The Effects of Marine Nutrients on 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and PCB-126 Toxicity in HEPA1-6 Cells

by

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Bergen, June 2023
Felicia Reyes

TABLE OF CONTENTS

ABBREVIATIONS

AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of Variance
ARNT	Aryl hydrocarbon receptor nuclear translocator
AUC	Area under curve
BSA	Bovine serum albumin
cDNA	Complementary DNA
Ctrl	Control
CYP	Cytochrome-P450
Cyto-roGFP	Cytosolic - Reduction-oxidation sensitive green fluorescent protein
DHA	Docosahexaenoic acid
DLCs	Dioxin like compounds
(dl-)PCB	**(**dioxin like-) Polychlorinated biphenyl
DMEM	Dulbeccos modified eagle's medium
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
FA	Fatty acid
FPS	Fetal bovine serum
HSP90	Heat shock protein 90
Matrix - roGFP	Mitochondrial matrix - reduction-oxidation sensitive green fluorescent protein
PBS	Phosphate buffered saline
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
POPs	Persistent organic pollutants
qPCR	Quantitative polymerase chain reaction
REP	Relative effect potency
ROS	Reactive oxygen species
RTCA	Real time cell analyser
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TEF	Toxic equivalency factor
TEQ	Toxic equivalency quotient
TWI	Tolerable weekly intake
VKM	Vitenskapskomiteen for mat og miljø

ABSTRACT

Dioxin like compounds (DLCs) are a group of persistent organic pollutants (POPs) that are associated with several adverse health effects. DLCs are lipophilic compounds that can bioaccumulate in the marine food chain, making seafood a major source of human exposure. However, this food group is also an important source of essential n-3 fatty acids, such as DHA and EPA. The European Food Safety Authority (EFSA) did a new risk assessment on DLCs in 2018, where they lowered the tolerable weekly intake (TWI) to 2 pg TEQ/kg bodyweight per week, which was 7-fold lower than the previous TWI for DLCs. The Norwegian Scientific Committee for Food and Environment (VKM) conducted a benefit and risk assessment in 2022 concluding that the benefits outweigh the risk from contaminants in fish and seafood. However, the same year VKM also published a risk assessment for DLCs concluding that the general dietary exposure in the Norwegian population exceeds the TWI. Knowledge regarding the interactions between n-3 fatty acids, such as DHA and EPA, and DLCs is still limited. Therefore, the aim of this thesis was to investigate whether marine n-3 fatty acids can have an effect on the DLCs, TCDD and PCB-126, in mouse hepatoma cells (HEPA1-6 cells).

In this study, we examined cell viability, gene expression, CYP1a1 protein expression and ROS induction. The methods used to assess these endpoints were cell impedance assay and gene- and protein expression analysis. Additionally, the cells were transiently transfected with roGFP to assess the redox changes in the cells. The results showed that n-3 fatty acids did not affect the toxicity of TCDD and PCB-126 to growth in HEPA1-6 cells. Further, the gene expression analysis of the TCDD exposed cells showed a significant upregulation of *Cyp1a1* and *Cyp1a2*, as well as reduced gene expression of *Cyp1a1* in EPA-incubated cells. Moreover, the gene expression results indicated that there was no upregulation of antioxidant markers (CAT, GPX and SOD). Due to methodological challenges, we were not able to measure CYP1a1 abundance and redox changes.

1. INTRODUCTION

1.1 DIOXINS AND dl-PCBs

Polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) are two groups of tricyclic planar compounds often referred to as dioxins. Although these compounds have structural and molecular similarities, individual dioxin congeners have different toxicity depending on the number and position of chlorine atoms (EFSA, 2018). There are 210 possible dioxin congeners, but only 17 congeners that consists of at least four chlorine atoms on each of the two benzene rings (2,3,7,8) are harmful to humans and animals (Pereira, 2004). Dioxins have not been manufactured for commercial or industrial use, but are unintentionally formed by-products of industrial combustion processes of chlorine-based compounds, i.e., bleaching of paper pulp, evaporation from chlorophenol wood preservatives (like pentachlorophenol (PCP)), and usage and manufacture of some pesticides. Even though these industrial processes are the main source of dioxins in the environment, they can also occur from natural processes such as forest fires and volcanic activities (Hoogenboom *et al.*, 1995; Baars *et al.*, 2004).

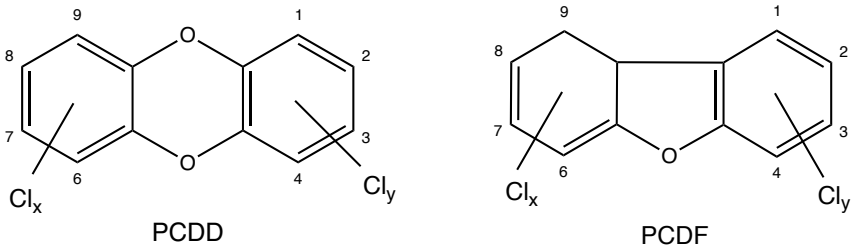

Figure 1.1 - General structure of PCDDs and PCDFs. General molecular structure of polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF). Adapted from EFSA (2018). Structures created in ChemDraw®

Polychlorinated biphenyls (PCBs), unlike dioxins, were industrially and commercially produced from the late 1920s due to their physical and chemical properties, such as chemical stability, non-flammability, high dielectric constant, to name a few (EFSA, 2018). Usually, they were produced as mixtures and rarely as individual congeners**.** However, in the late 1970s, they were banned in many countries due to their high persistency in the environment and toxicity to biota (Baars *et al.*, 2004). PCBs are chlorinated hydrocarbons consisting of two benzene rings. There are 209 possible congeners of PCB, based on the number of chlorine

atoms (1-10) and where on the two benzene rings they are substituted (Kulkarni, Crespo and Afonso, 2008). Their biochemical and toxicological properties can divide them into groups of dioxin-like PCBs (dl-PCBs) and non-dioxin-like PCBs (ndl-PCBs**)**. As the name suggests, dl-PCBs show similar toxicity to dioxins and these congeners contain at least four chlorine atoms which are either non-ortho or mono-ortho substituted. This constitutes 12 congeners that are of toxicological concern, however, most PCBs do not show dioxin like toxicity (EFSA, 2010).

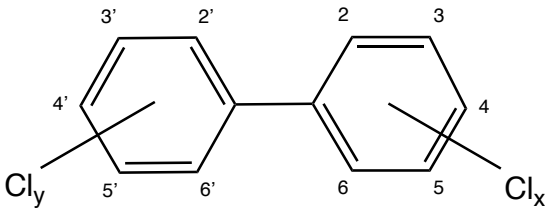

Figure 1.2 – General structure of PCB. General molecular structure of polychlorinated biphenyls (PCB). Adapted from EFSA (2018). Structure created in ChemDraw®

There are in total 29 congeners of dioxins and dl-PCBs that share similar physiochemistry and toxicity. Dioxins and dl-PCBs toxicity or effects are mainly mediated by binding to the aryl hydrocarbon receptor (AHR) and activating its gene expression. The dl-PCBs are coplanar, hence they can bind to the AhR and exhibit similar toxicity to dioxins (EFSA, 2010). The AHR is a member of the basic Helix-Loop-Helix Per-ARNT-Sim (bHLH-PAS) family and is a ligand-activated transcription factor. In a non-ligand bound state, AHR is located in the cytoplasm as a complex with two Heat Shock Protein 90 (HSP90), Hepatitis B Virus X-associated protein 2 (XAP2) and a co-chaperone p23 (Fujii-Kuriyama and Mimura, 2005). Upon ligand binding, by for instance dioxins or dl-PCBs, AHR is released from its complex and the AHR subunit is translocated into the nucleus. When entering the nucleus, the ligand bound AHR will form a heterodimer with its partner molecule ARNT (Aryl hydrocarbon receptor nuclear translocator). The ligand-AhR-ARNT complex can further bind to the dioxin response element (DRE) located in the promoter regions of the targeted gene and induce the expression of genes such as CYP1A1 and CYP1A2 (Mandal, 2005; Larigot *et al.*, 2018).

1.2 TOXIC EQUIVALENCY FACTOR

Toxic equivalency factors (TEF) are a concept that has been developed to compare the different toxicities of the different congeners of dioxins and dl-PCBs. The TEF concept assumes that the dioxins and dl-PCBs mediate their toxicological effects through binding of the AhR, where the

most toxic and potent of the dioxins is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Therefore, when determining an individual congeners TEF, their relative toxicity is compared against TCDD which has a TEF of 1 (Haws *et al.*, 2006; Van den Berg *et al.*, 2006). These TEF values are based on *in vivo* studies and are supported by *in vitro* studies (EFSA, 2018). The criteria for including a compound in the TEF-scheme are: (1) that they show structural similarities to dioxins, (2) the compounds ability to bind to the AHR, (3) to ability to elicit AHR-mediated biochemical and toxic response, and (4) persistence and accumulation in the food chain (Van Den Berg *et al.*, 1998). The TEF concept is established using the relative potency (REP) which is determined by comparing the potency of an individual dioxin or dl-PCB to a reference compound, usually TCDD (Haws *et al.*, 2006). This is similar to TEF but REP is based on a single study (EPA, 2010). The total toxic equivalent (TEQ) is based on TEF values as it estimates the total toxicity of a mixture. The TEQ value of a mixture is calculated by summing the concentration of each compound multiplied with their respective TEF values as most dioxins and dl-PCBs occurs in mixtures in the environment (Van den Berg *et al.*, 2006; Aune, 2007). These TEF-values are determined by the World Health Organization (WHO) expert panel and were last evaluated in 2005 by Van den Berg *et al*. However, there was an expert meeting in October of 2022 where the 2005 WHO TEFs were re-evaluated, and the details from the re-evaluation are expected to be published in 2023 (WHO, 2022).

Table 1.1 – Selected WHO_{2005} TEF – Values for corresponding dioxins and dl-PCBs. TEF values are retrieved from Van den Berg (2006).

Dioxins (PCDD and PCDF)		**dl -PCBs**	
Congener	WHO_{2005}-TEF	Congener	WHO_{2005}-TEF
2,3,7,8-TCDD	1	PCB 77	0.0001
1,2,3,7,8-PeCDD	1	PCB 81	0.0003
1,2,3,4,7,8-HxCDD	0.1	PCB 126	0.1
OCDD	0.0003	PCB 169	0.03
2,3,7,8-TCDF	0.1	PCB 105	0.00003
1,2,3,7,8-PeCDF	0.03	PCB 114	0.00003
1,2,3,4,7,8-HxCDF	0.03	PCB 118	0.00003
OCDF	0.0003		

1.3 TOXICOKINETICS AND TOXICODYNAMICS

Dioxins and dl-PCBs are hydrophobic and lipophilic, making them highly persistent in the environment, hence being categorized as persistent organic pollutants (POPs). Due to these characteristics, they can bioaccumulate and biomagnify into the food chain, making food the main source of exposure to humans. This accounts for approximately 90% of the total human exposure of dioxins and dl-PCBs (EFSA, 2018). Dioxins and dl-PCBs are mainly taken up by the body via intestinal tract. When entering the body, it is transported via the blood where they are bound to lipid particles and proteins. As dioxins and dl-PCBs are highly lipophilic and stable, they tend to be stored in adipose and liver tissues (EFSA, 2018). Additionally, a congener is more stable and lipophilic as the number of chlorine atoms increases (Aune, 2007), and these characteristics makes them difficult to metabolise within the body (Furue *et al.*, 2021). However they can, to some degree, be metabolized in the liver by hydroxylation (Phase I) followed by sulfation or glucuronidation (Phase II) (VKM, 2011). Both phases are part of the biotransformation of xenobiotics; the latter are chemicals which are foreign to the body (Burcham, 2014). Biotransformation is a metabolic process with the primary goal to excrete xenobiotics out of the body by making them more water soluble than their parent molecule. Enzymes such as CYP1A1 and CYP1A2 are important oxidizing enzymes in Phase I of biotransformation, and as mentioned previously are highly upregulated when AHR is activated (Burcham, 2014; EFSA, 2018).

The upregulation of CYP1a1 and limited metabolization of dioxins and dl-PCBs cause oxidative stress in cells (Stohs, 1990; Hassoun *et al.*, 2000).The Phase I and Phase II enzymes that are regulated by AHR can generate reactive intermediate in the oxidation process and the formation of reactive oxygen species (ROS). If the production of ROS is higher compared to the cellular antioxidant defenses, it results in oxidative stress. Consequently, it can do damage to the DNA, increase inflammation, cause lipid peroxidation, and apoptosis (Dalton, Puga and Shertzer, 2002). Some of the cellular antioxidant defenses, include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase 4 (GPX) (Dalton, Puga and Shertzer, 2002; Burcham, 2014). In the cell, SOD converts superoxides into O_2 and hydrogen peroxide (H_2O_2), which are more reactive than superoxide, but CAT and GPX converts H_2O_2 to H_2O and O_2 (Weydert and Cullen, 2010). TCDD has been shown to induce oxidative stress resulting in lipid peroxidation and DNA damage (Hassoun *et al.*, 2000). Therefore, oxidative stress indued by

dioxins and dl-PCBs is considered to be a mechanism that characterizes dioxin-mediated toxicity (Furue *et al.*, 2021).

1.4 MARINE N-3 FATTY ACIDS

N-3 (also known as ω-3) fatty acids (FA) are one of two classes of long-chained polyunsaturated fatty acids (PFUA) and have the first double bond located at carbon number three from the methyl end. There are four n-3 FAs: α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and docosapentaenoic acid (DPA, 22:5n-3) (VKM, 2011). EPA, DHA and DPA are also called marine n-3 FA as they are mainly derived from fish and seafood. The 'essential' fatty acids (EFA), i.e. linolenic acid and ALA cannot be synthesized by humans and animals and are therefore mainly obtained through the diet. However, the n-3 FAs can be synthesized in the body from ALA but the synthesis is very limited and it is therefore important to supplement from the diet as they are important for normal development and function (Spector, 1999; Calder, 2015). The n-3 FAs have been shown to reduce cardiovascular disease, have anti – inflammatory effects, and improve cognitive health (Calder *et al.*, 2010; VKM *et al.*, 2011). N-3 FAs are also important for growth and development in infants, as DHA have been associated with positive infant neurodevelopmental (Cetin and Koletzko, 2008; Calder *et al.*, 2010). In the cells, DHA and EPA can be incorporated to the glycerophospholipids in the cell membrane altering its fluidity and flexibility (Hishikawa *et al.*, 2017).

1.4.1 MOLECULAR MECHANISMS OF MARINE N-3 FATTY ACIDS

EPA and DHA can be catalyzed by three enzymatic pathways, cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450). These enzymes can metabolize DHA and EPA into metabolites that acts anti-inflammatory (Gurr, Harwood and Frayn, 2002; Dyall, 2015). For instance, when EPA is metabolized by COX it synthesizes the production of prostaglandins (PGs), thromboxane's (TX), and prostacyclin (PGI) that are less inflammatory than the ones that are derived from arachidonic acid (Pavel flachs, 2009).

1.5 DIRECTIVES

In 2018, the European Food Safety Authority (EFSA) published a new a risk assessment on dioxins and dl-PCBs in feed and food. In this report, they set a new Tolerable Weekly Intake

(TWI) at 2 pg TEQ/kg bodyweight per week, based on a decreased sperm concentration in men, as this new TWI protects reduced sperm quality. As there was new available knowledge, including the new TWI, the Norwegian Scientific Committee for Food and Environment (VKM) did two assessments in 2022, a new benefit and risk assessment of contaminants in the Norwegian diet and a risk assessment of dioxins, furans, and dl-PCBs in food in Norway. The reports concluded that the benefits from eating the recommended intake of fish of two to three times per week did outweigh the risk from contaminants from fish and seafood. However, the risk assessment of dioxins and dl-PCBs concluded that the general dietary exposure in the Norwegian population exceeds the TWI (VKM, 2022; VKM, 2022). Additionally, the TEF of PCB-126 was discussed as it might be too high, as studies have shown that PCB-126 is less potent in humans than rodents (EFSA, 2018). Establishment of TEF-values have mainly been based on animal studies, hence the high TEF value of 0.1 for PCB-126. Further, PCB-126 contributes to major part of the total TEQ-exposure from seafood, even though this congener only constitutes a minor part of the total dioxin concentrations (Nøstbakken *et al.*, 2021).

1.6 INTERACTIONS

n-3 FA have a range of health benefits for human health and development and are mostly obtained from fatty fish and seafood (Calder *et al.*, 2010). However, there is a growing concern about fish and seafood consumption as these food groups also contains contaminants, such as dioxins and dl-PCBs (Bushkin-Bedient and Carpenter, 2010). There is still limited knowledge about the interactions between dioxins and dl-PCBs, and n-3 FA, though some studies have shown that, n-3 FA can have a protective effect against TCDD induced toxicity (Turkez *et al.*, 2012; Türkez, Geyikoglu and Yousef, 2012; Palanisamy *et al.*, 2015).

1.7 THE AIM OF THE STUDY

Marine n-3 fatty acids fish and seafood are known to have positive impact on our health as it contributes to different stages of development, cognitive health, and decrease in cardiovascular disease (Calder *et al.*, 2010). However, seafood is also one of the main sources of environmental contaminants, such as dioxins- and dl-PCBs. The main aim of this thesis is to investigate if marine n-3 FA can have an effect on dioxin-like compounds (DLCs) toxicity, such as TCDD and PCB-126. As well as improving our knowledge on TCDDs toxicity on liver cells. To investigate toxicity of TCDD and PCB-126 and the potential interactions with n-3 FA

HEPA1-6 cells were used. HEPA1-6 cells are mouse hepatoma cells carried in C57 leaden/J mice (Darlington, 1987). The sub aims of this study were :

i. Examine the effects of TCDDs and PCB-126 on HEPA1-6 cells viability, with and without marine n-3 fatty acids.
ii. Investigate the regulation of transcriptional markers in the cells after exposure to TCDD in combination with n-3 fatty acids.
iii. Explore TCDDs effect on CYP1a1 induction in combination with n-3 fatty acids.
iv. Establish cell expressing roGFP to investigate changes in the redox environment when exposed to TCDD and marine n-3 fatty acids.

2. MATERIALS

2.1 CHEMICALS AND REAGENTS

Table 2.1 – Chemicals and reagents used during the master thesis.

Name	Supplier	Catalogue number
10 x PBS	Bio-Rad	1610780
10 x TrisglycineSDS Buffer	Bio-Rad	1610732
100 % Ethanol	Antibac	200-578-6
100x Protease/Phosphatase Inhibitor Cocktail	Cell Signalling Technology	5872S
2 x Laemmli Sample Buffer	Bio-Rad	161-0737
5 x Transfer Buffer	Bio-Rad	10026938
Acetic acid	ACROS Organics	A0305248
Agar	Sigma-Aldrich	9002-18-0
Amersham™ ECL select™ Western Blotting Detection Reagent	Merck	RPN2235
Ampicillin Sodium Salt – Irradiated	GIBCO™ /Thermo Fisher	11593027
Anti-CYP1A1 antibody	Abcam	Ab235185
Coomassie Brilliant Blue R	Sigma-Aldrich	6104-59-2
Dimethyl Sulfoxide (DMSO)	Sigma-Alrdich	67-68-5
Dulbecco's Modified Eagle's Medium – High Glucose	Sigma-Aldrich	D6429
ECL™ Blocking Agent	Merck	RPN2125
Fetal bone serum (FBS)	Sigma-Aldrich	1943609-65-1
FuGENE® HD Transfection Reagent	Promega	E231A
Hydrogen Peroxide Solution	Sigma-Aldrich	7722-84-1
Methanol	Honeywell Research Chemicals	67-56-1
Paraformaldehyd (37%)	Sigma-Aldrich	104003
Penicilin-streptomycin	Sigma-Aldrich	P4083
Phosphate buffered saline tablet	Sigma-Aldrich	79382

Precision Plus Protein™ WesternC™ Blotting Standards	Bio-Rad	1610376
Precision Protein Step Tactin-HRP Conjugate	Bio-Rad	1610381
Rabbit CYP1a1 Polyclonal Antibody	MyBioSource	MBS9409697
RIPA Buffer	sigma	R0278
RNA 6000 Nano Ladder	Agilent	5067-1529
Sodium Acetate Buffer Solution	Sigma-Aldrich	126-96-5
Tris-EDTA buffer solution	Sigma-Aldrich	93302
Trypan Blue 0.4%	Bio-Rad	1450013
Trypsin – EDTA Solution	Sigma-Aldrich	T4049
Tryptone	Sigma-Aldrich	91079-40-2
TWEEN® 20	Sigma-Aldrich	9005-64-5
Yeast extract	Sigma-Aldrich	8013-01-2
β-mercaptoethanol	Bio-Rad	1610710

2.2 TOXICANTS

Table 2.2 – Compounds used in the HEPA1-6 cell experiment.

Compounds	Abbreviation	TEF*	Molecular Weight (g/mol)	Concentration (mg/ml)	Catalogue number
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	TCDD	1	321.96	0.05	1746-01-6
3,3',4,4',5-Pentachlorobiphenyl	PCB-126	0.1	326.40	1	57465-28-8

*TEF-values obtained from WHO's re-evaluation of TEF values from 2005 (Van den Berg *et al.*, 2006)

2.3 COMMERCIAL KITS

Table 2.3 – Commercial kits utilized during the master thesis.

Name	Use	Supplier	Catalogue number
Pierce 660 Protein Assay	Protein concentrations	Thermo Fisher	22662
Pierce Co-Immunoprecipitation Kit	Detection of specific proteins	Thermo Fisher	26149
QIAGEN Plasmid Midi Kit	Plasmid DNA extraction	Qiagen	12143
RNA 6000 Nano LabChip Kit	RNA quality	Agilent	5067-1511
RNeasy® Plus Mini Kit	RNA extraction	Qiagen	74134

2.4 EQUIPMENT AND INSTRUMENTS

Table 2.4 – Equipment and instruments utilized during the master thesis.

Name	Use	Supplier
Nikon Eclipse Ti	Fluorescent imaging	Nikon®
CFX Touch Real-Time PCR Detection System	RT-qPCR	Bio-Rad
Biomek 4000	Pipetting	Beckman Coulter
Forma™ Steri-Cycle™ CO_2 Incubator	Cell maintenance	Thermo Scientific
Jenco™ Inverted Compound Microscope	Cell maintenance	Merck
Mini-PROTEAN® Tetra System	Electrophoresis	Bio-Rad
Mini-PROTEAN TGX Stain-Free gel (4-15%)	SDS-PAGE	Bio-Rad
Nanodrop™ 1000	RNA quality and protein concentration	Thermo Fisher
Mr. Frosty™ Freezing Container	Cell preservation	Thermo Fisher

Nunc EasyFlask 72 cm^2	Cell cultivation	Thermo Fisher
E-plate 96-well	Cell viability assay	Agilent
Trans-Blot® Turbo™ Transfer system	Western blot	Bio-Rad
xCELLigence RTCA	Cell viability assay	Bio-Rad
Victor x5 Multilabel Plate Reader	Protein concentration	PerkElmer

2.5 SOFTWARE

Table 2.5 – Software utilized during the master thesis.

Software	Use	Supplier
Agilent 2100 BioAnalyzer	RNA quality	Agilent Technologies
Biorender	Figures	Biorender
Bio-Rad CFX Maestro	qPCR data analysis	Bio-Rad
ChemDraw®	Figures	PerkinElmer Informatics
Excel	Data treatment	Microsoft
GraphPad Prism 9	Figures and statistics	Graphpad Software Inc.
Perkin Elmer working software 2030	Spectrophotometry	Perkin Elmer
Statistica 13	Statistics	Stat Soft
NIS-Elements AR 4.51.01	Imaging of transfected cells with roGFP	Nikon®
Image Lab 6.0.1	Detecting gels and blots	Bio-Rad
PowerPoint	Figures	Microsoft
RTCA Software 1.2.1	Cell viability	Agilent Technology

2.6 BUFFERS

Table 2.6 – Buffers used in SDS-PAGE and Western Blot

Buffers	Content
RIPA-Protease Lysis Buffer	1 µl of 100x Protease/Phosphatase Inhibitor cocktail 1 ml RIPA Buffer

Sample Buffer	950 µl Laemmli sample buffer 50 µl β-mercaptoethanol
1x Running buffer	100 ml 10x TriglycineSDS buffer 900 ml dH_2O
Washing Buffer (0.05% PBS-Tween)	100 ml 10xPBS 900 ml dH_2O 500 µl Tween-20
1x Transfer Buffer	200 ml 5x Transfer Buffer 600 ml dH_2O 200 ml 100% Ethanol
Blocking buffer (5% ECL)	1 g ECL-blocking reagent 20 ml Washing Buffer

2.7 LB-MEDIA AND COOMASIE BLUE STAIN SOLUTION

Medium and solution	Content
LB-Media	950 ml ddH_2O 10 g Tryptone 10 g Sodium Chloride (NaCl) 5 g Yeast Extract 10 mg/ml Ampicillin Sodium Salt – Irradiated
Coomasie blue stain solution	For 100 ml: 0.3 g Coomasie blue stain 45 ml methanol 10 ml acetic acid 45 ml dH_2O

2.8 PLASMIDS

Table 2.8 – Plasmids used for transfection.

Plasmid	Supplier	Catalogue number
Cyto-roGFP	addgene	49435
Matrix-roGFP	addgene	49437

2.9 CELL LINE AND CELL MAINTENANCE

Table 2.9.1 – Cell line utilized in this master thesis.

Cell line	Use	Supplier
HEPA1-6	Interaction studies between TCDD and marine nutrients	ATCC (American type cell collection)

Table 2.9.2 – Complete growth medium for cultivating HEPA1-6 cells.

Component	Concentration
Dulbecco's Modified Eagle's Medium – High glucose	1X
Fetal Bovine Serum (FBS)	10 %
Penicilin-Streptomycin	1%

2.10 REACTION MIX AND SET-UPS USED FOR qPCR

Table 2.10.1 cDNA reaction mix and reaction set-up for cDNA.

Reaction mix		Set-up	Time	Temperature
RNase free H_2O	8.9 µl	Incubation	10 min	25 °C
10X TaqMan RT Buffer	3 µl	Reverse transcription	60 min	48 °C
25 mM $MgCl_2$	11 µl	Reverse Transcriptase Inactivation	5 min	95 °C
10 mM deoxyNTPs Mixture	10 µl			
50 µM Randome Hexamer primers	2.5 µl			
RNase Inhibitor (20U/µl)	0.6 µl			
Multiscribe Reverse Transcriptase	1 µl			

Table 2.10.2 – Reaction mix and reaction set-up for qPCR

Reaction mix		**Set-up**	**Time**	**Temperature**
ddH_2O	2.8 µl	Pre-incubation	5 min	95°C
Forward primer (50µM)	0.1 µl	Amplification		
		Denaturing	10 sec	95°C } x45
		Annealing	10 sec	60°C
		Extension	10 sec	74°C
Reverse primer (50µM)	0.1 µl	Melting curve	5 sec	95 °C
			1 min	65 °C
				97 °C
SYBRGreen PCR Master Mix (2X)	5 µl	Cooling	10 sec	40 °C

2.11 qPCR PRIMERS

Gene	**Primer direction**	**Nucleotide Sequence (5´ – 3´)**	**Supplier**
Tbp	F	ACC CTT CAC CAA TGA CTC CTA TG	INVITROGEN™
	R	ATG ATG ACT GCAA GC AAA TCG C	
β-actin	F	ATG GGT CAG AAG GAC TCC TAC G	
	R	AGT GGT ACG ACG ACC AGA GG	
Calnexin	F	GCA GCG ACC TAT GATT GA CAA CC	
	R	GCT CCA AAC CAA TAG CAC TGA AAG G	
Eef1a1	F	ACG AGG CAA TGT TGC TGG TGA C	
	R	GTG TGA CAA TCC AGA ACA GGA GC	
Ahr	F	CCA TGT ATC AGT GCC AGC	
	R	AGC TGT CGA AAG CCC TTA CC	
Cyp1a1	F	CCT CTT TGG AGC TGG GTT TG	
	R	TGC TGC GGG GGA TGG TGA AG	
Cyp1a2	F	AAG ACA ATG GCG GTC TCA TC	
	R	GAC GGT CAG AAA GCC GTG GT	

Cox1	F	AGT CGG TCC ACC TTA TCC
	R	CCG CAG GTG ATA CTG TCG TT
Cox2	F	GAC TGG GCC ATG GAG TGG
	R	CAC CTC TCC ACC AAT GAC C
Cat	F	CGG CAC ATG AAT GGC TAT GGA TC
	R	AAG CCT TCC TGC CTC TC CAAC A
Gpx4	F	GCA GGA GCC AGG AAG TAA TC
	R	GGC TGG ACT TTC ATC CAT TT
Sod1	F	AAC CAG TTG TGT TGT CAG GAC
	R	CCA CCA TGT TTC TTA GAG TGA GG

3. METHODS

3.1 CELL MAINTENANCE

The cell line used in this thesis was maintained in their complete growth medium (Table 2.6.2) in an incubator at 37°C with 5% CO_2. All the chemicals and reagents used for the cell culture were prewarmed to 37°C or room temperature and handled under sterilized conditions in a fume hood**.** The cells were split when reached approximately 80% confluency with a ratio between 1:3 to 1:6.

The cells were thawed and transferred to a 15 ml Nunc tube containing 9 ml of complete growth medium. The cell-medium mixture was centrifuged at 130 x g for 5 minutes. The supernatant was carefully discarded and resuspended in 5 ml medium. This was transferred to a 75 cm^2 T-75 Nunc cell cultivation flask and 10 ml of complete growth medium was added for a final volume of 15 ml. The flask was placed in an incubator in humid conditions at 37°C with 5% CO_2.

3.1.2 SPLITTING OF HEPA 1-6 CELLS

The cells were washed three times with PBS to ensure that any residue from the growth medium was removed before adding 1.5 ml of trypsin and placing it back in the incubator for 2-3 minutes. The cells were examined under a microscope to assure that the cells had properly detached from the flask. The detached cells were resuspended with complete growth medium and distributed to T-75 cell flasks. The flasks with cells were then placed back in the incubator.

3.1.3 LONG TERM STORAGE

The HEPA1-6 cells were used at different times throughout this thesis, and for preservation reason the cells were kept frozen in liquid nitrogen. The cell flasks were washed with PBS and treated with trypsin, as previously mentioned, and transferred to a 15 ml tube containing 8.5 ml growth medium. This was then centrifuged at 130 x g for 5 minutes forming a cell pellet at the bottom of the tube. The supernatant was discarded, and the cell pellet was resuspended in 1 ml freeze medium (95% complete growth medium and 5% DMSO (v/v)) and transferred to a 1.8 ml Nunc CryoTube. The CryoTubes were placed into a Mr.Frosty™ and stored in a -80°C freezer overnight. The following day the CryoTubes were placed into liquid nitrogen.

3.2 xCELLigence REAL TIME CELL ANALYSIS

The xCELLigence Real-Time Cell Analyser (RTCA), is a non-invasive and label free cell-based assay. The xCELLigence system consists of the RTCA Control Unit (a computer with the RTCA software), RTCA Analyser, RTCA single plate (SP) station and a disposable E-plate 96. The E-plate 96 is integrated with gold microelectrode biosensors at the bottom of each well, which measure the electrical impedance through a bulk solution (i.e growth medium) to measure cell activity. The RTCA uses a unitless parameter called Cell Index (CI) to represent the changes in impedance of electron flow when cells are present and absent thus can represent cell growth in each well (Agilent, no date).

3.2.1 CELL SEEDING

Prior to the exposure of TCDD, PCB-126, and fatty acids, the optimal cell density for the experiment were determined by using the xCELLigence system. The cells were detached from the flasks and were manually counted by mixing 100 µl of the disperse cells and 100 µl of trypan blue and transferring it over to a counting chamber. The volume of the desired cell density was calculated and diluted in complete growth medium. Before starting an xCELLigence experiment, 100 µl of medium was added to each well for background impedance measurements. A cellular concentration ranging from 1 000 – 64 000 cells per well were seeded into the E-plate. The cells were monitored by the RTCA system with one swipe every 30 min for 96 hours in the incubator at $37^{O}C$ and 5% CO_2.

3.2.2 TCDD AND PCB-126 DOSE-RESPONSE

To determine the concentrations of TCDD and PCB-126 prior to exposure in combination with fatty acids, a dose-response experiment was carried out. The appropriate dose should have an impact on the cells, but not enough to cause extensive cell death. The dose response relationship was also established using the xCELLigence system. The HEPA 1-6 cells was seeded in an E-plate with a concentration of 8000 cells per well. After approximately 48 hours the cells were exposed to TCDD- and PCB-126 with concentrations ranging from 100 pM – 500 nM and 1 nM – 5000 nM, respectively**.** The cells were monitored by the RTCA system for additional 72 hours with one swipe (electrical impedance) every 30 min.

3.2.3 INTERACTION BETWEEN FATTY ACIDS, AND DLCs

The marine fatty acids used in this experiment were pre-coupled with fatty acid free-bovine serum albumin (BSA) before use as described by (Ghioni *et al.*, 1997) by a previous master student. The coupling of fatty acids to BSA improves the fatty acids solubility making them more receptable to be taken up by the cells as fatty acids are highly lipophilic (Alsabeeh *et al.*, 2018).

Prior to TCDD and PCB-126 exposure the HEPA 1-6 cells were pre-incubated with DHA, EPA and BSA (a control). A stock of DHA, EPA, and BSA was mixed with complete growth medium to a final concentration of 60 μM. Further, 100 μl of each stock with n-3 FA and BSA was transferred into an E-plate to measure the background. The HEPA1-6 was then seeded to the E-plate with a concentration of 8000 cells per well.

3.3 REAL TIME – QUANTITATIVE PCR

Real-Time quantitative Polymerase Chain Reaction (RT- qPCR) is a fluorescent-based method used for detection and quantification of PCR-product (Taylor *et al.*, 2010). SYBR® Green, for example, is a DNA binding cyanine dye that activates when bound to the minor groove of double-stranded DNA. The amplification of the fluorescence can be detected and hence measures DNA (Wilson and Walker, 2010).

3.3.1 RNA-EXTRACTION

The RNA – extraction from the HEPA1-6 cells was done by using the RNeasy® Plus Mini Kit and following the manufactures protocol. Pre-incubated HEPA1-6 cells were seeded to 6-well plates and exposed to TCDD when a confluence of around 80% was reached. The cells were harvested approximately 24 hours after TCDD-exposure. The cells were washed three times with PBS before adding 30 ml of trypsin which were then incubated at 37 °C with 5% CO_2 for 2-3 min. After the incubation, 100 ml of PBS was added, and the dispersed cells were transferred to 1.5 ml Eppendorf tubes before being centrifuged at 20 817 x g for 5 min at 4°C. The supernatant was discarded, and the cell pallet was resuspended with 600 μl of the Buffer RLT Plus. The mixture was vortexed until homogenized before transferring the homogenized lysate to a gDNA Eliminator spin column and centrifuged for 30 s at 10 000 x g. This step was to remove genomic DNA from the sample, leaving the total RNA in the flow-through. Further,

600 µl of 70% ethanol was added to the flow-through and mixed well by pipetting. The samples were then transferred to a RNeasy spin column, where the RNA binds to the membrane. By adding RW1 and RPE buffer through the spin column it provides a rinsing of impurities as it passes through by centrifuging. Finally, RNA was re-diluted with 50 ml of RNase-free water.

The concentration and purity of RNA was measured using NanoDrop™ 1000. The nanodrop is a spectrophotometer that uses a molecules absorption of light at a specific wavelength to determine the concentration and purity of a sample. RNA, DNA, and nucleic acids absorb light at a wavelength of 260nm. Therefore, the $A_{260/230}$ and $A_{260/280}$ ratio were used to determine the purity of the samples. A sample was considered "pure" if the $A_{260/280}$ was around 1.8 for DNA and 2.0 for RNA. Additionally, if the $A_{260/230}$ ratio was lower than 1.7 this indicates that there might be contaminants (i.e carbohydrates or phenols) present in the samples. Before measurements on the nanodrop, 1 µl of a "blank" sample, preferably the solution that the samples were dissolved in were pipetted to the pedestal. The blank was then removed, and 2 µl of each sample was measured.

3.3.2 PRECIPITATION OF RNA

Samples with an $A_{260/230}$ ratio lower than 1.7 were precipitated to assure removal of contaminants. This was done by adding a ratio of 0.1:1 of sodium acetate and 2.5:1 ratio of 100% ethanol to the applicable samples. The samples were incubated at -80 °C overnight. The following day, the samples were centrifuged at 12 000 x g at 4 °C and the supernatant was carefully discarded. Further, an additional wash step was performed by adding 75% ethanol to the samples and centrifuged. The supernatant was carefully removed, and the RNA pellet was left to airdry. The pellet was resuspended with RNase-free water.

3.3.3 RNA QUALITY - BIOANALYZER

The RNA quality was analysed by using the BioAnalyzer and RNA 6000 Nano LabChip Kit, following the manufactures protocol. Twelve samples were randomly chosen for this analysis. Briefly, the RNA Nano dye was vortexed for 10 sec, before adding 0.5 µl of the RNA Nano dye to 32.5 µl of the gel-matrix. The gel-dye mixture was centrifuged at 13 000 x g for 10 min. Meanwhile, the twelve RNA-samples were denatured at 70 °C for 2-3 min. Further, RNA Nano Chip was placed in the Chip Priming station, and the gel-dye mixture, RNA Marker, RNA ladder, and the RNA samples was added to their respective wells. The RNA Nano Chip was

then centrifuged for 1 min at 24 00 rpm before placing it in the BioAnalyzer to be analysed. The Bioanalyzer provides an RNA Integrity Number (RIN) which is an objective measurement of RNA quality, where 1 is the most degraded and 10 is the most intact RNA (Mueller *et al*, 2004).

3.3.4 cDNA

The RNA-samples were diluted with RNase-free water to a final concentration of 50 ng/µl ± 5%. A mix of all the samples were made to make a six series dilution consisting of the concentrations 100, 50, 25, 6.25, and 3.125 ng/µl. This dilution series was later used to make a standard curve for downstream analysis. The reaction mix was prepared as shown in Table 2.7.1 and 40 µl of the reaction mix was added to a 96-well cDNA plate. Further, 10 µl of each RNA-sample was added to the cDNA plate in duplicates and the standard curve was added in triplicates. Additionally, a no amplification control consisting of RNA-mix with a final concentration of 50 µg/µl with the reaction mix without the Multisubscibe Reverse Transcriptase, as well as a non-template control consisting of the reaction mix and dH_2O were added to the cDNA plate. The cDNA was covered and centrifuged at 50 x g for 1 min before placing it in T100 Thermal Cycler with the condition specified in Table 2.10.1 The cDNA plate was stored at -20°C for further usage.

3.3.5 qPCR

The cDNA plate was thawed on ice and centrifuged at 1000 x g for 1 min before being vortexed at 1300 rpm for 3 min. The cDNA plate was diluted 1:1 with dH_2O by using the Biomek 4000 robot and centrifuged at 1200 x g for 1 min and vortexed at 1500 rpm for 5 min. Meanwhile, reaction mix described in Table 2.10.2 was prepared for each primer. By the help of the Biomek 4000 robot, 8 µl of the reaction mix and 2 µl of the cDNA was added to a 384-well qPCR plate. Next, the qPCR plate was centrifuged at 1500 x g for 2 min before and qPCR was carried out in CFX Touch Real-Time PCR Detection System with the conditions specified in Table 2.7.2. The results of the first qPCR run had high efficiencies, therefore the cDNA was diluted again in a 1:1 ratio with dH*2*O. The procedure proceeded on as usual.

3.4 PLASMID PURIFICATION

Redox-sensitive green fluorescent protein (roGFP) is a green fluorescent protein that is sensitive to changes in a redox environment (Waypa *et al.*, 2010). The roGFP contains thiol groups with cysteine residues that change conformation in response to redox changes, and hence changes in fluorescent properties. The redox ratio in the cells can be measured by using the roGFPs two emission points at two different excitation wavelengths, which vary in intensity according to the redox changes in the cell (Hanson *et al.*, 2004). The plasmids used in this study were roGFP expressed in the cytosol (cyto-roGFP) and mitochondrial matrix (matrix-roGFP).

3.4.1 PREPARATION OF LURIA BERTANI MEDIUM

Each component to the Luria Bertani (LB) medium were mixed to a final volume of 1L (Table 2.7). The pH solution was balanced with sodium hydroxide (NaOH) to reach a pH around 7. Further, 200 ml of the LB-medium was transferred to another container and 4 g of agar were mixed into the medium. The LB-medium and the LB-medium containing agar was autoclaved at 120°C for 20 min. Ampicillin was added with a concentration of 10mg/ml when the medium(s) had reached a temperature of ≤ 50 °C. The LB-medium containing agar was added to petri dishes with a volume of approximately 20 ml which were placed at room temperature without lid for 2 hours before being placed upside down at 4 °C.

The following day, the bacteria was streaked onto the petri dishes by using an inoculating loop and stored at 37 °C for 24 hours. The following day, single colonies from the petri dish were inoculated into 10 ml liquid LB medium in a shaking incubator at 190 rpm at 37 °C for another 24 hours, forming an overnight liquid culture. The overnight liquid culture should appear cloudy indicating growth and was further used for plasmid extraction and long-term storage.

3.4.2 LONG TERM STORAGE

The overnight liquid bacteria culture was frozen in a glycerol stock for long-term storage by mixing 2 ml of the overnight culture with 300 µl of 100% glycerol until homogenous. This was transferred into Nunc CryoTubes and placed in a - 80°C freezer.

3.4.3 EXTRACTING THE PLASMID

The plasmids were extracted by using the QIAGEN's plasmid Midi Kit and following the manufactures protocol. To describe briefly, the overnight culture was centrifuged at 6000 x g for 15 min at 4 °C. The bacterial pallet was resuspended in Buffer P1 and Buffer P2 and mixed thoroughly. This was incubated at room temperature for 5 min before adding Buffer P3 and centrifuged at 20 000 x g for 30 min in 4 °C. This was placed in a QIAGEN-tip and centrifuged. The QIAGEN-tip was washed 2 times with Buffer QC and the DNA was eluted with Buffer QF. Further, the DNA was precipitated with isopropanol, centrifuged, and the supernatant was carefully removed. The DNA pellet was washed with 70% ethanol and left to air-dry. The air-dried DNA pallet was redissolved in 100 µl TE-buffer. The concentrations of the DNA were measured using the Nano Drop™ 1000.

3.4.4 TRANSFECTION

Transfection is the process of introducing a foreign genetic material (DNA or/and RNA) into a (eucaryotic) cell. The genetic modified cells can be used as an analytical tool to study gene and protein function (Kim and Eberwine, 2010). There are two main ways of transfecting a genome into the cells; stable transfection and transient transfection. Stable transfection is when the foreign DNA is integrated into the host cells genome. In contrast, transient transfection is not integrated into the host genome and [often] expressed for a limited period of time (Recillas-Targa, 2006).

3.4.5 FIXATING THE CELLS

The HEPA1-6 cells were seeded into 24-well plate containing a coverslip at the bottom of each well with fatty acids. The cells were transfected when they reached approximately 70-80% by using FuGENE HD® transfection reagent and following the manufactures protocol. On the day of transfection, the plasmid DNAs was diluted in serum free medium (DMEM). A transfection mix was prepared by mixing each diluted plasmid DNA with FuGENE® HD transfection reagent with a ratio of 2.5:1. The transfection mixes was incubated at room temperature for 10 min before adding 5 µl of the transfection mixes to each well and placed back into the incubator set at 37°C with 5 % CO_2 for 48 hours. The cells were observed in a microscope with a blue

filter to detect if there were any fluorescent in the cells. After the 48 hours, the cells were exposed to TCDD with the concentrations 0.1 nM and 10 nM for 24 hours.

After approximately 24 hours, the cells that had not been exposed to TCDD were treated with H_2O_2 with concentrations ranging from 0.176 µM to 0.544 µM, to later make a standard curve for comparison. H_2O_2 was incubated for 20 min before preparing the cells for fixation with 4% paraformaldehyde. The cells were washed three times with 1X PBS, and 1000 µl of 4% PFA was added to each well following incubation at room temperature for 15 min covered. The cells were washed again three times with 1X PBS. The coverslips were carefully picked up and placed on a microscope slide on a drop of ProLong™ Gold antifade reagent containing DAPI.

3.4.6 FLUORESCENCE MICROSCOPY

The Nikon Eclipse Ti Microscope was used to image the transfected cells by using cyto-roGFP and matrix-roGFPs excitation points at 400 nm and 484 nm with both having an emission point at 525 nm. DAPI has an excitation point of 345 and an emission point at 455 nm. Therefore, the redox ratio can be measured by using these emission and excitation points:

$$\frac{emittion\ at\ exitation\ point\ 4884nm}{emittion\ at\ exitation\ point\ 400\ nm} = \text{redox ratio}$$

3.5 WESTERN BLOT

Western blot is a method that can detect specific proteins in a sample. This method includes separating the proteins by weight using methods like SDS-PAGE (see section 3.5.3). The separated proteins can then be transferred to a PVDF-membrane and primary- and secondary-antibodies can be used to detect the specific protein of interest.

3.5.1 CELL LYSIS

HEPA1-6 cells were seeded in a 6-well plate with complete growth medium and exposed to 0.1 nM and 10 nM TCDD when reached approximately 80% confluency. The following day, the cells were harvested by washing each well three times with 1xPBS and adding 200 µl RIPA-Protease Lysis Buffer. The cells were then manually scraped from the wells using a cell scraper

and transferred to a 1.5 ml Eppendorf tube. Further, a cycle of freezing and thawing the cells and pipetting in between was repeated until homogenised. The samples were then centrifuged at 20 817 x g for 30 min at 4°C. The supernatant was collected and transferred to CryoTubes and stored in a -80 °C freezer for further use.

3.5.2 TOTAL PROTEIN CONCENTRATION.

The total protein concentration of each sample was determined using the Pierce™ 660 nM Protein assay while following the manufactures protocol. The kit provided a set of 7 pre-diluted standard which were used to make a standard curve to extrapolate the concentration of the protein samples.

10 µl of each standard solution, the protein samples and RIPA-Protease lysis buffer (blank sample) were transferred into a 96 well plate and adding 150 µl of the Protein Assay Reagent to each well. The plate was covered with aluminium foil and placed on a shaker for 1 min followed by an incubation at room temperature for another 5 min. The plate was placed in the VICTOR Multilabel Plate Reader which were programmed to read the absorbance at 660 nm. The protein concentration was determined from the standard curve of the BSA standards by plotting the 660 nm measurements for each BSA standard against its concentration in µg/mL.

3.5.3 SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

A 1:1 ratio with protein sample and sample buffer were mixed in Eppendorf tubes to prepare the proteins for sodium Dodecylsulphate-Polycrylamide Gel Electrophoresis (SDS-PAGE). Further, each protein/sample buffer-mix were heated at 95 °C for 5 minutes and subsequently placed on ice. The SDS-PAGE was carried out by using BioRads Mini PROTEAN Tetra Cell. The gel cassettes were placed into the buffer tank and the chambers were filled with 1x Running buffer. The calculated volume (20 ng) of the protein samples and 2 µl Precision Plus Protein™ Western C™ Standard was applicated to the wells. The Mini PROTEAN Tetra Cell was set to a current at 200 V for approximately 30 minutes. The gels were then imaged using ChemiDoc™ XRS+ system set to stain free gel imaging with faint bands.

3.5.4 WESTERN BLOT

To assemble a blotting sandwich, the filter pads were soaked in transfer buffer and placed in a cassette. A PDVF-membrane was soaked in methanol for activation and placed on top of the filter pads, followed by the gel and another filter pads soaked with transfer buffer. Any bubbles were removed by carefully pressing them out with a roller. The cassette was placed in a Trans-Blot® Turbo™ Transfer Apparatus and was set at a standard program of 1.3A/22V for 7 minutes. The membrane was washed 3 x 10 min with washing buffer and incubated with blocking buffer for at least 3 hours whilst shaken at room temperature. This was followed by 3 x 10 min wash with washing buffer. The membrane was covered in 5% ECL blocking buffer containing anti CYP1a1 antibody and Precision Protein Step Tactin-HRP Conjugate. A ratio of 1:1000, 1:2000 was tested to find the optimal ratio of antibodies and a 1:10 000 for the HRP Conjugate. This was shaken for two hours before incubation overnight at 4°C. The following day, each membrane was washed 5 x 10 min in washing buffer before detecting the proteins. The ECL Prime Western Blotting Detection Reagents were mixed 1:1 and 2 ml was added to the membrane then covered for 3 min. An additional, 1:500 ratio of antibodies was blocked on the membrane that had been previously blocked with 1:1000 ratio. The blot was imaged using ChemiDoc™ XRS+ system with chemi-blot imaging and using the default setting for faint bands.

3.6 PIERCE IMMUNOPRECIPITATION (IP)

Since we were unable to detect CYP1a1 proteins using Western Blot, an additional test to detect the protein was done using Pierce Immunoprecipitation (IP). IP is a method used to isolate proteins by immobilizing the antibodies on a solid substance such as agarose resin. Further, the protein binds to the antibodies, where the bound protein can be eluted and analyzed (Thermo Fisher Scientific, 2016). However, as a result of the anti-CYP1a1 antibody being depleted, a different CYP1a1 antibody was used for the immunoprecipitation.

The protein samples for the IP were prepared the same way as for SDS-PAGE and Western blotting. The Pierce™ 660 nm Protein assay was also used for protein concentration quantification for the IP protein samples.

The IP was preceded by using the Pierce Co-Immunoprecipitation kit according to the manufactures protocol. The process consists of antibody immobilization, binding of the antibody to the target protein, removal of unbound protein, and elution of bound protein. To briefly describe, the AminoLink Plus Coupling Resin was gently swirled before adding 50 µl of the resin to a Pierce Spin Column which were placed in a microcentrifuge tube. This was centrifuged at 1000 x g for 1 min followed by two washes and centrifuge with 200 µl 1X Coupling Buffer. The flow through was discarded after each centrifuge. Furter, 10 µg of antibody and 1X coupling Buffer was adjusted to a final volume of 200 µl which were added to the spin column. In addition, 2 µl of the Sodium Cyanoborohydride Solution was added for every 200 µl of antibody-solution. This was incubated on a mixer for 120 min. Afterwards, the column was centrifuged and the flow through was collected for further analysis. The column was washed and centrifuges with 1X Coupling buffer before adding Quenching buffer and Sodium Cyanoborohydride Solution. This was left to incubate for another 15 min. The column was washed six times with Wash Solution and the flow through was discarded after each centrifuge.

The protein samples were prepared by diluting the samples with IPlysis/Wash Buffer to a final concentration of 1 mg/ml and a final volume of 150 µl. The columns were washed twice with IP Lysis/Wash Buffer and centrifuged after each wash. The flow through was discarded. Next, the diluted protein samples were added in the spin column and placed on a mixer for 3 hours before incubating it overnight at 4°C. The following day, the column was centrifuged and the flow through was collected. The column was washed 3 times with IP Lysis/Wash Buffer and centrifuged. The flowthrough was collected after each wash and the last wash should not contain any protein. Lastly, for the elution step, the column was placed in a collecting tube and 50 µl of Elution Buffer was added and centrifuged. The elution and the flow throughs were stored at -80 °C. Further, SDS-PAGE was performed to check if the chosen proteins had been eluted from the samples.

The SDS-polyacrylamide gel was incubated with Coomassie blue stain for 1 hour at room temperature whilst shaken. After incubation, the gel was distained by soaking it with a washing solution (Coomassie stain solution without Coomassie blue) for 2 hours whilst being shaken. The washing solution was discarded and changed every 30-45 min. The gel was imaged using ChemiDoc™+ XRS system set to stain free gel imaging with white light transilluminator.

3.7 DATA PROCESSING

3.7.1 xCELLigence data

The data from the xCELLigence was normalized to a given time, which was 55 hours after seeding the cells. This time was approximately 7 hours after TCDD or PCB-126 exposure, when the cells and medium had re-stabilized. In the figures, this time is set as 0 hours. Values that clearly displayed as outliers were removed.

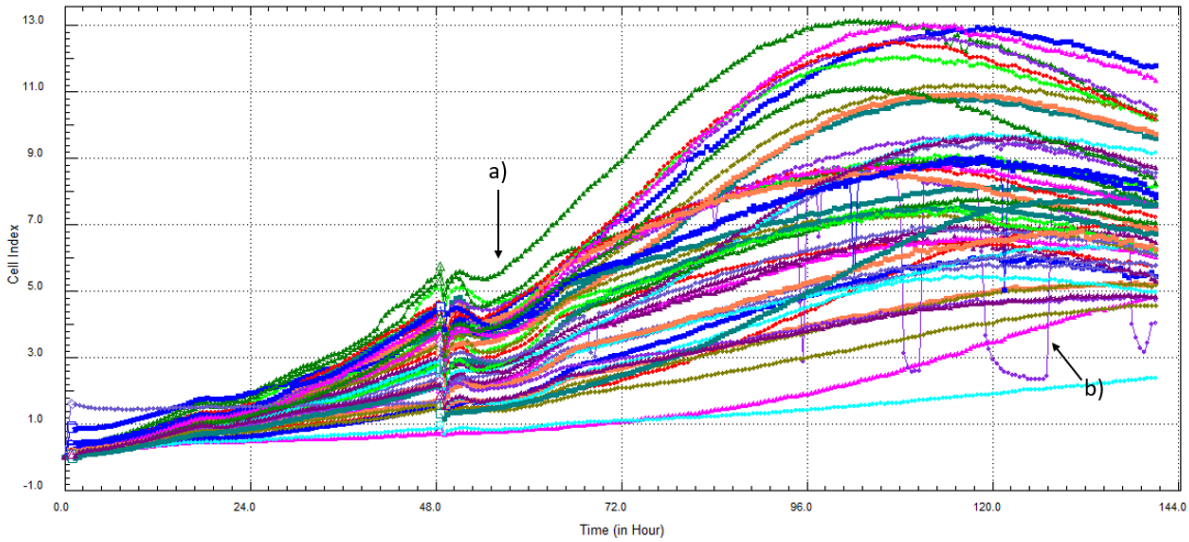

Figure 3.7.1 – xCELLigence data before normalization. a) The arrow shows the time of normalization, which is set as 0 hours in the Figures in the result section; b) Shows an example of values in the data that were excluded from the analysis.

3.7.2 qPCR data

The qPCR data assessed in the CFX Maestro software were evaluated by efficiency (E)-values, R^2, and the slope. The qPCR data should have an E-value between 90% and 110%, a $R^2 \sim 1$, and a slope between -3.58 and -3.10. Outliers of the standards were removed to improve the E-value, R^2, and the slope of the different genes. COX2 were excluded from the analysis due to large variations in the results.

GeNorm is a software used to find normalization factors based on the M-value which was used to normalize target genes. Firstly, the reference genes were analyzed in the reference Gene Selection Tool in the CFX Maestro software to determine the gene stability. Reference genes with an M-value < 0.05 were considered ideal for normalization. All reference genes were used to normalize the target genes.

3.8 STATISTICAL ANALYSES

Microsoft excel version 2303 was used to plot and process raw data. The statistical analysis was done with the software Graphpad 9 and Statistica 13, and the Figures were made in Graphpad 9.

All data sets were evaluated for homogeneity of variance using Spearman's test for heteroscedasticity. If the assumptions were met, the data were further analyzed by Two-way analysis of variance (ANOVA) followed by the post hoc test Tukey's multiple comparisons. The two-way ANOVA was used to test if there were any significant difference between the main factors of TCDD and fatty acids, and if there were any interactions between these factors. If the assumptions of homogeneity of variance were not met by the Spearman's test, the data were either log-transformed, tested homogeneity of variance using the Levenes test, or BOX-COX transformed, to further be analyzed by a two-way ANOVA, as mentioned. However, if the homoscedasticity were still not met, a non-parametric test was performed using the Kruskal-Walli's test followed by a Dunn's test for multiple comparisons.

The dose-response data for TCDD and PCB-126 exposure were analyzed using Brown-Forsythe test, as well as a Bertlett's test to test for homogeneity of variance. If the assumptions were met, the data were analyzed using one-way ANOVA followed by a post hoc Dunnett's test, as well as a non-linear regression analysis. If the assumptions of homogeneity were not met, the Kruskal-Walli's test was used followed by Dunn's test for multiple comparisons.

All results are presented as means ± standard deviation (SD), and with a significance level of $p<0.05$. Differences in significance are illustrated with different letters in the results.

4. RESULTS

4.1 CELL VIABILITY

4.1.1 TCDD

The dose-response relationships for TCDD in HEPA1-6 cells are presented in Figure 4.1, where the cells are exposed to TCDD concentrations ranging from 0.1 nM to 500 nM with control groups (0 nM and DMSO ctrl). A decrease in cell growth was observed in cells exposed to TCDD compared to the controls, but no lethal dose was detected. The results from a one-way ANOVA followed by Dunnett's multiple comparisons showed a significant difference for 10 and 50 nM when comparing the two concentrations to 0 nM ($p=0.0367$ and $p=0.0190$). Additionally, a non-linear regression analysis showed a significant decreasing trend ($R^2=0.1072$). The dose-response relationship of TCDD was deemed sufficient for investigating interactions between TCDD and FA. The concentrations used to investigate these interactions were 0.1 nM and 10 nM.

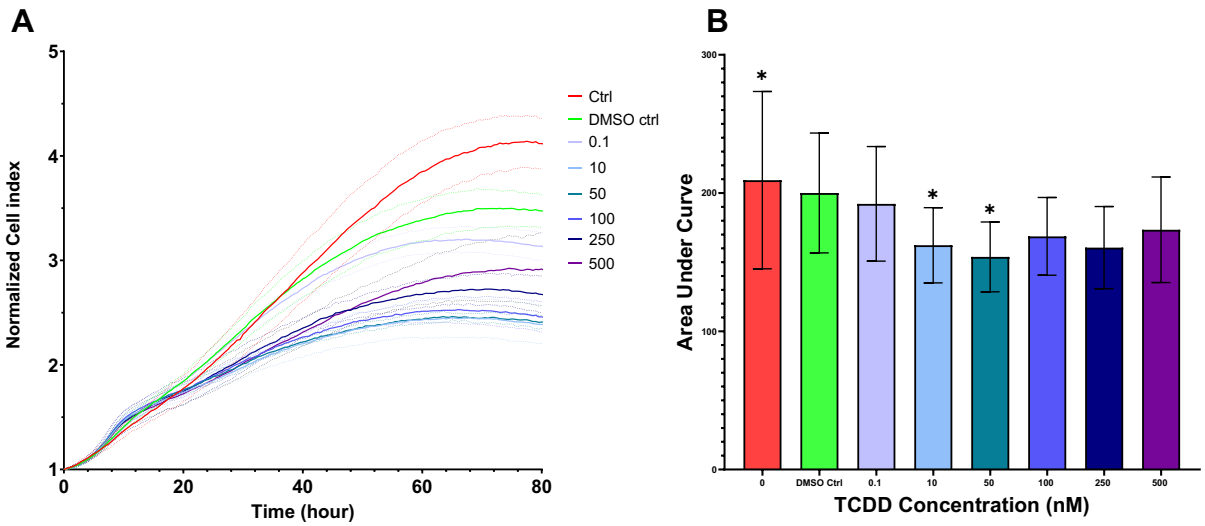

Figure 4.1 – TCDD dose-response in HEPA1-6 cells. (**A**) Normalized cell index shows the cell growth after 80 hours of TCDD exposure with concentrations ranging from 0.1 nM to 500 nM with control groups (0 nM and DMSO ctrl). (**B**) Area under curve (AUC) of the normalized cell index. All results are shown as means ± SD (n=4). Statistical significance ($p<0.05$) between 0nM and the TCDD-concentrations is illustrated with asterisk.

4.1.2 EFFECTS ON TCDD AND FATTY ACIDS

The effects of TCDD and n-3 fatty acids (FA) on HEPA1-6 cell viability are shown in Figure 4.2 and Figure 4.3. A dose-response effect for the TCDD-concentrations was observed for all cases of n-3 FA-treatments (Figure 4.2). The two-way ANOVA analysis showed a significant difference in TCDD concentrations ($p<0.0001$) where all the groups were significantly different from each other. Further, there was no observed effect of n-3 FA ($p>0.05$) on HEPA1-6 cells viability. The effects of TCDD and n-3 FA were also used to inspect if there were any interactions, but the two-way ANOVA showed no interactions between TCDD and n-3 FA.

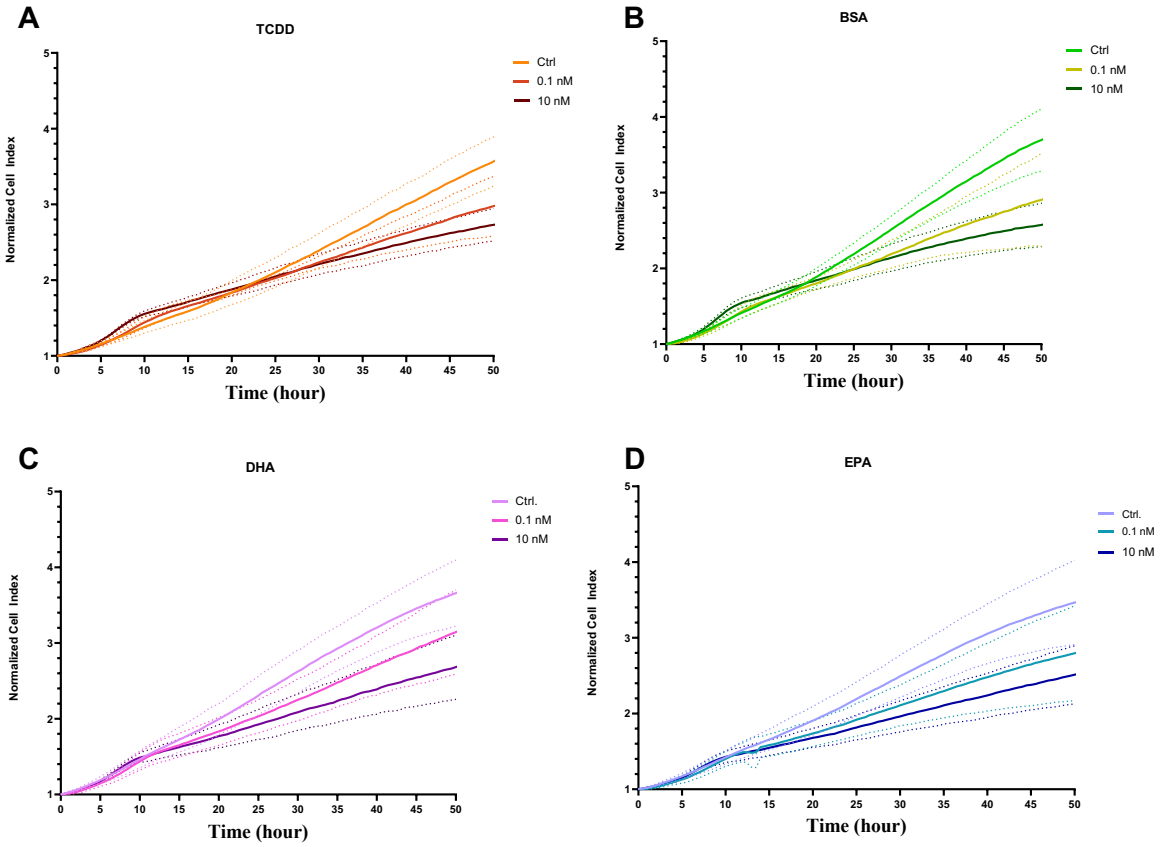

Figure 4.2 – Effects of TCDD and n-3 fatty acids in HEPA1-6 cells viability. The normalized cell index shows the cell growth of HEPA 1-6 pre incubated with (**A**) no n-3 fatty acids, (**B**) BSA, (**C**) DHA, and (**D**) EPA after 50 hours of TCDD exposure with 0, 0.1, and 10 nM. All results are shown as mean cell index ± SD (n=8). The control (ctrl) was not incubated with n-3 fatty acids.

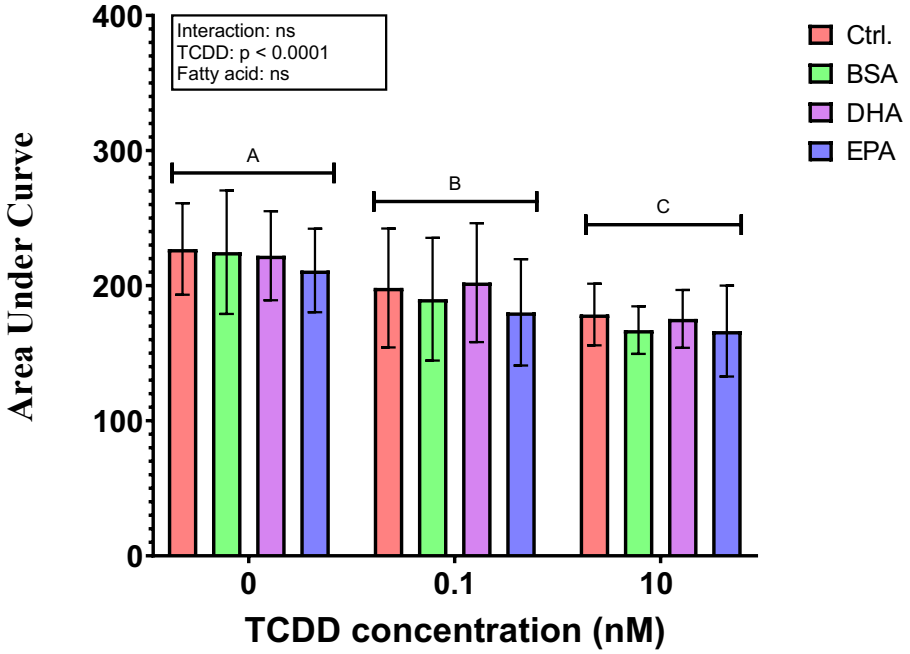

Figure 4.3 – Area under curve of effects of TCDD and n-3 fatty acids in HEPA1-6 cell viability. The area under curve is shown as mean ± SD (n=8). The ctrl contains no n-3 fatty acid. The box in the figure denotes p-values from a two-way ANOVA for the main factor TCDD. Different uppercase letters represent statistical significance ($p<0.05$) in the main factor TCDD. The statistics were done on log transformed data.

4.1.3 PCB-126

The PCB-126 data are presented as TEQ. The normalized cell index over time reflects the overall cell growth of HEPA1-6 cells when exposed to PCB-126 concentrations ranging from 0.1 nM to 500 nM with control groups (0 nM and DMSO-ctrl) (Figure 4.4). A dose-response was observed as the cell growth decreased when the PCB-126 concentrations increased. However, the results from the Kruskal-Walli's test did not show any significant difference between the concentrations when compared to 0 nM. A non-linear regression analysis showed a significance in a decreasing trend ($R^2 = 0.1718$). The dose-response relationship of PCB-126 was deemed sufficient for investigating interactions between PCB-126 and n-3 FA. The PCB-concentrations used to investigate this were the same as those used for the study of effects of TCDD and n-3 FA (0.1 nM and 10 nM).

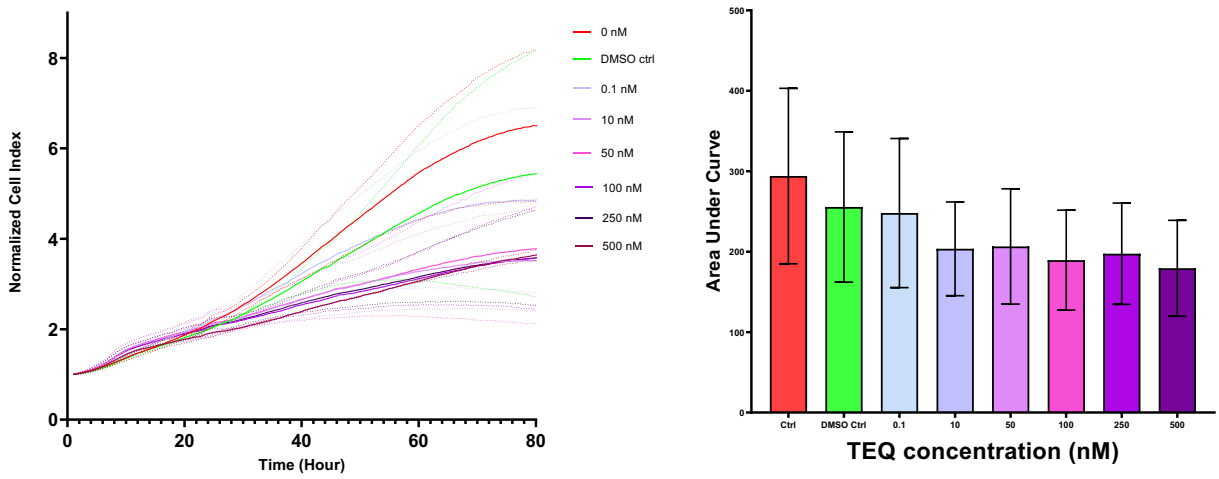

Figure 4.4 – PCB-126 dose-response in HEPA1-6 cell viability. (**A**) Normalized cell index shows the cell growth after 80 hours of PCB-126 exposure with TEQ-concentrations ranging from 0.1 nM to 500 nM with control groups (0 nM and DMSO ctrl) (**B**) The normalized area under curve (AUC) of the normalized cell index. All results are shown as means ± SD (n=4). The Kruskal-Wallis test did not show any significant differences between the concentrations compared to 0 nM ($p>0.05$)

4.1.4 EFFECTS OF PCB-126 AND n-3 FATTY ACIDS

The effects of PCB-126 and n-3 FA on HEPA1-6 cell viability are shown in Figure 4.5 and 4.6. A dose-response can be observed in Figure 4.5 as the cell viability decreases when TEQ-concentrations increases. The result from the two-way ANOVA confirms the dose-response relationship as there is significant difference in the main factor of TEQ-concentrations ($p<0.0001$) where the concentrations differ from each other. The FA had no effect on HEPA1-6 cell viability ($p>0.05$). The effects of PCB-126 and n-3 FA was also used to inspect if there were any interactions, however the two-way ANOVA showed no interactions between PCB-126 and n-3 FA.

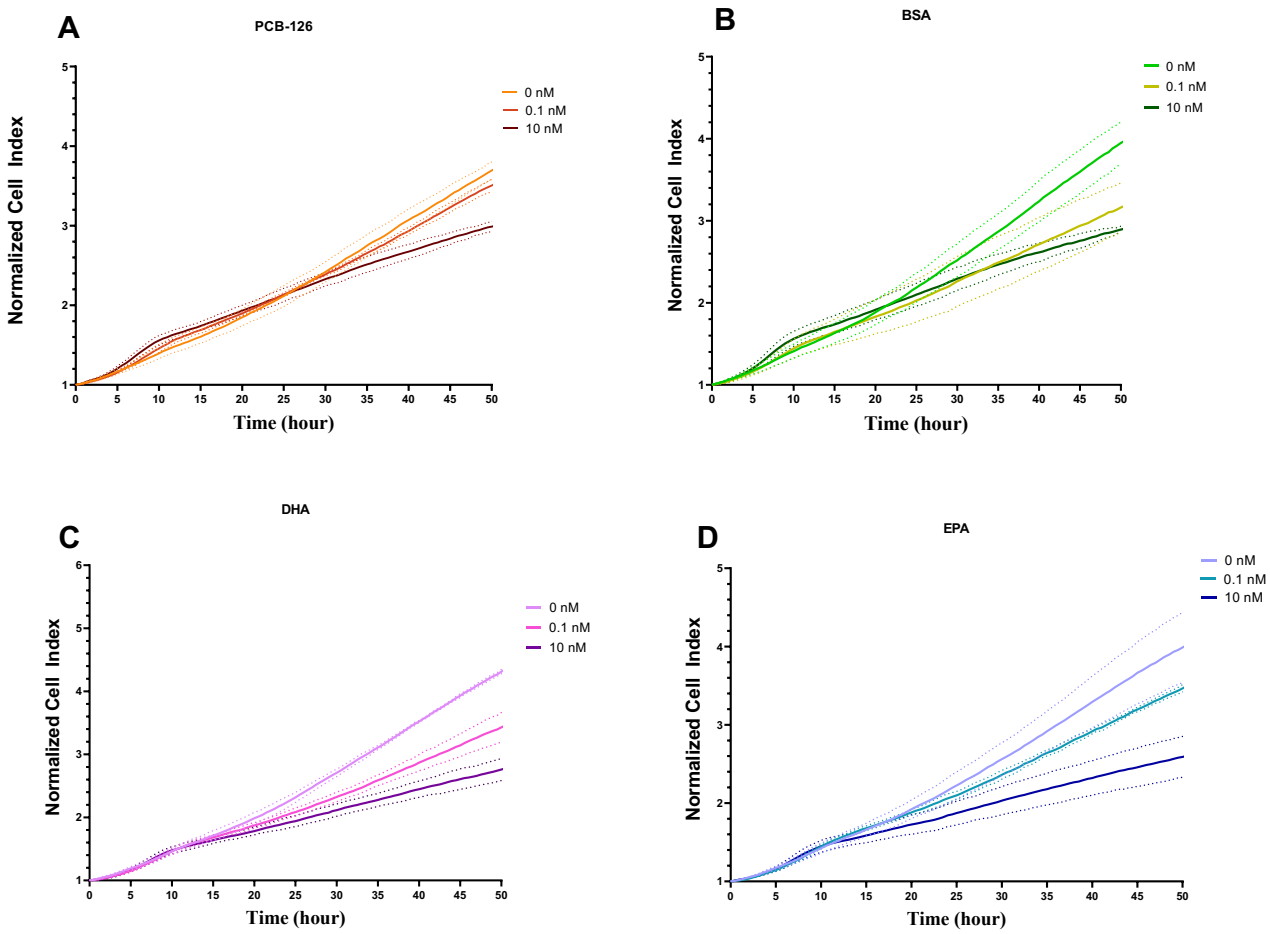

Figure 4.5 – Effects of PCB-126 and n-3 fatty acids on HEPA1-6 cell viability. The normalized cell index shows the growth of HEPA1-6 cells which were pre-incubated with (**A**) no n-3 fatty acids, (**B**) BSA, **C**) DHA, and (**D**) EPA after 50 hours of PCB-126 exposure with 0, 0.1 and 10 nM. All results are shown as mean cell index ± SD (n=8). The control (ctrl) was not incubated with n-3 fatty acids.

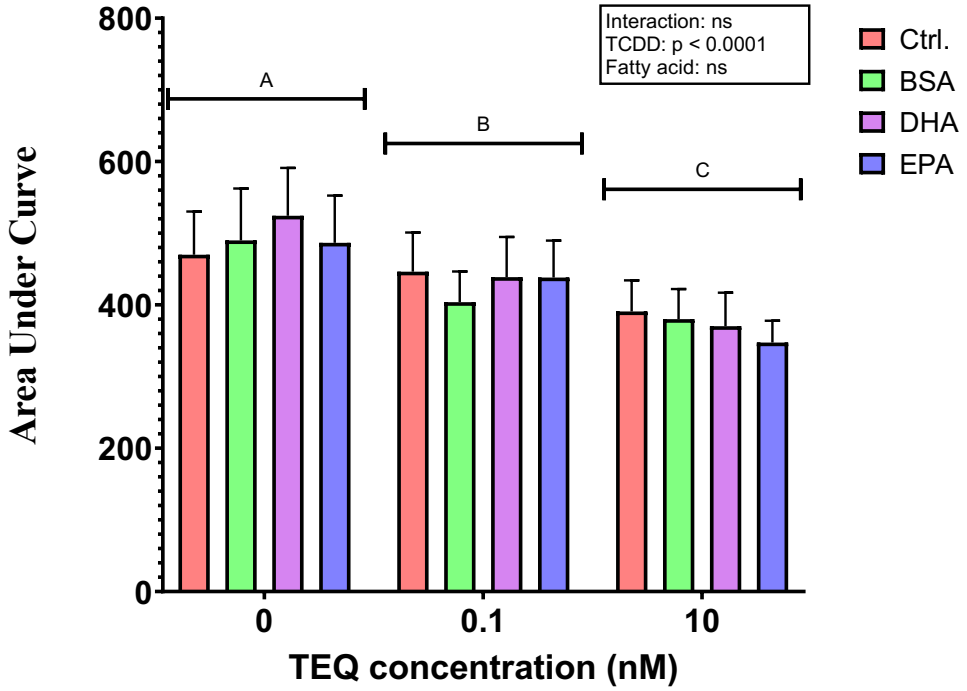

Figure 4.6 – Area under curve of effects of PCB-126 and n-3 fatty acids HEPA1-6 cell viability. The normalized area under curve is shown as mean ± SD (n=8). The ctrl contains no n-3 fatty acids. The box in the figure denotes p-values from a two-way ANOVA for the main factor TEQ (PCB-126). Different uppercase letters represent statistical significance in the main factor TEQ (PCB-126) ($p<0.05$).

4.1.5 TCDD vs. PCB126

As mentioned previously the PCB-126 concentrations are presented as TEQ, making it possible to compare PCB-126 and TCDDs effect on HEPA1-6 cell viability (Figure 4.7). The concentrations were normalized to the controls (0 nM and DMSO ctrl) as they were not significantly different. The data was BOX-COX transformed and a two-way ANOVA analysis showed significance in the main factor for concentrations ($p<0.0001$) and congeners ($p<0.0001$). The multiple comparisons showed that all concentrations were significantly different from the ctrl. An additional correlation analysis showed that TCDD had a higher effect on HEPA1-6 cell viability than PCB-126 ($r=0.72$ and $p<0.0001$).

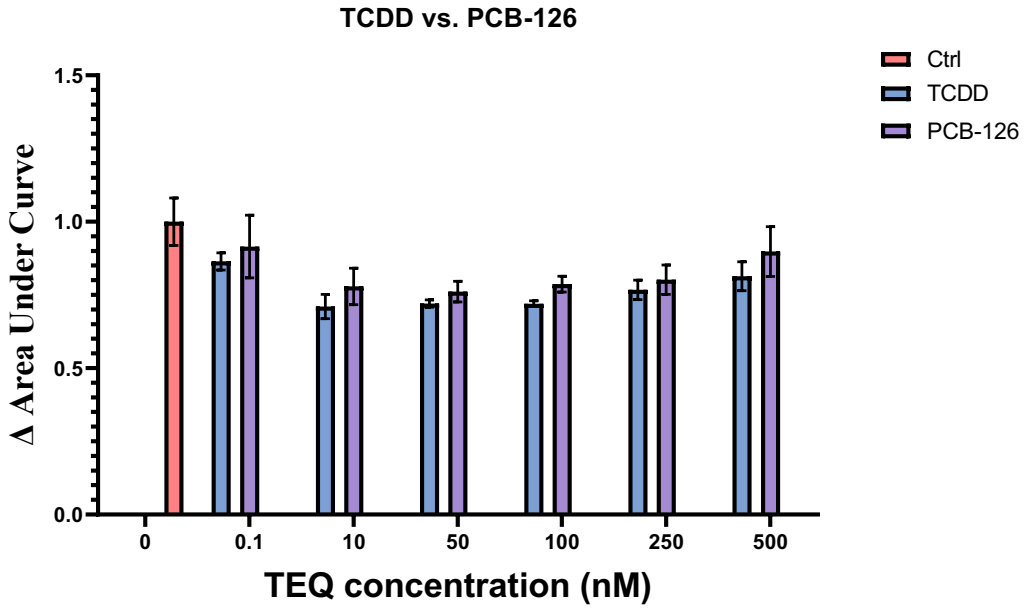

Figure 4.7 – Delta (Δ) area under curve of normalized cell growth after normalization to controls (DMSO ctrl and 0 nM). All results are shown as means ± SD (n=4). The statistical analysis was done on BOX-COX transformed data, and a two-way ANOVA analysis showed a significance for the main factor of concentration ($p<0.0001$) and congeners ($p<0.0001$). Additionally, a correlation analysis showed a significance ($p<0.0001$) and a $r=0.72$

4.2 GENE EXPRESSION

The relative gene expression of *Ahr*, *Cyp1a1, Cyp1a2,* and *Cox1* were examined after TCDD exposure in n-3 FA-incubated HEPA1-6 cells (Figure 4.8). The *Cyp1a1* gene was shown to be highly upregulated when exposed to 0.1 nM and 10 nM TCDD with an average of roughly 40-folds higher than for 0 nM. A main n-3 FA effect was detected between Ctrl and EPA ($p=0.0332$). For the *Cox1* gene, the results from the two-way ANOVA showed a significant for the main factor TCDD, where the gene was upregulated for 0.1 nM and 10 nM TCDD compared to 0 nM ($p=0.0003$). The statistical analysis used on both *Ahr* and *Cyp1a2* was a Kruskal-Walli's test followed by Dunn's multiple comparisons. The results showed that concentrations of 0.1 and 10 nM had an effect on the regulation of *Cyp1a2* compared to 0 nM ($p < 0.0001$ and $p=0.0007$, respectively). There was no significant difference between the regulation of the gene for 0.1 and 10 nM TCDD. No significant regulation of *Ahr* was observed after exposure to TCDD and FA. There were no interactions detected for the effects on TCDD and n-3 FA in any of the genes tested.

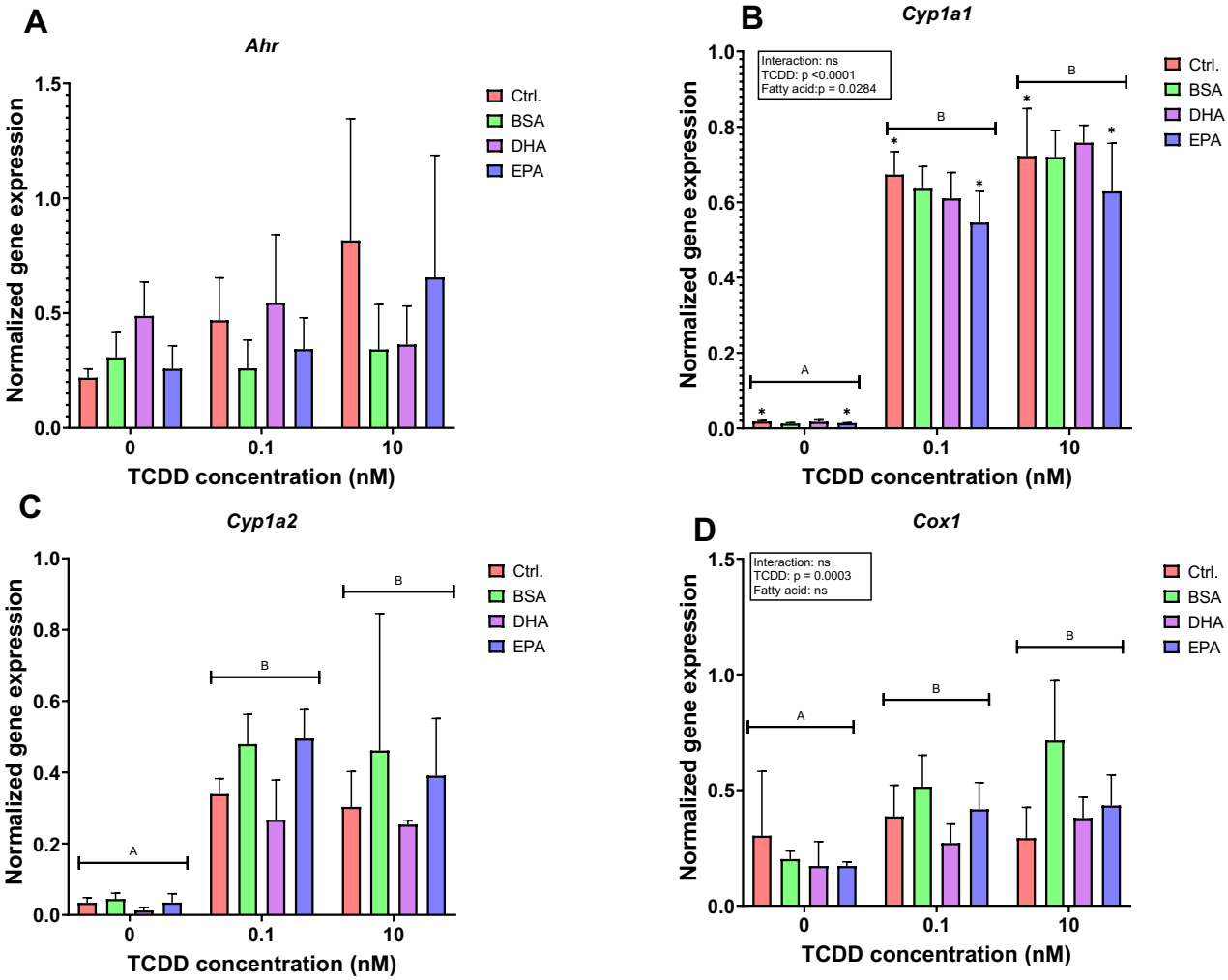

Figure 4.8 – Relative gene expression of *Ahr, Cyp1a1, Cyp1a2,* and *Cox1* in HEPA1-6 with n-3 fatty acid and TCDD. Mean normalized gene expression ± SD (n=3). Gene expression was detected by qPCR analysis. All genes were normalized using the four reference genes normalizing factors given by the geNorm algorithm. The box in the figure denotes p-values from a two-way ANOVA showing TCDD (main factor), or fatty acid (main factor). The statistical analysis was done on log-transformed data for *Cyp1a1* and *Cox1*. The figures without a box were analyzed using Kruskal-Walli's test followed by Dunn's multiple comparisons. Different uppercase letters represent statistical significance in the main factor TCDD, and (*) represent a statistical significance in the marked groups.

The gene expression of *Cat, Sod1,* and *Gpx4* was also tested to see if there were any oxidative stress caused by TCDD or/and n-3 FA (Figure 4.8). The two-way ANOVA results showed significance for the main factors of TCDD (p=0.0001) and FA (p=0.0396) for the regulation of gene expression of *Cat*. There was a higher regulation detected for the 0.1 nM TCDD as it was significantly different compared to 0 nM (p=0.0021) and 10 nM (p=0.0002). The multiple comparisons showed no significance for n-3 FA even though the two-way ANOVA indicated

that there was a significant difference present. There was no significance of gene regulation for *Sod1* and *Gpx4* for the main factors TCDD and n-3 FA. There were no interactions detected for *Cat, Sod1,* and *Gpx4* for the effects of TCDD and n-3 FA.

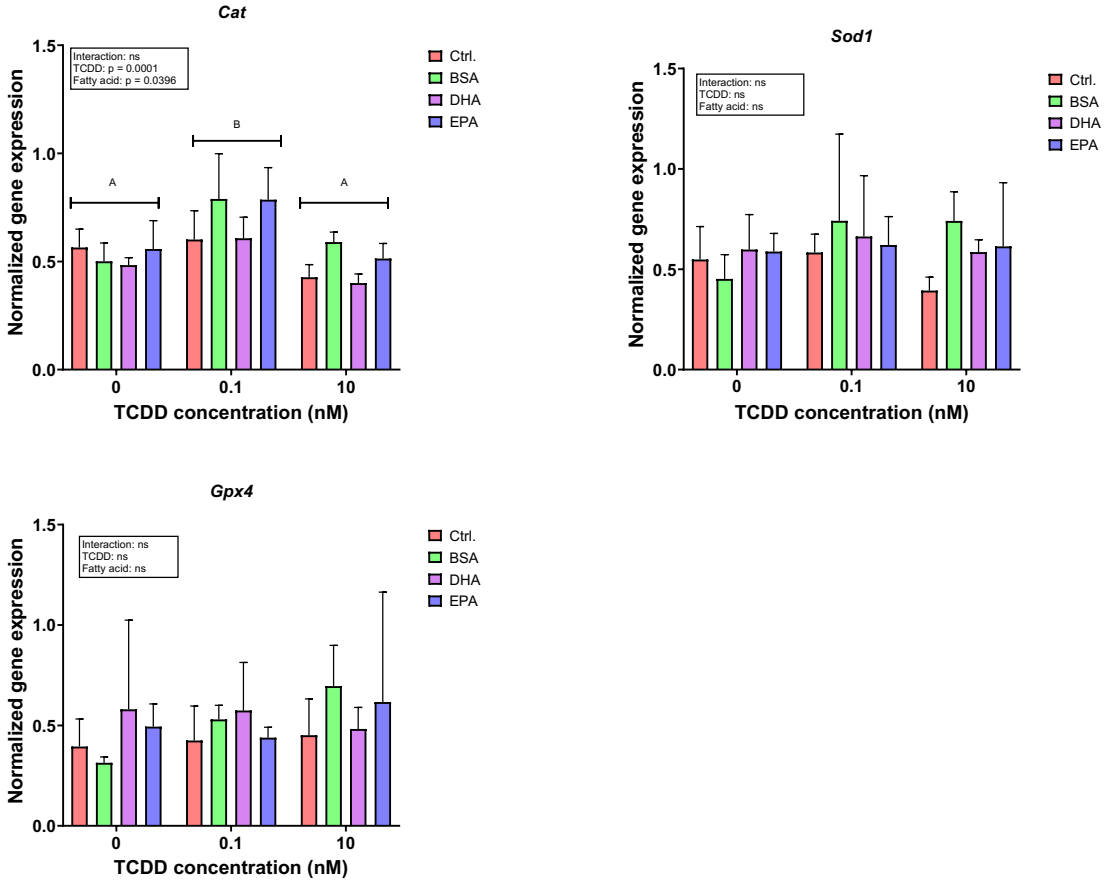

Figure 4.9 - Figure 4.8 – Relative gene expression of *Cat, Sod1,* and *Gpx4* in HEPA1-6 with n-3 fatty acid and TCDD. Mean normalized gene expression ± SD (n=3). Gene expression was analyzed using qPCR. All genes were normalized using the four reference genes normalizing factors given by the geNorm algorithm. The box in the figure denotes the results from a two-way ANOVA. Different uppercase letters represent statistical significance for the TCDD effect. The statistical analysis was done on log-transformed data for *Sod1* and *Gpx4*.

4.3 CYP1a1PROTEIN EXPRESSION

The HEPA1-6 cells were analyzed for CYP1a1 protein expression after TCDD exposure using western blot (Figure 4.10). There was an incomplete transfer of proteins from the SDS-Polyacrylamide gel to the membrane after Western Blot transfer (Figure 4.9). Most of the standard had been transferred from the SDS-polyacrylamide gel when comparing the before and after transfer to the PDVF-membrane. Additionally, there are observed a transfer of protein from the SDS-polyacrylamide gel in Figure 4.10C/D

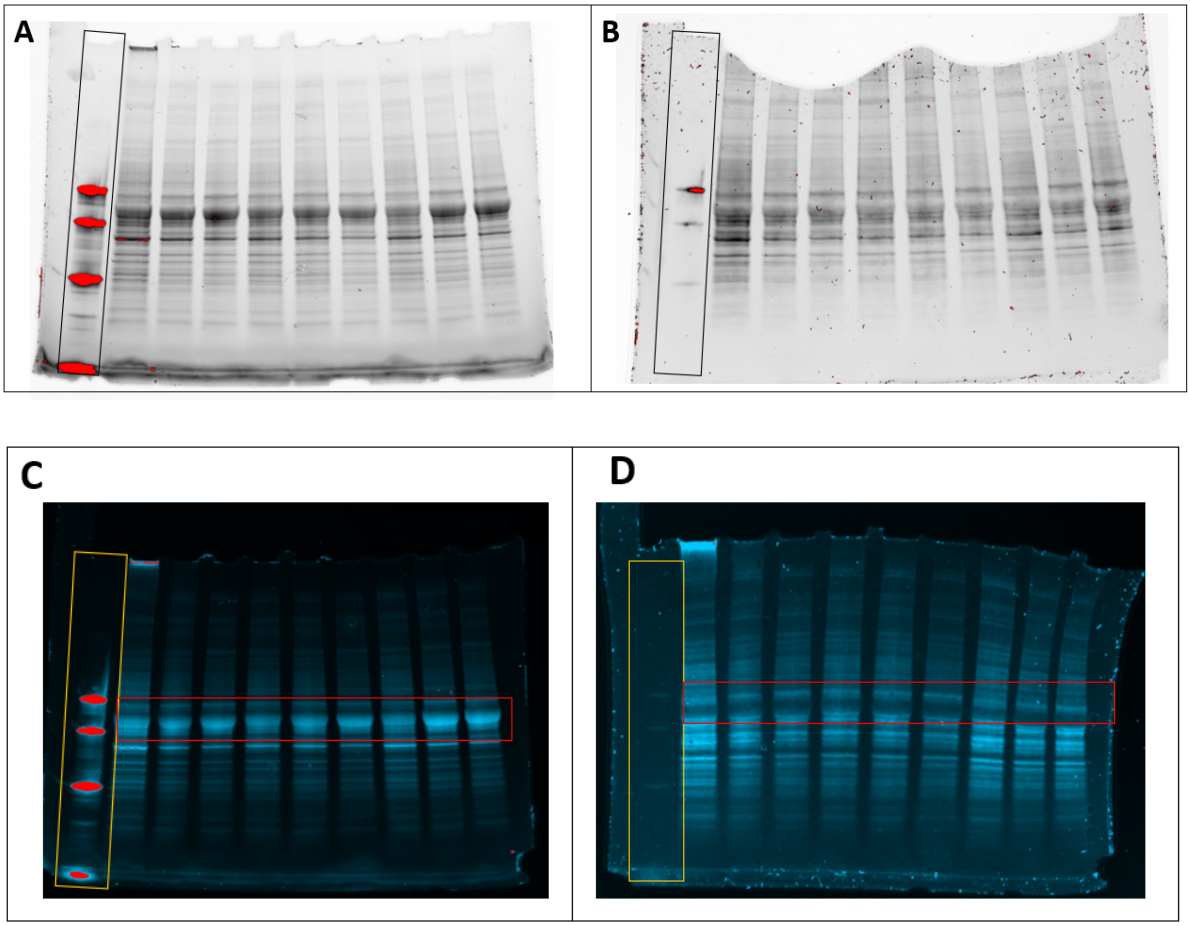

Figure 4.10 - SDS-polyacrylamide gel before and after Western blot transfer onto PDVF-membrane. The SDS-polyacylamide gels (A) and (B) are the same gels, as are (C) and (D). (A)/(B) and (C)/(D) were taken with different colour settings on the ChemiDoc™+ XRS system. The placement of the Precision Plus Protein™ WesternC™ Standard is indicated by the black and yellow boxes. The red box highlights where a band of proteins has been transferred.

The CYP1a1 proteins was blocked with different anti CYP1a1 antibody concentrations with ratios of 1:1000 and 1:2000 (Figure 4.11A/B). Further, an additional blocking of membrane (**A**) was done using a ratio of 1:500 (Figure 4.11C). Very faint bands were detected in membrane (**A**) between 50 and 75 kDa. For 1:500 (Figure 4.11C) multiple bands were detected. There was no CYP1a1 protein band detected for membrane (B).

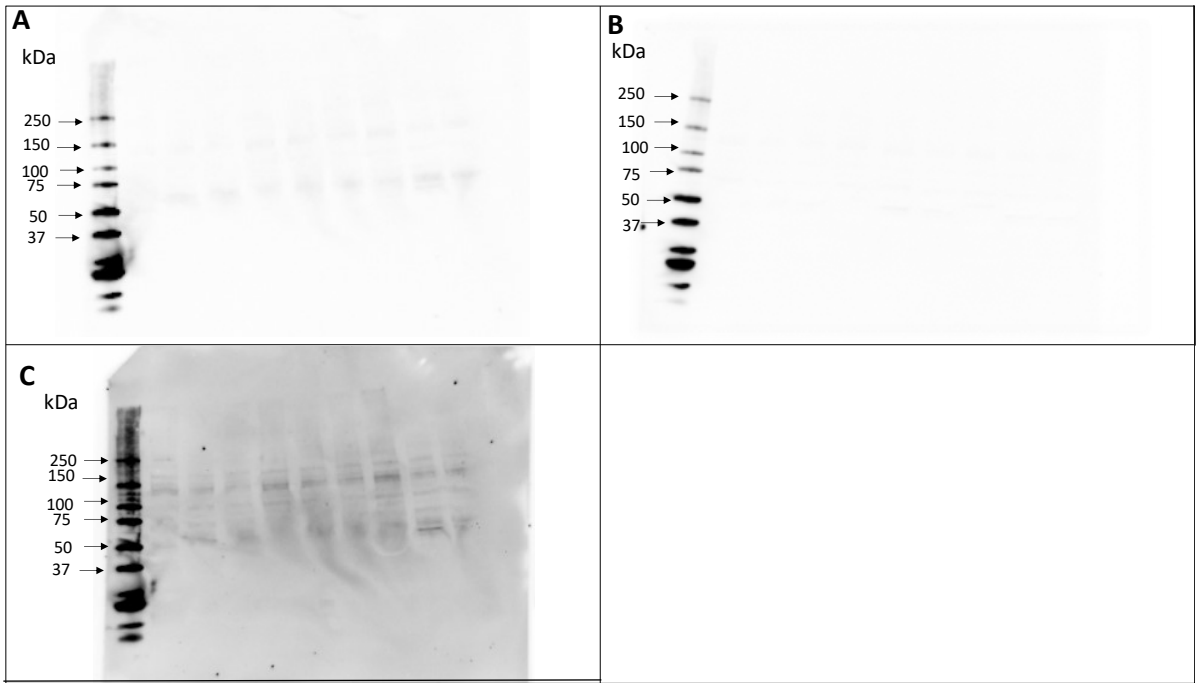

Figure 4.11 - Western blot PDVF-membrane blocked with different CYP1a1 antibody concentrations following a detection with ECL Prime Western Blotting Detection Reagent. The following PDVF membrane had been blocked with a CYP1a1 antibody concentration on (**A**) 1:1000, (**B**) 1: 2000, and (**C**) 1:500. The membranes were pictured by using the ChemiDoc™+ XRS system.

As the western blot showed multiple bands, an additional immunoprecipitation (IP) was utilized. The SDS-polyacrylamide gel with elution from the IP (Figure 4.11A) shows a faint band in the wells for 0.1 nM, 100 nM and one of the 0 nM. An additional Coomassie stain was subsequently executed to rule out detection errors using non-stain-based methodology, but there were no bands detected. However, the standard ladder was clearer after the Coomassie stain (Figure 4.11B).

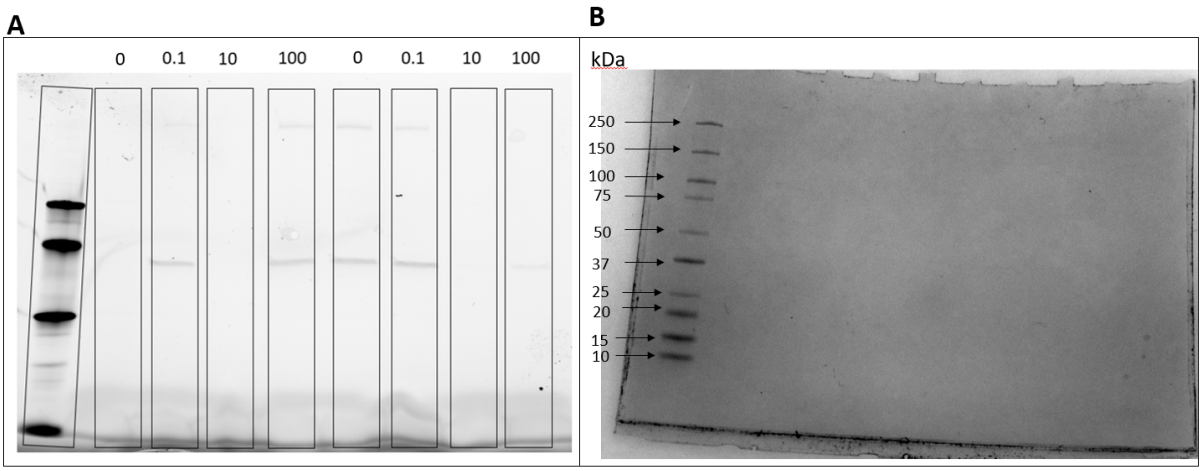

Figure 4.12 - Immunoprecipitation (IP) samples on SDS-polyacrylamide gel. (**A**) SDS-Polyacrylamide gel taken with stain free gel-application using ChemiDoc™ XRS+ System (**B**) the same gel stained with Coomasie.

4.4 CYTO- AND MATRIX-ROGFP

The HEPA1-6 cells were successfully transiently transfected with cytosolic-roGFP and mitochondrial matrix-roGFP plasmids as fluorescence was observed in the cells (Figure 4.13). Additionally, the cells were stained with DAPI to visualize the nuclei. These cells were transfected to investigate TCDDs effect on the redox environment in the cells, however, this was not possible due to complications which is further discussed in the discussion section.

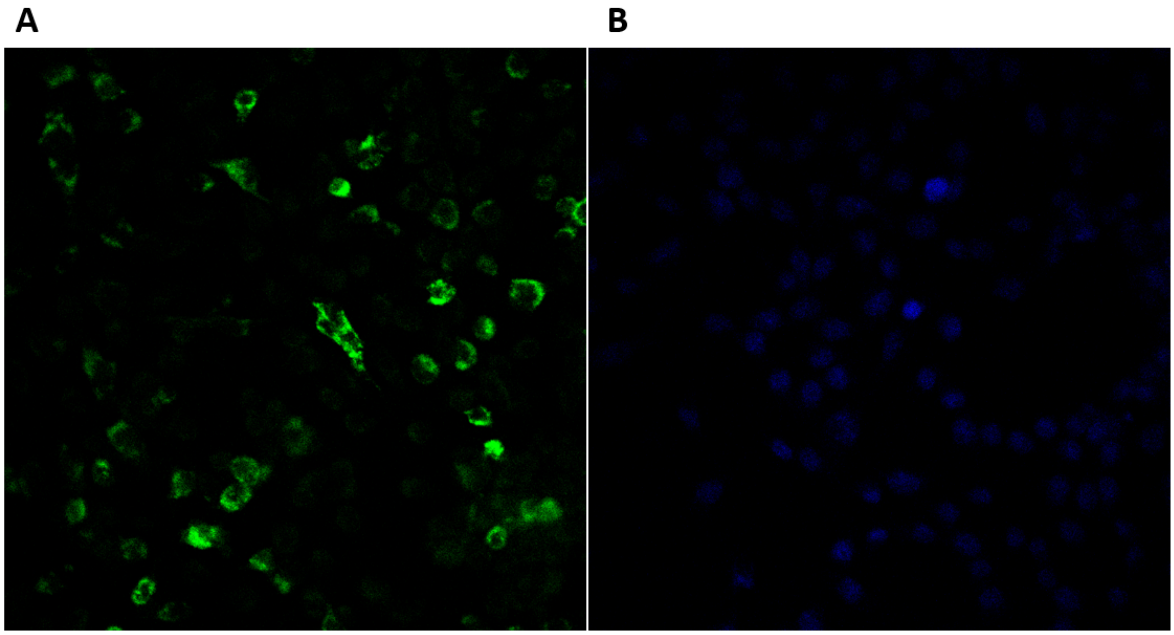

Figure 4.13 – Image of HEPA1-6 cells transiently transfected with cyto-roGFP. (**A**) Shows the fluorescence for excitation point at 488 nm. (**B**) Nuclei stained with DAPI taken at 405 nm.

5. DISCUSSION

In this thesis we have shown that TCDD and PCB-126 can reduce on HEPA1-6 cell growth and/or viability. Additionally, the HEPA1-6 cells were pre- incubated with n-3 FA to investigate the interaction effects between n-3 FA and the DLCs, however, no interactions were observed. The effects of PCB-126 and TCDD on HEPA1-6 cell growth were compared, and TCDD had a slightly more toxic effect on cell growth. Our study also showed that TCDD highly upregulated the expression of *Cyp1a1* and *Cyp1a2*. However, there was no upregulation of the antioxidative markers, suggesting no ROS-induction by TCDD and n-3 FA. There was also observed a reduced ability to upregulate *Cyp1a1* gene expression in EPA-incubated cells compared to the ctrls.

5.1 FATTY ACID HAD NO EFFECT ON HEPA1-6 CELL VIABILITY – TCDD INDUCED DOSE-RESPONSE.

In this study we investigated the effect of TCDD and PCB-126 on HEPA1-6 cell viability in combination with and without the n-3 FA. We observed a dose dependent response for TCDD up to 50 nM, where the curve flattened with the higher concentrations. This is consistent with previous findings in the HepG2 cell line as there was a dose-dependent decrease in cell viability at lower concentrations, contra higher (i.e. 50 nM and 100 nM) (Palanisamy *et al.*, 2015). A possible explanation could be that the higher concentrations saturate AHR and occupying the availability in the cells.

Attenuation of the dose response can be explained by a large variation, as indicated by the R^2. It could have been beneficial to repeat the experiment with an increased number of replicates and a narrower concentration interval. However, we proceeded with the available data, as our primary focus was to investigate the interactions between DLCs and n-3 FA. However, n-3 FA showed no effect on HEPA1-6 cell growth, nor were there any interaction observed between DLCs and n-3 FA. This is in contrast to a previous study by Palanaisamy et al, in which EPA were shown to have a protective effect against TCDD induced toxicity on cell viability (Palanisamy *et al.*, 2015).

5.1.1 TCDD vs. PCB-126

In this study we also included PCB-126, a dl-PCB with a TEF value of 0.1, which is the highest TEF amongst the dl-PCBs. This was of interest because of PCB-126 contributes a major part of the total TEQ-exposure from seafood, even though the congener only makes up a minor part of the total dioxin concentrations (Nøstbakken *et al.*, 2021). This was also highlighted in EFSAs report were they showed that PCB-126 had a high impact on the total TEQ and recommended a re-evaluation of the TEF (EFSA, 2018). In this study, PCB126 concentrations are presented as TEQ enabling comparison between TCDD and PCB-126 effect on HEPA1-6 cell viability. Our results showed that TCDD had a slightly higher effect on the cell viability compared to PCB-126, indicating that the current TEF-value for PCB-126 are accurate. However, some studies suggest that PCB-126 are less sensitive in humans than rodents (Zeiger *et al.*, 2001; Connor and Aylward, 2006; Larsson *et al.*, 2015), suggesting that the TEF-value are too high when transferring this to humans.

5.2 REGULATION OF TRANSCRIPTIONAL MARKERS WHEN EXPOSED TO TCDD AND N-3 FATTY ACIDS

TCDD is a known inducer of AHR and upon ligand binding it induces the expression of detoxification genes, such as *Cyp1a1* and *Cyp1a2* (Mimura and Fujii-Kuriyama, 2003), and has been observed to induce *Cyp1a1* and *Cyp1a2* gene regulation in different cell types (Nohara *et al.*, 2006; Zhang *et al.*, 2006). Our results showed a significant increase in both *Cyp1a1* and *Cyp1a2*, and notably a higher expression of *Cyp1a1* (average of 40-folds higher than 0 nM TCDD). This upregulation of *Cyp1a1* and *Cyp1a2* indicates that TCDD has been taken up by the cells and activated AHR, hence induced the regulations of these genes. As TCDD is poorly metabolized it can cause oxidative stress because of sustained induction of CYP1a1 and CYP1a2 (Shertzer *et al.*, 1998). In our study there were no increases in the antioxidative gene markers, *Sod1* and *Gpx4*, suggesting that there were no oxidative stress present, as these genes are upregulated when ROS are present (Weydert and Cullen, 2010). This is consistent with the findings in the liver of male mice fed with n-3 PUFA diet which did not show any increase in oxidative stress markers (Wiest *et al.*, 2016). Conversely, Turkez et.al (2012) did a study on cultured rat hepatocytes, where they demonstrated that TCDD-induced oxidative stress was mediated by the cells inability to scavenge ROS and suggesting that TCDD decreases antioxidant enzymes activity. However, in the present study we were not able to measure the redox changes in the cells, but TCDD-induced toxic effects have been shown to

be mediated by an imbalance in redox environment (Wan *et al.*, 2014). Notably, oxidative stress has been shown to downregulate *Cyp1a1* gene expression as the AHR-ARNT transcriptional complex acts in synergy with nuclear factor I/CCAAT (NFI/CTF) for the *Cyp1a1* expression in HepG2 cells, which is a redox-sensitive transcription factor (Morel *et al.*, 1999; Barouki and Morel, 2001). This might also be another indication that ROS was not present in our study as an increase of *Cyp1a1* gene expression was observed. Additionally, studies have reported that ROS can inhibit CAT-activity and its ability to inhibit O_2^- (Kono and Fridovich, 1982), conversely in our study it was observed a small increase for *Cat* gene expression. However, it might be more likely that this observation is a consequence of methodological reasons (section 5.5.2), as the increase was only detected for 0.1 nM TCDD.

TCDD induced gene expression has been shown to be both dose- and time-depended (Santostefano *et al.*, 1998). The cells in our study were harvested 24 hours post TCDD exposure, and as observed in the cell viability results, there is a clearer dose-response around 40 hours post TCDD exposure than after 24 hours (Figure 4.2 and 4.5). Turkez *et. al* (2012) reported an increase in total oxidative stress in rat hepatocytes exposed to TCDD for 48 hours. There could have been a higher regulation of the different gene markers such as the antioxidative markers if we had harvested the cells around 40 hours post TCDD exposure.

5.2.1 EPA REDUCES CYP1A1 GENE EXPRESSION

Our study showed a fatty acid-effect on *Cyp1a1* gene regulation for EPA-incubated cells, indicating that EPA had reduced the regulation of the gene. This is consistent with the findings of mRNA expression of *Cyp1a1* in liver cells from C57BL/6J male mice (Wiest *et al.*, 2016). Wiest *et al.* (2016) fed TCDD-exposed mice with n-3 PUFA diet with a high EPA concentration, and observed a reduced mRNA expression of *Cyp1a1*, as well as a reduction in CYP1a1 protein expression, suggesting that n-3 PUFA can prevent TCDD induced CYP1a1 mediated injuries. Additionally, EPA has also been shown to have protective effects against TCDD-induced toxicity (Turkez *et al.*, 2012; Palanisamy *et al.*, 2015). Both studies observed an increase in TCDD-induced ROS, where Palanisamy *et. al*. (2015) showed a correlation between increased CYP1a1 activity and ROS activity. Further, demonstrating that EPA had a protective effect against TCDD- induced ROS by reducing the CYP1a1 activity (Palanisamy *et al.*, 2015). However, to my knowledge, the mechanism behind n-3 FA on reduced Cyp1a1 gene expression are still unknown.

5.3 CYP1a1 PROTEIN EXPRESSION

In addition to the effect of TCDD on gene expression we wanted to assess whether the combination with n-3 FA influenced gene regulation. However, this was not possible due to methodological complications. TCDD have been established to induce CYP1a1 activity in hepatocytes (Xu *et al.*, 2000; Zacharova *et al.*, 2003). We can speculate that the upregulation of *Cyp1a1* gene expression in our results would lead to an upregulation of CYP1a1 protein expression, as an induction in both mRNA and protein expression of *Cyp1a1* has been previously reported in cell lines (Nohara *et al.*, 2006; Neri *et al.*, 2008).

5.4 INTERACTIONS BETWEEN TCDD AND N-3 FATTY ACIDS

In this thesis, it was not observed a distinct interaction between the DLCs and n-3 FA. However, the gene expression analysis, more specific the reduced *Cyp1a1* protein expression by EPA can indicate that an interaction does exist. Similarly to other studies have suggested that n-3 FA have a protective effect against TCDD induced toxicity (Türkez *et al.*, 2012; Palanisamy *et al.*, 2015; Wiest *et al.*, 2016). Even though, we did not observe any effect of DHA in this thesis, other studies have shown that DHA have ameliorative effects on TCDD induced toxicity in rat liver (Türkez, Geyikoglu and Yousef, 2012). A plausible reason for not detecting an interaction can be due to the experimental set up as it might not have been sensitive enough to observe these interactions. It is possible that the observes outcomes could be time-dependent, thereby indicating that an alternative time point for sampling may have had other observed results.

5.5 METHODOLOGY

In this study, there were certain methodological challenges that hindered the investigation of the aims. However, the results were still included in this thesis, as the troubleshooting became an important part of the thesis and may give insight for future investigations.

5.5.1 CYP1A1 PROTEIN DETECTION (WESTERN BLOTTING AND IMMUNOPRECIPITATION)

It occurred complications on the way which resulted in an insufficient detection of CYP1a1 bands. Before immunoprecipitation we tested blocking with different CYP1a1 antibody concentrations, increasing blocking time, and blocked with 2% and 5% ECL blocking buffer.

But as seen from the results there was multiple bands detected after using western blot and blocking with different antibody concentrations. It was detected a very faint band with 1:1000 antibody concentration, which was why we blocked this membrane in additional 1:500 concentrations to increase the intensity of the bands. However, multiple bands were detected instead, suggesting that the antibody did not bind specifically to the CYP1a1 protein. On the other PVDF-membrane that had been incubated with 1:2000 ratio of antibody did not detect any bonds. This might be due to the incomplete transfer of proteins from the SDS-polyacrylamide gel to PVDF-membrane. As seen in Figure 4.10, there were still proteins left on the SDS-polyacrylamide gel after western blot transferring, but the standard ladder did transfer which indicate that some transferring occurred. It was detected a lower voltage than the setting was set to be (1.3/22V), indicating that the insufficient transfer could be due to a lower voltage from the cathode to anode, as sufficient voltage is required for protein transferring. Due to the challenges encountered in detecting specific bonds, immunoprecipitation was employed as an additional approach to detect CYP1a1 protein expression. The SDS–polyacrylamide gel with the elution samples from the immunoprecipitation exhibited more distinct bands. However, the observed bands did not align with our expectations, as some of the bands was not detected in the samples exposed to TCDD and a band was detected for 0 nM which should not have a high expression of CYP1a1. Due to difficulties in interpreting the standard ladder, we were unable to measure the kDa values of the bands. Therefore, we opted to stain the SDS-gel with Coomassie blue to potentially enhance the intensity of the bands and standard ladder. However, this was not the case as the protein bands disappeared upon Coomassie blue stain, but the standard ladder was enhanced. This could be because of low protein concentrations in the bands, enabling Coomassie blue to bind and stain the proteins. Due to time constrains, we were not able to repeat the experiment.

5.5.2 qPCR

The qPCR analysis presented in this thesis utilized a cDNA that was additionally diluted with a 1:1 ratio with dH_2O. This problem-solving was due to the high efficiency observed in the initial qPCR run, suggesting that inhibitors were present in the cDNA sample. Even though the efficiency did improve as a result of this dilution, it is important to be aware of the factors that can affect the outcome. Such as a reduced concentration of the target genes, which can affect the quantification of these genes, as well as cause increased variation. A suggestion for future work, is to use an enzyme that is more robust against inhibitors in the cDNA.

5.5.3 REDOX MEASUREMENTS

In this thesis, one of the aims was to establish roGFP in the liver cells to investigate changes in the redox environment following exposure to TCDD, and subsequently examine this response in combination with n-3 FAs. The cells were transiently transfected with cyto-roGFP and matrix-roGFP, which was successful as fluorescence was detected in the cells. Because the cells were stained with DAPI, it was not possible to assess the redox ratio as the emission and excitation points for DAPI interfered with cyto- and matrix-roGFP. Moreover, it would be preferably to repeat this experiment (without DAPI), also with n-3 FA-incubated cells, but due to time constrains this was not possible.

6. CONCLUSION

The main aim of this present study was to investigate if marine n-3 fatty acids had an effect on TCDD and PCB-126 toxicity in HEPA1-6 cells. In this present study we have observed that the dioxin like compounds, TCDD and PCB-126 showed a dose-dependent response in the HEPA1-6 cell growth. However, when the cells were incubated with n-3 fatty acids, there were no observed effect on cell viability when exposed to TCDD and PCB-126. An additional comparison of TCDD and PCB-126 showed that TCDD had a slightly more toxic effect on cell growth than PCB-126. Further, TCDD exposed cells had a highly induced *Cyp1a1* and *Cyp1a2* gene expression, and EPA-incubated cells reduced *Cyp1a1* gene expression compared to the ctrls. Moreover, TCDD exposure did not upregulate antioxidative markers, indicating that no oxidative stress was present at the time of sampling. Lastly, due to methodological challenges and time constrains, the effect of TCDD and n-3 FA in HEPA1-6 cells on CYP1a1 protein expression, as well as the redox changes was not possible to observe, due to methodological challenges. In this study, distinct interaction between n-3 FA and TCDD/PCB-126 was not observed. However, it is likely that the interactions exist but was not observed in our experimental set up. Suggesting that this thesis can give a pinpoint to areas and mechanisms to assess when studying the interactions between DLCs and marine n-3 FA.

7. FUTURE PERSPECTIVES

Due to methodological challenges that arose, the experiments with western blot, immunoprecipitation, and measuring of redox ratio should be repeated. The un-identified bonds detected by western blotting and immunoprecipitations should be further examined, for instance by DNA-sequencing to fully identify the proteins. Additionally, it could have been interesting to use ethoxyresorufin-O-deethylase (EROD) assay to investigate the CYP1a1 activity in combination with n-3 FA, and perhaps gain insight to a possible ROS induction.

A finding in this thesis was the reduced effect of *Cyp1a1* gene regulation after TCDD-exposure in EPA incubated cells. It could be interesting to further investigate other aspects of EPA metabolism when exposed to DLCs, such as the production of prostaglandins.

In this study PCB-126 had similar effect on cell viability as TCDD, due to workload and time constrains, TCDD was used to further investigate the interactions. However, it would be interesting to explore the same aspects for PCB-126. Moreover, PCB-126 have been shown to produce higher concentrations of superoxide anion in brain tissue than in liver tissue in rat (Hassoun *et al.*, 2001). Therefore, it could be interesting to study the effects of DLCs and n-3 FA on brian cells.

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