functional level of the two key proteins in the steroidogenesis (StAR and P450scc) with immunochemical analysis (western blot) and also the enzyme activity of four selected antioxidant enzymes were performed spectrophotometrically. Finally, the potential neurotoxicity of metformin was investigated by the determination of acetylcholine esterase (AChE) activity. This study identified overall uptake of metformin which was found to be the highest in gills, but still at a relatively low concentration level. Metformin caused temporarily increase in the gene expression of StAR and P450scc gene, but without change in protein expression. The two biotransformation enzymes cyp1a1 and cyp3a were either inhibited or increased depending on time exposure, but without showing much concentration dependence. No toxicity by accumulation of exogenous and endogenous compounds was apparent due to absence of important inhibition for the two multi-xenobiotic resistance proteins p-gp and multi-R. The organic anion transporters were not affected by metformin. It appears that oxidative stress was not induced upon metformin treatment. Finally, metformin did not induce neurotoxicity in Juvenile Atlantic salmon. Overall, metformin seems to cause some biological response in juvenile Atlantic salmon without potential biological effects. Further study is required to confirm the biological and experimental variations.

CONTENTS

Preface and acknowledgments.....i
Abstract..... iii
CONTENTS.....vi
1 Introduction 1
1.1 Pharmaceutical and personal care products in the environment .1
1.2 Metformin.....4
1.2.1 Presence in the environment 7
1.3 Toxicity 7
1.4 Specific uptake and distribution.....8
1.5 Physiological changes 9
1.5.1 Metabolism in toxicity 9
1.5.2 Multi-xenobiotic (drug) resistance proteins (MXR) 15
1.5.3 ABC transporters 16
1.5.4 Steroidogenesis 18
1.5.5 Oxidative stress.....20
1.5.6 Neurotransmitters.....25
1.6 Juvenile Salmon as Model.....26
1.7 Studies outline and objectives 26
2 Materials and Methods 28
2.1 Chemicals and reagents.....28
2.2 Experimental design.....28
2.2.1 Fish.....28
2.2.2 cDNA synthesis 29
2.2.3 Exposure 31

2.2.4 Sampling 31
2.3 Chemical analysis for bioaccumulation 31
2.4 Gene expression analysis 33
2.4.1 RNA Isolation 33
2.4.2 RNA concentration and its quality 36
2.4.3 Real-time polymerase chain reaction 38
2.4.4 Primer testing 40
2.4.5 Normalization of data 42
2.5 Biochemical analysis 43
2.5.1 Preparation of post-mitochondrial fractions (PMF) 44
2.5.2 Bradford 45
2.5.3 Determination of enzyme activity 46
2.5.4 Acetylcholine esterase activity 53
2.5.5 Western Blotting 54
2.6 Statistical analysis 57
3 Results 59
3.1 Biometric parameters 59
3.2 Metformin uptake and accumulation 60
3.2.1 Uptake and accumulation of metformin 60
3.3 Bioconcentration factor (BCF) 61
3.4 Physiological parameters 62
3.4.1 Phase I and II biotransformation 62
3.4.2 Membrane transporters and Multidrug Resistant Protein 64
3.4.3 Steroidogeneisis proteins 67
3.5 Western blot 69
3.6 Oxidative stress responses 71
3.6.1 Acetylcholine esterase (AchE) 79
4 Discussion 80
4.1 Exposure and methods 80

4.1.1 Exposure 80
4.1.2 RNA and its quality 81
4.1.3 Primers 81
4.1.4 Normalization and quantification 82
4.2 Statistical analysis 82
4.3 Experimental data....85
4.3.1 Length and weight....85
4.3.2 Uptake and accumulation of metformin 86
4.3.3 Effects on Steroidogenesis....87
4.3.4 Effects on biotransformation system 91
4.3.5 Effect on multi-xenobiotic membrane and organic anion transporters 94
4.3.6 Effect on oxidative stress....97
4.4 Neurotoxic effects 104
5 Future studies....105
6 Conclusion....106
7 References 108

Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
cDNA	Complementary deoxyribonucleic acid
CDNB	1-Chloro-2,4-dinitrobenzene
CoA	Coenzyme A
Ct	Cycle threshold
CYP1A1	Cytochrome P450 1
CYP3A	Cytochrome P450 3
ddH2O	Double-distilled water
dw	Dry weight
dH2O	Distilled water
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DTNB	Ellman's Reagent, 5,5'-Dithiobis-(2-Nitrobenzoic Acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GPx	Glutathione peroxidase
GR	Glutathione reductase

GSH	Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
KH_2PO_4	Potassium phosphate (K-phosphate)
MDR	Multi-drug resistance
MXR	Multi-xenobiotics resistance
mM	millimoles L-1
mRNA	Messenger ribonucleic acid
MQ-water	Milli-Q water
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nano meters
·O2	Superoxide anion
·OH	Hydroxyl radical
PCR	Polymerase chain reaction
P450scc	Cytochrome P450 side-chain cleavage
RNA	Ribonucleic acid RNase RNA nuclease
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
StAR	Steroidogenic acute regulatory protein
Tm	Melting temperature
Vtg	Vitellogenin

1 Introduction

The world is facing a constant increase of environmental contamination by a wide range of pollutants. Environmental contaminants are described as any potentially hazardous substances that are introduced into the environment either by accident or deliberately and may have the capacity to harm wildlife, plants, humans (2016). There are two main sources of contaminants, natural and anthropogenic. The first type encompasses different natural processes including harmful gases emitted upon volcanic eruption, toxic components that are washed out of ore during floods and natural excretion of toxic substances from all kinds of organisms due to metabolic activity. On the other hand, human activities in the form of urbanization, industry and transport have been reported as a significant contributor to pollution. Therefore, the ecological balance is harmed due to accumulation of these potential toxic substances in the environment (Facek 1990). Moreover, many contaminants are considered as emerging contaminants due to their persistence into natural habitat which could cause either acute or chronic toxic effects on the wild life. Moreover, due to their potential ability to bioaccumulate in plants and aquatic organisms, they may make their way back to the food chain. Thus, such pollutants have a higher chance of causing harmful effects to animals and/or humans through the food chain (Donaldson, Van Oostdam et al. 2010).

1.1 Pharmaceutical and personal care products in the environment

Emerging contaminants, such as pharmaceuticals and personal care products (PPCPs), and several additives such as detergents, plasticizers, flame-retardants continuously contaminates the environment (Eggen,

Heimstad et al. 2013). PPCPs are chemical pollutants that are used in various applications including medicine, household and agricultural products (Corcoran, Winter et al. 2010). Pharmaceuticals are known to be used mainly in the treatment of human or animal diseases. On the other hands, care products are established to define chemicals intended for personal use either as for hygiene or cosmetics such as deodorants, toothpastes and shampoos (Boxall, Rudd et al. 2012). PPCPs reach the environment as the parent compound or metabolites through different pathways (Figure 1) (Boxall, Rudd et al. 2012). Moreover, their use has intensified with time, leading to unavoidable augmentation of surface and ground water contaminations and consequently potential adverse effect on aquatic wildlife and water value (Boyd, Reemtsma et al. 2003). Pharmaceuticals are commonly found at concentration ranging from ng/L to µg/L with some exceptions in several countries (Boyd, Reemtsma et al. 2003). In general, the more a drug is prescribed - the more it is predominant in WWTW effluents and river water (Corcoran, Winter et al. 2010). However, this is not the only reason for the detection of pharmaceuticals concentration but also the degree of metabolism upon ingestion by the patient, degradation rates in the wastewater treatment and in the water where end up the chemical, and how the compound partitions into the water column/sediments. In hospital, care facilities or pharmacies, a lot of prescribed pharmaceuticals are unfortunately unused and somehow discarded either directly or indirectly into wastewater. That represents a cost of about 1 billion dollars each year in the US. These substances are produced for defined goals in humans or livestock. Upon transfer to other habitat, they can be biologically active in non-target wildlife species (Corcoran, Winter et al. 2010). Thus, despite the low toxicity of most of the pharmaceuticals products towards humans, or

livestock, the risk of being harmful for other non-target species is of reasonable concern (Arnold, Brown et al. 2014). In fact, due to differences in physiology and biochemistry between different species, the result of a specific drug might not be identical, and, it might also lead to side effect toward the non-target species (Corcoran, Winter et al. 2010). Among organisms within the aquatic environment, fish show to be the most likely to be affected by pharmaceuticals initially targeted for mammals or livestock. The principal reason for that are the similarities between fish and mammals from a physiological point of view. (Corcoran, Winter et al. 2010). The fact that fish have a lower capacity to metabolise xenobiotic than human, requires the need for more studies on the effect of PPCPs (Corcoran, Winter et al. 2010). Fish can be exposed to pharmaceuticals at different life stages such as egg, juveniles and adult stages. However, they might not have the same response towards the pharmaceutical (Arnold, Brown et al. 2014). In addition, even though the level of pharmaceuticals in the environment is too low to induce clinical effect, there is a lack of characterization for their risk to wild fish and consideration about possible chronic exposures or mixture scenarios (Corcoran, Winter et al. 2010). However, living organisms are believed to develop a strong defence system against pollutants. Those who are repetitively exposed to pollutants have developed efficient detoxification system. These systems allow them to survive and continue to prosper in such highly contaminated environments by a number of different contaminants such as described previously (Ferreira, Costa et al. 2014).

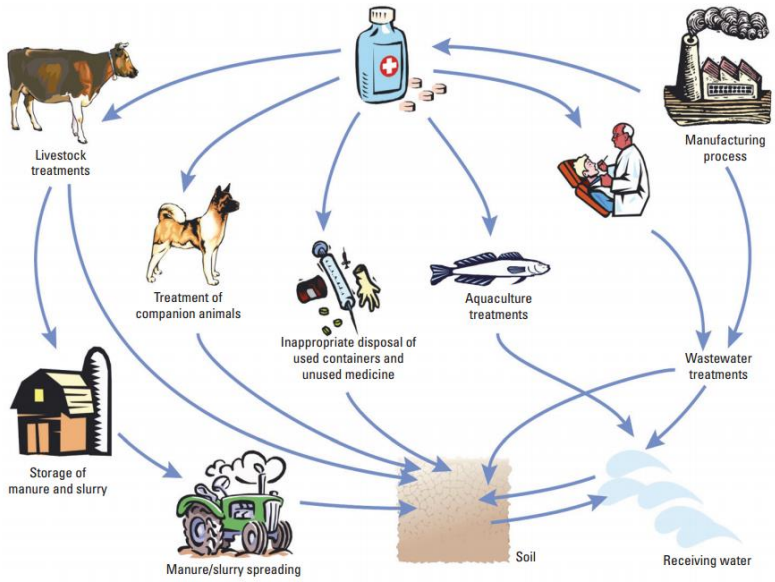

Figure 1 Schematic representation of the main pathways that allow PPCPs to reach the environment. Figure 1 obtained from (Boxall, Rudd et al. 2012)

1.2 Metformin

Among a vast number of PPCP products, metformin is one of the most prescribed PPCPs and is considered as emerging environmental contaminants since 1990 (Eggen and Lillo 2012). Indeed, it is one of the most available pharmaceutical in WWTP effluent with concentration ranging from 1 - 47µg/L, as well as in surface-waters at concentrations from 0.06 - 3µg/L (Niemuth, Jordan et al. 2015) Thus, it seems to be one of the most discharged PPCPs into the aquatic habitat (Niemuth, Jordan et al. 2015).

Metformin is an anti-diabetic drug, hydrophilic, polar and water soluble and is prescribed worldwide to patient suffering from diabetes (Scheurer, Michel et al. 2012). Additionally this drug is also prescribed in cases of cancer, endocrine disorder and polycystic ovary syndrome (Niemuth,

Jordan et al. 2015). It is reportedly biologically stable, this explains why it is not metabolized in the organism and therefore excreted to the treatment facilities via urines and faeces. This drug is therefore transferred to the environment unchanged and thus able to moves through the food chain in its parent chemical form. It has been shown that metformin is present in the environment at relevant concentrations to produce consequences to the natural ecosystem and biota (Scheurer, Michel et al. 2012, Trautwein, Berset et al. 2014). As already mentioned, fish are likely to be one of the most affected aquatic organisms by PPCP's. Unfortunately, there is a currently huge knowledge gap about toxicokinetics, toxicodynamics and possible effects of PPCPs on fish (Boxall, Rudd et al. 2012). Therefore, strong knowledge about PPCPs chemical properties, uptake, distribution and physiological effects on organisms needs to be assessed. This information will be important to perform health risk assessments which are for now, compromised, due to insufficient concerning fish, and particularly with metformin (Arnold, Brown et al. 2014, Trautwein, Berset et al. 2014).

Metformin is an anti-diabetic drug II, belonging to the class of biguanides, that is widely prescribed worldwide, mainly for people suffering from non-insulin-dependent diabetes mellitus (Scheurer, Michel et al. 2012). This drug is known to be not metabolized upon administration to humans and thus, it is excreted unmodified in urine and faeces (Scheurer, Michel et al. 2012). This is due to its movement across the body without passing the gut wall which then allows the chemicals to be released in the environment under an intact form. In addition it has been reporter that metformin is absorbed in the body with a rate of less < 20% (Corcoran, Winter et al. 2010).

Upon administration of metformin, the substance is localized in the liver, and triggers an inhibition of the mitochondrial respiratory chain, the complex I of the electron transport chain. Thus, due to drop of the energy availability, the synthesis of glucose through gluconeogenesis decreases in order to create a balance. Two steps are necessary to create this balance. Firstly, the simultaneously drop of ATP levels and rise of AMP amount helps to inhibit gluconeogenesis. Secondly, the high concentration of AMP plays a key inhibitor role in the suppression of AMP-activated protein kinase A signalling, cAMP-PKA signalling as well as fructose-1,6-bisphosphatase (FBPase) which is an essential enzyme required during the synthesis of glucose. It will also trigger the activation of 5' adenosine monophosphate-activated protein kinase (AMPK). Thus, the production of glucose and lipid/cholesterol is arrested (Rena, Pearson et al. 2013). The action of Metformin is described in the simple schema in Figure 2.

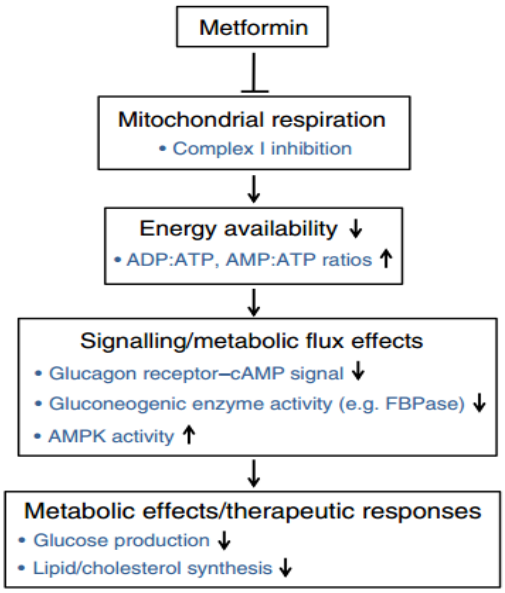

Figure 2 Simplified scheme illustrating the action of Metformin in the body obtained from (Rena, Pearson et al. 2013).

1.2.1 Presence in the environment

As mentioned above, metformin is not metabolized by the body and therefore released in the urine and faeces which is able to reach the waste treatment facilities where it cannot be filtered out completely. This is due to the stability of metformin from common water treatment techniques such as UV light irradiation, ozonation, etc (Trautwein, Berset et al. 2014). Moreover, this substance is highly stable in aqueous solution with a degradation of only 10% after 8 days at a temperature ranging from 30 to 70°C (Niemuth, Jordan et al. 2015). As such, it can be considered as persistent pollutant. Thus, metformin is very likely to be able to reach the food chain and brought back to humans. Among PPCP's, metformin is considered to be one of the most abundant substances found in high amount in the environment and seems to represent half of the total pharmaceutical released into the sewage treatment plants. This might be due to a combination of its prescription and chemical stability, thereby placing it as a high potential persisent environmental contaminant in 2006 (Trautwein, Berset et al. 2014).

1.3 Toxicity

Any chemicals may enter a biological system and produce certain toxic effect. These foreign chemicals are known as xenobiotics. Various steps exist from the entrance of the xenobiotics into the organism and its downstream biological effects (Figure 3) (Boelsterli 2007). Firstly, xenobiotic may first enter the cell and its potential to induce toxicity will mainly depend on the toxicokinetic and toxicodynamic factors. Toxicokinetics corresponds to the changes of the amount of a chemical in the organism over time. In other word, one can describe it as being the fate of a compound upon its entrance in the organism. On the other hand, toxicodynamic consist of the dynamic interactions of a compound with a

biological target and its subsequent biological effects. In other word, what effects can cause the compound on the body (Boelsterli 2007).

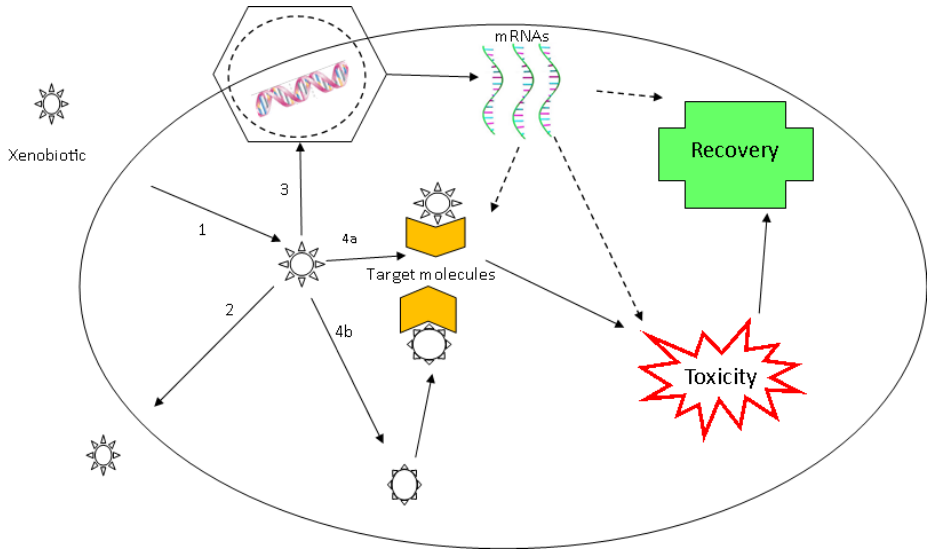

Figure 3 Representation of the possible pathways for xenobiotics and its effects on the cell. 1) The xenobiotics enter the cells and can then go through 4 different pathways. First, it can be directly be excluded out of the cell (2), or induce regulation of gene expression (3). It can also bind to target molecules whether under unmodified profile (4a) or modified (4b), which will possibly cause toxicity. Toxicity can also be provoked through gene regulation indirectly or directly. This potential toxicity can then in some cases be recovered in order to avoid adverse effects for the organism. Figure 3 was drawn based on (Boelsterli 2007).

1.4 Specific uptake and distribution

Specific uptake and distribution of pharmaceutical are widespread in fish and can occur through different ways such as dermal, and gill surfaced. Despite the use of the octanol/water partition coefficient (K_{ow}) in order to approximately determine uptake of neutral organic substances, it has not shown to be a good indicator concerning the uptake of pharmaceuticals.

Pharmaceuticals that are mainly ionisable have shown to be extremely sensitive to variations of pH. It seems that the pH-corrected octanol-water partition coefficient (D_{ow}) could be a better way to determine approximately the uptake of pharmaceuticals. Still, the determination of uptake can be further enhanced by other coefficient calculation such as the pH corrected liposome-water (D_{lipw}). Some suggest using the Volume of Distribution (V_D) to determine the uptake of pharmaceuticals in aquatic species. The V_D is defined as the quantity of the drug in the human body. Moreover, in addition to the physico-chemical properties of drugs that can be affect its uptake by organisms; biological factors can also influence it. Such biological factors are reproduction type, source of oxygen, habitat, age, and sex (Meredith-Williams, Carter et al. 2012).

1.5 Physiological changes

1.5.1 Metabolism in toxicity

Organisms have the important capacity to establish a defence system against toxic chemicals. To do so, they attempt to stop the toxic compound from being harmful by first modifying the chemical and then removing it quickly from the body. The first step is referred as detoxification. A four step cascades is involved in the mechanism of reducing harmful effects of foreign chemicals upon its passage through the body. The various steps of this cascade of reactions are absorption, distribution, metabolism and excretion, and are referred as ADME. Prior to the possible efflux of endogenous or exogenous compounds out the organism, a chemical reaction is required in order to convert them into more hydrophilic products and thus facilitating their clearance. Thus, the metabolism of the substance is performed in order to augment its

solubility in water that will facilitate its excretion via urine or faeces. So, it is important to understand the metabolism step that contain itself two phase, the phase I and II. Figure 4 represents in a simplified way how exogenous compounds can be process upon entrance into the body (Plant 2003).

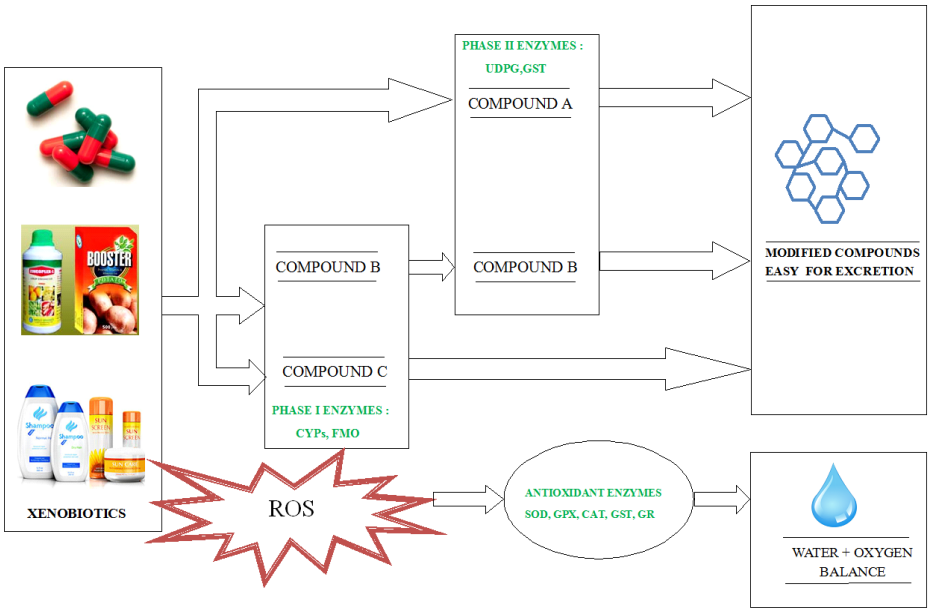

Figure 4 Representation of the possible pathway for xenobiotics upon entrance into the body and its possible consequences. Upon entrance in the organism, exogenous compounds can be metabolized via phase I biotransformation (compound B and C) or directly metabolized via phase II biotransformation (Compound A). After phase I, modified compounds can either be further metabolized through phase II or directly excreted out of the body. After phase II biotransformation for compounds, they can also be excreted. When xenobiotics are metabolized by phase I enzymes (CYPs, FMO), they may produce reactive oxygen species (ROS) which are then reduced to water by antioxidant system defence. Figure 4 was drawn based on (Plant 2003)

Concerning the phase I biotransformation reaction, it is the moment at which reactive groups are added to the initial compound in order to be in the phase II eventually conjugated to a second group that is more

reactive. This mechanism is illustrated in a more details manner in Figure 5. It is possible to notice that upon entering of the chemicals X through the membrane, it might be excreted by the transporter proteins known as ATP-binding cassette transporters (ABC transporters) depending on its property. But in parallel, it can process through the phase I of metabolism where enzymes such as CYP family will add a group to the compound. Then the compound can either be evacuated from the cell by the transporter protein or further modified via the metabolism phase II. In phase II, different enzymes are involved such as Glutathione-S-transferase (GST). Following the phase II, the modified chemical can be removed from the cell by a different transporter than previously which is often referred as multidrug resistance proteins (MRP) (Ferreira, Costa et al. 2014). The two phases are further described in the two next paragraphs.

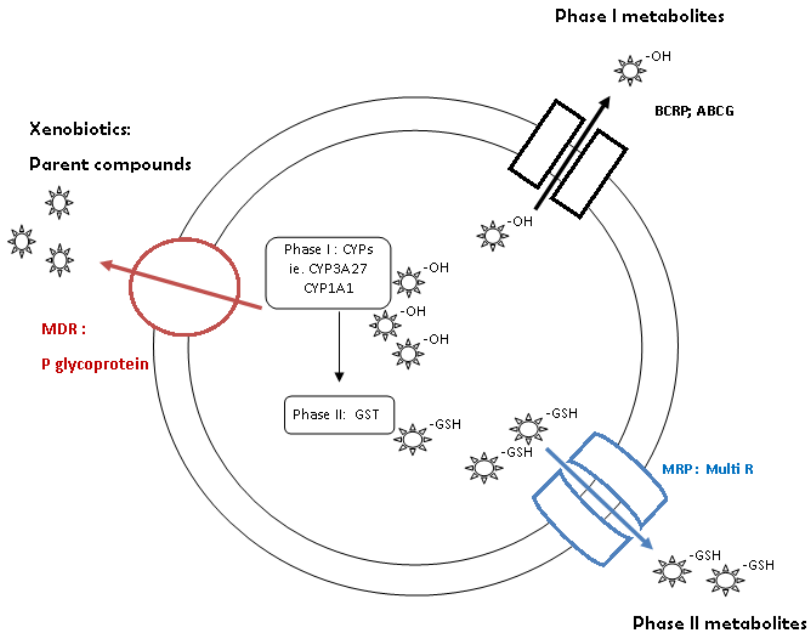

Figure 5 Handling of xenobiotic upon entrance in the organism. Drawn based on (Ferreira, Costa et al. 2014).

1.5.1.1 Phase I and II

The main role of the phase I metabolism is to increase the polarity of endogenous or exogenous compounds in order to facilitate their clearance. The main family of enzyme implied in this process is the cytochrome P450 but another one, the Flavin mono-oxygenase (FMO) can also take part of this process**.** Xenobiotics are render more hydrophilic by either hydrolyses, reduction or oxidation which is carried out by phase I enzymes. However, in general, oxidation is preferred by these enzymes, and FMO as well as cytochrome P450 follow similar reaction as described in Equation 1. For instance, cytochrome P450 enzymes will in a typical reaction, use oxygen and the cofactor NADPH to add a reactive group such hydroxyl radical (OH) to the foreign chemical as described in Equation 1. This reaction is not without repercussions, because during the step of detoxification, still reactive molecules that are produced. Thus, the phase II discussed in the next part is very important in order to avoid damage to protein, RNA and DNA (Plant 2003).

Equation 1 Representation of oxidation reactions that can be performed by Cytochrome P450 enzymes or Flavin mono-oxygenase (FMO) from (Plant 2003).

$$RH + NADPH + H^{+} + O_2 \longrightarrow ROH + NADP + H_2O$$

Other enzymes including cytochrome P450 reductase, cytochrome b5 also often intervene within this mechanism. Cytochrome P450 is involved in the reduction of chemicals such as drug into reduced compounds. On the other hand, cytochrome b5 adds chemical reactive groups and globally increase the polarity of molecules. However, the excretion rate will often be insufficient to properly evacuate this

intermediate. Thus, further supplementation of large polar groups is necessary that will take place during the phase II. In addition, the intermediates that are produced in the xenobiotic metabolism can be toxic for the cell because of their reactivity and therefore require attention. The detoxification process of chemicals has to be performed in a safe way for the organism itself. Thus, those are the reasons why Phase II metabolism is so crucial for the whole organism survival (Plant 2003).

The phase II metabolism further add large polar group in order to supplement the phase I that previously already added polar group but unfortunately usually not sufficient to cause large excretion of the compounds out of the cells (Plant 2003). The function of the phase II metabolism is to add a specific moiety to the xenobiotic in order to evacuate it out of the cell. This is done via a coenzyme that is often a transferase. This is a process of conjugation where there is formation of conjugate that unfortunately also might prove to be toxic for the cell. Among those reactions the most common of them are executed via methylation, acetylation, sulfation, glucoronidation, glutathione and amino acid conjugation. (Jancova, Anzenbacher et al. 2010) This phase is challenging due to the need to produce the conjugate prior to its addition to the compound (Plant 2003). Thus, the phase II metabolism involves different enzymes that are widespread in all tissues but highly concentrated in the liver. The three main families are glutathione-S-transferase, acetyltransferase and methyltransferase. The normal metabolism activity might increase the level reactive oxygen species called ROS (superoxide radical, hydrogen peroxide) through oxidative reactions such as cellular oxidative reactions. For instance, the action of cytochrome P450 will form ROS during the phase I and have to be removed to avoid irreversible consequences (Figure 4). Thus, through

several pathways, wide range chemicals can undergo the phase II metabolism that ensures a quick and powerful elimination of harmful compounds, as well as reactive oxidative species. Glutathione conjugation is a well-example of such system capable to keep the cell safe in case of oxidation. Thus, glutathione-S-transferases (GST), is one of the predominant enzyme that is involved in both the metabolism of xenobiotics (phase II) and in the safeguard of the cells against oxidative stress. To proceed, as described on the figure 6, GST form the glutathione-S-conjugate by conveying a thioether conjugate between the xenobiotic and the tripeptide glutathione (Jancova, Anzenbacher et al. 2010). GST enzymes are also found in fish. Other antioxidant enzymes play a role in metabolism such as catalase, glutathione peroxidase, glutathione reductase that are further discuss in the following paragraphs (Plant 2003). Besides its main role, the detoxification of toxic compounds, GST is able to metabolize other compounds such as steroids (Jancova, Anzenbacher et al. 2010).

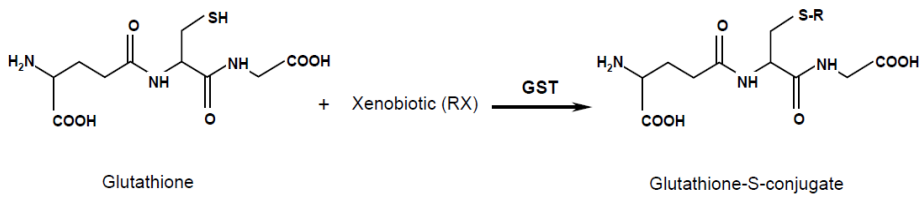

Figure 6 The transformation of xenobiotic (RX) via glutathione conjugation

One more remarkable phenomenon is the careful controlled balance between the enzymes in the phase I and II. This is very important and essential for the survival of organisms due to the production of harmful intermediates during both phase of the metabolism activity that are removed during the phase II as explained above. So, there is a well-

established coordination between the expression of the gene coding for both the enzymes in the phase I and II (Smart and Hodgson 2013).

1.5.2 Multi-xenobiotic (drug) resistance proteins (MXR)

The multidrug resistance (MDR) phenomenon come from its discovery in mammalian tumour cell lines that were resistant to a single agent as well as several anti-cancer drugs that seemed to be structurally and functionally distinct. The primary cause of this resistance was due to a transmembrane protein called P-glycoprotein (MDR1) due to its ability to efflux the drugs out of the cell via its ATP dependant pump (Litman, Druley et al. 2001). In addition, this P-glycoprotein belongs to the ATP binding cassette ABC family and is encoded by the ABCB1 gene. Nevertheless, it is not the only protein within the ABC transporter, members of the subfamily. ABCC and ABCG subfamilies are also part of this family (Ferreira, Costa et al. 2014). Against all odds, those multidrug resistance proteins are also found in healthy cells. Thus, this multidrug resistance system is an important concern in the medical and environmental areas. Another term is also mentioned in toxicology literature, the multi xenobiotic resistance (MXR) which refers to the capacity of organisms to eliminate xenobiotic compounds in order to survive in contaminated environment with for instance pharmaceutical products, industrial products, and metal. Moreover, MXR is well conserved through the evolution and is notably found widely spread in aquatic organisms which allowed marine species to be adapted to toxic compound and then survive in highly polluted places (Doussantousse, Pelletier et al. 2011). There is evidence of the presence of multidrug transporters in aquatic organism including fish protecting them against toxic compounds (Ferreira, Costa et al. 2014). Thus, this system is considered as the primary system of defence against harmful substances

(Nornberg, Batista et al. 2015). The MDR and MXR are related to each other (Bard 2000). Therefore, the proteins belonging to the ATP binding cassette ABC family found in the MDR system also play a key role in the MXR. Moreover, it is a process that does not require specificity of the substrate for the transporters due to that a wide range of toxic compounds that is taken care by a small number of transporter proteins.

1.5.3 ABC transporters

ABC transporters also called ATP-binding cassette transporters are used primarily as a transport system in order to decrease the concentration of drugs in organisms, and for the uptake of compounds. In order to perform these actions, the transporters are using their ability via the ATPase subunits to use the energy of adenosine triphosphate. Thus, this system is important in order to ensure the survival of organisms against toxic compounds called xenobiotics. However, they also have two other functions such as the importation and the translation of messenger RNA, and DNA repair. This study focus on the main action of the ABC transporters that is to effectively pump any toxic substances out of the cell of living organisms from prokaryotes to humans. The ABC transporters are classified through different subfamilies of proteins such as ABCB, ABCC and ABCG that have been created based on the sequence and organisation of their ATP binding cassette (ABC) domains. Some of those proteins are very important for the elimination of a many xenobiotics (Ferreira, Costa et al. 2014, Nornberg, Batista et al. 2015).

In a general rule, ABC transporters are composed of two intracellular nucleotide binding domains and two trans-membrane regions consisting of 12 trans-membrane segments. However, some make exception of the rule and possess additional trans-membrane domains (MRPs) or they

have a single nucleotide binding domain hence the name of half transporters (BCRP). In summary, the three main ABC transporters are the p-glycoprotein (ABCB1), the Multi Resistance Associated Proteins (MRP1-9; ABCC sub family) and the Breast Cancer Resistance Protein (BCRP; ABCG sub family). The first group transports, for instance, organic cations, weak organic bases, mainly non modified xenobiotics whereas the two other groups mainly transport modified compounds (Ferreira, Costa et al. 2014). It has been suggesting that ABCG group preferably transport xenobiotics and steroids conjugated to GSH as well as glucoronide metabolites (Ferreira, Costa et al. 2014). Thus, those three groups have different specificity for chemicals (Smart and Hodgson 2013). One main and important difference between MRP and P-glycoprotein is that contrary to p-glycoprotein, p-gp, MRP is able to transport drug metabolite in addition to unmodified drugs. BCRP have been identified in cells that did not express p-gp or MRP1. BCRP handle a lot of substrate that overlaps with the 2 other main group of ABC transporters (Smart and Hodgson 2013).

Moreover, the ABC transporters are highly multi specificity for substrates, and are found in several kingdoms. That might be a confirmation of their good conservation in the ABC sequence of the cassette through evolution. The integral membrane proteins probably have evolved independently many times which explain the different number of families for the ABC transporters. In addition there is the evidence of the expression of these transporters protein in aquatic species due to confirmation by immunochemical analysis. As a result, the ABC transporters are of great interest in the influence of the uptake, distribution and excretion of drugs. And within the different proteins serving as transporters, p-glycoprotein is one of the best known and

characterized. Thus, this 170 kDa trans-membrane glycoprotein is one of the main commonly studied ABCB superfamily transporters due to its relevance in aquatic organism to resist the potential effects of polluted environments. In fact, it has been found and described in a lot of marine organisms such as mussels, fish, sponges, worms. This protein is encoded by the ABCB1 gene. In resistant cell, this phosphorylated protein will mainly eliminate the accumulation of drugs and preferably the one that are not metabolized as seen in the previous Figure 5. The first eukaryotic ABC member that has been discovered is the ABCB1 also known as MDR1 due to their effect during chemotherapy that renders the cancer cells resistant to the anti-cancer drug. This capacity to offer such characteristics to the cells is due to the amazing non specificity of the P glycoprotein for the substrates. Nevertheless, the substrates have some shared features such as size, charge and hydrophobicity level (Ferreira, Costa et al. 2014). But still the p-pg transports mainly large and moderate hydrophobic cations. Concerning the mechanism of this transporter protein, various steps are involved in order to avoid accumulation of endogenous compounds, xenobiotic in the cells exposed to them. Firstly, the substrate binds to the p-glycoprotein and modifies the structure of the nucleotide-binding-domain (NBDs) via hydrolysis of ATP. Thus, this stimulates the transport of the drug out of the cell. However it has been demonstrated that the presence of NBDs are not mandatory to the well-functioning of the p-gp (Loo and Clarke 2005).

1.5.4 Steroidogenesis

The steroidogenic acute regulatory protein (StAR) is responsible for the transport of cholesterol into the mitochondria and thus the control of the speed of the steroid hormones synthesis (Figure 7). Therefore, they are found in steroidogenic tissues such as testis, ovary and adrenal cortex.

Inside the mitochondria, the cholesterol side-chain cleavage enzyme (P450scc) cleave the cholesterol into pregnenolone, an endogenous steroid hormone (Figure 7) (Kusakabe, Todo et al. 2002, Arukwe 2005). Pregnenolone is a precursor of the progestogens and estrogenes amongs other steroid hormones. Steroid hormones are of a capital importance for the good functioning of the body because it is involved in vital physiological functions including sexual differentiation, carbohydrate homeostasis, immune system function, reproduction, and stress managing (Arukwe 2005). In the cell, there are inhibitors that can suppress the activity of those enzymes and thus supress the steroid hormone formation by inhibiting the movement of cholesterol. But this can be cancelled if the mitochondrial membrane is broken (Christenson and Strauss 2001).

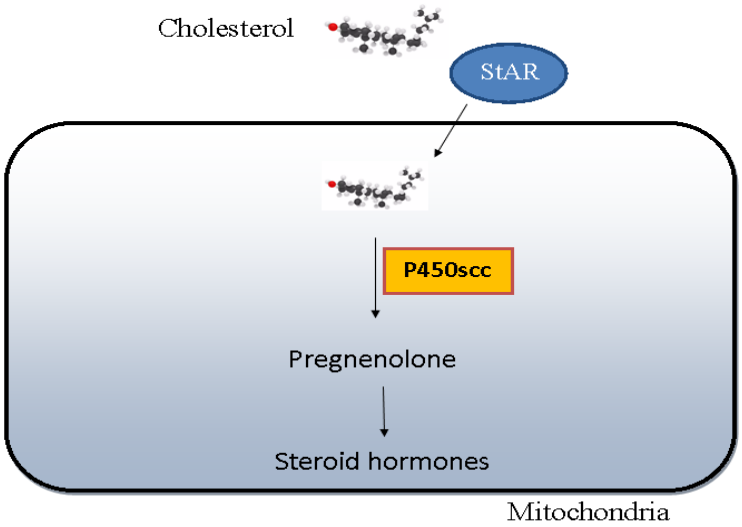

Figure 7 *Scheme representation of the steroidogenesis mechanism. The cholesterol is transported into the mitochondria by the steroidogenic acute regulatory protein StAR and then cleaved into pregnenolone by cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone, can then be further transformed to several steroid hormones. Figure 7 drawn based on (Christenson and Strauss 2001).*

1.5.5 Oxidative stress

Many xenobiotics often cause direct toxic effects in the affected organism. However, the metabolites of the parent substance, ROS or reactive nitrogen species can also be the cause of toxic effects (Smart and Hodgson 2013). ROS are continuously formed under normal aerobic metabolism where oxygen is used to produce energy. Therefore, a system of defence in order to deal with ROS and avoid possible damage to the cellular macromolecules is required for the survival of cells and so on. There are three levels of protection; 1) the anticipation of ROS formation, 2) the cessation of the ROS using free radical scavengers or antioxidant enzymes, and 3) the healing of injured cellular components. Two systems exist such as the enzymatic and non-enzymatic defences. Among the methods to overcome oxidation stress, glutathione (He and Lin) is seen as one of the most essential (Storey 1996).

Unfortunately, it happens that these defence systems are submerged and therefore cannot overcome too high amount of ROS in the cell. This creates an imbalance where there is a higher quantity of oxidants than antioxidants. And, that is denominated as oxidative stress. Thus, ROS formation, might lead to oxidative stress in cells and damaged biological macromolecules comprising DNA, proteins and lipids (Smart and Hodgson 2013).

As mentioned, xenobiotics and other molecules such as dioxygen (O_2) and nitrogen oxide (NO) may become highly reactive towards endogenous molecules due to toxication. Toxicity is known as the process that renders substances dangerous for the organism (Liu, Goyer et al. 2008). These molecules become more reactive through conversion to either electrophiles, free radicals, nucleophiles or redox active

reactants. The two discussed here are electrophiles and free radicals because they seem to be often the result of toxication. Concerning electrophiles, one can define it as molecules encompassing an atom with a deficient electron and a partial or full positive charge. Due to these properties, these molecules can react by distributing electron pairs with electron rich in nucleophiles. Cytochrome P450s have the capacity to form many electrophilic metabolites. On the other hand, free radicals are molecules that contain at least one unpaired electron in its outer orbital. Free radicals can be created via three main processes. Firstly, by accepting an electron from reductases, thus several xenobiotics are known to be able to generate radicals by accepting an electron that is then transfer to a molecule of dioxygen O_2. This result in the formation of superoxide anion radical ($\cdot O_2^-$), and renewal of the parent xenobiotic, which is ready to gain a new electron. Thus, a unique xenobiotic molecule is able to form a considerable number of ($\cdot O_2^-$) via this so-called "redox cycling". This superoxide anion radical (·O2-) can then be involved into two different pathways where it starts toxication pathway. The first pathway is the formation of hydrogen peroxide H_2O_2 and potentially (·OH-), and the second pathway with the formation of ONOO· and finally ·NO2 and CO2-·. These properties render superoxide anion radical (·O2-) of a capital importance in toxicology (Storey 1996, Liu, Goyer et al. 2008) Moreover, this radical as already mentioned is also produced by the mitochondria electron transport chain. Secondly, free radicals can be formed by the loss of electrons of some nucleophilic chemicals like phenols, aromatic amines, thiols and xenobiotics via a reaction catalysed by peroxidases. Thus, due to loss of an electron by its transfer from a molecule to another one, radicals are formed. One important free radical to be produced by this mechanism is the hydroxyl

radical ($\cdot OH^-$). Hydrogen peroxide (H_2O_2) is reduced by superoxide by the Haber-Weiss reaction (Figure 8). This is a two-steps procedure where transition metals such as Cu (I) or Fe (Brooks and Doswell III) are oxidized (Figure 8), known as Fenton reaction, and give rise to the harmful ($\cdot OH^-$). Thus the presence of such metals ions will determine the toxicity levels of hydrogen peroxide and superoxide anion (Storey 1996, Livingstone 2001). Moreover, the location of these metals within the cell determinate/influence the type of injury and its level (Storey 1996).

$Fe^{3+} + \cdot O2^-$ $Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2$ $Fe^{3+} + OH^- 0 + \cdot OH^-$ Fenton reaction

$\cdot O_2^- + H_2O_2$ $O_2 + OH^- + \cdot OH^-$ Haber-Weiss reaction

Figure 8 Production of the hydroxyl radical from H_2O_2 via the Haber-Weiss reaction, a two-step process catalyzed by transition metals (Fe3+) and involving the Fenton reaction. Drawn based on equation from (Storey 1996) .

Among the Reactive oxygen species, it is important to note that ·O2- and H_2O_2 are very poor reactive molecules but nevertheless represent real danger due to their ability to give rise to the highly reactive ·OH- which in turn leads to serious damages for cellular macromolecules (Storey 1996).

1.5.5.1 Antioxidants

Various antioxidant enzymes exist in order to reduce oxidative stress in cells and by then limit the number of damages to macromolecules. The most important includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR). These enzymes have a specific role in the process of maintaining the redox homeostasis. Their cellular activities are strongly co-ordinated. They are

very important because they prevent the formation of hydroxyl radical (·OH^-) which is usually not destroyed by enzymes because of its extreme reactivity and short half-life (Storey 1996). Thus, the process by which ·OH^- production is prevented is described in more detailed next and represented in Figure 9.

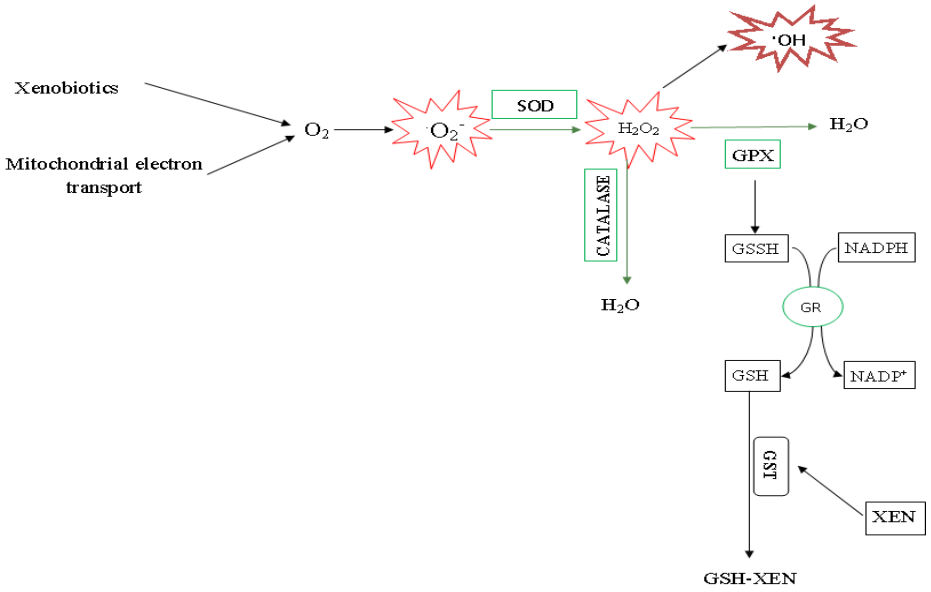

Figure 9 Diagram representation of the principle behind the mode of action of antioxidant defence system found in aquatic organisms. Xenobiotics or normal mitochondrial respiratory chain produces dioxygen (O2) which is then transformed to superoxide anion ($\cdot O_2^-$). (SOD) can produce hydrogen peroxide (H_2O_2) from $\cdot O_2^-$. Two antioxidants, Catalase (Cat) and Glutathione peroxidase (GPx) reduce (H_2O_2) to water (H_2O). During this reduction by GPx, Glutathione reductase is used to replenish glutathione levels due to its oxidation to GSSG during the reaction with GPx. Finally, Glutathione-S-transferase (GST) catalyze the conjugation of GSH with xenobiotics. Drawn based on information in (Storey 1996).

Dioxygen (O_2) produced either by normal mitochondrial electron transport or xenobiotics entrance into cells, may give rise to reactive oxygen species such as superoxide anion (·O2-) which in turn can be

dismutated to hydrogen peroxide (H_2O_2) by the superoxide enzymes SOD. However, H_2O_2 despite its relatively low reactivity, it has the potential to be further transformed to the mostly reactive species known as hydroxyl radical (·OH-). The production of ·OH- will be overcome by other antioxidant enzymes including catalase and glutathione peroxidase (GPX) by reducing it to water. GPX is a selenium containing enzyme that when removing H_2O_2 will oxidize glutathione (He and Lin) to Glutathione disulfite (GSSG). This leads to the reduction of the ratio GSH/GSSH. Thus Glutathione Reductase (GR) is needed to replenish the cell with GSH to keep its level stable. To do that GR needs $NADPH_2$ as energy source. Finally, a secondary enzyme called Glutathione-S-transferase is also important in the detoxification of reactive compounds. And, it works with Glutathione (He and Lin), one of the most important antioxidant within organisms, where GST catalyses the GSH attachment to toxic compounds such as xenobiotics. GST is also able to response against the production of ROS by altering cellular levels in GSH. To be more detailed, Glutathione-S-transferase is issue from a superfamily of enzymes that take part of the phase II metabolism. Those enzymes are found in the cytoplasm as well as in mitochondria but as a small fraction. They consist of six different classes and according to that, they will have preference for a specific substrate and therefore detoxify different molecules (Smart and Hodgson 2013). Moreover, GST consist of a tripeptide thiol that is able to add glutathione, GSH, to electrophilic substrates like ROS damaged cellular components and xenobiotics (Smart and Hodgson 2013). Thus, it serves to remove the toxic effect of xenobiotic, reactive metabolites, by render them more soluble and easier to be evacuated out of the body via renal excretion. In addition, due to its peroxidase activity, it prevents oxidative stress and thus following side

effects for the cell (Smart and Hodgson 2013). Moreover, GST can transform the H_2O_2 into fatty acids, phospholipids, cholesterol (Liu, Goyer et al. 2008). On the other hand, they are able to bio activate many chemicals such as halogenated compounds.

1.5.6 Neurotransmitters

Neurotransmitters are signal molecules that are released by the presynaptic neuron at a chemical synapse. These molecules are then received by the postsynaptic cell and allow signal relay between different neurons which cause either excitatory or inhibitory response. The type of response is determined by the receptor type. Various neurotransmitters exist such as dopamine, acetylcholine, epinephrine, norepinephrine, serotonin, etc. Neurotransmitters are important for the daily life of living organisms including humans, fish and other terrestrial animals. Acetylcholine is one among the various neurotransmitter to be used in toxicology studies because it is an important signaling molecule and mainly abundant in the central nervous system. However it is also found in many other organs at the peripheral nervous system. This neurotransmitter is necessary for diverse body function such as skeletal muscle movement, regulation of smooth and cardiac muscle, and may also be part of the learning and memory functions (Cooper, Bloom et al. 2003). Concerning the process, choline and acetylcoenzyme A are necessary for the synthesis of acetylcholine (ACh) that is then stored at the presynaptic neurons. Upon neuronal stimulus, ACh is released and transported to the postsynaptic neuron where it will bind to specific receptors. Two types of receptor exist such as muscarinic and nicotinic and are both present in the brain and involved in the respiratory control and cardiovascular system. However, when the stimulus is ended, the acetylcholine signal must be stopped. Thus, acetylcholine esterase

degrades acetylcholine into choline. Afterwards, ACh can be restored at the presynaptic neuron and be ready for a new stimulus action. In the case of inactivation of the enzyme AChE, there is a high risk of augmentation of ACh and subsequent toxic effects on the organism (Boelsterli 2007).

1.6 Juvenile Salmon as Model

Atlantic salmon is intensely used in aquaculture from the 1990's and more particularly in Norway, the United Kingdom and Chile. However, other countries such as the United States have also seen their salmon farming industries growing (Asche and Bjorndal 2011). On the other hand, there are the wild salmon that have a complex life cycle and migrate from freshwater to saltwater depending on their life stage (trust 2016) Salmon are a sensitive species to environmental changes and chemicals entering rivers are seen as obvious pollutants (TRUST 2016) Thus, the constant increase of contaminants such as drugs, detergents, cosmetic products and several other persistent chemical compounds into aquatic environment could have potential adverse effects on salmonoids, In addition, Atlantic salmon are located relatively at high level in the food chain and highly consumed by humans which will increase risks for upward mobility of contaminants through the food chain (Renzoni, Zino et al. 1998).

1.7 Studies outline and objectives

Despite the awareness of the presence of PPCPs in the environment, few studies, especially for the anti-diabetic II drug metformin, have been performed until today. Metformin can be considered as a persistent xenobiotic because it is continuously found in the environment due to increased and continuous prescription of this pharmaceutical (Trautwein, Berset et al. 2014). It is important to perform health risk assessment on

such xenobiotic in order to obtain sufficient scientific knowledge which will enable to facilitate establish risk assessment. Therefore, the present study investigated the uptake, organ distribution and physiological effects of metformin in Juvenile Atlantic Salmon (Salmo Salar).

In the present study, possible effects on the uptake, distribution and physiological pathways in Juvenile Atlantic salmon as a result of exposure to various concentration of metformin was investigated.

The hypothesis of this thesis is that the exposure of metformin to Juvenile Atlantic salmon S. Salar, produce organ specific uptake and distribution, and subsequent effects on physiological pathways and oxidative stress?

2 Materials and Methods

2.1 Chemicals and reagents

Metformin was purchased from Sigma-aldrich. Trizol reagent was purchased from Invitrogen Life technologies (Carlsbad, CA, USA). Chloroform, isopropanol, ethanol-absolute, DMSO, Methanol and Gel Red were purchased from VWR. iScript cDNA synthesis kit and Tween 20 were purchased from Bio-Rad Laboratories. DTNB, NADPH, MOPS, SDS, APS, Trizma base, agarose, EDTA, BSA, H_2O_2, Acetylthiocholine iodide, Glutathione (He and Lin), Glutathione reductase (GR), L-Glutathione oxidase, catalase and CDNB were purchased from sigma. SYBR Green supermix was purchased from Roche. Immunostar kit was purchased from Bio-Rad.

2.2 Experimental design

2.2.1 Fish

Juvenile, sexually immature Atlantic Salmon (*S. Salar*) were obtained from Lundamo hatchery (Sør-Trondelag, Norway). The age of fish was identical and they had an average body weight of 79.0 ± 2cm and an average length of 21.2 ± 0.2cm. Fish were kept at holding facilities of Norwegian University of Science and Technology (NTNU-Sealab) and were firstly acclimatized in fresh water for 5 days at 9 °C and under natural light cycle 12:12 hours light:dark. These two parameters were then maintained during the all exposure time. In addition, fish did not receive any food during both the acclimatization and the experiment duration. During the experiment, a good level of oxygen to about 84% was supplied to the tanks as well as a proper average pH of 7.9.

2.2.2 cDNA synthesis

Nowadays, reverse transcription polymerase chain reaction (RT-PCR) has become routinely used in laboratories in order to produce cDNA from isolated total RNA. This technique uses a reverse transcriptase enzyme from retroviruses as for example the modified Moloney Murine Leukemia Virus. The use of cDNA for the future analysis is practical because of it is stability when stored at low temperature for a long period while RNA has short life time and is easily degraded by enzymes as RNase. In addition, the quantification of cDNA by following PCR reflect directly the initial amount of mRNA present in a tissue (Bustin 2000)**.** These are the reasons why cDNA was used for gene expression analysis in this toxicology study.

Different possibilities exist for producing cDNA from RNA that include the so-called one-step and two-step method. In the first case, the technique regroups both, the RT and PCR reactions in a unique tube. On the other hand, for the two-step method, these two reactions are performed in two separate tubes. The main advantage of the two-step-methods is that upon cDNA synthesis, it is possible to store the left genetic material and perform future and various gene expression analyses for different genes on well conserved cDNA. Thus, the two-step method was the technique of choice in this case, despite the disadvantages that included the risk of pipetting errors, contamination due to more handling of the genetic material (Bustin 2000).

Protocol

iScript cDNA synthesis kit from Bio-Rad was used for the production of cDNA from total RNA. This kit contained oligo dT primers that during RT reaction, will bind to the poly A tail of mRNA molecules. Moreover,

this kit contained also nonspecific hexamer primers which are advantageous on the presence of secondary structure in mRNA that inhibits the binding of poly dT primers to the RNA molecule and thus compromising the obtaining of cDNA and efficiency of future analysis (Bustin 2000, Fleige and Pfaffl 2006). Concerning the procedure for cDNA synthesis, at first, mastermix for the cDNA synthesis reaction was prepared as described in Table 1. Then, the RNA volume to add to the reaction in order to get 1µg in a total of 5µL was determined based on prior RNA concentration measurements. Thus, the RNase free water volume necessary to add for get a final volume of 5µL was deduced. Then, at this stage, it was possible to begin the preparation of the RT step. 15µL of mastermix was added to the plate, followed by the addition of the calculated RNase free water. And, RNA volume required was added to the plate. Some extra RNA samples were added in the plate for establishing a cDNA that was used for performing primer testing. The plate was vortex and spin down shortly at 600rpm for a minute. Finally, the plate was placed inside the thermocycler and set up at 25ºC for 5 minutes, 42ºC for 30 minutes and 85 ºC for 5 minutes.

Table 1 Composition of the mix for synthesis of cDNA

Mastermix compounds	**Volume for one reaction**
5x iScript reaction mix	4µL
iScript reverse transcriptase	1µL
Nuclease free water	10µL
1µg total RNA/Nuclease free water	5µL
Total	**15µL**

2.2.3 Exposure

In total 96 fish were exposed for 3, 7 and 10 days to different concentrations of metformin including 0, 5, 50 and 500 µg/L. Four tanks were filled with 50 litres of fresh water, air pump and air stone. Then, each tank was supplied with adequate amount of stock solution to get the four different final concentrations of metformin. 24 fish were transported in each tank containing 50L of fresh water from the laboratory tap water.

2.2.4 Sampling

Fish were sampled at 3, 7 and 10 days after immersion in whether fresh water alone or fresh water containing metformin. Six fish from each tank were blowed on the head, and their length and weight were measured. Organs such as liver, gill, kidney, and brain were collected to be instantly frozen in nitrogen liquid. But in parallel about 50mg of liver were kept to be directly added to a separated tube containing 500µL of trizol reagent and crashed before freezing in nitrogen liquid. This step allowed conservation of the genetic material from organs. The remaining carcass was frozen at -80°C as well as all of the organs in order to process further analysis on them. Concerning the blood samples, it was kept on ice until their centrifugation at 5000 rpm for 5 minutes to get their plasma. The plasma was also conserved at -80°C for potential further analysis.

2.3 Chemical analysis for bioaccumulation

At first, fish carcasses were weighted and cut in small pieces to be dried using a moisture analyser MA45 (Satorius, Göttingen, Germany). The dried fish was grinded and added to 50ng caffeine and homogenized. Then 10ml of Millipore water/methanol to 1/3 was pored over the homogenate before the extraction step that was performed using a vortex Reax2000 (Heidolph, Germany) for 30 seconds followed by ultrasonic

bath (“Branson 5200”, Scientific Support, USA) for 20 minutes. Next, the samples were centrifuged at 5000rpm for 10 minutes before evaporation of the supernatant to 1ml using a TurboVap II device Biotage (Uppsala, Sweden). Afterwards, dispersive cleanup was performed using 100mg of C18 sorbent (Merk) and 50mg $CaSO_4$ powder to remove lipophilic components. A second centrifugation was carried out for at 10000rpm for 10 minutes in order to recover the supernatant and diluted with 500µL acetonitrile (ACN). This extract was conserved at 5 °C overnight before an ultimate centrifugation at 11000rpm for 10 minutes.

The samples were analyzed next by HPLC-MS-MS (Agilent 1260 HPLC-QTrap5500, SCIEX, Darmstadt, Germany) by direct injection of 5µL of samples to the HPLC-MS-MS analyzer. A reversed HPLC columns Ascentis Express C18 with the dimensions of 10cm*3mm ID, 2.7 µm particle size from Supelco, Germany was used for the separation of the samples. The gradient of elution was set at a flow rate of 300 µL/min and the analysis was started with 75% solution A made of water and 0.1mmol ammonium acetate, and 25% solution B consisting of acetonitrile. 5 to 9min at 50% solution A and between 9.1 to 12 minutes a 75% of solvent A.

Finally the mass spectrometric analysis was carried out by using a positive electrospray ionization of 5.5kV and an ion source temperature of 400°C. MRM (Multiple Reaction Monitoring) was applied for mass analysis detecting substance typical ion transitions listed in Table 2.

Table 2, Ion transitions used for the mass analysis (MRM-mode) of metformin and the internal standard caffeine (IST) as well as method characteristics (f.w.= fresh weight, d.w.= dry weight)

	Ion transition (m/z)	Collision energy (V)	Retention time (min)	Method limit of quantification	Relative standard deviation
metformin	130 → 60	17	1.2	24 ng/g f.w. 80 ng/g d.w.	± 6 % (n=4) (5 µg/sample)
	130 → 71	29			
caffeine (IST)	194 → 138	27	1.5	10 ng/g f.w. 20 ng/g d.w.	± 16 % (n=5) (50 ng/sample)
	194 → 110	31			

2.4 Gene expression analysis

Genes of eukaryotic cell are not all equally expressed at a specific time or under specific conditions. Changes of their environment for instance, require the ability of the cell/organism to adapt in order to survive. Thus, it is necessary for the cell/ organism to switch on and off expression of genes when it is needed. Therefore, Quantitative PCR is an easy, widely used technique that monitors the changes in expression of specific genes at a given time. In addition, it is now developed and indispensable in toxicology studies, where one can accurately, precisely determine the changes in the expression of specific genes upon specific exposures. (Arukwe 2006)

2.4.1 RNA Isolation

RNA quality is essential for the success of gene expression analysis (Fleige and Pfaffl 2006, Wieczorek D 2012). Thus, prior to RNA isolation, it is primordial to be aware of the fragility of RNA molecules to avoid procedures that may increase their degradation (Copois, Bibeau et al. 2007). Otherwise, non-reliable results may arise due to errors during

RNA isolation. In order to avoid the emergence of wrong biological differences in gene expression, and decrease possible contaminations of the isolated RNA, several measures are need to be taken during the procedure of isolation. Firstly, it is possible to decrease this potential problem by avoiding the presence of enzymes such as ribonucleases (RNases) that would degrade RNA. And the samples can also be kept cool, protected from any pollution, and adding strong inhibitor of Rnases such as guanidium thicyanate (Wieczorek D 2012).

Protocol

The isolation of RNA was both processed on liver and brain samples by using the trizol isolation technique currently done in this laboratory. The procedure for the RNA isolation was followed from ambion by life technologies. About 50mg of Liver/ Brain samples were added to 500µL Trizol and then homogenized using Polytron® PT3000 mechanical homogenizer from Kinematica AG. 500µL trizol were added and tissue was homogenized one more time. When the second 500µL Trizol were added, the homogenizer was washed with the trizol to ensure that no tissue was left on it and thus lost. Before the first use and between each sample within an exposure group and therefore between each exposure group, the homogenizer was systematically washed with distilled water, then 70% ethyl alcohol and finally with RNase-free diethylpyrocarbonate (DEPC) water. DEPC-treated water was used to avoid any contamination by RNase. At the end of the homogenization, the samples were incubated at room temperature for 5 minutes. This incubation allowed the nucleoprotein complex to be entirely dissociated. 200µL of chloroform were then added to the tubes followed by vigorous shaking by hand for 15 seconds. After an incubation time for 2 to 3 minutes at room temperature, the samples were centrifuged at 12000g, 4°C for 15

minutes. This step permits to separate the RNA from the DNA and proteins. Thus, three phases are formed such as the lower red phenol chloroform phase, the interphase, and the limpid upper aqueous phase which contains the RNA. So, after obtainment of the different phases, the aqueous phase of the sample were removed and placed in a new clean tube before the addition of 500µL of 100% isopropanol. The remove of the upper phase was done delicacy in order to not contaminate with DNA that is found in the interphase. The tubes were then reversed 3 to 5 times before a new incubation at room temperature for 10 min to allow RNA to precipitate and the following centrifugation at12000g, 4°C for 10 minutes. This first part of the protocol represented the separation of phase, where after this centrifugation the RNA is found in the pellet that is kept. The supernatant of the tube was thrown. The next step of the RNA isolation is the washing step. Firstly, the pellet is washed with 1ml of 75% ethanol in DEPC-treated water and vortex briefly before to be centrifuged at 7500g, 4°C for 5 minutes. The supernatant is then removed and discarded. This washing step was repeated 2 times for an optimal wash. At the end of the washing procedure, the pellet was dried by using a needle connected to a vacuum pump. The needle was changed between every sample to avoid contamination between samples. To make sure that the drying step was optimal, the tubes were left opened under hood for 2 minutes. However, the pellet was tried to not be too dried to avoid loss of solubility. Finally, the RNA was eluted in 150µL or 200µL of DEPC-treated water depending on the size of the pellet. Prior to freeze at -80ºC, the tubes were incubated at 60°C for 10 minutes in order to get complete dissolved pellets.

2.4.2 RNA concentration and its quality

Before the accomplishment of any further steps in the gene expression study, it is primordial to determine the concentration of total RNA isolated and to make sure of its proper purity and quality. The lack of good quality can significantly reduce the accuracy, precision of gene expression analysis and thus might not reflect the real level of expression of studied genes. Thus, bad purity and bad quality will equal to non-reliable study. RNA is considered as pure if there is absence of contaminant such as DNA, proteins or other chemicals such as phenol and guanidinium isothiocyanate. On the other hand, RNA with a good quality is qualified as being not degraded when having both 18S and 28S subunits of the ribosomal RNA. In other term, the integrity of RNA is reflecting degradation level of the samples. The quality is also generally based on the ratio of 28S:18S rRNA (Wieczorek D 2012).

Concentration and purity of RNA

Firstly, NanoDrop Uv visible spectrophotometry ND 2000c was used in order to determine the RNA concentration of the samples. This allowed thereafter getting the same final concentration of cDNA for all samples. In order to determine the concentration of RNA, the absorbance at 260nm was measured. The measure of the absorbance at this wavelength is a good indicator to know the concentration of RNA because it is recognized that a reading of 1.0 at 260nm is equal to approximately 40 µg/ml of RNA (Wieczorek D 2012).

The liver samples were diluted due to high concentration of RNA which was ranging from 1100ng/ µL to 4900ng/µL. However the brain samples were ranging from 244ng/µL to 1030ng/µL and thus did not need to be diluted. The purity of RNA was checked via the measurement of optical density at different wavelengths by checking A260/280 and

A260/230 ratios. The absorbance measured at 260 and 280nm is specific to nucleic acid and protein respectively. The A260/280 ratio is telling to the user if there is any contamination of the RNA with protein, however the A260/230 ratios is indicating possible contamination due to other chemicals such as phenol, guanidinium isothiocyanate. Thus, RNA is considered as pure if the A260/280 ratio is ranging from 1.8 to 2.2 and A260/230 ratio is superior to 1.7 (Wieczorek D 2012).

Quality assessment

The RNA integrity cannot be determined by the reading of the absorbance at 260nm and therefore need other methods such as agarose or acrylamide gel electrophoresis and bioanalyser technique (Wieczorek D 2012). Thus, the quality was checked by running an electrophoresis gel where 1µg RNA from 10 randomly selected samples was separated at 70 V for 10 min and 50V for 2 hours. Then the presence of the two bands was checked under UV as shown in the next Figure 10

Firstly, the gel was prepared by melting together in a beaker, 2.4776g of agarose, 210ml of 10xMOPS and, 180ml of DEPC-treated water. When it was melted and reaches a temperature around 50°C, 0.9ml of 37% formaldehyde and 5µL of GelRed was added. Then, the mix was transferred into gel electrophoresis equipment to be allowed to thick for about 30 minutes. Secondly, 5µL of 10 random samples were added to 10uL of sample buffer and denatured at 65°C during 4minutes. Then the samples were applied in each well of the gel. Finally, the gel was applied to a current of 70volts for 10 minutes and then at 50V for two hours. The gel was observed under UV lamp and took in picture with G box software.

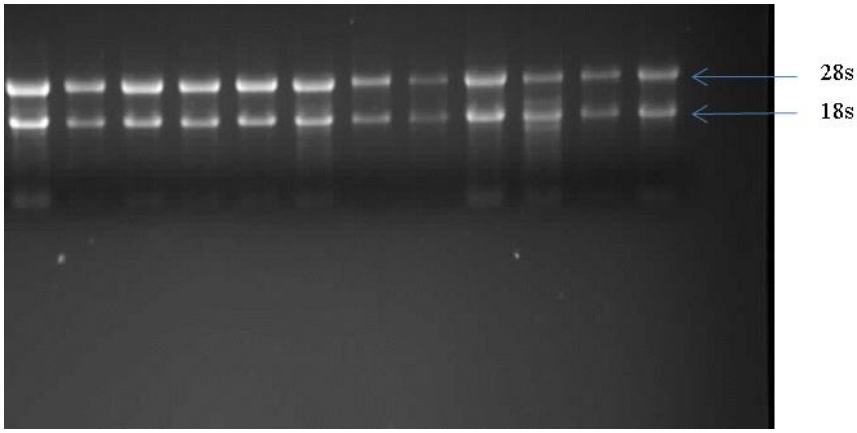

Figure 10 The total RNA was separated by acrylamide gel electrophoresis. The presence of two intact bands allowed to show the presence of 18S and 28S ribosomal RNA that represent about 80% of total RNA. Thus, this gel confirmed that the RNA was acceptable in order to continue with the gene expression analysis. Total RNA was separated by formaldehyde gel.

2.4.3 Real-time polymerase chain reaction

In order to detect and quantify mRNA, one can use the reverse transcription followed by polymerase chain reaction known as PCR. This technique shows good reproducibility as well as great sensitivity (Bustin 2000). This type of PCR is not an end point technique as traditional PCR is due to its capacity to measure the level of PCR product at the end of each reaction cycle. To do that, it uses a probe such as SYBR green that upon binding to double stranded DNA will emit fluorescence at 520nm. In theory, there is proportionality between the level of fluorescence signal and the amount of PCR product generated in the exponential phase of the PCR reaction.

During the real-time PCR reaction, one can notice the exponential augmentation of the fluorescence until it exceeds the background fluorescence. Thus, the Cycle threshold or Ct value was determined by

using this property and in addition of a standard curve served to calculate the copies number of mRNA of a specific gene present in the sample.

Protocol

In this study, real time PCR was performed with Mx3000P real-time PCR system (Stratagene, La Jolla, CA). Prior to the real time PCR, the primers, diluted cDNA at 1/6 and Light Cycler SYBR Green master mix were thawed on ice. And the 96 well-plate containing the diluted cDNA was spined down at 600rpm for one minute. Due to light sensitivity of the SYBR Green mix, this product was protected with aluminium foil. After having vortex and spin primers, and tilt the SYBR Green up and down, the master mix was prepared as described in the table 3. Next, 15µL of the master mix was added to a new well labelled PCR plate followed by the addition of 5µL of cDNA with an eight-channel pipet. Finally, the plate is cover with a plastic seal and spin down at 600rpm for one minute before the run of the real time PCR. It was important here to verify the absence bubbles in the plate. The thermal profile setup for the real-time PCR was set up as shown in the Table 4.

Table 3 Mastermix composition for real-time PCR

Mastermix compounds	**Volume for one reaction**
SyberGreen supermix	10µL
Primer (Reverse)	1µL
Primer (Forward)	1µL
H_2O	3µL
Total	**15µL**

Table 4 Program for real-time PCR

Temperature (°C)	Time	Number of Cycles
95	10 minutes	1
95	30 seconds	
60	15 seconds	40
72	15 seconds	
95	1 minute	
65	30 seconds	1
95	30 seconds	

In order to identify the melting temperature of the DNA, the dissociation curve has been generated by adding a final step where the temperature augmented gradually form 65 to 95°C. It is known that the Tm is sequence specific and thus it serves to check that the amplification of a single, specific cDNA was successful, by checking the presence of a single peak in the melting curve. Therefore, after each real-time PCR reaction, it was checked that only one peak was present which gave the affirmation that only a single sequence was amplified. However, if the dissociation curve displayed multiple peaks, this would have indicated the presence of non-specific product of primer dimers.

2.4.4 Primer testing

When receiving new primers, the annealing temperature is unknown, thus, it is necessary to test the primers on some samples before using them routinely. This primer testing served also to ensure of the specificity of the primers for the samples. The primer testing can be done either by

using classical PCR or using Q (RT)-PCR. In this case, it was performed by using Q (RT)-PCR and a cDNA pool produced beforehand. Thus, a Mastermix with the SYBR Green, primers, autoclaved water and the cDNA pool was prepared as seen in the table 3. The total volume of the reaction was 25 µL. The real-time PCR program used was the one that is used ordinarily. Only the annealing temperature varies. The real-time PCR program is described in the Table 4.

The melting curve that is produced at the end of the reaction, indicated on whether or not the primers were binding specifically the cDNA at a specific temperature. By checking the presence of a single pick on the melting curve, it was possible to determine the correct annealing temperature for primers. So the new primers including multi-R, OAT1d1, OAT, and P-glycoprotein were tested on cDNA pool. However, the annealing temperatures for CYPA1, CYPA3, GPX, GR, GST, Catalase, StAR, and P450scc were already known due to their routinely use in this laboratory. The primers used for this study are grouped in the Table 5.

Table 5 List of used primers in this study

Target name	Primer sequence 5'-3' Forward	Primer sequence 5'-3' Reverse	Annealing temperature (°C)	Size (bp)
P-glycoprotein (p-gp)	CTGCTTTGGACAAGGTGAGA	GCTGTGTGTTCCCAGCTCTA	60	126
Multidrug resistant (MDR) protein (Multi R)	CCCAGCACTCTCTTCAACAA	AGCTGTCTATCCAATGCAG	60	111
Organic anion transport (OAT)	CTATTGTCTGTACGGCCTGG	ATTGTTGTTCCCATTGGCCT	60	-
Organic anion transport (OAT1d1)	GGTGCTGTACCTGCAACTGT	GTCCCGGTCATCTTTGTTCT	60	145
Steroidogenic acute regulatory protein (StAR)	AGGATGGATGGACCACTGAG	GTCTCCCATCTGCTCCATGT	63	163
Cholesterol side-chain cleavage enzyme(P450scc)	CTCTGCCAGCATGTGGACTA	TCTGGACCGTACTGCTGTTG	60	96
Catalase (CAT)	CATCCAGAAACGTTGGGTTC	GAGGCACCTCTACGGGTGTA	60	129
Glutathione peroxidase (GPx)	GTGGGGAGTGGAAATCATGT	ATTTGTTGAATGGGGAGCTG	60	131
Glutathione reductase (GR)	AGGAAGCTGGCACACAGACT	CTTCTCCTCTTTGCCCACAC	60	248
Glutathione-S-transferase (GST)	CGCATTGACATGATGTGTGA	TGTCGAGGTGGTTAGGAAGG	57	121
Biotransformation enzyme phase I (cyp1a1)	GAGTTTGGGCAGGTGGTG	TGGTGCGGTTTGGTAGGT	60	76
Biotransformation enzyme phase I (cyp3a)	ACTAGAGAGGGTCGCCAAGA	TACTGAACCGCTCTGGTTTG	60	146

2.4.5 Normalization of data

Housekeeping genes are normally known to have a constant expression that does not vary with experimental conditions. In order to correct possible variations between samples that might occur during real-time PCR, Scientifics use often housekeeping gene as an internal control gene called standard. This is known as normalization where the mRNA copy numbers of the target is determined. However, some toxicological studies with aquatic organisms exposed to chemicals have shown that housekeeping genes changed significantly. Thus, this leaded to possible errors and not suitable interpretations of the results (Arukwe 2006). This

is the reason why, for this study, normalization to plasmid containing gene reference specific to the same primers than the template was chosen. So it is possible, upon real time PCR reaction to determine precisely the copy numbers of mRNA by simply reporting the Ct to the obtained standard curve.

Plasmids containing the different genes used in this study were diluted in a manner to obtain a dilution series. They served to obtain a standard plot of Ct values versus the logarithm of copy number leading to a linear equation. This linear equation of a specific standard curve was used to normalize the gene expression of the corresponding gene. However, because of the lack of some plasmids, ready standard curve from previous gene expression analysis was used instead. The plasmids missing included OAT, OAT1d1, p-glycoprotein, multi-R and GST. These dilution series were run at the same time than the samples.

Furthermore, normalized results were transformed in percentage of control by dividing the exposure groups over the control and where each day had their own corresponding control.

2.5 Biochemical analysis

Gene expression analysis can be compliment by protein analysis, using either qualitative or quantitative methods. Upon transcription of genes to RNA, further modification may intervene such as post-transcriptional modification, translational or again alternatives processes that lead to functional changes in proteins. Thus, the presence or inactive protein is not all the time well reflected by the mRNA transcript (Ekins, Nikolsky et al. 2005). This is the main reason of both gene and protein expression studies when toxicological studies are performed.

During this project, different enzymatic assays were performed. Firstly the activity of molecules involved in defence of organisms against

reactive oxygen species was assayed including catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR), Glutathione (He and Lin). Secondly the potential neurotoxicity effect of metformin was studied by determination of Acetylcholine esterase activity. Finally, immunochemical analysis, western blot was performed on the steroidogenesis key proteins StAR and P450scc. The spectrophotometry instrument synergy TM HT multidetection microplate (Winnoski, Vermot, USA) was used in order to determine all of these activities.

2.5.1 Preparation of post-mitochondrial fractions (PMF)

Prior to perform the enzymatic assays, it was necessary to extract total proteins. Therefore, post-mitochondrial fractions protocol was followed as following described. About 300mg of liver kept carefully on ice to slowly thaw and was added to cold 0.1M homogenizing phosphate buffer at pH7.4 (Table 6). Then, the tissue was crushed by using a pre-chilled tissue homogenizer. Afterwards, the samples were transferred into tubes and centrifuged at 12000 g for 20 minutes at 4°C. Finally, the supernatant was collected and poured into new tube by avoiding the pipetting of the pellet and the floating lipid layer. The content of the new tube was aliquot in several 500ml ependorf tube. The post-mitochondrial fractions were store at -80°C for further analysis.

Table 6 Composition of the 0.1M homogeniser buffer pH 7.4.

Compounds	Quantity	Volume
NaH_2PO_4	13.8g	
KCl	11.2g	
Distilled water		500ml
EDTA (ethylene diamine tetra acetic acid)	0.372g	
DTT (1,4-Dithiothreitol)	0.154g	
Glycerol		115ml
Distilled water		To 1L

2.5.2 Bradford

The total amount of protein was measured before further biochemical analysis on the samples. Bradford, a commonly used method was performed in this purpose.

Protocol

40ml of coomassie was added to 40ml of phosphoric acid was prepared. Next, the standard calibration curve was prepared through three different steps. Firstly, BSA stock solution of 10mg/ml was diluted to 0.2mg/ml for be measure in nanodrop at 280nm. This allowed determining the concentration of the diluted BSA and using it as a factor (Equation 2) in calculating the concentration of the standards.

Equation 2 Calculation for factor

$$\frac{Abs\ 280\ BSA}{0.667}$$

Where 0.667= Extinction coefficient of BSA standard of 1mg/ml

Then the samples were diluted in PMF buffer at 1/600. The BSA standard was diluted according to the Table 7 with first the water and

BSA. Next, 50µL of diluted samples were applied in duplicate to a 96 well plate. Then the 3ml of coomassie was added to the BSA tubes followed by a mixing. Finally, 350uL of standards tubes were added to the plate. After 5 minutes of incubation at room temperature, the plate was read in the plate reader at 595nm.

Table 7 Composition of the BSA standards

Tube	µL BSA	µL H_2O	Coomassie ml
1	0	500	3
2	10	490	3
3	20	480	3
4	30	470	3
5	50	450	3
6	75	425	3
7	100	400	3
8	150	350	3
9	200	300	3

2.5.3 Determination of enzyme activity

2.5.3.1 Catalase activity

Catalase reduces the hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) in order to protect the cell against damage of macromolecules. In this study, the activity of catalase was determined by using a colorimetric method by using a standard range made of formaldehyde solution. By measuring the absorbance of both the samples and the standard at 540nm, it was possible to determine the activity of catalase.

At first, a formaldehyde standard solution was prepared in sample buffer (Table 8) to get a range of different final concentrations from 0 to 75µM formaldehyde in a volume total of 170µL. Secondly; the plate was filled

by 100µL of assay buffer (Table 8) and 20µL of the samples and standard. Next, 20µL of catalase were added to the CAT control wells. Then, in the all plate, 30µL of methanol followed by 20µL of diluted hydrogen peroxide (H_2O_2) were added. Upon addition of hydrogen peroxide, the reaction was immediate, so, it was important to start the timer after its addition in the first column. Afterwards, the plate was covered with aluminium foil and incubated at room temperature for 20 minutes under shaking at around 500. After incubation, 30µL of potassium hydroxide was added to strop the reaction. Thus, at this step, the timer was stopped when the first column of the plate received the potassium hydroxide in order to have the accurate reaction time. Before similar second incubation of the plate during 10 minutes, 30µL of Purpald was added to start colour production. At the end of incubation, 10µL of potassium periodate (KIO_4) was added to the whole plate in order to stop the colour production. Finally, a last incubation of the plate for 5 minutes was performed prior to the reading of absorbance at 540nm using the plate reader.

Table 8 Composition of the Assay and Sample buffer for catalase

	Compounds	**Quantity**	**Volume**
Assay buffer (pH 7)	KH_2PO_4	6.80g	500ml
	Milli-Q water		
Sample buffer (pH 7.5)	KH_2PO_4	3.4022 g	
	Milli-Q water		200ml
	EDTA (ethylene diamine tetra acetic acid)	0.3722g	
	Milli-Q water		200ml
	BSA	10g	
	Distilled water		To 1L

The catalase activity of the 60 samples was calculated as following:

First, the standard curve was established by plotting the rectified absorbance in function of the final concentration in a total volume of 170µL reaction. Thus, the equation y= ax + b was obtained and used to calculate the activity of catalase for each sample. It was important to take in account the dilution factors. Then the calculation as showing bellow (Equation 3) served to determine the catalase activity in nmol/min/mg protein.

Equation 3 Calculation for catalase activity in nmol/min/mg protein

$$\frac{\left(\frac{\text{Corrected Abs} - \text{b}}{\frac{\text{a}}{\text{reaction time}}}\right) * dilution\ factor}{mg\ prot/ml}$$

Where

Corrected Abs= Sample Absorbance-Standard without formaldehyde

Reaction time: the time of reaction with hydrogen peroxide to reduce NADPH to $NADP^+$

Dilution factor = 1700

Mg/protein= amount of proteins in each sample determined by Bradford method.

2.5.3.2 Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) has the capacity to reduce hydrogen peroxide (H_2O_2) as well as organic compounds. Upon reduction of hydrogen peroxide, there is formation of oxidized glutathione (GSSG) and thus diminution of glutathione (He and Lin) that is then re-established by GR. Gr uses a dependent NADPH activity in order to recycle the GSSG. Therefore, the activity of glutathione peroxidase was determined indirectly by a coupled reaction with glutathione reductase

(GR). To be more detailed, GPX activity was measured by assessing consumption of NADPH at 340 nm and at 25°C.

Firstly, 120µL of assay buffer (Table 9) was added in the blank wells also referred as non-enzymatic wells of the 96 microtiter plate, and 100µL to the sample wells. Secondly 20µL of sample were added to the all wells except the blank wells. Prior to that, the samples were dilute to 1/500 in sample buffer (Table 9). Then, 20µL of GSH (10mM), GSSG (2.4U/ml), NADPH (1.5mM), and H_2O_2 were added to each well. The reaction starts immediately upon addition of the hydrogen peroxide, so it was important to add it as fast as possible. The plate is carefully checked for a few second to mix the content. And, the absorbance is directly read once every minute for a duration total of 5 minutes at 340 nm using plate reader. The initial absorbance of the samples should be between 0.5 and 1.2. The entire samples were in this range. Afterwards, the GPx activity was determined as described in the Equation 4 and expressed as nmol/min/mg protein.

Equation 4 Calculation for GPx activity in nmol/min/mg protein

$$\frac{\left(\frac{\frac{\Delta A340}{\min}}{0.003730}\,\mu M * \frac{0.2}{0.02}\,mL * \text{sample dilution}\right)}{mg\ prot/ml}$$

Where:

ΔA340/min = change in absorbance per minute. This value for each sample was directly obtained from the software of the plate reader instrument and then substrate by the ΔA340/min of the blank samples.

0.003730 = fixed extinction coefficient for NADPH because the usual extinction coefficient is 0.00622 but was adjusted for cuvette size 0.6 instead of 1

0.2ml = total volume of the assay per well

0.02ml = volume of NADPH per well

Mg protein/mL= amount of proteins in each sample determined by Bradford method.

Table 9 Composition of the Assay and Sample buffer for GPx.

	Compounds	Quantity	Volume
Assay buffer (pH 7.6)	Tris (hydoxymethyl) aminomethane	3.03g	
	Milli-Q water		500ml
	EDTA (ethylene diamine tetra acetic acid)	0.93g	
Sample buffer (pH 7.6)	Assay buffer		400ml
	BSA	0.4g	

2.5.3.3 Glutathione-S-transferase (GST) activity

GST has the capacity to form the conjugation of glutathione with compounds that inhibit electrophilic site of a reactive molecule (Storey 1996). In this assay, 1-chloro-2.4-dinitrobenzene (CDNB) was used as substrate for the enzyme GST to catalyse the conjugation of glutathione with compounds. The activity of GST was determined by measuring the absorbance of samples at 340nm.

At the start of the assay, 10µL of sample and 10µL of 0.1M homogeniser buffer used for PMF (Table 6) was added to a 96 well microtiter plate. In addition, 20µL of homogeniser buffer (Table 6) was added to separate wells as blank samples. Then, 200µL of a solution containing assay buffer with CDNB (100mM) as substrate, and reduced glutathione (1mM) was added to all wells. Finally, the plate was read on the plate reader at 340nm every minute for 5 minutes. Afterwards, the

GST activity was determined as described in the Equation 5 and expressed as nmol/min/mg protein.

Equation 5 Calculation for GST activity in pmol/min/mg protein

$$\frac{\frac{\Delta A340}{min}}{\frac{0.96 * 0.6 * 0.22}{(\mu L \text{ buffer PMS} * \text{mg prot/ml})}}$$

Where:

ΔA340/min= change in absorbance per minute. This value for each sample was directly obtained from the software of the plate reader instrument and then substrate by the ΔA340/min of the blank samples. (Background corrected rate (samples abs-blank abs)

0.96= extinction coefficient for the conjugate

0.6= path length for 0.22mL

0.22= total volume of each well

$\mu L \text{ PMS} * \text{mg prot/mL}$= Normalization with protein concentration in the PMS

2.5.3.4 Glutathione Reductase (GR) activity

Because of the consumption of NADPH during the reduction of GSSG to GSH by GR, it was possible to determine the GR activity based on this property. Thus, GR activity was determined by measuring the consumption of NADPH at 340nm at room temperature. Afterwards, the GR activity was determined as described in the Equation 6 and expressed as nmol/min/mg protein.

Equation 6 Calculation for GR activity in nmol/min/mg protein

$$\frac{\left(\frac{\frac{\Delta A340}{\min}}{0.003730}\,\mu M * \frac{0.19}{0.02}\,mL * \text{sample dilution}\right)}{mg\ prot/ml}$$

Where:

ΔA340/min = change in absorbance per minute. This value for each sample was directly obtained from the software of the plate reader instrument and then substrate by the ΔA340/min of the blank samples.

0.003730 = fixed extinction coefficient for NADPH because the usual extinction coefficient is 0.00622 but was adjusted for cuvette size 0.6 instead of 1

0.2ml = total volume of the assay per well

0.02ml = volume of NADPH per well

Mg prot/mL= amount of proteins in each sample determined by Bradford method.

Protocol

Firstly 120 and 100µL of GR assay buffer (Table 10) was added to the non-enzymatic wells and sample wells respectively, of a 96 well microtiter plate. Next, 20µL of sample was applied in the sample wells of the plate followed by 20µL of GSSG (9.5mM) and 50µL of NADPH (1.5mM) in all wells. Finally the plate was carefully shaken for a few second and directly read every minute for 5 minutes at 340nm with the plate reader.

Table 10 Composition of GR assay buffer

	Compounds	Quantity	Volume
Assay buffer (pH 7.5)	K_2HPO_4	4.355g	
	Milli-Q water		500ml
	EDTA (ethylene diamine tetra acetic acid)	0.186g	

2.5.4 Acetylcholine esterase activity

Acetylcholine is a neurotransmitter found in most of species and thus in fish. It is important for the proper functioning of muscle and the behaviour of the organism (Modesto and Martinez 2010). This neurotransmitter can be hydrolysed by the acetylcholine esterase and thus this enzyme shown to be a good biomarker for neurotoxicity (Ellman, Courtney et al. 1961). In this study, the activity of the enzyme acetylcholine esterase has been accessed because it is an important indicator for the neurotoxicity in organisms. Acetylthiocholine, the substrate for the enzyme acetylcholine esterase is transformed to thiocholine and acetate. Then, in presence of dithiobisnitrobenzoate, the thiocholine reacts and result to a yellow colour production. This was, thus, measured by the plate reader at 340nm and the activity of acetylcholine esterase was determined by following the rate of yellow colour formation. (Ellman, Courtney et al. 1961)

Protocol

Beforehand, the samples were diluted to 1 over 10 in phosphate buffer (0.1M pH 8). The first step of the assay started by the addition of 100 and 150µL phosphate buffer (0.1M pH 8) in the sample wells and blank well respectively of a 96 well plate. Next, 50µL of dithiobisnitrobenzoate, DTNB (0.5mM) were added to all the plate, followed by 50µL of

samples only in sample wells. At this step it was important to cover the plate with aluminium foil because DTNB is reactive to the light. Finally 50µL of the substrate acetylthiocholine (1mM) was added to every well of the plate as fast as possible. Finally, after agitation for few seconds, the plate was read by the plate reader every minute for 5 minutes at 412nm. Afterwards, the AChE activity was determined as described in the Equation 7.

Equation 7 Calculation for AChE activity in nmol/min/mg protein

$$\frac{\left(\frac{\Delta A340}{\min}\frac{1}{8490000}\,\mu M * \frac{0.250}{0.05}\,mL * \text{sample dilution}\right)}{mg\ prot/ml}$$

ΔA340/min = change in absorbance per minute. This value for each sample was directly obtained from the software of the plate reader instrument and then substrate by the ΔA340/min of the blank samples.
8490000 = fixed extinction coefficient for dithiobisnitrobenzoate (DTNB). The usual extinction coefficient is 0.00622 but it was adjusted for cuvette size 0.6 instead of 1
0.250ml = total volume of the assay per well
0.05ml = volume of DTNB per well
Mg prot/mL= amount of proteins in each sample determined by Bradford method.

2.5.5 Western Blotting

In order to supplement the gene expression analysis previously performed by real time PCR, the study of the level of protein expression

has been performed by Western Blotting technique. Western blotting is a powerful technique to detect numerous proteins, especially those who are present in small quantity. In addition, Western blot is a useful tool in order to detect and assess the protein expression (Kurien and Scofield 2006). By testing samples with Western Blotting, it was possible to directly study specific proteins including StAR and P450scc and determine their levels between different conditions of exposures to metformin.

Protocol

Firstly the samples were prepared by adding the determined amount of sample buffer and the samples in order to get a final amount of 50µg per 20µL volume total. Next the proteins were denatured by a bath at 95ºC for 3 to 4 minutes. This allowed the linearization of proteins and thus exposing the negative charged to allow migration into SDS-polyacrylamide gel-electrophoresis. Afterward, a SDS-polyacrylamide gel-electrophoresis was loaded with 5µL of standard in the first well and 20µL of samples in the next wells. Once, the gel loaded and placed in chambers, bloating buffer was added and electrical field was applied to the gel. At First, 70V was applied for 10 min, then 150V for about 40 minutes. The migration of proteins was stopped when the blue front line reached the bottom of the gel to allow good separation of the proteins while avoiding their escape from the gel. Then, the gels were exposed to bloating buffer for 10 minutes. The next step consisted to the transfer of the proteins from gels to PVDF membranes by the so-called sandwich technique. Membranes were activated with pure methanol prior to the transfer technique. First a sponge soaked with transfer buffer was placed in the cassette, followed with 2 papers socked with transfer buffer were

placed on top of the sponge. The gel was then placed on top of the rest before to be carefully covered with the membrane. It was important at this stage to make sure that no bubbles were present between the gel and membranes and if necessary to remove bubbles, it was possible to use roller. Afterwards, two other papers soaked with transfer buffer were place on top of the membrane, followed by a new soaked sponge. The cassettes were then placed into the blotting cell with its negative pole facing the negative pole of the container and filled up with transfer buffer and a frozen cooler. The transfer was performed during an hour at 100volts-400mA at 4ºC. Afterwards, the membranes were placed in TTBS-buffer for 5 minutes and 2 times before to be left in blocking buffer for an hour in order to block the unreacted binding sites on the membranes. Next, the primary antibody solution was poor on the membrane and left for incubation overnight at 4ºC. The next day, the membranes were washed with TTBS for 5 minutes and 6 times before to be incubated with the secondary antibodies for one hour at room temperature. An ultimate wash with TTBS as before was performed. Finally, 3ml of prepared immunostar detection kit reagent was applied on the membranes and left for 3 minutes. The membranes were then covered and developed in Kodak machine. The composition of the different solutions including running and blotting buffer, TTBS, Blocking solution is referred in Table 11.

Table 11 Composition of Running and Blotting buffer, TTBS, and blocking solution for western blot

	Composition	Quantity	Volume
Running buffer (pH 8.3)	Trizma base	6.0g	
	Glycine	28.8g	
	Sodium Dodecyl Sulfate (SDS)	2g	
	Distilled water		2L
Blotting buffer	Trizma base	3.03g	
	Glycine	14.14g	
	Distilled water		800ml
	Methanol		200ml
TTBS (pH 7.5)	Trizma base	4.84g	
	NaCl	58.48g	
	Distilled water		2L
	Tween-20		1ml
Blocking solution	TTBS		100ml
	Dry non-fat milk	5g	

2.6 Statistical analysis

Gene expression, protein analysis, biometric parameters of fish including length, weight, Fulton's condition factor CF and accumulation data have been statistically analysed with IBM SPSS software, version 13. First, the normality of the data was checked with Shapiro-Wilk test as well as using boxplot and linear curve for visual observation, followed by the homogeneity of variance tested with Levene's test. Afterwards, the statistical analysis could begin with statistical tests that were appropriate for each data set. Thus, when data did not follow the normality

requirement, they were attempting to be transformed in order to become normally distributed. In the case of impossible transformation to render the data normal, non-parametric test was applied for the statistical test such as Kruskal-Wallis followed by Mann-Whitney U test. On the other hand when the data were normal, and following the homogeneity of variance, one-way ANOVA followed by Tukey post hoc test with Bonferonni was used. If the data were normal but lacking homogeneity or not equal in size sample, Welch test with Games Howell test were used. Statistical test were performed in order to detect significant differences between control and exposure groups or between the exposure groups at a specific day. In addition, the statistical tests were also used to identify significant differences between groups for a specific concentration and different days. The p-value was set to 0.05.

In addition to the statistical test, outliers were detected by after observation of the data and manually detection of suspicious data, Grubb's test, chauvenet test were both performed. Thus, in case of presence of extreme outliers, they were removed from the data analysis and graph.

3 Results

3.1 Biometric parameters

Concerning the length and weight of the fish, there were no significant differences detected during the experimental period. The Fulton's condition factor CF, an indicator for fish health and growth was determined using the equation 100*(weight/length^3) (Table 12). K factor showed significant differences at day 3 between 0 and 50, 0 and 500 as well as between 5 and 50, 5 and 500µg/L with the non-parametric Mann-Whitney U test and p-value of 0.05. The exposure experiment did not lead to mortalities.

Table 12 Weight, length and Fulton's condition factor K at day 3, 7 and 10, and for all concentration of metformin. Weight and length are specified in grams and cm. Juvenile Atlantic Salmon were exposed to various concentration of metformin during different days. Fish were sacrificed and measured before the calculation of Fulton's condition factor K. All values are represented in this table with the mean and standard error of mean (SEM)

		Weight (g)		Length (cm)		Fulton's condition factor, K	
		Mean	SEM	Mean	SEM	Mean	SEM
Day3	0 µg/L	76.24	4.75	20.20	0.41	0.92	0.04
	5 µg/L	93.66	7.27	21.74	0.76	0.91	0.02
	50 µg/L	86.90	9.25	22.80	1.64	0.76	0.08
	500 µg/L	91.56	11.60	22.84	0.81	0.76	0.03
Day7	0 µg/L	69.70	6.26	20.26	0.56	0.83	0.02
	5 µg/L	79.38	5.17	20.88	0.38	0.87	0.02
	50 µg/L	74.58	4.69	20.96	0.42	0.81	0.01
	500 µg/L	81.38	6.58	21.44	0.70	0.82	0.02
Day10	0 µg/L	67.88	3.51	20.320	0.22	0.81	0.03
	5 µg/L	77.44	4.75	20.940	0.21	0.84	0.05
	50 µg/L	74.16	5.77	20.680	0.51	0.84	0.04
	500 µg/L	74.86	7.28	20.980	0.55	0.80	0.03

3.2 Metformin uptake and accumulation

3.2.1 Uptake and accumulation of metformin

Overall no significant differences in metformin uptake and accumulation were detected between exposure groups in carcass and gills. However, a significant difference was observed between the control and carcass at day 10 with 500µg metformin/L (Figure 11). Despite the absence of significant differences, a higher accumulation pattern of metformin in gills was observed, compared with the carcasses at all sampling days. Overall, the detected amount of the drug measured in both carcasses and gills was relatively low compared to the nominal concentration of metformin added to the tanks.

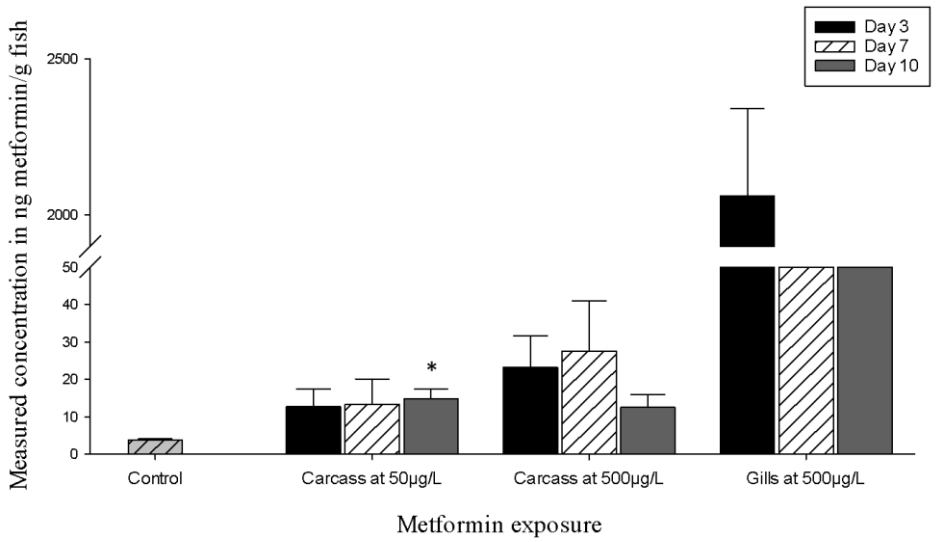

Figure 11 Determination of the accumulated metformin in carcass and gills of Juvenile Atlantic salmon (Salmo salar) after 3, 7 and 10 days. Only the carcass at 50 and 500µg/L as well as gills at 500µg/L were analysed with n=3 for all carcass group and n=3 for gills at day 3 and n=1 at day 7 and 10. The control were only analysed at day 3. The asterisk denotes the significant difference between group exposure and control.

3.3 Bioconcentration factor (BCF)

The control group displayed a high BCF that was demonstrated to be much higher than the carcass at both 50 and 500µg/L exposure groups (Figure 12). However, at the short exposure time of 3 days, a slightly higher BCF than the control was observed. The BCF decreased for gills when fish were exposed for a longer period at day 7 and 10 (Figure 12).

.

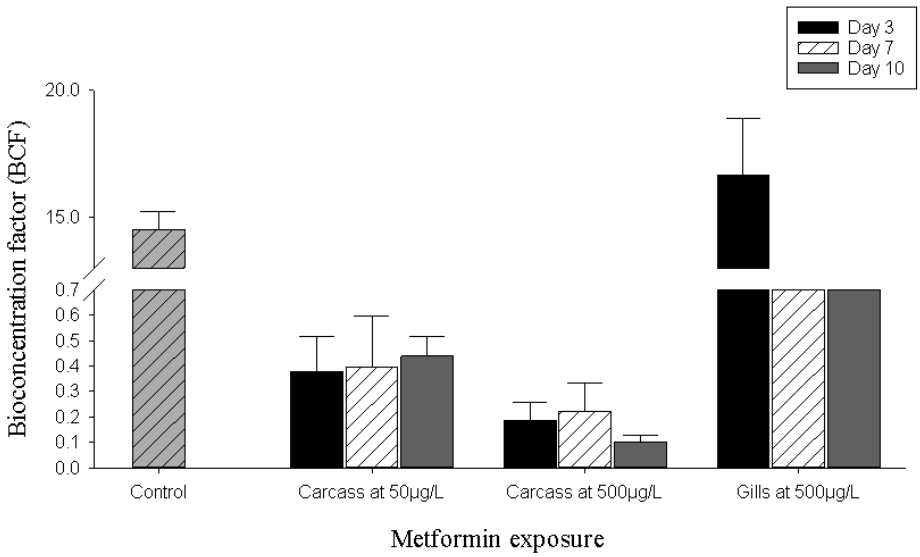

Figure 12 Determination of the bioconcentration factor for fish carcass at 50 and 500µg/L, and gills at 500µg/L. Only the carcass at 50 and 500µg/L as well as gills at 500µg/L were used with n=3 for all carcass groups and n=3 for gills at day 3 and n=1 at day 7 and 10.

3.4 Physiological parameters

3.4.1 Phase I and II biotransformation

cyp1a1

Gene expression of cyp1a1 did not show significant differences between the various exposure groups or between the control and exposure groups, at both day 3 and 7 (Figure 13A). However, at day 10, a significant repression of cyp1a1 mRNA was observed when fish were exposed to 50 and 500µg metformin/L, compared to the control (Figure 13A). One could observe a clear increase of the transcript expression after 7 days of exposure followed by a decrease after 10 days for all the three groups which turned out to be significant for 500µg/L. Thus, fish that were exposed to 500µg metformin/L seemed to show higher cyp1a1 mRNA variation over time than the other exposed groups (Figure 13A).

cyp3a

Overall, no significant alterations were detected for cyp3a mRNA within days. Nevertheless, a slight decrease was apparent after 3 days exposure to metformin at 5 and 50µg/L groups. At day 7, a small increase was visible and especially at 5 and 50µg/L exposure groups (Figure 13B). An increase of cyp3a mRNA was observed between day 3 and 7 for all the exposure groups. However, this increase was only significant for 5µg/L and 50µg/L groups (Figure 13B). After 10 days, the transcript level of cyp3a seemed to reach back to the basal level, but remained significantly higher for 5 and 50µg/L exposure groups, compared to same concentrations at day 3 (Figure 13B)

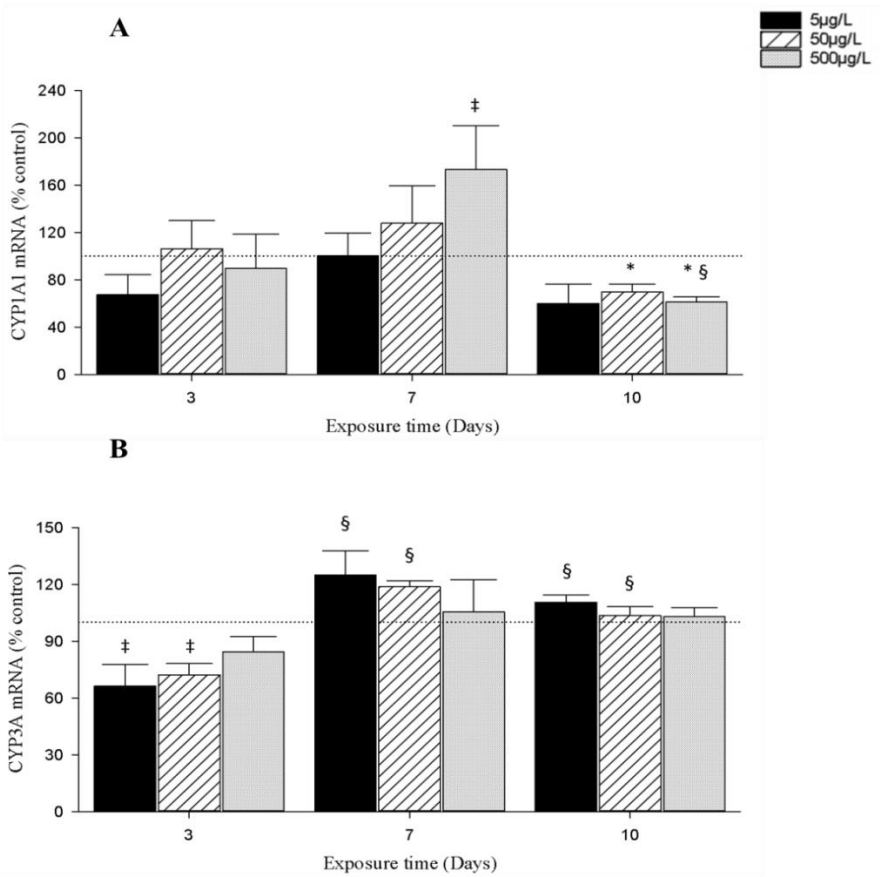

Figure 13 Expression of cyp1a1 (A) and cyp3a (B) mRNA in liver of Juvenile Atlantic Salmon exposed to metformin. Fish were exposed to metformin (0, 5, 50 and 500µg/L) and sampled after 3, 7 and 10 days. The data are set as mean expressed as % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%. Two outliers were removed for cyp1a1 (A) at day 3-5µg/L and day 10-0µg/L, and one for cyp3a (B) at day 7-0µg/L. Asterisks denote significant differences between control group and exposure group within a same day. Bars without asterisk display no significant difference from any other column within a day. Symbols show significant differences for a same concentration at different days. Symbols are absent when no significant differences were detected. Welch with Games-Howell post-hoc test ($p<0.05$) for cyp1a1 at day 10, and for the concentration 500µg/L at different days. One-way ANOVA followed by Tukey post-hoc test ($p<0.05$) and bonferroni for cyp3a between days at a specific concentration for both 5 and 50µg/L.

3.4.2 Membrane transporters and Multidrug Resistant Protein

P-glycoprotein

Overall, the exposure of fish to metformin did not significantly alter the P-glycoprotein mRNA level either at a same day or between days (Figure 14A). Nevertheless, the mRNA levels of the three exposure groups showed an increasing trend at day 7 to slightly become above control level and decrease back after 10 days in order to reach the basal level. However, at day 10, 500µg/L exposure group was slightly decreased, compared to the control and the other exposure groups. At day 7, the increase of P-glycoprotein mRNA seems to be more pronounced at the lowest exposure concentration of 5µg/L (Figure 14A).

Multidrug Resistant (MDR) protein (Multi-R)

At day 7, an increase of MDR expression was observed for the three exposure groups, which was significant for 50µg/L, compared to the control (Figure 14B). Exposure to metformin led to an increase of Multi-R mRNA levels over time, in the three exposure groups, followed by a decrease back to the basal level. However, the expression of this gene at the highest concentration of 500µg/L stayed slightly increased at day 10, compared to the other exposure groups. Concerning the apparent time-dependent increase and decrease of Multi-R mRNA, this was proved to be significant when fish were exposed to 50µg metformin/L (Figure 14B). One could notice that 50µg/L which is highly increase at day 7, returned back the control level at day 10 while the 500 µg/L exposure groups did not manage to get back to basal level (Figure 14B).

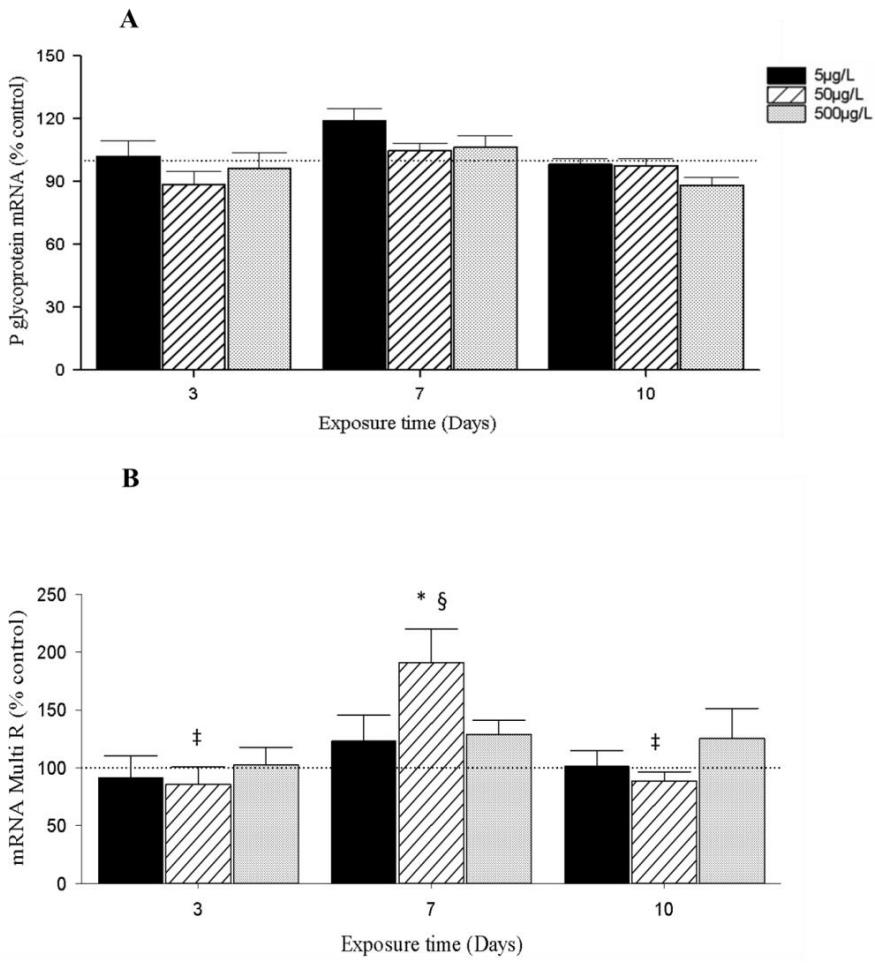

Figure 14 Expression of P glycoprotein (A) and Multi-R (B) mRNA in liver of Juvenile Atlantic Salmon exposed to metformin. Fish were exposed to metformin (0, 5, 50 and 500µg/L) and sampled after 3, 7 and 10 days. The data are set as mean expressed as % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%. Asterisks denote significant differences between control group and exposure group within a same day. Bars without asterisk display no significant difference from any other column within a day. Symbols show significant differences for a same concentration at different days. Symbols are absent when no significant differences were detected. Symbols are absent when no significant differences were detected. Welch with Games-Howell post-hoc test ($p<0.05$) for MDR within day 7 and between the three days at 50µg/L.

Anionic transporter, OAT and OAT1d1

The expression of both OAT and OAT1d1 did almost not vary between exposure and control groups as well as over the different days (Figure 15A-B). At day 3, the OAT mRNA for the three exposure groups lower than the control but only 10% for the most extreme one, while for day 7, they were basically all at control level (Figure 15A). At day 10, very small increase of the exposure group at 500µg/L (Figure 15A). Exactly the same trend was observed for OAT1d1 (Figure 15B).

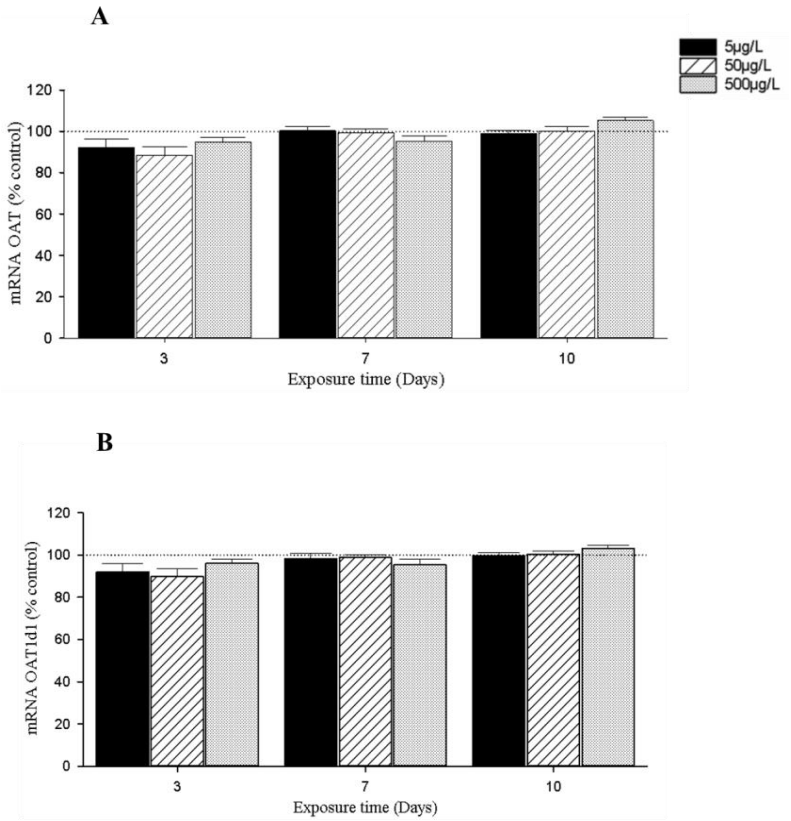

Figure 15 Expression of OAT (A) and OAT1d1 (B) mRNA in liver of Juvenile Atlantic Salmon exposed to metformin. Fish were exposed to metformin (0, 5, 50 and 500ug/L) and sampled after 3, 7 and 10 days. The data are set as mean expressed as % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%.

3.4.3 Steroidogeneisis proteins

StAR

Exposure to 5, 50 and 500µg/L of metformin produced changes in StAR mRNA level when fish were exposed for 3 days (Figure 16A). However, only the 50µg metformin/L group displayed a significant increase compared to the control as well as compared to 5µg/L group. For longer time period including 7 and 10 days, no clear changes in the gene expression of StAR were noticeable. At day 10, StAR expression in the middle concentration of 50µg/L seemed to slightly decrease, compared to the two other concentrations, as well as the control. The same observation was also seen at day 7, but for 500µg/L in this case (Figure 16A). In addition, regardless the concentration of metformin whose fish were exposed to, the expression of StAR mRNA decreased significantly at both day 7 and 10 compared to day 3 (Figure 16A).

P450scc

The expression of p450scc followed the same trend than StAR. A general increase of gene expression for the three exposure groups at day 3 was observed and which is moreover significant for the 50µg/L group when compared to the control and the 500µg metformin/L group (Figure 16B). Also in this case, there was restoration of the gene expression to control level after 7 and 10 days. In addition, at both 7 and 10 days, when fish were exposed to 50µg/L metformin, the expression of P450scc mRNA significantly decreased compared to the day 3 (Figure 16B).

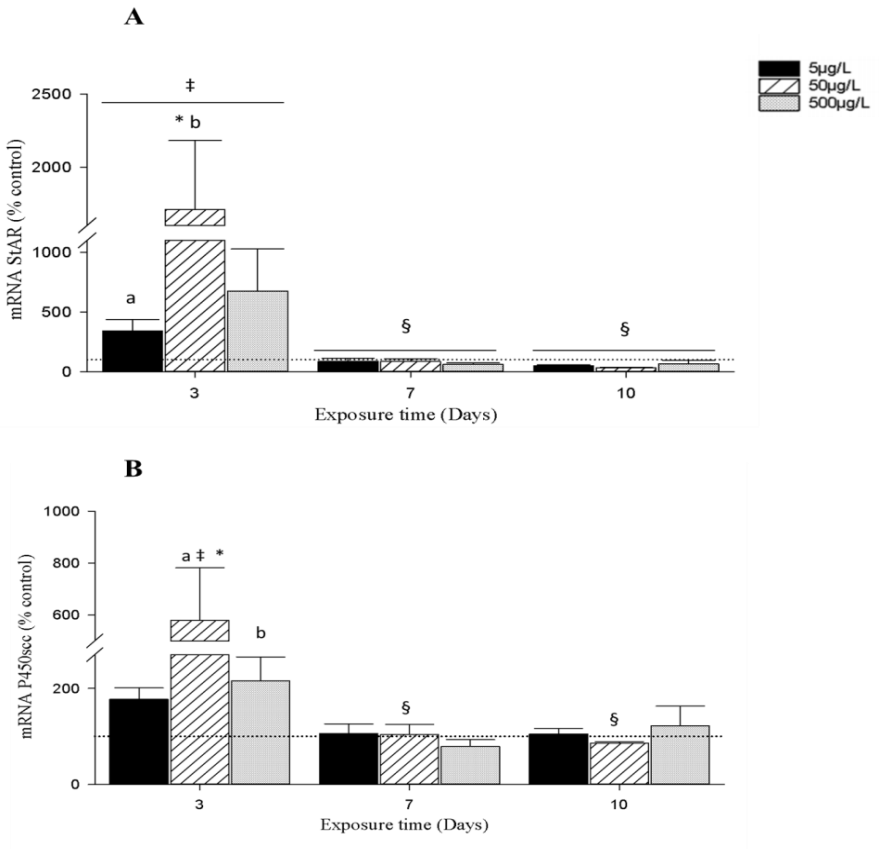

Figure 16 Expression of P450scc and StAR mRNA in brain of Juvenile Atlantic Salmon exposed to metformin. Fish were exposed to metformin (0, 5, 50 and 500µg/L) and sampled at 3, 7 and 10 days. The data are set as mean expressed in % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%. One outlier were removed for StAR (A) at day3-0µg/L, and two for P450scc (B) at day 3-0µg/L and 10-0µg/L. Asterisks denote significant differences between control group and exposure group within a same day. Bars without asterisk display no significant difference from any other column within a day. Letters represents significant differences between different concentrations at a specific day. Bars without letters in a day represent no significant difference for that day. Symbols show significant differences for a same concentration at different days. Symbols are absent when no significant differences were detected. Mann-Witney U test ($p<0.05$) for P450scc mRNA at day 3 and at 500µg/L between days. Welch with Games-Howell post-hoc test ($p<0.05$) for StAR mRNA at day 3. One-way ANOVA followed by Tukey post-hoc test ($p<0.05$) and bonferroni for StAR mRNA at 5, 50 and 500µg/L between day.

3.5 Western blot

Concerning StAR protein, the simple eyes observation of the immunochemical analysis seemed to show about the same protein band intensity when compared between day groups and control or between day groups (Figure 17A1). At day 10, the first StAR protein from the left seemed to be slightly lighter than the other StAR protein. However, it was challenging to describe the results by simple eyes observation (Figure 17A1).

The P450scc protein at day 3 displayed a smaller intensity than for the control but which was less visible for the first band of day 3 from the right (Figure 17A2). The protein intensities at day 3 seemed also to be less pronounced than the ones at day 7 and one of the two at day 10. One could by simple observation see that the two proteins at day 7 were more intense than the control sample. At day 10, the second protein from the left of the picture was really intense compared to the other replicate, and the protein control. (Figure 17A2)

The densitometric analysis, overall, did not revealed large visible differences between exposure groups or between control and exposure groups for StAR and P450scc (Figure 17B1-B2). Exposure to metformin slightly decreased the amount of both proteins at day 3 (Figure 17B1-B2). However, for StAR when compared to the day 3 as well as the control, the amount of the proteins slightly increased at day 7 to after 10 days to keep barely the same amount of protein than day 7 (Figure 17B1). For P450scc, an increase was also visible at day 7 compared to the day 3 and control, followed by slight decrease at day 10 (Figure 17B2). The densitometric analysis for StAR and P450scc did not correspond perfectly to the membrane visualisation by eyes (Figure 17). For StAR protein, the densitometric analysis showed slight variation of protein

expression between control and exposure groups, as well as between exposure groups (Figure 17B1) while no apparent differences was observed on the membrane (Figure 17A1). The comparison between membrane (Figure 17.A2) and densitometric analysis (Figure 17.B2) showed relatively same profile.

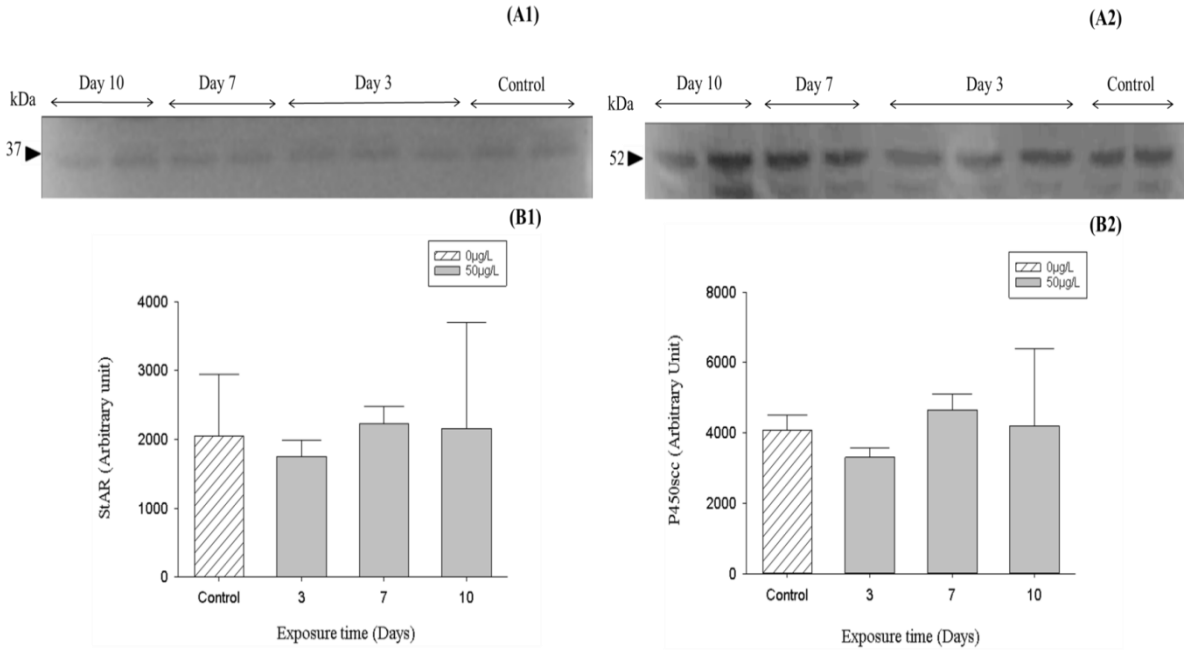

Figure 17 Western blotting was used in order to detect StAR (A1-B1) and P450scc (A2-B2) proteins in Atlantic salmon when exposed to 50µg/L of metformin during different times. The StAR and P450scc proteins were detected on membrane PVDF under chemiluminescence and shown as A1 and A2 respectively. The corresponding quantification of this immunochemical detection was expressed as arbitrary unit for StAR and P450scc as shown in B1 and B2 respectively. Only two samples were used for the control and the group at day 3.

3.6 Oxidative stress responses

Catalase

The only significant change in catalase mRNA compared to the control was for the decrease at day 7 and 500µg/L (Figure 18A). At day 3, exposure to 5 and 50µg/L of metformin slightly repressed the gene expression of catalase while this one remained stable for 500µg/L, compared to the control (Figure 18A). At day 7, all exposure groups were decreased, but either slightly for 5 and 50µg metformin/L or significantly for 500µg/L exposure group, compared to the control. After 10 days, exposure to metformin at all the different concentration induced slight increase in mRNA level (Figure 18A). Overtime, the two lowest exposure groups have the same trend in the variation of mRNA levels, which remained relatively stable from day 3 to 7 and then increased at day 10. An opposite expression pattern was observed for 500µg/L which significantly decreased after 7 days. However, this exposure group also increase after 10 days in order to become slightly increased (Figure 18A).

For catalase enzyme activity, at day 3, the catalase activity decreased at about the same proportion at all exposure groups, compared to the control with only the lowest concentration showing significant difference compared to the control (Figure 18B). At day 7, the catalase activity had increased compared to the day 3 but still whether under or at the basal level. The activity of catalase further increased after 10 days of metformin exposure in order for most of groups to get back to the control level. Thus, metformin exposure led to an overall overtime increased of the catalase activity (Figure 18B).

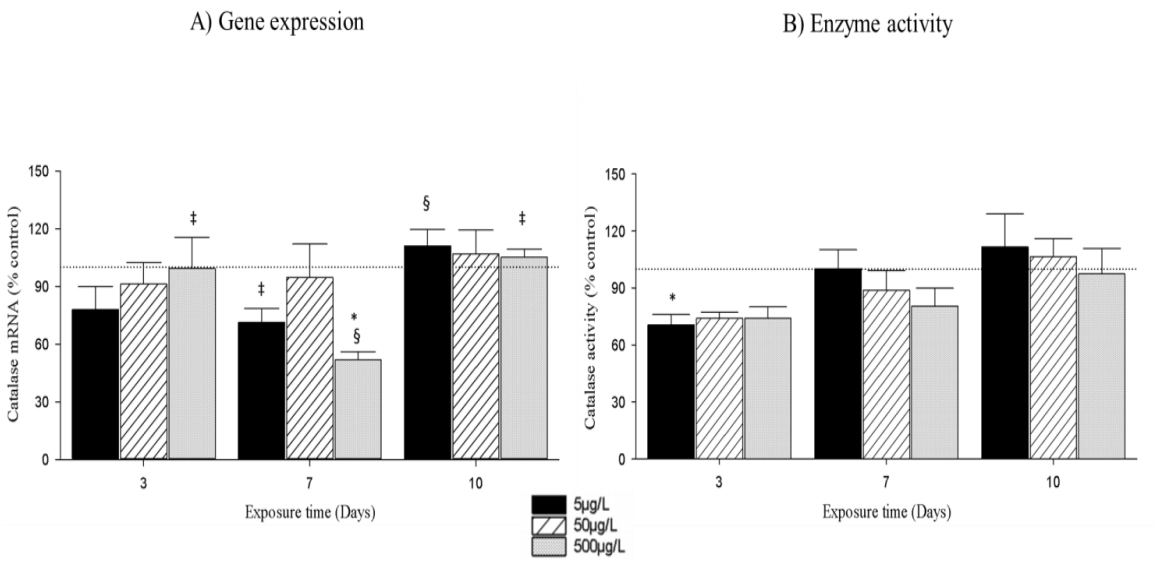

Figure 18 A) Expression of catalase mRNA in liver of Juvenile Atlantic Salmon exposed to metformin and B) changes in catalase activity. Fish were exposed to metformin (0, 5, 50 and 500µg/L) and sampled at 3, 7 and 10 days. The data are set as mean expressed in % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%. The activity of catalase was determined spectrophotometrically in the liver. A value was removed at day 10 and 50µg/L for catalase activity due to bias in the laboratory analysis. Asterisks denote significant differences between control group and exposure group within a same day. Bars without asterisk display no significant difference from any other column within a day. Symbols show significant differences for a same concentration at different days. Symbols are absent when no significant differences were detected. One-way ANOVA followed by Tukey's post-hoc test ($p<0.05$) and bonferroni for catalase mRNA at day 7 + between days at 5 and 500 µg/L. Welch with Games-Howell post-hoc test ($p<0.05$) for catalase activity at day 3.

GPx

Overall, there were no significant differences in the expression of GPx gene whether in day groups or overtime (Figure 19A). Nonetheless, at day 3, a tendency of increase was noticed for all exposure groups compared to the control, which was especially pronounced for 5 and 500µg/L groups. Overtime, metformin exposure led to the decrease of GPx mRNA for the three groups (Figure 19A). At day 10, the gene expression of GPx was lowered when fish were exposed to 5 and 50µg metformin/L compared to the control. However, this was not the case for the 500µg/L exposure group with its GPx mRNA level similar to the control (Figure 19A).

Overall, only fish exposed to 500µg/L of metformin significantly increased GPx activity, compared to both the control and other exposure groups including 5 and 50µg/L at day 7 (Figure 19B). This increase was also present at day 3 and 10, but to a lesser extent. Similarities between day 3 and 10 for the different exposure groups were observed. Overall, a similar tendency was observed between gene expression of GPx and its enzymatic activity. However, an exception was noticed with 500µg/L at day 7 with increased activity (Figure 19A), while mRNA was similar to the control (Figure 19B).

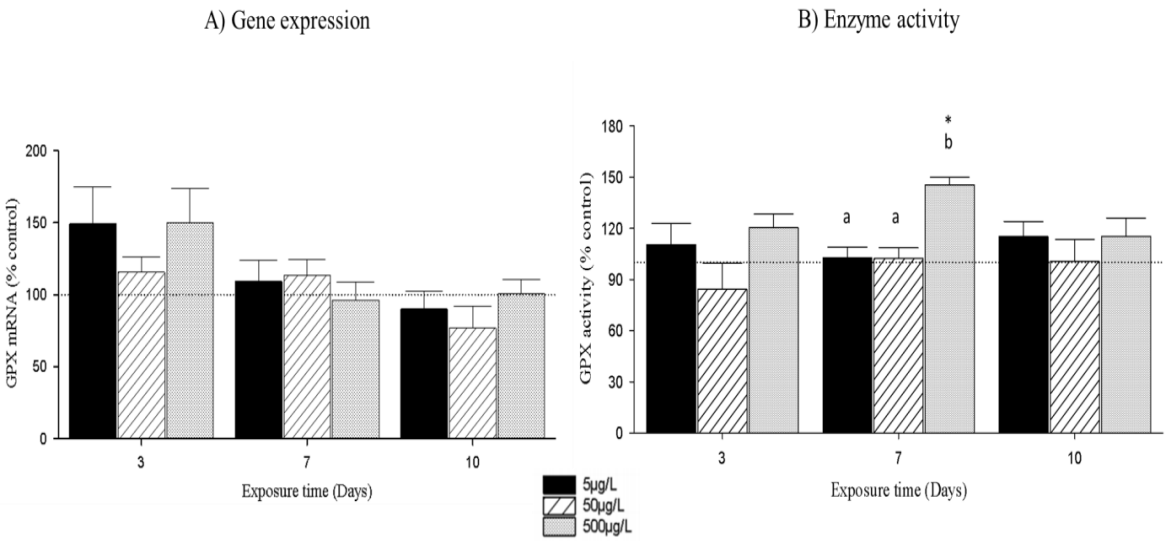

Figure 19 A) Expression of GPX mRNA in liver of Juvenile Atlantic Salmon exposed to metformin and B) changes in GPX activity. Fish were exposed to metformin (0, 5, 50 and 500µg/L) and sampled at 3, 7 and 10 days. The data are set as mean expressed in % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%. The activity of GPX was determined spectrophotometrically in the liver. Asterisks denote significant differences between control group and exposure group within a same day. Bars without asterisk display no significant difference from any other column within a day. Letters represent significant differences between exposure groups at a defined day. Day groups without letter display no significant difference between exposure groups at a given day. One-way ANOVA followed by Tukey post-hoc test (p=0.05) and bonferroni for GPX at day 7

Glutathione reductase (GR)

At day 3, exposure to metformin led to more pronounce decrease of GR mRNA level for fish exposed to 50µg metformin/L, than other exposure groups compared to the control. Overall, the GR expression of the exposure groups decreased after 7 days and increased after 10 days until reaching the control or slightly increased (Figure 20A). Fish that were exposed to the highest concentration of metformin showed a clearer decrease as well as increase compared to other exposure groups. At day 7, a decrease of the mRNA level which was significant only for the

highest concentration compared to control and the lowest concentration was observed. Moreover, the increase of mRNA level after 10 days in fish exposed to 500µg metformin/L was significant (Figure 20A).

At day 3, despite the lack of significant variations between the different exposure groups or between groups and control, there was some decrease of GR activity, which was clearer for the 50µg/L concentration (Figure 20B). After 7 days, in comparison to the group of fish exposed to 50µg/L, the GR activity was significantly lower for fish exposed to 500µg metformin/L. At day 10, despite the lack of significant differences between groups, lower activity was apparent for the 50µg/L group, compared to both the control and the group exposed to 5µg metformin/L (Figure 20B). Metformin seemed to produce different pattern for different exposure group (Figure 20B).

With the exception of the 500µg/L exposure group, the two other groups showed an opposite time-specific trend of the protein activity than for the gene expression, with an increase in GR activity at day 7 of exposure to metformin (Figure 20A-B). Moreover, at day 10, the enzyme activity has decreased for 50µg/L group, opposing the gene expression pattern. But the increased pattern found after 10 days of exposure for gene expression was also observed for 5 and 500µg/L of the enzyme activity (Figure 20A-B).

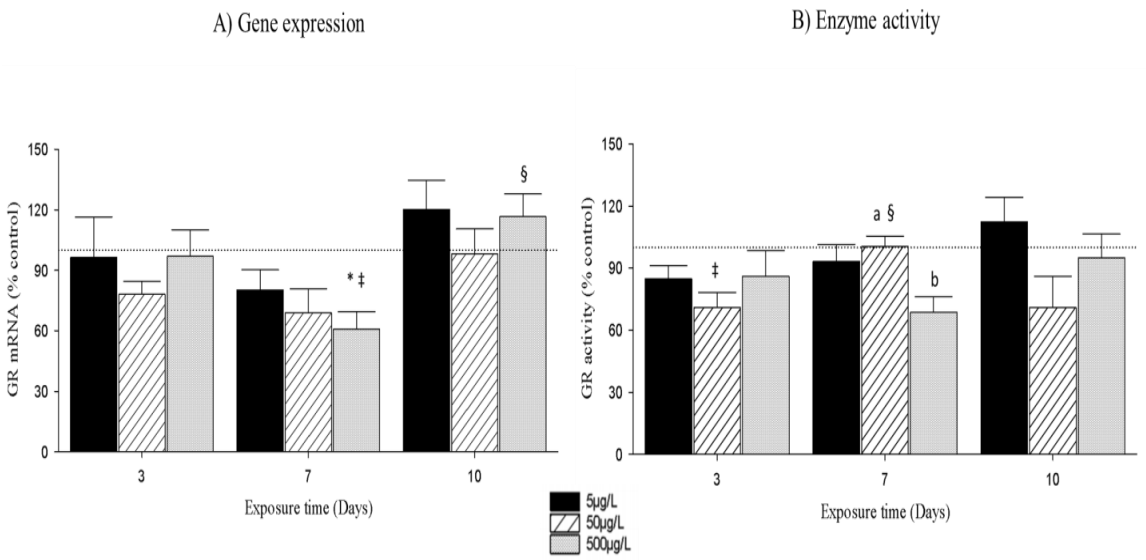

Figure 20 A) Expression of GR mRNA in liver of Juvenile Atlantic Salmon exposed to metformin and B) changes in GR activity. Fish were exposed to metformin (0, 5, 50 and 500µg/L) and sampled at 3, 7 and 10 days. The data are set as mean expressed in % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%. The activity of GR was determined spectrophotometrically in the liver. One outlier was removed for GR mRNA (A) at day 10-500µg/L and one for GR activity (B) at day 7-50µg/L. Asterisks denote significant differences between control group and exposure group within a same day. Bars without asterisk display no significant difference from any other column within a day. Letters represent significant differences between exposure groups at a defined day. Day groups without letter display no significant difference between exposure groups at a given day. Symbols show significant differences for a same concentration at different days. Symbols are absent when no significant differences were detected. Welch with Games-Howell post-hoc test ($p<0.05$) for GR activity at day 7 + at 50µg/L between day 3 and 7. Mann-Witney U post hoc ($p<0.05$) for gene expression of GR at day 7. One-way ANOVA followed by Tukey post-hoc test ($p<0.05$) and bonferroni for gene expression of GR at 500µg/L between day 7 and 10.

Glutathione-S-Transferase (GST)

Despite lack of significant differences at day 3, a decrease GST mRNA was observed when fish where exposed to 5, 50 and 500µg metformin/L, compared to the control (Figure 21A). At day 7 and 10, the gene expression of GST was at the basal level for all exposure groups with the exception of 5µg/L groups which did show slight increase compared to the control. One could have observed an increase of gene expression at day 7 and 10, compared to day 3 to either reach back or exceed slightly the basal level of expression, which was significant for fish exposed to 5µg metformin/L between day 3 and 7. But also the 500µg/L exposure groups showed significant increase overtime, after 7 and 10 days compared to day 3 (Figure 21A).

At day 3, the activity of GST increased significantly for the 500µg/L exposure group, compared to 50µg/L (Figure 21B). An increase was also evident between control group and 500µg/L as well as for 5µg/L. However, the activity at 50µg/L was observed to be very close to the control activity (Figure 21B). Concerning day 7, a low enzyme activity was observed for all exposure groups compared to the control. At day 10, a slight increase of the activity for the 5 and 50µg/L groups was visible (Figure 21B). Moreover, a more important increase was seen for the exposure group 500µg/L compared to the control. On the other hand, the 500µg/L exposure group displayed an initial decrease followed by a significant increase of GST activity from day 3 to 10 (Figure 21B). This was also true for other exposure groups but to a lesser extend (Figure 21B).

Different patterns were observed between the gene expression and enzyme activity for GST (Figure 21A-B). The main differences were seen at day 3 and 10 with the 500µg/l group and at day 7 for all exposure groups. Moreover, the highest concentration of metformin showed more important consequences in the enzyme activity than gene expression (Figure 21A-B).

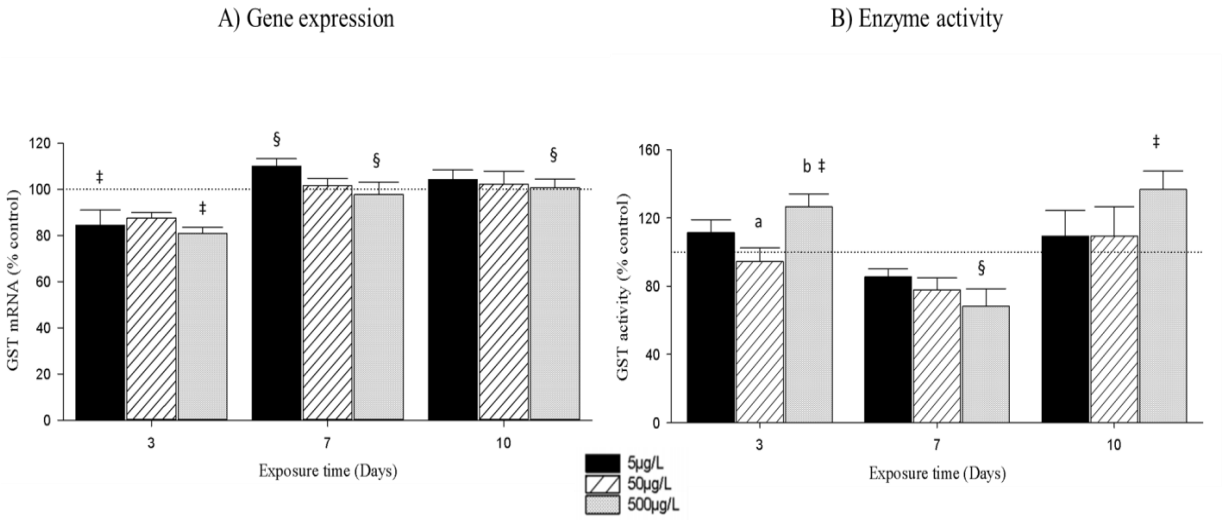

Figure 21 A) Expression of GST mRNA in liver of Juvenile Atlantic Salmon exposed to metformin and B) changes in GST activity. Fish were exposed to metformin (0, 5, 50 and 500µg/L) and sampled at 3, 7 and 10 days. The data are set as mean expressed in % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%. The activity of GST was determined spectrophotometrically in the liver. Asterisks denote significant differences between control group and exposure group within a same day. Bars without asterisk display no significant difference from any other column within a day. Letters represent significant differences between exposure groups at a defined day. Day groups without letter display no significant difference between exposure groups at a given day. Symbols show significant differences for a same concentration at different days. Symbols are absent when no significant differences were detected. One-way ANOVA followed by Tukey post-hoc test ($p<0.05$) and bonferroni for gene expression of GST at 5 and 500µg/L between days. One-way ANOVA followed by Tukey post-hoc test ($p<0.05$) and bonferroni for GST activity at day 3, and at 500µg/L between days.

3.6.1 Acetylcholine esterase (AchE)

No significant differences in the activity of acetylcholine esterase were observed between exposure groups and control within each day (Figure 22). The only significant result was observed at day 10, where one could observe a significant increase in AchE activity when fish were exposed to 500µg/L, compared to 50µg/L (Figure 22). However, at day 3, there was quite high AchE amount for the 500µg/L exposure group. Overtime, exposure to metformin seemed to affect AchE activity mostly when the fish were exposed to 500µg/L. The high enzyme activity at day 3, decreased after 7 days to increase again after 10 days (Figure 22).

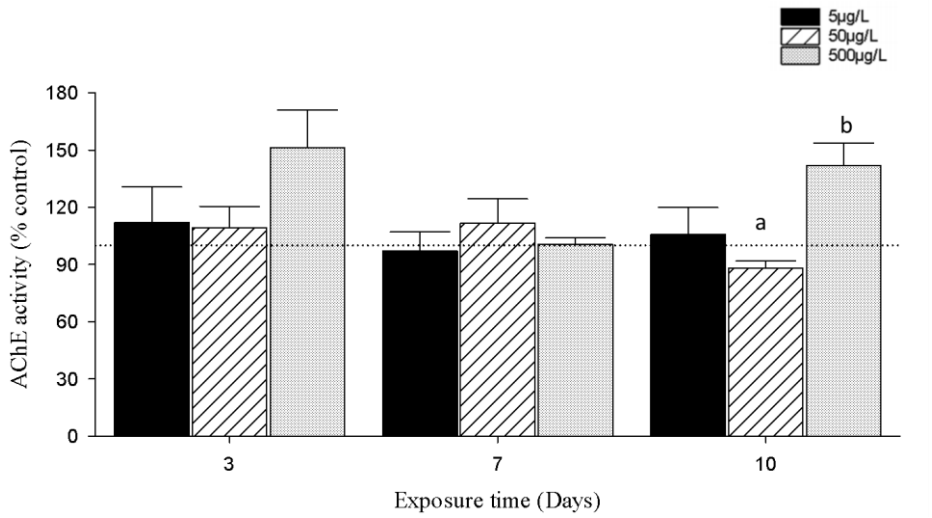

Figure 22 Changes in acetylcholine esterase activity measured in liver of Juvenile Atlantic Salmon exposed to metformin. Fish were exposed to metformin (0, 5, 50 and 500µg/L) and sampled at 3, 7 and 10 days. The data are set as mean expressed in % of control ± standard error of mean (SEM). The control is represented by the dotted line at 100%. The activity of acetylcholine esterase was determined spectrophotometrically in the liver. Letters represent significant differences between exposure groups at a defined day. Day groups without letter display no significant difference between exposure groups at a given day. One-way ANOVA followed by Tukey post-hoc test ($p<0.05$) and bonferroni for gene expression of GST at day 10.

4 Discussion

The success in medical and pharmaceutical field promoted an increase usage of pharmaceutical and medical products. Medical and pharmaceutical fields are not alone; there is also great amount of products such as cosmetics, cleaning products. Thus, PPCPs are widely found in the environment where potentially risks further increase in quantity. Metformin, an anti-diabetic II drug is one of the most prescribed drug and very little is known about it in terms of ecotoxicological effects. It is unknown if metformin due to its non-metabolism property in human and being persistency in the environment could have impact on non-target species such as fish and other aquatic organisms. There is a need to study the presence of metformin in the environment as it could potentially make its way back in the food chain with negative effect on human health. With limited amount of studies in relation to this drug, it is deemed necessary to perform toxicology study for metformin, as only few studies have previously investigated the effects of metformin in aquatic organisms.

4.1 Exposure and methods

4.1.1 Exposure

For the exposure, 20 Juvenile Atlantic salmon were placed per tank with 50L of freshwater. The tanks were big enough due to small size of the fish. The tanks were carefully protected with a net that avoided jumping, and this turned out to be successful as no fish were found outside the tanks. Daily checks were performed in order to monitor the pH, temperature and oxygen level of the water as well as potential mortality if any. The water was changed during the experiment which turned out to be challenging because the fish can easily be stressed and be the

consequence of errors in the future results. Thus, the renewal of freshwater and metformin was limited to 3 times in total.

4.1.2 RNA and its quality

During dissection and sampling of the fish, it was practically not possible to have a total sterile environment. However, the sampling of organs was done as optimal as possible in order to avoid degradation of RNA. First, the sampling was performed with clean material and by using protection equipment such as laboratory coat and gloves. The sampling of organs was done as fast as possible and instantly frozen in liquid nitrogen, and the organs used directly for the molecular analysis were added in trizol followed by freezing step in liquid nitrogen. This greatly prevented the degradation of the tissues and especially avoided the degradation of RNA by RNases. Moreover, as the fish were not fed during the experiment, this decreased any further risk of RNA degradation. Upon purity and quality analysis of the isolated RNA, it was possible to perform RT-QPCR as the samples displayed good purity and quality

4.1.3 Primers

Before performing the real-time PCR, one needs to know the correct annealing temperature to use. Thus, when the Tm of primers to be tested is unknown it was necessary to perform primer testing on some experimental samples before gene expression analysis of all the samples.

In parallel of the primers already present in the laboratory, four new primers were ordered such as MDR (multi-R), OAT, OAT1d1 and P-glycoprotein. All were tested with real-time PCR protocol, by providing the melting curve to determine the appropriate annealing temperature for each primer. The first temperature tested was 60ºC and both primers P-glycoprotein, OAT, OAT1d1 provided a single peak in the melting curve.

However, for multi-R, the melting curve was less optimal than for the rest of the primers. Nevertheless, after several other tests with various temperatures, 60ºC was found to be the best one.

Furthermore, the multi-R displayed a big Ct of about 38 in average and with few samples higher than 40 while the Ct of OAT, OAT1d1 and P-glycoprotein were ranging from 17 to 25. Thus, the high Ct for multi-R might be result from extremely low amount of the target sequence and led to difficulty of getting a perfect melting curve.

4.1.4 Normalization and quantification

The mRNA quantification experiments often lead to variations between samples which can be corrected by various methods (Arukwe 2006). Housekeeping genes are genes that are known to be expressed continuously regardless the condition, and thus expected to have constant expression in cells. Therefore, they are often used in gene expression analysis for the normalization of the obtained data. However, it has been demonstrated that they might lead to miss-interpretation of the results due to their demonstrated variation through different experimental conditions as discussed by Arukwe (Arukwe 2006). In order to avoid the problem of unreliable housekeeping genes, the normalization was done against a plasmid containing gene reference specific to the same primers than the template.

4.2 Statistical analysis

The main difficulty was the rejection of outliers as it is common to happen in such experiment where natural biological variations between individuals is possible that can results to an “false outlier” (Alvarez, Castilla et al. 2003). Depending on the test achieved the operator may remove a data or measurement that considered to be abnormal, however

caution must be exercised on what to remove with possibility of performing additional test to ensure whether the data point is an outlier. Thus, during this study, not only grubb's test was taken in account, but also chauvenet test and a simple visual test where a data could not be rejected if this one was not at least the double of the mean of all the value in a specific group. In addition, because of small sample size, only the data that were said to be possible to be rejected by the three different tests was removed from the data set. Also only a single data was removed in a same group.

The other difficulty was to decide on either the data set was normally enough distributed because it turned out that Shapiro-Wilk test was not all the time perfect according to the linear graph and boxplot. Thus, it was decided afterward to use in addition of Shapiro-Wilk test and to support it, boxplot and linear graph visualisation. On the other hand, when data could not be transformed, alternative statistical test with lower power was used, however this may led to potentially less robust results. Thus, during the statistical process it was crucial to ensure that the data were normally distributed in order to do the appropriated test and avoid unreliable results.

Some groups in gene expression or enzyme activity did not show significant differences as one would have expected when visualising the graphs. However, if the p-value is augmented up to 0.01 for instance, then there will be significant differences detected. Also, some groups were almost significant differences and thus if p-value was set at 0.06 there will be some more groups significantly different. Still for some, it will not be the case and that was probably due to unequal sample size in some groups. Thus, in future studies and in term of statistical it will be

recommended to perform first statistic test with p-value of 0.05 and then to increase the P-value up to 0.1 or decreasing up to 0.01, 0.001 depending on how the study drives. In this study, the p-value was set only at 0.05, however, not only significant results but also the non-significant ones were taken in account and discussed in the next parts.

In addition, some exposure groups were not significantly different with control whereas one could see and expect to have significant difference when looking at the graphs. Before to explain why it was not significant even if one will think so, it is important to notice that the exposure groups of a specific day were normalized with control of the same day. Thus, the control was presented as dotted line on each graph to easily visualise the possible effects induce on the gene and protein by metformin. And, the standard error of mean was practically not possible to be added for the control groups, but during the analysis of the data, the statistical test took in account the variations for the group exposed to metformin but also the control groups.

In toxicology studies, one should expect to have variations in the results for a same condition due to natural biological variations in a group of individuals, laboratory materials, and the operator. That is the reason why, the material in the laboratory such as the pipettes were renewed because it has been noticed during the start of the analysis that they were not anymore well calibrated. During the analysis of the results, that's was also the reason why outlier and rejection tests was performed on the data before to decide to remove any suspicious data little to moderately far from others.

Finally, it has been decided to apply statistical test between groups within day as well as between a specific concentration and the different days.

4.3 Experimental data

4.3.1 Length and weight

The length, weight and the Fulton's CF were presented for all exposure groups at the three days. Concerning the weight and length, no significant differences were detected between the various exposure groups within a day or between the different days. Nevertheless, from the Table 12, one could notice that free-metformin fish had a slightly lower weight than the one exposed to metformin and that is especially visible at day 3 leading to the possibility effect from metformin. One previous study showed significant variations in weight, length and condition factor between treated adult female fathead minnows over a longer time period than the present study (Niemuth and Klaper 2015). In addition to that, at day 3, control fish or exposed to 5µg metformin/L showed significant differences for the Fulton's CF and that may reveal some effect from the exposition to the drug. However, this result might be due biological variations between individuals as well as potential measurement errors. Thus, to investigate the development of Juvenile Atlantic salmon over long period of time as for instance counting in months in future studies may gave further clues about the effect of metformin on the development, maturation, differentiation in female and male (Niemuth and Klaper 2015). In order to observe changes from the start and end of the experiment on the biometrics parameters, it would have been preferable to take the dimensions of each fish at the start of the exposure experiment. Unfortunately, this was practically not possible in our animal facilities due to lack of a recognition method of each fish.

4.3.2 Uptake and accumulation of metformin

Due to the very small number of previous studies about the toxicological effect of metformin in aquatic organism, none were found with the assessment of the bioaccumulation of this drug in fish. Thus, this was the first time where the uptake and accumulation of metformin in fish was determined. The measurement was performed on remaining carcasses after removal of the head and two organs such as gill and liver. However, only the highest exposure concentrations were used such as 50 and 500µg/L for livers and 500µg/L for gills. The number of samples per groups was restricted as well as the number of group tested due to transport feasibility. Fish in free-metformin water were nearly drug-free. This small detected amount might be due to presence of metformin at environmental relevance as tap water from the laboratory was used for the exposure experiment. Also, because higher concentration of metformin detected in fish control compared to the experimental water, it reveals the possible uptake of metformin by the fish earlier in their life, at the hatchery for instance (Crago, Bui et al. 2016). The BCF, for control fish was surprisingly higher than the ones for exposure groups. Because of the very small amount of metformin detected in both fish and water, this probably increased the sensitivity of the BCF calculation. A greater BCF than 1 was obtained when fish contained more metformin than in the water. However, no metformin was added to the control tank which leads to think that the amount in water must come from the environment. And the higher concentration found in fish could have been uptake before their arrival to our animal facility, and in addition during the acclimatization time. To counter this doubt, it will be important in future studies to perform chemical analysis in order to determine the concentration in tissues of fish before the start of the experiment. This

could tell us if the fish already had accumulated metformin from its previous habitat. In addition, not only control water where fish was but also tap water that has not been in contact with fish should also be tested.

The final measured amount of metformin was lower compared to the initial added quantity of metformin. Several reasons for that can listed such as fish uptake and distribution in different organs, evaporation of the chemical, adsorption to the tank walls, transformation. In our case, all are probable. However, due to the evidence of bacterial transformation of metformin to its metabolite guanylurea in the environment (Trautwein, Berset et al. 2014) and the relatively high stability in water for a period of several days, this cause may be further suggested. This stability was demonstrated to be between 30 and 70ºC and being degraded only at an estimation of 10% after more than 8 days (Crago, Bui et al. 2016). Thus, the questions to ask in the case of future studies with metformin are whether this chemical is or is not stable at low temperature, and what is the period for its stability when using similar species such as salmon. The water analysis was carried months after the experiment and this was an error because and it is not sure that metformin was well conserved at 4ºC during all this time or degraded.

4.3.3 Effects on Steroidogenesis

Metformin increased the expression of Steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage (P450scc) mRNA at day 3. This might have reflected an increased transportation of cholesterol into mitochondria followed by an increase of its cleavage to pregnenolone, the precursor for other steroid hormones. Thus, augmentation in the synthesis of pregnenolone is expected (King and Stocco 2011). As these two proteins were demonstrated to works in

cooperation, this same effect upon metformin exposure seems to be coherent (Arukwe 2008)**.** However, this increases in the gene expression of these two key proteins for steroidogenesis was only transient with the return of mRNA level to basal level after 7 and 10 days, indicating probably a return to the pregenolone to normal level. StAR and P450scc, the two main proteins involved in the early production of steroid hormones and essential for their regulation can be modify by environmental chemicals (Arukwe 2005). Thus, both StAR and P450scc were shown to be the targets for environmental endocrine disruptor chemicals, EDCs (Arukwe 2005). Metformin being structurally different from xenoestrogens, hormone like compounds, should not in theory induce estrogenic activity by binding to the estrogen receptor. Nevertheless, a study by (Niemuth and Klaper 2015) has demonstrated the potential capacity of metformin to act as EDCs at environmental relevant concentrations in adult fathead minnows. Moreover, an additional study suggested that metformin may cause estrogenicity through secondary pathways, including steroidogenesis alterations, inhibition and diminution of both Phase I and II biotransformation enzymes that may damage clearance of E2, or alterations to the hypothalamus-pituitary–gonadal axis of the fish (Crago, Bui et al. 2016). However, in a third study, no changes in StAR and P450scc after 28 days exposure of minnow adult fish exposure to 40µg/L of metformin was reported by (Niemuth, Jordan et al. 2015). In addition, they suggested in this previous study that endocrine disruption does not seem to rely on upstream steroidogenesis changes (Niemuth and Klaper 2015). Our study did not show the same outcome than this previous study with increase of gene expression of both StAR and P450scc at day 3. This present study was performed in a different fish species, at different life cycle stage and

over a shorter timer period where effects were visible compared to this previous study. Nevertheless, in the present case, the mRNA levels for StAR and P450scc went back to the control levels after longer time as 7 and 10 days. Thus, the results at longer period of time seem to re-join the same results of the previous study (Niemuth and Klaper 2015). It is important to be aware that fish according to their development stage will show different responses against EDC's (Jin, Shu et al. 2011). Again, it seems that fish are more sensitive to these substances in in early development and particularly during sexual differentiations (Niemuth and Klaper 2015). Thus, this make challenging of establishing strong confirmation, correlation between this new study and the previous ones as mentioned above. In addition, the middle concentration of metformin seems to affect more StAR and P450scc genes than the 500µg/L. Suggestions are not simple to be done in this case, but one might be able to think that higher metformin amount led to faster recovery of normal expression of these two genes.

On the other hands, the analysis of StAR and P450scc protein with western blot did not reveal any relevant changes in the amount of proteins between different groups. The same trend was seen for these two proteins which might as for gene expression be coherent due to cooperation of the two proteins (Arukwe 2008).

A study of (Geslin and Auperin 2004) revealed that at least StAR can be regulated at various control point such as transcription, post-transcription, translation, post-translation. In addition, for toxicological studies, it is preferable to consider transcriptional responses as biomarkers of exposure, and enzyme activity level as biomarkers of effect (Giuliani et al., 2013). This is mainly due to the fact that despite the lack of

functional information from mRNA, it is known to be sensitive to pollution. On the other hand, protein activity is a good indicator of the protein function (Giuliani, Benedetti et al. 2013). Due to the absence of apparent changes in protein amount where a clear transcriptional responses was seen at day 3 for both StAR and P450scc, this suggests that metformin on a short time period have a biological response in juvenile Atlantic salmon without potential biological effect.

Concerning the practical side of the western blot analysis, only control of the day 3 was applied to the gel. However, it will be judicious and more reliable to add control from each day for future studies. Upon the immunochemical detection of the proteins, several bands corresponding to other proteins than StAR and P450scc were visible on the membrane. As the protocol was followed correctly and with efficient and numerous washing step between antibodies incubation and before the detection of the protein, that let presume to the presence of imperfect specificity of the antibodies for the investigated proteins.

Vitellogenin (Vtg), a glycolipophosphoprotein and precursor of egg yolk in aquatic organism and synthesis by the liver in response to estrogen stimulation has been shown to be an early and sensitive indicator of exposure to endocrine disrupting compounds (Niemuth, Jordan et al. 2015). Moreover, Vtg mRNA was studied in previous study and has shown to be significantly increased upon exposure to metformin in juvenile fathead minnows, and a trend for increased of Vtg mRNA in adult male (Niemuth, Jordan et al. 2015). Therefore, to investigate the expression of Vtg in future studies with juvenile Atlantic salmon may give further clues about possible endocrine disruption upon metformin exposure.

4.3.4 Effects on biotransformation system

As mentioned in the introduction part, xenobiotics can be metabolised through Phase I and II biotransformation by enzymes including CYPs generally used at phase I and in several cases by GST at phase II. Thus, CYPs are involved in the metabolism of foreign chemicals when they enter into the body. They make compound more water-soluble in order to excrete them. However, as this metabolite often stills not enough polar, enzymes from phase II are needed such as GST to further render it more hydrophilic. In addition cyp1a1 and cyp3a are also involved in the steroid metabolism (Arukwe 2005).

Concerning cyp1a1, metformin induced an increase of its gene expression overtime from day 3 to 7 and then decrease to become repressed at day 10. Very high concentration of metformin shown the biggest effect on this phase I metabolism enzyme. Due to the capacity of CYP1 family to metabolically activate compound to carcinogens that can then form DNA and or protein adducts, they are considered as the most linked metabolism enzymes with toxic responses (Plant 2003). Thus, in the present study it is hard to confirm whether the induction of cyp1a1 mRNA is reflecting detoxification or toxication of metformin. In some case, such as with PAH and PCH contaminants, fish has the ability through biotransformation of these compounds to more soluble, to adapt to such pollutants (Regoli, Pellegrini et al. 2002). However upon exposure to other chemicals, they might go through the same process and but producing more active electrophilic compounds that can be harmful for the organism (Boelsterli 2007). After 10 days of exposure to metformin, inhibition of cyp1a1 was identified and with significance for 50 and 500µg/L groups. This leads to propose the non-involvement of cyp1a1 in the metabolism of metformin after longer time period. This

outcome is consistent with the suggestion of (Attia, Rainey et al. 2001, Do, Kim et al. 2014) previous studies about the capability of metformin to decrease this cyp1a1 in mammals.

On the other hand, cyp3a group is leading to detoxification and is in theory not involved in the metabolic activation of substance to toxins. Nevertheless, because of its wide range of substrate-specificity, one could except to see some examples where cyp3A will render compounds toxic (Plant 2003). Upon a short exposure with the three concentration ranges of metformin, one could see a repressible effect for cyp3a. This gene increased after 7 days of exposure as presented in the results. One of the rare previous study with metformin, have shown in adult fathead minnows the same trend for cyp3a126 than cyp3a in this current study with increase of its gene expression at day 7 (Crago, Bui et al. 2016). However it has been reported to significant in the study of while it was not the case for this present study. Moreover, in contrary of the other study, (Crago, Bui et al. 2016), this study showed an inverse trend as the up-regulation of cyp3a was negatively correlated with metformin concentration increases.

As already mentioned, cytochrome P450 also serves to metabolize steroids, in addition it can also metabolize other endogenous compounds like arachinodic acid and prostaglandins (Simpson 1979). If the metabolism of these substances is affected due to for instance inhibition of biotransformation enzymes, there is a risk of harmful effects on important biological processes including development, reproduction, immune responses (Braunbeck, Hinton et al. 1998). And in this present study, cyp1a1 mRNA expression was inhibited at day 10 when fish were exposed to metformin regardless the concentration and this may indicate

potential arrest of the metabolism of not only xenobiotics as explained in previous paragraph, but also the metabolism of the previous cited endogenous compounds and may lead to side effects for fish. However, cyp3a3 was not inhibited at day 10 and as it is also used in the metabolism of molecules, may propose that this enzyme function is less sensitive regarding metformin than cyp1a1. Thus, same studies as here could be renew in the future by prolonging the exposure time in order to get further insight about these phase I enzymes mRNA levels and whether cyp1a1 still inhibited or not, and whether or not cyp3a will be potentially also inhibited. Thus, these experiments could direct towards the identification of potential effects of chronic metformin exposure in fish development, reproduction and immune response. In addition, at day 3, cyp1a1 and cyp3a showed a similar pattern for 5 and 500µg metformin/L group with visible inhibition of their gene expression.

The co-activation of pregnane X-receptor (PXR) was used for sensing the presence of foreign toxic substances, with the steroid receptor co-activator 1 (SCR1) was demonstrated to be disrupted and therefore resulting in decreased expression of cyp3a4 in human (Krausova, Stejskalova et al. 2011). Cyp3a4 serves for the metabolism of drug as well as synthesis of steroids hormones. (Krausova, Stejskalova et al. 2011) The results of this present experiment with metformin and fish, by showing decrease of cyp3a may indicate possible reduction of metabolism activity as well as PXR and SCR1 disruption as in the other study done in human. However, when fish were exposed to a longer period of time to metformin, the opposite results was observed with increase of cyp3a levels at day 7. This may indicate a possible restoration of PXR and SCR1 activation. Thus, future studies including the gene

expression analysis of both PXR and SCR1 will able to answer this suggestion.

For these two metabolism enzymes, only the gene expression was analysed. However, another good indicator for the cyp1 activity, the ethoxyresofurin-0-deaslkylase (EROD) has been demonstrated in previous studies (Smith, Iftikar et al. 2012). Thus, in future studies, EROD should be used in complementation of mRNA expression analysis for cyp1a.

4.3.5 Effect on multi-xenobiotic membrane and organic anion transporters

Aquatic organisms possess a mechanism that allowed them to survive in highly contaminated environment and have shown to have sometime less contaminants level in their body than in the environment (Van der Oost, Beyer et al. 2003). This capacity of adaptation to polluted environment is known to be mediated by the MXR phenomenon These transporter membranes eliminate both exogenous and endogenous compounds (Van der Oost, Beyer et al. 2003). In aquatic organisms, MXR is thus considered as a general biological first line defence system that enable them to have some protection against environmental pollutants (Kurelec 1995, Smital and Kurelec 1997). But, despite this good protective mechanism, this system of defence is relatively fragile. Various chemical classes can inhibit MXR system and are referred as chemosensitizers (Kurelec 1995, Smital and Kurelec 1998, Van der Oost, Beyer et al. 2003). This group of compounds are able to block this basic biological defence system and subsequently are placed at high rank for environmental hazardous compounds because by blocking this basic biological and may raise side effects on the natural resistance to

pathologies. (Kurelec 1995) Thus it is important to control the possibility of pharmaceuticals such as metformin to inhibit MXR.

Despite the lack of studies with metformin for MXR mechanism in fish, some studies have demonstrated the inhibition of multi-xenobiotic transporter membranes upon other pharmaceuticals in aquatic organisms (Smital, Luckenbach et al. 2004, Caminada, Zaja et al. 2008). However, this present study did not show such as drastic results for both P-glycoprotein and multi-R which suggests that metformin does not have an inhibitory action on these transporter membranes. Moreover, the inhibitory effect of several contaminants such as insecticide, oil on MXR has shown to increase the bioaccumulation of xenobiotics in organisms followed by increase of the toxin level in cells (Smital and Kurelec 1997, Van der Oost, Beyer et al. 2003, Ferreira, Costa et al. 2014). This may cause genotoxicity, cytotoxicity and neurotoxicity for animals. (Van der Oost, Beyer et al. 2003) However, in this present study, no apparent strong inhibition was detected for both p-gp and multi-R when compared exposed groups to control group. Thus, metformin does not appear to increase the accumulation of xenobiotics in juvenile Atlantic salmon and thus no potential increase of cytotoxicity, genotoxicity or neurotoxicity. Moreover, p-gp in addition of having the capacity of reducing drug accumulation, can also cause drug resistance (Smital and Kurelec 1997). One could think that metformin might not induce this drug resistance property as the p-gp mRNA was relatively stable regardless concentration of metformin exposure and over time. In opposite, at day 7, multi-R mRNA for the group exposed to 50µg metformin/L showed a significant increase compare to control fish and this may propose a possible increase of the cellular pump action.

Previous studies in aquatic organisms, suggested that p-gp and cyp1a might not be co-ordinately regulated in fish (Bard 2000). However, these studies proposed that they may be complementary to each other in detoxification processes (Ferreira, Costa et al. 2014). The problem is that the relation between p-gp and biotransformation enzymes in marine animals is still unclear in addition of scarce knowledge about the other ABC efflux transporters (Ferreira, Costa et al. 2014). Thus, different studies showed different outcomes with some demonstrating that when p-gp is induced, cyp1a is also induced while other studies showed that when provoking inhibition of p-gp, cyp1a still expressed. In addition, other studies showed possible induction of cyp1a followed by no activation of p-gp (Van der Oost, Beyer et al. 2003). This present study displayed at day 10, and 500µg metformin/L, a small decrease of p-gp and inhibition of cyp1a. However, multi-R was seen to be increase by little at day 10 and 500µg/L which may in the inverse of p-gp indicate increase of xenobiotic efflux out of the cells by this MXR. On the other hand cyp3a was not inhibited and may metabolised metformin. Based on these finding, it is challenging to do affirm whether cyps and MXR are co-ordinated and/or complementary to each other due to the differential responses toward metformin exposure.

And one could notice also that the CYPs did also increase after 7 days as p-gp and multi-R maybe we can link both CYPs and multi R induction especially at day 7 because multi R is used to excrete modified xenobiotics while P-glycoprotein which did not show such variations does serve to eliminate only parent compounds. As cyp1a1 and cyp3a mRNAs showed the same trend as for multi R with increase of their gene expression when fish were exposed for 7 days, this probably indicate were more expressed than the control at day 7.

The little increase of P-glycoprotein and bigger increase for MDR may indicate that some of the metformin have been modified through phase I and II metabolism.

The exposure of metformin did clearly not reveal any potential effect on the two organic anion transporters, OAT and OAT1d1. Thus, this present study indicates that metformin may not affect the regulation of these two organic anion transporters at gene expression level.

4.3.6 Effect on oxidative stress

Antioxidants are widely used as biomarkers in toxicological studies to assess their biological effect when animals exposed to environmental pollutant because of their good responsiveness to chemical effects in aquatic organisms (Valavanidis, Vlahogianni et al. 2006, Giuliani, Benedetti et al. 2013).

Previous studies about metformin and oxidative stress responses were not found. Thus, this present study is at the forefront concerning the potential effect of metformin towards oxidative stress in aquatic organisms.

When aquatic organisms exposed to environmental contaminants, their cellular antioxidant defence systems may decrease or increase to redress the imbalance led by oxidative stress. In general, measurement senses depletion of enzyme levels but in some cases it will detect increase. Studies suggested when depletion are measured one can use it as biomarker for adverse effect of xenobiotics. Numerous studies were performed in aquatic organism and on antioxidant defences. However, the regulation of exogenous and endogenous pollutants source of ROS is still not well known (Valavanidis, Vlahogianni et al. 2006).

In this study, both gene expression and enzyme activity for biomarkers to oxidative stress has been performed and analysed. Generally, toxicology studies do involve only one of these analyses and often choose to perform gene expression studies which substitute the enzyme activity analysis. This has been recently demonstrated to be wrong due to probable modification of proteins after the stage of transcription point control which is considered as one of the most important. The current state of the art publications suggest a very few number of studies that include both gene expression and enzyme reactions in toxicology studies (Giuliani, Benedetti et al. 2013). Therefore, this motived the current study to examine and perform both analyses.

4.3.6.1 Catalase

Catalase is widely used as biomarker in oxidative stress studies upon exposure of organisms to pollutants. However, catalase has been shown to not be an optimum biomarker in toxicological studies due to demonstration of both increase or decrease of its activity after exposure to various contaminants (Peixoto, Alves-Fernandes et al. 2006). Nevertheless, based on the result of both gene expression and enzyme activity, the catalase variations upon metformin exposition is discussed next.

Although transcriptional changes for *catalase* were generally differential, a significant diminution in mRNA level were visible in 500µg metformin/L at day 7 as well as slight diminution in 5 and 50µg/L groups at both 3 and 7 days were noticeable. Concerning the catalase activity, a clear diminishment occurred after 3 days under exposure to the three different metformin amounts. This decrease was clearer at the gene level. However, it was less clear at day 7 where the enzyme activity was not

that much low compared to the gene expression**.** Thus, catalase showed differential responses between gene and protein analysis and therefore one would prefer to affirm any possible biological effect upon metformin exposure based on the observation of the activity of catalase (Giuliani, Benedetti et al. 2013).

These diminutions both at gene level and catalase activity could rise from various reasons. One would be possible low level of H_2O_2 in the way that fish did not need longer expression of the catalase gene. (Polidoros and Scandalios 1999) study showed that in maize, while high doses of H_2O_2 appeared to increase catalase mRNA levels, low doses of H_2O_2 did show reduction of catalase mRNA and this seem to correspond with our study. Thus, this study may indicate a potential low level of H_2O_2 in the cells. If this is the case, then that may indicate potential prevention of these reactive species by metformin. This would be in accordance with the finding that metformin reduced ROS production in mammals as described by (Pintana, Apaijai et al. 2012). However, because of the different animal species this cannot be confirmed by this unique study and thus would need further investigations.

A second reason may be the opposite of the previous one with the presence of too high amount of hydrogen peroxide (H_2O_2) that would potentially have overwhelmed the antioxidant and rise direct oxidative toxicity toward catalase protein. This proposition is consistency with the findings of the author (Giuliani, Benedetti et al. 2013).

In addition, a study showed of the treatment of carp and catfish with dichlorvos, a known oxidative stress inducer, showed a reduction of the catalase activity (Zhang, Wang et al. 2004). This may indicate a possible presence of oxidative stress upon metformin exposure over a short period

such as 3 days. But, in this present study, both gene expression and the catalase activity seemed to rise back to nominal level after 10 days of exposure, which in case of potential oxidative stress lead to think that this oxidative stress is reversible and no that alarming. In fact, the study of (Kono and Fridovich 1982) demonstrated through an experiment where a large amount of superoxide radicals were generated could provoked the inhibition of catalase. Thus, the decrease of catalase activity in this present study may be due to superflux of superoxide radicals. In this particular hypothesis, at day 10, the reason why both gene and protein expression were re-established to control level could be due to decrease of superoxide anion by SOD. In future studies, SOD activity should be tested in order to determine whether or not this suggestion will be confirmed and support the study by (Kono and Fridovich 1982)

However, based on the possibility that prior to day 10, a too high amount of ROS was present, it is possible to suggest due to increase of catalase mRNA after 10 days exposed to metformin regardless of the concentration, that fish need some time to adapt themselves to the possible augmentation of hydrogen peroxide. One interesting future study will be to expose Atlantic salmon to metformin over a longer time period in order to see if the catalase would or would not be further induced.

4.3.6.2 Glutathione peroxidase (GPx), Glutathione reductase (GR) and Glutathione-S-transferase (GST)

The results between gene expression and enzyme activity of GPx are tighter than for catalase in the exception of the highest concentration of metformin. Based on the GPx activity results, the main outcome in this case was a higher observed effect for the highest exposure group with a significant augmentation of GPx activity at day 7. Despite significant outcomes for the gene expression of GPx, one important noticeable

element was the relatively up-regulation at day 3 for all groups but more clear for 5 and 500µg/L, which overtime decreased. The activity of GPx showed same trend but with lower activity than mRNA levels at day 3 and slightly higher activity than mRNA at day 10 for all exposure group. But the most remarkable point was with the exposure group 500µg/L that instead of decreasing after 7 days compared to day 3 for gene expression, it has increased after 7 days for the enzyme activity. Furthermore, this increase was significant and findings might indicate possible high removal of hydrogen peroxide by GPx. GPx in opposite of catalase which showed some increase in its activity. Both GPx and catalase are able to reduce H_2O_2 into water. However, it has been demonstrated that when only low concentration of H_2O_2 is present, GPx seems to play a more important role than catalase. On the other hand, catalase will increase under significant oxidative stress. (Bagnyukova, Vasylkiv et al. 2005). Therefore, this may indicate and further strengthen the suggestion in the catalase part with presence of low hydrogen peroxide (H_2O2) upon metformin exposure. Based on these finding, it seems that metformin may no induce augmentation of this potential ROS. Moreover, in contrary to catalase, GPx activity was not inhibited and if possible presence of superoxide anion as suggested previously would have be able to inhibit catalase, it is apparently not valid for GPx. (Kono and Fridovich 1982). It will be judicious in future studies, to expose fish for a longer period of time in order to see if this group tend to continue to decrease and maybe becoming depleted at a certain stage because one can see a decrease at day 10 of the enzyme activity for the 500µg/L exposure group compared to day 7.

The expression of Glutathione reductase (GR) was significantly depleted for the 500µg/L group at day 7, and seems to be dependent to

concentration. This depletion in GR mRNA at day 7 and 500µg/L was also seen in the GR activity. However, the depletion of GR activity at day 7 and 500µg/L was not significant compared to the control, but it was when compared to 50µg/L. When looking back at GPx activity which showed significant increase for the 500µg/L at day 7, it is possible to note that GR activity has an inverse trend. This may indicate of the incapacity for GR to replenish the cells with GSH after oxidation of GSH to GSSH and thus probably induced consequences on the GSH/GSSH ratio by lowering it dramatically. This finding is consistency with the published article (Storey 1996) (Espinosa-Diez, Miguel et al. 2015) The problem could be that if GR cannot refill GSH level at the normal, GPx will be after a prolonged time in the inability to reduce ROS and thus leading to a danger for the organism (Storey 1996).

Nevertheless, after 10 days of metformin exposure, the level of GR seems to go back to more normal level and indicate in a possible adaptation of fish or due to GPx activity that also went back to control level. Theoretically, if GPx is not active, GSH level might be stable and thus GR may not be required as there is no need of GSH replenishment (Storey 1996). This may indicate further possible reversibility action of the antioxidant system.

GST a phase II enzymes which conjugate organic compounds to glutathione in order to help to eliminate xenobiotics, is also part of the antioxidant system (Storey 1996, Giuliani, Benedetti et al. 2013). Both, responsiveness and non-responsiveness of either transcriptional or enzymatic activities have been reported in mollusc (Giuliani, Benedetti et al. 2013). Although transcriptional changes for *GST* were generally not differential compared to control groups, a small down-regulation was

apparent at day 3 for all exposure groups. This suggest to a lower conjugation rate compare to the control at day 3 or to the other groups at 7 and 10 days. So after long enough time, the action of GST might be stopped for both its metabolism activity and antioxidant responses. However, the activity of GST showed different trend than transcriptional analysis. The 500µg/L group turn out to be the main responsive one. GST activity suggests inducing conjugation at higher level for both day 3 and 10 at 500µg metformin/L. As there are various isoform of GST, for the determination of enzyme activity CDNB was used and measured the total activity of different GST isoform. On the other, gene expression analysis did target a specific GST. Thus this might lead to differences between the two methodologies (Giuliani, Benedetti et al. 2013).

As GST is also a phase II enzymes, one could make the relation between this GST and the multi-R, a multi-xenobiotic resistance protein MXR, because this transporter membrane protein serves to excrete metabolite phase II. The gene profile of GST and multi-R are pretty similar with the exception of multi-R mRNA which is higher than GST mRNA at day 10 compared to the control.

In the opposite of other environmental pollutants (Radi, Matkovics et al. 1985, Sayeed, Parvez et al. 2003), overall metformin does not seem to highly induce GPx and GST activity in juvenile Atlantic salmon. The study of (Sayeed, Parvez et al. 2003) concluded to potential protection against ROS upon deltamethrin exposure in Channa punctatus Bloch, a freshwater fish due to detection of elevated GPx and GST in liver. However, in this present study overall both of these enzymes were not to this high level and probably in the inverse of this previous study did not serve in the protection against ROS. This finding is further supported by

(Giuliani, Benedetti et al. 2013) study affirming that reactive species may induce transcription of GPx as well as other antioxidant genes such as GST.

4.4 Neurotoxic effects

The main change observed in acetylcholine esterase activity was its increase at day 3 and 10 compared to the control when fish were exposed to 500µg metformin/L. Overall, metformin did not cause inhibition of the enzyme activity. In order to identify signal of toxicity of environmental contaminant as early as possible, AChE activity is a good indicator (Lionetto, Caricato et al. 2013). It seems that this biomarker has been used mainly for study the effect of pesticides in aquatic organism (Rhee, Kim et al. 2013).

AChE and GST are considered as being good biomarkers for contaminations with xenobiotics (Radi, Matkovics et al. 1985). Despite the lack of studies with metformin, it has been demonstrated that persistent pollutant such as pesticides, insecticides can affect AChE enzyme (Rhee, Kim et al. 2013). For instance, Organophosphorus insecticides, is well known to be an inhibitor for this enzyme and causes subsequent toxicity for organisms (Radi, Matkovics et al. 1985, Fulton and Key 2001). Metformin exposure did not show any potential hazardous inhibition effect on the fish. Thus indicating the absence of abnormal accumulation of the neurotransmitter acetylcholine as the level of its cleavage enzyme did not drastically decrease. In this present study, compared to previous studied on other xenobiotics that causes dramatically inhibition of AChE, did not show such results upon metformin exposure (Fulton and Key 2001). In this present case the increase of this enzyme activity indicates higher amount of acetylcholine

esterase in liver at 500µg metformin/L for both day 3 and 10. This leads to think that fish exposed to environmental concentration of metformin does not show potential risk of side effects due to the relatively higher tested concentration than found in aquatic habitat. In addition this suggestion is possible as the only important visible change was noticed at very high concentrated metformin dose that has not been detected in the environment (Scheurer, Michel et al. 2012, Niemuth, Jordan et al. 2015).

5 Future studies

To confirm the results, it will be important to renew this study by increasing the number of fish in order to avoid any interpretation errors due to biological variability between individuals. Moreover, as mentioned it will be judicious to extend the exposure time over 10 days to understand the effect of metformin over longer time period.

Improvement for the experimental set-up will include control of the metformin concentration at both the start of the exposure and at the final day, or between water changing.

It will be interesting to determine the lipid peroxidation level in Juvenile Atlantic salmon as it seems also to be a powerful biomarker for oxidative stress.

6 Conclusion

State of the art study demonstrated the presence of various Pharmaceuticals Personal Care Products (PPCPs) in the aquatic environment including metformin an anti-diabetic II drug. Metformin is considered as emerging contaminant and therefore require study about its impact in non-target species. This study was unique with the respect to examining potential effects of metformin in juvenile Atlantic salmon. Hence, it was not directly possible to compare with other studies as different drugs may interact differently with different organisms at different life stages.

In conclusion this study firstly demonstrated an overall uptake of metformin which was noted to be the highest in gills, but still at relatively low concentration level. Secondly, it demonstrated that metformin only transiently disturbed the expression of the two genes StAR and P450scc that regulate the synthesis of steroid hormones. This study could not confirm the presence of endocrine disruption upon metformin exposure in juvenile Atlantic salmon.

Metformin led to either inhibition or increase of the phase I biotransformation enzymes including cyp1a1 and cyp3a depending mainly on the time of exposure, rather than concentration. As no drastically inhibition was noticed for the two multi-xenobiotic resistance proteins, metformin does not seem to cause potential direct toxicity for the cell by accumulation of exogenous and endogenous compounds. Organic anion transporter was clearly not affected upon metformin exposure.

Despite the lack of previous studies with antioxidant enzymes in aquatic organisms exposed to metformin, this compound did not induce severe

oxidative stress responses. However, more studies will be needed to confirm and validate this present finding. The AChE enzyme was not significantly affected by metformin exposure, regardless the concentration.

7 References

(2016, 05/2016). "Environmental contaminants." from http://www.hc-sc.gc.ca/ewh-semt/contaminants/index-eng.php.

Alvarez, C., et al. (2003). "Biological variation of seminal parameters in healthy subjects." Human Reproduction **18**(10): 2082-2088.

Arnold, K. E., et al. (2014). "Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems." Phil. Trans. R. Soc. B **369**(1656): 20130569.

Arukwe, A. (2005). "Modulation of brain steroidogenesis by affecting transcriptional changes of steroidogenic acute regulatory (StAR) protein and cholesterol side chain cleavage (P450 SCC) in juvenile Atlantic salmon (Salmo salar) is a novel aspect of nonylphenol toxicity." Environmental science & technology **39**(24): 9791-9798.

Arukwe, A. (2006). "Toxicological housekeeping genes: do they really keep the house?" Environmental science & technology **40**(24): 7944-7949.

Arukwe, A. (2008). "Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc)-regulated steroidogenesis as an organ-specific molecular and cellular target for endocrine disrupting chemicals in fish." Cell biology and toxicology **24**(6): 527-540.

Asche, F. and T. Bjorndal (2011). The economics of salmon aquaculture, John Wiley & Sons.

Attia, G. R., et al. (2001). "Metformin directly inhibits androgen production in human thecal cells." Fertility and sterility **76**(3): 517-524.

Bagnyukova, T. V., et al. (2005). "Catalase inhibition by amino triazole induces oxidative stress in goldfish brain." Brain research **1052**(2): 180-186.

Bard, S. M. (2000). "Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms." Aquatic Toxicology **48**(4): 357-389.

Boelsterli, U. A. (2007). Mechanistic toxicology: the molecular basis of how chemicals disrupt biological targets, CRC Press.

Boxall, A. B., et al. (2012). "Pharmaceuticals and personal care products in the environment: what are the big questions?" Environmental Health Perspectives **120**(9): 1221.

Boyd, G. R., et al. (2003). "Pharmaceuticals and personal care products (PPCPs) in surface and treated waters of Louisiana, USA and Ontario, Canada." Science of the total Environment **311**(1): 135-149.

Braunbeck, T., et al. (1998). Fish ecotoxicology, Springer Science & Business Media.

Brooks, H. E. and C. A. Doswell III (2001). "Normalized damage from major tornadoes in the United States: 1890-1999." Weather and Forecasting **16**(1): 168-176.

Bustin, S. A. (2000). "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays." Journal of molecular endocrinology **25**(2): 169-193.

Caminada, D., et al. (2008). "Human pharmaceuticals modulate P-gp1 (ABCB1) transport activity in the fish cell line PLHC-1." Aquatic Toxicology **90**(3): 214-222.

Christenson, L. K. and J. F. Strauss (2001). "Steroidogenic acute regulatory protein: an update on its regulation and mechanism of action." Archives of medical research **32**(6): 576-586.

Cooper, J. R., et al. (2003). The biochemical basis of neuropharmacology, Oxford University Press, USA.

Copois, V., et al. (2007). "Impact of RNA degradation on gene expression profiles: assessment of different methods to reliably determine RNA quality." Journal of Biotechnology **127**(4): 549-559.

Corcoran, J., et al. (2010). "Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish." Critical reviews in toxicology **40**(4): 287-304.

Crago, J., et al. (2016). "Age-dependent effects in fathead minnows from the anti-diabetic drug metformin." General and comparative endocrinology.

Do, M. T., et al. (2014). "Metformin suppresses CYP1A1 and CYP1B1 expression in breast cancer cells by down-regulating aryl hydrocarbon receptor expression." Toxicology and applied pharmacology **280**(1): 138-148.

Donaldson, S., et al. (2010). "Environmental contaminants and human health in the Canadian Arctic." Science of the total Environment **408**(22): 5165-5234.

Doussantousse, É., et al. (2011). "Multixenobiotic resistance in coelomocytes from three echinoderm species." AQUATIC BIOLOGY **12**(1): 81-96.

Eggen, T., et al. (2013). "Uptake and translocation of organophosphates and other emerging contaminants in food and forage crops." Environmental Science and Pollution Research **20**(7): 4520-4531.

Eggen, T. and C. Lillo (2012). "Antidiabetic II drug metformin in plants: uptake and translocation to edible parts of cereals, oily seeds, beans,

tomato, squash, carrots, and potatoes." Journal of agricultural and food chemistry **60**(28): 6929-6935.

Ekins, S., et al. (2005). "Techniques: application of systems biology to absorption, distribution, metabolism, excretion and toxicity." Trends in pharmacological sciences **26**(4): 202-209.

Ellman, G. L., et al. (1961). "A new and rapid colorimetric determination of acetylcholinesterase activity." Biochemical pharmacology **7**(2): 88-95.

Espinosa-Diez, C., et al. (2015). "Antioxidant responses and cellular adjustments to oxidative stress." Redox biology **6**: 183-197.

Facek, Z. (1990). "Contaminants and the environment." Rostlinná Výroba **36**(4): 337-344.

Ferreira, M., et al. (2014). "ABC transporters in fish species: a review." Front. Physiol **5**: 266.

Fleige, S. and M. W. Pfaffl (2006). "RNA integrity and the effect on the real-time qRT-PCR performance." Molecular aspects of medicine **27**(2): 126-139.

Fulton, M. H. and P. B. Key (2001). "Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects." Environmental Toxicology and Chemistry **20**(1): 37-45.

Geslin, M. and B. Auperin (2004). "Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (Oncorhynchus mykiss)." General and comparative endocrinology **135**(1): 70-80.

Giuliani, M. E., et al. (2013). "Transcriptional and catalytic responses of antioxidant and biotransformation pathways in mussels, Mytilus galloprovincialis, exposed to chemical mixtures." Aquatic Toxicology **134**: 120-127.

He, Y. and C.-C. Lin (2007). Development and validation of a mean value engine model for integrated engine and control system simulation, SAE Technical Paper.

Jancova, P., et al. (2010). "Phase II drug metabolizing enzymes." Biomedical Papers **154**(2): 103-116.

Jin, Y., et al. (2011). "Environmental cues influence EDC-mediated endocrine disruption effects in different developmental stages of Japanese medaka (Oryzias latipes)." Aquatic Toxicology **101**(1): 254-260.

King, S. R. and D. M. Stocco (2011). "Steroidogenic acute regulatory protein expression in the central nervous system." Frontiers in endocrinology **2**.

Kono, Y. and I. Fridovich (1982). "Superoxide radical inhibits catalase." Journal of Biological Chemistry **257**(10): 5751-5754.

Krausova, L., et al. (2011). "Metformin suppresses pregnane X receptor (PXR)-regulated transactivation of CYP3A4 gene." Biochemical pharmacology **82**(11): 1771-1780.

Kurelec, B. (1995). "Inhibition of multixenobiotic resistance mechanism in aquatic organisms: ecotoxic consequences." Science of the total Environment **171**(1): 197-204.

Kurien, B. T. and R. H. Scofield (2006). "Western blotting." Methods **38**(4): 283-293.

Kusakabe, M., et al. (2002). "Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout." Endocrinology **143**(6): 2062-2070.

Lionetto, M. G., et al. (2013). "Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives." BioMed research international **2013**.

Litman, T., et al. (2001). "From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance." Cellular and Molecular Life Sciences CMLS **58**(7): 931-959.

Liu, J., et al. (2008). "Casarett and Doull's toxicology: the basic science of poisons."

Livingstone, D. (2001). "Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms." Marine Pollution Bulletin **42**(8): 656-666.

Loo, T. and D. Clarke (2005). "Recent progress in understanding the mechanism of P-glycoprotein-mediated drug efflux." The Journal of membrane biology **206**(3): 173-185.

Meredith-Williams, M., et al. (2012). "Uptake and depuration of pharmaceuticals in aquatic invertebrates." Environmental pollution **165**: 250-258.

Modesto, K. A. and C. B. Martinez (2010). "Effects of Roundup Transorb on fish: hematology, antioxidant defenses and acetylcholinesterase activity." Chemosphere **81**(6): 781-787.

Niemuth, N. J., et al. (2015). "Metformin exposure at environmentally relevant concentrations causes potential endocrine disruption in adult male fish." Environmental Toxicology and Chemistry **34**(2): 291-296.

Niemuth, N. J. and R. D. Klaper (2015). "Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish." Chemosphere **135**: 38-45.

Nornberg, B. F., et al. (2015). "ABCB1 and ABCC4 efflux transporters are involved in methyl parathion detoxification in ZFL cells." Toxicology in Vitro **29**(1): 204-210.

Peixoto, F., et al. (2006). "Toxicological effects of oxyfluorfen on oxidative stress enzymes in tilapia Oreochromis niloticus." Pesticide Biochemistry and Physiology **85**(2): 91-96.

Pintana, H., et al. (2012). "Effects of metformin on learning and memory behaviors and brain mitochondrial functions in high fat diet induced insulin resistant rats." Life sciences **91**(11): 409-414.

Plant, N. (2003). Molecular toxicology, Garland Science.

Plant, N. (2003). Molecular toxicology. London, BIOS Scientific Publishers.

Polidoros, A. N. and J. G. Scandalios (1999). "Role of hydrogen peroxide and different classes of antioxidants in the regulation of catalase and glutathione S-transferase gene expression in maize (Zea mays L.)." Physiologia Plantarum **106**(1): 112-120.

Radi, A. A. R., et al. (1985). "Comparative antioxidant enzyme study in freshwater fish with different types of feeding behaviour." Comparative Biochemistry and Physiology Part C: Comparative Pharmacology **81**(2): 395-399.

Regoli, F., et al. (2002). "Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet (Mullus barbatus)." Marine Pollution Bulletin **44**(9): 912-922.

Rena, G., et al. (2013). "Molecular mechanism of action of metformin: old or new insights?" Diabetologia **56**(9): 1898-1906.

Renzoni, A., et al. (1998). "Mercury levels along the food chain and risk for exposed populations." Environmental Research **77**(2): 68-72.

Rhee, J.-S., et al. (2013). "Effect of pharmaceuticals exposure on acetylcholinesterase (AchE) activity and on the expression of AchE gene in the monogonont rotifer, Brachionus koreanus." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **158**(4): 216-224.

Sayeed, I., et al. (2003). "Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, Channa punctatus Bloch." Ecotoxicology and Environmental safety **56**(2): 295-301.

Scheurer, M., et al. (2012). "Occurrence and fate of the antidiabetic drug metformin and its metabolite guanylurea in the environment and during drinking water treatment." Water research **46**(15): 4790-4802.

Simpson, E. R. (1979). "Cholesterol side-chain cleavage, cytochrome P450, and the control of steroidogenesis." Molecular and cellular endocrinology **13**(3): 213-227.

Smart, R. C. and E. Hodgson (2013). Molecular and biochemical toxicology, John Wiley & Sons.

Smital, T. and B. Kurelec (1997). "Inhibitors of the multixenobiotic resistance mechanism in natural waters: in vivo demonstration of their effects." Environmental Toxicology and Chemistry **16**(10): 2164-2170.

Smital, T. and B. Kurelec (1998). "The chemosensitizers of multixenobiotic resistance mechanism in aquatic invertebrates: a new class of pollutants." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **399**(1): 43-53.

Smital, T., et al. (2004). "Emerging contaminants—pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **552**(1): 101-117.

Smith, E. M., et al. (2012). "In vitro inhibition of cytochrome P450-mediated reactions by gemfibrozil, erythromycin, ciprofloxacin and fluoxetine in fish liver microsomes." Aquatic Toxicology **109**: 259-266.

Storey, K. B. (1996). "Oxidative stress: animal adaptations in nature." Brazilian Journal of Medical and Biological Research **29**: 1715-1733.

Trautwein, C., et al. (2014). "Occurrence of the antidiabetic drug Metformin and its ultimate transformation product Guanylurea in several compartments of the aquatic cycle." Environment international **70**: 203-212.

trust, A. s. (2016). Life Cycle of Atlatic Salmon and Wild %A Alvarez, C, ESHRE.

TRUST, S. (2016). Salmon Threats - Salmon in the Classroom, http://www.snh.org.uk/Salmonintheclassroom/salmon_threats.shtml.

Valavanidis, A., et al. (2006). "Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants." Ecotoxicology and Environmental safety **64**(2): 178-189.

Van der Oost, R., et al. (2003). "Fish bioaccumulation and biomarkers in environmental risk assessment: a review." Environmental toxicology and pharmacology **13**(2): 57-149.

Wieczorek D, D. L. a. S. T. (2012, October 2012). "Methods of RNA Quality Assessment." 2016, from

https://no.promega.com/resources/pubhub/methods-of-rna-quality-assessment/.

Zhang, J., et al. (2004). "Effects of water-soluble fractions of diesel oil on the antioxidant defenses of the goldfish, Carassius auratus." Ecotoxicology and Environmental safety **58**(1): 110-116.