Effects of North Sea Oil on Biotransformation and Immune Responses in Juvenile Atlantic Cod *(Gadus morhua)*

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Abstract

The purpose of this study was to characterise biotransformation and immune responses in juvenile Atlantic cod (*Gadus morhu*a) following exposure for 2, 8 and 16 days to the water-soluble fraction of North Sea crude oil in different concentrations. Biomarkers (ELISA, hepatic EROD activity and PAH metabolites in bile) were used to examine effects. The study also included oil-related effects on DNA by assessing DNA damage with the comet assay. A third aspect of the study was quantifying immune responses related to oil exposure, which was done by measuring respiratory burst in leukocytes. A pathogen challenge in the form of LPS was injected after 8 days to assess if the immunocompetence changed as a result of oil exposure.

The biomarkers of exposure were successful in linking oil treatments with elevated PAH metabolite levels, EROD activity and a change in CYP1A levels. There was no link between increased DNA damage and exposure to the WAF of oil. Furthermore, there were no treatment effects on respiratory burst. Injection of LPS did not appear to change immunocompetence.
In total, the study provided good insight into effects of the WAF of crude oil on biotransformation pathways in cod as well as assessing immune responses, although the latter may need further studies to successfully contribute to risk assessment.

Preface

This master degree has been a huge learning experience for me, one which I have found to be both interesting and, from time to time, challenging. I have truly enjoyed working on this project, especially as I have constantly had people around me that have motivated me. I want to direct my whole-hearted gratitude to my supervisor Ketil Hylland and my co-supervisor Tor Fredrik Holth for their continuous patience, support and guidance. Without them there would have been no project. Something that made this project even more exciting was that I was given the chance to travel not just once, but twice! I am very grateful to Halldór Pálmar Halldórsson and Ásdís Ólafsdóttir at the Sandgerði Marine Center in Iceland as well as Concepción Martínez-Gómez at Centro Oceanográfico de Murcia who were all tremendously welcoming and helpful. I highly enjoyed making their acquaintance and have learned a lot from both of them.
I also want to direct my appreciation to Merete Grung at NIVA for doing the HPLC analysis on the bile samples. It was truly helpful!

A special thanks goes to Audun Storset who has been my colleague and sparring-partner during the whole project, and who was always ready with a comforting hug or pat on the back when the statistics were not cooperating.
Lastly but not least, I could not have done this project without the unconditional support of my friends and family, so a great personal thanks to Karen Ribeiro, Johanne Pereira Ribeiro and Oscar Pereira-Laursen. Also, a big thanks to Estelle Grønneberg and Tor Sudmeyer who, in addition to their never ending encouragement, helped me greatly by proofreading the thesis.

Abbreviations

ANOVA – Analysis of variance
B[a]P - Benzo(a)pyrene
BSA - Bovine serum albumin
CCM – Cell culture medium
CYP1A - Cytochrome P4501A
DCM - Dichloromethane
DMSO – Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DTT - Dithiothreitol
EDTA - Ethylenediaminetetraacetic acid
ELISA - Enzyme-linked immunosorbent assay
EROD - Ethoxyresorufin-O-deethylase
GAR-HRP – Goat-anti-rabbit-horseradish peroxidase
HPLC - High-Performance Liquid Chromatography
ISD – Intern standard
KPO-buffer – Potassium phosphate buffer (K_2HPO_4/KH_2PO_4)
KRPG-buffer - Krebs Ringer's phosphate glucose buffer
LMP – Low melting point
LPS - Lipopolysaccharides
NADPH - Nicotinamide adenine dinucleotide phosphate
NIVA – Norwegian Institute for Water Research
NSC oil – North Sea crude oil
OD – Optical density
PAHs - Polycyclic aromatic hydrocarbons
PBS - Phosphate buffered saline
PMA - phorbol 12-myristate 13-acetate
RB - reaction buffer
Reagent A - Alkaline copper tartrate solution
Reagent B - Folin reagent
ROS – Reactive oxygen species
TE-buffer - (Tris-EDTA buffer)
TI – Tail intensity
TMB – Tetramethylbenzidine liquid substrate
TTBS - Tris-tween buffered saline

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1 Introduction

1.1 Concerns Regarding Oil in Marine Environments

Both fisheries and offshore activities involving oil and gas are huge industries, especially in Norway. It is important to keep the conflicts between these to a minimum by monitoring and controlling the contaminant inputs and effects (Hylland et al. 2008). The marine environments surrounding Norway have become an area of concern due to the high amount of petroleum-related activities and the localization of large oil and gas reserves in the Arctic (Nahrgang et al. 2010). Oil spills and extraction activities represent a threat to aquatic organisms, as spills to the marine environment expose them to the water-soluble fraction of the oil (Nahrgang et al. 2010). Marine organisms will often be exposed to low concentrations due to the dilution that occurs when the discharged products are mixed with the surrounding seawater (Hylland et al. 2008) but nonetheless, it is important to continue monitoring and further develop biomarkers for PAHs to investigate effects in organisms in exposed areas (Nahrgang et al. 2010). Further, when accidental oil spills occur, the concentration may be much higher than the background concentration present from continuous discharges from platforms. Discharges are usually in the form of produced water, which in addition to PAHs contain alkyphenols, metals and production chemicals (Hylland 2006).

1.2 Biomarkers and Immune Responses

Biomarkers to detect effects of PAHs have been increasingly used in studies during the last decades. Biomarkers generally function as a link between the contaminant and the biological effects of the contaminant; for this reason, they can be used to detect exposure and effects (Bucheli et al. 1995). Phase I enzymes such as hepatic EROD activity and CYP1A levels, as well as biotransformation products such as PAH metabolite levels in bile, have been proven to be valuable

and some of the most efficient fish biomarkers for environmental risk assessment (Van der Oost et al. 2003). Studies using biomarkers may continue to contribute to gaining further knowledge about exposure effects and routes but as organisms in their natural environments are continuously exposed to pathogens and parasites, a good immune system and immunocompetence is vital, and has been directly linked with minimized fitness costs (Owens et al. 1999, Graham et al. 2010). More insight into immune responses and their possible correlations to other biomarkers will further contribute to risk assessment, especially in aquaculture.

The immune system in fish and other vertebrates is divided into an innate and an adaptive part, both of which contain humoral, and cellular factors. The responses of the adaptive system are usually slower but more specific and can therefore make way for high variation of somatic antigen-binding receptors from only few genes (Litman 2010). In general cellular factors are immune cells such as macrophages, natural killer cells or granulocytes, while humoral factors may be the acute-phase proteins of the complement system that can be activated to attack intruding microorganisms, cytokines and chemokines, lysozyme or others (Segner et al. 2012). The humoral defence in fish contains certain non-specific factors that, even though they are generally not fundamentally different from those in higher vertebrates such as mammals, do contain some different features (Yano 1996). For instance, the optimal complement activity temperature in fish is different than that of mammals due to the temperature of the water surrounding them (Rijkers 1982, Koppenheffer 1987).

By using immune related biomarkers in ecotoxicological studies, some insight can hopefully be provided into how the immune system in marine species is affected by pollutants. Even though knowledge of the immune system in fish has grown during the last years, as of now, there is still limited understanding of the degree to which exposure to environmental pollutants leads to modulation of the immune system in marine fish species in aquaculture. Due to this, the understanding of how resistance to diseases is affected by environmental pollutants is correspondingly inadequate (Segner et al. 2012). Since modulation

of the immune system is an important parameter essential for fish populations, the area needs to be studied further. Perhaps one reason that there is still a lack of knowledge in this area is due to the complexity of the immune system, and the fact that immunotoxic effects need to be determined by specific techniques and endpoints. It is important to choose the right parameters and determine how the meaning of these may represent the individual's entire immunocompetence (Segner et al. 2012). International groups have investigated the effects of oil pollution, and the effects of PAHs in various fish species. One such study investigated the effects of oils (heavy fuel oil and light cycle oil) and pure PAHs on humoral immune parameters (lysozyme concentration and complement activity) in European sea bass, and demonstrated that several of them were modulating these immune parameters (Bado-Nilles et al. 2009). Moreover, a recent study has further linked exposure to light cycle oils with inflammatory phenomena in the same species (Bodo-Nilles et al. 2011). A study on acute effects of oil exposure, also in European sea bass, showed similar results, as well as indicating that even after a recovery period in clean sea water, contaminated fish still contained levels of substances such as naphthalene and benzo[a]pyrene high enough to pose a risk for human consumption (Danion et al. 2011). Adverse effects have also been demonstrated in other species such as in zebrafish *(Danio rerio)* exposed to produced water (a waste from oil and gas production that contains oil derived substances such as PAHs). These showed effects in several vital systems (nervous, respiratory and immune system) as well as possible reduction in gene transcription and other adverse effects (Holth et al. 2008). Many of the same results were also apparent in juvenile Atlantic cod exposed to oil and produced water, in a treatment that resulted in significantly induced protein changes even at low levels (Bohne-Kjersem et al. 2009). A study of PAHs effects on an endangered species of salmon (Chinook salmon) suggested that PAHs in the food chain are a potential source of injury to organisms (Yanagida et al. 2012). The project of this thesis has focused on how oil pollution affects both biotransformation and immune responses in juvenile Atlantic cod. It was coordinated with another project set to begin simultaneously: the MSc-project of Audun Storset, which looked into the same effects on the species turbot. This should allow for interspecies comparisons.

1.3 Experimental species

The species chosen for the study was juvenile Atlantic cod (*Gadus morhua*), obtained from Aquaculture Research Station, Marine Research Institute, S Grindavik, Iceland. Throughout its lifecycle it can be found in several different habitats, its lifecycle involves spawning periods in the first quarter of the year until spring season starts around April (Brander 1994). The eggs are planktonic and take 1-3 weeks to spawn. Larvae diet consists of zooplankton until a larger juvenile stage is reached where the diet then expands to also involve larger zooplankton such as small crustaceans like shrimp as well as worms (Hop et al. 1992). The juvenile cod reach a mature stage when they are 3-4 years old and the spawning cycle may start again. At the mature stage the Atlantic cod could be classified as a top predator with a diet that includes several species of forage fish in addition to previously mentioned organisms (Köster et al. 2001).

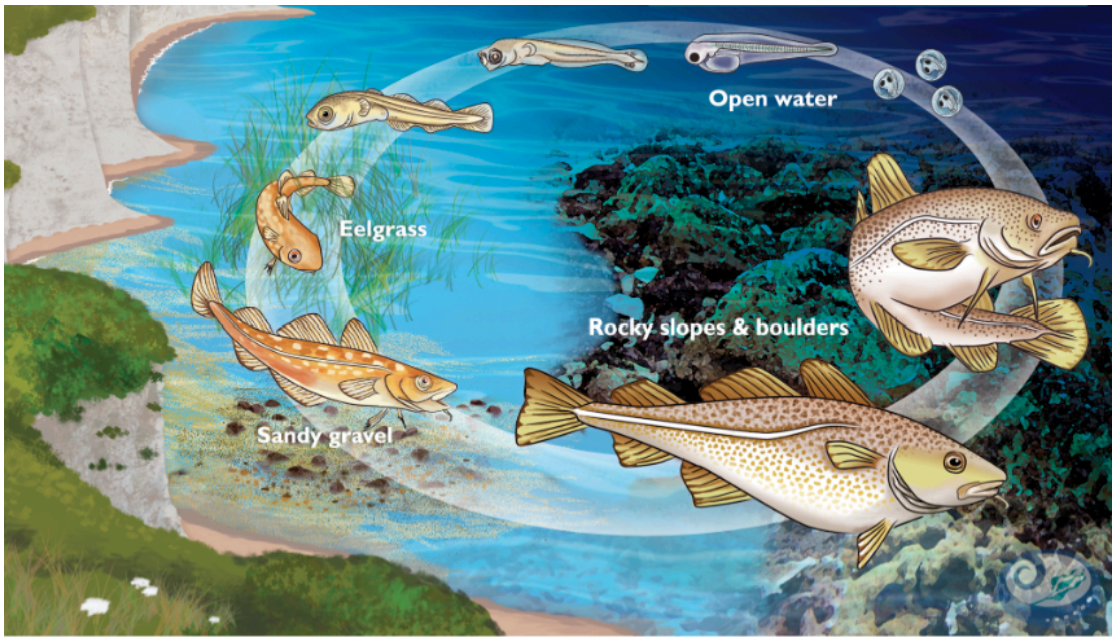

Figure 1.1 *Lifecycle of the Atlantic cod, image used with permission from artist (PISCO 2011).*

1.4 Aims

The project aimed to quantify biomarker responses in cod exposed to the WAF of NSC oil, as well as to identify selected immune responses in cod *(Gadus morhua)*. The project also aimed to quantify the effect of a pathogen (challenge) on the same species following exposure to the water-soluble fraction of crude oil.

The overall goals of the project and the null hypotheses related to each goal were as follows:

1) Quantify biomarker responses for exposure to oil and PAHs and the response pattern
   1.1) Quantifying EROD activity for different treatment levels and exposure times.
   H_0: *Hepatic EROD activity is not treatment related.*
   H_0: *Hepatic EROD activity is the same for each treatment at different exposure periods.*

   1.2) Quantifying CYP1A levels for different treatment levels and exposure times.
   H_0: *Hepatic CYP1A protein levels are not treatment related.*
   H_0: *Hepatic CYP1A protein levels are the same for each treatment at different exposure periods (there is no effect of varied exposure time).*

   1.3) Quantifying PAH metabolites in bile for different treatment levels and exposure times.
   H_0: *PAH metabolite levels in bile are not treatment related.*
   H_0: *PAH metabolite levels in bile are the same for each treatment at different exposure periods (there is no effect of varied exposure time).*

2) Investigating how oil exposure affects amount of DNA damage in leukocytes.

   H_0: *There is no difference in DNA damage between treated groups.*

   H_0: *DNA damage is not related to exposure period (there is no effect of different exposure times).*

3) Quantify immune responses following exposure to water-soluble components of crude oil.

   H_0: *There will be no differences in H_2O_2 production between PMA stimulated and non-stimulated leukocytes.*

   H_0: *There will be no treatment related effects on leukocyte H_2O_2 production.*

   H_0: *Treatment effects related to leukocytes ability to handle oxidative stress will be the same at each exposure period (there is no effect of varied exposure time).*

4) Determine if the immunocompetence changes after exposure to the WAF of oil by a challenge (pathogen).

   H_0: *There will be no effect of injection in biomarkers or immune responses.*

5) Investigate species differences in biotransformation and immune responses between Atlantic cod *(Gadhus morhua)* and turbot *(Scophthalmus maximus).*

   H_0: *There will be no species differences between Atlantic cod (Gadhus morhua) and turbot (Scophthalmus maximus).*

2 Materials and Methods

2.1 Exposure conditions

The experiments were conducted at the Marine University Centre in Sandgerði, Iceland. The North Atlantic and the Arctic Oceans that surround Iceland are naturally clean and have low background levels of PAHs compared to the Baltic Sea (Witt 1995, Lohmann et al. 2009). The seawater is pumped from a borehole extending 20 m. into the ground and is naturally filtered through the porous lava. It is aerated upon arrival at the marine station and is free from particles and anthropogenic material (Halldórsson 2014)

Juvenile Atlantic cod (*Gadus morhua*) was obtained from Aquaculture Research Station, Marine Research Institute, Grindavik, Iceland.

During the exposure experiment, the fish were fed with small pieces of defrosted shrimp following a feeding schedule where the animals were fed until 2 days prior to sampling. The animals were starved during the last 2 days before each sampling to avoid emptying of the bile.

2.2 Experiment setup

The fish were exposed to three different concentrations of the water-soluble fraction of North Sea crude oil (n=6). The control group (n=6) was kept in clean seawater that flowed through the same type of column as the exposed groups. The tanks were 20L, whole-glass aquaria measuring 23 x 36 x 26 cm. Each tank contained 4 cod, giving an overall number of 96 fish used for the experiment. As the project was coordinated with the M.Sc. projects of Audun Storset and Ásdís Ólafsdóttir, each tank also contained 4 juvenile turbot (*Scophthalmus maximus*) and 6 blue mussels (*Mytilus edulis*). Additionally, 8 fish were sampled as 0-samples before the experiment started. These were acclimated and fed the same

way as the fish used in the experiment, but were sampled from a larger main tank.

Table 1.1 *The various treatments used with their relative concentration of NSC oil.*

Treatment	Concentration
Control	0 g NSC kg^{-1} gravel
Low	6 g NSC kg^{-1} gravel
Medium	12 g NSC kg^{-1} gravel
High	24 g NSC kg^{-1} gravel

The oil was pre-treated by mixing it with the solvent dichloromethane (DCM) and water on a magnetic stirrer for 24 h to simulate weathering (Holth et al. 2014). Gravel was spread out and treated with the appropriate amount of oil using and then left to dry for 24 h (table 1). The gravel was then filled into cylinders (d x h: 75 x 350 mm) and each one connected to a tank that would then be connected to the flow-through system where the input flow of clean seawater was set to 200 mL min^{-1} and the exposure flow set to 50 mL min^{-1}. The exposure flow was led by tubes from the pumps and through the relative column associated with each tank. Together the input of clean and contaminated water was thus 250 mL min^{-1}. The flow of water into the tanks was measured twice a week to ensure stable exposure conditions throughout the experiment. Seawater was pumped through the cylinders and into the tanks. The tubes connected to the peristaltic pumps were inspected for leaks every day. Samples were taken after 2, 8 and 16 days of exposure.

2.3 Sampling

Each fish was carefully removed from its tank, and euthanized by a sharp blow to the head, behind the eyes. Fish weight and head-to-tail length was measured and mucus collected from the area in front of the dorsal fin using a cell scraper. All surfaces and dissection tools were thoroughly cleaned and gloves were changed between every fish sample to avoid cross-contamination. Collected mucus was

diluted in 5x tris-buffer in a cryo-tube and flash-frozen on liquid nitrogen. Peripheral blood (0.2 mL) was extracted from the caudal vein using syringes prepared with heparin to avoid blood clotting within the syringe while extracting the blood. After extraction, the cannula was removed and the blood diluted in 0,3 mL cell culture media (CCM) that was prepared by adjusting Leibovitz L-15 medium to 380 mOsm by adding 1.5M NaCl, then adding 1 ul heparin stock (10 kU/mL) per mL osmolality adjusted L-15 medium, giving 10 U/mL, which again had 10 uL antibiotics solution added pr. mL. The CCM would then contain 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 ug/mL amphotericin B. Tissue and bile samples were collected after opening the abdomen. The entire gall bladder was collected in an eppendorf tube, using a surgical scissor while tweezing together the bile duct to avoid spillage of bile and contamination of the remaining organs. The liver was then excised and the area between the portal vein entrances sampled. The head kidney was excised using tweezers and collected by ventral incision.

2.4 Pathogen challenge study

After 13 days of exposure there were 2 fish remaining in each tank. One of these was injected intraperitoneally with a sham pathogen (lipopolysaccharide LPS). The amount of injected pathogen was 20 ul/g with an initial concentration of 1 mg/mL. However, as the volume required relative to the weight turned out to be quite high and caused some discomfort to the fish (which showed in reduced swimming ability), the concentration was doubled to 2 mg/mL to reduce the volume needed for injection to 10 ul/g. The fish were injected carefully so as to not puncture any internal organs, but nevertheless as swiftly as possible to reduce the amount of stress on the animal. To further reduce stress, the head region of the fish was protected with a moist cover until the injection was over. For detecting eventual effects of the procedure on biomarkers, the remaining fish were injected with phosphate buffered saline solution (PBS) using the same approach. A small fin-clip was done on the dorsal fin of all individuals injected with LPS.

2.5 Extraction of leukocytes from blood

The samples of diluted blood were layered on top of a percoll gradient calculated according to (Sørensen et al. 1997, Munoz et al. 1999, Espelid et al. 2003, Øverland et al. 2010). The gradient was made in a 15 mL Falcon centrifugation tube with 2,5 mL percoll 1,070 g/L (0.490 mL percoll + 1.5M NaCl + 0.410 mL H_2O_2) in the bottom layer and 2,0 mL percoll 1,050 g/L (0.337 mL percoll + 1.5M NaCl + 0.563 mL H_2O_2) in the upper layer, making the total volume of the tube 5 mL. The tube was placed in a swing-out rotor centrifuge and centrifuged at 400 x *g* for 40 min. at room temperature.
Leukocytes then became visible as a white band in the 1.050-1.070 percoll interface and were collected using a plastic Pasteur pipette, transferred to a marked eppendorf tube and diluted 2x in CCM. The tubes were centrifuged for 5 min. at 400 x *g* in room temperature. A pellet of cells was obtained in the bottom of the tubes and was resuspended in 1,5 mL CCM.
To count and assess viability of the cells, 15 µL of each sample was added 15 µL Trypan blue and loaded onto a slide for measurement with an automated cell counter (Invitrogen Countess® Automated Cell Counter). Samples were diluted to 1*10^6 cells/mL with CCM.

2.6 Extraction of leukocytes from kidney

The extracted head kidney tissue was added to 1 mL CCM, which was minced through a 100 µm nylon cell strainer using another 4 mL of CCM, giving a total volume of 5 mL before adding the suspended kidney leukocytes to a tube with the same percol gradient as previously mentioned. Following, the process was the same as described in section 2.5.

2.7 Respiratory burst

The assay was based on the Invitrogen Amplex Red Hydrogen assay, where peroxidase reacts with the added substrate Ampliflu Red and forms the very fluorescent compound resorufin. H_2O_2 stock (20 mM) was diluted to 10 uM with reaction buffer and a 2x dilution series with 7 concentrations was then made. The 2.5 uM (dilution no 3) was discarded. One-hundred uL blank assay solution (0.1 U mL^{-1} HRP in KRPG-buffer containing 145 mM NaCl, 4.86 mM KCl, 5.7 mM $NaH_2PO4x2H_2O$, 0.54 mM $CaCl_2$, 1.22 mM MgSO4x7H2O, 5.5 mM glucose and pH adjusted to 7.4) was added to wells A1-A3 of a NUNCLON 96-well microtiter plater. Assay working solution (50 uM Amplex Red; 0.1 U mL^{-1} HRP in KRPG-buffer was prepared and added to wells B1-H3 and rows B, D, F and H on the microtiter plate (100 uL in each well). Standard curve (H_2O_2 dilution series) was added in triplets for each concentration in wells C1-H3 and blank (RB) was in wells B1-B3. Twenty uL of samples diluted to $1*10^6$ cells mL^{-1} with CCM were added to wells A4-H12. Each sample was added in 2 triplets. A random sample was also added to wells A1-A3 to test that there would be no reaction when Amplex Red was not added.
Finally an assay working solution, containing PMA (assay working solution + 0.12 ug PMA per mL^{-1}) was added to rows A, C, E and G (coloumn 4-12).
The fluorescence of the product was then read in a plate reader (BioTek Synergy MX) at 530 nm/590 nm. As the the reactions flattened out after 15 min., the fluorescence was read immediately after all assay components were added to the plate.

2.8 Comet assay

After dilution, 10 µL of each sample was suspended in 90 µL LMP agarose (0,75% in PBS) on GelBond films. When the agarose had solidified, the films were put in boxes containing prechilled lysis buffer (2.5 M NaC, 100 mM Na_2 EDTA, 10 mM Trizma base, 1% Triton X-100 with pH 10) at 4 °C. The samples were rinsed briefly in electrophoresis buffer (300 mM NaOH, 1 mM EDTA with pH approx.

13.2) for unwinding and denaturation of DNA. The samples were then transferred to a fresh electrophoresis buffer in the electrophoresis chamber for a total of 20 min. Horizontal electrophoresis was run at 4°C for 20 min. with 25V applied. The films were removed and rinsed in a neutralisation buffer (0.4 M Trizma base in dH_2O adjusted to pH 7.5) at room temperature and then stored in a fresh neutralisation for 15 min. After a brief rinse with first dH_2O and then 96% ethanol, the films were fixed for a minimum of 1 ½ hours in 96% ethanol and then air dried for storage until staining would take place.

The films were evaluated by staining them in a darkroom for 20 min. using 50 mL TE-buffer (10mM Tris-Hcl with PH 8 and 1mM EDTA with pH 8 the buffer was finally adjusted to pH 8 before use) containing 40 µL SyberGold (prediluted 10.000x from stock in DMSO). To make sure the dye was distributed to the entire film, the staining boxes were placed on a rocking table during the staining period. After rinsing the films with dH_2O, they were placed on plexi-glass plates, and cover slides were applied with dH_2O, while avoiding air bubbles.
The films were then scored using the CometAssay IV software (Perceptive Instruments), linked to a fluorescence microscope (Zeiss). Scoring was done using 20x magnification and 50 cells were scored for each sample.

2.9 PAH metabolites from bile

The bile samples were stored at -20°C until the analysis commenced, at which point they were thawed on ice in the dark. Twenty µL of extracted bile was pipetted and mixed with 50 µL dH_2O in a small eppendorf tube. The weight of the bile was recorded using a balance of precision. 10 µL of standard (Trifenylamine 10 mg pr. 25 mL methanol) was added to the tube and the weight was recorded. 20 µL of the enzyme β-glucuronidase/aryl sulphatase was added and the weight was recorded. The samples were then incubated for 60 min. at 37°C.
To stop the reaction, 200 µL of methanol was added to each tube, and the contents were thoroughly mixed. Samples were then cooled on ice. The samples were centrifuged for 10 min. at 4°C at 4000 *g*. The supernatant from each tube

was transferred to HPLC tubes and all samples were analysed by Merete Grung at NIVA, using high-performance liquid chromatography (HPLC) with fluorescence detection. The samples were stored at -20°C until analysis. The HPLC analysis was done in a PAH C18 column with a precoloumn (Vydac 201TP5415, 5 µm particle size, 4,6x250 mm). Fluorescence detection was done at wavelengths shown in table 1.2.

Table 1.2 HPLC Fluorescence detection; excitation and emission for metabolites.

Metabolite	Excitation nm	Emission nm
Naphtalene	325	358
Phenatrene	251	364
Pyrene	246	384
B[a]P	380	430
ISD picene	282	375
ISD trifenylamin	300	360

Injection volume was 25 ul and the column temperature was 30°C. The samples were analysed with 2 mobile phases, first a 40:60% w/w acetonitrile and water and second a 100% acetonitrile mobile phase. The flow was 1 mL/min and the reservoir was degassed continuously with helium (25 ml min^{-1}) during the analysis. The tops of each produced curve was used to quantify the amount of metabolite.

2.10 Preparation of cytosolic and microsomal extract from liver

The frozen liver samples (approx. 0.1 g) were each transferred to a 2-ml tube containing approx. 20 homogenisation beads (Precellys 24 Soft Tissue homogenizing 1.4 mm ceramic beads, Bertin Technologies) and a homogenisation-buffer (0.1 M NaPO buffer, 0.15M KCL, 1mM DTT, glycerol (5%w/w) and pH adjusted to 7.8). The liver was then homogenized in a Precellys homogenizer (Bertin Technologies). The machine was set up to run 3 rounds of 10 sec. each at 6000 rpm with 5 sec. break between each round. Sample

temperature was kept stable at 4°C by pumping cold air, cooled by liquid nitrogen onto the samples using a Cryolys cooler (Bertin Technologies). The homogenized samples were transferred to eppendorf tubes, while at the same time removing the plastic beads from the sample by pipetting.
The samples were centrifuged for 30 min. at 4°C at 10.000 *g* in a Heareus Multifuge 3 S-R centrifuge.
Using a Pasteur glass pipette, the fatty layer was carefully penetrated and the supernatant was collected and transferred to a centrifugation tube. The supernatant was centrifuged for 60 min. at 4°C at a speed of 100.000 x *g* (Thermo Scientific Sorvall mTX150 Micro-Ultrasentrifuge). The supernatant was removed and the microsomal pellet transferred to an eppendorf tube along with 200 µL of microsomal buffer (0.1 M NaPO buffer with glycerol (20% w/w) added and pH adjusted to 7.5). The sample was then homogenized using a pellet pestle. For each sample, 30 µL was collected for ELISA analysis, 30 µL was collected for protein quantification and at least 60 µL was collected for EROD analysis.

2.11 Protein analysis

Samples were defrosted from -80°C and diluted with 0.1M Tris buffer (pH 8.0 at 4°C). BSA standard (bovine gamma globuline protein standard) 15 mg/mL was also defrosted from -80°C and diluted to 1000 µg/mL with 0.1M Tris buffer. A 2x dilution series was included, and a reference sample of approx. 0,4 µg/mL was included in all analyses. All work was done on ice. Four replicates of blank (0.1M Tris buffer), 3 replicates of each standard, 3 replicates of each diluted sample and 3 replicates of reference sample were then pipetted onto a 96 well microtiter plate with 10 µL in each well.
Twenty-five µL of room-tempered reagent A (BioRad (kit. No. 5000-0111)) was then pipetted into all wells, using a multipipette. Next, 200 µL of reagent B was pipetted into all wells. The plate was gently agitated by hand and set to incubate at room temperature for 15 minutes. After the incubation period, absorbance was read at 750 nm using a BioTek Synergy MX plate reader and the Gen 5

software (ver. 4.0). Samples outside the standard curve were diluted further and reanalysed.

2.12 Ethoxyresorufin *O*-deethylase (EROD) activity

Microsomes were diluted on ice in 0.1M potassium phosphate buffer (pH 8.0) to 1 mg/mL protein for EROD analysis. The reaction solution was prepared at room temperature in the dark (as both 7-ethoxyresorufin and resorufin are light sensitive) using 30 mL KPO-buffer (0.1M, pH 8.0) and 180 uL 0.5mM 7-ethoxyresorufin (in DMSO). The standard (1mM resorufin in DMSO) was defrosted from -20°C, and diluted to 0.64 µM in two steps (first 100x to 10 µM in potassium phosphate buffer, then to 0.64 µM in reaction solution). The 0.64 µM standard was then further diluted in a 2x dilution series to give the following concentration series: 0.64, 0.32, 0.16, 0.08, 0,04, 0.02, 0.01 and 0.00. Eight replicates (50 ul) of potassium phosphate buffer (blank) were distributed to a NUNC 96-well microtiter plate (Thermo Scientific). Resourufin-standards were then pipetted in duplicates to the plate (75 µL was added to each well). Diluted samples and reference samples (cod samples from the M.sc. project of Lene Fredriksen that had been diluted 10x) were then pipetted onto the plates with 6 replicates for each sample, 50 µL in each well. Three replicates of each sample and reference then had 10 µL 0,32 µM resorufin added to them. Reaction solution was distributed to all wells (200 µL) except for wells containing resorufin standards. The reaction was started by the addition of NADPH solution (2,4 mM NADPH diluted in potassium-phosphate buffer), 25 µL per well. Fluorescence was read in a platereader (BioTek Synergy MX) immediately thereafter with the following settings: Excitation 530 nm, emission 590 nm, with 20 nm slit width and optics position set to “top”. Sensitivity was automatically related to the fluorescence of the highest concentration of resorufin and the plate reader (BioTek Synergy MX) was set to shake for 10 sec at medium intensity before the first kinetic read. There were a total of 8 read intervals and the total read time was 5 min.

2.13 Concentration of CYP1A

Liver microsomes were diluted to 10 µg protein/mL in carbonate-bicarbonate buffer (0.05M, pH 9.6 at 25°C). One hundred µL of coating buffer were pipetted into all wells in the 1st column of the plates. 4 of these would later have primary antibody added to them while the rest would be left with no primary antibody to monitor non-specific binding. 4 replicates of each diluted sample were then distributed to 4 wells on a 96-well microtiter plate, with 100 µL in each well. Additionally, a reference sample was added with 4 replicates (each containing 100 µL). The plate was incubated overnight at 4°C with tape covering each well to avoid evaporation.

On day 2 the plate was washed (using the Scanwasher 300) on a setting that washed out the plate 3 times with TTBS consisting of 20mM Tris-buffer pH 8.5 with 0.5M NaCl all diluted in 10 L dH_2O with 5 mL Tween-20 added. A blocking solution (TTBS with 1% w/w BSA) was then added to all wells (300 µL in each well) and set to incubate for 60 min. at room temperature. The plate was washed another 3x before primary antibody (rabbit anti-fish CYP1A antibody diluted 1:1000 in antibody buffer containing TTBS with 0.1% w/w BSA) was pipetted to all wells (100 µL in each well) containing samples. The primary antibody was also distributed to the 4 replicates in the first column of the plate.

The ScanWasher 300 aspirated all liquid from the plate so the antibody was added to empty, but coated wells. The plate was then set to incubate overnight at 4°C with tape as per the first day.

After the overnight incubation, plates were recovered from 4°C and washed 3x with the Scanwasher 300 using TTBS. 100 µL of diluted (1:3000 in antibody buffer) secondary antibody (goat anti-rabbit GAR-HRP) was added to all wells. The plates were then set to incubate for 7 ½ hours at 4°C with tape covering them. After the incubation period plates were washed 5x. One hundred µL of colouring buffer (TMB) was added to all wells in a darkroom and the plates were incubated at room temperature until colour developed (this took 18 min.). When an appropriate amount of colour had developed, the reaction was stopped by

adding 100 ul 0.18M H_2SO_4 to all wells. The absorbance was read in the plate reader (BioTek Synergy MX) at 450 nm.

2.15 Statistical Analyses

Results were analysed using a set of rules that were followed consequently. All data was log10 transformed for continuity in the data representation and easier comparison between groups. Performing a Levene's test assessed the variances in the data. This is a statistical test where the hypothesis is that the variance in the data is equal. If the p-value of this test is significant (<0,05), it means that the variance is not equal and measures must be taken to achieve equal variances. When this was not possible, it was necessary to run a non-parametric analysis (Kruskal-Wallis test). In most cases, however, the already transformed data had equal variance so a parametric one-way ANOVA could be run, comparing treated groups to the control group. Should there be a significant difference between these, a Dunnett's test could be run additionally to assess *which* treatment groups were significantly different from the control. In the case of the respiratory burst assay, there was more than one parameter, so a two-way ANOVA was used. Statistical analyses were performed using JMP (version 11.0 by SAS Institute Inc) software and graphs were made using GraphPad PRISM (version 6.0).

3 Results

3.1 Size and Weight

The fish were weighed and their head-to-tail length measured and recorded for reference. Average weight was 30.8 g and average length was 15.6 cm.

3.2 Hepatic 7-ethoxyresorufin-O-deethylase (EROD) Activity

Control groups for all exposure days (with the control for 16 days split into 2 groups, one injected with LPS and the other injected with PBS) were tested against the zero samples. As Levene's test showed equal variances, each control group was tested against the zero samples with a one-way ANOVA. None of the control groups were significantly different from the zero samples (p-value = 0.9617). R^2 for the test was 0.028.

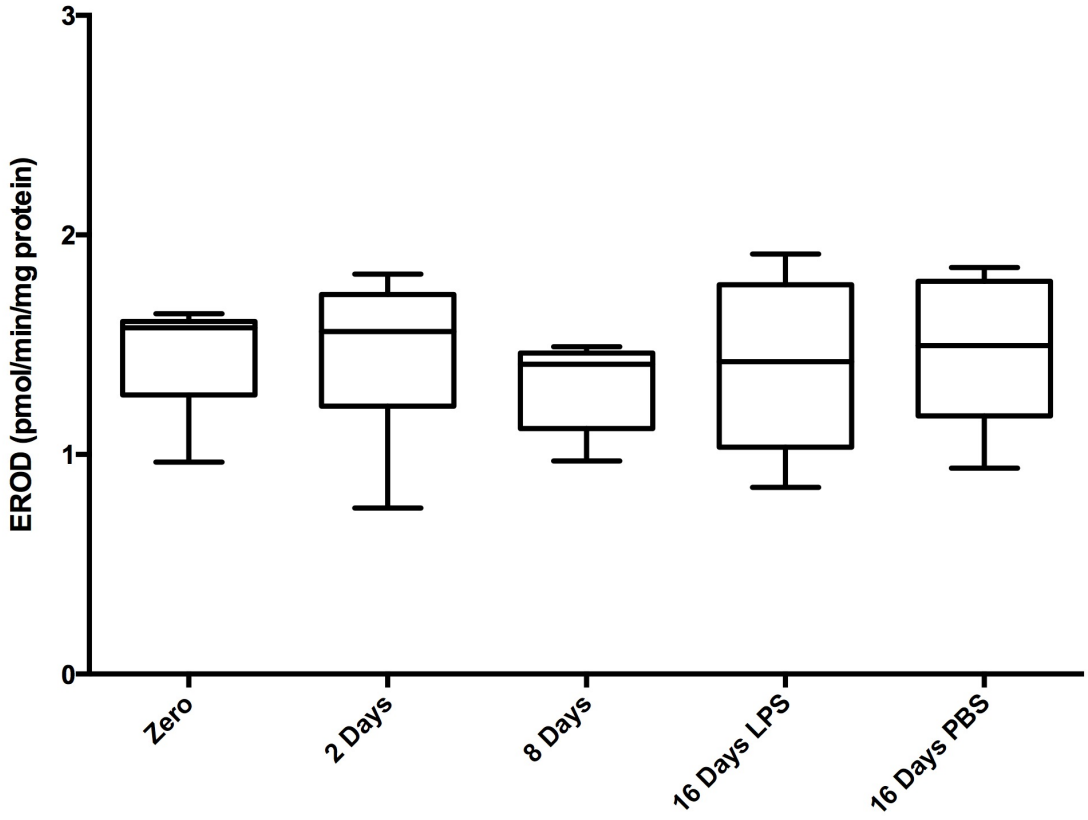

Figure 3.1 *Control groups for 2, 8, 16 days with LPS and 16 days with PBS compared to zero samples; median, quartiles, minimum and maximum.*

Following 2 days of exposure, the Levene's test showed equal variances (p-value = 0.2942) so a one-way ANOVA was run yielding a significant p-value (p-value =0.0284*). A Dunnett's test showed that the low (p-value = 0.0160*) and medium (p-value = 0.0415*) treatment groups were significantly different from the control group. R^2 for the test was 0.358.

The data for 8 days of exposure had equal variances, and a one-way ANOVA was done. The ANOVA had a significant Prob > F value (p-value = < 0.0001*) which indicated a significant difference between treated groups and the control group. A Dunnett's test showed that all treatment groups had significant p-values (low: < 0.0001*, medium: 0.0003* and high: 0.0008*). R^2 for the test was 0.645.

For the 16 days exposure injected groups were analysed separately, as one group had been injected with LPS and the other with PBS. For the LPS group, the Levene's test showed that the data had equal variances so a one-way ANOVA was done. The test statistic for the ANOVA showed a significant test statistic (p-value = 0.0477*) and a Dunnett's test indicated that the High treatment group was significantly different from the control group (p-value = 0.0393*). R^2 for the test was 0.445. The PBS injected group showed equal variances in the data on the Levene's test. The one-way ANOVA was not significant (p-value = 0.1448) and indicated no differences in the treatment groups relative to the control group. R^2 for the test was 0.330.

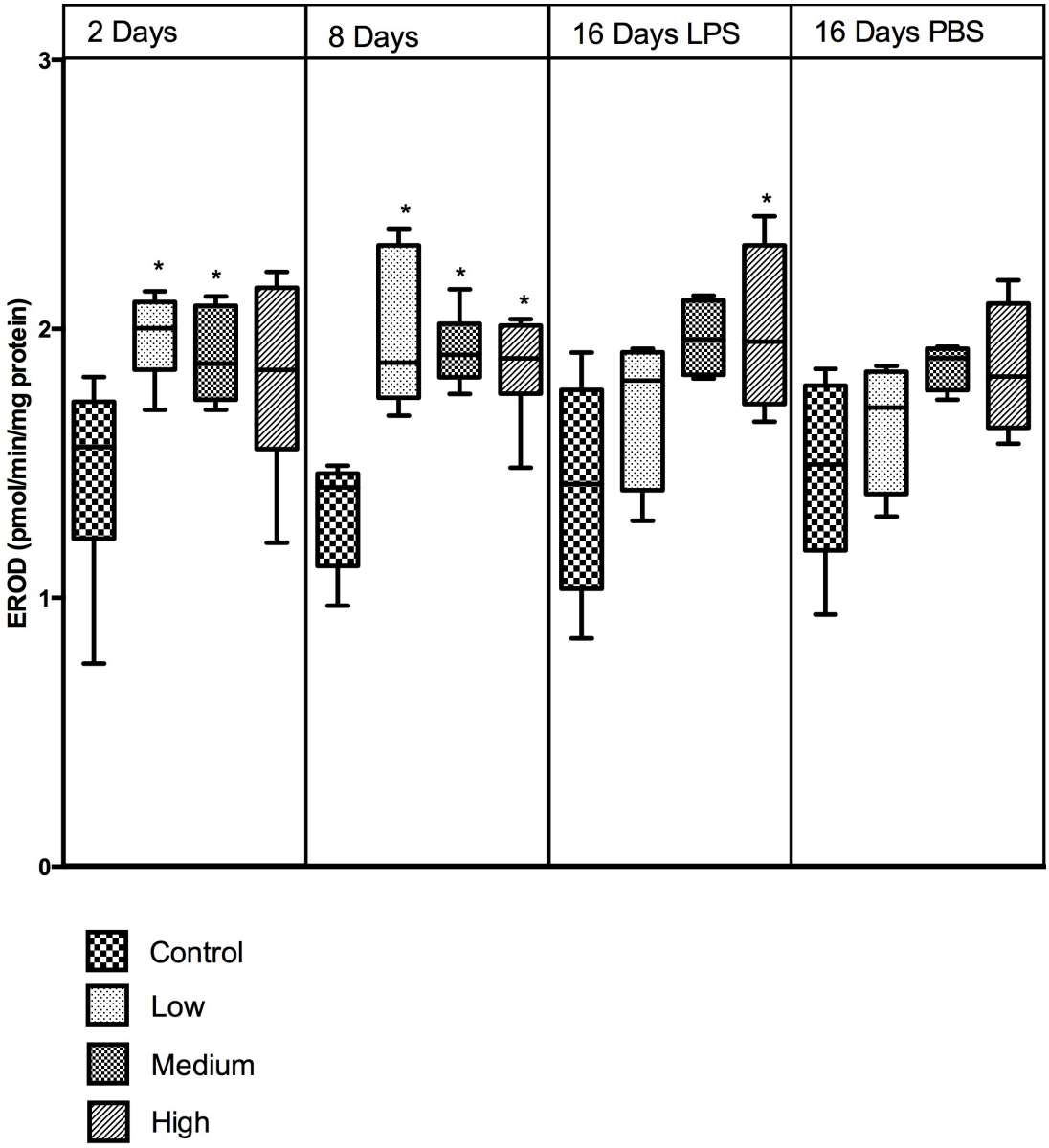

Figure 3.2 *EROD activity in cod exposed to treatment groups for all exposure times (2, 8 and 16 days). Results for 16 days of exposure depicted with only LPS injected samples, and only PBS injected samples. Graph shows medians, quartiles, minimum and maximum. Asterisks indicate treatment groups with significant (< 0.0500) p-values.*

3.3 Concentration of CYP1A

Control groups for all exposure days were tested against the zero samples with a one-way ANOVA after a Levene's test had showed equal variances in the data. The ANOVA was not significant (p-value = 0.9403), showing that none of the control groups were significantly different from the zero samples.
R^2 for the test was 0.030.

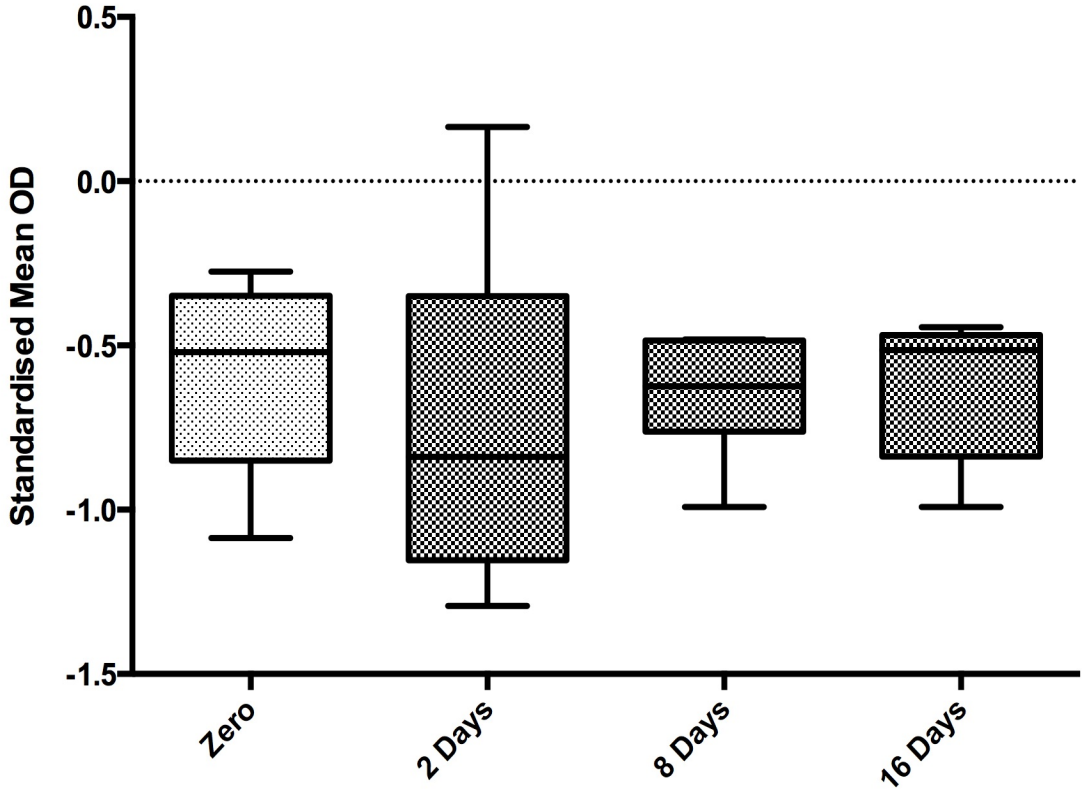

Figure 3.3 *CYP1A concentrations in control groups for all exposure times compared to the zero-samples group. Graph shows medians, quartiles, minimum and maximum.*

For 2 days of exposure the Levene's test showed equal variance. A one-way ANOVA was used to compare the treated groups against the control. The ANOVA was significant (p-value = 0.0024*) and a Dunnet's test showed that the low (p-value = 0.0035*) and the high (p-value = 0.0017*) treatment groups were significantly different than the control group. R^2 for the test was 0.505. The data for 8 days of exposure showed equal variance with Levene's test and a one-way ANOVA further showed that there was a significant difference between

treatment groups and the control (p-value = <0.0001*). A Dunnett's test revealed significant p-values in all treatment groups (low: 0.0014*, medium: 0.0004* and high: <0.0001*). R^2 for the test was 0.66. The PBS injected group exposed for 16 was used to represent the 16 days of exposure group. Levene's test showed equal variances so a one-way ANOVA was performed, and showed a significant treatment effect (p-value = 0.0046*). Dunnet's test further showed that all treatment groups were significantly different from the control group (p-values = 0.0172*, 0.0031* and 0.0137* for low, medium and high treatment group respectively). R^2 for the test was 0.621.

For 16 days of exposure and injected with LPS, the Levene's test showed equal variances in the data. A one-way ANOVA gave a significant test value (p-value = 0.0093*) and a Dunnett's test revealed that all treatment groups were significantly different from the control (p-value = 0.0363*, 0.0056* and 0.0263* for low, medium and high treatment groups respectively). R^2 for the test was 0.580.

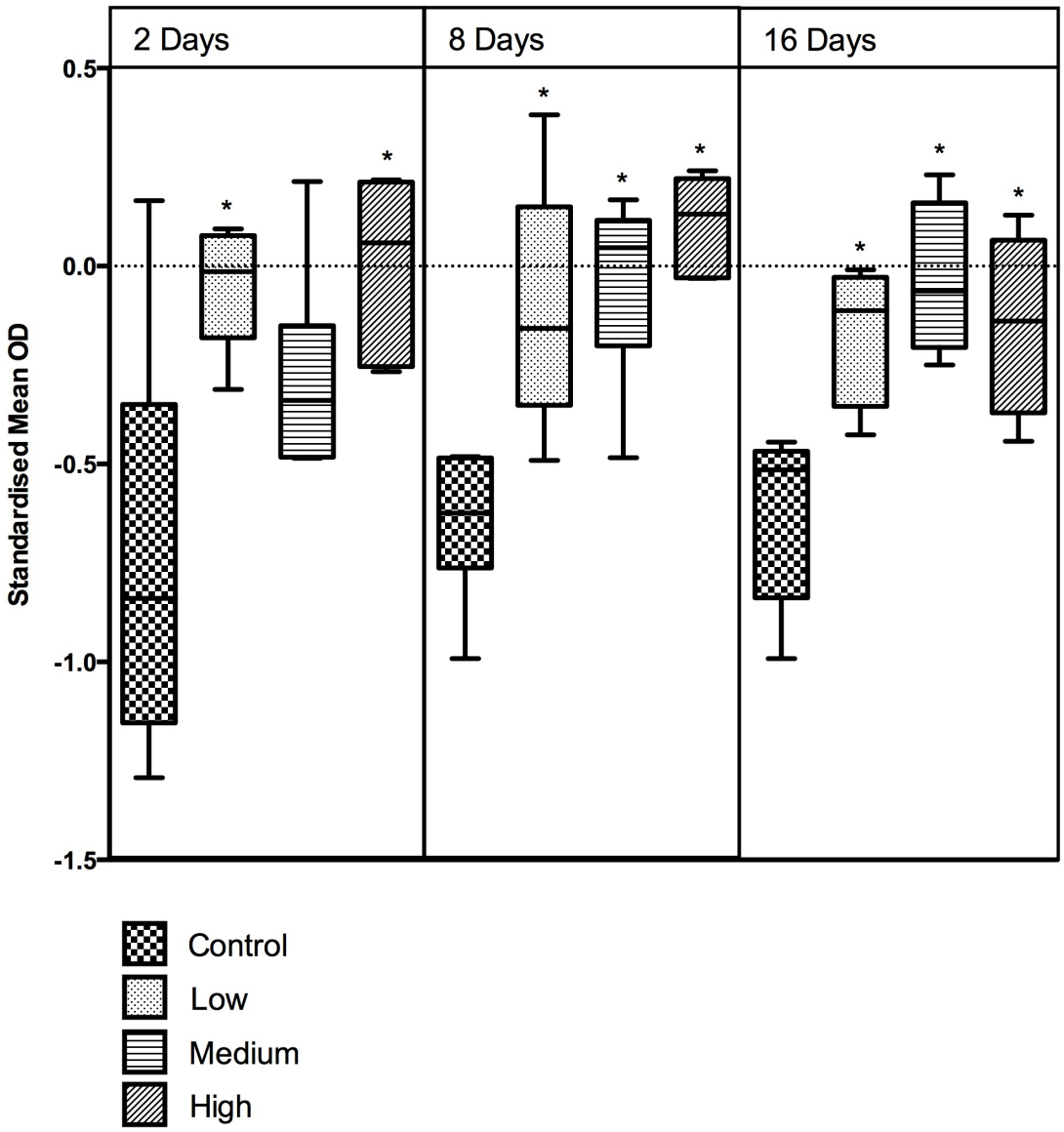

Figure 3.4 *CYP1A concentrations in treated groups for all exposure times (2, 8 and 16 days). Results for 16 days of exposure depicted with PBS injected sample group. Graph shows medians, quartiles, minimum and maximum. Asterisks indicate treatment groups with significant (< 0.0500) p-values.*

3.4 Comet Assay

Zero time samples were compared to the control from each exposure time in each tissue, with the 16 days exposure group split in two depending on injection. For blood the Levene's showed unequal variances in the transformed data, so a non-parametric approach was used and a Kruskal-Wallis test was performed

(Prob > ChiSq = 0.0164*). The control groups for 8 days (mean-mean0/std0 = -1.063), 16 days with LPS injection (mean-mean0/std0 = -1.669), and 16 days with PBS injection (mean-mean0/std0 = -1.002) were significantly different from the zero samples (mean-mean0/std0 = 0.446), whereas the control group for 2 days were not (mean-mean0/std0 = 3.085).

For kidney, the Levene's test also showed unequal variances, so a Kruskal-Wallis test was utilized again for this data. The test statistics showed that there were no significant differences between the groups (Prob > ChiSq = 0.9611).

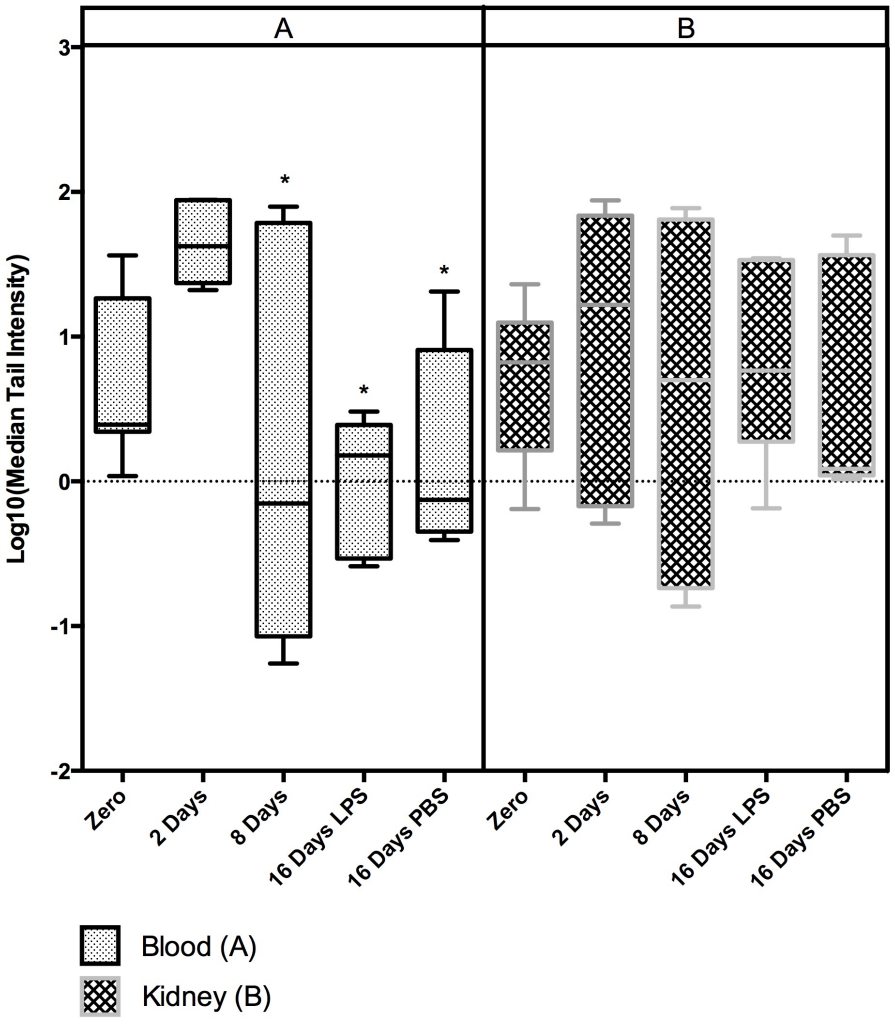

Figure 3.5 *DNA damage in control groups compared to the zero-group for blood (A) and kidney (B); medians, quartiles, minimum and maximum. Asterisks indicate control groups that are significantly (p-value < 0.0500) different from the zero samples*

Furthermore, there were no significant differences between treatment groups and the control at any exposure times in neither the data from obtained from blood samples, nor in the data obtained from kidney samples.

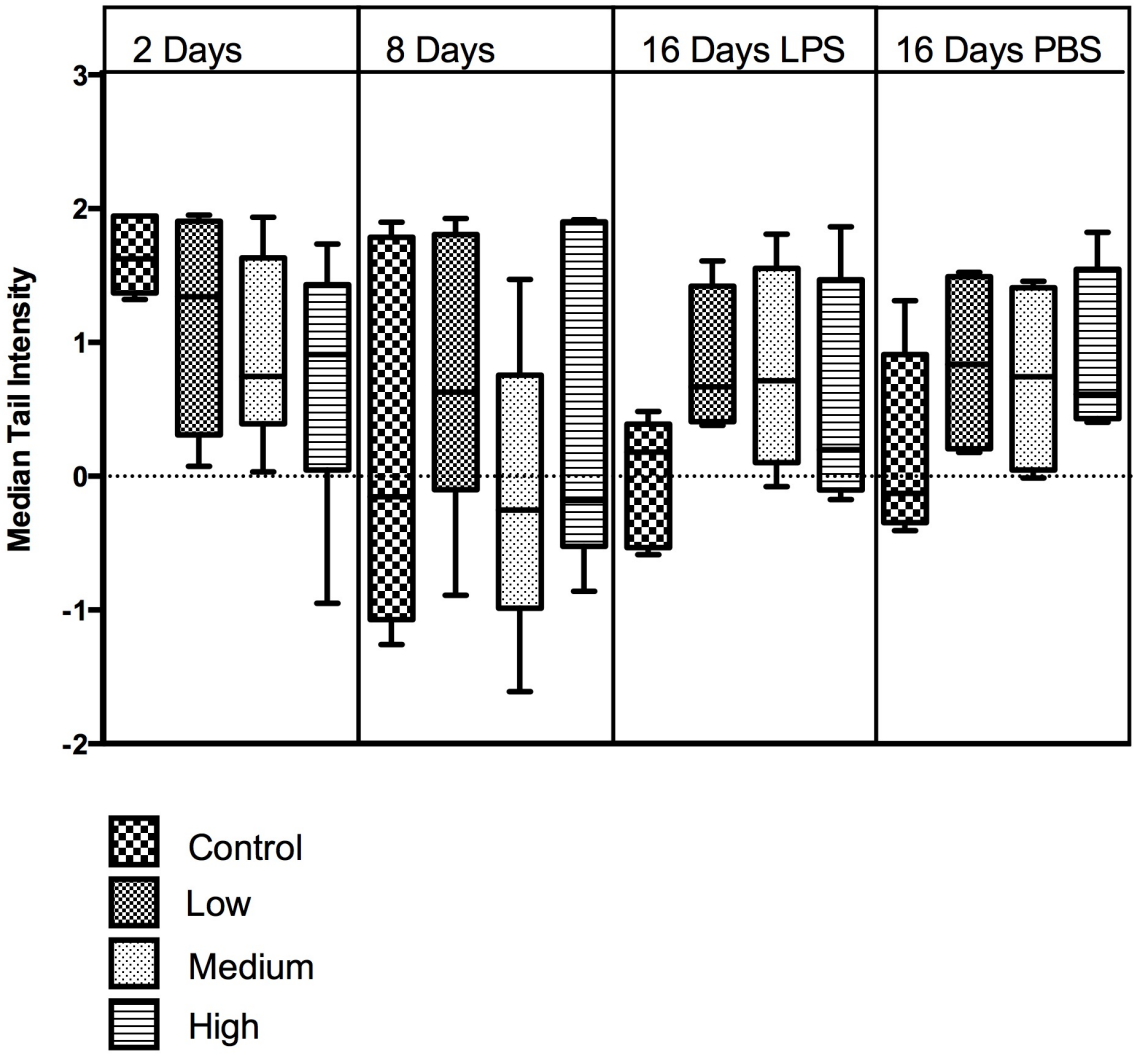

Figure 3.6 *DNA Damage in blood: Every treatment groups for all exposure times. Graph shows medians, quartiles, minimum and maximum.*

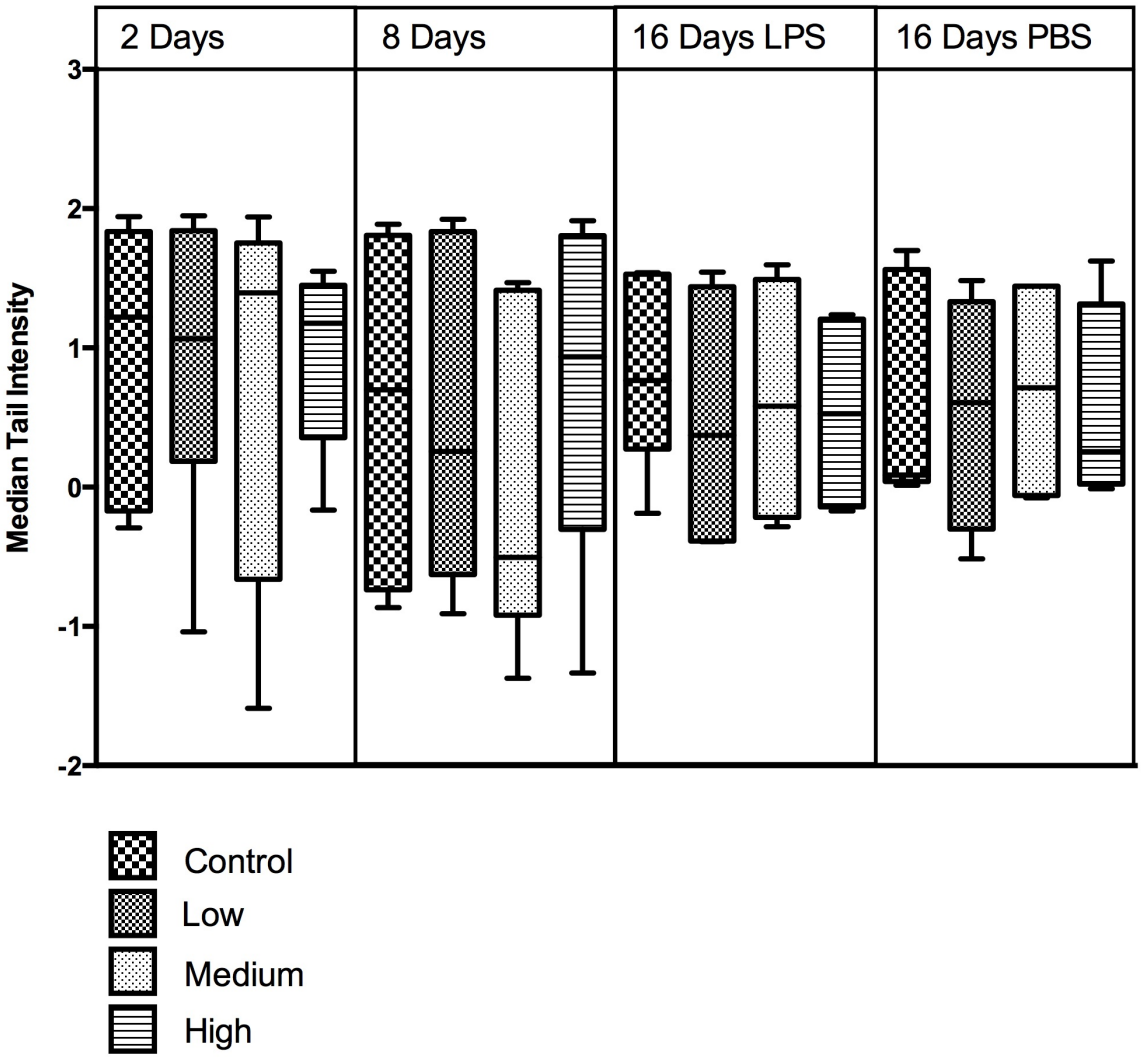

Figure 3.7 *DNA Damage in kidney: Every treatment groups for all exposure times (2, 8 and 16 days with LPS and with PBS). Graph shows medians, quartiles, minimum and maximum.*

3.5 Respiratory Burst

Due to lost replicates, the data from 8 days of exposure unfortunately had to be excluded from the analysis. Zero samples were first analysed with Levene's test, which showed equal variance in the data. A one-way ANOVA was then carried out to assess the effect of the stimulant factor (with PMA/without PMA). This showed that there was no significant effect of the stimulant (Prob > F = 0.9359 and R^2 = 0.000478) on the zero samples. Stimulated and non-stimulated control groups for 2 and 16 (with PBS and LPS) were compared to their respective zero sample group. As Levene's test showed equal variances in the data for both stimulated and non-stimulated cells. A one-way ANOVA was run on each group. There were no significant differences between neither of the control groups and the zero samples (p-value = 0.3441 for PMA stimulated and 0.5787 for non-stimulated).

For the data from 2 days of exposure, a 2-way ANOVA was done, which showed a significant effect of the stimulant (Prob > F = 0.0277) alone, but no significant effect from treatment (Prob > F = 0.0954) or from stimulant and treatment combined (Prob > F = 0.5755). R^2 for the test was 0.369. For the data from 16 days of exposure with injection of LPS, a 2-way ANOVA was done. There was no significance of stimulant (Prob > F = 0.3137), treatment (Prob > F = 0.8918) or stimulant and treatment combined (Prob > F = 0.9576). R^2 for the test was 0.074. For the data from 16 days of exposure with injection of PBS, a 2-way ANOVA was done. There was no significance of stimulant (Prob > F = 0.6420), treatment (Prob > F = 0.2083) or stimulant and treatment combined (Prob > F = 0.9502). R^2 for the test was 0.172059. A 2-way ANOVA was also used to assess if the injection of LPS and PBS had any effect. The analyses showed that there were no significant effects on H_2O_2 production by neither LPS nor PBS injection. (p-value = 0.9535 for PMA stimulated replicates and p-value = 0.8701 for non-stimulated replicates).

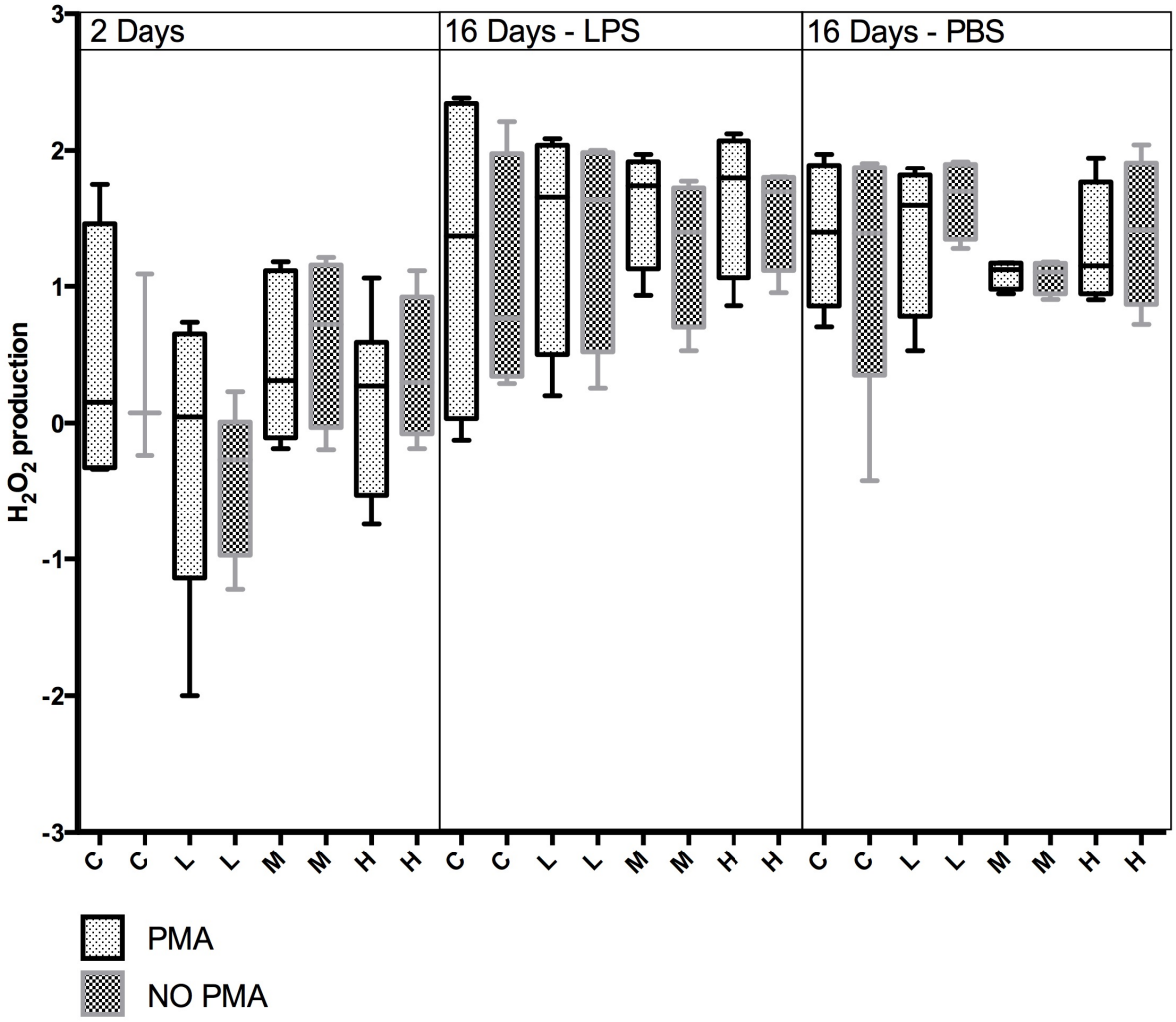

Figure 3.8 *H_2O_2 production in both PMA stimulated and non-stimulated groups in zero samples and for all treatments after 2 and 16 days exposure. Graph shows medians, quartiles, minimum and maximum.*

3.6 PAH Metabolites in Bile

3.6.1 OH-Pyrene

First, a comparison between the control group from each exposure time, and the zero samples group was done. A Levene's test showed that there were equal variances in the data, so a one-way ANOVA was done. The test statistic was not significant (p-value = 0.1867) indicating that none of the control groups were significantly different from the zero samples group.

For the data from 2 days of exposure a Levene's test showed that variance was unequal. As the data had already been transformed, a non-parametric approach was used to analyse the data and a Kruskal-Wallis test was done. X^2 was not significant (p-value = 0.4559) indicating that there was no effect of the treatments. The data for 8 days of exposure showed equal variances with a Levene's test and a one-way ANOVA was done. This showed that there was a significant difference in the treatment groups relative to the control (p-value = 0.0323*). A Dunnet's test showed that the high treatment group was significantly different from the control (p-value = 0.0185). R^2 for the test was 0.40. For the data from 16 days of exposure, a Levene's test showed that the variance was equal, and a one-way ANOVA was done. This showed that there were significant differences in the treatment groups (p-value = 0.0069*). A Dunnet's test showed that all of the treatment groups were significantly different from the control (p-values 0.0134*, 0.0083* and 0.0211* for the low, medium and high treatment groups respectively). R^2 for the test was 0.654319.

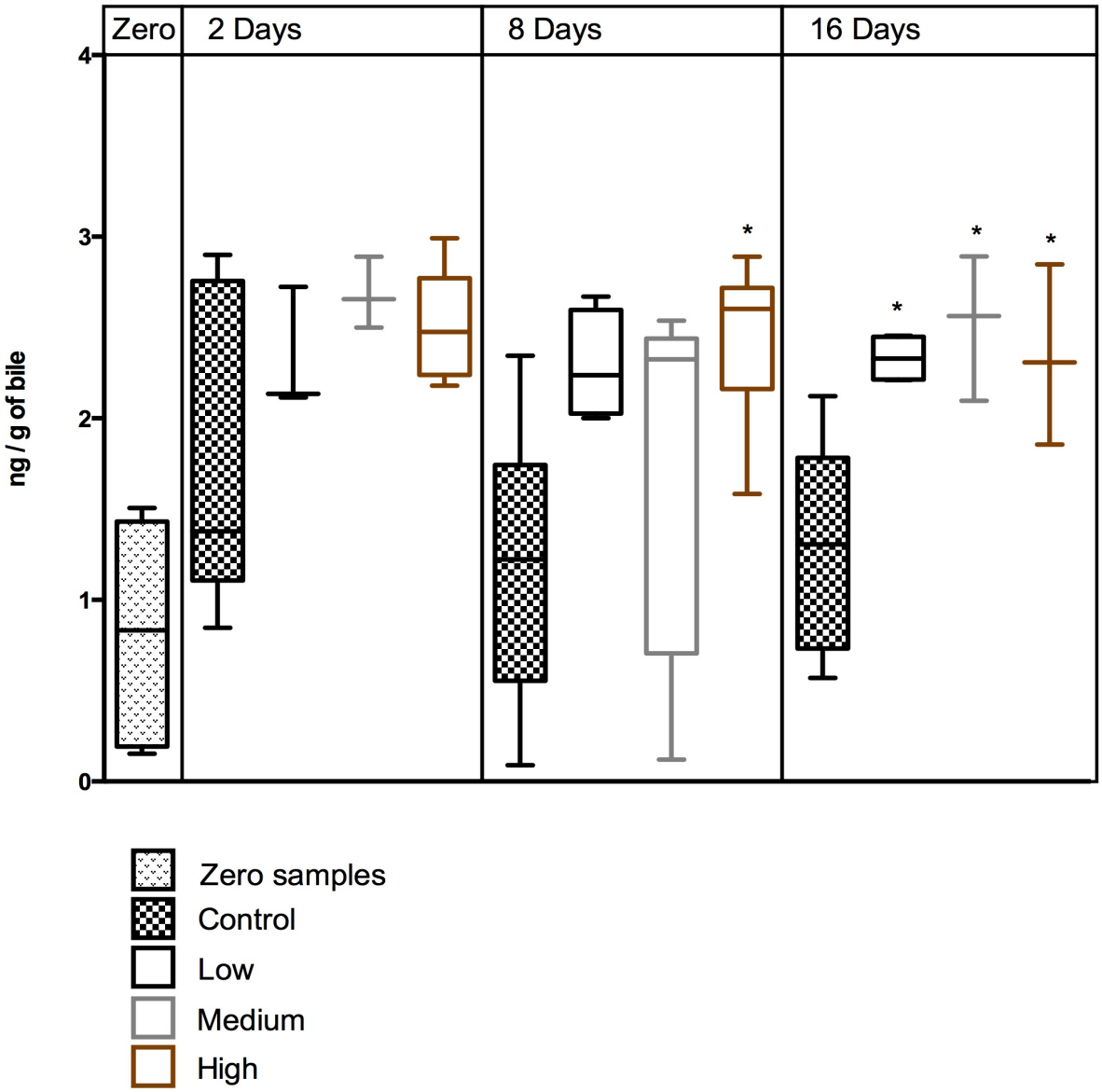

Figure 3.9 *PAH metabolites (pyrene): Every treatment groups for all exposure times (2, 8 and 16 days). Results for 16 days of exposure depicted with PBS injected sample group. Graph shows medians, quartiles, minimum and maximum. Asterisks indicate treatment groups with significant (< 0.0500) p-values.*

3.6.2 OH-Phenatrene

A comparison between the control group from each exposure time, and the zero samples group was done. A Levene's test showed that there were equal variances in the data, so a one-way ANOVA was performed. The test statistic was not significant (p-value = 0.1684) indicating that none of the control groups were significantly different from the zero samples group.

For the data from 2 days of exposure a Levene's test showed that variance in the data was equal. A one-way ANOVA was done. This showed that there was a significant difference in the treated groups (p-value = <.0001). A Dunnet's test showed that all of the treatment groups were significantly different from the control (p-values 0.0002, <0.0001 and <0.0001 for the low, medium and high treatment groups respectively). R^2 for the test was 0.842.

For the data from 8 days of exposure, a Levene's test showed that the data had equal variances and a one-way ANOVA was done. This showed that there was a significant difference in the treated groups (p-value = <.0001*). A Dunnet's test showed that all of the treatment groups were significantly different from the control (p-values were <0.0001 for both the low, medium and high treatment groups). R^2 for the test was 0.826.

For the data from 16 days of exposure, a Levene's test showed that there were equal variances in the data and a one-way ANOVA was done. This showed that there was a significant difference in the treated groups (p-value = <.0001*). A Dunnet's test showed that all of the treatment groups were significantly different from the control (p-values were <0.0001* for both the low, medium and high treatment groups). R^2 for the test was 0.89337.

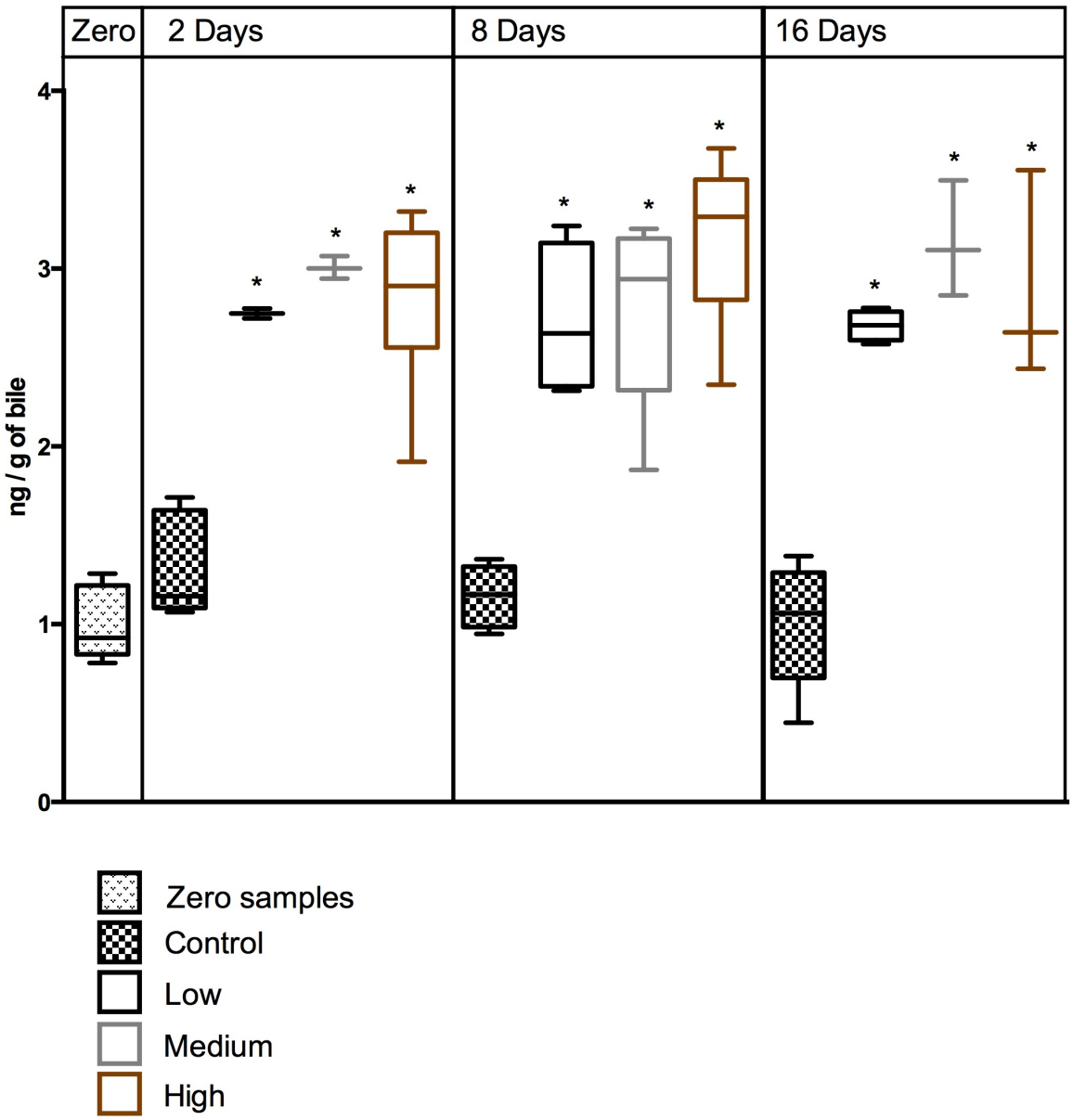

Figure 3.10 *PAH metabolites (phrnatrene): Every treatment groups for all exposure times (2, 8 and 16 days). Results for 16 days of exposure depicted with PBS injected sample group. Graph shows medians, quartiles, minimum and maximum. Asterisks indicate treatment groups with significant (< 0.0500) p-values.*

3.7 Correlations

The correlations analysis was done using a multivariate method and testing with Spearman's p test.

Table 3.1 Correlation data. Only correlated variables are shown. Asterisks indicate significant (< 0.05) p-values.

2 days of exposure			
Variable	**By variable**	**Speaman's p**	**p-value**
OH-Phenatrene	OH-Pyrene	0.6029	0.01*
Respiratory burst NO PMA	Respiratory burst PMA	0.5676	0.02*
8 days of exposure			
Variable	**By variable**	**Speaman's p**	**p-value**
OH-Phenatrene	OH-Pyrene	0.8312	<.0001*
ELISA mean OD	OH-Pyrene	0.4390	0.05*
ELISA mean OD	OH-Phenatrene	0.6675	0.0009*
EROD pmol/min/mg protein	OH-Phenatrene	0.5013	0.02*
EROD pmol/min/mg protein	ELISA mean OD	0.8078	<.0001*
Respiratory burst PMA	ELISA mean OD	-0.5676	0.02*
Respiratory burst PMA	Weight	0.6194	0.01*
Respiratory burst PMA	Comet median TI blood	0.5147	0.04*
Comet median TI kidney	Comet median TI blood	0.7391	<.0001*
Comet median TI kidney	Respiratory burst PMA	0.6412	0.007*
Respiratory burst NO PMA	ELISA mean OD	-0.6118	0.01*
Respiratory burst NO PMA	Weight	0.6785	0.004*
Respiratory burst NO PMA	Length	0.6667	0.005*
Respiratory burst NO PMA	Respiratory burst PMA	0.7588	0.0007*
16 days of exposure with LPS injection			
Variable	**By variable**	**Speaman's p**	**p-value**
OH-Phenatrene	OH-Pyrene	0.7500	0.0008*
EROD pmol/min/mg protein	OH-Phenatrene	0.9375	<.0001*
Respiratory burst NO PMA	OH-Pyrene	0.6118	0.01*
Respiratory burst NO PMA	Respiratory burst PMA	0.8971	<.0001*
16 days of exposure with PBS injection			
Variable	**By variable**	**Speaman's p**	**p-value**
OH-Phenatrene	OH-Pyrene	0.8857	<.0001*
ELISA mean OD	OH-Pyrene	0.5214	0.05*
ELISA mean OD	OH-Phenatrene	0.6643	0.007*
EROD pmol/min/mg protein	ELISA mean OD	0.5539	0.02*
Respiratory burst NO PMA	Respiratory burst PMA	0.8848	<.0001*

4 Discussion

4.1 PAH metabolites in bile

Fish may be exposed to PAHs via the gills or through their diet (Grung et al. 2009). PAH metabolites in bile are the results of compounds that enter and interact with the biological system where it induces various reactions associated with biotransformation. The presence of elevated levels of metabolites shows that the organism has been exposed to PAHs through its environment (Van der Oost et al. 2003, Grung et al. 2009, Beyer et al. 2010). The data analysis focused on the metabolites 1-OH-phenantrene and 1-OH-pyrene. Phenanthrene consists of three aromatic rings while pyrene consists of four (Hylland 2006). Three, four and five-ring compounds are most abundant in the distribution of PAHs (Page et al. 1999), and this should support this analysis main focus on phenanthrene and pyrene.

The results related to phenatrene accumulation were very defined and the results illustrate a clear correlation between treatment and elevated concentrations of phenatrene in the bile. After 8 days of exposure there was a visible dose-response pattern with median metabolite amount increasing with treatment.
For 2 and 16 days of exposure the median for the High treatment was slightly lower, but close to that of the medium treatment, which could indicate that the initial conditioning and response to the oil exposure peaks at the medium concentration, perhaps due to higher concentrations being a redundant factor for making the fish maximize their biotransformation responses.
The results for pyrene showed that after 2 days of exposure there were no significant differences in the treated groups and after 8 days of exposure, only the High treatment group was significantly different. The data for 16 days of exposure showed the same tendency as the phenatrene data for 2 and 16 days of exposure with a higher median for the medium treatment.
The elevated amount of phenatrene compared to pyrene, is most likely due to the

PAH content in the WAF of oil containing higher amounts of two- and three-ring components and lower levels of heavier PAHs such as pyrene (Hylland 2006).

This temporal tendency in the results seems to oppose previously observed temporal patterns in fluorescence (Aas et al. 2000), where the PAH levels increase rapidly in the beginning of the exposure period and more steadily for the rest of the period. In most of the studies where this has been observed, it has been with caged fish in suspected polluted areas. Such a pattern would make sense in an uncontrolled exposure environment such as in an oil spill area, where the amount of PAHs available for accumulation will decrease over time. A constant input-flow was maintained in this experiment, so the PAHs would not decrease over time. This seems to be in accordance with the observed pattern where there were still high concentrations of metabolites after the longest exposure period.

4.2 Hepatic ethoxyresorufin *O*-deethylase (EROD) Activity

The correlation analysis indicated that EROD activity levels were correlated with PAH metabolite levels, more specifically OH-phenatrene concentrations for both 8 and 16 days of exposure. EROD activity is a pathway for the formation of PAH metabolites (Aas et al. 2001), which gives further support to the correlation observed. Other studies, such as that carried out by McDonald et al. (1995), have previously documented a connection between phenatrene metabolites and elevated EROD activity levels. Additionally, the high fat percentage in cod liver (40-70%) makes the organ especially susceptible to accumulation of lipophilic contaminants (Husøy et al. 1996). It has been suggested that this may contribute to an inhibition of EROD induction (Bernhoft et al. 1994) and may help explain why CYP1A induction has been unevenly distributed in some studies (Husøy et al. 1994).

The pattern observed in EROD activity from this study indicated that mean EROD activity was highest in cod from the low treatment after 2 days of exposure and then decreased with treatment. For 8 days of exposure the median EROD activity

did not seem to differ very much between the treatments, and at 16 days of exposure injected with both LPS and PBS, the mean EROD activity peaked at the medium treatment, while the low and high treatment EROD activity levels remained lower. This pattern shows some similarities with results obtained in studies with polar cod (*Boreogadus saida*) that used related oil concentrations (Nahrgang et al. 2010, Nahrgang et al. 2010). The oil treatments described in these studies as medium and high treatment correspond to the concentrations used in the present study for low and medium treatments. The pattern they observed after 0+ days corresponds to the pattern seen in this study after 2 days of exposure, while the pattern after 2 weeks in the two studies corresponds to 8 and 16 days of exposure in this study (although not as clearly visible after 8 days). Therefore, there seems to be compliance with previously observed patterns in EROD activity.

The shift in the pattern between the early stages of the experiment and the later stages could possibly be connected to elevated stress factors during the first days of the experiment, but as there is no data to support this assumption it will not be further discussed here. It is also possible that the change in pattern could have been related to the injection of a pathogen challenge thirteen days into the experiment, as a change in hepatic EROD activity has previously been observed in carp *(Cyprinus carpio)* that had been intraperitoneally injected with LPS. In carp, the injection caused a down regulation of CYP1A in liver and spleen, but at the same time an increase in hepatic EROD activity levels (Marionnet et al. 1998). Although there are species differences to consider, it has been established that LPS triggers biotransformation processes and may affect induction of biotransformation enzymes (Marionnet et al. 1998). The general trend in LPS and PBS injected fish in this study was similar and statistically, there were no significant differences between each treatment group when LPS and PBS injected groups were compared. Although the high treatment group among the LPS injected fish showed to be significantly different from the control, the same treatment group among the PBS injected fish was not significant, indicating that the LPS injection may have induced higher EROD activity in the fish that were also subjected to elevated stress from the highest exposure to oil.

4.3 CYP1A concentrations

There was a clear correlation between EROD activity and CYP1A levels after 8 days of exposure, in addition to a correlation between OH-phenatrene, OH-pyrene and CYP1A levels. The same correlation was present after 16 days of exposure. CYP1A plays a major role in the biotransformation of PAHs (Stegeman et al. 1991) so the correlation seems to support potential effects from PAHs in the oil. Previous studies have already established a correlation between exposure to PAHs and elevated EROD activity and CYP1A levels measured by the ELISA assay, specifically in Atlantic cod (Gadus morhua) and European flounder (Platichthys j' ksus) that had been exposed to (b-naphthoflavone) (Husøy et al. 1996) and in Atlantic cod and corkwing wrasse (*Symphodus melops*) exposed to naphthalene, pyrene and benzo[a]pyrene (Aas et al. 2001). The pattern in the data did not exactly resemble a dose-response relationship. In a previous study where cod was exposed to other contaminants (PCB-105), it was suspected that unevenly distributed data could be connected to early samplings, resulting in contaminant levels not being substantial enough to induce elevated EROD and CYP1A levels (Bernhoft et al. 1994). The flow used in this system was stable, but accumulation extensive enough to cause a response could be somehow delayed in this experiment as well. However, the results do not indicate that there was a tendency to higher mean OD in the samples exposed for the longest time period (16 days). In contrast, the data for 8 days of exposure seems generally slightly higher in at least the medium and high treatment groups, so delayed uptake as an explanation to the non-dose dependent pattern observed remains questionable.

4.4 DNA strand breaks

This study was looking into whether or not there would be significant DNA damage as a result of the oil exposure. The study showed that there were no significant results for any treatment groups after the different exposure periods. The data was characterized by high variance, and when analysing the variances, R^2 for most of the exposure times indicated that the model was only to a very low degree able to explain the variances in the data.

A relationship between exposure to PAHs and DNA damage has previously been described in a study using polar cod (*Boreogadus saida*). DNA damage measured using the comet assay was correlated with PAH metabolites; pyrene and benzo(a)pyrene (Nahrgang et al. 2010). There were no correlations between DNA damage and PAH metabolites in this study and no apparent effect from the oil treatment. However there was a strong correlation observed between DNA damage in blood and DNA damage in kidney, as well as a relationship between DNA damage in both blood and kidney and the PMA stimulated response in the respiratory burst assay. The head kidney is the organ where leukocytes are matured and then distributed to the rest of the system, so an increase in DNA damaged leukocytes in this organ could account for the distribution of damaged cells to the bloodstream. Damage to the DNA strand may be caused by toxic metabolites (Gravato et al. 2003) or may occur after increased ROS production (Regoli et al. 2003). PAHs are also known to induce the production of ROS (Nahrgang et al. 2010)which may explain the observed correlation between DNA damage in blood and kidney and the response on the respiratory burst assay.

4.5 Effects on immune responses

Respiratory burst plays an important role in the immune system because it is a reaction that occurs in phagocytes and is involved in the degradation of internalized particles and bacteria (Nikoskelainen et al. 2006). The degradation is connected to catalase enzymes which catalyse the breakdown of reactive oxygen species to water and oxygen and, therefore, plays an important part in protecting the cell from oxidative stress and damage (Chelikani et al. 2004). The cells in this study were activated with PMA, and the production of H_2O_2 was measured to assess the individual ability to induce an immune response. The data showed a significant effect of treatment only for 2 days of exposure. Although the exposure periods could not be statistically compared, the data pattern suggest that median H_2O_2 production is higher after 16 days of exposure. Furthermore, as the statistic analyses also verified, the pattern shows that there is no obvious effect of PMA stimulation (median H_2O_2 production is very close for stimulated and non-stimulated replicates in all treatment and exposure groups). The comparison between the LPS and the PBS injected groups for 16 days of exposure further showed that the injection had no effect on respiratory burst. These results contradict a previous long-term study by Hamoutene et al. (2011) where cod were exposed to PAHs through produced water, and where the fish were also injected with either an LPS or PBS. The study examined both respiratory burst of whole blood, which was not affected, and respiratory burst in head kidney, which was significantly decreased after the fish had been injected with LPS. The decrease was strongest in fish that had been exposed to the highest concentrations. Inhibition of the immune responses was argued to be a result of the vast effects of LPS on the natural immune cells' reactions. It is contradictory because, in the present study, the pattern shows that the lower and medium treatments generally have more elevated medians than the higher ones. It is however, likely that the acute and longer-term effects could be very different. A study on stress effects in rainbow trout (Oncorhynchus mykiss) argued that acute stress would act as a stimulant on the innate immune system rather than inhibiting it, in an attempt to protect the fish (Demers et al. 1997).

Moreover, it is possible that the immune system would adapt to the chronic conditions after a period of time, which could explain the significant treatment effect after 2 days but not after 16 days.
The correlation analysis showed that the size of the fish was correlated with the immune response. The fish used were all juveniles with the same age, but there were some weight and length variances. Magnadóttir et al. (1999) have previously shown that humoral immune parameters in cod are influenced by its size, and it has been argued that this may be due to a primitive regulatory system (Magnadóttir et al. 1999).
The absence of significant immune responses related to oil treatments and LPS/PBS injection does not seem logical; however, it has previously been established that inducing immune responses in cod is challenging (Espelid et al. 1991, Schrøder et al. 1992). In that sense, the results of the present study are consistent with existing literature and not entirely anomalous.

4.6 Interspecies Comparisons

There were quite substantial species differences when it came to the biomarkers of exposure. Although turbot showed significant differences on EROD activity at all treatments, this was only the case for 2 days of exposure. There were no significant results for CYP1A levels at any times of exposure either. Results for PAH metabolites in bile were more substantial; for phenatrene turbot had significant differences relative to the control in all treatment groups at all exposure times, and for pyrene the medium treatment was significantly different for the medium treatment at 8 and 16 days of exposure. Accumulated PAHs in the bile in turbot indicates that the oil was in fact bioavailable and accumulating in turbot as well. It should be noted that hardly any of the turbot were feeding during the experiment. Due to this, the gall bladder was very full, and the absence of bile removal from the gall bladder during the entire experiment could possibly explain a higher accumulation of PAHs in the bile. The presence of elevated PAH metabolite levels in both species indicate that the different CYP1A levels and EROD activity are species specific. There were no significant

differences in DNA damage between the treatment groups in turbot either, and no significant results in respiratory burst related to the oil exposure, which could point to similarities in the way the immune system responds in both species, or it could be due to methodology.

4.7 Conclusions

The biomarkers of exposure utilized in this study were successful in linking exposure to the oil treatments to metabolic reactions. EROD activity was increased and there was a clear connection between elevated CYP1A levels in treated groups compared to the control. There was a strong relationship between treated groups and elevated PAH metabolite levels in bile. Together, these biomarkers showed that there was an effect of the oil on biotransformation pathways.

The study did not reveal any differences in DNA damages between the treated groups, and the oil exposure did thus not appear to affect this parameter. Neither did the oil seem to have any significant effect on the immune responses, as there were no treatment effects on respiratory burst. This may be connected to the fact that stimulation of the leukocytes proved ineffective in generating a cellular response. It is possible that these methods need further study and optimization before successful results may be developed and new links between oil exposure and immune responses established. Major effects of the injected challenge could not be conclusively determined, although there were some indications that some of the biomarkers were somewhat affected. It is possible that a more specific study into the effects of other immune parameters would yield more conclusive results.

It was not possible to compare exposure times directly due to the experiment design, although the trends in the data did appear to show some effects related to exposure period.

For EROD activity, the pattern did not show differences between specific treatments at different exposure periods although there was some observable variation in mean concentration. CYP1A levels appeared to show a relative

difference for each treatment at different exposure periods, especially between the first and the last sampling. Although it could not be statistically verified, it could indicate some effect of exposure period. PAH metabolites levels in bile were similar in each treatment group at different times of exposure. The pattern in amount of DNA damage did not seem to vary in relation to exposure period either, which is consistent with the finding that the oil did in fact not affect amounts of DNA damage.
The immune responses showed a trend of generally higher mean respiratory burst in all treatments for the longest exposure period compared to the shortest exposure period, which could indicate that longer exposure would be more likely to trigger an immune function. In regards to the injected challenge, the EROD activity in the LPS injected high treatment group was significantly different from control, but not significantly different from the high treatment group injected with PBS. Furthermore, there were no significant differences in immune responses measured. From this it can be gathered that injection in it self did not change the immunocompetence in this study.

There were species differences in EROD activity and quite substantial differences in CYP1A levels, which in turbot did not seem affected by the oil treatments like they were in cod. The PAH metabolites in bile revealed that both species had bioaccumulated high amounts of PAHs, so the differences in CYP1A levels and EROD activity appeared to be species specific. There were no significant differences in DNA damage between the treatment groups in turbot either, and no significant results in respiratory burst related to the oil exposure. This could point to similarities in the way the immune system responds in both species, or it could be due to an unknown error in methodology.

It is important to note that in their natural habitat, organisms may be exposed to more than one contaminant, which may at any given time interact with and change the single effect of one another. The WAF of crude oil contains several other components that PAHs and the interaction between contaminants is an important factor. It is essential to assess exactly which contaminants may be acting upon the organisms so that their interactive effects may be determined. In

that regard, further studies assessing other components of the oil would be environmentally relevant, although assessing the properties of individual components like this study has done with PAHs is equally important, as it contributes to further understanding the results of possible interaction effects.

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Appendix

Attachment 1: Physiological data for the fish.

Sample	Treatment	Exposure (days)	Weight (grams)	Length (cm.)
0T1	Zero	0	28	14
0T2	Zero	0	20	13
0T3	Zero	0	17	14
0T4	Zero	0	34	16
0T5	Zero	0	35	15
0T6	Zero	0	31	14.5
0T7	Zero	0	22	13.3
0T8	Zero	0	23	13
1T1	Medium	2	31	15.5
1T2	Low	2	33	15.5
1T3	High	2	21	14
1T4	Medium	2	54	19
1T5	High	2	21	14
1T6	Low	2	20	13
1T7	Low	2	18	13.5
1T8	High	2	28	15.5
1T9	Control	2	35	16
1T10	Low	2	58	18.5
1T11	High	2	30	16
1T12	High	2	70	19
1T13	Medium	2	41	18
1T14	Control	2	22	15
1T15	Low	2	23	14
1T16	Control	2	29	15.5
1T17	Low	2	57	19.3
1T18	Medium	2	31	16
1T19	Control	2	18	13
1T20	Control	2	26	14.5
1T21	Medium	2	18	13
1T22	High	2	57	19
1T23	Control	2	25	14.5
1T24	Medium	2	28	15
2T1	Medium	8	44	17
2T2	Low	8	34	17.7
2T3	High	8	25	14.7
2T4	Medium	8	25	16
2T5	High	8	32	16
2T6	Low	8	44	18
2T7	Low	8	20	14

2T8	High	8	26	15
2T9	Control	8	22	14
2T10	Low	8	25	14.5
2T11	High	8	29	14.5
2T12	High	8	26	16
2T13	Medium	8	38	16.5
2T14	Control	8	61	20
2T15	Low	8	46	18
2T16	Control	8	32	16
2T17	Low	8	39	17
2T18	Medium	8	34	17
2T19	Control	8	48	18.5
2T20	Control	8	26	15
2T21	Medium	8	21	14
2T22	High	8	26	15.5
2T23	Control	8	20	14
2T24	Medium	8	20	14.5
3T1-i	Medium	16	23	14
3T1-0	Medium	16	31	16
3T3-i	High	16	31	16
3T3-0	High	16	23	15
3T5-i	High	16	25	14
3T5-0	High	16	54	19
3T6-i	Low	16	29	16
3T6-0	Low	16	25	15
3T7-i	Low	16	18	14
3T7-0	Low	16	13	13
3T9-i	Control	16	18	13
3T9-0	Control	16	37	17
3T10-i	Low	16	22	14.5
3T10-0	Low	16	21	14
3T11-i	High	16	21	15
3T11-0	High	16	44	18.5
3T12-i	High	16	51	18.5
3T12-0	High	16	21	14.5
3T13-i	Medium	16	29	15.5
3T13-0	Medium	16	25	14.5
3T15-i	Low	16	33	16
3T15-0	Low	16	24	15
3T16-i	Control	16	25	15
3T16-0	Control	16	32	16
3T19-i	Control	16	21	13.5
3T19-0	Control	16	25	14.5
3T20-i	Control	16	45	18

3T20-0	Control	16	21	14
3T21-i	Medium	16	32	16.5
3T21-0	Medium	16	20	14.5
3T23-i	Control	16	42	18
3T23-0	Control	16	49	18
3T24-i	Medium	16	49	18.5
3T24-0	Medium	16	29	15.5

Attachment 2: Data for PAH metabolites in bile show as log10-transformed concentrations.

Sample	Treatment	Exposure (days)	OH-Pyrene	OH-Phenatrene
0T1	Zero	0		
0T2	Zero	0	1.506288508	0.781827153
0T3	Zero	0	1.405482963	1.28474647
0T4	Zero	0	0.907303849	0.972063916
0T5	Zero	0		
0T6	Zero	0	0.758381942	0.846027675
0T7	Zero	0	0.206015877	0.875061263
0T8	Zero	0	0.153509989	1.195733648
1T1	Medium	2		
1T2	Low	2	2.724218506	2.776425123
1T3	High	2	2.29992113	2.888112069
1T4	Medium	2	2.890426608	3.001430812
1T5	High	2	2.475424195	3.162026432
1T6	Low	2		
1T7	Low	2	2.134718841	2.720473536
1T8	High	2	1.473735068	1.91392506
1T9	Control	2	0.846151477	1.157879674
1T10	Low	2		
1T11	High	2	2.179034466	2.917867708
1T12	High	2	2.551230355	2.771183127
1T13	Medium	2		
1T14	Control	2		
1T15	Low	2		
1T16	Control	2	2.899711095	1.713070326
1T17	Low	2	2.114977745	2.748374113
1T18	Medium	2	2.49938462	2.944102498
1T19	Control	2	1.368286885	1.114610984
1T20	Control	2	2.612709703	1.568201724
1T21	Medium	2		
1T22	High	2	2.991292544	3.320769228
1T23	Control	2	1.377852419	1.067256889
1T24	Medium	2	2.655455393	3.07044425
2T1	Medium	8	2.3253925	3.116972823

2T2	Low	8	2.098193626	2.312980862
2T3	High	8	1.583493495	2.346607217
2T4	Medium	8	2.339411687	2.941238049
2T5	High	8	2.659906697	3.443012511
2T6	Low	8	2.001387524	2.413366831
2T7	Low	8		
2T8	High	8	2.652081878	3.297979244
2T9	Control	8	0.089905111	1.364888545
2T10	Low	8	2.37817979	2.858928007
2T11	High	8	2.553506799	3.284633733
2T12	High	8	2.354300562	2.98311638
2T13	Medium	8	1.290857877	1.868662068
2T14	Control	8	1.541529322	1.098989639
2T15	Low	8		
2T16	Control	8	2.344470872	1.310990527
2T17	Low	8	2.670681785	3.240449399
2T18	Medium	8	2.537819095	3.22331419
2T19	Control	8	0.919757781	0.945862325
2T20	Control	8	1.524512957	0.996424104
2T21	Medium	8	0.120573931	2.764146232
2T22	High	8	2.890286853	3.676547296
2T23	Control	8	0.710286648	1.232894509
2T24	Medium	8		
3T1-i	Medium	16 LPS	2.195733482	2.878462622
3T1-0	Medium	16 PBS	2.09684052	2.849658916
3T3-i	High	16 LPS	2.093600864	2.812175766
3T3-0	High	16 PBS		
3T5-i	High	16 LPS	1.113215123	2.232085933
3T5-0	High	16 PBS	2.307987817	2.64234579
3T6-i	Low	16 LPS	2.248856075	2.410142616
3T6-0	Low	16 PBS	2.433529826	2.77825981
3T7-i	Low	16 LPS	2.293747079	2.481806538
3T7-0	Low	16 PBS	2.210184706	2.574864314
3T9-i	Control	16 LPS	1.035503382	1.091807597
3T9-0	Control	16 PBS	0.895129602	1.06228107
3T10-i	Low	16 LPS	0.964923878	2.065736525
3T10-0	Low	16 PBS	2.454234896	2.665637267
3T11-i	High	16 LPS	2.593568598	3.139289872
3T11-0	High	16 PBS	2.848416985	3.553955807
3T12-i	High	16 LPS		
3T12-0	High	16 PBS	1.855785587	2.437116093
3T13-i	Medium	16 LPS	2.364803026	2.959587496
3T13-0	Medium	16 PBS	2.891219565	3.496362205
3T15-i	Low	16 LPS	1.742847147	2.420297435
3T15-0	Low	16 PBS	2.224636639	2.699030801

3T16-i	Control	16 LPS	2.267503541	1.563955465
3T16-0	Control	16 PBS	1.305759672	0.446070936
3T19-i	Control	16 LPS	1.035689411	0.834166284
3T19-0	Control	16 PBS	1.443231686	1.198024426
3T20-i	Control	16 LPS	1.605738383	1.085647288
3T20-0	Control	16 PBS	2.121953584	1.383671774
3T21-i	Medium	16 LPS	2.223050998	2.637271527
3T21-0	Medium	16 PBS	2.562518877	3.104213884
3T23-i	Control	16 LPS	0.988645856	1.203386492
3T23-0	Control	16 PBS	0.570391144	0.950316164
3T24-i	Medium	16 LPS	2.290955595	2.931030486
3T24-0	Medium	16 PBS		

Attachment 3: Data for EROD activity shown as log10-transformed concentrations.

Sample	Treatment	Exposure (days)	EROD pmol/min/mg protein
0T1	Zero	0	0.965842708
0T2	Zero	0	1.599555591
0T3	Zero	0	1.608512222
0T4	Zero	0	1.581452594
0T5	Zero	0	1.365487985
0T6	Zero	0	1.572775267
0T7	Zero	0	1.239920365
0T8	Zero	0	1.641226736
1T1	Medium	2	1.902131404
1T2	Low	2	1.699206759
1T3	High	2	1.917711991
1T4	Medium	2	2.075183533
1T5	High	2	1.205091478
1T6	Low	2	2.140225125
1T7	Low	2	2.038262407
1T8	High	2	1.776658813
1T9	Control	2	1.821665151
1T10	Low	2	1.898939767
1T11	High	2	2.2122287
1T12	High	2	1.669476289
1T13	Medium	2	1.840443652
1T14	Control	2	1.698448856
1T15	Low	2	2.086967781
1T16	Control	2	0.755780868
1T17	Low	2	1.966573435
1T18	Medium	2	2.120718077
1T19	Control	2	1.591162576
1T20	Control	2	1.530176586

1T21	Medium	2	1.749817889
1T22	High	2	2.133785142
1T23	Control	2	1.374198258
1T24	Medium	2	1.699499353
2T1	Medium	8	1.758034256
2T2	Low	8	2.290279529
2T3	High	8	2.005277906
2T4	Medium	8	1.841174758
2T5	High	8	1.869013265
2T6	Low	8	1.787678908
2T7	Low	8	2.372460841
2T8	High	8	1.483913203
2T9	Control	8	1.436006539
2T10	Low	8	1.766351481
2T11	High	8	1.851416724
2T12	High	8	2.036036042
2T13	Medium	8	1.912045414
2T14	Control	8	1.385461088
2T15	Low	8	1.677443397
2T16	Control	8	1.167421183
2T17	Low	8	1.962340212
2T18	Medium	8	2.147094368
2T19	Control	8	0.970811611
2T20	Control	8	1.491476074
2T21	Medium	8	1.977439207
2T22	High	8	1.912228894
2T23	Control	8	1.45274578
2T24	Medium	8	1.896641461
3T1-i	Medium	16 LPS	2.124145824
3T1-0	Medium	16 PBS	1.904158148
3T3-i	High	16 LPS	2.419292682
3T3-0	High	16 PBS	1.8109714
3T5-i	High	16 LPS	1.918644262
3T5-0	High	16 PBS	1.573556562
3T6-i	Low	16 LPS	1.741356848
3T6-0	Low	16 PBS	1.638969997
3T7-i	Low	16 LPS	1.287186268
3T7-0	Low	16 PBS	1.302378187
3T9-i	Control	16 LPS	1.634081201
3T9-0	Control	16 PBS	1.727064305
3T10-i	Low	16 LPS	1.92633167
3T10-0	Low	16 PBS	1.777032467
3T11-i	High	16 LPS	1.986547813
3T11-0	High	16 PBS	1.83491388
3T12-i	High	16 LPS	1.655258803

3T12-0	High	16 PBS	2.180956951
3T13-i	Medium	16 LPS	1.871948594
3T13-0	Medium	16 PBS	1.878975211
3T15-i	Low	16 LPS	1.875749472
3T15-0	Low	16 PBS	1.86294085
3T16-i	Control	16 LPS	1.912506309
3T16-0	Control	16 PBS	1.851632408
3T19-i	Control	16 LPS	0.849993233
3T19-0	Control	16 PBS	1.496111114
3T20-i	Control	16 LPS	1.422798053
3T20-0	Control	16 PBS	0.938478449
3T21-i	Medium	16 LPS	1.815727984
3T21-0	Medium	16 PBS	1.736558972
3T23-i	Control	16 LPS	1.217834624
3T23-0	Control	16 PBS	1.414304688
3T24-i	Medium	16 LPS	2.050055386
3T24-0	Medium	16 PBS	1.934205226

Attachment 4: Data for CYP1A levels from the ELISA assay, shown as log10-transformed mean optical density.

Sample	Treatment	Exposure (days)	Mean optical density
0T1	Zero	0	-0.339134522
0T2	Zero	0	-0.274905479
0T3	Zero	0	-1.084738074
0T4	Zero	0	-0.37675071
0T5	Zero	0	-0.852840986
0T6	Zero	0	-0.417936637
0T7	Zero	0	-0.8403922
0T8	Zero	0	-0.623708419
1T1	Medium	2	-0.344110489
1T2	Low	2	-0.311580178
1T3	High	2	0.125262458
1T4	Medium	2	0.213783299
1T5	High	2	0.217747073
1T6	Low	2	-0.137074318
1T7	Low	2	0.094121596
1T8	High	2	0.210012629
1T9	Control	2	-1.295281401
1T10	Low	2	-0.034242807
1T11	High	2	-0.249491605
1T12	High	2	-0.266911532
1T13	Medium	2	-0.481400839
1T14	Control	2	-1.109237515
1T15	Low	2	0.006552455

1T16	Control	2	-0.521129586
1T17	Low	2	0.071570269
1T18	Medium	2	-0.271646218
1T19	Control	2	0.165541077
1T20	Control	2	-0.881769341
1T21	Medium	2	-0.485736387
1T22	High	2	-0.007382363
1T23	Control	2	-0.795875528
1T24	Medium	2	-0.335358024
2T1	Medium	8	-0.48366701
2T2	Low	8	0.072556091
2T3	High	8	0.178079194
2T4	Medium	8	0.018509341
2T5	High	8	0.214846871
2T6	Low	8	-0.305131898
2T7	Low	8	0.382377303
2T8	High	8	-0.029212977
2T9	Control	8	-0.599208655
2T10	Low	8	-0.262514591
2T11	High	8	0.085087852
2T12	High	8	-0.031050319
2T13	Medium	8	0.073806552
2T14	Control	8	-0.686879104
2T15	Low	8	-0.491061592
2T16	Control	8	-0.991319121
2T17	Low	8	-0.051098465
2T18	Medium	8	0.167317335
2T19	Control	8	-0.481223157
2T20	Control	8	-0.485969593
2T21	Medium	8	0.098989639
2T22	High	8	0.240433564
2T23	Control	8	-0.649751982
2T24	Medium	8	-0.106964377
3T1-i	Medium	16 LPS	-0.363725706
3T1-0	Medium	16 PBS	0.230704314
3T3-i	High	16 LPS	-0.091346232
3T3-0	High	16 PBS	-0.123976126
3T5-i	High	16 LPS	0.174563914
3T5-0	High	16 PBS	-0.442510451
3T6-i	Low	16 LPS	-0.177178355
3T6-0	Low	16 PBS	-0.084430599
3T7-i	Low	16 LPS	-0.162945697
3T7-0	Low	16 PBS	-0.140261434
3T9-i	Control	16 LPS	-1.059070902
3T9-0	Control	16 PBS	-0.445345186

3T10-i	Low	16 LPS	-0.079027576
3T10-0	Low	16 PBS	-0.009047887
3T11-i	High	16 LPS	-0.463436294
3T11-0	High	16 PBS	-0.154060899
3T12-i	High	16 LPS	-0.094646682
3T12-0	High	16 PBS	0.128669509
3T13-i	Medium	16 LPS	0.169968174
3T13-0	Medium	16 PBS	-0.07305998
3T15-i	Low	16 LPS	-0.165609917
3T15-0	Low	16 PBS	-0.425715638
3T16-i	Control	16 LPS	-0.46183173
3T16-0	Control	16 PBS	-0.490768475
3T19-i	Control	16 LPS	-0.401497569
3T19-0	Control	16 PBS	-0.684060856
3T20-i	Control	16 LPS	-0.65338317
3T20-0	Control	16 PBS	-0.514414577
3T21-i	Medium	16 LPS	0.019455633
3T21-0	Medium	16 PBS	-0.05207638
3T23-i	Control	16 LPS	-0.413118395
3T23-0	Control	16 PBS	-0.990355092
3T24-i	Medium	16 LPS	0.221722938
3T24-0	Medium	16 PBS	-0.249491605

Attachment 5: Data for DNA damage shown as log10-transformed median tail intensity (TI).

Sample	Treatment	Tissue	Injection	Exposure (days)	Median TI
0T1	Zero	Blood	-	0	1.5608381
0T2	Zero	Blood	-	0	0.353907107
0T3	Zero	Blood	-	0	0.036436755
0T4	Zero	Blood	-	0	0.393617633
0T5	Zero	Blood	-	0	1.388659866
0T6	Zero	Blood	-	0	0.896123884
0T7	Zero	Blood	-	0	0.340339325
0T8	Zero	Blood	-	0	0.390806453
1T1	Medium	Blood	-	2	1.529511741
1T2	Low	Blood	-	2	0.388144509
1T3	High	Blood	-	2	0.702288744
1T4	Medium	Blood	-	2	0.630964063
1T5	High	Blood	-	2	-0.94875419
1T6	Low	Blood	-	2	1.88975093
1T7	Low	Blood	-	2	1.122105469
1T8	High	Blood	-	2	1.11617234

1T9	Control	Blood	-	2	1.322129748
1T10	Low	Blood	-	2	0.074661508
1T11	High	Blood	-	2	1.330063467
1T12	High	Blood	-	2	0.377124553
1T13	Medium	Blood	-	2	0.510311778
1T14	Control	Blood	-	2	1.481575922
1T15	Low	Blood	-	2	1.562788835
1T16	Control	Blood	-	2	1.944228649
1T17	Low	Blood	-	2	1.952771522
1T18	Medium	Blood	-	2	0.032377731
1T19	Control	Blood	-	2	1.944634944
1T20	Control	Blood	-	2	1.768627809
1T21	Medium	Blood	-	2	0.862193688
1T22	High	Blood	-	2	1.736601282
1T23	Control	Blood	-	2	1.386397738
1T24	Medium	Blood	-	2	1.935169595
2T1	Medium	Blood	-	8	0.086866824
2T2	Low	Blood	-	8	0.849686819
2T3	High	Blood	-	8	-0.245399042
2T4	Medium	Blood	-	8	0.515642966
2T5	High	Blood	-	8	-0.412894224
2T6	Low	Blood	-	8	0.407715733
2T7	Low	Blood	-	8	0.163186992
2T8	High	Blood	-	8	-0.115862869
2T9	Control	Blood	-	8	0.101860642
2T10	Low	Blood	-	8	1.768086135
2T11	High	Blood	-	8	1.896052816
2T12	High	Blood	-	8	1.916637545
2T13	Medium	Blood	-	8	1.470687004
2T14	Control	Blood	-	8	1.898865406
2T15	Low	Blood	-	8	1.925451901
2T16	Control	Blood	-	8	1.748473948
2T17	Low	Blood	-	8	-0.888909985
2T18	Medium	Blood	-	8	-0.778086287
2T19	Control	Blood	-	8	-1.0084601
2T20	Control	Blood	-	8	-1.258837461
2T21	Medium	Blood	-	8	-0.591889226
2T22	High	Blood	-	8	-0.859911191
2T23	Control	Blood	-	8	-0.408139114
2T24	Medium	Blood	-	8	-1.60886624
3T1-i	Medium	Blood	LPS	16	0.786666898
3T1-0	Medium	Blood	PBS	16	1.263365469

3T3-i	High	Blood	LPS	16	1.863632964
3T3-0	High	Blood	PBS	16	1.823469552
3T5-i	High	Blood	LPS	16	0.115784498
3T5-0	High	Blood	PBS	16	0.403709659
3T6-i	Low	Blood	LPS	16	0.849306312
3T6-0	Low	Blood	PBS	16	0.280874628
3T7-i	Low	Blood	LPS	16	0.380435541
3T7-0	Low	Blood	PBS	16	1.524493094
3T9-i	Control	Blood	LPS	16	0.483053934
3T9-0	Control	Blood	PBS	16	0.505429873
3T10-i	Low	Blood	LPS	16	1.610172582
3T10-0	Low	Blood	PBS	16	1.389648725
3T11-i	High	Blood	LPS	16	0.280889394
3T11-0	High	Blood	PBS	16	0.714964589
3T12-i	High	Blood	LPS	16	-0.175114888
3T12-0	High	Blood	PBS	16	0.508485288
3T13-i	Medium	Blood	LPS	16	0.641963877
3T13-0	Medium	Blood	PBS	16	0.222991567
3T15-i	Low	Blood	LPS	16	0.487737168
3T15-0	Low	Blood	PBS	16	0.179006919
3T16-i	Control	Blood	LPS	16	-0.47978217
3T16-0	Control	Blood	PBS	16	1.312034044
3T19-i	Control	Blood	LPS	16	0.179529028
3T19-0	Control	Blood	PBS	16	-0.289510401
3T20-i	Control	Blood	LPS	16	0.297832554
3T20-0	Control	Blood	PBS	16	-0.406069678
3T21-i	Medium	Blood	LPS	16	1.809614379
3T21-0	Medium	Blood	PBS	16	-0.013086642
3T23-i	Control	Blood	LPS	16	-0.587163904
3T23-0	Control	Blood	PBS	16	-0.127424384
3T24-i	Medium	Blood	LPS	16	-0.076557913
3T24-0	Medium	Blood	PBS	16	1.457800123
0T1N	Zero	Kidney	-	0	1.119720426
0T2N	Zero	Kidney	-	0	0.846087513
0T3N	Zero	Kidney	-	0	1.361561944
0T4N	Zero	Kidney	-	0	0.035277769
0T5N	Zero	Kidney	-	0	1.028822952
0T6N	Zero	Kidney	-	0	0.797511304
0T7N	Zero	Kidney	-	0	-0.190507667
0T8N	Zero	Kidney	-	0	0.750156793
1T1N	Medium	Kidney	-	2	-1.588917266
1T2N	Low	Kidney	-	2	-1.037941971

1T3N	High	Kidney	-	2	1.550641908
1T4N	Medium	Kidney	-	2	1.687633153
1T5N	High	Kidney	-	2	1.176290932
1T6N	Low	Kidney	-	2	1.805524813
1T7N	Low	Kidney	-	2	1.479747966
1T8N	High	Kidney	-	2	1.349217294
1T9N	Control	Kidney	-	2	1.941491418
1T10N	Low	Kidney	-	2	1.948028252
1T11N	High	Kidney	-	2	-0.164327348
1T12N	High	Kidney	-	2	0.87666011
1T13N	Medium	Kidney	-	2	1.690653488
1T14N	Control	Kidney	-	2	1.800976679
1T15N	Low	Kidney	-	2	0.654048346
1T16N	Control	Kidney	-	2	1.093595394
1T17N	Low	Kidney	-	2	0.593784401
1T18N	Medium	Kidney	-	2	1.939853694
1T19N	Control	Kidney	-	2	-0.129769229
1T20N	Control	Kidney	-	2	-0.29201621
1T21N	Medium	Kidney	-	2	-0.351487584
1T22N	High	Kidney	-	2	
1T23N	Control	Kidney	-	2	1.347349612
1T24N	Medium	Kidney	-	2	1.103541357
2T1N	Medium	Kidney	-	8	1.468434907
2T2N	Low	Kidney	-	8	-0.908288142
2T3N	High	Kidney	-	8	1.456642197
2T4N	Medium	Kidney	-	8	-0.768808332
2T5N	High	Kidney	-	8	0.415230451
2T6N	Low	Kidney	-	8	0.254201637
2T7N	Low	Kidney	-	8	0.260951621
2T8N	High	Kidney	-	8	0.040547171
2T9N	Control	Kidney	-	8	1.747905908
2T10N	Low	Kidney	-	8	1.922473196
2T11N	High	Kidney	-	8	1.771805187
2T12N	High	Kidney	-	8	1.91242622
2T13N	Medium	Kidney	-	8	1.39585193
2T14N	Control	Kidney	-	8	1.782417891
2T15N	Low	Kidney	-	8	1.80681067
2T16N	Control	Kidney	-	8	1.888265669
2T17N	Low	Kidney	-	8	-0.533171063
2T18N	Medium	Kidney	-	8	-0.335133771
2T19N	Control	Kidney	-	8	-0.865521551
2T20N	Control	Kidney	-	8	-0.3478374

2T21N	Medium	Kidney	-	8	-0.671214201
2T22N	High	Kidney	-	8	-1.335361662
2T23N	Control	Kidney	-	8	-0.694688323
2T24N	Medium	Kidney	-	8	-1.372599473
3T1N-i	Medium	Kidney	LPS	16	-0.284072506
3T1N-0	Medium	Kidney	PBS	16	-0.013620652
3T3N-i	High	Kidney	LPS	16	-0.0531566
3T3N-0	High	Kidney	PBS	16	0.118502645
3T5N-i	High	Kidney	LPS	16	-0.17180914
3T5N-0	High	Kidney	PBS	16	0.391136311
3T6N-i	Low	Kidney	LPS	16	-0.392013103
3T6N-0	Low	Kidney	PBS	16	-0.513968248
3T7N-i	Low	Kidney	LPS	16	-0.372943448
3T7N-0	Low	Kidney	PBS	16	0.336875033
3T9N-i	Control	Kidney	LPS	16	1.540810157
3T9N-0	Control	Kidney	PBS	16	1.698401368
3T10N-i	Low	Kidney	LPS	16	1.544917026
3T10N-0	Low	Kidney	PBS	16	1.483538935
3T11N-i	High	Kidney	LPS	16	1.238497164
3T11N-0	High	Kidney	PBS	16	-0.010783741
3T12N-i	High	Kidney	LPS	16	1.108668497
3T12N-0	High	Kidney	PBS	16	1.62326155
3T13N-i	Medium	Kidney	LPS	16	1.597261646
3T13N-0	Medium	Kidney	PBS	16	1.441594232
3T15N-i	Low	Kidney	LPS	16	1.118363602
3T15N-0	Low	Kidney	PBS	16	0.877650929
3T16N-i	Control	Kidney	LPS	16	0.735993686
3T16N-0	Control	Kidney	PBS	16	1.42943146
3T19N-i	Control	Kidney	LPS	16	-0.186857347
3T19N-0	Control	Kidney	PBS	16	0.069509457
3T20N-i	Control	Kidney	LPS	16	1.51856762
3T20N-0	Control	Kidney	PBS	16	0.014277072
3T21N-i	Medium	Kidney	LPS	16	-0.01451081
3T21N-0	Medium	Kidney	PBS	16	-0.07648276
3T23N-i	Control	Kidney	LPS	16	0.765660522
3T23N-0	Control	Kidney	PBS	16	0.086175336
3T24N-i	Medium	Kidney	LPS	16	1.177289751
3T24N-0	Medium	Kidney	PBS	16	1.442789171

Attachment 6: Respiratory burst data shown as log10-transformed H_2O_2 production stimulated with PMA and without PMA.

Sample	Treatment	Exposure (days)	H_2O_2 production NO PMA	H_2O_2 production PMA
0T1	Zero	0	0.354108439	1.226342087
0T2	Zero	0	1.097604329	1.694780636
0T3	Zero	0	0.753583059	0.773786445
0T4	Zero	0	1.65040467	1.532372134
0T5	Zero	0	-0.26760624	0.649334859
0T6	Zero	0	0.252853031	0.413299764
0T7	Zero	0	1.230193379	1.266231697
0T8	Zero	0	2.564879018	2.452108584
1T1	Medium	2		0.506505032
1T2	Low	2	-1.22184875	-2
1T3	High	2		0.372912003
1T4	Medium	2	0.456366033	0.117271296
1T5	High	2	-0.187086643	0.434568904
1T6	Low	2	-0.214670165	0.56937391
1T7	Low	2	-0.721246399	0.737192643
1T8	High	2	0.252853031	-0.455931956
1T9	Control	2		0.598790507
1T10	Low	2	0.230448921	-0.27572413
1T11	High	2		-0.744727495
1T12	High	2	0.340444115	0.170261715
1T13	Medium	2		-0.080921908
1T14	Control	2		
1T15	Low	2		
1T16	Control	2		
1T17	Low	2	-0.26760624	0.045322979
1T18	Medium	2	-0.193820026	-0.187086643
1T19	Control	2	-0.236572006	-0.337242168
1T20	Control	2	0.075546961	-0.292429824
1T21	Medium	2	0.98811284	1.180985581
1T22	High	2	1.115610512	1.061829307
1T23	Control	2	1.091666958	1.744762237
1T24	Medium	2	1.211921084	1.0923697
2T1	Medium	8	0.477121255	0.722633923
2T2	Low	8	0.698970004	0.344392274
2T3	High	8	-0.055517328	-0.091514981
2T4	Medium	8	0.264817823	-0.431798276
2T5	High	8	0.526339277	1.055760465
2T6	Low	8	0.706717782	0.809559715
2T7	Low	8	-0.259637311	0.401400541
2T8	High	8	0.356025857	0.041392685

2T9	Control	8	0.041392685	0.645422269
2T10	Low	8	0.80685803	1.171141151
2T11	High	8	0.195899652	0.413299764
2T12	High	8	0.378397901	0.646403726
2T13	Medium	8	0.330413773	1.123524981
2T14	Control	8	1.053078444	1.546542664
2T15	Low	8	0.797959644	1.437274797
2T16	Control	8	0.774516966	1.172894698
2T17	Low	8		
2T18	Medium	8		
2T19	Control	8		
2T20	Control	8		
2T21	Medium	8		
2T22	High	8		
2T23	Control	8		
2T24	Medium	8		
3T1-i	Medium	16 LPS	1.769451179	1.97183228
3T1-0	Medium	16 PBS	1.178401342	1.081347308
3T3-i	High	16 LPS	0.954724791	0.859138297
3T3-0	High	16 PBS	1.308350949	1.079181246
3T5-i	High	16 LPS	1.780389329	1.916348652
3T5-0	High	16 PBS	2.040246215	1.944137073
3T6-i	Low	16 LPS	1.940616082	1.89580915
3T6-0	Low	16 PBS	1.915610863	1.869349081
3T7-i	Low	16 LPS	2.001647191	2.084111352
3T7-0	Low	16 PBS	1.547282308	1.532117116
3T9-i	Control	16 LPS	1.743744879	2.228528677
3T9-0	Control	16 PBS	-0.420216403	0.703291378
3T10-i	Low	16 LPS	0.255272505	0.198657087
3T10-0	Low	16 PBS	1.276691529	0.530199698
3T11-i	High	16 LPS	1.801952235	2.121756759
3T11-0	High	16 PBS	0.722633923	0.902546779
3T12-i	High	16 LPS	1.603144373	1.671450554
3T12-0	High	16 PBS	1.511616021	1.220369633
3T13-i	Medium	16 LPS	1.217483944	1.757623746
3T13-0	Medium	16 PBS	1.072984745	1.164055292
3T15-i	Low	16 LPS	1.32407658	1.40534636
3T15-0	Low	16 PBS	1.842921121	1.653115993
3T16-i	Control	16 LPS	2.211414245	2.383635868
3T16-0	Control	16 PBS	1.903903527	1.970579306
3T19-i	Control	16 LPS	0.290034611	-0.124938737
3T19-0	Control	16 PBS	1.120902818	1.009450896
3T20-i	Control	16 LPS	0.766412847	0.51054501
3T20-0	Control	16 PBS	1.388633969	1.395501124
3T21-i	Medium	16 LPS	0.530199698	0.934498451

3T21-0	Medium	16 PBS	1.136403448	1.172310969
3T23-i	Control	16 LPS	0.397940009	
3T23-0	Control	16 PBS	1.845779967	1.810098041
3T24-i	Medium	16 LPS	1.574147064	1.713490543
3T24-0	Medium	16 PBS	0.903632516	0.946452265

Attachment 7: Chemicals

Chemical	**Product No**	**Producer**
Bovine serum albumin	A7030	Sigma-Aldrich
Carbonate-bicarbonate buffer (capsule)	C3041	Sigma-Aldrich
Copper tartrate solution (reagent A)	500-0113	Bio-Rad
Dichloromethane		
Dimethyl sulfoxide	0231	Amresco
DL-Dithiotreitol	43819	Fluka Analytic
Ethylenediaminetatraacetic acid solution	03690	Fluka Analytic
Folin reagent (reagent B)	500-0114	Bio-Rad
Glutaraldehyde		Sigma-Aldrich
Glycerol	G5516	Sigma-Aldrich
Goat anti-rabbit IgG conjugated with HRP, GAR-HRP	L99496153-001	Biosense Laboratories
Heparin	H3393	Sigma-Aldrich
Horseradish peroxidase (HRP)	P8125	Sigma-Aldrich
Hydrogen peroxide solution (30%)	216763	Sigma-Aldrich
L15 Leibovitz	L4386	Sigma-Aldrich
LMP agarose	A9414	Sigma-Aldrich
Methanol	322131	Sigma-Aldrich
Percoll	17-0891-01	GE Healthcare
Phorbol 12-myristate 13-acetate (PMA)	P8139	Sigma-Aldrich
Potassium chloride	1.04936.1000	Merck
Potassium phosphate dibasic	221317	Sigma-Aldrich
Potassium phosphate monobasic	P5379	Sigma-Aldrich
Rabbit-anti-fish CYP1A antibody	CO2401201-500	Biosense Laboratories AS
Resorufin ethyl ether	E3763	Sigma-Aldrich
Resorufin sodium salt	R3257	Sigma-Aldrich
Sodium dihydrogenphosphate	71504	Sigma-Aldrich
Sodium phosphate dibasic	S5136	Sigma-Aldrich
SyberGold		
Tetramethylbenzidine liquid substrate (TMB)	4395L	Kem En Tec Diagnostics
Triphenylamine	92930	Fluka Analytics
Triton X-100	1001054580	Sigma-Aldrich
Trizma base	T-1503	Sigma-Aldrich
Trizma hydrochloride	T3253	Sigma-Aldrich
Tryphan-blue		Invitrogen

Tween-20	P1379	Sigma-Aldrich
β-glucoronidase/aryl sulphatase	1.04114.0002	Merck
β-NADPH reduced tetrasodium salt	N1630	Sigma-Aldrich