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MASTER'S THESIS

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Study of treated and untreated oil-based drilling waste exposure in Atlantic salmon (*Salmo salar*) using a biomarker approach: EROD and oxidative stress parameters

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ABSTRACT

This thesis presents an *in vitro* study evaluating if detoxification and oxidative stress enzymes ethoxyresorufin-O-dethylase (EROD), glutathione S-transferase (GST) and catalase (CAT) can reflect the effect thermally treated and untreated oil-based drilling waste has on Atlantic salmon parr (*Salmo salar*), and how these biomarkers can be used in future monitoring of drilling waste discharges. Fish were exposed for 3, 7 and 14 days to high (1 ppm oil) and low (0.1 ppm oil) doses of treated and untreated drilling waste, followed by a one week recovery period. EROD analysis was performed fluorometrically using NADPH as a substrate. GST and CAT activities were determined photometrically utilising CDNB and H_2O_2 as substrates. Values from all three assays were protein normalised. The results showed that EROD activity in fish exposed to the high dose of untreated drilling waste peaked after 3 days of exposure reaching an average of 3.7 ± 4.2 nmol/min/mg protein. A secondary rise was observed after 14 days, continuing post-recovery. EROD activity in fish exposed to the high dose of treated waste peaked at 4.0 ± 4.3 nmol/min/mg protein after 14 days of exposure. The low dose treated tank reached the highest value at 4.1 ± 3.9 nmol/min/mg protein, after 3 exposure days. The late EROD responses after 14 days of exposure support a theory about a delayed biological metabolisation of PAHs, judged from PAH metabolite concentrations in the fish bile. GST activity in the high dose untreated tank was at its highest after 7 days of exposure with 0.030 ± 0.004 U mg protein. The high dose treated tank reached its peak after 3 days of exposure with 0.032 ± 0.012 U mg protein. CAT activity was at its highest in all tanks including control at 3 days into the exposure, with 0.619 ± 0.087 U mg protein in the high dose untreated tank and 0.567 ± 0.216 U mg protein in the high dose treated tank. The low dose tanks, treated and untreated, reached 0.570 ± 0.186 and 0.550 ± 0.066 U mg protein respectively. Although some responses were consistent with other biomarkers in the study, it was concluded that the enzyme parameters were not significantly reflective of the effect the drilling waste had on the fish. Too many unidentifiably caused responses in the low dose and control tanks masked the moderate effects seen in the high dose tanks. EROD, GST and CAT alone would therefore not be sensitive enough for biomonitoring drilling waste discharges to the level of contamination used in this study.

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ABBREVIATIONS

AhR – Ah receptor

ARNT – Ah receptor nuclear translocator

BaP – Benzo(a)pyrene

BKME – Bleached kraft mill effluent

BSA – Bovine serum albumin

CAT – Catalase

CDNB – 1-chloro-2,4-dinitrobenzene

CF – Condition factor

CFS – Continuous flow system

CYP1A – Cytochrome P450

DMSO – Dimethyl sulfoxide

EDTA – Ethylenediamine tetraacetic acid

EPA – Environmental Protection Agency

ERA – Environmental Risk Analysis

EROD – Ethoxyresorufin-O-deethylase

ETHA – Ethacrynic acid

FL – Fluoranthene

GSH – Reduced glutathione

GST – Glutathione S-Transferase

HSP90 – Heat shock protein 90

HTHP – High temperature high pressure

IP – Indenol[1,2,3-cd]pyrene

LOEC – Lowest Observable Effect Concentration

LSI – Liver somatic index

MFO – Mixed-function oxidase system

mRNA – Messenger ribonucleic acid

β-NADPH – Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt

NPD – Naphthalene, Phenanthrene and Dibenzothiophene

Nph – Naphthalene

OBM – Oil based mud

OCP - Organochlorine pesticides

OSPAR – Oslo Paris Commission

PAH – Polycyclic aromatic hydrocarbons

PCB – Polychlorinated biphenyls

PCDD – Polychlorinated dibenzop-dioxins

PCDF – Polychlorinated dibenzofurans

PEC – Predicted Environmental Concentration

PLONOR – Pose Little or No Risk (to the Environment)

PNEC – Predicted No-Effect Concentration

Pyr – Pyrene

ROS – Reactive oxygen species

SBM – Synthetic based mud

SOP – Standard Operating Procedure

TCC – Thermomechanical cuttings cleaner

TD – Time drive

WBM – Water based mud

1. INTRODUCTION

Pollutants are continuously being released into the environment from various sources. This is particularly concerning for the aquatic environment, which functions as an ultimate sink for pollutants. This is a consequence of direct discharges of pollutant chemicals, along with atmospheric and hydrologic processes.

There is concern about pollution from oil and gas exploration and production. Drilling activities produce waste, such as drill cuttings, oily water and atmospheric gases. Disposal of the waste produced is an issue; e.g. produced water increases as oil fields mature, and drill cuttings accumulate. Current disposal methods are costly, moreover incorrect disposal may cause pollutant chemicals to leach out into the environment.

Pollution and its consequential impacts on living organisms need to be controlled through environmental monitoring. Biomonitoring is frequently used as an environmental monitoring approach. This includes the use of transplant organisms, studying their health conditions and biomarker responses. Changes to these conditions can potentially be used as assessment criteria.

1.1 PURPOSE OF THESIS

The **principal objective** in this thesis is to evaluate the biological response in freshwater salmon subjected to oil-based treated and untreated drilling waste exposure by the use of enzyme biomarkers. The **secondary objective** is to evaluate how these biomarkers can be used in future monitoring of discharges.

This thesis is a toxicity related part of a larger project by the Polish-Norwegian collaboration under the EEA: "*Conception of reuse of the waste from onshore and offshore drilling in the aspect of environmental protection*".

The research for the thesis was completed at the International Research Institute of Stavanger (IRIS) and at the University of Stavanger (UiS).

2. THEORY

This chapter presents theory related to drilling waste, xenobiotics, biomarkers and their applications.

2.1 DRILLING WASTE

During the drilling process, drilling fluids (muds) are used to control formation pressure and prevent formation loss. The mud lubricates and cools down the drill bit, and helps carry drill cuttings up to the surface (Bilstad, 2014).

Drilling wastes contain a mixture of oil, drilling fluids and solids, fragments of rock (drill cuttings), sediments and chemicals used in drilling exploration, appraisal and production wells (Breuer, Shimmield, and Peppe, 2008). A typical composition is 70% minerals, 15% water and 15% oil (Bilstad, 2014). The toxicity level of the waste depends largely on the composition of the formation rock and the type of drilling fluid used (Leonard and Stegemann, 2010). Drilling fluids consist of three main components: liquids, solids and soluble salts. They can be dealt into two categories: aqueous and non-aqueous muds, with the latter having three sub-categories: oil-based mud (OBM), synthetic-based mud (SBM) and diesel (Onwukwe and Nwakaudu, 2012).

Water-based mud (WBM) is a combination of Iron (III) oxide, $CaCO_3$, $BaSO_4$, bentonite clay, polymers, lignosulfate deflocculant, viscosifier and various salts. OBM consists of water, emulsifiers, weighting agents, mineral oil or diesel and various (often undisclosed) yellow and red of the list of chemicals that Pose Little Or No Risk to the environment (PLONOR). Due to this, OBM is of greater environmental concern than WBM (Bilstad, 2014). Synthetic based mud (SBM) was introduced in 1990 as a more environmentally friendly alternative to OBM. Consisting of internal olefins, esters, linear alpha-olefins and linear paraffin's they share some of the desirable drilling fluid properties of OBMs but without polycyclic aromatic hydrocarbons (PAHs) (Gagnon and Bakhtyar, 2003). SBMs have lower toxicity, faster biodegradability and lower bioaccumulation potential than OBM (Onwukwe and Nwakaudu, 2012).

Selecting whether to use WBM, OBM or SBM depends on the nature of the reservoir. In reservoirs with high temperatures and high pressures (HTHP) polymers crack. HTHP is

common in deep well reservoirs. In the case of shale based reservoirs, OBM is used as it does not react with formation clay, something that can make shale instable. SBM has the disadvantage that it may in deep-water wells or cold conditions develop undesirably high or low viscosities (Mason and Gleason, 2003). Also, due to its hydrophobicity, OBM has better accuracy. This makes it easier to control the spreading of OBMs, rather than WBMs, which are hydrophilic, mixing well in with water, and potentially spreading uncontrollably (Nilsen et al., 2010).

Oil-based drilling waste requires extensive treatment before disposal. During this treatment the oil is removed from the waste, reducing the leachability of other contaminants present. Treatment and disposal methods include combustion, thermal desorption, mechanical separation, distillation, stabilisation, bioremediation *in situ*, bioreactors, land farming, re-injection and re-spreading. Post-treatment recycling is still largely prevented by the presence of contaminants in large volumes (Al-Ansary and Al-Tabbaa, 2004). Contaminants present are both organic, (e.g. aliphatic hydrocarbons, PAHs and PCBs) and inorganic with heavy metals such as lead (Pb), barium (Ba), zinc (Zn), mercury (Hg), chromium (Cr), arsenic (As) and nickel (Ni), as well as chloride (Cl^-) compounds (Leonard and Stegemann, 2010).

The rapidly increasing amounts of drilling wastes and stricter disposal regulations have encouraged research on drill cuttings reuse options. Drill cuttings recycling proposals include their use in construction (e.g. as concrete or cement, aggregates, blocks and bricks, making pipe beddings, roads and paths), composting (as top soil admix) and as fuel (Al-Ansary and Al-Tabbaa, 2004).

2.2 XENOBIOTICS

A xenobiotic is an organic chemical unexpectedly found in an organism. Xenobiotics are of apprehension as they are potentially harmful to the organism and its surroundings. Examples of concerning xenobiotics include PAHs, polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polychlorinated dibenzofurans (PCDFs) and dibenzop- dioxins (PCDDs) (van der Oost et al., 2003). In oil-based drilling waste, the major xenobiotic concerns are PAHs (Leonard and Stegemann, 2010).

2.2.1 POLYCYCLIC AROMATIC HYDROCARBONS

PAHs are a group of over 100 different chemicals formed during the incomplete combustion of fossil fuels or garbage, and are known to be widespread pollutants. They are also naturally present in crude oil. Most PAHs are planar molecules consisting of three or more benzene rings attached to each other (Walker et al., 2012).

In fish, absorption of PAHs takes place through ingestion, inhalation, and dermal/gill contact. The PAHs enter the blood and lymph stream, ending up in the liver for metabolisation. Due to their lipophilic nature, non-metabolised PAHs can accumulate in the adipose tissue of organisms (Essumang, Dodoo and Adjei, 2012).

PAHs are of particular concern to health due to their carcinogenic and genotoxic properties (Walker et al., 2012). However they do require metabolic activation. This happens when microsomal enzymes yield reactive epoxides that react with DNA (Pashin and Bakhitova, 1979). Sixteen PAHs are listed on the US Environmental Protection Agency's (EPA) priority pollutant list (Figure 1). These are based on the PAH's toxicity, potential for human exposure and frequency of occurrence at hazardous waste sites (Bojes and Pope, 2007).

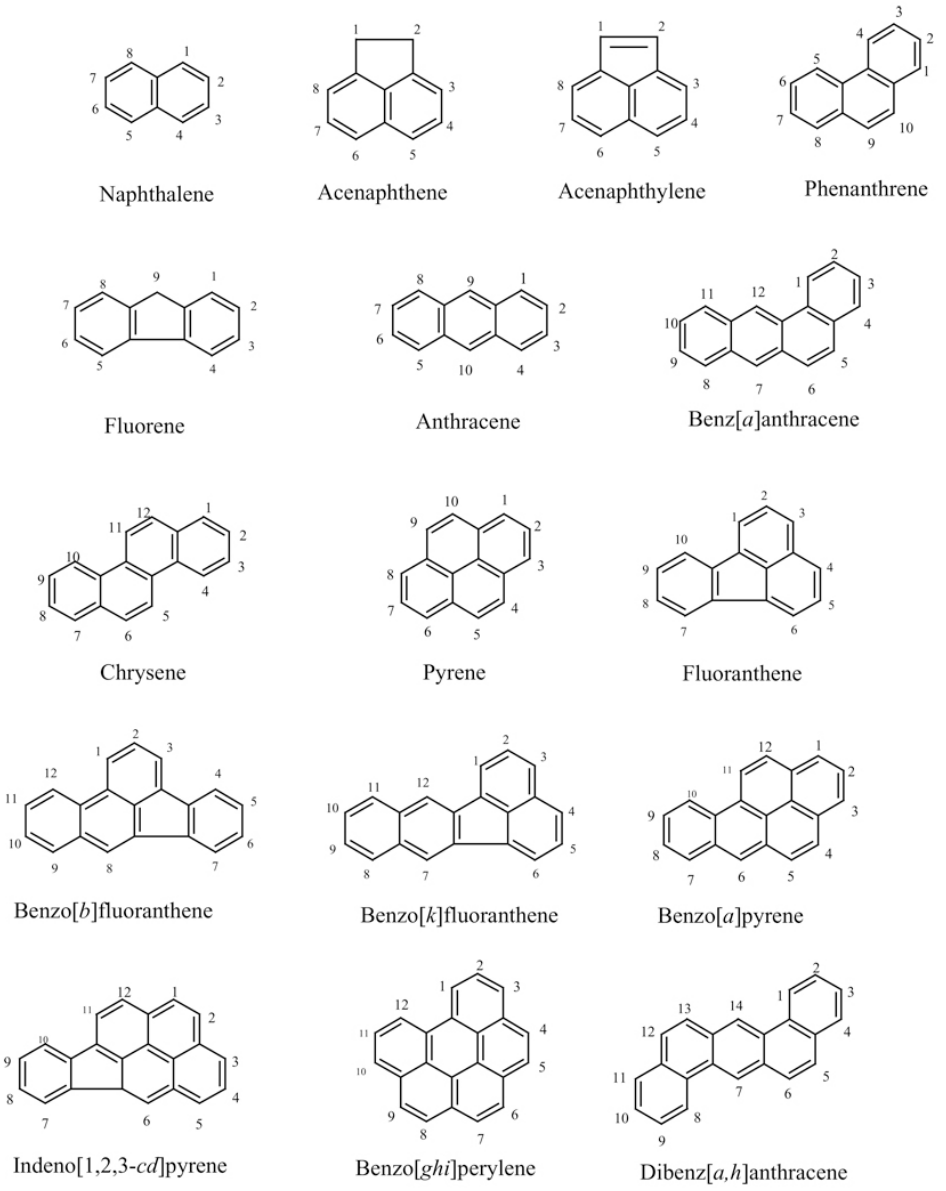

Figure 1. EPA listed priority pollutant PAHs (Yan et al., 2004).

Out of the 16 listed PAHs, 7 are considered carcinogenic; benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene and dibenz(a,h)anthracene. The carcinogenic PAHs have a higher molecular weight as well as lower solubility constants and vapour pressure compared to the non-carcinogenic PAHs (Bojes and Pope, 2007).

When PAHs first enter the aquatic environment they follow their hydrophobic nature, accumulating in fine grained sediments and suspended particles. Eventually, they remobilise, becoming bioavailable to aquatic organisms. When accumulated in fish, PAHs have the

potential to interfere with the cellular membrane functions and their linking enzyme reactions (Zhonghua et al., 2014).

2.2.2 HEAVY METALS

Heavy metals are another concern with oil-based drilling waste. A heavy metal is any metal of environmental concern. The bioavailability of metals largely determines how damaging they are to the environment. Metal solubility in water increases as the pH lowers. Metals are non-biodegradable and cannot be broken down into less harmful compounds (Walker et al., 2012).

The lipid tissues of organisms store inorganic pollutants. Cadmium (Cd) is particularly bioaccumulating, being assimilated quickly and excreted slowly. Fish are generally most sensitive to metal exposure in their embryonic and larval stages. Manganese (Mn), iron (Fe), copper (Cu), and Zn are essential micronutrients in the correct amounts. Exceeding these, they become toxic. Hg, Pb and Cd are not required by any living organism, and are always considered pollutants (Lenntech, 2014; Walker et al., 2012).

2.3 THERMOMECHANICAL CUTTINGS CLEANERS

One way of treating oil-based drilling waste is by using a thermomechanical cuttings cleaner (TCC). The TCC is a machine designed to deal with drill cuttings. It works by hammers causing constant friction and heating up to above the boiling points of water and oil. At these temperatures water and oil are released from the cuttings, leaving them with values as low as <1% oil. This limit is acceptable for disposal both onshore and offshore. The vapours remaining after the combustion are condensed and recovered as heavy oil, light oil and water (Halliburton, 2013).

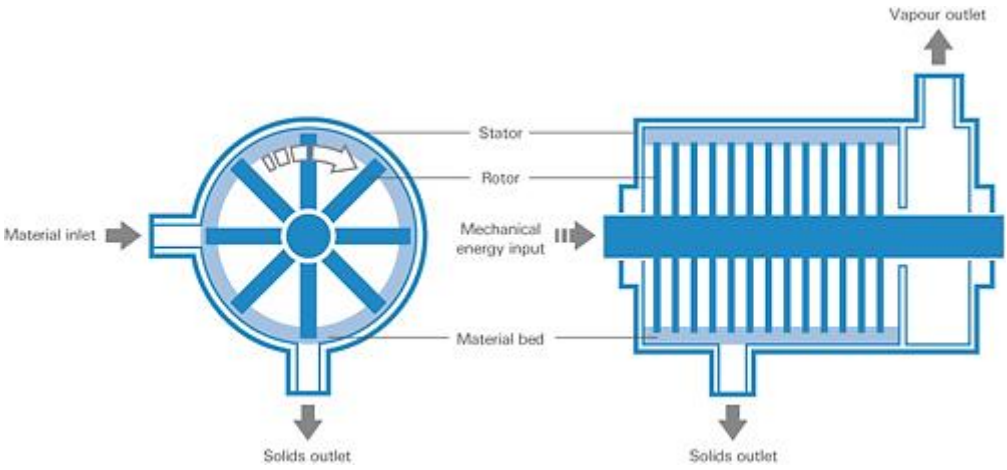

Figure 2. Scheme of thermomechanical cuttings cleaner (Thermtech, 2014).

The downside with the TCC is that it cannot remove inorganic pollutants. The effectivity of PAH removal has yet not been confirmed. Tests by Vik et al. (2013) found PAH removal to vary between 66-99%.

2.4 BIOMARKERS

Biomarkers in environmental research are as defined by van Gestel and van Brummelen (1996) as sub-individual level changes resulting from being exposed to a given substance. Hence, biomarkers measure interactions between the biological system of an organism and a potential hazard. These measurements are performed using the body fluid, cells or tissue of the organism in question, to search for the presence of toxicants or host responses (NRC, 1987). When toxicants are present in the organism, they spread through the body causing noxious effects.

Biomarkers are divided up into hierarchical organisation levels: metabolite, biomolecular, organelle, cellular, tissue, organ system and organism (Walker et al., 2012).

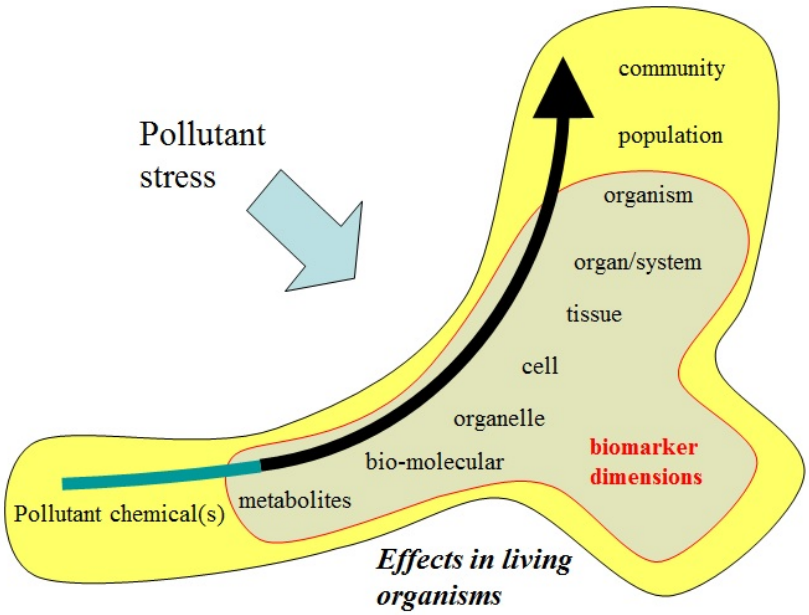

Figure 3. The biomarker hierarchy (modified from Walker et al., 2012; illustrated by Beyer, unpublished).

The higher in the hierarchy the pollutant stress effects show, the more levels are affected by the pollutant, i.e. the lower levels must be affected first before climbing the hierarchy. Changes on the lower levels can be used as early-warning biomarkers, signaling further xenobiotic exposure will result in changes at higher response levels, causing more serious and more likely irreversible damages (Walker et al., 2012).

Response times in organisms vary. Some have to go past the homeostasis stage to respond. Even then, biological factors such as species, size, age, gender and reproductive status may influence the response (van der Oost et al., 2003).

For accuracy purposes, readings should be taken on several biomarker parameters (van der Oost et al., 2003). In this study, the focus is on phase I and II detoxification, and oxidative stress enzyme responses on a metabolite level; measuring increases in ethoxyresorufin-O-deethylase (EROD), glutathione S-transferase (GST) and catalase (CAT) activities in livers of Atlantic salmon exposed to treated and untreated drilling waste.

2.5 ENZYME BIOMARKERS

The majority of xenobiotic chemicals in fish biotransform in the liver, meaning the liver activity may indicate the presence of organic pollutants. The fate of xenobiotics in the liver cell follows one of two paths. Path 1 is the mechanism for detoxification or toxication, while path 2 is the mechanism for enzyme induction.

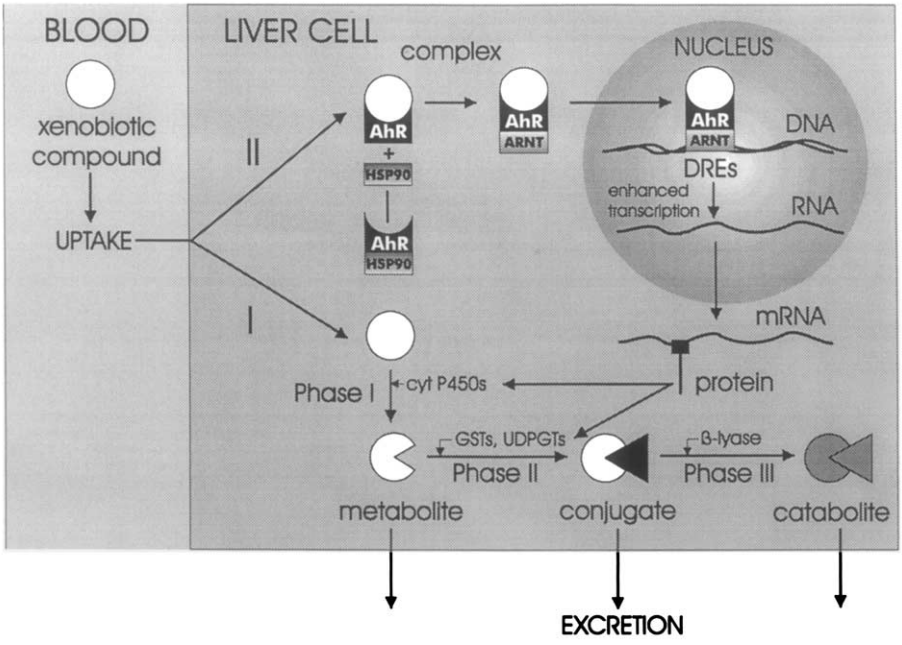

Figure 4. Fate of xenobiotics in liver cells (van der Oost et al., 2003).

Path 1 biotransformation can be subdivided into phases I, II and III. Biotransformation enzymes are either induced or inhibited when exposed to toxic xenobiotics. In phase I the foreign molecule is catalysed by the mixed-function oxidase (MFO) system through oxidation, reduction or hydrolysis, i.e. a non-synthetic modification. Conjugation of the modified molecule takes place in phase II, followed by enzymatic catabolisation in phase III by peptidases, hydrolases and blyase (Commandeur, Stijntjes and Vermeulen, 1995).

Environmental pollutants and their metabolites can cause oxidative stress. Over time, the detoxification systems of organisms have evolved to using antioxidant enzymes such as GST and CAT to combat oxyradical formations (Winston and Di Giulio, 1991).

The enzyme cytochrome P450 (CYP1A) catalyzes most biotransformations in fish. Cytochrome P450 consists of a membrane bound protein, and is predominantly located in the endoplasmic reticulum of the liver (hepatic cytochrome P450) (van der Oost et al., 2003). CYP1A can oxidise highly unreactive compounds like PAHs. The oxidation results in an epoxide which is a highly reactive electrophilic group. The epoxides are then hydrolysed into hydroxyl groups, and coupled with glucuronic acid; producing water-soluble compounds that can be excreted. Sometimes epoxides do not react on hydrolysation, but with DNA instead, binding to the genetic material (Lodish et al., 2000).

Path 2 enzyme induction takes place through the binding of a certain xenobiotic and a protein complex containing the Ah receptor (AhR) and heat-shock protein 90 (HSP90). The HSP 90 is released, while the AhR binds to aryl hydrocarbon nuclear transferase (or Ah receptor nuclear translocator, ARNT), migrating to the cell nucleus. The ARNT then binds to the DNA recognition sequence upstream of the cytochrome P450 genes. The promoter region of the CYP1A gene can now be accessed by the transcription factors. Due to this, messenger RNA (mRNA) synthesis increases, elevating the hepatic protein levels. Elevated protein levels can therefore indicate the presence of ingested xenobiotics (van der Oost et al., 2003).

2.5.1 ETHOXYRESORUFIN-O-DEETHYLASE (EROD)

Ethoxyresorufin-O-deethylase (EROD) activity is a phase I enzymatic reaction where oxidation catalysed by the cytochrome CYP1A causes substrate 7-ethoxyresofin to transform into the reaction end product resorufin (figure 5).

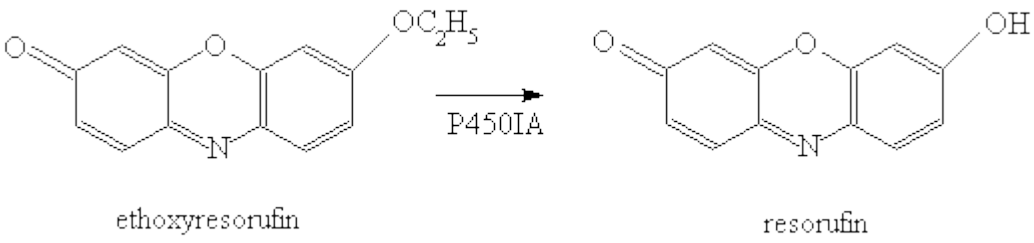

Figure 5. Dealkylation of ethoxyresorufin (Friedli, 1996).

Through this reaction, EROD can be used to measure CYP1A activity in the fish liver. EROD

activity is considered a sensitive catalytic probe for analysing the inductive response of CYP1A in fish and is therefore used as a biomarker to assess exposure of various xenobiotics (Goksøyr and Førlin, 1992; van der Oost et al., 2003). Substances that increase CYP1A catalytic activities include planar PAHs and PCBs, PCDDs and PCDFs, as well as some heavy metals (Jung, Klaus and Fent, 2001).

EROD activity is used as a biomarker for detoxification.

2.5.2 GLUTATHIONE S-TRANSFERASE (GST)

GST is a family of eukaryotic and prokaryotic phase II enzymes. GSTs are mostly soluble, and primarily found in the cytosolic fraction of the liver. GSTs are divided into isoenzymes, sharing ~30% sequence identity. Each isoenzyme has a different function depending on the compound being metabolised. The total number of isoenzymes in fish is yet unknown (Henson, Stauffer and Gallagher, 2001). As a whole, GST aids detoxification in several ways. GST speeds up the linking of xenobiotics with glutathione (GSH), and helps transporting organic anions and other hydrophobic compounds (Townsend and Tew, 2003). The GST conjugate also functions as a downstream signal for phase III of detoxification (Habig et al., 1974).

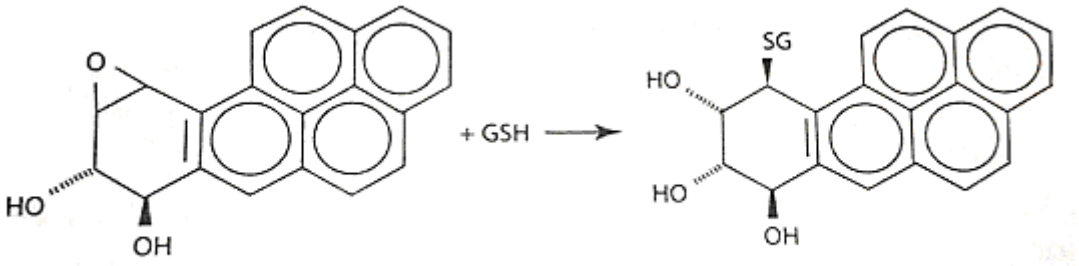

Figure 6. GST biotransformation (Walker et al., 2012).

During catabolism GST is involved in intracellular transport (heme, bilirubin and bile acids) and biosynthesising leukotrienes and prostaglandins. In this way it also protects against oxidative damage and peroxidative products of DNA and lipids. Due to its multiple purposes, GST is considered a very important enzyme of the phase II family for detoxification of xenobiotics (van der Oost et al., 2003).

GST activity is considered a biomarker for both detoxification and oxidative stress.

2.5.3 CATALASE (CAT)

CAT is a widespread hematin-containing enzyme, part of the antioxidant system of an organism, with a function of metabolising hydrogen peroxide (H_2O_2) through the following reaction: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Hydrogen peroxide forms in animal peroxisomes found in the liver and kidneys following oxidative stress, potentially from xenobiotics.

Catalase is a tetramer with four polypeptide chains. These chains are each more than 500 amino acids long. CAT contains four porphyrin iron groups allowing the removal process of hydrogen peroxide. (van der Oost et al., 2003). Catalase also oxidises toxins such as phenols, formic acid, formaldehyde and alcohols by using hydrogen peroxide. The complete mechanism of catalase is still unknown, yet it is believed to occur in two stages:

$$H_2O_2 + Fe_{(III)}\text{-}E \rightarrow H_2O + O{=}Fe_{(IV)}\text{-}E \quad (1)$$

$$H_2O_2 + O{=}Fe_{(IV)}\text{-}E \rightarrow H_2O + Fe_{(III)}\text{-}E \quad (2)$$

(Boon, Downs and Marcey, 2007)

CAT belongs to the antioxidant system and is used as a biomarker for oxidative stress.

2.6 ATLANTIC SALMON (*Salmo salar*)

The model organism used for this biomarker study was the Atlantic salmon (*Salmo salar*).

The Atlantic salmon is an andramonous fish that spends its juvenile phase in freshwater before migrating to the seas to feed and grow, and returning to its birthplace to spawn. The salmon has seven life cycle phases: eggs, alevins, fry, parr, smolt, adult salmon and kelt. Four phases are possible for toxicity testing with salmon as a freshwater species: eggs, alevins, fry and parr (figure 7) (Jensen and Frodesen, 1968; MII, 2007). The fish used in this study were in their parr phase as this phase was considered the most practical. This phase is widely available in Norway due to fish farming. Parr is the last phase before smoltification takes place, with salmon adapting salt regulation mechanisms and preparing for life in seawater. Therefore, by using parr, the results are not only representative for freshwater fish, but also as close as possible to what would be expected in marine adapted salmon. Parr is also the last

phase before sexual maturation takes place, leaving out complications caused by hormone cycles. Using alevins or fry for biomarker measurements would be difficult due to their small sizes, for instance in order to have enough sample materials for biomarker analyses. Yet they may be more sensitive to pollutants, which will not be included in the present study (Sanni, *pers. comm*., 2014).

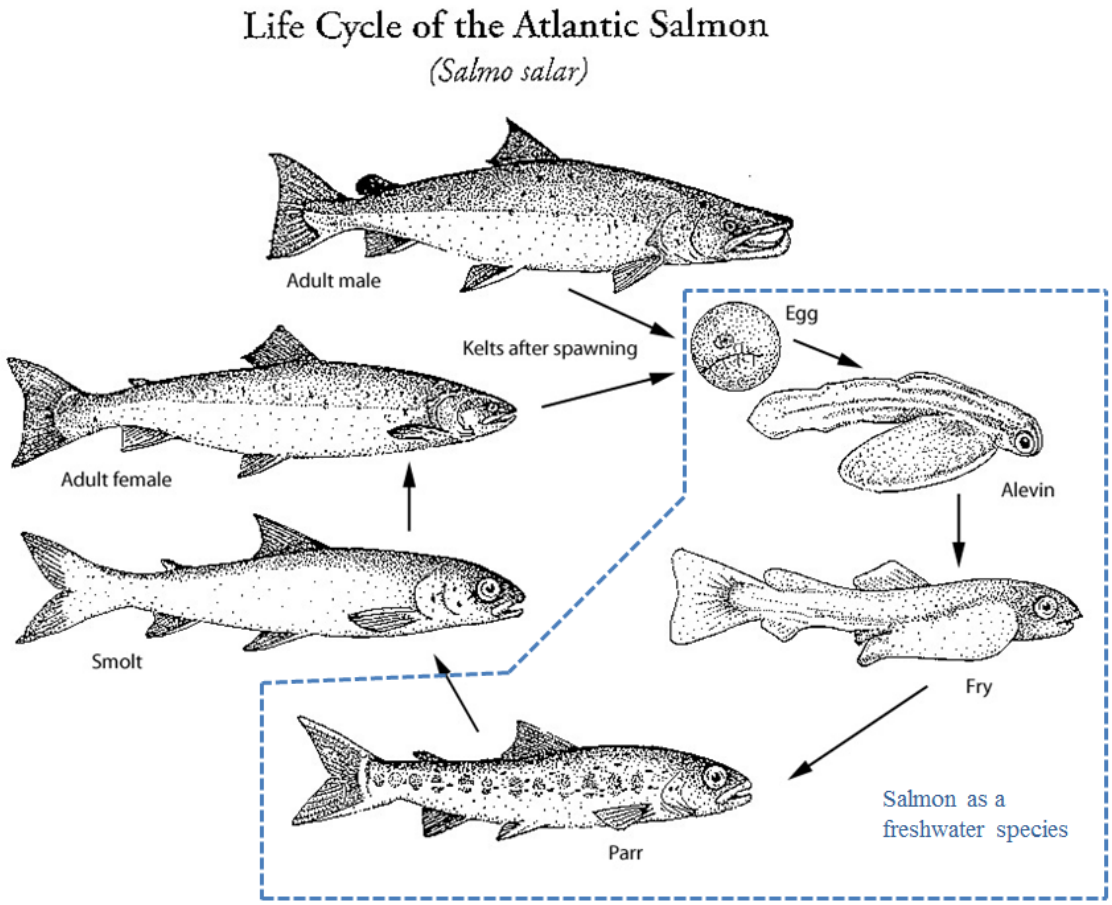

Figure 7. Atlantic salmon life cycle (modified from U.S Fish and Wildlife Service, 2011).

2.7 CONDITION FACTOR AND LIVER SOMATIC INDEX

In analyses involving fish, physical ratios such as condition factor (CF) and liver somatic index (LSI) can be used to understand the general health of the fish.

CF is the general condition of the fish being compared. CF of salmonids is normally calculated and scored using Fulton's formula and K-index (table 1), measuring the ratio between the length and the weight of the fish:

$$\mathbf{CF\ (K) = \frac{100\ \times body\ weight\ (g)}{(length\ (cm))^3}}$$

Table 1 – Fulton's K- index

CF/K-value	Condition
1.41 - 1.60+	Excellent
1.21 - 1.40	Good
1.01 - 1.20	Fair
0.81 - 1.00	Poor
≤ 0.80	Extremely poor

Fish CF is affected largely by the availability of food and food consumption. Feeding is impaired when fish are stressed, giving a poor K-value (Barnham and Baxter, 2003). Other parameters that affect CF are season, disease and nutritional value of food available (van der Oost et al., 2003).

LSI (or hepatosomatic index) shows the correlation between the body weight of the fish and the size (weight) of the liver and is determined using the formula below. The scoring is based on comparison of LSI in healthy fish of the same age and species.

$$\mathbf{LSI = \frac{100\ \times liver\ weight\ (g)}{body\ weight\ (g)}}$$

A relationship between liver enlargement and chemical pollutant exposure has been found by several studies. Slooff et al. (1983) conducted biochemical and histochemical research on bream from polluted sites, finding that their increased liver size was due to hypertrophy, the increase in cell size. Poels et al. (1980) studied juvenile rainbow trout experimentally exposed to polluted river water; results showed that liver enlarged due to hyperplasia, the increase in cell numbers. It had been suggested that the age of the fish caused the different findings. The rapidly growing liver in juvenile fish will respond more readily to hyperplasia than then liver of adult fish (van der Oost et al., 2003).

LSI had proven to respond to a number of pollutants such as PAHs, PCBs, bleached kraft mill effluent (BKME), OCPs and PCDDs. These may increase or decrease the LSI (van der Oost et al., 2003). Exposures to high levels of cadmium and zinc have been seen to have an effect on lowering the LSI. A decreased liver size may also indicate low energy reserves in the fish (EDP, 2007).

2.8 BIOMARKERS IN ENVIRONMENTAL RISK ASSESSMENT

Biomarkers have the potential to be used in environmental risk assessments (ERA). An ERA is a comprehensive system of assessing the scale, potential and probability of adverse environmental effects from anthropogenic activities or natural disasters. Typically, an ERA is categorised in two sets: environmental risk analysis, the scientific process of determining the magnitude and probability of effects; and environmental risk management, which looks at management strategies deciding how to handle the effects determined in the risk analysis (van der Oost et al., 2003). Biomarkers can play a role in steps in both categories.

Environmental risk analysis can be divided into steps such as hazard identification, effect assessment, exposure assessment and risk characterisation. Environmental risk management involves steps in communication, risk management and occasionally in ecological monitoring (figure 8) (van der Oost et al., 2003).

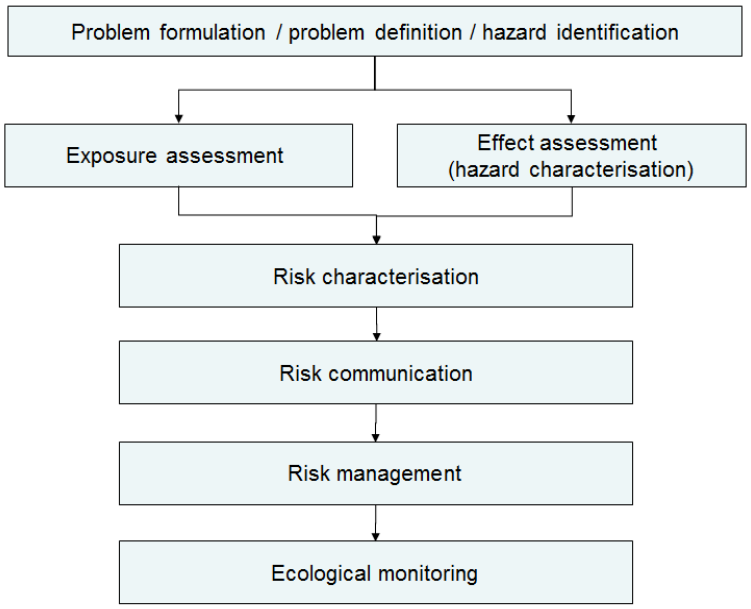

Figure 8. Steps in a total environmental risk assessment (modified from van der Oost et al., 2003).

Biological effect endpoints such as biomarkers are commonly used in effect assessment for determining the dose-response relationships of the environmental stressor and predict no effect concentration (PNEC). The PNEC is required in risk characterisation, where it is

plotted against the predicted environmental concentration (PEC). If PEC/PNEC > 1, additional measures are required (Walker et al., 2012). Ecological monitoring is another step where biomarkers can be valuable. Regular biomarker testing can work as part of a monitoring scheme to observe the environmental health of the assessment site, and draw attention to any pollutant discharges (van der Oost et al., 2003).

3. MATERIALS AND METHODS

The methods were conducted in three parts: drilling waste exposure and sampling, sample preparation, and sample analyses. The first part with exposure and sampling took place at IRIS; while the last two parts with sample preparation and analyses were completed using the laboratories at both IRIS and UiS.

3.1 EXPOSURE PREPARATION

The drilling waste used for the exposure was provided by Halliburton, derived from an offshore reservoir using OBM. One set was untreated drilling waste, and the other a set of TCC treated drill cuttings (figure 9).

Figure 9. From the left, untreated and treated drilling waste.

Before the start of the experiment the waste contents were analysed by Intertek West Lab, an independent laboratory specialising in onshore and offshore fluid analyses. The findings are displayed in tables 2 and 3.

Table 2: Oil, mercury and PAH content in exposure drilling waste

Component	Unit	Untreated	Treated
Oil in sand	mg/kg DM	160000	960
Oil in sand (wt%)	wt % DM	16	0.096
Dry matter content	wt %	66.0	84.6
Mercury in dry matter	mg/kg DM	0.37	0.49
Naphthalene	mg/kg DM	5.0	0.043
Acenaphthylene	mg/kg DM	1.7	< 0.05
Acenaphthene	mg/kg DM	3.3	< 0.01
Fluorene	mg/kg DM	2.0	0.038
Phenanthrene	mg/kg DM	2.1	0.13
Anthracene	mg/kg DM	0.37	0.014
Fluoranthene	mg/kg DM	0.26	0.021
Pyrene	mg/kg DM	1.2	0.061
Benzo(a)anthracene	mg/kg DM	0.26	0.028
Chrysene	mg/kg DM	0.30	0.046
Benzo(b)fluoranthene	mg/kg DM	0.15	0.041
Benzo(k)fluoranthene	mg/kg DM	0.017	< 0.01
Benzo(a)pyrene	mg/kg DM	0.12	0.031
Indeno(1,2,3-c,d)pyrene	mg/kg DM	0.037	0.022
Dibenz(a,h)anthracene	mg/kg DM	0.031	0.015
Benzo(g,h,i)perylene	mg/kg DM	0.16	0.098
Sum 16 EPA-PAH	mg/kg DM	17	0.59

* DM = Dry Matter

Five of the PAH present are considered particularly concerning; BaP, chrysene, pyrene (Pyr), phenanthrene and naphthalene (Nph). These PAHs are of concern due to their bay region attracting pollutants. Epoxides located in the bay make the PAH reactive and mutagenic (Walker et al., 2012).

Table 3: Metals present in exposure drilling mud

Component	Unit	Untreated	Treated
Cadmium, Cd	mg/kg DM	0.22	0.35
Cromium, Cr	mg/kg DM	22	26
Copper, Cu	mg/kg DM	74	78
Nickel, Ni	mg/kg DM	22	36
Lead, Pb	mg/kg DM	64	70
Zink, Zn	mg/kg DM	100	120

The drilling waste density was measured at IRIS, finding that the untreated waste had a density of 1.65 kg/L and the treated cuttings 1.27 kg/L. The freshwater flow into each tank was set at 4.0 ± 0.5 L/min (due to shared flow between five tanks finer accuracy was not possible).

3.2 EXPOSURE

A total of about 300 Atlantic salmon parr were collected from EWOS fish research centre in Dirdal, Rogaland, Norway. Upon arrival at IRIS Environment in Mekjarvik, Rogaland, Norway, the fish were acclimatised in five 100 cm x 100 cm x 60 cm 600 L glass fiber tanks for 14 days. The water used was tap water, filtrated through 5 L of Aqua Medic activated carbon. A continuous flow system (CFS) was applied, with equal parts of water flowing in and out of the tanks. Water parameters flow rate, temperature and oxygen content were measured daily, and the fish were fed *ad libitum*. The tanks were cleaned daily of feces and leftover pellets.

As drilling waste exposure commenced, two 15 L tanks containing the treated and untreated drilling waste were added to the CFS, along with two peristaltic pumps (models Watson Marlow 505U and 520S). Requiring homogenisation, the waste tanks had propellers moving continuously. The CFS was placed above the tanks to make use of gravity, with neoprene tubes transporting drilling waste into the tanks (figure 10). One tank received no waste as this was used as a negative control tank.

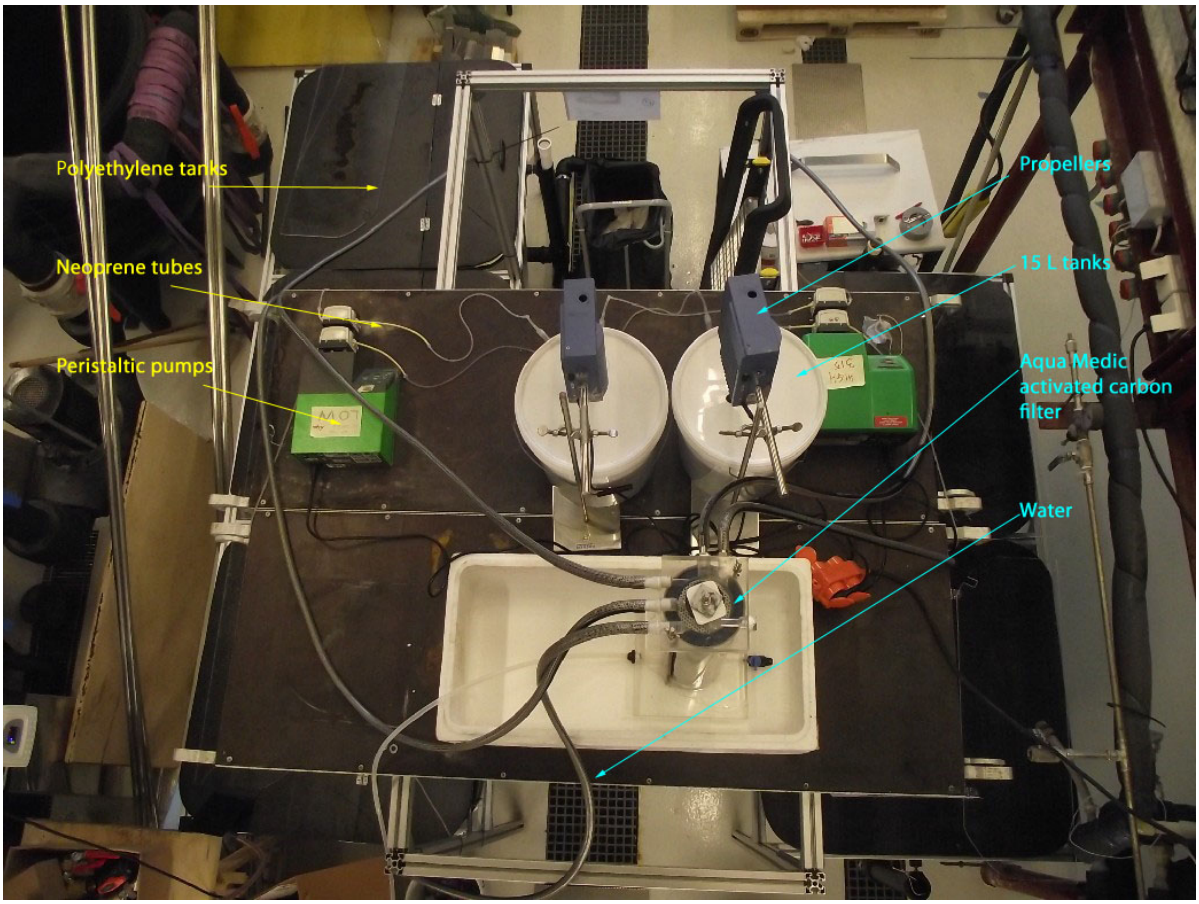

Figure 10. Set up of continuous flow exposure system.

Tanks 1 and 2 received high concentrations of drilling waste with 1 ppm oil. Tank 1 was given treated waste, while tank 2 received untreated waste. Tanks 3 and 4 received low doses of waste with 0.1 ppm oil. Tank 3 had the untreated and tank 4 the treated. Tank 5 was used as the negative control. The drilling waste exposure period lasted for 2 weeks, followed by a 1 week recovery period. The oil concentration calculations were based on PAH levels, ensuring a sub-lethal concentration (calculations in appendix A).

Daily care of fish was amended during the exposure period. Feeding was stopped to ensure bile production. Water flow rate was measured every three days to avoid excessive fish disturbance. Oxygen and water temperature were measured daily. In addition, the neoprene tubes were checked daily for clogging or rupture.

3.3 FISH SAMPLING

Fish sampling for analysis took place four times during the exposure period: 3, 7 and 14 days into the exposure, and after the 1 week recovery. Ten fish from each tank were sacrificed during each sampling. The fish were anaesthetised using metomidate hydrochloride (Aquacalm 50 mg/L). Fish length and weight was measured. Blood was drawn from the tail vein. Fish were then sacrificed with a blow to the head. The fish were dissected by cutting open the abdomen. Livers were cut out and weighed. Using cryovials, liver samples were swiftly put on ice after removal. The lengths, body and liver weights were used to calculate CF and LSI (appendix B).

3.4 SAMPLE PREPARATION FOR ENZYME BIOMARKER ANALYSIS

Supernatant fractions S100 and microsomes were required for EROD, GST, CAT and Bradford analyses and were extracted from hepatic tissue samples collected in the above chapter 3.3. The sample preparation was completed in two parts using IRIS standard operating procedure (SOP) *Preparation of S12, microsomes and S100 by differential centrifugation.* To obtain supernatant 100 (S100) and microsomes, supernatant 12 (S12) preparations were a pre-requisite.

Equipment used:
Tweezers
Pipettes
Homogenisation tube (glass)
Cryogenic 2.0 mL Eppendorf tubes
Cryogenic 1.5 mL Eppendorf tubes
Centrifugation tubes
Pasteur pipettes
Homogeniser (IKA Euro ST-P CV)
Teflon pistil
Table centrifuge with cooling 12 000 g (Eppendorf AG 580R)
Ultracentrifuge with cooling 100 000g (Beckmann vacuum centrifuge)
Pre-cooled centrifuge rotor (70.1 TI)
Pre-cooled, labelled cryovials

pH meter (WTW series inoLab 730)
Scales (Sartorius LE6202P)
Weighing trays
Ice

Chemicals (supplied by Merck and Sigma-Aldrich):
$NaH_2PO_4xH_2O$
KCl
Ethylenediamine tetraacetic acid (EDTA)
NaOH (2M)
Glycerol (100%)
Distilled water

Part 1: S12 preparation

The chemicals were used to make a homogenisation buffer consisting of $NaH_2PO_4xH_2O$ (0.1 M), KCL (0.15 M) and EDTA (1 mM), pH 7.4. This buffer is referred to as buffer A.

The centrifuge was switched on, and set to a temperature of 4°C to allow cooling. Tissue samples were brought out of the freezer and let thaw on ice, keeping them cold throughout the procedure. Eppendorf tubes were labelled and placed in the freezer to cool. One by one the thawed livers were weighed, and using tweezers, transferred into a homogenisation tube. With a pipette, weight correlating amounts of ice cold buffer solution was added (ratio: 4 mL buffer per 1 g hepatic tissue). The livers were then homogenised using five slow strokes of the Teflon pistil, assuring no air-intrusion. Liver homogenate was transferred into the two pre-cooled 2.0 mL centrifugation tubes. The tubes were placed in the centrifuge rotor with approximate same volumes of analyte facing opposite each other, ensuring steady balance. The samples were centrifuged at 12 000 g at 4°C for 20 minutes. Using a Pasteur pipette, supernatant was transferred into the pre-cooled 1.5 mL Eppendorf vials, ensuring no pellet material was included. The vials, kept on ice, were then placed in the -80°C freezer to prevent biodegradation while in storage.

Part 2: S100 and microsome preparation:

Suspension buffer required for post-ultracentrifugation and S100 and microsome preparations was made following the same recipe as buffer A, but with the addition of 200 mL of glycerol. This is referred to as buffer B.

The ultracentrifuge was switched on, allowing vacuum and cooling to 4°C. The S12 samples made in the previous step were taken out of the freezer, put on ice and let thaw. An amount of 600 µL S12 was pipetted into ultracentrifugation tubes. The centrifugation tubes containing the sample, lids and O-rings were weighed, and paired up with samples of same weight (< 0.01 g weight difference) for centrifuging. Samples with no weight matching pairs were balanced using small amounts of buffer A. The centrifuge rotor was brought out of the cooling room, and sample pairs were placed opposite each other in the rotor sockets. The ultracentrifuge was run at 100 000 *g* (37 500 rpm) at 4°C for one hour. The cytosolic fraction, making up S100 was transferred into labelled aliquot cryovials using a Pasteur pipette. The remaining microsome layer in the centrifugation tube was re-suspended using 250 µL of buffer B. With a Pasteur pipette; the microsomes were transferred into an Eppendorf tube and homogenised using a handheld micro-pistil until no visible fragments were present. The homogenate was then pipetted into aliquot cryovials. The cryovials containing the S100 and microsomes were placed in the - 80°C ultra-freezer for storage.

3.5 BRADFORD PROTEIN ASSAY

To be able to determine total EROD, GST and CAT activity, as they are protein normalised, knowing the protein contents of the analytes was necessary. The calculations for the enzymatic reactions were based on the mg (/mL) of protein in the test samples. The protein contents were derived with a Bradford protein assay. The Bradford protein assay is a colorimetric technique based on Coomassie Brilliant Blue G-250 dye binding proportionally to proteins. Coomassie dye absorbs at 595 nm, allowing for optical density measurements. Protein concentrations are determined with a comparison to a standard curve based on protein standards that exhibit a linear absorbance profile. The most commonly used protein standard for comparison is Bovine Serum Albumin (BSA) (Bradford, 1976).

Bradford protein analysis was completed using the S100 and microsome samples prepared in chapter 3.4, following the IRIS SOP *Bradford on microplates* based on the methods by Bradford (1976).

Equipment used:
Plate reader (Tecan Infinite F200 PRO)
Operator PC
Microplates
Pipettes

Chemicals:
Bovine Serum Albumin (BSA) (5% solution, 10 mg/mL)
Bio-Rad Protein Assay Dye Reagent (Coomassie Brilliant Blue 1)
Distilled water

Procedure:

The S100 and microsome samples prepared in chapter 3.4 were brought out from the freezer and let thaw on ice. The plate reader and operator PC were switched on, opening Magellan 7.1 software. Readings were set to 595 nm and flashes to 25. To construct a calibration curve, a 1 mg/mL working solution was made by diluting 100 µL of 10 mg/mL BSA stock with 900 µL of distilled water. From this working solution and further dilutions, four reference samples of concentrations 0.1, 0.2, 0.3 and 0.4 mg/mL protein were made. While marking the plate layout on a calculation sheet; 10 µL of distilled water and 10 µL reference samples were transferred into four individual microplate wells. 10 µL of each unknown S100 and microsome hepatic sample were pipetted into own wells. 200 µL of dye reagent was added to each microplate well sample, and air bubbles were popped using a clean pipette tip. The microplate was placed in the dark to incubate for 10 minutes, with absorbance increasing over time. Post-incubation, absorbance was measured at 595 nm using the microplate reader. Values gained were exported into Microsoft Excel, plotting a calibration curve from the distilled water and BSA reference sample readings. The curve was checked for linearity, and the readings from the unknown S100 and microsome samples were interpreted using the curve equation.

3.6 ETHOXYRESORUFIN-O-DEETHYLASE (EROD)

EROD activity is measured in pmol resorufin/min/mg protein. The presence of resorufin in the analyte is detected using spectrofluorometry. The fluorophore spectra changes as a function of the concentration of EROD metabolites at excitation 535 nm and emission 585 nm (So and Dong, 2002).

The microsome samples prepared in chapter 3.4 were used in the EROD cuvette method. The method followed the IRIS SOP *EROD cuvette method* based on methods used by Nilsen et al. (1998). Appropriate in-house reference samples were used for analysis quality control.

Equipment used:
Spectrofluorometer (Perkin Elmer LS-50B)
Spectrophotometer (Perkin Elmer Lambda 2S)
Plastic cuvettes (10mm light path)
Pipettes (10 µL, 20 µL and 1 mL)
pH-meter (WTW series inoLab PH730)
Glassware
Parafilm

Chemicals (supplied by Merck and Sigma-Aldrich):
$NaH_2PO_4xH_2O$
7-Ethoxyresorufin
Resorufin
Dimethyl sulfoxide (DMSO)
Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (β-NADPH)

The chemicals were used to make up five buffers and solutions:

1. EROD buffer (0.1M Na phosphate buffer): 13.8 g $NaH_2PO_4xH_2O$ was dissolved in 950 mL distilled water, and pH adjusted to 7.4. Distilled water was added to give an end volume of 1000 mL.
2. 7-Ethoxyresorufin solution: 1 mg 7-ethoxyresorufin dissolved in 10 mL DMSO.
3. Resorufin stock solution: 1 mg resorufin dissolved in 50 mL DMSO.
4. Resorufin internal standard solution: stock solution diluted 1:3 in DMSO, with absorbance measured at 572 nm using a spectrophotometer. Resorufin concentration

(mM) is calculated by dividing the OD_{572} value on the resorufin extinction coeffiecient (73.2 mM^{-1} cm^{-1}).

5. NADPH stock solution (9 mM NADPH): 5 mg β-NADPH (4 x H_2O) is dissolved in 600 µL distilled water.

All buffers were made in advance and kept in appropriate cold storage: 4°C for buffers/solutions 1, 2, 4 and 5; - 20°C for solution 3.

Procedure:

The spectrofluorometer and computer were switched on. On the computer, the software Luminous was selected, establishing communication between the fluorometer and software, setting excitation and emission to 535 nm and 585 nm respectively. The spectrophotometer was switched on and wavelength set to 572 nm. The absorption of the resorufin internal standard was measured against a DMSO blank. In-house control samples and the unknown tissue samples prepared in chapter 2.4 were brought out of the freezer and let thaw on ice, keeping them cold throughout the procedure. The EROD buffer was taken out of the fridge and let adapt to room temperature (20°C). For each of the analytes 1.96 mL of the EROD buffer, 10 µL 7-Ethoxyresorufin substrate solution and 20 µL microsome fraction was added into a cuvette and mixed by inverting the cuvette 2-3 times. The cuvette was placed in the spectrofluorometer, recording the baseline signal. Using a pipette, 10 µL of NADPH was added into cuvette solution, mixing again by inverting cuvette 2-3 times. The cuvette was placed back in the spectrofluorometer, where change in fluorescence until a continuous linear response was observed. Once more using a pipette, 10 µL resorufin internal standard solution was added to the cuvette solution, yet again mixing by inverting cuvette 2-3 times. Back in the spectrofluorometer, the rise in fluorescence level of the analyte was recorded. The fluorescence change per amount (pmol) of resorufin added was calculated, as well as the specific enzymatic activity (pmol/min/mg protein) of each measured sample. The formula used for the latter:

$$\textbf{pmol resorufin / min / mg protein} = F_S / \text{min} \times R / F_R \times 1 / V_S \times 1 / C_S$$

Where:

F_S / min Increase in sample fluorescence per minute

R pmol resorufin added as internal standard

F_R Increase in fluorescence due to the addition of the resorufin standard

V_S Volume of sample (0.02 mL)

C_S Protein concentration in analytical mix (mg/mL)

3.7 GLUTATHIONE S-TRANSFERASE (GST)

Total GST activity is measured spectrophotometrically at 340 nm using a substrate such as 1-chloro-2, 4-dinitrobenzene (CDNB) or ethacrynic acid (ETHA), and excess glutathione (GSH.) The formation of the GST-CDNB (or ETHA) conjugate induces the increase in absorbance (Novoa-Valinas et al., 2001). GST is expressed as international enzyme units (U) per mg protein (1 U = 1 μmol / min) and normalised against the total protein (mg/mL) content of the sample. Lambert-Beer's law is used to calculate molar enzyme activities using the GST activity extinction coefficient (ϵ) = 9.6 mM^{-1} cm^{-1}.

S100 samples prepared in chapter 3.4 were utilised in the GST analysis. The method followed the IRIS SOP *GST cuvette method* based on methods determined by Habig et al. (1974) via GSH-CDNB conjugation. Appropriate in-house reference samples were used for analysis quality control.

Equipment used:
Spectrophotometer (Perkin Elmer Lambda 2S)
Quartz cuvettes (10 mm light path)
Pipettes (50 µL, 150 µL and 1 mL)
pH-meter (WTW series inoLab PH730)
Eppendorf tubes
Glassware

Chemicals (supplied by Merck and Sigma-Aldrich):
KH_2PO_4
K_2HPO_4
1-chloro-2, 4-dinitrobenzene (CDNB)
Dimethyl sulfoxide (DMSO)
Reduced GSH
Distilled water
MilliQ

The chemicals were used to make up three buffers and solutions:

1. Phosphate buffer (100mM, pH 7.0 / 7.4): 17.42 g KH_2PO_4 was dissolved in 1000 mL distilled water. pH was adjusted to 7.0 and 7.4 using 13.65 g/L K_2HPO_4 (mixed in distilled water).
2. CDNB solution: 4.0 mg of CDNB was dissolved in 1 mL of DMSO. Aliquots were kept wrapped in foil due to their light sensitivity.
3. GSH solution: 6.1 mg of GSH was dissolved in 1 mL MilliQ.

Buffers were kept in appropriate storage: Buffer 1 at 4°C, solution 2 at - 20°C and solution 3 made fresh daily.

Procedure:

The S100 samples were brought out of -80°C and put to thaw on ice. The spectrophotometer and connected PC were switched on, and Lambda 2 software was selected. Time drive (TD) mode was chosen, with absorbance set to 340 nm with 60 second readings and 1 second intervals. The thawed S100 samples were diluted 1:4 with 50 µL sample and 150 µL ice cold pH 7.4 phosphate buffer. Two cuvettes were filled with 1800 µL room temperature pH 7.0 phosphate buffer, and used to auto zero the spectrophotometer. To commence the measurements, a blank consisting of 1800 µL pH 7.0 phosphate buffer, 100 µL CDNB solution and 100 µL GSH solution was measured. One by one, the diluted samples were added to the cuvette following an order of 1700 µL pH 7.0 phosphate buffer, 100 µL CDNB solution, 100 µL GSH solution and 100 µL of cytosol. Within 10 seconds of the addition of cytosol, the cuvette was capped, mixed by inversion and placed in the spectrophotometer. The increase in absorbance was recorded for 60 seconds for each sample. To confirm results, samples were analysed twice, using the mean value for further calculations. The cuvette was rinsed with distilled water between each sample. GST activity was calculated using the equation below. To obtain the net slop, the mean of the blank slope was subtracted from all the sample measurements.

$$\textbf{GST activity (U mg protein)} = \frac{\textbf{Net slope (A340/min)}}{\textbf{9.6} \times \textbf{mg/mL protein}} \times \textbf{Sample dilution}$$

3.8 CATALASE (CAT)

CAT activity is determined spectrophotometrically at a wavelength of 240 nm, and defined by moles of H_2O_2 consumed per minute per mg protein in sample. CAT is expressed as U per mg protein (1 U = 1 μmol/min). The molar extinction coefficient (ϵ) for H_2O_2 which is 0.04 mM^{-1} is used for calculating the activity.

S100 samples prepared in chapter 3.4 were used for the CAT analysis, following the methods given in the IRIS SOP *Catalase* based on Claiborne (1985). Appropriate in-house reference samples were used for analysis quality control

Equipment used:
Spectrophotometer (Perkin Elmer Lambda 35)
Quartz cuvettes (10 mm and 50 mm light path)
Pipettes (30 μL, 150 μL and 1 mL)
pH meter (WTW series inoLab PH730)
Eppendorf tubes
Glassware

Chemicals (supplied by Alfa Aesar and Merck):
KH_2PO_4
K_2HPO_4
Hydrogen peroxide (H_2O_2) 27%
Distilled water

The chemicals were used to make up two buffers:

1. Phosphate buffer (100mM, pH 7.4): 6.81 g KH_2PO_4 was dissolved in 1000 mL distilled water. pH was adjusted to 7.4 using a 11.41 g/L K_2HPO_4 solution (mixed in distilled water).
2. Hydrogen peroxide buffer: 0.62 mL of 27% hydrogen peroxide was dissolved in 10 mL of 100 mM phosphate buffer.

Buffer 1 was stored at 4°C, and buffer 2 was fresh made every day.

Procedure:

The spectrophotometer and operator PC were switched on, opening Lambda 35 software. Selecting TD mode, readings were set to 240 nm with 30 second readings and 1 second intervals. The S100 samples were brought out of the ultra-freezer to thaw on ice. Once thawed, they were diluted 1:5 with 30 µL cytosol and 120 µL ice cold phosphate buffer. The spectrophotometer was auto-zeroed by placing phosphate buffer in both cuvettes. Starting the measurements, a blank consisting of 2850 µL phosphate buffer and 150 µL H_2O_2 buffer was measured in the 50 mm cuvette. The samples were measured individually, added to the 50 mm cuvette in the order of 2700 µL phosphate buffer, 150 µL H_2O_2 buffer and 150 µL of diluted cytosol. The cuvette was capped, and mixed by inversion. Within 10 seconds of the addition of cytosol, the cuvette was placed in the spectrophotometer and recordings of increase in absorbance were noted. Due to the linear signal obtained, the samples were analysed two additional times. Between samples, the cuvette was rinsed with distilled water. The CAT activity was calculated using the formula below.

$$\textbf{Catalase activity (U mg proteins)} = \frac{\Delta OD/min}{0.040 \times mg/mL \text{ protein in cuvette}}$$

3.9 STATISTICAL ANALYSIS

The data was analysed using Microsoft Excel and SAS JMP statistical software. One-way ANOVA plots were created to enable variable comparison between control and exposure tanks. Dunnett's tests with $P < 0.05$ were completed to compare statistically significant differences in the individual treatment tanks against the control tank (Dunnett, 1955).

4. RESULTS

The results include the morphological parameters CF and LSI, activity response in phase I enzyme EROD and phase II enzyme GST, and responses in antioxidant enzyme CAT. All enzyme analyses are based on results from the Bradford protein assay completed in chapter 3.5. The hepatic protein content for the samples can be found in the appendix C.

4.1 CONDITION FACTOR

The CF measurements were based on 10 fish from each tank per analysed sampling. The results obtained from these measurements are displayed in figure 11.

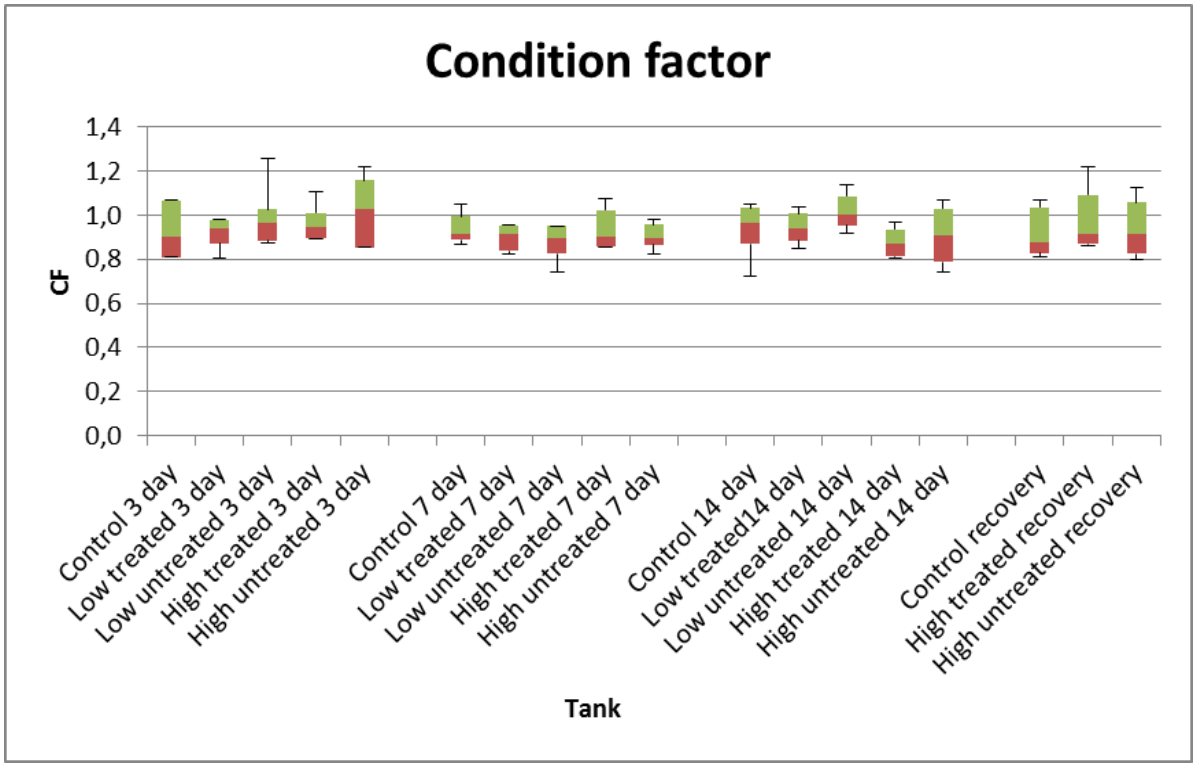

Figure 11. CF of fish sampled. The red bars display the 10 percentile to the median, and the green bars the median to 90 percentile. The vertical lines represent the minimum and maximum values.

The CF graph (figure 11) indicates there were little morphological changes in the fish over the exposure period. The maximum CF found was 1.26, occurring in the low dose untreated tank at 3 days into the exposure. The lowest CF established was 0.72 in the 14 day control tank.

4.2 LIVER SOMATIC INDEX

The LSI measurements were as CF, based on the 10 fish sampled per tank per sampling. LSI results are displayed in figure 12 below.

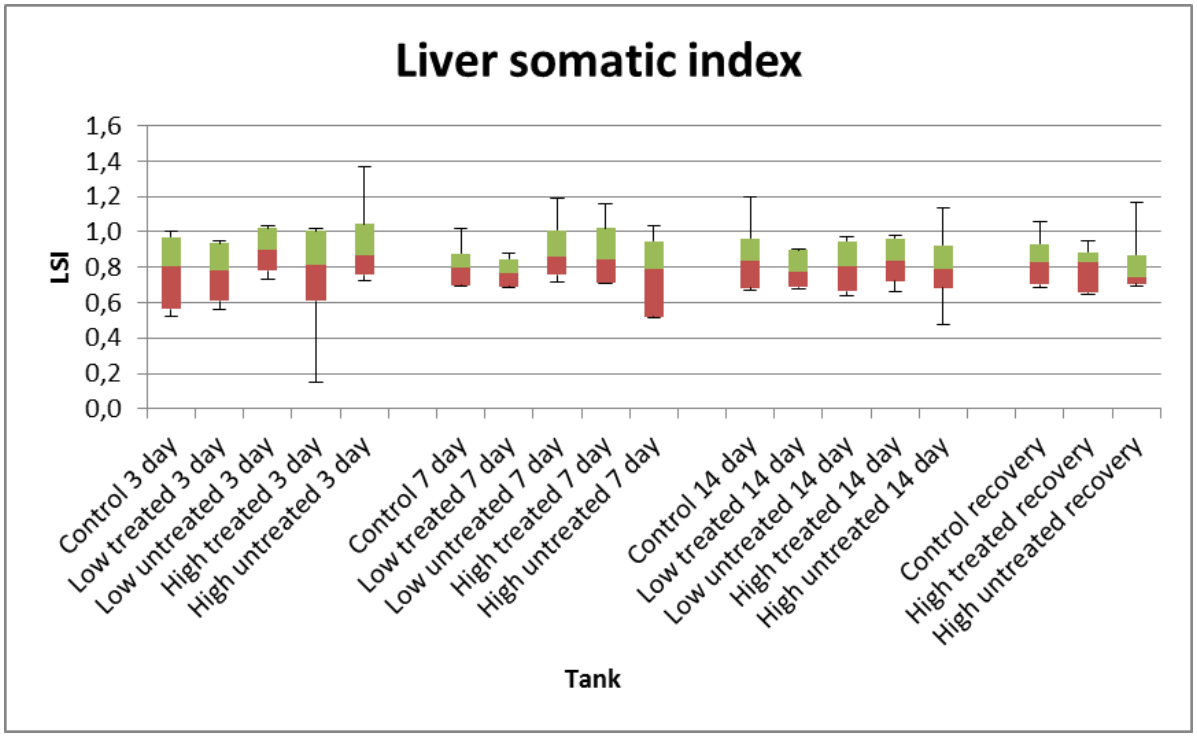

Figure 12. LSI of sampled fish. (10, 50 and 90 percentiles, min and max values displayed, see figure 11.)

Median changes in liver sizes throughout the exposure were vague (figure 12). The highest LSI value measured was 1.37 in the high dose untreated tank 3 days into the exposure. The lowest LSI measured, 0.15, occurred in the high dose treated tank 3 days into exposure.

4.3 ETHOXYRESORUFIN-O-DEETHYLASE (EROD)

EROD activity was measured fluorometrically from 7 fish per tank per sampling from 3, 7 and 14 day exposures, and from the control and high dose tanks post-recovery.

EROD results are presented in nmol instead of the commonly used pmol. This is due to the low values obtained. Large variations were observed within the values in each measurement group. This is common when dealing with low values.

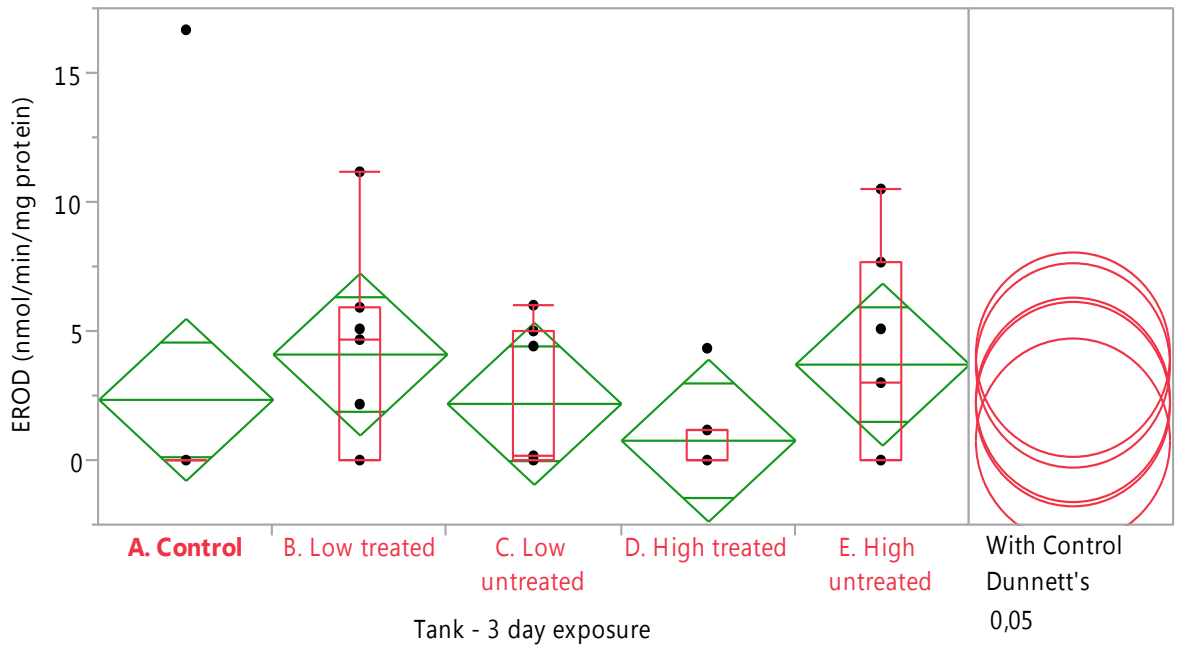

Figure 13. Hepatic EROD activity in fish sampled 3 days into exposure.

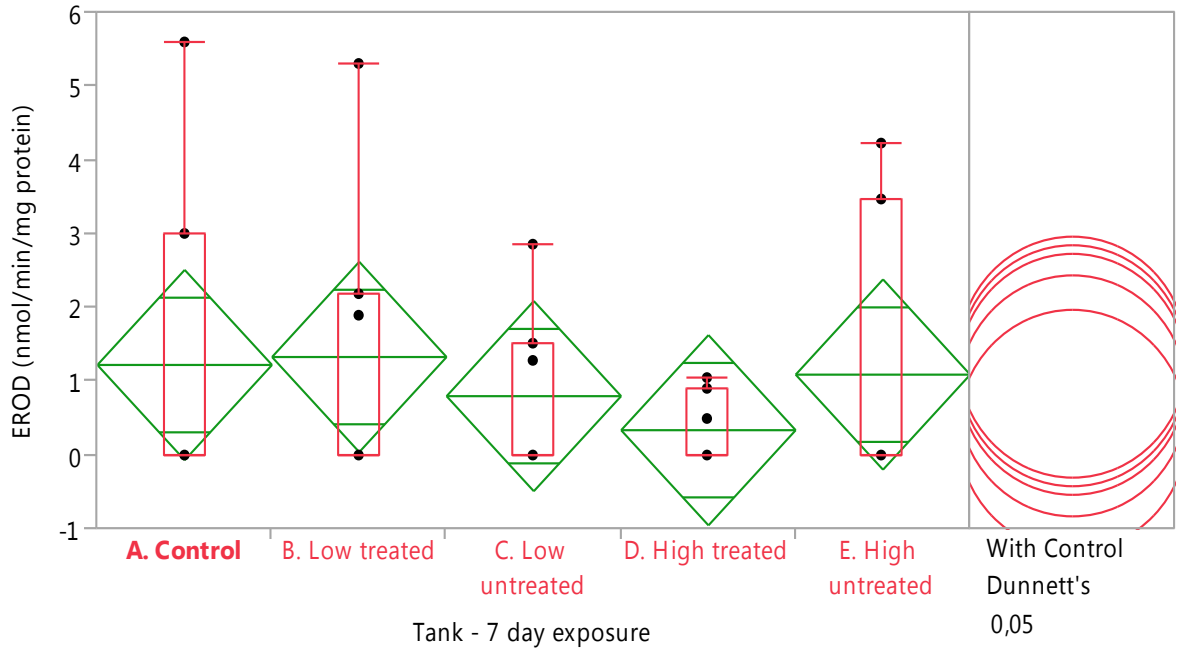

Figure 14. Hepatic EROD activity in fish sampled 7 days into exposure.

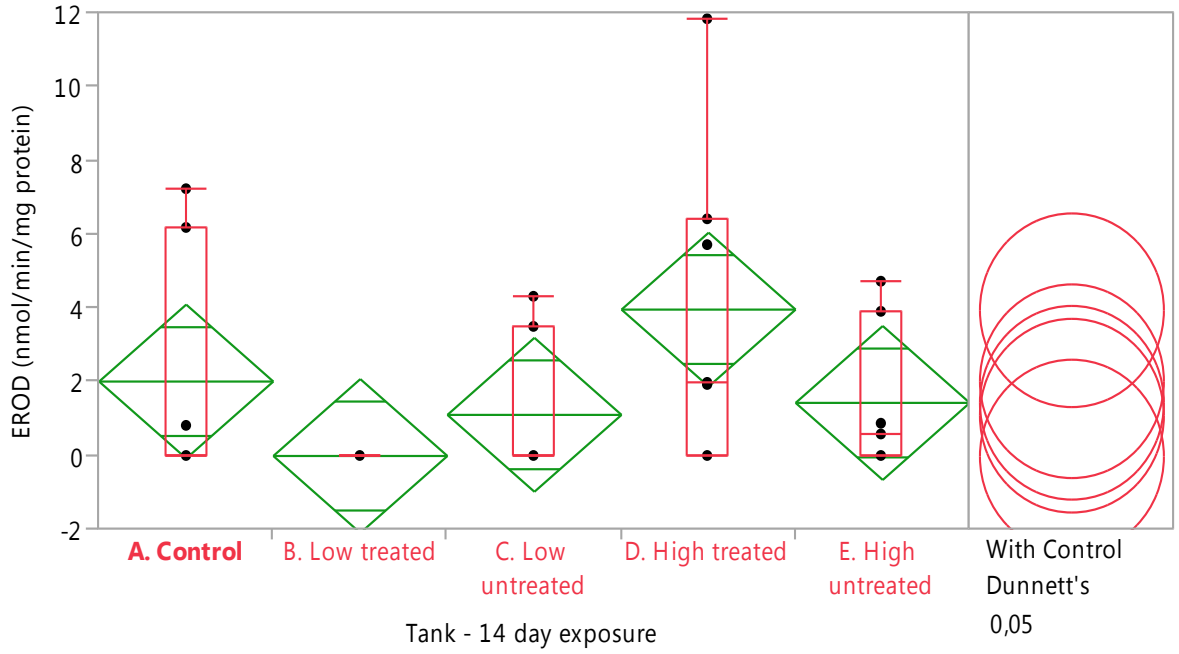

Figure 15. Hepatic EROD activity in fish sampled 14 days into exposure.

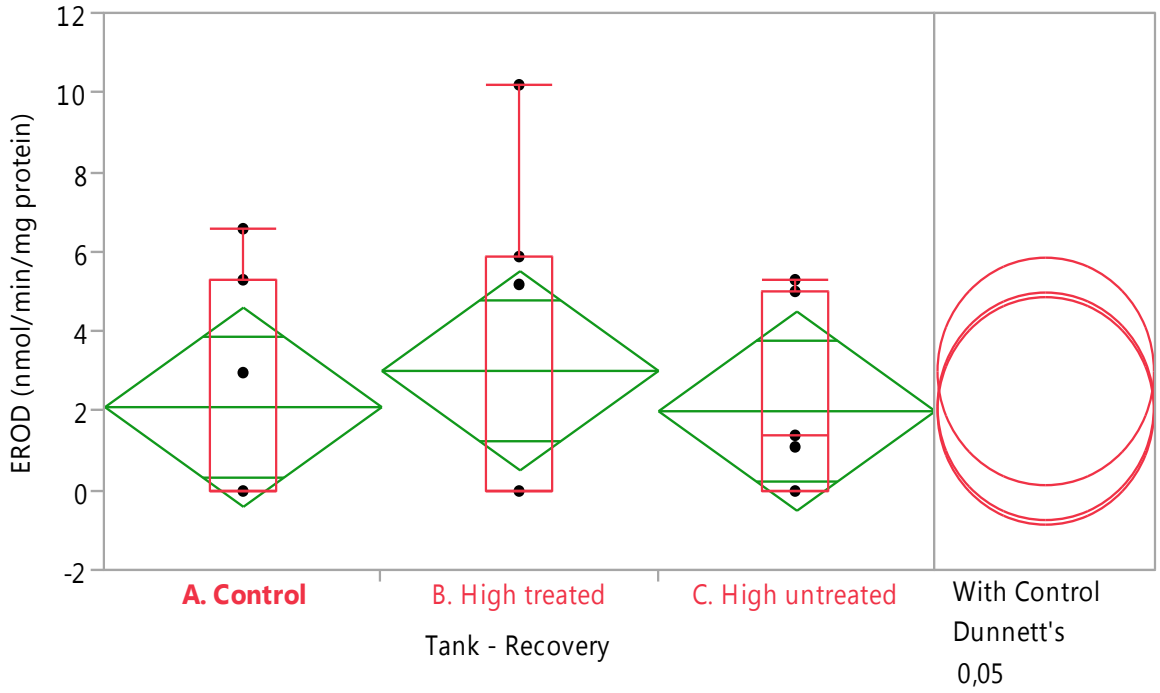

Figure 16. Hepatic EROD activity in fish sampled after a one week recovery period.

The box represents the 10 to 90 percentile with the horizontal line showing the median. The diamond displays the mean and standard deviations. The vertical lines reflect the minimum and maximum values. Points not included are considered outliers.

EROD activity was shown to be at maximum in the high dose untreated tank 3 days into the exposure with a mean and standard deviation of 3.7 ± 4.2 nmol/min/mg protein (figure 13). After 7 days the activity decreased, picking up again at 14 days, reaching an average of 1.4 ± 2.0 nmol/min/mg protein (figure 14 and 15). The high treated tank had low activity until 14 days into the exposure, when it increased significantly, peaking with a mean value of 4.0 ± 4.3 nmol/min/mg protein (figure 15). The low dose untreated tank reached its highest values 3 days into exposure at 2.2 ± 2.8 nmol/min/mg protein, decreasing at 7 days and increasing slightly 14 days in (figures 13, 14 and 15). The low dose treated tank peaked 3 days into exposure with 4.1 ± 3.9 nmol/min/mg protein, decreasing significantly after 7 days and displaying no EROD activity after 14 days (figures 13, 14 and 15). The increased EROD activity in the high dose tanks after 14 days of exposure led to the choice to analyse the high dose recovery samples. As seen in figure 16, the activity in the high treated tank started decreasing (3.0 ± 4.1 nmol/min/mg protein) while the high untreated tank activity continued increasing (2.6 ± 2.5 nmol/min/mg protein).

4.4 GLUTATHIONE S-TRANSFERASE (GST)

GST activity was determined photometrically from 7 fish per control and high dose tank from 3, 7 and 14 days of exposure and one week of recovery.

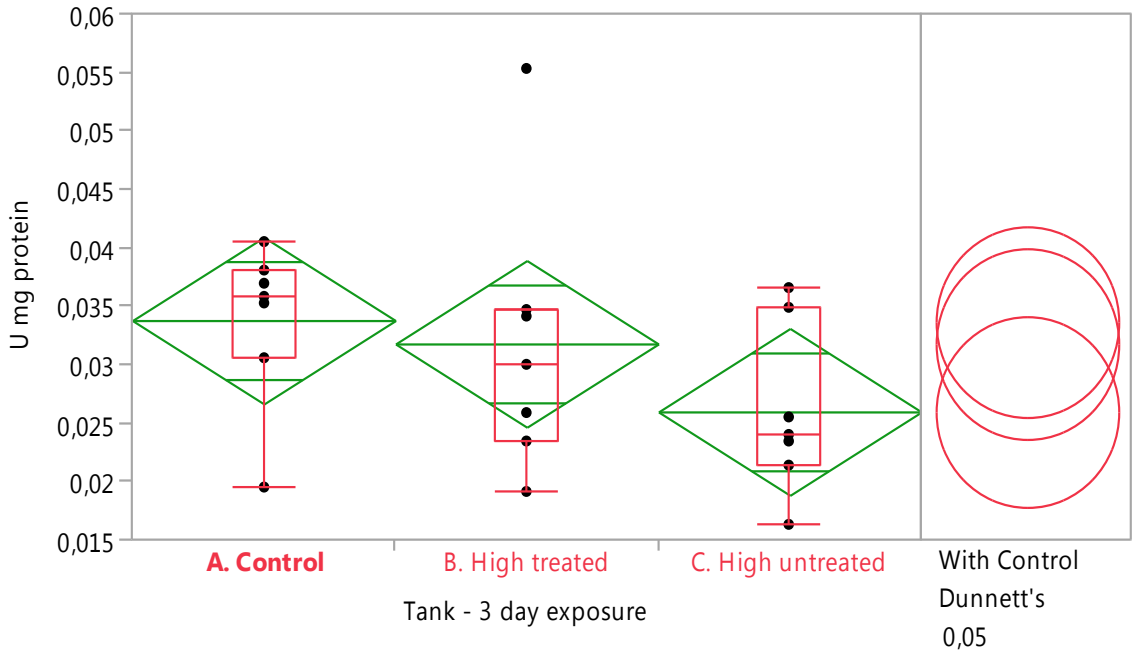

Figure 17. Hepatic GST activity in the control and high dose tanks in fish sampled 3 days into the exposure.

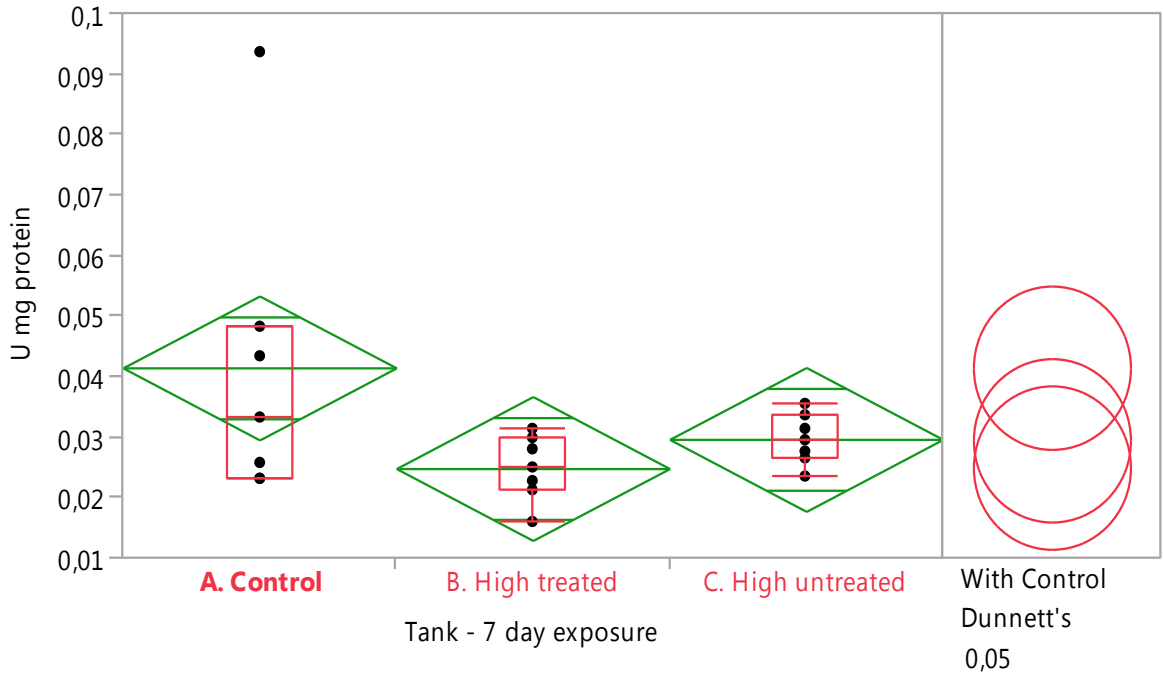

Figure 18. Hepatic GST activity in the control and high dose tanks in fish sampled 7 days into the exposure.

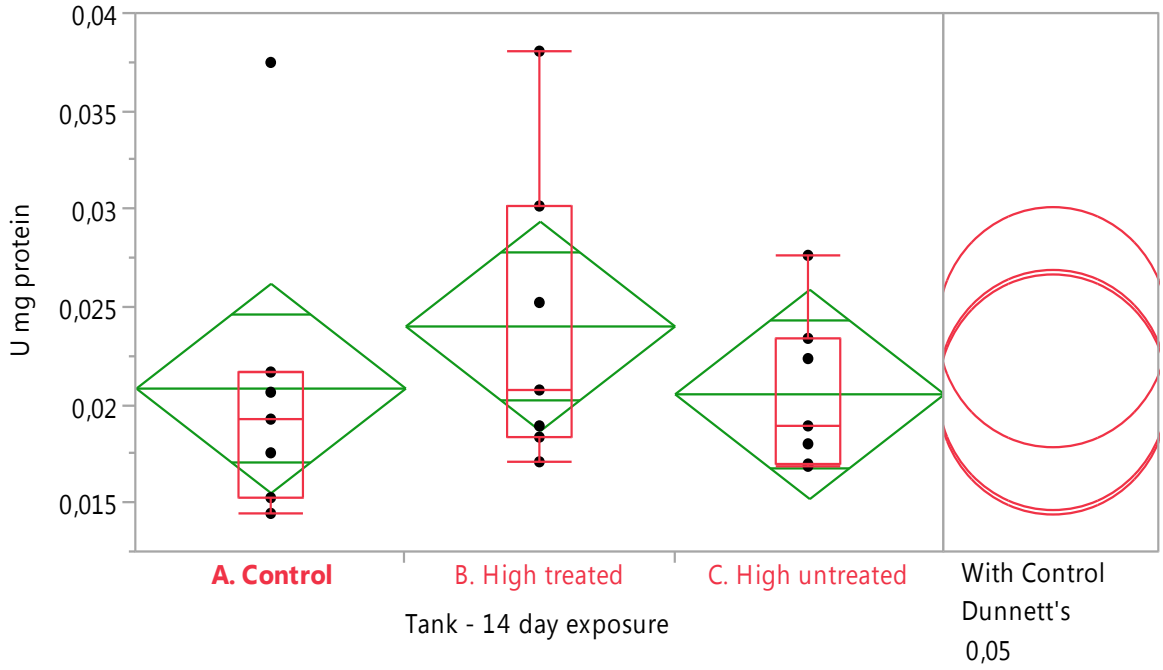

Figure 19. Hepatic GST activity in the control and high dose tanks in fish sampled 14 days into the exposure.

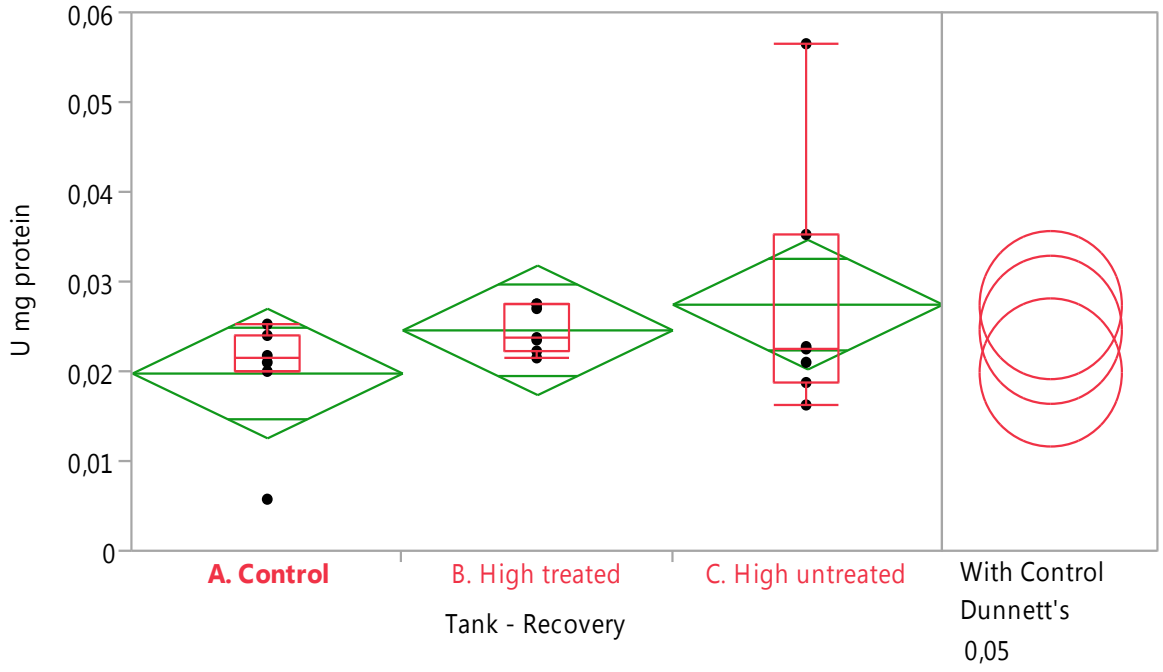

Figure 20. Hepatic GST activity in the control and high dose tanks in fish sampled after the one week recovery period.

Mean, standard deviations, 10, 50 and 90 percentiles, min and max values displayed. Points not included considered outliers.

The highest GST activity was found in the 3 and 7 day control tanks (0.034 ± 0.007 and 0.042 ± 0.025 U mg protein respectively, figures 17 and 18). Disregarding the GST control tanks, GST activity was found to be the highest in the high dose treated tank after 3 days of exposure at 0.032 ± 0.012 U mg protein (figure 17). This activity reduced after 7 days and picked up again after 14 days (figure 18 and 19). The activity in the high dose untreated tank was at its highest after 7 days of exposure reaching 0.030 ± 0.004 U mg protein, reducing at 14 days and picking up in the recovery period (figures 18, 19 and 20).

4.5 CATALASE (CAT)

CAT activity was alike GST determined photometrically from 7 fish per tank per sampling from 3, 7 and 14 day exposures, and from the control and high dose tanks post-recovery.

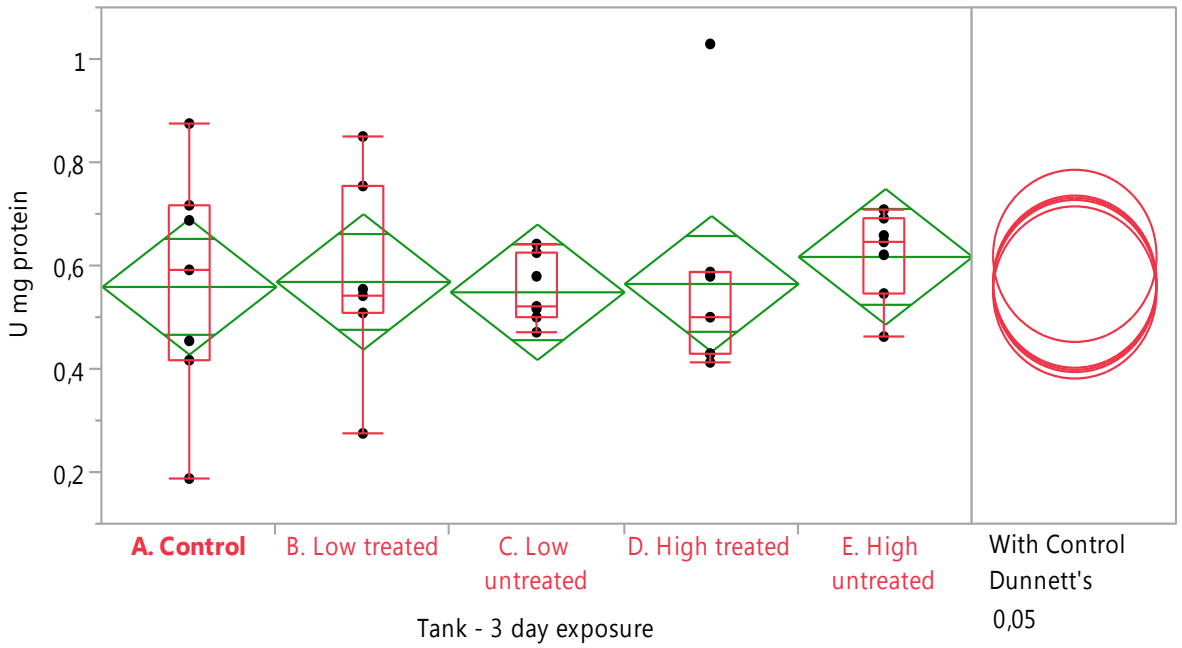

Figure 21. Catalase activity in fish sampled after 3 days of drilling waste exposure.

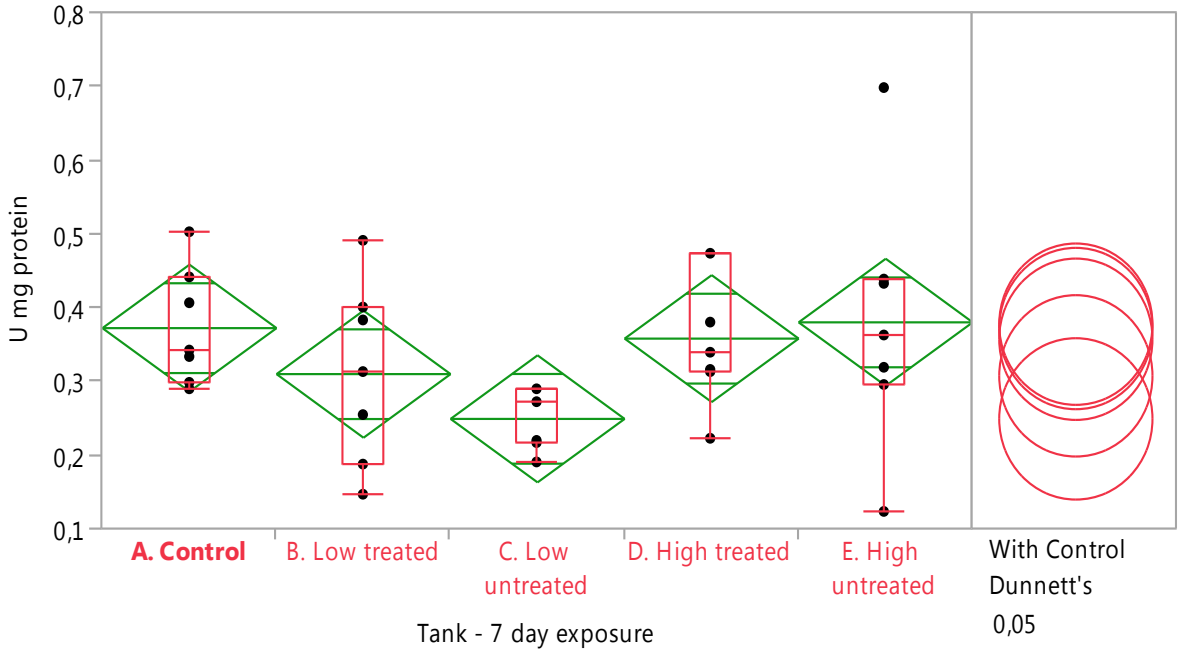

Figure 22. Catalase activity in fish sampled after 7 days of drilling waste exposure.

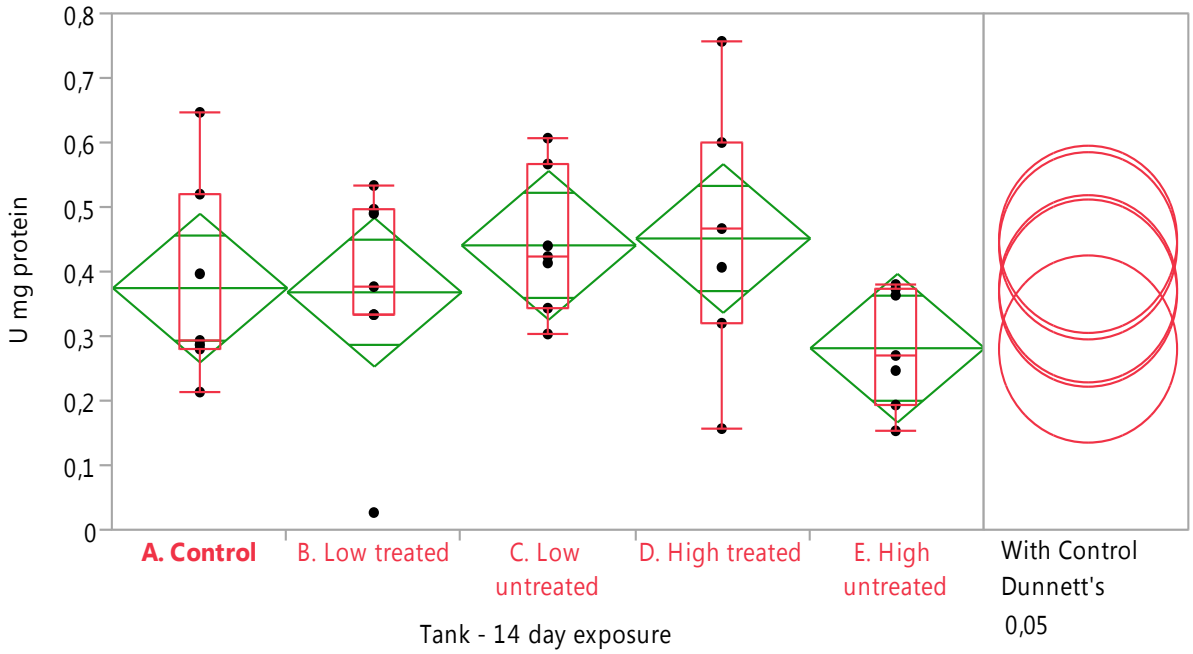

Figure 23. Catalase activity in fish sampled after 14 days of drilling waste exposure.

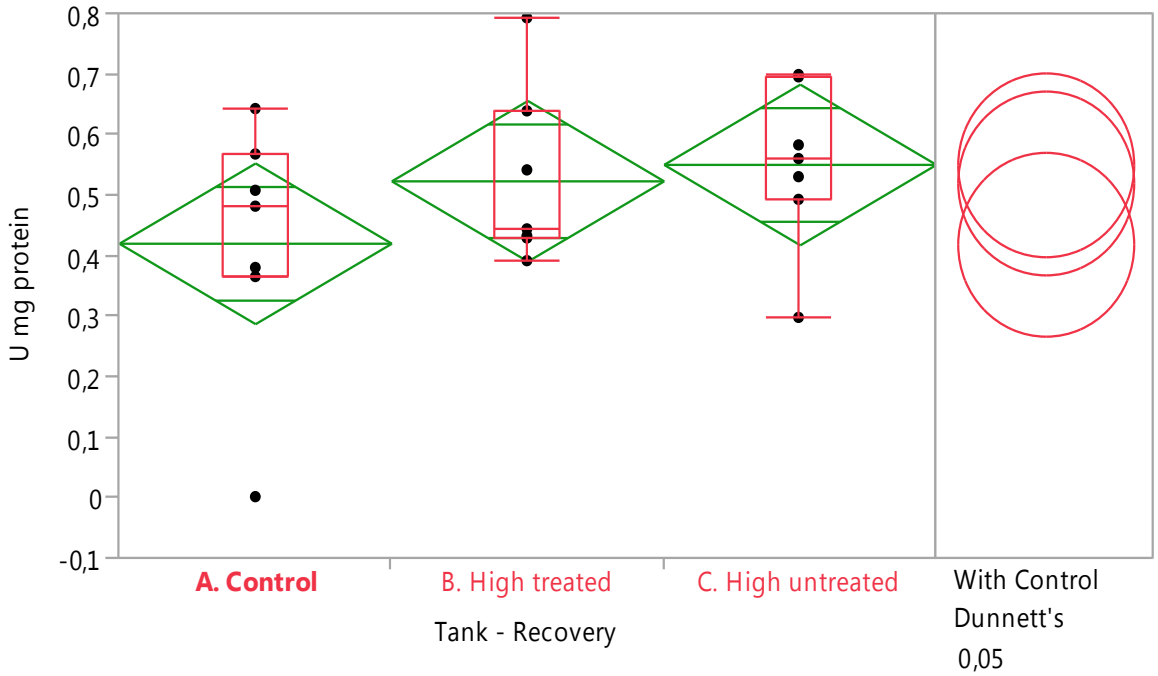

Figure 24. Catalase activity in fish sampled after a one week recovery period.

Mean, standard deviations, 10, 50 and 90 percentiles, min and max values displayed. Points not included considered outliers.

CAT activity was found to be the highest in the beginning of the exposure in all tanks, including control. The highest CAT activity was found in the 3 day exposure high dose untreated tank with 0.619 ± 0.087 U mg protein (figure 21). The activity in this tank sank during the 7 and 14 days, accumulating again in the recovery period (figures 22, 23 and 24). The CAT activity in the high dose treated tank was at its highest 3 days into the exposure (0.567 ± 0.216 U mg protein), sinking after 7 days and increasing again after 14 days and further after the recovery period (figures 21, 22, 23 and 24). The low dose tanks both reached their highest CAT activity in the 3 day exposure (treated: 0.570 ± 0.186 U mg protein, untreated: 0.550 ± 0.066 U mg protein), decreasing after 7 days and increasing again after 14 days (figures 21, 22 and 23). CAT activity measurements were not performed on the low dose tanks.

5. DISCUSSION

The primary objective of this thesis was to evaluate if detoxification and oxidative stress enzymes EROD, GST and CAT can reflect the effect treated and untreated drilling waste has on Atlantic salmon. The results displayed in chapter 4 show that biotransformations in the fish did occur, however, correlating these to the drilling waste exposure proves challenging. This makes their use in discharge monitoring debatable.

5.1 MORPHOLOGICAL PARAMETERS

The CF is indicative of health by reflecting feeding conditions, energy consumption and metabolism of the fish. The fish used in this study scored between good and extremely poor on the Fulton index. It must be noted that the Fulton index comes with limitations such as assuming linear growth and not accounting for the age of the fish (Barnham and Baxter, 2003). Therefore, it can be argued that the fish used in this study, due to their young age, do not fit the Fulton index. Disregarding this, the results from the CF and calculations show that there was no significant change in the morphology of the fish exposed to drilling waste in comparison to the fish in the control tank. The sizes and weights of the fish stayed within a similar ratio throughout the whole exposure period. This was expected due to the short time span of the experiment and general growth rates of Atlantic salmon. No significant effects in CF are common in short term studies in salmon. For significant changes in CF to occur, the exposure time span generally has to be over several months (Hoque et al., 1998).

The LSI links the relative liver size and the hepatic enzyme activity occurring during detoxification, indicating the biotransformation of xenobiotics (Ensibi et al., 2013). Also the LSI stayed stable all through the exposure. This is likely to be for the same reasons as for CF, the short time period of the exposure and Atlantic salmon being a slow growing fish. It is documented that LSI can either increase or decrease when the organism is exposed to pollutant chemicals. As the fish studied were in their juvenile phase, findings of hyperplasia such by Poels et al. (1980) could have been expected if the study was of a longer duration. Alternatively, liver size could have decreased due to low energy reserves from the lack of food, and exposure to Zn and Cd. LSI is generally considered more effective in experiments taking place somewhere subjected to annual and seasonal changes (Hoque et al., 1998).

5.2 ETHOXYRESORUFIN-O-DEETHYLASE (EROD)

Results obtained from the EROD assay provided both expected results, with an increase in EROD activity early in the exposure, and unexpected results with an increase in activity at the end stage of the exposure. EROD is considered an early stage biomarker, being at the lower end of the biomarker hierarchy. Expectations were therefore that the highest EROD activity would be found in the 3 day exposure samples, with lower yet increased response in 7 day samples. This was the case in the high dose untreated tank, which peaked after 3 days, dipping at 7. The activity however picked up again increasing considerably between 7 and 14 days into the exposure, and further during the recovery period. The high dose treated tank showed low activity until it markedly peaked after 14 days of exposure. At 14 days, increases in EROD activity showed in all tanks except the low dose treated tank, which exhibited no activity. This late increase in EROD activity proposes that there was a delayed biological metabolisation. If this proposal was to be correct, it could be suggested that the rise in EROD activity experienced after only 3 days of exposure was rather due to other factors than PAH and heavy metal exposure. This proposal is supported by how the low dose treated tank experienced highly increased EROD activity in this period. Both low dose tanks showed higher EROD activity than the high dose treated tank. One factor that could have exerted the increase in EROD activity 3 days into exposure is the sudden introduction of particles and/or some of the associated contaminants to the tanks when the exposure commenced (Sanni, *pers. comm*., 2014). Still, the EROD activity in the control tanks varied noticeably throughout the exposure. The reason for this is unknown.

The two EROD peaks were studied in detail, with an individual Dunnett's test performed comparing the peak to the control values as both non-log and log transformed plots (figures 25 and 26).

3 day exposure:

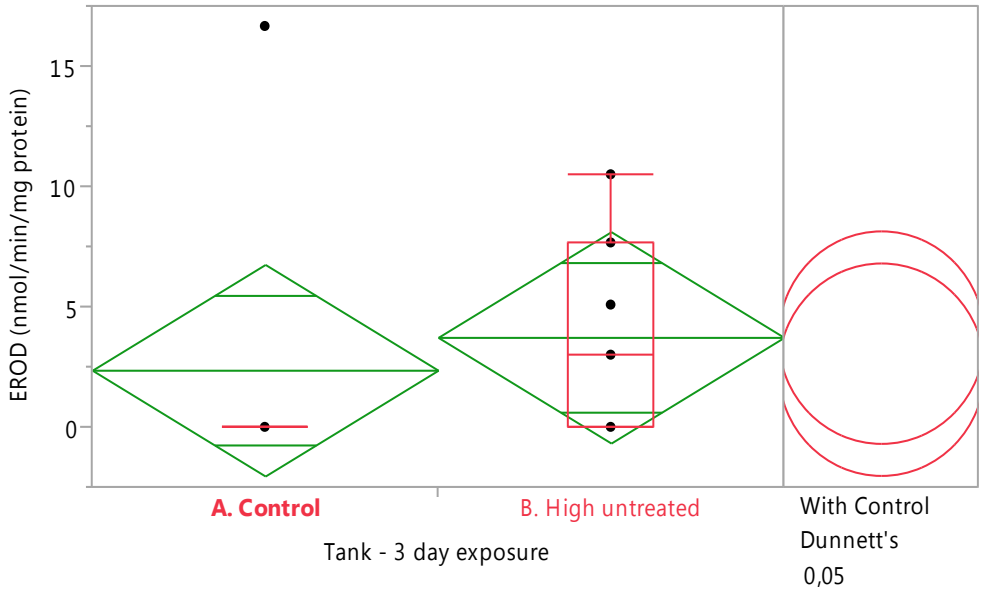

Figure 25a. Non-log transformed comparison of control tank and high untreated peak.

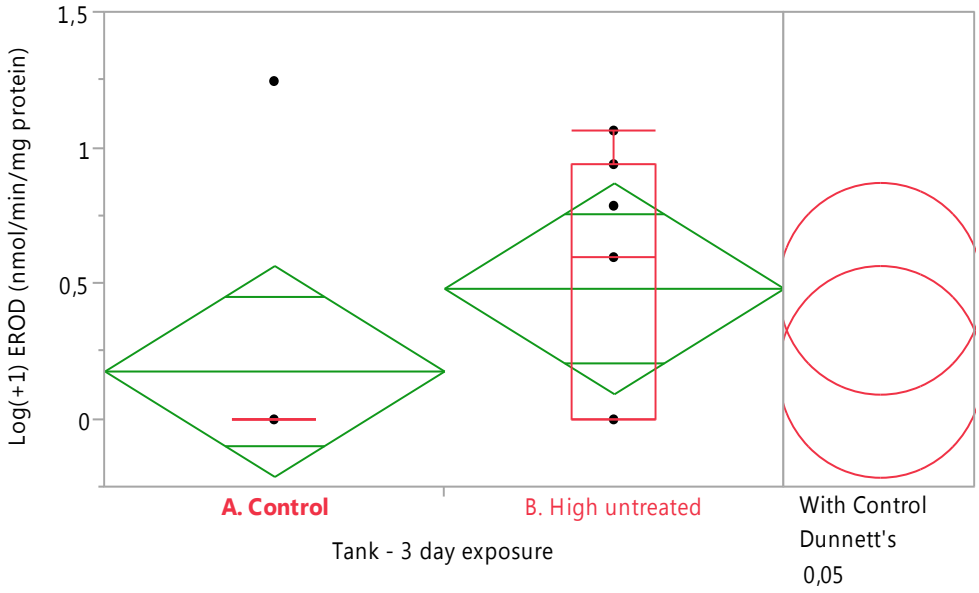

Figure 25b. Log transformed comparison of control tank and high untreated peak.

14 day exposure:

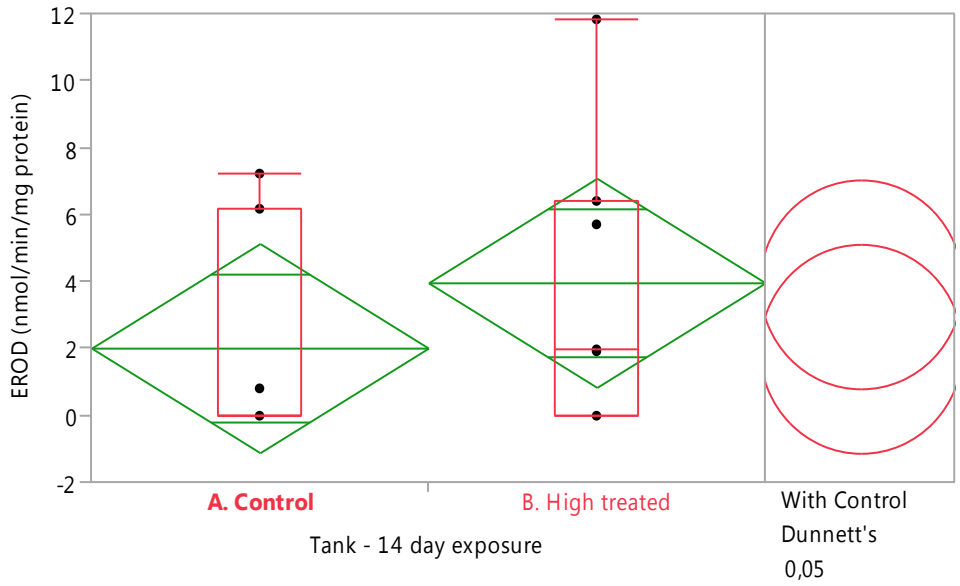

Figure 26a. Non-log transformed comparison of control tank and high treated peak.

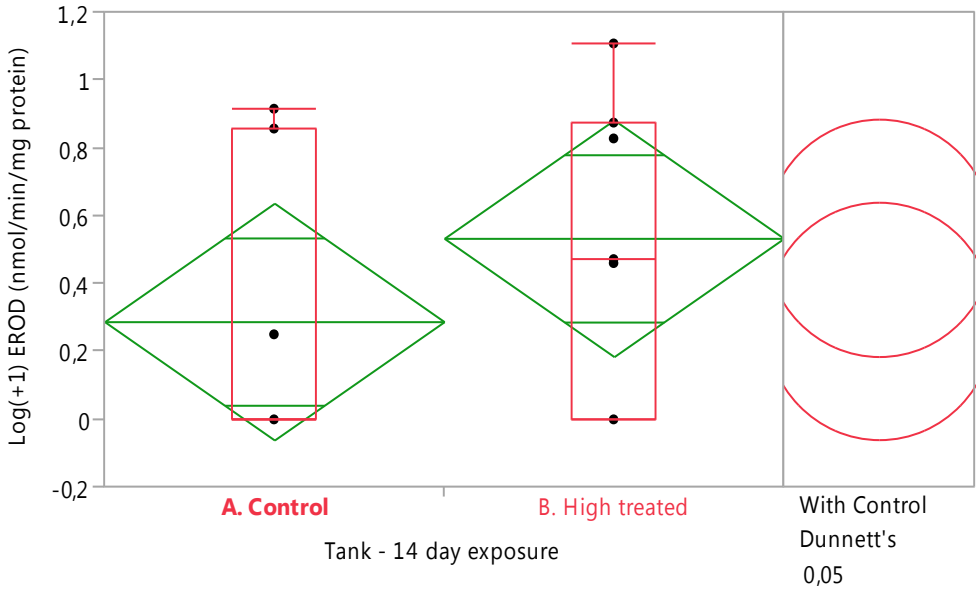

Figure 26b. Log transformed comparison of control tank and high treated peak.

Mean, standard deviations, 10, 50 and 90 percentiles, min and max values displayed. Points not included considered outliers.

The visual inspections in figures 25 and 26 showed that the non-log transformed data was more normally distributed in both cases. The log transformed plots show skewed results. In all

cases the Dunnett's test showed that the experimental mean is not statistically different from the control mean at a 5% level.

A study on Atlantic salmon smolt in seawater exposed to dispersed oil in the range of 0 to 750 ppb over a time period of 4 weeks was conducted at IRIS Environment in 2009 (Sanni *pers. comm*., 2014). Relating the numerical results found in this thesis with the findings of the 2009 study, the EROD values obtained vary greatly, meaning the values are not comparable. Yet, the two studies share patterns of relative EROD activity, such as the fluctuations. In the 2009 study, EROD activity increased slightly between control and 15 ppb PAHs, and significantly with 60 ppb PAHs. At 120 ppb the EROD activity however sank to below the 15 ppb level, to then rise at 250 ppb and further 750 ppb. The study also found relatively high values of EROD activity occurring in the control tanks throughout the exposure period. It was concluded that the trigger of EROD activity in the control tanks was the presence of an unknown variable.

Hepatic EROD activity is one of the pre-stages for bile production. Bile is produced in the liver during metabolism, and through the common hepatic duct, transported into the gall bladder for storage. Bile can contain xenobiotic metabolites following ingestion. The EROD results found were compared with a study by Goonewardene (2014) on PAH metabolite types Nph, Pyr and BaP, run parallel with this study, using bile from the same salmon parr as hepatic samples utilised in this study. Looking at the PAH metabolites from the study, there is an increase in Nph and Pyr metabolites from 3 to 7 days into the exposure (figure 27). This correlates well with the peaking EROD activity in the 3 day high dose untreated exposure tank. The metabolisation of the PAHs from the liver to gall bladder has taken place during the four days between the 3 and 7 day samplings. At 7 days of exposure, the EROD activity in the high untreated tank is low, as the metabolites are now in the bile. This transfer of PAH metabolites from liver to bile discredits the proposal about the increased EROD activity in the 3 day exposure tanks being from particles, suggesting it is in fact from a PAH uptake, at least for the high untreated tank.

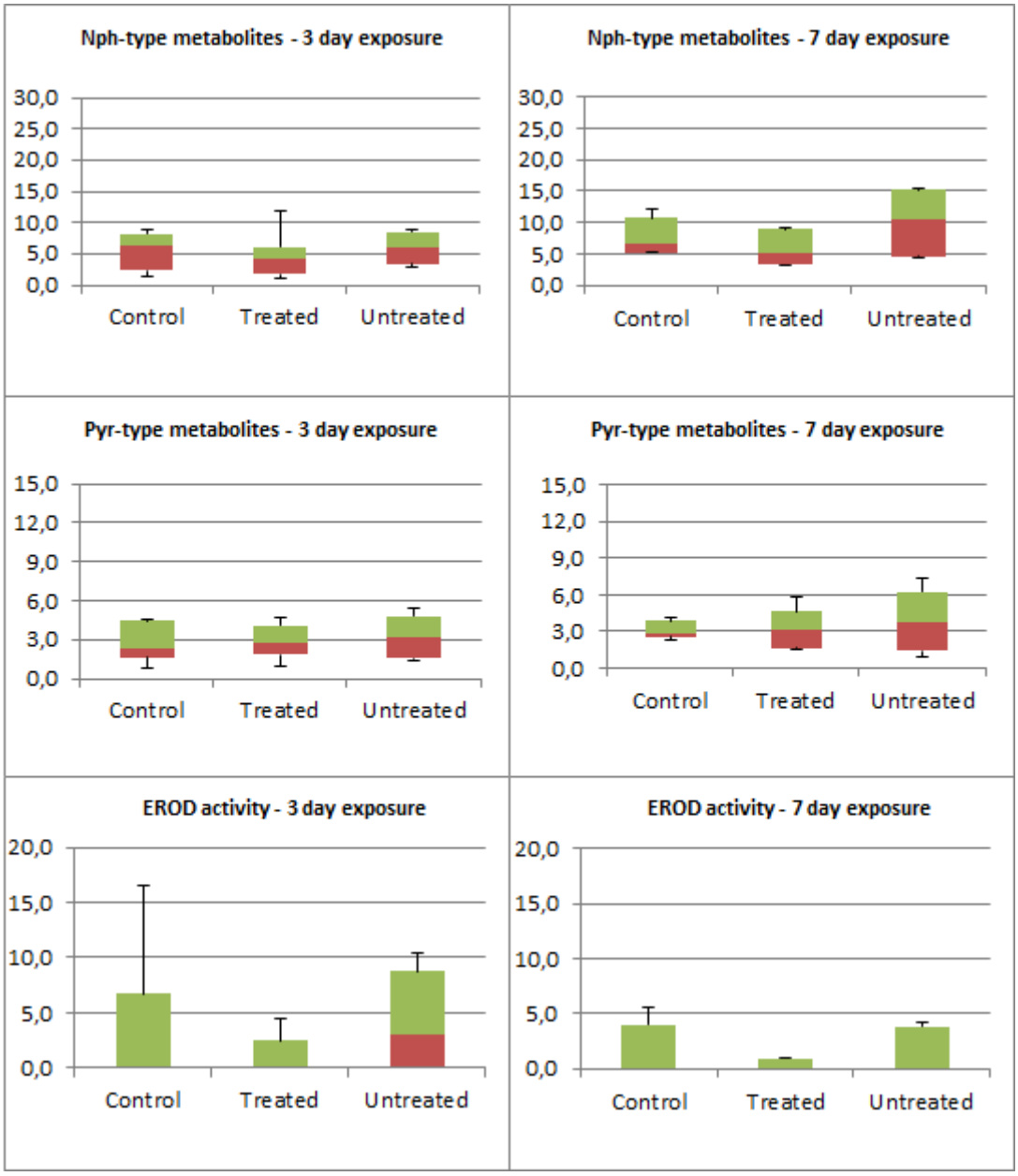

Figure 27. Above and middle: PAH metabolite concentrations (µg/mL) in fish bile after 3 and 7 days of drilling waste exposure (Sanni, *pers. comm*., 2014). Below: Hepatic EROD activity (nmol/min/mg protein) after 3 days of drilling waste exposure. 10, 50 and 90 percentiles, min and max values displayed.

The study by Goonewardene (2014) also supports the theory of a delayed biological metabolisation. As shown in figure 28 below, EROD activity and PAH metabolites both increased from 7 to 14 days and kept increasing in the high untreated tank after the one week purification period.

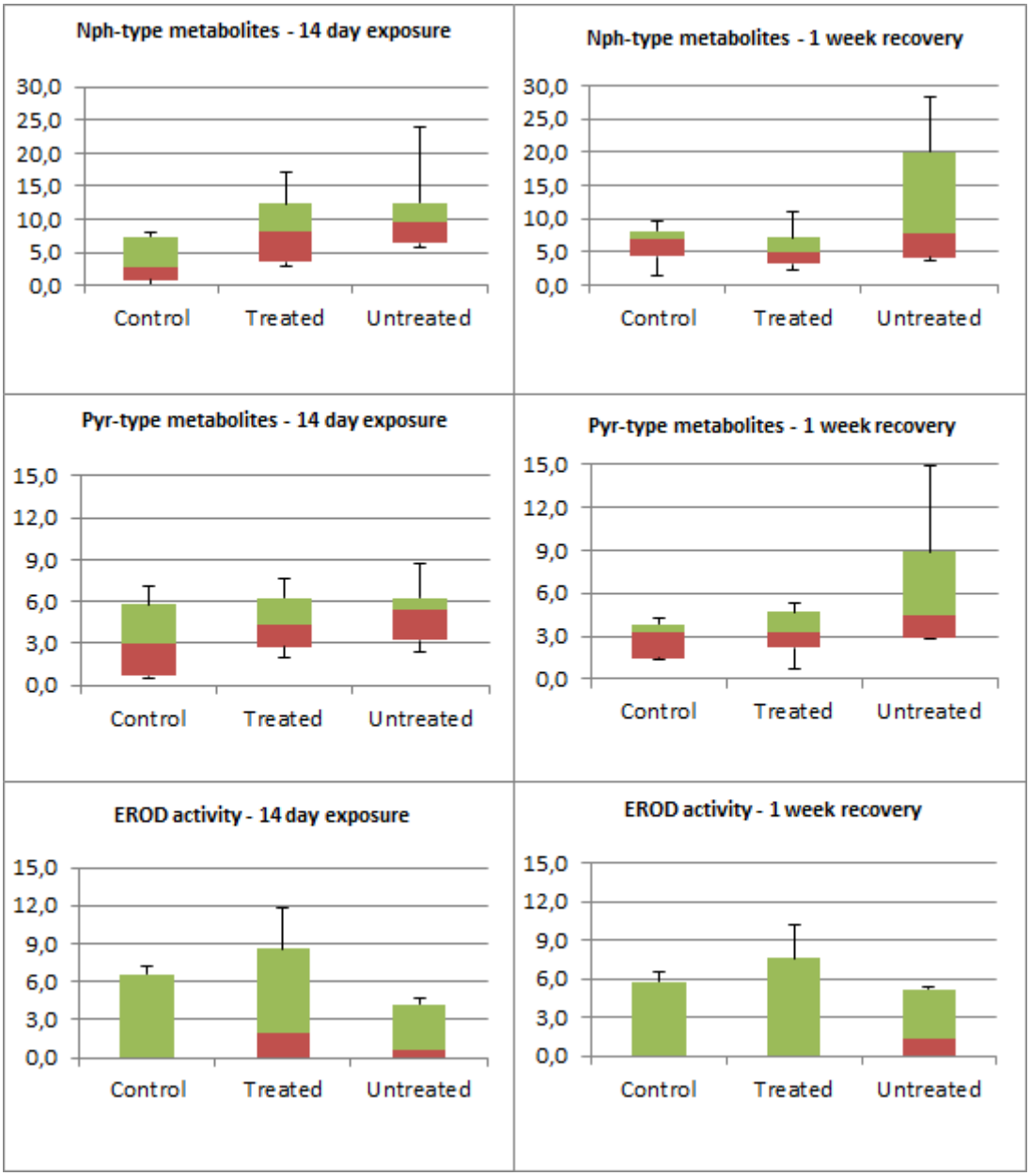

Figure 28. Above and middle: PAH metabolite concentrations (µg/mL) in fish bile after 14 days of drilling waste exposure and after one week of recovery (Sanni, *pers. comm*., 2014). Below: Hepatic EROD activity (nmol/min/mg protein) after 14 days of drilling waste exposure and one week of recovery. 10, 50 and 90 percentiles, min and max values displayed.

The EROD activity in the high dose treated tank peaked after 14 days of exposure and started decreasing during the one week recovery (figure 28). The PAH metabolites in this tank follow a similar pattern in the 14 days of exposure. The EROD corresponding PAH metabolite increase suggest that untreated drilling waste responds earlier than treated drilling waste. This is however difficult to prove as it is unknown whether the increase in the 3 day high untreated

tank was solely due to PAHs or a combination of particle release and/or heavy metal exposure.

When comparing this with the EROD – PAH metabolite dynamic observed between the 3 and 7 day exposures, it could be suggested that there is a more constantly heightened EROD activity later in the exposure, hence both EROD and PAH metabolites are high on 14 days and post-recovery. This is especially likely if the first EROD increases were in fact particle induced. More constant EROD activity could propose that the fish have developed a certain level of adaptation to the pollutants (Sanni, *pers. comm*., 2014).

A lot of the samples measured showed no EROD activity, giving median values of zero. There are many reasons for zero readings when using EROD as a biomarker. Firstly, EROD activity is vulnerable to several correlating factors such as species variation, season, gender, sexual maturation, temperature changes and inhibition. Atlantic salmon has shown to have a lower EROD response than a variety of other fish such as cod and flounder (Sanni, *pers. comm*., 2014). As the fish were kept in a laboratory environment, seasonal changes can be considered irrelevant. To avoid issues with gender and sexual maturation, juvenile fish with undeveloped gonads were used. The water in the tanks was kept at a steady temperature of 6.6 ± 0.5ºC. Inhibition of EROD activity can be considered a possibility. Han et al. (2013) found in their study of PAHs and heavy metals on EROD activity in *Mossambica tilapia* that Cd, Cu and Hg inhibited the EROD activity when exposed alone or co-exposed with indenol[1,2,3-cd]pyrene (IP) and fluoranthene (FL), pollutants all present in the drilling waste used in this experiment. The Intertek West Lab results showed that the treated drilling waste contained 0.021 mg/kg FL and 0.022 mg/kg IP, and the untreated 0.26 mg/kg FL and 0.037 mg/mg IP. The amounts of Cd, Cu and Hg were higher in the treated cuttings with 0.35, 78 and 0.049 mg/kg respectively. The untreated waste contained 0.22 mg/kg Cd, 74 mg/kg Cu and 0.37 mg/kg Hg. Of course, pollutant influences are complex with regards to inhibition and induction mechanisms and every fish has an individual response time.

Due to the complexity of EROD activity induction, EROD measurements are often completed on more than just one organ. Aside from the liver, EROD activity can be successfully detected in the kidneys and gills of fish. A study by Andersson (2007) evaluating biomarker responses in fish compared EROD activity in gills, liver and kidneys in rainbow trout (*Oncorhynchus mykiss*), finding the readings from the gills being constantly higher, therefore suggesting it is more sensitive as a biomarker than the liver. Similar findings were reported in the study by

Abrahamson (2007), who studied gill EROD activity in rainbow trout as a biomarker for waterborne Ah-receptor agonists. Abrahamson (2007) suggests it is due to absorption and metabolism in the liver taking time, meaning it takes the liver longer to reach the same concentrations obtained in the gills. Nahrgang, Jonsson and Camus (2010) found the opposite when studying EROD activity in liver and gills of polar cod (*Boreogadus saida*) exposed to waterborne and dietary crude oil. The hepatic EROD results were higher than the gill EROD from both waterborne and dietary crude oil. All three studies compared were of similar time frames as this study (21 days). A significant difference with the Andersson (2007) and Abrahamson (2007) studies compared to Nahrgang, Jonsson and Camus (2010) was the fish species examined; rainbow trout and polar cod. Atlantic salmon and rainbow trout are both salmonid species, suggesting the two have similar physiologies. This could to a degree explain the delayed biological responses seen in this study. Absorption and metabolism of xenobiotics in the salmonid liver are likely to differ from the cod liver, and could imply rates are slower in salmonids than in cod. This could also suggest that EROD would have been visible sooner in the gills than in the liver.

Due to the potentially affecting co-variables present with EROD responses, EROD analysis alone is not considered a decisive biomarker. EROD is a biomarker for exposure. For biomonitoring purposes, a variety of biomarkers are suggested to be used (Cajaraville et al., 2000).

5.3 GLUTATHIONE S-TRANSFERASE (GST)

The GST activity was based on the results from the EROD activity, and focused only on the high dose tanks and the control. The activity varied in both the high dose treated and untreated tanks. In the untreated tank the activity was at its highest 7 days into exposure, decreased at 14 days, rising after the one week recovery. For the treated tank the GST activity was at its highest 3 days into the exposure, decreasing at 7 days, increasing at 14 days and decreasing again after a week of recovery. Abnormally high GST activity was registered in the 7 day control tank (figure 18). The reason for this is unknown. Excluding the 7 day control tank, the 3, 14 and recovery control tanks registered an evenly varying GST activity which is normal. The earlier mentioned 2009 study also compared Lowest Observable Effect Concentrations (LOEC) for biomarker response in four types of fish exposed to 0.1 µg naphthalene, phenanthrene and dibenzothiophene (NPD) per litre, of which Atlantic salmon was one. The

salmon GST values were predicted to be at 0.08 U mg protein for a LOEC. Correlating this to the results found in this thesis it could be suggested that the GST activity was not affected by the exposure drilling waste, which contained a higher amount of PAHs (treated: 0.32 µg/NPD/L, untreated: 1.0 µg/NPD/L) than what was used in the 2009 exposure. This would mean the observed activity is merely the natural metabolism of the fish.

Perez-Lopez et al. (2002) found alike this study, increases in GST activity even in the control tanks. In their study, the control tank fish were however exposed to corn oil (through injection), which they concluded as the reason for the heightened GST activity. GST activity increases from oils as they contain a high proportion of polyunsaturated fatty acids, which again activate the peroxisomal fatty acid oxidation system leading to oxidative stress. Fish feed pellets contain high amounts of oil, which in theory could affect GST activity. The relevance of the fish food in this study is debatable, as feeding was stopped when the exposure commenced.

GST is part of the antioxidant system, with the role of protecting tissues from oxidative stress through GST mediated conjugation. Xenobiotics can both increase and inhibit this response; this makes GST debatable as a biomarker of exposure (Ensibi et al., 2013). Olawale and Onwurah (2007) discuss in their article that GST often displays no activity at high levels of contaminants due to the shock effect on the cell metabolism when there is a sudden high dose of pollutants. Studies have been done as an attempt to validate the usefulness of GST induction as a biomarker of exposure. One of these is by Martinez-Lara et al. (1996) on individual GST isoenzymes. The findings by Martinez-Lara et al. (1996) showed that GST isoenzyme pattern is characteristic for each xenobiotic, suggesting monitoring total GST activity is not enough. Petrivalksy et al. (1997) ran a study on rainbow trout, measuring GST activity with CDNB as a substrate. They determined very weak increases of cytosolic GST enzymes after exposing the fish to a number of xenobiotics (cocktail effect). Due to the low increase, their study concluded that GST is not a suitable indicator of xenobiotic contamination in fish.

An induction of one form GST isoenzyme may be masked by the inhibition of another, something that is not visible when total cytosolic GST activity is measured. More research on the induction pattern of piscine induction dynamics is required. So far it is only HPLC profiling, such as completed by Martinez-Lara et al. (1996) that is reliable when using GST activity to determine xenobiotic contamination.

5.4 CATALASE (CAT)

Cytotoxic reactive oxygen species (ROS) were expected in the exposure tanks. A CAT assay was chosen to study the antioxidant defence system response. The results obtained from the CAT analysis showed highest CAT activity in all tanks 3 days into the exposure. The results are questionable though due to the high CAT activity found in the control tank. As the control tank, low dose tanks and the high dose treated tank all follow the same pattern, no definite link between CAT activity and biotransformation owing to drilling waste exposure can be made between the four tanks. The high untreated tank however follows a different pattern, going from high to low as the exposure goes on. This could suggest a heightened activity at the start of the exposure, with the fish antioxidant system trying to combat the sudden oxidative stress from the drilling wastes. As the activity lowers and the exposure goes on, either the CAT defence system is giving up, or the fish develop a certain degree of adaptation to the oxidative stress they are constantly under (Sanni, *pers. comm.*, 2014).

Similar results were obtained by Ensibi et al. (2013), where CAT activity increased at 4 days after exposure (with concentrations 0.4 and 0.8 µg/L deltamethrin), decreased and then increased again at 15 days (0.4 µg/L).

CAT studies are often vague due to the complexity of the correlating factors. Even in a pollutant-free aquatic environment, dissolved oxygen and temperature affects oxidative stress (van der Oost et al., 2003). This alone creates a challenge for interpreting CAT results. Adding a laboratory environment to the factor, further challenges occur. In addition, the test fish being andramonous presents an own possible concern. A study by Kolayli and Keha (1999) found significant differences in the antioxidant systems in rainbow trout, depending on if they are adapted to seawater or freshwater. While they obtained significant CAT readings in both freshwater and seawater rainbow trout, they concluded that their results implicate that the antioxidant capacities in andramonous fish adapt to the physical and chemical characteristics of the environment, and cannot be compared. CAT activity can also increase from heavy metal exposure (Atli et al., 2006). In their study CAT activity increased by 25% from exposure to Cr at concentrations of 1.5 mg/L, which is lower than the amount of Cr found in both the treated and untreated drilling waste used in this study.

5.5 ENZYME BIOMARKER EVALUATION

Comparing the findings from EROD, GST and CAT, all values found were quite low. The response was low even though the PAH metabolites in fish bile increased more than the chemical concentration should indicate. All three biomarkers share the pattern of increased activity after the one week recovery period in the high dose untreated tank, again supporting the delayed biological metabolisation of drilling waste pollutants. Another correlation is the activity in the control tanks. In all three biomarkers, the control tank values varied in a similar form as in the exposure tanks, making it difficult to interpret what increases and decreases have occurred due to drilling waste in the exposure tanks.

In all three biomarker assays, abnormalities occurred. Values that were significantly higher or lower than what was seen in the rest of the group. Graphically these were plotted as outliers. The two most extreme outliers were found in the EROD 3 day control tank and GST 7 day control tank. Three mild outliers found in the 3 day high dose treated GST and CAT tanks, and in the high dose untreated tank at 7 days were considered of interest and individual samples studied. The three outliers turned out to stem from two individual samples that both had unusually low protein contents when compared to the other examined samples (3 day high treated protein average: 20.5 mg/mL, median: 21.5 mg/mL, outlier: 11.6 mg/mL; 7 day high untreated protein average: 21.3 mg/mL, median: 22.6 mg/mL, outlier: 13.4 mg/mL). A closer look was taken at this, finding an additional sample with low protein content in the high dose treated tank post-recovery with 11.8 mg/mL. What all three low protein samples had in common is unusually high CAT activity: 1.03, 0.79 and 0.70 U mg protein, following the order of lowest to highest protein content. Subsequently, individual samples with unusually high protein contents (> 39 mg/mL) were examined. Six samples were found: one from the low dose treated tank at 7 days (protein average: 27.0 mg/mL, median 22.2 mg/mL, sample 39.2 mg/mL) and four from the 14 day sampling, with two from the control tank (protein average: 30.7 mg/mL, median: 28.5 mg/mL samples: 40.3 and 42.4 mg/mL), two from the high dose treated tank (protein average: 32.1 mg/mL median, 25.9 mg/mL samples: 39.0 and 64.8 mg/mL) and one from the high dose untreated tank (protein average: 30.1 mg/mL, median: 33.1 mg/mL, sample: 39.1 mg/mL). These were compared with the GST and CAT activity which was below average for the tanks. When the GST and CAT values were calculated without protein normalisation, the values obtained were more similar to those of the test group. A study by Jesuthasan (2014) on erythrocytic nuclear aberrations run parallel with this study on the same fish found micronuclei in three of the samples (low dose treated

and controls), nuclear buds in five samples (excluding one control) and binucleations in three samples (low dose treated, control and high dose treated). The findings suggest there is a correlation between high liver protein content and low detoxifying enzyme response. Whether the outcomes of the Jesuthasan (2014) study are related to drilling waste exposure or sporadic abnormalities is unknown, but could advise that erythrocytic nuclear abberations prevent the hepatic detoxification system from functioning correctly.

Elevated protein levels could be a sign of increased mRNA synthesis. CYP1A protein levels increase from organic environmental pollutant exposure through the path 2 route of xenobiotic fate. PAHs are one of the pollutants which have in numerous studies shown very strong increases; Goksøyr (1991), Goksøyr and Larsen, (1991) and Stagg et al. (2000) all performed studies with PAHs at pollutants, finding elevated CYP1A protein levels. These were sometimes increased > 500%.

5.6 TREATED VERSUS UNTREATED DRILLING WASTE

It is yet unclear whether treated or untreated drilling waste causes the most harm in an experiment like this. A toxicity study run by Randrianarimanana (2014) on zooplankton with the same drilling wastes as used for this study, found TCC treated drilling waste to be significantly more acutely toxic by EC50 values than untreated drilling waste. This was believed to be due to the bioavailability of the heavy metals being increased in the treated waste. The exact reason for this is unsure, yet the treated waste had higher amounts of heavy metals per mg/kg of dry matter than the untreated waste. The study performed by Randrianarimanana (2014) was an acute batch test, while this fish study was a CFS test. This means the same metal bioavailability cannot be assumed, as they will not accumulate in the water in a CFS in the same way as in a batch test. In this study on Atlantic salmon, there was not a clear distinction of the effects of the drilling waste based on their treatment methods, and there was little correlation between the enzyme biomarker responses and the nominal oil and PAH concentrations. It was clear from the EROD activity peaks and PAH metabolites in the high dose tanks that PAH uptake was high regardless of the waste being treated or not. Water chemistry analysis performed after 14 days of exposure by Intertek West Lab, found the high dose treated and untreated tanks had 0.32 µg NPD/L and 1.0 µg NPD/L respectively. The increased EROD activity and PAH metabolites in both high dose tanks would suggest

these NPD concentrations were way beyond the LOEC. This questions the effectivity of TCC in removing PAHs.

5.7 ENZYME BIOMARKERS IN ENVIRONMENTAL RISK ASSESSMENT

The practicality of enzyme biomarkers for discharge monitoring as part of an ERA can be debated due to numbers of occurring complications associated with them, particularly in field settings. Jimenez et al. (1990) performed a field study on EROD response in fish. While the variations between season and gender occurred they also found that the fish from the most polluted sites did not show the highest EROD responses. This was understood to be due to hepatotoxic liver damage. The hepatotoxic damage would in this case be yet another limitation of the usefulness of EROD measurements in the field. There is also the possibility of an overload on the CYP1A system, causing EROD responses to be inhibited, such as seen in the studies by Sturve et al. (2005) and Jonsson et al. (2010). Forbes et al. (2005) argue that biomarkers are generally only useful for hypothesis generation in carefully controlled experiments. Enzyme biomarkers in the field as part of a discharge monitoring programme come with high risks of false positives and negatives causing environmental and financial damage, and raising the question of what level of organism changes should be reached before action is taken. Also, the mechanism of induction and inhibition of enzyme activity posed a problem with masking effects as pollutants usually come in cocktails, rather than single-chemical exposures. Some pollutants induce enzyme biomarker activity, while others inhibit it.

Enzyme biomarkers in ERA can be relevant in the ecological monitoring step of the ERA proceedings (van der Oost et al., 2003).

EROD activity and CYP1A protein levels are used in ERA sections determining dose-response relationships and ecological monitoring. As long as experimental design take into consideration factors that influence the activity and protein levels, and pollutants that inhibit the activity, useful information on e.g. toxic mechanisms of xenobiotics, toxicity screening, exposure identification and impact, early warning effects and health of ecosystem may be obtained (van der Oost et al., 2003). Key examples of ERA related projects incorporating phase I enzyme biomarkers are the Norwegian Environmental Directorate's water column monitoring programme, studying whether fish along the Norwegian coast are affected by

produced water and other pollution from the petroleum sector (Hylland et al., 2008); and the Joint Assessment and Monitoring Program of the Oslo and Paris Commission (OSPAR), monitoring EROD activity in fish in large Norwegian lakes (NIVA, 2003).

The use of GST in ERA related ecological monitoring of drilling discharges is more questionable, due to its limited sensitivity to the associated pollutants exposure. It is incorporated in the water column monitoring, but has shown to have limited success (Hylland et al., 2008). However, being such an important part of the detoxification system of an organism it could prove useful in studying toxic mechanisms of xenobiotics. It is clear that further studies are required to improve the usefulness of GST with regards to ERA purposes. Studies such as the fore mentioned Martinez-Lara (1996) study on GST isoenzymes articulates that there is potential, and that GST isoforms rather than total GST activity, are probably better as indicators of exposure or effects.

Using CAT in steps of an ERA is in general not a viable option, and not for monitoring of drilling discharges either. Further research on CAT mechanism is required. CAT is commonly less responsive than both phase I and II detoxification enzymes, and the correspondence between contaminants and CAT response is yet not well enough established (van der Oost et al., 2003).

Comparing the pollutant dosage in this study with the actual practice of drilling waste disposal in an aquatic environment is a challenge. It is impossible to know the lake or sea dilution factor, and therefore how long the polluting substances are found around the disposal site or when they have drifted away. There is reason to believe that the concentrations used in this experiment would be high environmental concentrations (Sanni, *pers. comm.*, 2014). With the results obtained in this study, it could be anticipated that a combination of EROD, GST and CAT activity would not be sensitive or clear enough for monitoring discharges to the level of contamination.

6. CONCLUSION

It can be concluded that the detoxification enzyme parameters did not appear reflective of the effect the drilling waste had on the fish. Too many unknown caused responses in the low dose and control tanks masked the possible responses seen in the high dose tanks. It appeared as if the treated drilling waste gave the same enzymatic biomarker response effect as the untreated waste when the fish were exposed to it at high enough doses. This response appeared later than with the untreated waste. Dose is critical when disposing of drilling waste, treated or untreated. EROD, GST and CAT alone for biomonitoring would not be sensitive enough for monitoring discharges to the level of contamination used in this study. It is questionable if they would contribute positively in a suite of biomarkers for biomontoring drilling waste discharges.

7. FURTHER RECOMMENDATIONS

Suggested future studies would be a marine version of this study, with the drilling waste exposure completed on seawater fish and/or invertebrates. A marine biomarker study could prove useful for the offshore oil and gas industry, as reservoirs requiring the use of OBM are generally found in deep sea conditions (Mason and Gleason, 2003). Another study of interest for the offshore industry would be comparing long term low dose (chronic) exposure with short term high dose exposure. This type of study would investigate how long a chronic exposure can take place before environmental damage is done. This would be of relevance, because it generally takes longer for a low dose chronic exposure to occur in the field. In addition, it could be interesting to see if EROD readings on gills would improve accuracy.

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APPENDIX

A. Exposure calculations

B. Sampling data, condition factor and liver somatic index

C. Bradford protein assay results

D. EROD results

E. GST results

F. CAT results

Appendix A – Exposure calculations

Oil calculations

Flow dimentioning drill cuttings experiment:			"muddy water" = mud suspended in water in header tank				
Calculation of flows based on untreated mud:							
dry weight (solids) % of mud	wt %	66	%	Weight of added wet mud in header tank:		0,200	kg wet mud
water content % in mud	wt %	34	%	Dry Weight (=solids) of added wet mud in h		0,132	kg dry mud
density of wet mud	d1	1,65	kg/dm3 wet mud	Concentration of wet mud in header tank:		16,7	g wet mud/dm3 (L)
assumed approx. density of muddy water	d2	1,0	kg/dm3 muddy water	Concentration of particles in header tank:		10,98	g particles/dm3 (L)
oil conc. in dry mud	c1 (DW	160 000	mg oil/kg dry mud	oil conc. in wet mud	c1 (WW)	105 600	mg oil/dm3 (L) wet mud
Volum of wet mud in header tank	V1	**0,121**	dm3 (L) wet mud				
oil conc. in muddy water	c2	1 065	mg oil/dm3 (L) muddy water				
Volum of muddy water	V2	12	dm3 (L) muddy water				
Target nominal conc. in high conc. tank:	cH	1,66	mg oil/L (= appr. ppm)				
Target nominal conc. in low conc. tank:	cL	0,55	mg oil/L (= appr. ppm)				
Freshwater flow into tanks:	Fw	**4,0**	L/min.				
Muddy water flow into tank H:	FmwH	**6,23**	mL/min.				
Muddy water flow into tank L:	FmwL	**2,08**	mL/min.				
sum flow from header tank:	Fmw	8,31	mL/min.				
Flow of muddy water out of header tank pr. 24h:	Fmw	**12,0**	L/24h				
Exposure duration:	Tt	14	d				
wet mud usage in total:	Vt	**1,694**	dm3 (L) wet mud				
Calculation of amounts and concentrations of treated mud (assuming equal flow as for untreated mud):							
dry weight % of mud	wt %	85	%	Weight of added wet mud in header tank:		**0,156**	kg wet mud
water content % in mud	wt %	15	%	Dry Weight (=solids) of added wet mud in h		0,132	kg dry mud
density of wet mud	d1	1,27	kg/dm3 wet mud	Concentration of wet mud in header tank:		13,0	g wet mud/dm3 (L)
assumed approx. density of muddy water	d2	1,0	kg/dm3 muddy water	Concentration of particles in header tank:		10,98	mg particles/dm3 (L)
oil conc. in dry mud	c1 (DW	960	mg oil/kg dry mud	oil conc. in wet mud	c1 (WW)	812	mg oil/dm3 (L) wet mud
Volum of wet mud in header tank	V1	**0,123**	dm3 (L) wet mud* ->	*25,5 mL of extra wet mud added to obtain the same particle concentration (as in untreated mud)			
oil conc. in muddy water	c2	6,39	mg oil/dm3 (L) muddy water				
Volum of muddy water	V2	12	dm3 (L) muddy water				
Target nominal conc. in high conc. tank:	cH	0,010	mg oil/L (= appr. ppm)				
Target nominal conc. in low conc. tank:	cL	0,003	mg oil/L (= appr. ppm)				
Freshwater flow into tanks:	Fw	**4,0**	L/min.				
Muddy water flow into tank H:	FmwH	**6,23**	L/min.				
Muddy water flow into tank L:	FmwL	**2,08**	L/min.				
sum flow from header tank:	Fmw	8,31	L/min.				
Flow of muddy water out of header tank pr. 24h:	Fmw	**12,0**	L/24h				
Exposure duration:	Tt	14	d				
wet mud usage in total:	Vt	**1,717**	dm3 (L) wet mud				

PAH calculations

Flow dimentioning drill cuttings experiment:			"muddy water" = mud suspended in water in header tank				
Calculation of flows based on untreated mud:							
dry weight (solids) % of mud	wt %	66	%	Weight of added wet mud in header tank:		0,186	kg wet mud
water content % in mud	wt %	34	%	Dry Weight (=solids) of added wet mud in h		0,123	kg dry mud
density of wet mud	d1	1,65	kg/dm3 wet mud	Concentration of wet mud in header tank:		15,5	g wet mud/dm3 (L)
assumed approx. density of muddy water	d2	1,0	kg/dm3 muddy water	Concentration of particles in header tank:		10,25	g particles/dm3 (L)
TPAH conc. in dry mud	c1 (DW	17	mg TPAH/kg dry mud	TPAH conc. in wet mud	c1 (WW)	11	mg TPAH/dm3 (L) wet mud
Volum of wet mud in header tank	V1	0,113	dm3 (L) wet mud	oil conc. in dry mud	c1 (DW)	160 000	mg oil/kg dry mud
TPAH conc. in muddy water	c2	0,1056	mg TPAH/dm3 (L) mu	Percentage of TPAH in the oil		#####	
Volum of muddy water	V2	12	dm3 (L) muddy water				
Target nominal conc. in high conc. tank:	cH	0,165	µg TPAH/L (= appr. pp	0,1925			
Target nominal conc. in low conc. tank:	cL	0,055	µg TPAH/L (= appr. ppb)				
Freshwater flow into tanks:	Fw	4,0	L/min.				
Muddy water flow into tank H:	FmwH	6,25	mL/min.				
Muddy water flow into tank L:	FmwL	2,08	mL/min.				
sum flow from header tank:	Fmw	8,33	mL/min.				
Flow of muddy water out of header tank pr. 24h:	Fmw	12,0	L/24h				
Exposure duration:	Tt	14	d				
wet mud usage in total:	Vt	1,581	dm3 (L) wet mud				
Calculation of amounts and concentrations of treated mud (assuming equal flow as for untreated mud):							
dry weight % of mud	wt %	85	%	Weight of added wet mud in header tank:		0,144	kg wet mud
water content % in mud	wt %	15	%	Dry Weight (=solids) of added wet mud in h		0,122	kg dry mud
density of wet mud	d1	1,27	kg/dm3 wet mud	Concentration of wet mud in header tank:		0,0	g wet mud/dm3 (L)
assumed approx. density of muddy water	d2	1,0	kg/dm3 muddy water	Concentration of particles in header tank:		10,17	mg particles/dm3 (L)
TPAH conc. in dry mud	c1 (DW	960	mg TPAH/kg dry mud	TPAH conc. in wet mud	c1 (WW)	812	mg TPAH/dm3 (L) wet mud
Volum of wet mud in header tank	V1	0,114	dm3 (L) wet mud* ->	*25,5 mL of extra wet mud added to obtain the same particle concentration (as in untreated mud)			
TPAH conc. in muddy water	c2	5,96	mg TPAH/dm3 (L) muddy water				
Volum of muddy water	V2	12	dm3 (L) muddy water				
Target nominal conc. in high conc. tank:	cH	9,318	mg TPAH/L (= appr. ppm)				
Target nominal conc. in low conc. tank:	cL	3,106	mg TPAH/L (= appr. ppm)				
Freshwater flow into tanks:	Fw	4,0	L/min.				
Muddy water flow into tank H:	FmwH	6 250,00	L/min.				
Muddy water flow into tank L:	FmwL	2 083,33	L/min.				
sum flow from header tank:	Fmw	8 333,33	L/min.				
Flow of muddy water out of header tank pr. 24h:	Fmw	12 000,0	L/24h				
Exposure duration:	Tt	14	d				
wet mud usage in total:	Vt	1,591	dm3 (L) wet mud				

Appendix B – Sampling data, condition factor and liver somatic index

Fish Number	Tank	Weight (g)	Length (cm)	Liver weight (g)	Condition Factor	Liver somatic index
1	Control	96,00	22,00	0,5	0,90	0,52
2	Control	103,3	22,50	0,95	0,91	0,92
3	Control	85,90	21,50	0,73	0,86	0,85
4	Control	126,30	25,00	1,22	0,81	0,97
5	Control	129,90	23,00	0,74	1,07	0,57
6	Control	96,30	21,00	0,84	1,04	0,87
7	Control	113,40	22,00	0,87	1,06	0,77
8	Control	86,10	22,00	0,56	0,81	0,65
9	Control	110,70	23,00	0,82	0,91	0,74
10	Control	90,00	22,00	0,9	0,85	1,00
11	High treated	86,50	21,00	0,64	0,93	0,74
12	High treated	102,50	22,00	1,03	0,96	1,00
13	High treated	111,70	23,00	0,99	0,92	0,89
14	High treated	102,70	22,00	0,68	0,96	0,66
15	High treated	134,60	23,00	0,2	1,11	0,15
16	High treated	121,80	23,00	0,95	1,00	0,78
17	High treated	120,00	23,00	0,98	0,99	0,82
18	High treated	95,00	22,00	0,84	0,89	0,88
19	High treated	99,20	22,00	0,8	0,93	0,81
20	High treated	123,70	24,00	1,26	0,89	1,02
21	High untreated	118,1	24,00	1,02	0,85	0,86
22	High untreated	90,80	22,00	0,69	0,85	0,76
23	High untreated	95,90	21,00	0,92	1,04	0,96
24	High untreated	87,30	21,00	0,72	0,94	0,82
25	High untreated	97,40	20,00	1,33	1,22	1,37
26	High untreated	99,00	21,00	0,72	1,07	0,73
27	High untreated	92,60	21,00	0,89	1,00	0,96
28	High untreated	92,00	20,00	0,93	1,15	1,01
29	High untreated	81,10	20,00	0,71	1,01	0,88
30	High untreated	97,40	21,00	0,79	1,05	0,81
31	Low untreated	94,20	22,00	0,96	0,88	1,02

32	Low untreated	121,30	23,00	1,13	1,00	0,93
33	Low untreated	106,60	22,00	1,05	1,00	0,98
34	Low untreated	127,40	24,00	1,03	0,92	0,81
35	Low untreated	104,70	22,00	0,92	0,98	0,88
36	Low untreated	88,00	21,00	0,69	0,95	0,78
37	Low untreated	86,20	19,00	0,79	1,26	0,92
38	Low untreated	92,40	21,00	0,68	1,00	0,74
39	Low untreated	112,20	23,00	1,16	0,92	1,03
40	Low untreated	93,10	22,00	0,77	0,87	0,83
41	Low treated	81,50	21,00	0,76	0,88	0,93
42	Low treated	86,00	21,00	0,63	0,93	0,73
43	Low treated	97,30	21,50	0,69	0,98	0,71
44	Low treated	102,20	22,00	0,63	0,96	0,62
45	Low treated	117,70	23,00	0,66	0,97	0,56
46	Low treated	104,50	22,00	0,99	0,98	0,95
47	Low treated	113,70	23,00	0,95	0,93	0,84
48	Low treated	114,60	23,00	0,8	0,94	0,70
49	Low treated	85,70	22,00	0,77	0,80	0,90
50	Low treated	82,70	21,00	0,76	0,89	0,92
51	Control	92,20	22,00	0,64	0,87	0,69
52	Control	131,70	24,00	1,34	0,95	1,02
53	Control	112,20	23,00	0,91	0,92	0,81
54	Control	91,60	21,00	0,76	0,99	0,83
55	Control	103,60	22,50	0,82	0,91	0,79
56	Control	83,60	21,00	0,72	0,90	0,86
57	Control	97,00	21,00	0,68	1,05	0,70
58	Control	88,60	21,50	0,66	0,89	0,74
59	Control	97,00	22,00	0,7	0,91	0,72
60	Control	111,30	23,00	0,89	0,91	0,80
61	High treated	68,60	20,00	0,69	0,86	1,01
62	High treated	127,90	24,50	0,91	0,87	0,71
63	High treated	99,60	21,00	0,77	1,08	0,77
64	High treated	103,50	22,50	0,83	0,91	0,80
65	High treated	83,40	21,00	0,76	0,90	0,91
66	High treated	73,50	20,50	0,69	0,85	0,94
67	High treated	102,60	22,50	0,73	0,90	0,71
68	High treated	83,80	21,00	0,63	0,90	0,75
69	High treated	106,40	23,00	1,23	0,87	1,16
70	High treated	123,70	23,00	1,09	1,02	0,88

71	High untreated	94,40	22,00	0,79	0,89	0,84
72	High untreated	87,60	22,00	0,59	0,82	0,67
73	High untreated	83,20	21,00	0,78	0,90	0,94
74	High untreated	92,60	22,00	0,79	0,87	0,85
75	High untreated	104,20	22,50	0,64	0,91	0,61
76	High untreated	123,40	24,00	1,07	0,89	0,87
77	High untreated	116,00	23,00	0,86	0,95	0,74
78	High untreated	112,00	22,50	1,16	0,98	1,04
79	High untreated	94,20	21,50	0,49	0,95	0,52
80	High untreated	114,30	23,50	0,59	0,88	0,52
81	Low untreated	128,00	24,00	1,01	0,93	0,79
82	Low untreated	90,20	23,00	0,75	0,74	0,83
83	Low untreated	88,40	21,50	0,79	0,89	0,89
84	Low untreated	133,40	25,00	1,32	0,85	0,99
85	Low untreated	79,20	21,00	0,7	0,86	0,88
86	Low untreated	96,40	22,00	1,15	0,91	1,19
87	Low untreated	85,40	21,00	0,61	0,92	0,71
88	Low untreated	94,60	21,50	0,72	0,95	0,76
89	Low untreated	87,90	21,00	0,76	0,95	0,86
90	Low untreated	82,90	21,50	0,71	0,83	0,86
91	Low treated	83,90	21,00	0,61	0,91	0,73
92	Low treated	140,10	24,50	0,96	0,95	0,69
93	Low treated	84,90	21,00	0,75	0,92	0,88
94	Low treated	114,10	24,00	0,96	0,83	0,84
95	Low treated	98,00	22,00	0,8	0,92	0,82
96	Low treated	100,00	22,00	0,81	0,94	0,81
97	Low treated	81,70	21,00	0,67	0,88	0,82
98	Low treated	124,50	24,00	0,87	0,90	0,70
99	Low treated	77,80	21,00	0,55	0,84	0,71
100	Low treated	101,40	22,00	0,7	0,95	0,69
101	Control	113,20	25,00	-	0,72	-
102	Control	81,20	20,00	0,61	1,02	0,75
103	Control	97,30	21,00	0,65	1,05	0,67

104	Control	105,40	22,00	1,26	0,99	1,20
105	Control	97,50	22,00	0,67	0,92	0,69
106	Control	141,30	24,00	1,28	1,02	0,91
107	Control	147,20	25,00	1,29	0,94	0,88
108	Control	94,40	22,00	0,8	0,89	0,85
109	Control	128,80	24,00	1	0,93	0,78
110	Control	95,50	21,00	0,8	1,03	0,84
111	High treated	119,50	24,50	0,87	0,81	0,73
112	High treated	117,60	23,00	1,15	0,97	0,98
113	High treated	86,20	21,00	0,57	0,93	0,66
114	High treated	125,80	25,00	1,21	0,81	0,96
115	High treated	103,50	23,00	0,82	0,85	0,79
116	High treated	117,70	23,50	1	0,91	0,85
117	High treated	109,90	23,50	1	0,85	0,91
118	High treated	156,60	26,00	1,43	0,89	0,91
119	High treated	101,20	23,00	0,84	0,83	0,83
120	High treated	82,30	21,00	0,68	0,89	0,83
121	High untreated	109,40	24,00	0,89	0,79	0,81
122	High untreated	145,20	25,00	1,23	0,93	0,85
123	High untreated	98,00	22,50	1,11	0,86	1,13
124	High untreated	95,00	21,00	0,7	1,03	0,74
125	High untreated	91,50	22,00	0,67	0,86	0,73
126	High untreated	102,30	22,00	0,84	0,96	0,82
127	High untreated	144,40	25,00	1,02	0,92	0,71
128	High untreated	79,20	22,00	0,38	0,74	0,48
129	High untreated	130,00	23,00	1,17	1,07	0,90
130	High untreated	109,10	23,00	0,84	0,90	0,77
131	Low untreated	111,90	23,00	1,02	0,92	0,91
132	Low untreated	91,20	20,00	0,89	1,14	0,98
133	Low untreated	102,00	22,00	0,65	0,96	0,64
134	Low untreated	115,00	22,00	0,93	1,08	0,81
135	Low untreated	102,00	22,00	0,68	0,96	0,67
136	Low untreated	106,00	22,00	1	1,00	0,94
137	Low untreated	92,90	21,00	0,74	1,00	0,80

138	Low untreated	112,50	22,00	0,85	1,06	0,76
139	Low untreated	108,30	22,00	0,92	1,02	0,85
140	Low untreated	106,80	22,00	0,81	1,00	0,76
141	Low treated	126,60	24,00	0,99	0,92	0,78
142	Low treated	95,80	21,00	0,65	1,03	0,68
143	Low treated	90,10	22,00	0,62	0,85	0,69
144	Low treated	74,90	20,00	0,57	0,94	0,76
145	Low treated	95,90	22,00	0,86	0,90	0,90
146	Low treated	115,00	23,00	0,82	0,95	0,71
147	Low treated	86,80	21,00	0,72	0,94	0,83
148	Low treated	94,70	22,00	0,78	0,89	0,82
149	Low treated	93,00	21,00	0,68	1,00	0,73
150	Low treated	101,20	22,00	0,91	0,95	0,90
151	Control	97,00	22,00	1,03	0,91	1,06
152	Control	93,90	22,00	0,74	0,88	0,79
153	Control	113,70	22,00	0,81	1,07	0,71
154	Control	99,00	22,50	0,73	0,87	0,74
155	Control	82,50	20,00	0,73	1,03	0,88
156	Control	111,00	23,00	1,02	0,91	0,92
157	Control	129,60	25,00	0,89	0,83	0,69
158	Control	116,70	24,00	1,02	0,84	0,87
159	Control	119,00	24,00	1,09	0,86	0,92
160	Control	105,30	23,50	0,74	0,81	0,70
161	High treated	131,90	24,00	0,85	0,95	0,64
162	High treated	126,30	24,50	1,06	0,86	0,84
163	High treated	106,00	23,00	0,87	0,87	0,82
164	High treated	93,30	21,50	0,78	0,94	0,84
165	High treated	97,50	20,00	0,64	1,22	0,66
166	High treated	105,70	23,00	0,93	0,87	0,88
167	High treated	82,20	21,00	0,78	0,89	0,95
168	High treated	101,40	22,50	0,77	0,89	0,76
169	High treated	73,40	19,50	0,56	0,99	0,76
170	High treated	80,00	19,50	0,69	1,08	0,86
171	High untreated	110,20	24,00	0,76	0,80	0,69
172	High untreated	83,30	19,50	0,97	1,12	1,16
173	High untreated	105,30	22,00	0,75	0,99	0,71
174	High untreated	114,6	23,00	0,86	0,94	0,75
175	High untreated	114,10	22,50	0,88	1,00	0,77
176	High untreated	84,00	20,00	0,62	1,05	0,74

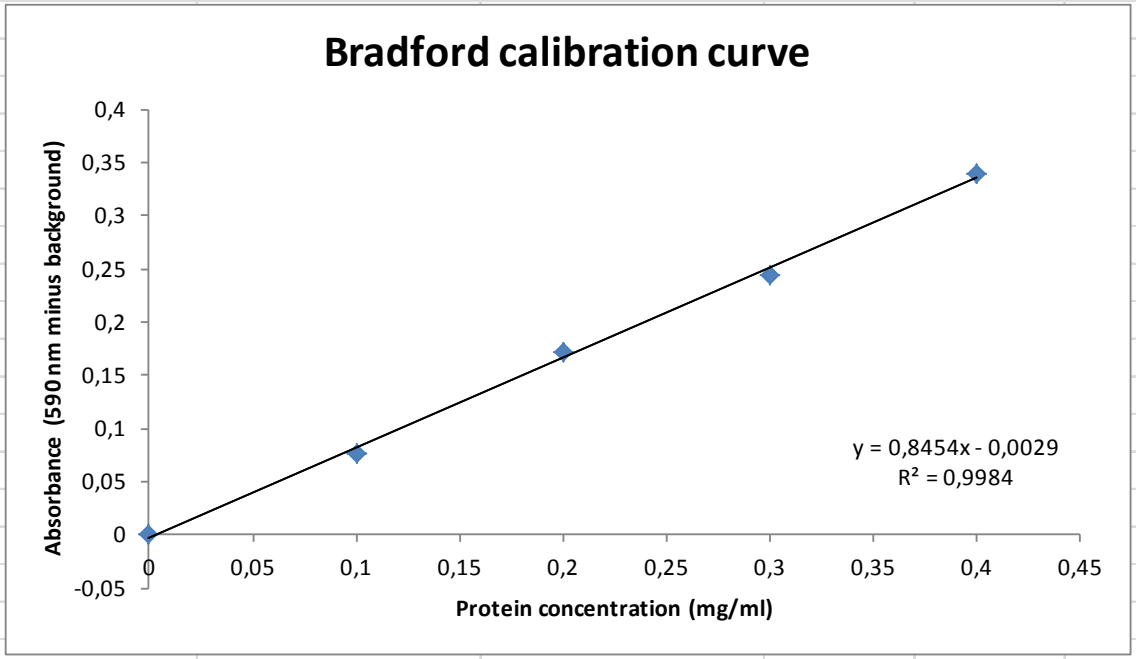
177	High untreated	115,10	23,50	0,89	0,89	0,77
178	High untreated	82,30	21,00	0,69	0,89	0,84
179	High untreated	88,00	22,00	0,63	0,83	0,72
180	High untreated	90,50	22,00	0,64	0,85	0,71
181	Low untreated	100,50	23,00	0,8	0,83	0,80
182	Low untreated	88,30	22,00	0,67	0,83	0,76
183	Low untreated	101,80	23,00	0,8	0,84	0,79
184	Low untreated	145,10	25,50	1,07	0,88	0,74
185	Low untreated	89,90	21,00	0,81	0,97	0,90
186	Low untreated	91,80	21,50	0,84	0,92	0,92
187	Low untreated	104,90	23,00	0,95	0,86	0,91
188	Low untreated	98,50	22,00	0,73	0,93	0,74
189	Low untreated	142,80	20,00	1,01	1,79	0,71
190	Low untreated	80,00	21,00	0,67	0,86	0,84
191	Low treated	117,50	22,00	0,97	1,10	0,83
192	Low treated	87,50	21,00	0,67	0,94	0,77
193	Low treated	81,30	21,00	0,63	0,88	0,77
194	Low treated	125,10	23,00	0,86	1,03	0,69
195	Low treated	104,40	23,00	0,70	0,86	0,67
196	Low treated	98,40	23,00	0,80	0,81	0,81
197	Low treated	85,00	21,00	0,62	0,92	0,73
198	Low treated	97,70	22,00	0,70	0,92	0,72
199	Low treated	86,00	21,50	0,57	0,87	0,66
200	Low treated	80,60	21,00	0,85	0,87	1,05

Appendix C – Bradford protein assay results

Bradford protein assay data:

Calibration curve data:

Conc. (mg/ml)	Abs (nm)	Abs (- bkground)
0	0,2849	0
0,1	0,3616	0,0767
0,2	0,4560	0,1712
0,3	0,5289	0,2440
0,4	0,6239	0,3391



3 day exposure		S100		Microsomes	
Sample #	**Tank**	**Abs (- bkground)**	**Protein conc. (mg/ml)**	**Abs (- bkground)**	**Protein conc. (mg/ml)**
2	Control	0,1328	16,05	0,1728	20,78
4	Control	0,1925	23,11	0,1601	19,28
5	Control	0,1341	16,21	0,1207	14,63
6	Control	0,1194	14,47	0,1982	23,79
7	Control	0,1152	13,97	0,1720	20,69
9	Control	0,1707	20,53	0,1591	19,16
10	Control	0,1559	18,78	0,1965	23,58
12	High treated	0,1791	21,53	0,1679	20,21
13	High treated	0,2293	27,46	0,1732	20,83
16	High treated	0,1556	18,75	0,1872	22,49
17	High treated	0,1883	22,62	0,1891	22,72
18	High treated	0,1864	22,40	0,1865	22,40
19	High treated	0,0948	11,56	0,1986	23,83
20	High treated	0,1569	18,91	0,1860	22,34
21	High untreated	0,1442	17,40	0,1444	17,43
23	High untreated	0,1963	23,57	0,1734	20,86
25	High untreated	0,1675	20,16	0,2548	30,49
26	High untreated	0,1635	19,69	0,1335	16,14
27	High untreated	0,1508	18,19	0,1163	14,10
28	High untreated	0,1452	17,52	0,1912	22,96
30	High untreated	0,1484	17,90	0,1156	14,02
31	Low untreated	0,1829	21,98	0,1595	19,21
32	Low untreated	0,1912	22,96	0,1644	19,79
33	Low untreated	0,1670	20,09	0,1188	14,40
34	Low untreated	0,1849	22,22	0,1855	22,29
35	Low untreated	0,1577	19,00	0,1821	21,88
37	Low untreated	0,1614	19,44	0,1554	18,73
39	Low untreated	0,1223	14,81	0,3019	36,05
41	Low treated	0,1740	20,92	0,2692	32,18
43	Low treated	0,1420	17,14	0,2934	35,05
46	Low treated	0,1602	19,29	0,3179	37,95
47	Low treated	0,1893	22,74	0,1521	18,34
48	Low treated	0,1511	18,22	0,0852	10,42
49	Low treated	0,1488	17,94	0,2067	24,79
50	Low treated	0,1886	22,65	0,3652	43,54

7 day exposure		**S100**		**Microsomes**	
52	Control	0,2239	26,83	0,2100	25,18
53	Control	0,1709	20,56	0,1749	21,03
54	Control	0,1821	21,89	0,3147	37,57
55	Control	0,1793	21,56	0,1956	23,48
56	Control	0,1343	16,23	0,0961	11,71
59	Control	0,1959	23,51	0,2442	29,23
60	Control	0,2047	24,56	0,3272	39,05
62	High treated	0,1887	22,67	0,0404	5,12
63	High treated	0,2025	24,30	0,1748	21,02
64	High treated	0,2446	29,28	0,2379	28,48
65	High treated	0,1824	21,92	0,5720	68,00
67	High treated	0,2219	26,59	0,2817	33,67
69	High treated	0,1996	23,95	0,2179	26,12
70	High treated	0,1478	17,83	0,2332	27,93
71	High untreated	0,1691	20,34	0,2016	24,19
73	High untreated	0,2045	24,53	0,2388	28,59
74	High untreated	0,1368	16,52	0,2875	34,35
75	High untreated	0,1104	13,41	0,1380	16,67
76	High untreated	0,2270	27,19	0,3337	39,82
77	High untreated	0,2019	24,22	0,2112	25,33
78	High untreated	0,1884	22,63	0,1739	20,91
81	Low untreated	0,2524	30,20	0,2507	30,00
82	Low untreated	0,2038	24,45	0,2130	25,54
83	Low untreated	0,2660	31,80	0,1905	22,88
84	Low untreated	0,2541	30,40	0,3008	35,92
86	Low untreated	0,2203	26,41	0,1916	23,01
88	Low untreated	0,1995	23,94	0,2821	33,71
89	Low untreated	0,2340	28,02	0,2194	26,30
92	Low treated	0,2622	31,35	0,1193	14,45
93	Low treated	0,3216	38,39	0,2740	32,75
94	Low treated	0,3282	39,17	0,1500	18,09
95	Low treated	0,1736	20,88	0,1145	13,89
96	Low treated	0,1851	22,24	0,1469	17,72
98	Low treated	0,1639	19,73	0,2715	32,46
100	Low treated	0,1447	17,46	0,1069	12,98

14 day exposure		S100		Microsomes	
104	Control	0,2378	28,47	0,2273	27,23
105	Control	0,2062	24,73	0,1514	18,25
106	Control	0,1789	21,51	0,2271	27,21
107	Control	0,3376	40,27	0,3439	41,02
108	Control	0,3553	42,38	0,1925	23,12
109	Control	0,2950	35,24	0,2196	26,32
110	Control	0,1884	22,63	0,1720	20,69
111	High treated	0,2069	24,82	0,2114	25,35
112	High treated	0,3268	39,00	0,2838	33,92
114	High treated	0,5448	64,79	0,2099	25,18
115	High treated	0,2308	27,64	0,2172	26,04
116	High treated	0,1530	18,44	0,1554	18,73
117	High treated	0,2163	25,93	0,1190	14,42
119	High treated	0,2036	24,42	0,2267	27,16
121	High untreated	0,3278	39,12	0,2225	26,66
122	High untreated	0,2765	33,05	0,3186	38,03
123	High untreated	0,2040	24,47	0,2637	31,54
126	High untreated	0,2235	26,78	0,1744	20,97
127	High untreated	0,2851	34,07	0,2142	25,68
129	High untreated	0,2892	34,56	0,1071	13,01
130	High untreated	0,1542	18,58	0,2924	34,94
131	Low untreated	0,3192	38,10	0,2424	29,02
132	Low untreated	0,1597	19,24	0,1827	21,96
134	Low untreated	0,2577	30,83	0,2530	30,27
136	Low untreated	0,2853	34,09	0,2191	26,26
138	Low untreated	0,2889	34,51	0,1703	20,49
139	Low untreated	0,2505	29,97	0,1790	21,51
140	Low untreated	0,1711	20,58	0,2053	24,63
141	Low treated	0,2827	33,78	0,2871	34,30
145	Low treated	0,2695	32,23	0,1681	20,22
146	Low treated	0,1924	23,11	0,1091	13,25
147	Low treated	0,2198	26,34	0,1452	17,52
148	Low treated	0,2070	24,83	0,1218	14,76
149	Low treated	0,3146	37,56	0,0910	11,11
150	Low treated	0,3058	36,52	0,2450	29,33

1 week recovery		S100		Microsomes	
151	Control	0,2008	24,0961	0,1383	16,70
153	Control	0,0408	5,1701	0,0518	6,47
156	Control	0,2198	26,3475	0,1370	16,55
157	Control	0,2239	26,8325	0,1891	22,72
158	Control	0,2478	29,6516	0,2196	26,32
159	Control	0,2171	26,0242	0,1348	16,29
160	Control	0,1991	23,8911	0,1792	21,55
161	High treated	0,2074	24,8768	0,1513	18,24
162	High treated	0,1945	23,3548	0,1318	15,93
163	High treated	0,2303	27,5816	0,1710	20,57
164	High treated	0,2661	31,8202	0,1895	22,76
166	High treated	0,2063	24,7506	0,2045	24,54
167	High treated	0,1888	22,6806	0,0969	11,80
168	High treated	0,2146	25,7324	0,1689	20,32
171	High untreated	0,2030	24,3524	0,1820	21,87
172	High untreated	0,2422	28,9971	0,1620	19,51
173	High untreated	0,1800	21,6357	0,1235	14,95
174	High untreated	0,1591	19,1675	0,1680	20,22
175	High untreated	0,2627	31,4181	0,1756	21,12
177	High untreated	0,1551	18,6864	0,1395	16,84
178	High untreated	0,1345	16,2497	0,1684	20,27

Appendix D – EROD results

Key: HC: House control - HT: High treated - HU: High untreated - LT: Low treated - LU: Low untreated

House control:

House control

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
HC	High1	0,0006	1,7908	1,7902	124,6	0,02	7,89	0,264643201
HC	High2	0,0007	1,9199	1,9192	122,4	0,02	9,87	0,226158062
HC	High3	0,0007	1,5996	1,5989	121,3	0,02	6,34	0,418811198
HC	High4	0,0006	1,2963	1,2957	120,9	0,02	10,09	0,277429047
Median		0,00065	1,6952	1,69455			8,88	0,271036124
Mean		0,00065	1,65165	1,651			8,55	0,296760377
SD		5,7735E-05	0,2709881	0,270961			1,77329026	0,084234401

Exposure:

3 day exposure

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
Control	2	0,0001	1,803	1,8029	124,6	0,02	20,78	0,016625943
Control	4	0	1,6414	1,6414	124,6	0,02	19,28	0
Control	5	0	1,4848	1,4848	124,6	0,02	14,63	0
Control	6	0	1,6568	1,6568	124,6	0,02	23,79	0
Control	7	0	1,5653	1,5653	124,6	0,02	20,69	0
Control	9	0	1,6965	1,6965	124,6	0,02	19,16	0
Control	10	0	1,7338	1,7338	124,6	0,02	23,58	0
Median		0	1,6568	1,6568			20,69	0
Mean		1,42857E-05	1,6545143	1,6545			20,27	0,002375135
SD		3,77964E-05	0,1057011	0,105678			3,11	0,006284016

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
HT	12	0	1,8101	1,8101	124,6	0,02	20,21	0
HT	13	0	1,7024	1,7024	124,6	0,02	20,83	0
HT	16	0	1,6807	1,6807	124,6	0,02	22,49	0
HT	17	0,000008	1,8207	1,820692	124,6	0,02	22,72	0,001205059
HT	18	0	1,7413	1,7413	124,6	0,02	22,40	0
HT	19	0,00003	1,7997	1,79967	124,6	0,02	23,83	0,004357702
HT	20	0	1,9163	1,9163	124,6	0,02	22,34	0
Median		0	1,7997	1,79967			22,40	0
Mean		5,42857E-06	1,7816	1,781595			22,12	0,00079468
SD		1,12377E-05	0,0805091	0,080507			1,22	0,00163407

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
HU	21	0	1,8291	1,8291	124,6	0,02	17,43	0
HU	23	0,00002	2,0107	2,01068	124,6	0,02	20,86	0,002971423
HU	25	0	1,6868	1,6868	124,6	0,02	30,49	0
HU	26	0,00004	2,0107	2,01066	124,6	0,02	16,14	0,007679353
HU	27	0,00004	1,6868	1,68676	124,6	0,02	14,10	0,010474395
HU	28	0	1,8031	1,8031	124,6	0,02	22,96	0
HU	30	0,00002	1,752	1,75198	124,6	0,02	14,02	0,005073437
Median		0,00002	1,8031	1,8031			17,43	0,002971423

Mean		1,71429E-05	1,8256	1,825583			19,43	0,003742658
SD		1,79947E-05	0,1372287	0,137224			5,91	0,004187324

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
LU	31	0,00003	1,6289	1,62887	124,6	0,02	19,21	0,00597401
LU	32	0,000001	1,9967	1,996699	124,6	0,02	19,79	0,000157628
LU	33	0,00002	1,969	1,96898	124,6	0,02	14,40	0,004395605
LU	34	0,00003	1,6646	1,66457	124,6	0,02	22,29	0,005037239
LU	35	0	1,5006	1,5006	124,6	0,02	21,88	0
LU	37	0	1,5962	1,5962	124,6	0,02	18,73	0
LU	39	0	1,8326	1,8326	124,6	0,02	36,05	0
Median		0,000001	1,6646	1,66457			19,79	0,000157628
Mean		1,15714E-05	1,7412286	1,741217			21,76	0,002223497
SD		1,45127E-05	0,1927517	0,192752			6,81	0,002762884

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
LT	41	0,00009	1,5549	1,55481	124,6	0,02	32,18	0,011205386
LT	43	0,00005	1,511	1,51095	124,6	0,02	35,05	0,005882018
LT	46	0,00002	1,5403	1,54028	124,6	0,02	37,95	0,002131522
LT	47	0	1,335	1,335	124,6	0,02	18,34	0
LT	48	0	1,8241	1,8241	124,6	0,02	10,42	0
LT	49	0,00003	1,6186	1,61857	124,6	0,02	24,79	0,004657271
LT	50	0,00006	1,6981	1,69804	124,6	0,02	43,54	0,005056119
Median		0,00003	1,5549	1,55481			32,18	0,004657271
Mean		3,57143E-05	1,5831429	1,583107			28,90	0,004133188
SD		3,30944E-05	0,1538101	0,153809			11,65	0,003923731

7 day exposure

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
Control	52	0	1,6208	1,6208	122,4	0,02	25,18	0
Control	53	0	1,879	1,879	122,4	0,02	21,03	0
Control	54	0,00003	1,6322	1,63217	122,4	0,02	37,57	0,002994492
Control	55	0,00004	1,8617	1,86166	122,4	0,02	23,48	0,005600084
Control	56	0	1,7479	1,7479	122,4	0,02	11,71	0
Control	59	0	1,7974	1,7974	122,4	0,02	29,23	0
Control	60	0	1,7419	1,7419	122,4	0,02	39,05	0
Median		0	1,7479	1,7479			25,18	0
Mean		0,00001	1,7544143	1,754404			26,75	0,001227797
SD		1,73205E-05	0,1015445	0,101544			9,54	0,002227687

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
HT	62	0	1,8115	1,8115	122,4	0,02	5,12	0
HT	63	0,000003	1,7892	1,789197	122,4	0,02	21,02	0,000488168
HT	64	0,000007	1,6969	1,696893	122,4	0,02	28,48	0,000886433
HT	65	0,00002	1,7453	1,74528	122,4	0,02	68,00	0,001031288
HT	67	0	1,8641	1,8641	122,4	0,02	33,67	0
HT	69	0	1,7904	1,7904	122,4	0,02	26,12	0
HT	70	0	1,9315	1,9315	122,4	0,02	27,93	0
Median		0	1,7904	1,7904			27,93	0
Mean		4,28571E-06	1,8041286	1,804124			30,05	0,000343698
SD		7,40977E-06	0,0766426	0,076647			19,06	0,00045839

Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
HU	71	0,00003	1,7995	1,79947	122,4	0,02	24,19	0,004217048
HU	73	0	1,6696	1,6696	122,4	0,02	28,59	0
HU	74	0	1,9287	1,9287	122,4	0,02	34,35	0
HU	75	0	1,7371	1,7371	122,4	0,02	16,67	0
HU	76	0,00004	1,7739	1,77386	122,4	0,02	39,82	0,003466007

	HU	77	0	1,5479	1,5479	122,4	0,02	25,33	0
	HU	78	0	2,0384	2,0384	122,4	0,02	20,91	0
Median			0	1,7739	1,77386			25,33	0
Mean			0,00001	1,7850143	1,785004			27,12	0,001097579
SD			1,73205E-05	0,1617728	0,161773			7,91	0,00188697

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	LU	81	0,00002	1,4341	1,43408	122,4	0,02	30,00	0,002844787
	LU	82	0	1,844	1,844	122,4	0,02	25,54	0
	LU	83	0	1,7927	1,7927	122,4	0,02	22,88	0
	LU	84	0	0,3539	0,3539	122,4	0,02	35,92	0
	LU	86	0,00001	1,75	1,74999	122,4	0,02	23,01	0,001519727
	LU	88	0,00001	1,4271	1,42709	122,4	0,02	33,71	0,001272052
	LU	89	0	1,9947	1,9947	122,4	0,02	26,30	0
Median			0	1,75	1,74999			26,30	0
Mean			5,71429E-06	1,5137857	1,51378			28,20	0,000805224
SD			7,86796E-06	0,5527371	0,552737			5,15	0,001116677

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	LT	92	0	1,7729	1,7729	122,4	0,02	14,45	0
	LT	93	0	1,8135	1,8135	122,4	0,02	32,75	0
	LT	94	0,00001	1,801	1,80099	122,4	0,02	18,09	0,00187876
	LT	95	0	1,8148	1,8148	122,4	0,02	13,89	0
	LT	96	0	1,8751	1,8751	122,4	0,02	17,72	0
	LT	98	0,00002	1,7288	1,72878	122,4	0,02	32,46	0,002181256
	LT	100	0,00002	1,7803	1,78028	122,4	0,02	12,98	0,005294829
Median			0	1,801	1,80099			17,72	0
Mean			7,14286E-06	1,7980571	1,79805			20,34	0,001336406
SD			9,5119E-06	0,0451069	0,045113			8,59563512	0,001992526

14 day exposure

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	Control	104	0,000007	2,0211	2,021093	121,3	0,02	27,23	0,000771407
	Control	105	0	1,9779	1,9779	121,3	0,02	18,25	0
	Control	106	0	1,971	1,971	121,3	0,02	27,21	0
	Control	107	0,00008	1,9205	1,92042	121,3	0,02	41,02	0,006159409
	Control	108	0	2,0048	2,0048	121,3	0,02	23,12	0
	Control	109	0	1,98	1,98	121,3	0,02	26,32	0
	Control	110	0,00005	2,0354	2,03535	121,3	0,02	20,69	0,00719997
Median			0	1,98	1,98			26,32	0
Mean			1,95714E-05	1,9872429	1,987223			26,26	0,002018684
SD			3,23206E-05	0,0379294	0,037941			7,36	0,003210591

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	HT	111	0,00009	1,8181	1,81801	121,3	0,02	25,35	0,01184405
	HT	112	0,00002	1,8375	1,83748	121,3	0,02	33,92	0,001946299
	HT	114	0	1,7684	1,7684	121,3	0,02	25,18	0
	HT	115	0,00005	2,0467	2,04665	121,3	0,02	26,04	0,005690927
	HT	116	0,00001	1,7125	1,71249	121,3	0,02	18,73	0,001891304
	HT	117	0	2,0541	2,0541	121,3	0,02	14,42	0
	HT	119	0,00005	1,7384	1,73835	121,3	0,02	27,16	0,006422072
Median			0,00002	1,8181	1,81801			25,35	0,001946299
Mean			3,14286E-05	1,8536714	1,85364			24,40	0,003970664
SD			3,33809E-05	0,1410949	0,141096			6,25	0,004299478

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	HU	121	0,00003	1,7473	1,74727	121,3	0,02	26,66	0,003906147
	HU	122	0,00001	1,8635	1,86349	121,3	0,02	38,03	0,000855892

	HU	123	0	1,8794	1,8794	121,3	0,02	31,54	0
	HU	126	0	1,9664	1,9664	121,3	0,02	20,97	0
	HU	127	0	2,0791	2,0791	121,3	0,02	25,68	0
	HU	129	0,00002	1,9695	1,96948	121,3	0,02	13,01	0,004733102
	HU	130	0,000006	1,8109	1,810894	121,3	0,02	34,94	0,00057521
Median			0,000006	1,8794	1,8794			26,66	0,00057521
Mean			9,42857E-06	1,9023	1,902291			27,26	0,001438622
SD			1,16456E-05	0,1112742	0,111281			8,55	0,002009819

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	LU	131	0,00003	1,7867	1,78667	121,3	0,02	29,02	0,003509123
	LU	132	0	1,9149	1,9149	121,3	0,02	21,96	0
	LU	134	0,00004	1,8604	1,86036	121,3	0,02	30,27	0,004308521
	LU	136	0	1,8837	1,8837	121,3	0,02	26,26	0
	LU	138	0	1,8867	1,8867	121,3	0,02	20,49	0
	LU	139	0	1,8264	1,8264	121,3	0,02	21,51	0
	LU	140	0	1,8712	1,8712	121,3	0,02	24,63	0
Median			0	1,8712	1,8712			24,63	0
Mean			0,00001	1,8614286	1,861419			24,88	0,001116806
SD			1,73205E-05	0,0426258	0,042635			3,81	0,001921219

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	LT	141	0	1,6219	1,6219	121,3	0,02	34,30	0
	LT	145	0	1,7744	1,7744	121,3	0,02	20,22	0
	LT	146	0	1,7846	1,7846	121,3	0,02	13,25	0
	LT	147	0	1,8336	1,8336	121,3	0,02	17,52	0
	LT	148	0	1,6268	1,6268	121,3	0,02	14,76	0
	LT	149	0	1,98	1,98	121,3	0,02	11,11	0
	LT	150	0	1,7329	1,7329	121,3	0,02	29,33	0
Median			0	1,7744	1,7744			17,52	0
Mean			0	1,7648857	1,764886			20,07	0
SD			0	0,123835	0,123835			8,66	0

Recovery (1 week)

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	Control	151	0	1,8291	1,8291	120,9	0,02	27,23	0
	Control	153	0,00004	2,0107	2,01066	120,9	0,02	18,25	0,00658856
	Control	156	0,00004	1,6868	1,68676	120,9	0,02	27,21	0,005268921
	Control	157	0	1,8031	1,8031	120,9	0,02	41,02	0
	Control	158	0,00002	1,752	1,75198	120,9	0,02	23,12	0,002984986
	Control	159	0	1,8154	1,8154	120,9	0,02	26,32	0
	Control	160	0	1,8432	1,8432	120,9	0,02	20,69	0
Median			0	1,8154	1,8154			26,32	0
Mean			1,42857E-05	1,8200429	1,820029			26,26	0,002120352
SD			1,90238E-05	0,0997167	0,099715			7,36	0,002846329

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	HT	161	0	2,0818	2,0818	120,9	0,02	25,35	0
	HT	162	0,00006	2,0663	2,06624	120,9	0,02	33,92	0,005175332
	HT	163	0	1,8884	1,8884	120,9	0,02	25,18	0
	HT	164	0	1,9109	1,9109	120,9	0,02	26,04	0
	HT	166	0,00006	1,8991	1,89904	120,9	0,02	18,73	0,010199336
	HT	167	0	1,9478	1,9478	120,9	0,02	14,42	0
	HT	168	0,00005	1,899	1,89895	120,9	0,02	27,16	0,005859551
Median			0	1,9109	1,9109			25,35	0
Mean			2,42857E-05	1,9561857	1,956161			24,40	0,00303346
SD			3,04725E-05	0,0828142	0,082814			6,25	0,004097392

	Tank	Sample	Slope Fs/min	Slope FR	FR	R (pmol)	Vs (ml)	Cs (mg/ml)	EROD
	HU	171	0,00004	1,7413	1,74126	120,9	0,02	26,66	0,005208938
	HU	172	0,00006	1,8079	1,80784	120,9	0,02	38,03	0,005275973
	HU	173	0,00001	1,7649	1,76489	120,9	0,02	31,54	0,001085958
	HU	174	0,00003	1,7201	1,72007	120,9	0,02	20,97	0,00502695
	HU	175	0,00001	1,7104	1,71039	120,9	0,02	25,68	0,001376428
	HU	177	0	1,8951	1,8951	120,9	0,02	13,01	0
	HU	178	0	1,7199	1,7199	120,9	0,02	34,94	0
Median			0,00001	1,7413	1,74126			26,66	0,001376428
Mean			2,14286E-05	1,7656571	1,765636			27,26	0,00256775
SD			2,26779E-05	0,0662775	0,066278			8,55	0,002488629

Appendix E – GST results

3 day exposure

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
	Blank	0,0008	0,0032	0,0000	0	0
Control	2	0,0178	0,0712	0,0680	16,05	0,0353
Control	4	0,0143	0,0572	0,0540	23,11	0,0195
Control	5	0,0182	0,0728	0,0696	16,21	0,0358
Control	6	0,0173	0,0692	0,0660	14,47	0,0380
Control	7	0,0163	0,0652	0,0620	13,97	0,0370
Control	9	0,0196	0,0784	0,0752	20,53	0,0305
Control	10	0,0236	0,0944	0,0912	18,78	0,0405
Median						0,0358
Mean						0,0338
SD						0,0070

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
HT	12	0,0228	0,0912	0,0880	21,53	0,0341
HT	13	0,0165	0,0660	0,0628	27,46	0,0191
HT	16	0,0203	0,0812	0,0780	18,75	0,0347
HT	17	0,0167	0,0668	0,0636	22,62	0,0234
HT	18	0,0182	0,0728	0,0696	22,40	0,0259
HT	19	0,02	0,0800	0,0768	11,56	0,0554
HT	20	0,0178	0,0712	0,0680	18,91	0,0300
Median						0,0300
Mean						0,0318
SD						0,0118

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
HU	21	0,0199	0,0796	0,0764	17,40	0,0366
HU	23	0,0123	0,0492	0,0460	23,57	0,0163
HU	25	0,0137	0,0548	0,0516	20,16	0,0213
HU	26	0,015	0,0600	0,0568	19,69	0,0240
HU	27	0,0147	0,0588	0,0556	18,19	0,0255
HU	28	0,0131	0,0524	0,0492	17,52	0,0234
HU	30	0,0195	0,0780	0,0748	17,90	0,0348
Median						0,0240
Mean						0,0260
SD						0,0073

7 day exposure

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
	Blank	0,0008	0,0032	0,0000	0	0
Control	52	0,0193	0,0772	0,0740	26,83	0,0230
Control	53	0,0151	0,0604	0,0572	20,56	0,0232
Control	54	0,0292	0,1168	0,1136	21,89	0,0433
Control	55	0,0613	0,2452	0,2420	21,56	0,0936
Control	56	0,0243	0,0972	0,0940	16,23	0,0483
Control	59	0,0191	0,0764	0,0732	23,51	0,0259
Control	60	0,0252	0,1008	0,0976	24,56	0,0331
Median						0,0331
Mean						0,0415
SD						0,0250

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
HT	62	0,0198	0,0792	0,0760	22,67	0,0279
HT	63	0,0238	0,0952	0,0920	24,30	0,0315
HT	64	0,0180	0,0720	0,0560	29,28	0,0159
HT	65	0,0148	0,0592	0,0560	21,92	0,0213
HT	67	0,0188	0,0752	0,0720	26,59	0,0226
HT	69	0,0188	0,0752	0,0720	23,95	0,0251
HT	70	0,0167	0,0668	0,0636	17,83	0,0297
Median						0,0251
Mean						0,0249
SD						0,0054

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
HU	71	0,0176	0,0704	0,0672	20,34	0,0275
HU	73	0,0239	0,0956	0,0924	24,53	0,0314
HU	74	0,0175	0,0700	0,0668	16,52	0,0337
HU	75	0,0151	0,0604	0,0572	13,41	0,0356
HU	76	0,0223	0,0892	0,0860	27,19	0,0264
HU	77	0,0179	0,0716	0,0684	24,22	0,0235
HU	78	0,0209	0,0836	0,0804	22,63	0,0296
Median						0,0296
Mean						0,0297
SD						0,0042

14 day exposure

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
	Blank	0,0008	0,0032	0,0000	0	0
Control	104	0,0131	0,0524	0,0492	28,47	0,0144
Control	105	0,0151	0,0604	0,0572	24,73	0,0193
Control	106	0,0250	0,1000	0,0968	21,51	0,0375
Control	107	0,0257	0,1028	0,0996	40,27	0,0206
Control	108	0,0231	0,0924	0,0892	42,38	0,0175
Control	109	0,0169	0,0676	0,0644	35,24	0,0152
Control	110	0,0155	0,0620	0,0588	22,63	0,0217
Median						0,0193
Mean						0,0209
SD						0,0078

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
HT	111	0,0196	0,0784	0,0752	24,82	0,0252
HT	112	0,0229	0,0916	0,0884	39,00	0,0189
HT	114	0,0411	0,1644	0,1612	64,79	0,0207
HT	115	0,0258	0,1032	0,1000	27,64	0,0301
HT	116	0,0219	0,0876	0,0844	18,44	0,0381
HT	117	0,0141	0,0564	0,0532	25,93	0,0171
HT	119	0,0142	0,0568	0,0536	24,42	0,0183
Median						0,0207
Mean						0,0241
SD						0,0077

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
HU	121	0,0219	0,0876	0,0844	39,12	0,0180
HU	122	0,0229	0,0916	0,0884	33,05	0,0223

HU	123	0,0133	0,0532	0,0500	24,47	0,0170
HU	126	0,0160	0,0640	0,0608	26,78	0,0189
HU	127	0,0247	0,0988	0,0956	34,07	0,0234
HU	129	0,0183	0,0732	0,0700	34,56	0,0169
HU	130	0,0162	0,0648	0,0616	18,58	0,0276
Median						0,0189
Mean						0,0206
SD						0,0040

Recovery (1 week)

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
	Blank	0,0008	0,0032	0,0000	0	0
Control	151	0,0164	0,0656	0,0624	24,10	0,0216
Control	153	0,0017	0,0068	0,0036	5,17	0,0058
Control	156	0,0197	0,0788	0,0756	26,35	0,0239
Control	157	0,0183	0,0732	0,0700	26,83	0,0217
Control	158	0,0232	0,0928	0,0896	29,65	0,0252
Control	159	0,0163	0,0652	0,0620	26,02	0,0199
Control	160	0,0159	0,0636	0,0604	23,89	0,0211
Median						0,0216
Mean						0,0199
SD						0,0065

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
HT	161	0,0168	0,0672	0,0640	24,88	0,0214
HT	162	0,0164	0,0656	0,0624	23,35	0,0223
HT	163	0,0235	0,094	0,0908	27,58	0,0274
HT	164	0,0271	0,1084	0,1052	31,82	0,0276
HT	166	0,0185	0,074	0,0708	24,75	0,0238
HT	167	0,0192	0,0768	0,0736	22,68	0,0270
HT	168	0,0189	0,0756	0,0724	25,73	0,0234
Median						0,0238
Mean						0,0247
SD						0,0026

Tank	Sample	Slope 15 sec	Slope 1 min	Net slope	Protein (mg/ml)	GST activity
HU	171	0,0127	0,0508	0,0476	24,35	0,0163
HU	172	0,0171	0,0684	0,0652	29,00	0,0187
HU	173	0,0156	0,0624	0,0592	21,64	0,0228
HU	174	0,0333	0,1332	0,1300	19,17	0,0565
HU	175	0,0219	0,0876	0,0844	31,42	0,0224
HU	177	0,0125	0,05	0,0468	18,69	0,0209
HU	178	0,018	0,072	0,0688	16,25	0,0353
Median						0,0224
Mean						0,0276
SD						0,0141

Appendix F – CAT results

3 day exposure

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
Control	**2**	0,1006	0,5598	16,0526	0,7152
Control	**4**	0,1073	0,5266	23,1143	0,4535
Control	**5**	0,0911	0,5375	16,2103	0,6885
Control	**6**	0,1037	0,6097	14,4715	0,8741
Control	**7**	0,0181	0,2508	13,9668	0,4165
Control	**9**	0,1677	0,6521	20,5317	0,5898
Control	**10**	0,4036	0,5452	18,7811	0,1885
Median					0,5898
Mean					0,5609
SD					0,2273

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
HT	**12**	0,0633	0,4312	21,5332	0,4271
HT	**13**	0,0786	0,5306	27,4633	0,4115
HT	**16**	0,0584	0,4936	18,7535	0,5802
HT	**17**	0,167	0,6211	22,6175	0,5019
HT	**18**	0,0614	0,587	22,3967	0,5867
HT	**19**	0,0574	0,5328	11,5616	1,0280
HT	**20**	0,046	0,3721	18,9072	0,4312
Median					0,5019
Mean					0,5666
SD					0,2158

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
HU	**21**	0,0567	0,5494	17,3971	0,7080
HU	**23**	0,046	0,4809	23,5677	0,4613
HU	**25**	0,1155	0,6716	20,1611	0,6896
HU	**26**	0,0668	0,4957	19,6879	0,5446
HU	**27**	0,116	0,5866	18,1857	0,6469
HU	**28**	0,0935	0,5296	17,5233	0,6222
HU	**30**	0,0961	0,5688	17,8978	0,6603
Median					0,6469
Mean					0,6190
SD					0,0874

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
LU	**31**	0,1107	0,619	21,9827	0,5781
LU	**32**	0,1713	0,6033	22,9645	0,4703
LU	**33**	0,0688	0,4701	20,0940	0,4993
LU	**34**	0,1665	0,6305	22,2153	0,5222
LU	**35**	0,2948	0,6867	19,0019	0,5156
LU	**37**	0,2382	0,7244	19,4356	0,6254
LU	**39**	0,0661	0,4464	14,8145	0,6418
Median					0,5222
Mean					0,5504
SD					0,0655

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
LT	**41**	0,0772	0,5395	20,9220	0,5524
LT	**43**	0,1517	0,7347	17,1369	0,8505
LT	**46**	0,2347	0,6531	19,2936	0,5421
LT	**47**	0,2495	0,7121	22,7397	0,5086
LT	**48**	0,1199	0,6684	18,2212	0,7526

LT	**49**	0,2301	0,5961	17,9412	0,5100
LT	**50**	0,6583	0,9092	22,6530	0,2769
Median					0,5421
Mean					0,5704
SD					0,1855

7 day exposure

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
Control	52	0,3691	0,6789	26,8325	0,2886
Control	53	0,279	0,5526	20,5554	0,3328
Control	54	0,3775	0,7644	21,8881	0,4419
Control	55	0,3736	0,8081	21,5569	0,5039
Control	56	0,2539	0,4469	16,2300	0,2973
Control	59	0,3736	0,7559	23,5125	0,4065
Control	60	0,4151	0,7515	24,5614	0,3424
Median					0,3424
Mean					0,3733
SD					0,0800

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
HT	62	0,4079	0,7515	22,6688	0,3789
HT	63	0,3846	0,714	24,3011	0,3389
HT	64	0,3762	0,7459	29,2771	0,3157
HT	65	0,4399	0,8541	21,9235	0,4723
HT	67	0,4703	0,8038	26,5919	0,3135
HT	69	0,3591	0,5717	23,9502	0,2219
HT	70	0,5156	0,8526	17,8269	0,4726
Median					0,3389
Mean					0,3591
SD					0,0906

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
HU	71	0,4431	0,7939	20,3424	0,4311
HU	73	0,4824	0,8383	24,5298	0,3627
HU	74	0,5063	0,7955	16,5218	0,4376
HU	75	0,2605	0,6354	13,4069	0,6991
HU	76	0,4431	0,5761	27,1913	0,1223
HU	77	0,445	0,7551	24,2223	0,3201
HU	78	0,4504	0,7181	22,6254	0,2958
Median					0,3627
Mean					0,3812
SD					0,1757

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
LU	81	0,5531	0,8185	30,2037	0,2197
LU	82	0,4717	0,7561	24,4549	0,2907
LU	83	0,3698	0,6138	31,8045	0,1918
LU	84	0,4843	0,8363	30,3969	0,2895
LU	86	0,4429	0,7307	26,4066	0,2725
LU	88	0,3767	0,583	23,9384	0,2154
LU	89	0,5258	0,8318	28,0193	0,2730
Median					0,2725
Mean					0,2504
SD					0,0403

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
LT	92	0,5703	0,8901	31,3550	0,2550
LT	93	0,4229	0,6491	38,3891	0,1473
LT	94	0,5255	0,8192	39,1698	0,1875
LT	95	0,4351	0,8441	20,8787	0,4897
LT	96	0,3773	0,6556	22,2350	0,3129
LT	98	0,4531	0,7558	19,7313	0,3835
LT	100	0,2589	0,5387	17,4602	0,4006
Median					0,3129
Mean					0,3109
SD					0,1228

14 day exposure

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
Control	104	0,041	0,3603	28,4727	0,2804
Control	105	0,0479	0,5626	24,7309	0,5203
Control	106	0,1476	0,7026	21,5056	0,6452
Control	107	0,0498	0,5215	40,2738	0,2928
Control	108	0,0641	0,4244	42,3754	0,2126
Control	109	0,0404	0,4425	35,2427	0,2852
Control	110	0,0141	0,3731	22,6293	0,3966
Median					0,2928
Mean					0,3762
SD					0,1553

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
HT	111	0,2154	0,6775	24,8216	0,4654
HT	112	0,1188	0,6177	38,9963	0,3198
HT	114	0,1262	0,5293	64,7908	0,1555
HT	115	0,0587	0,5081	27,6447	0,4064
HT	116	0,0854	0,6441	18,4380	0,7575
HT	117	0,0621	0,5465	25,9335	0,4670
HT	119	0,0827	0,6686	24,4233	0,5997
Median					0,4654
Mean					0,4531
SD					0,1928

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
HU	121	0,3424	0,645	39,1186	0,1934
HU	122	0,4224	0,7499	33,0544	0,2477
HU	123	0,3549	0,7109	24,4746	0,3636
HU	126	0,2919	0,7005	26,7812	0,3814
HU	127	0,5666	0,7759	34,0716	0,1536
HU	129	0,3361	0,7082	34,5566	0,2692
HU	130	0,4585	0,7356	18,5839	0,3728
Median					0,2692
Mean					0,2831
SD					0,0917

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
LU	131	0,2734	0,736	38,1013	0,3035
LU	132	0,1972	0,6342	19,2384	0,5679
LU	134	0,1888	0,6994	30,8266	0,4141

LU	136	0,1498	0,7477	34,0874	0,4385
LU	138	0,2618	0,7353	34,5132	0,3430
LU	139	0,2475	0,7547	29,9710	0,4231
LU	140	0,0784	0,5775	20,5830	0,6062
Median					0,4231
Mean					0,4423
SD					0,1103

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
LT	141	0,1422	0,6511	33,7838	0,3766
LT	145	0,2241	0,6519	32,2264	0,3319
LT	146	0,0686	0,5622	23,1064	0,5341
LT	147	0,0831	0,5979	26,3435	0,4885
LT	148	0,1178	0,6097	24,8334	0,4952
LT	149	0,6723	0,7136	37,5572	0,0275
LT	150	0,2441	0,7315	36,5202	0,3337
Median					0,3766
Mean					0,3696
SD					0,1716

Recovery (1 week)

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
Control	151	0,0312	0,4103	16,70	0,5674
Control	153	-0,0770	-0,0770	6,47	*0,0000*
Control	156	0,1562	0,4757	16,55	0,4828
Control	157	0,1272	0,5888	22,72	0,5080
Control	158	0,1262	0,5271	26,32	0,3807
Control	159	0,0337	0,4527	16,29	0,6429
Control	160	0,0166	0,3314	21,55	0,3653
Median					0,4828
Mean					0,4210
SD					0,2098

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
HT	161	0,0936	0,5586	18,24	0,6373
HT	162	-0,0250	0,2253	15,93	0,3928
HT	163	0,1454	0,4984	20,57	0,4291
HT	164	0,1144	0,5183	22,76	0,4437
HT	166	0,0413	0,4663	24,54	0,4330
HT	167	0,0347	0,4090	11,80	0,7929
HT	168	0,0537	0,4938	20,32	0,5414
Median					0,4437
Mean					0,5243
SD					0,1451

Tank	Sample	OD min	OD max	mg/ml protein	Catalase activity
HU	171	0,1442	0,4046	21,87	0,2976
HU	172	0,0919	0,5300	19,51	0,5614
HU	173	-0,0290	0,3895	14,95	0,6997
HU	174	0,0795	0,5506	20,22	0,5826
HU	175	0,0276	0,4428	21,12	0,4915
HU	177	0,0106	0,4798	16,84	0,6965
HU	178	0,0291	0,4588	20,27	0,5300
Median					0,5614
Mean					0,5513
SD					0,1370