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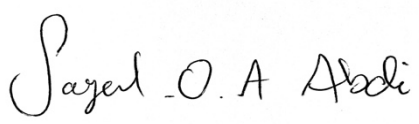
Author: Sagal Omar Ali Abdi	 (signature of author)
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Abstract

A series of oil-contaminated sediment samples (drill cuttings) have been analyzed for their toxicity using *in vitro* bioassay based on fish cell lines. Drill cuttings are pieces of rock that come out of a well, (a deep hole made in the ground, to locate oil) when a well is drilled to an oil or gas reservoir. During drilling operations, a fluid known as drilling mud is used to lubricate the drill bit and carry the drill cuttings to the surface, where they are separated from the mud and cleaned. The mud is reused where possible, and the drill cuttings are either discharged or taken ashore for further treatment and disposal. Unfortunately, up until the mid-1990s, discharge of cuttings to the seabed was permitted in most countries. Therefore, they have formed piles on the seabed and also settled on and between some of the oil storage cells. The evaluation of the toxicity of the drill cuttings and in particular their potential adverse effects in marine organisms is of known importance. The present work focuses on the use of *in vitro* cell based bioassay to evaluate the ecotoxicity of drill cuttings. By using fish cell line (i.e., PLHC-1 and RTgill-W1), the quality of these sediment types were assessed through assays for general cytotoxicity, reactive oxygen species production (i.e., oxidative stress) and EROD activity (a PAH related metabolism enzyme). The obtained results were compared with data from natural fjord sediment samples. The cell viability, EROD activity, and ROS production showed an adverse effect to the oil-contaminated drill cutting for PLHC-1 in comparison to the natural fjord sediments. While RTgill-W1 showed an adverse effect in cell viability and ROS production for oil-contaminated drill cuttings in contrast to the natural fjord sediments.

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List of abbreviations

Abbreviation	Long form
ANOVA	Analysis of variance
ATCC®	American Type Culture Collection
BCA	Bicinchoninic acid assay
bNF	β-naphtholflavone
CO_2	Carbon dioxide
CYP1A	Cytochrome P450 1A
DCF	2', 7'-dichlorofluorescein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid i
EDTA	Ethylenediamine tetraacetic acid
EPA	Environmental Protection Agency
EROD	7-ethoxy-resorufin-O-deethylase
H_2DCF-DA	2′7′-dichloro-dihydrofluorescein diacetate
H_2O_2	Hydrogenperoxide
L-15	Leibovitz's L-15 medium
MEM	Eagle’s minimum Essential Medium
OBM	Oil-based mud
PAH	Polycyclic aromatic hydrocarbon
Pb	Lead
PBS	Phosphate buffered saline
RFU	Relativ Fluorescence unit
ROS	Reactive Oxygen Species
SBM	Synthetic-based mud
UiS	University of Stavanger
UPV/EHU	University of the Basque Country
WBM	Water-based mud

1. Introduction

1.1. Background

Jarod Daimond once said, "*People often ask, 'What is the single most important environmental problem facing the world today?' The single most important problem is our misguided focus on identifying the single most important problem! ... because any of the dozen problems, if unsolved, would do us great harm and because they all interact with each other."* [1] .

One of these problems in the world today is the increasing amount of chemical pollutants in the environment due to anthropogenic activities. In synergy with the growing human population, urbanization and industrial development foreign chemicals (xenobiotics) such as polycyclic aromatic hydrocarbons (PAHs), are entering into the environment, having a potential detrimental effect on the ecosystem. [2].

During the past decades an estimated 2.4 million tons crude oil have annually been released into the aquatic environment as a result of worldwide oil exploration and use of oil products [3]. The marine sediments especially, acts as optimal sinks and sources for such pollutants. This type of contamination poses a serious hazard because of its large content of PAHs, a highly toxic component of crude oil known for its carcinogenic potential[4].

Understanding the impact of petroleum deriving compounds in sediments, however, remains a challenge due to their presence in a complex mixture. Traditional environmental monitoring approaches such as chemical analysis are not sufficient to assess the sediment quality alone. Some of shortcomings are not taking the bioavailability of the chemical in consideration or several compounds combined (antagonistic/synergetic) effect on organisms [5].

Developing new approaches to assess the environmental quality of sediments is hence necessary in order to characterize the overall health status of the ecosystem, and as result implement measures to minimize the adverse effects the pollutants might have.
Cell-based bioassay approach, an analytical method using biological response mechanism to exposure, is an such an approach which in newer time which have shown positive results [6]–[9].

1.2. Polycyclic aromatic hydrocarbons

PAHs constitutes a large group of organic pollutants with more than 600 compounds listed that are continuously released into the environment in high quantities, mostly due to human activities. Many of these ubiquitous organic compounds are non-polar planar molecules composed of two or more aromatic rings fused by alternating double and single bonds through sharing of carbon pair atoms (Figure 1.2.1). Because of their hydrophobic nature, molecules with the higher molecular weight (more aromatic rings) are known to be the less water soluble. While lower molecular weight (fewer aromatic rings) makes the PAHs molecules more water soluble. The relationship between hydrophobicity and molecular weight can be characterized by the octanol/water partition coefficient $K_{OW,}$ as seen in Table.1.1. Partitioning describes the tendency of organic pollutants to bind to biota (lipid compartment in organisms, particulate organic matter, and sediments). A higher partition coefficient indicates that the PAHs is less soluble, which contributes to the degree and rate PAHs are taken up by the organism, also known as bioavailability, leading to decrease in biodegradability and consequently bioaccumulation in biota [10][11][12].

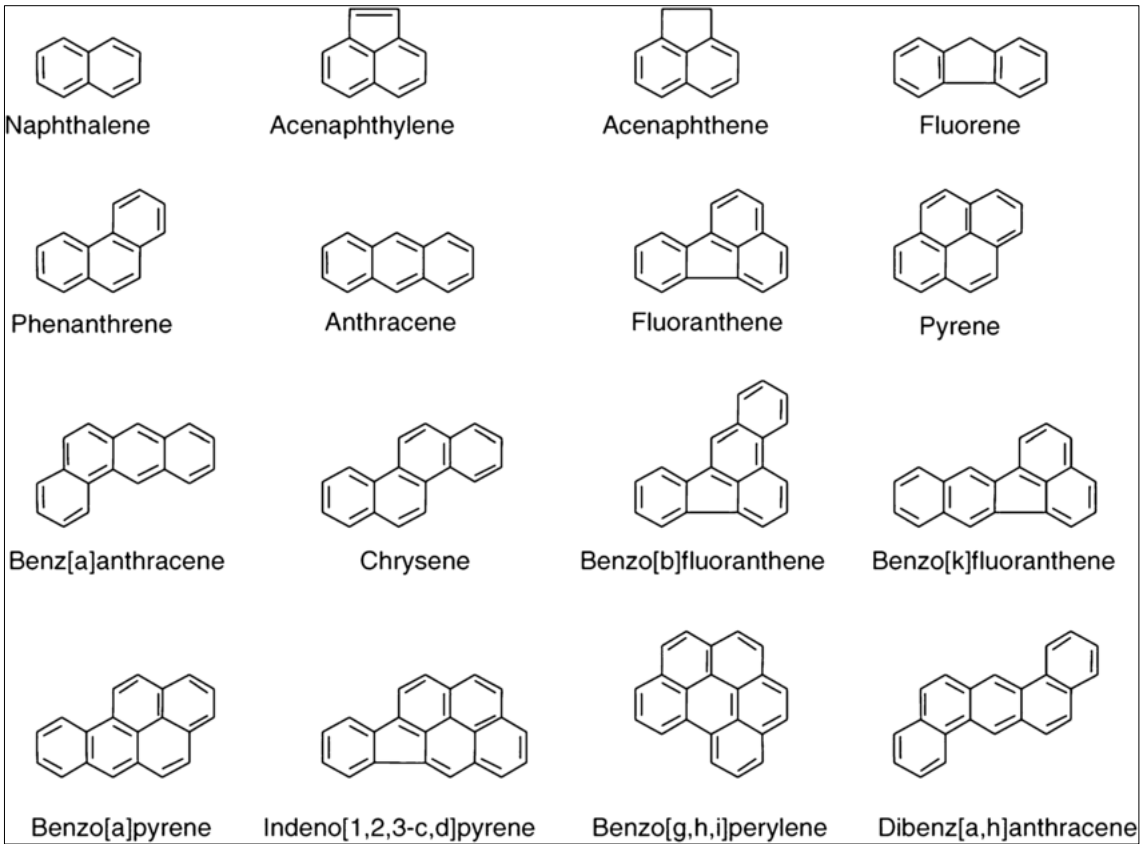

Figure 1.2.1 Molecular structure of US-EPA 16 priority pollutant PAHs [13].

Table.1.1 US-EPA 16 priority pollutant PAHs and selected properties [14].

PAH compounds	Molecular weight	Log K_{OW}*
Naphthalene	128	3.37
Acenaphthylene	152	4.00
Acenaphthene	154	3.92
Fluorene	166	4.18
Phenanthrene	178	4.57
Anthracene	178	4.54
Pyrene	202	5.22
Fluoranthene	202	5.18
Benzo[*a*]fluoranthene	228	5.91
Chrysene	228	5.75
Benzo[*b*]fluoranthene	252	5.8
Benzo[*k*]fluoranthene	252	6.0
Benzo[*a*]pyrene	252	6.04
Dibenzo[*a,h*]fluoranthene	276	6.58
Indeno[1,2,3-*cd*]pyrene	278	6.75
Benzo[*ghi*]pyrene	276	6.50

*K_{OW} :Octanol/water partition coefficient

PAHs are a concern to the aquatic organisms [14][15]. Studies have proven exposure to some PAHs affect growth, the reproductive system and cause oxidative stress on the organisms [15][17]. Other PAHs are known to be carcinogenic meaning they induce cancer development [18]. For these reasons, the US Environmental Protection Agency (EPA) and European Union (EU) established priority lists containing PAH parent compounds and their derivatives known to have toxic effects [19].

In the marine environment PAHs are present in complex mixtures from natural (e.g., forest fires and oil seeps) and anthropogenic (e.g., combustion of fossil fuels, vehicle emissions, petroleum activities) sources (Figure 1.2.2) [2][4]. Anthropogenic PAHs are divided into pyrogenic and petrogenic, classified by their origin. Pyrogenic PAHs are formed as a result of rapid incomplete combustion of organic matter [21]. While petrogenic PAHs are present in crude oil and oil products [22].

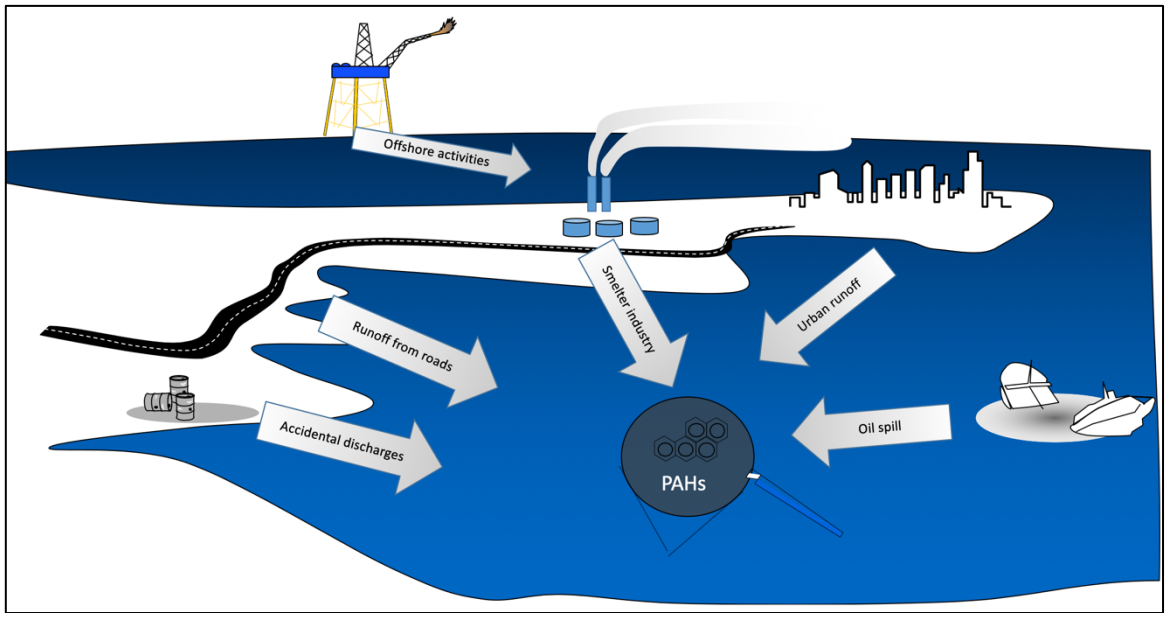

Figure 1.2.2 Sources of anthropogenic PAHs[4].

In offshore operations, the most significant volume of waste produced derives from drilling mud and drill cuttings. The drilling mud is an essential part of the operation as it is used to lubricate the drill bit and transfer while circulating drill cuttings back to the surface. The mud is then reused after removal of drill cuttings. Drilling mud is generally divided into three types of fluids: water-based mud (WBM), synthetic-based mud (SBM) and oil-based mud (OBM) with increasing toxicity depending on their chemical compositions and additives, making them

concern in regards ecological and health risk. Oil-based mud (OBM) is the fluid of highest concern because of its concentration of PAHs [23].

Drill cuttings are particles of the sedimentary rock that are excavated by the drill bit in order to locate oil. Present procedure after separation of drill cuttings and drilling mud is to clean them and transport to land for further treatment. However, up until mid-1990 discharge of drill cuttings into the seabed was permitted in most countries. This accumulation of oil in the sediments over longer time has led to the persistence of oil constitutes in the aquatic environment. Thus, monitoring of the seabed underneath platforms shows there are to this day cutting piles contaminated with oil still present at the sea bed and in close vicinity [3][19].

1.3. Environmental monitoring

Present, there is a general agreement that chemical pollutants from anthropogenic sources released into the marine environment pose a risk to the ecosystem. Therefore as a means to protect the ecosystem from harm environmental monitoring is conducted by determining the biological effect of these anthropogenic pollutants [2].

Traditionally, oil impact assessment of pollutants in the aquatic environment was determined through chemical monitoring where concentrations of single compounds were measured in the water column and biota. Nonetheless, these procedures in newer times are considered to be to nonrepresentative for the biological effect that occurs [24]. To understand the effect of the pollutants on the organisms, factors such as bioavailability and bioactivity must also be considered together with the chemical analyses. Pollutants interacting in a mixture also affect the uptake by causing additive and synergistic/antagonistic effects [25].

Newer studies on marine spills in European waters have investigated applying an combination of bioassays and biomarkers to complement the chemical analysis showing positive results [26]–[28].

1.4. Bioassay

Bioassay is a biomonitoring approach more often seen newer in studies of sediment quality assessment [6][8]. Rand and Petrocelli define the term bioassay as “a test used to evaluate the relative potency of a chemical by comparing its effect on a living organism with the effect of a

standard preparation on the same type of organisms." [20]. There are two ways of performing these tests, bioassay done *in vivo* (on whole organisms) or *in vitro* (on tissues and cells).

In environmental risk assessment of pollutants, cell-based bioassays are used to indicating the presence and potential effect of contamination by utilizing the fact that interaction between pollutants and biota initially transpires at cellular and molecular levels. As a result, the measurable response such as toxicity, oxidative stress and endocrine disruption effect can be used as endpoints (biomarkers) for exposure of specific pollutants [29].

The advantages of using bioassays is the ability to reflect the mode of action of pollutant to detect toxic effect of a mixture of chemical compounds in the environment. Cell-based bioassays are also cost efficient in comparison large field monitoring approach by covering multiple endpoints in a laboratory setting [24].

1.5. Aim of study

The aim of this thesis was to assess the potential toxic effects of oil contaminated sediments such as drill cuttings using bioassays based on fish liver cells (PLHC-1) and gill cells (RTgill-W1). A natural sediment collected in a clean area in the Stavanger (South of Norway) was used as a reference. The selected battery of biomarkers included general cytotoxicity (i.e. measured as cell viability), reactive oxygen species production (ROS) and 7-ethoxyresorufin-O-deethylase (EROD) activity.

2.Materials and methods

2.1. Materials

Table 2.1 Sediment extraction chemicals

Substances	Product number	Supplier	Origin
Dichloromethane	-	Sigma Aldrich	Oslo, Norway
n-hexane	34484	Sigma Aldrich	Oslo, Norway
Acetone	A4206	Sigma Aldrich	Oslo, Norway
Petroleum ether	77399-1L	Honeywell Riedel-de Haen®	Seelze,Germany

Table 2.2 Cell growth medium and supplements

Substances	Product number	Supplier	Origin
Eagle's Minimum Essential Medium (MEM)	L0430-500	Biowest	Nuaillé, France
Leibovitz's L-15 Medium (L-15)	21083027	LIFE TECHNOLOGIES	Bleiswijk Netherlands
Fetal bovine serum	-	Biowest	Nuaillé, France
L-glutamine	G8540	Sigma Aldrich	Oslo, Norway
Penicillin-streptomycin,	15140122	Life Technologies AS (Invitrogen Dynal AS)	Oslo, Norway

Table 2.3 Bioassay chemicals, reagents and buffers

Reagents	**Product number**	**Supplier**	**Origin**
Phosphate buffered saline (PBS)	-	LIFE TECHNOLOGIES	Bleiswijk Netherlands
Trypsin–EDTA	T4049	Sigma Aldrich	Oslo, Norway
7-Ethoxyresorufin,	16122-NOR	Cayman Chemical Company	Ann Arbor USA
7-hydroxyresorufin sodium salt	B21187.06	Alfa Aesar	Oslo, Norway
β-Naphtoflavone (bNF)	A18543.03	Alfa Aesar	
Dimethyl sulfoxide (DMSO)			
2′7′-dichloro-dihydrofluorescein diacetate (H_2DCF-DA)	D399	Invitrogen™ Molecular Probes™	Bleiswijk Netherlands
Resazurin	B21187	Thermo Fisher	Karlsruhe; Germany

Table 2.4 Commercial kit

KIT	Description	Product number	Supplier	Origin
Pierce(R) BCA Protein Assay	Total protein determination	23221	Thermo Fisher Scientific/ Pierce Biotechnology	Rockford, USA

Table 2.5 Instruments

Instruments	Description
Muse® Cell Analyzer	Cell counting
SpectraMax Paradigm Multi-Mode Microplate Reader	Plate reader
Class II Biological Safety Cabinet	Cell culture sterile workbench

Table 2.6 Software programs

Software name	Description	Supplier
Excel 2010	Statistical analyses and graphs	Microsoft
SoftMax Pro	Plate reading	

2.2. Fish cell line culturing

When experimenting with cell lines executing correct sterile techniques as well as providing optimal growth conditions is vital for assay results to be reliable. For this purpose, I attended a course held by Miren P. Cajaraville and Alberto Katsumit on "*in vitro* toxicity testing" at the University of the Basque Country (UPV/EHU). The course provided a theoretical introduction to primary and continues cell lines how they are used for research, and laboratory practical on how to culture cells lines for toxicity assay purposes (Figure 2.2.1) [32].

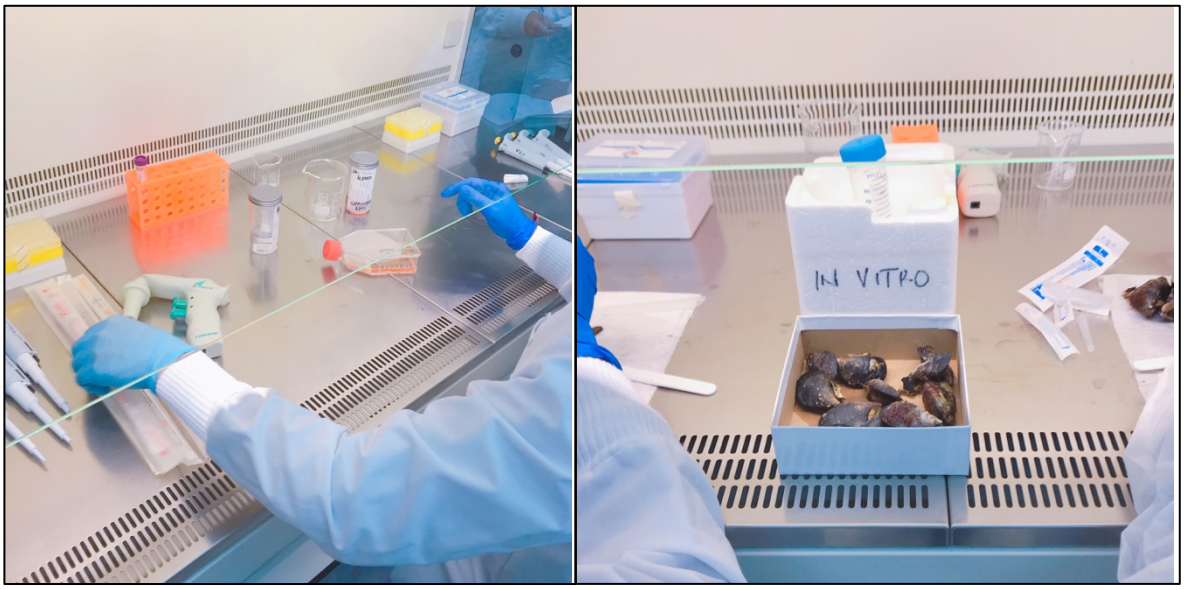

Figure 2.2.1 Laboratory practical experience at the "*in vitro* toxicity testing" course (private picture).

For this thesis, cell lines PLHC-1 liver cells from topminnow (*Poeciliopsis lucida*) and RTgill-W1 gill cells from rainbow trout (*Oncorhynchus mykiss*) were used. Materials were provided by the dCod.1 project and the PhD fellow Eystein Opsahl UiS, respectively.

2.2.1. Sub culturing

Sub culturing, also known as splitting, was done by aspiring culture medium, washing cells with 5 mL PBS, detaching cells with 4 mL of trypsin-EDTA and adding 6 mL of culture medium, in order to inhibit trypsin when all cells were detached. To keep the cell density in optimal conditions, a dilution of 1:2, 1:5 or 1:10 with culture medium was done before transferring cells into a new flask. All cell culture related work was done under sterile conditions in a level II biosafety cabinet.

Table 2.7 Description of cell lines

Cell line	Fish species	Tissue	Culture conditions	Source	Reference
PLHC-1	Topminnow *(Poeciliopsis lucida*)	Liver	Eagle's minimum Essential Medium (MEM) + 5 % fetal bovine serum in a 5% CO_2 humidified incubator at 30°C.	dCod.1 project	Huuskonen et al. [33]
RTgill-W1	Rainbow trout, (*Oncorhynchus mykiss*)	Gill	Leibovitz's L-15 medium (L-15) in an incubator without CO_2 at 18°C.	UiS, Eystein Opshal	Bols and Lee [34]

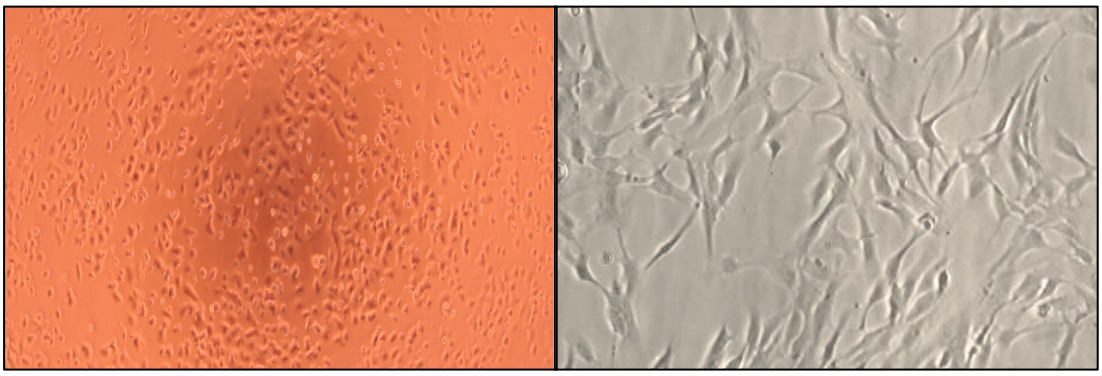

Figure 2.2.2 Microscopic picture of PLHC-1(at 10x magnification) and RTgill-W1(at 20x magnification) cells.

2.2.2. PLHC-1

PLHC-1 cell line were cultured in 10 mL culture medium supplemented with fetal bovine serum, L-glutamine, penicillin and streptomycin, according to protocols from ATCC®[35] as reported in Table 2.7. The PLHC-1 cells were grown in a filter-cap T75 tissue culture flasks in 5% CO_2 humidified incubator at 30°C (Figure 2.2.3). Culture medium was changed every other day throughout the experiment. While sub culturing was done when the cells were at 90% confluency of the flask area.

2.2.3. RTgill-W1

The RTgill-W1 were cultured in 11-15 mL Leibovitz's L-15 medium supplemented with fetal bovine serum, L-glutamine, penicillin and streptomycin, according to protocols from ATCC® as reported in Table 2.7. Cells were grown in a close-cap T-75 tissue culture flasks in an incubator without CO_2 at 18°C (Figure 2.2.3). Culture medium was changed every other day

throughout the experiment. While sub culturing was done when the cells where at 90% confluency of the flask area.

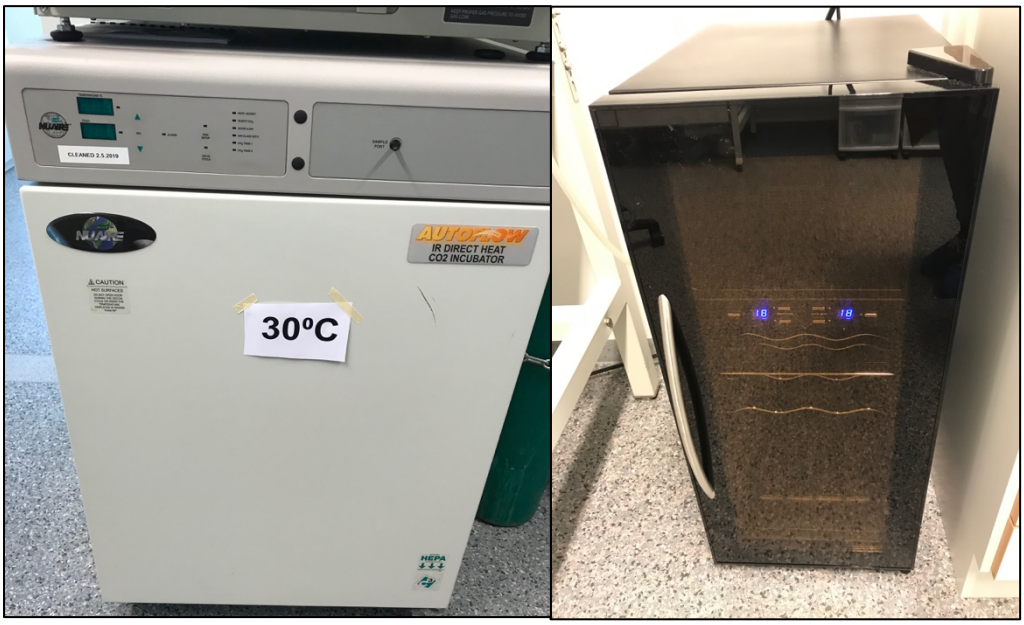

Figure 2.2.3 Incubators with CO_2 at 30°C (to the left) and without CO_2 at 18°C (to the right).

2.2.4. Plate seeding

The seeding for both cell lines was performed with a cell density of 40 000 cells/mL, which was determined using the Muse® Cell Analyzer from an 90% confluent culture flask. All experiments where conducted with a confluent monolayer in clear 96-well plates. After 24 h incubation, culture medium was replaced with appropriate medium containing sample extracts at concentrations 5, 10, 20, 40, 60, and 120 mg eQsed/mL, the positive controls 100 µM H_2O_2 was used in the cell viability and ROS assays and β-naphtholflavone (bNF) in the EROD assay. Four replicates was done for each concentration of sediment extract, control cell culture (0 mg eQsed/mL) and positive control (n = 32) in one experiment.

2.3. Cell viability

To assess if the sediment extracts in this experiment affected viability of the cells, conversion of resazurin to fluorescence compound resurin was used to monitor metabolic activity as an endpoint. The assay was performed using a minor modified version described by Blanco et al.[8] and cells were seeded as explained in 2.3.

Metabolic activity was monitored using 484μM resazurin fluorescence dye. Resazurin stock solution was prepared in a 50 mL centrifuge tube by dissolving 4.86 mg of powder in 40 mL PBS, filtered (0.25 μm) and covered with foil to prevent exposure to light. This stock solution can be stored in a fridge (4°C) for up to 3 months or until change in colour is observed.

Under dark working conditions, 20 μL of resazurin solution was added to wells with cells, the wells with only culture medium to correct for background fluorescence and wells with cells exposed to 100μM H_2O_2 as a positive control. The dye was mixed thoroughly using pipette to allow it to reach all cells, and the plates were covered with aluminum foil to protect against light. They were, thereafter, incubated for 4 h at the 30°C/18°C, respectively in accordance to culture conditions. Using the microplate reader (SpectraMax Paradigm Multi-Mode) fluorescence was read at 570/585 nm emission/excitation. Relative fluorescence unit (RFU) was converted to percentage cell viability relative to the control cells with no exposure medium.

2.4. Reactive oxygen species

Generation of reactive oxygen species (ROS) measured in fish cells exposed to sediment extracts was achieved by measuring the fluorescence of oxidized DCF as an end point using a slightly modified version described by LeBel et al. [36]. Cells were seeded as explained in 2.3 and exposed as explained bellow.

A stock solution of 10 M 2′7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) probe was freshly prepared by dissolving 5 mg of powder in an eppendorf tube with 940 µL DMSO. From the stock solution, a working solution was made by diluting 22 µL of stock in a 15 mL centrifuge tube with 11 mL PBS for the final concentration of 20µM.

For the exposure experiment culture medium was removed, cells were rinsed with 200 µL PBS and 100 µL of 20 µM H2DCF-DA probe was added. With the probe, cells were incubated for 30 mins at 30°C (PLHC-1) and 18°C (RTgill-W1) followed by two additional washings with PBS and exposure media diluted in PBS to final concentrations. The cells with exposure medium was incubated a further 60 mins and fluorescence emitted due to oxidation of H2DCF-DA was read in the microplate reader at 485/528 nm excitation/emission. The results were expressed as percentage change in fluorescence relative to the control cells.

2.5. 7-Ethoxyresorufin-O-deethylase

The induction of EROD activity in the presence of the sediment extracts was measured as the fluorescence molecule resorufin derived from deethylation of 7-Ethoxyresorufine as the end point. The assay was performed as indicated in Pérez-Albaladejo et al.[6], with slight modifications. The cells were seeded as explained in 2.3.

Two μM 7-ethoxyresorufin probe was made by diluting one tube 40 μL of 2000 μM 7-ethoxyresorufin and in 40 ml 50 mM Na-phosphate buffer (pH 8.0).

After 24 h incubation, cells were able to attach fully forming a monolayer. Culture medium was aspirated, followed by washing with 200 μL PBS. The cells were then exposed to the 200 μL different concentrations of sediment extracts and 1 μM b-naphthoflavone (bNF) as positive control and incubated for a further 24 h. Immediately after incubation, the exposure medium was removed and 200 μL of 2 μM 7-ethoxyresorufin in 50 mM Na-phosphate buffer pH 8.0 was added to each well containing cells and one row of just probe to correct for background fluorescence. After incubation at 30°C/18°C for 15 mins fluorescence was read at 537/583 nm emission/excitation.

Quantification of the activity was performed by calibration of 7-hydroxyresorufin were fluorescence signal from the assay was used to calculate conversion of pmol resorufin formed per minute.

For the total protein determination, cell lysing was performed by removing all fluids from the plates and freezing down for 48 h with one thawing in between. After re-thawing 50 μL of 50 mM Na-phosphate buffer was aliquoted into each well and followed by scraping with a pipette tip to mix the supernatant. Twenty-five μL of lysed cells were used to determine total cellular proteins using the BCA kit with bovine serum albumin as a standard. Final results were expressed as pmol of resorufin formed per minute and per milligram of protein (pmol/min/mg protein).

A summary of all bioassays is given in Figure 2.5.1.

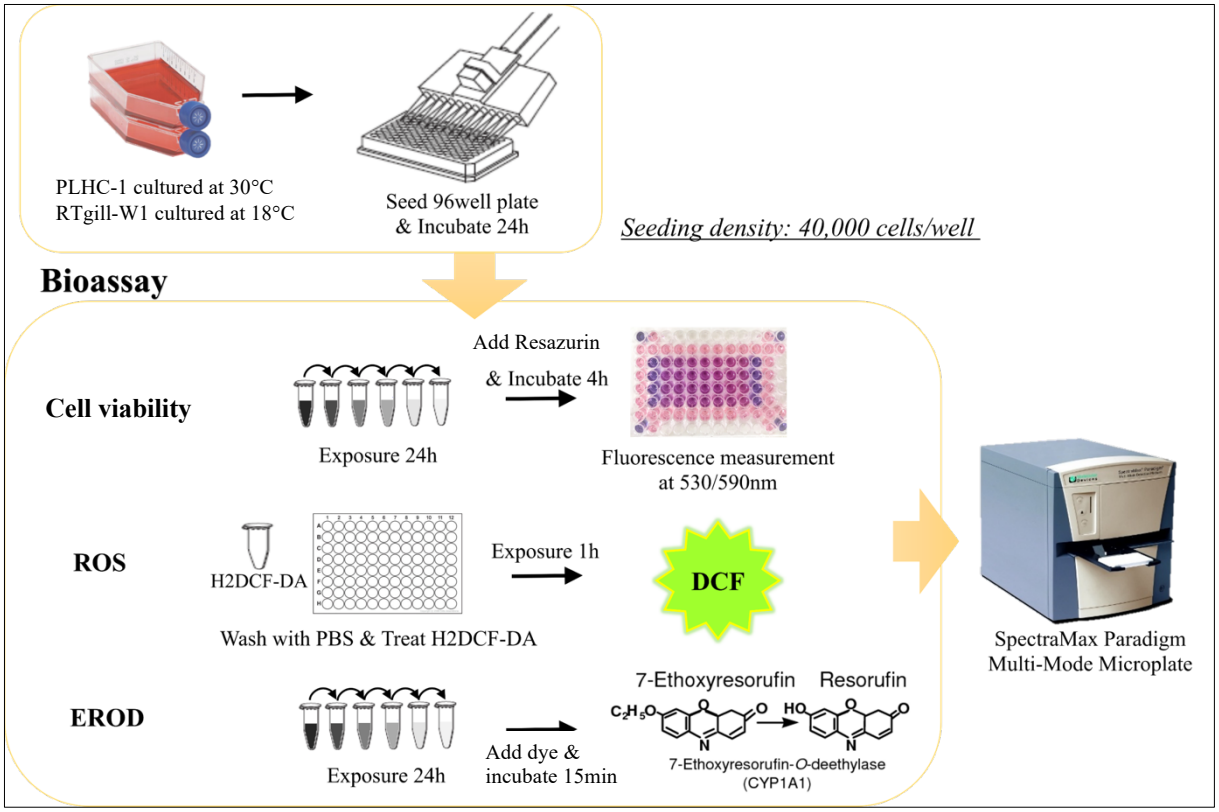

Figure 2.5.1 reports a schematic summary of the cell assays.

2.6. Sample preparation

The predominant method on processing environment sediment samples for exposure studies on cell line is mechanical extraction of the sediments organic soluble fraction using a range of solvent [6], [8], [9], [37]. Previous research using Soxtec to extract lipophilic compounds from solid matters has proven significant recovery with less time and solvent consumption then other methods such as Soxhlet, microwave assisted extraction (MAE) etc. [23] [24]. Therefore, in this thesis the two methods Soxtec and mechanical extraction has been chosen.

2.6.1. Soxtec

The Soxtec method was used to extract the organic soluble fraction of drill cutting and treated drill cutting sediments.

Six gr of freeze-dried sediment (grain size 1-2 mm) were transferred into thimbles (Figure 2.6.1) and plugged with cotton pads to prevent fall out. Two parallels of each sample were prepared. In pre-cleaned cups, 40 mL of petroleum ether solvent was added, and the cups were positioned in the apparatus (Figure 2.6.1). The thimbles containing samples went through an extraction process by boiling the solvent at 120°C for 1 h, followed by 1 h of rinsing where residual solvent and extracts dripped into the cups. Extracts were transferred into a glass bottle, left to evaporate to complete dryness in a hood, and reconstituted into 500 µL of DMSO. After reconstitution, the stock concentration was equivalent to 12 g dry weight sediment extract (eQsed)/mL. For the assays, extracts were serial diluted in proper medium to desired concentrations.

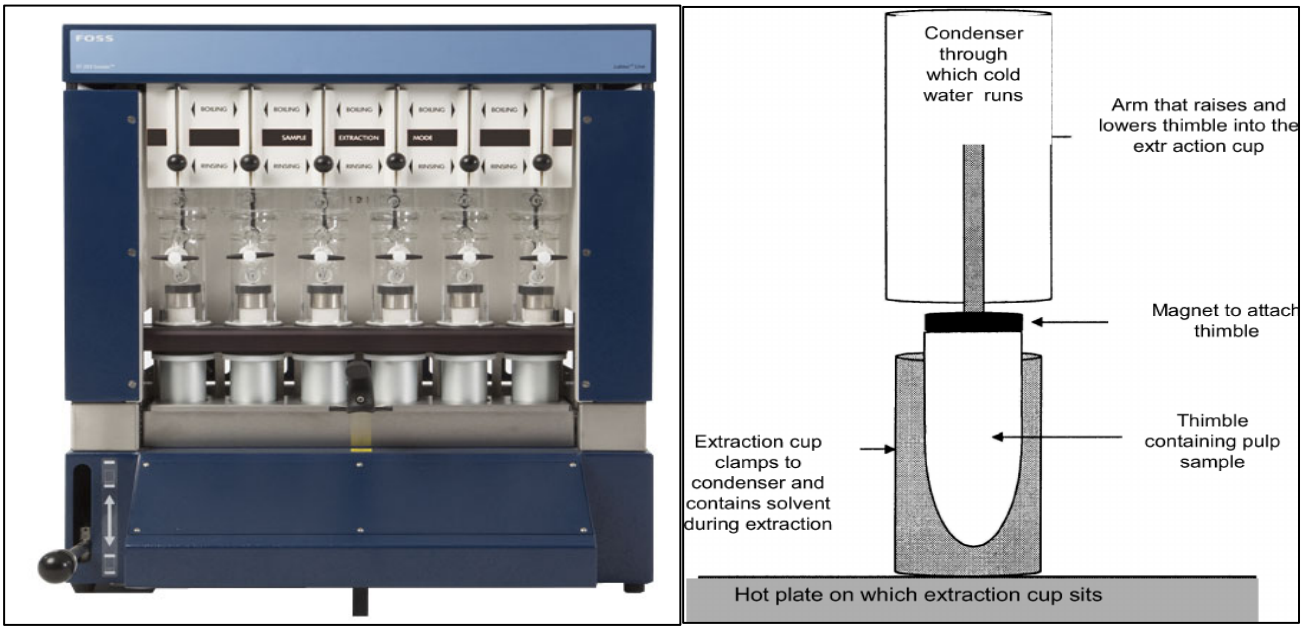

Figure 2.6.1 Soxtec™ apparatus (to the left)[40] and a schematic of a single extraction unit (to the right)[41].

2.6.2. Mechanical extraction

On the natural fjord sediment mechanical extraction method was performed.

To extract the sediments mechanically, 6 g of freeze-dried sediment were extracted twice with 20 mL dichloromethane/hexane (1:1, v/v), followed by an extraction with dichloromethane/acetone (1:1, v/v). For each extraction step, the sample was sonicated 10 min at room temperature and centrifuged 10 min at 2500 rpm. The extracts were combined in a glass bottle, evaporated to complete dryness under a hood, and reconstituted into 500 µL of DMSO. Similar to Soxtec extraction, the stock concentrations were equivalent to 12 g dry weight sediment extract (mg eQsed/mL) after reconstitution. For exposure assays extracts were serial diluted in proper medium to desired concentrations.

Table 2.8 Description of sediment samples.

Type	Name	Description	Extraction method	Final concentration
Drill cuttings	Untreated drill cuttings	From the North Sea	6 g of samples extracted using the Soxtec system.	Reconstituted in 500 µL DMSO, stock concentrations equivalent to 12 g dry weight extract (eQsed)/mL.
	Thermal threated drill cuttings	Cuttings treated for reduction of oil content		
Fjord	Boknafjord, Stavanger	Clean control sediment	6 g of samples extracted according to similar studies.	

Table 2.9 Concentrations of environmental pollutants in the sediments, expressed as mg/kg. EPA-PAHs = Environmental Protection Agency – polycyclic aromatic hydrocarbons.

mg/kg	Drill cutting	Treated drill cutting	Natural fjord sediment
Sum 16 EPA-PAHs	0.71	0.61	0.13
Hg	0.1	<0.05	0.02
Cd	0.59	<0.02	0.007
Cr	31	<0.02	12
Cu	66	<0.03	7.7
Fe	32000	0.44	9800
Mn	5000	100	160
Ni	34	0.1	10
Pb	19	<0.1	16
Zn	92	0.1	29

2.7. Statistical analysis

Comparisons between contaminated sediments and the natural sediment reference sample were made using one-way ANOVA followed by multiple independent group comparison (Dunnett and Tukey's test). All statistical analyses were performed with the software package SPSS 15.0 (SPSS Inc., Chicago, IL) and p-values lower than 0.05 were considered statistically significant.

3. Results and discussion

In this thesis, toxicity screening of sediments containing a mixture of chemicals was done by using bioassays with multiple endpoints in order to understand the modes of action these chemical mixtures take in an organism. Following results are based on two different cell types, PLHC-1 and RTgill-W1.

3.1. Cell viability

3.1.1. PLHC-1

Cell viability results for PLHC-1 cells are summarized in Figure 3.1.1 and 3.1.2. A significant decrease in viability was detected in PLHC-1 cells exposed to drill cutting extracts at 20 to 120 mg eQsed/mL compared to the control cells. Exposure to treated drill cutting extracts exhibited fluctuating viability with significant increase at the concentration 20 to 40 mg eQsed/mL and decrease in viability at 120 mg eQsed/mL relative to the control cells. For natural fjord sediment extract, no toxic effect in viability was recorded, however a significant increase was observed from 40 to 120 mg eQsed/mL (Figure 3.1.1).

A statistically significant difference between contaminated sediments extract and the natural fjord sediments was observed at concentrations 20, 40 and 120 mg eQsed/mL.

With viability being an indicator of the overall health of the cells, the significant differences between sediments (Figure 3.1.2**Error! Reference source not found.**) seen from 10 mg eQsed/ml up to 60 mg eQsed/ml indicate a toxic effect by exposure to drill cutting to the PLHC-1 cells. This suggests that even low concentration of both drill cutting and treated drill cutting sediments will have an undesired effect on and shouldn't be subjected to the aquatic environment.

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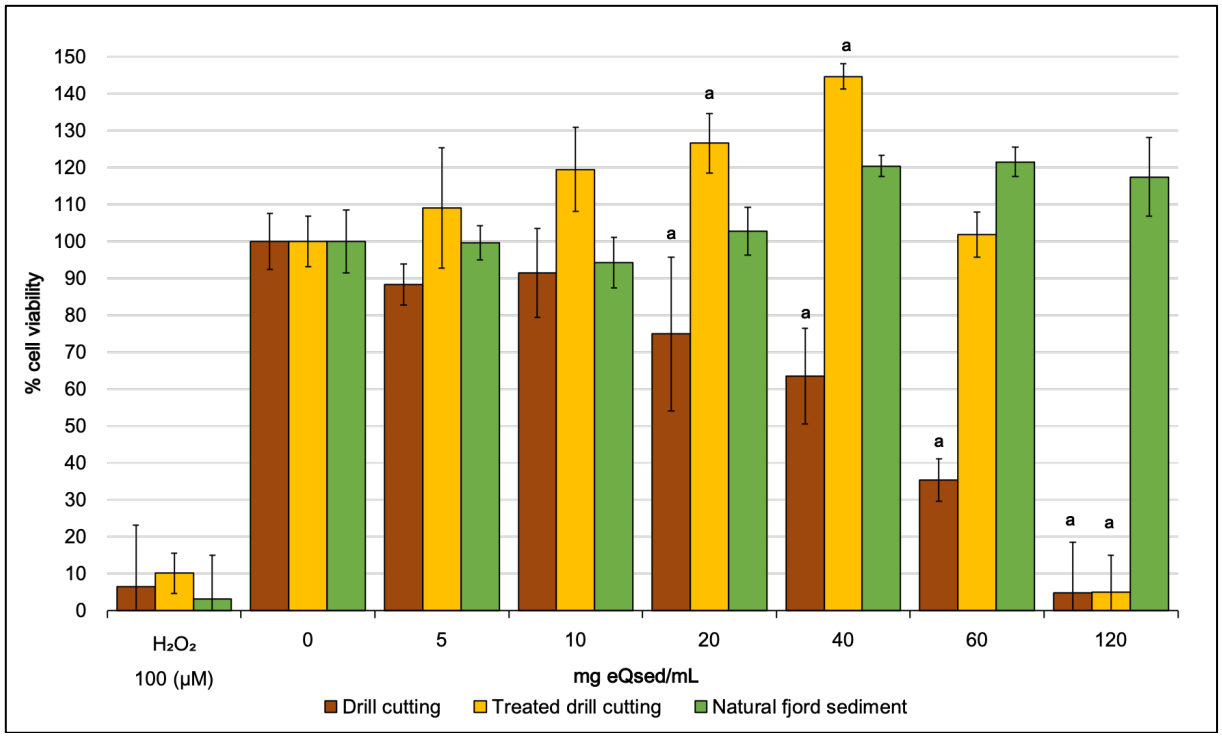

Figure 3.1.1 Cell viability in PLHC-1 cells after 24 h exposure to sediments extracts and positive control H_2O_2 (100 µM). Values are expressed as mean ± SD (n=3). [a]Statistically significant ($p < 0.05$) difference in cell viability relative to control cells (0 mg eQsed/mL).

5 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	n.s.	
Treated drill cutting	n.s.		
Drill cutting			

40 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	*	
Treated drill cutting	*		
Drill cutting			

20 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	*	
Treated drill cutting	*		
Drill cutting			

60 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	*	
Treated drill cutting	*		
Drill cutting			

10 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s	
Treated drill cutting	*		
Drill cutting			

120 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	*	
Treated drill cutting	n.s.		
Drill cutting			

Figure 3.1.2 Statistical comparisons of the different sediments cytotoxic effect on PLHC-1 cells at each concentration were done using the post hoc Tukey-test and results are reported as; * $p \leq 0.05$. and n.s. not significant.

3.1.2. RTgill-W1

Cell viability results for RTgill-W1 cells are summarized in Figure 3.1.3 and Figure 3.1.4.A significant increase in viability compared to the control cells was recorded for both drill cutting and treated drill cutting at 60 and 120, and 40 and 60 mg eQsed/mL respectively (Figure 3.1.3). A significant difference between both drill cuttings and natural fjord sediments was observed at the concentrations 60 and 120 mg eQsed/mL.

The results suggest that drill cutting samples where not toxic to the tested gill cells. On the other hand, it is showing an induced viability for the oil-contaminated sediment extracts, where treated drill cutting had a 4-fold increase at 60 mg/ml compared to the control samples. Similar regenerative effect of gill cells in *Oreochromis niloticus* fish has been recorded by Atta et al. in exposure to lead (Pb) at 0.025 mg/L [42]. Comparable results were also reported for cat fish by Olojo et al., where the authors suggested that low concentrations of Pb induces irregular proliferation [43]. The significant difference between the oil-contaminated sediment extracts and the natural fjord sediment extracts suggests that they may have adverse effect on the organisms, thus should be further tested.

Previous studies have also proven that RTgill-W1 cell line is a reliable model for determining cell viability with exposure to PAHs, where visible reduction in viability was seen. Arguments for this was that it avoids the metabolism of PAHs by the CYP1A gene expression due to the low amount in gill cells [34][44]. Roux also suggested to use different parameters, such as higher cell density and longer incubation time for gill cell line [45]. Further testing with additional parameters is therefore suggested for future studies.

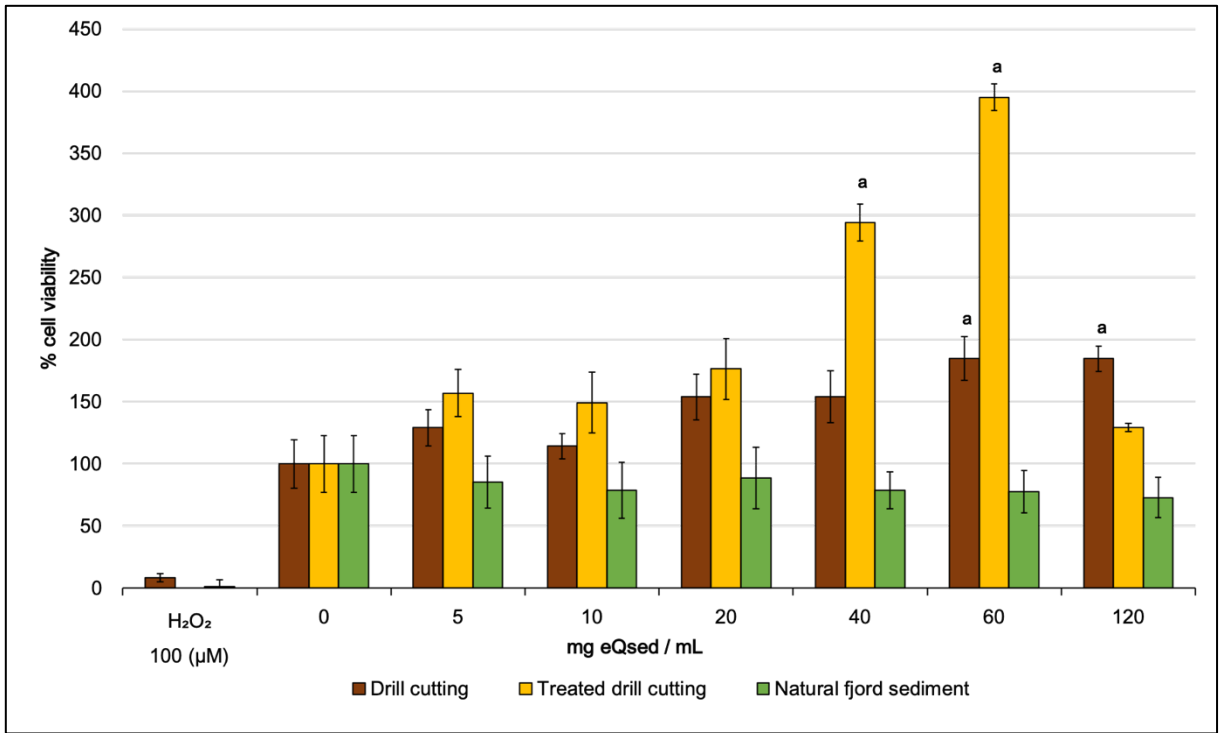

Figure 3.1.3. Cell viability in RTgill-W1 cells after 24 h of exposure to different sediments extracts and positive control H_2O_2 (100 µM). Values are expressed as mean ± SD (n = 3). [a]Statistically significant ($p < 0.05$) difference in cell viability relative to control cells (0 mg eQsed/mL).

5 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	*	
Treated drill cutting	n.s.		
Drill cutting			

40 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	*	
Treated drill cutting	*		
Drill cutting			

10 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	*	
Treated drill cutting	n.s.		
Drill cutting			

60 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	*	
Treated drill cutting	*		
Drill cutting			

20 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	*	
Treated drill cutting	n.s.		
Drill cutting			

120 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	*	
Treated drill cutting	*		
Drill cutting			

Figure 3.1.4 Statistical comparisons of the different sediments cytotoxic effect on RTG-W1 cells at each concentration were done using the post hoc Tukey-test and results are reported as; * $p \leq 0.05$. significant and n.s. not significant.

3.2. EROD activity

3.2.1. PLHC-1

EROD activity results for PLHC-1 cells are summarized in Figure 3.2.1. When comparing the two drill cutting sediment extracts, a significant increased activity was observed only at concentration 20 mg eQsed/mL. While a significant increase for natural fjord sediment extracts was measured in concentrations 20 to 60 mg eQsed/mL in comparison to both drill cutting samples. No significant induction of EROD activity was observed for treated drill cutting sediment extracts.

The results indicate low induction of monooxygenase of the liver cells when exposed to drill cutting extracts compared to the natural fjords sediment extracts. Chemical analysis of the sediments shows the presence of PAHs in the drill cutting and natural fjord sediments to be 0.71 and 0.13 mg/kg respectively. PAHs are known to upregulate the protective CYP1A gene that induces metabolism of planar hydro carbons [46]. These concentrations of PAHs could be expected to give higher EROD activity. However, such a relationship between the concentrations of PAHs and activity was not observed as the natural fjord sediments produced overall higher EROD activity.

It is also important to note the missing values from 40 to 60 mg eQsed/mL. This may be a result of losing cells from the washing step which affected the total protein concentration negatively.

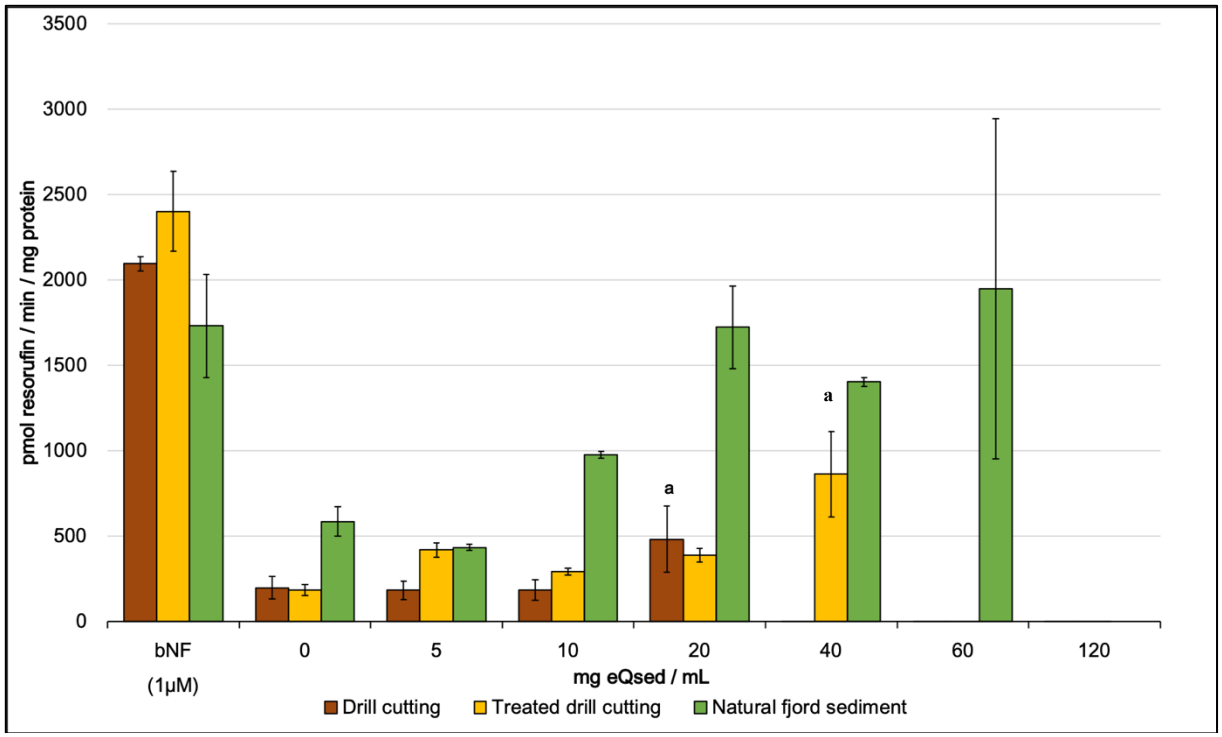

Figure 3.2.1 EROD activity in PLHC-1 cells exposed for 24 h to different sediments extracts and positive control β-Naphthoflavone (1 µM). Values are expressed in pmol/min/mg protein, as mean ± SD (n = 3). [a]Statistically significant ($p < 0.05$) difference relative to the control cells (0 mg eQsed/mL).

3.2.2. RTgill-W1

EROD activity results for RTgill-W1 cells are summarized in Figure 3.2.2. Generally, a no significantly different EROD activity was found in both drill cutting and treated drill cutting. With only a significant increase detected at 120 mg eQsed/mL. The natural fjord a significant increase was observed at 20 mg eQsed/mL.

Similar to the results for the PLHC-1(liver cells), no significant difference was observed for both drill cuttings extracts in all except at 120 mg eQsed/mL compare to the control cells. No relation between concentration of PAHs in the sediments (Table 2.9) and activity is seen. The PLHC-1 EROD activity were 3-fold than RTgill-W1. Reason for this can be explain by studies described by Schirmer. The study deem RTgill-W1 cell line as inadequate for detection of AhR agonist compounds due to its lack of CYP1A enzyme [44] in comparison to liver cells, thus explaining this low activity observations [46]. In exposure to natural fjord extracts on the other hand, similar to the liver cells, EROD activity levels were significantly high at 120 mg eQsed/mL.

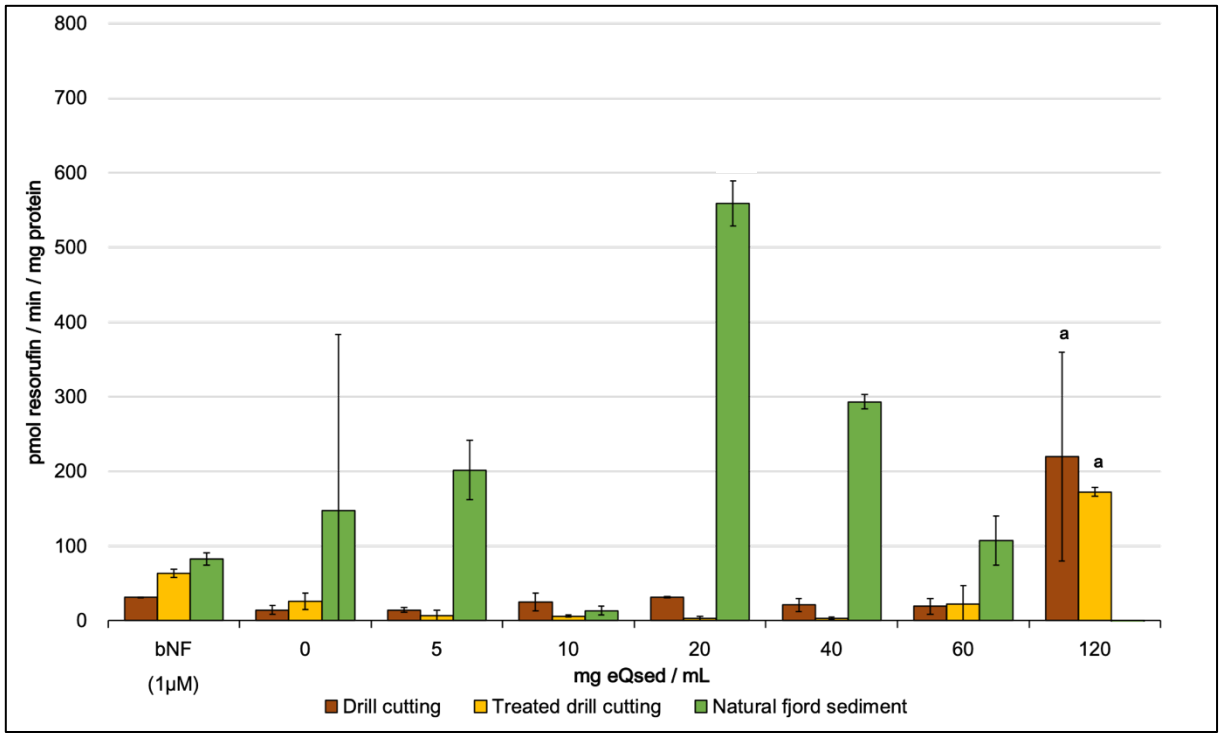

Figure 3.2.2 EROD activity in RTgill-W1 cells exposed for 24 h to different concentrations of sediments extracts and positive control β-Naphthoflavone (1 µM). Values are expressed in pmol/min/mg protein, as mean ± SD (n = 3). [a]Statistically significant ($p < 0.05$) difference relative to control cells (0 mg eQsed/mL).

3.3. Oxidative stress

3.3.1. PLCH-1

ROS production results for PLHC-1 cells are summarized in Figure 3.3.1 and Figure 3.3.2. In general, both drill cutting samples showed a significant concentration-depended increase in oxidative species at 40 to 120 mg eQsed/mL. While treated drill cuttings expressed a significant increased at 10, 20 and 120 and natural fjord sediments only at 120 mg eQsed/mL. A significant difference between both drill cutting and natural fjord sediment extracts was recorded at most concentrations (10 to 60 mg eQsed/mL).

ROS production is known to increase when exposed to anthropogenic compounds that undergo redox cycle, such as PAHs [47]. In PLHC-1 the significant increase suggest that such compounds are present in drill cutting sediment extracts at high concentrations. The significant difference between sediments indicates release of drill cuttings into the environment may lead to have a potential negative effect, possibly to biomolecules such as DNA [48].

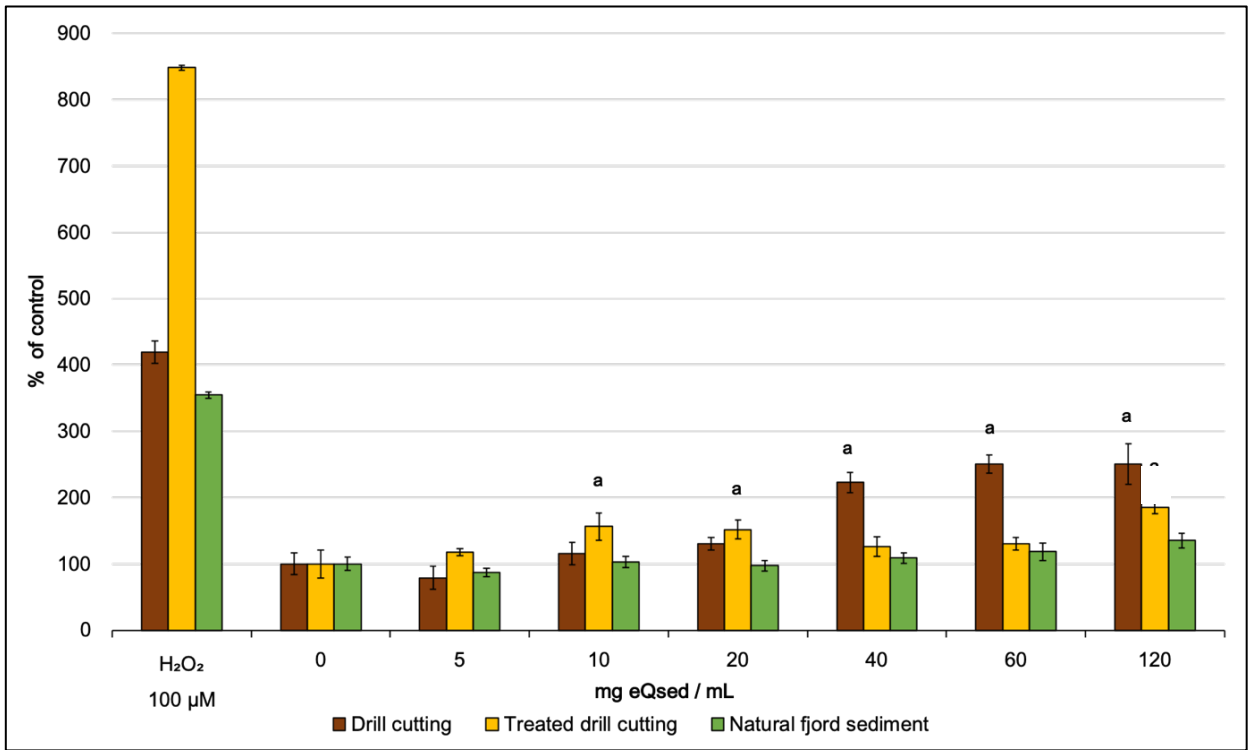

Figure 3.3.1 ROS production in PLHC-1 cells after 60 min of exposure to different sediment extracts and positive control H_2O_2 (100 µM). Values are expressed as mean ± SD. [a]Statistically significant ($p < 0.05$) increase in ROS production relative to control cells (0 mg eQsed/mL).

5 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	n.s.	
Treated drill cutting	n.s.		
Drill cutting			

40 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s.	
Treated drill cutting	n.s.		
Drill cutting			

10 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s.	
Treated drill cutting	*		
Drill cutting			

60 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s.	
Treated drill cutting	*		
Drill cutting			

20 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s.	
Treated drill cutting	*		
Drill cutting			

120 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	n.s.	
Treated drill cutting	n.s.		
Drill cutting			

Figure 3.3.2 Statistical comparisons of the different sediments production of ROS on PLHC-1 cells at each concentration were done using the post hoc Tukey-test and results are reported as; * $p \leq 0.05$. and n.s. not significant.

3.3.2. RTgill-W1

ROS production results for RTgill-W1 cells are summarized in Figure 3.3.3 and Figure 3.3.4. Results showed significant induction of ROS for both drill cutting samples at concentrations from 40 to 120 mg eQsed/mL. The drill cutting was more efficient in oxidizing H_2DCF then the treated drill cutting, as the production of ROS significantly increased approximately 3.8-fold 8.1-fold and 7.1-fold compared to the control cells for each concentration respectively. A significant induction was also detected in natural fjord sediments at 120 mg eQsed/mL compared to the control cells (Figure 3.3.3). Data in Figure 3.3.4 showed significant difference between both drill cutting samples and natural fjord sediments at exposure concentrations 10 to 60 mg eQsed/mL.

ROS production is known to increase when exposed to anthropogenic compounds that undergo redox cycle such as PAHs [47]. The dose-dependent significant increase suggests that such compounds are promoting the production of ROS. The significant difference between sediments indicates release of drill cuttings into the environment may have a potential negative effect, possibly to biomolecules such as DNA [48].Consequently it is likely that this increasing in ROS production may lead to oxidative stress.

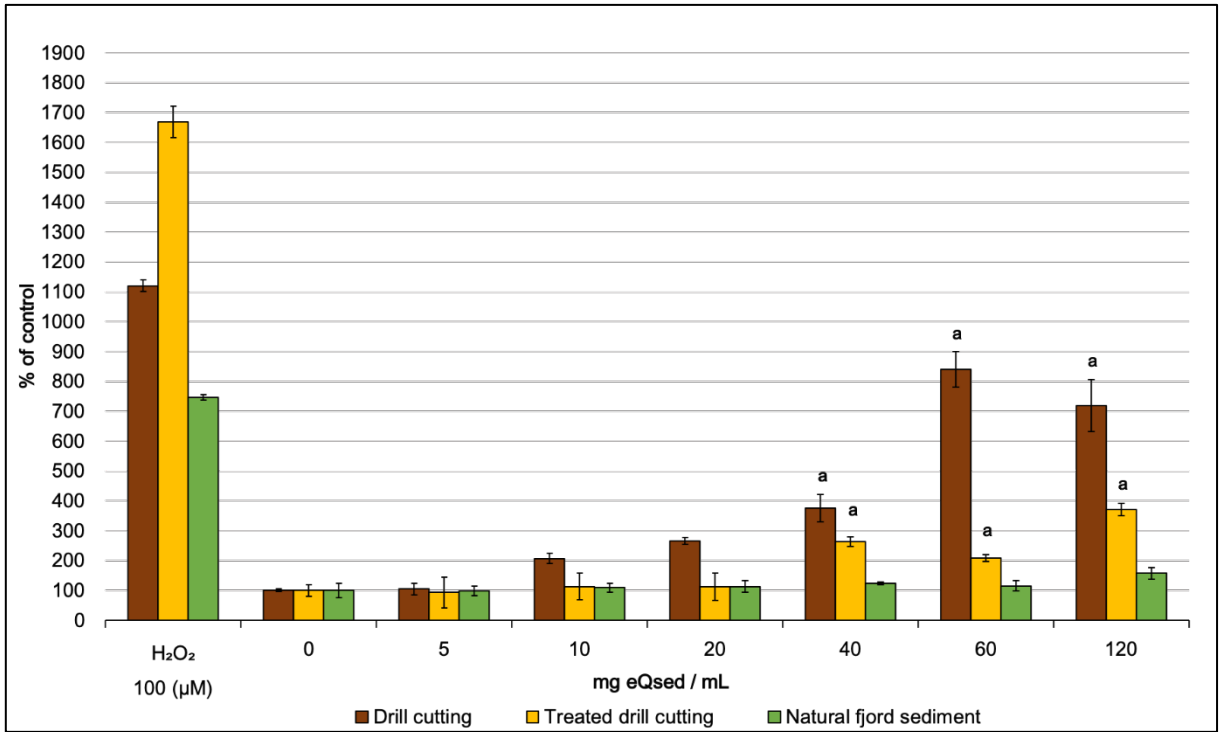

Figure 3.3.3. ROS production in RTgill-W1 Cells after 60 min of exposure to different sediments extracts and positive control H_2O_2 (100 µM). Values are expressed as mean ± SD. [a]Statistically significant ($p < 0.05$) increase relative to control cells (0 mg eQsed/mL).

5 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	n.s.	
Treated drill cutting	n.s.		
Drill cutting			

40 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s.	
Treated drill cutting	n.s.		
Drill cutting			

10 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s.	
Treated drill cutting	*		
Drill cutting			

60 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s.	
Treated drill cutting	*		
Drill cutting			

20 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	*	n.s.	
Treated drill cutting	*		
Drill cutting			

120 mg eQsed/mL	Drill cutting	Treated drill cutting	Natural fjord sediment
Natural fjord sediment	n.s.	n.s.	
Treated drill cutting	n.s.		
Drill cutting			

Figure 3.3.4 Statistical comparisons of the different sediments production of ROS in RTG-W1 cells. Comparisons between each concentration were done using the post hoc Tukey-test and results are reported as; * $p \leq 0.05$. and n.s. not significant.

4. Conclusion

Overall results show good potential of the *in vitro* bioassays in detecting cytotoxicity, EROD activity and ROS species in drill cuttings. Of the cell lines, PLHC-1 cells stood out as more sensitive and efficient in detecting cytotoxicity and EROD activity than the gill cells. However, RTgill-W1 cells exhibited highest ROS production. Both cell lines could be used for further testing of parameters, as suggested in the previous section.

Cell viability assay results for PLHC-1 proved to be an efficient model when exposed to drill cutting. A dose-depended sub-lethal effect was observed in comparison to the cells exposed natural fjord sediments. The cell viability assay for RTgill-W1 cells showed a regenerative effect, and no toxic effect from drill cutting.

EROD activity assay in PLHC-1 upregulated EROD activity, pointing to the presence of planar aromatic hydrocarbon. RTgill-W1 cells exposed to drill cutting sediment extracts at highest concentrations exhibited a significant increase in EROD activity, in comparison to natural fjord sediments. When comparing RTgill-W1 to PLHC-1, over all EROD activity was lower for the gill cells in exposure to drill cuttings. Interestingly, natural fjord sediments had the highest EROD induction, suggesting the presence of other inducing pollutant in the natural fjord which should be further evaluated. For this bioassay, RTgill-W1 may not be an optimal model cell line based on the obtained results.

The ROS production for both cell lines showed significantly increase when exposed to drill cutting and treated drill cutting samples compared to the natural fjord sediment. In conclusion, the ROS bioassay as a tool for detecting PAHs showed positive potential.

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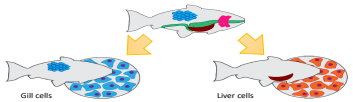
Appendix



Evaluation of oil contaminated sediment toxicity using *in vitro* bioassay based on fish cell lines

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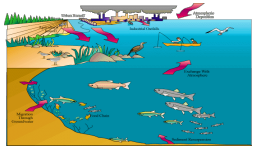
Background

In the aquatic environment there are many pollutants that are having an undesired effect on the aquatic organisms, such as PCBs, PAHs, metals etc. Of these many, petroleum products are a great concern and often the major contributor to contamination containing petrogenic polyaromatic hydrocarbons (PAHs) that are known for their toxic effect and carcinogenic potential [1].

In offshore operations, the most significant volume of waste produced derives from drilling mud and drill cuttings. Drill cuttings are particles of the sedimentary rock that are excavated by the drill bit in order to locate oil. These rocks are usually coated/saturated with oil and production chemicals from the drilling mud, making them an environmental concern. Present procedure, after separation of drill cuttings and drilling mud, is to clean the cuttings and transport to land for further treatment. However, up until mid-1990 discharge of drill cuttings into the seabed was permitted in most countries [2].



To evaluate the impact of contaminated sediment, including drill cuttings, laboratory experiments are usually performed. These studies of toxic effect are often done by *in vivo* exposure, assessing the dose-response relationship and their biotransformation / metabolisation [3].
Nonetheless, as a result of limitations both ethically and economically, the use alternative methods, such as cell culture *in vitro*, is rising with a focus on limiting the number of animals used.

To explore the potential toxic effects of oil-contaminated sediments, such as drill cuttings and sediments collected from a different location in Norway, bioassays based on fish liver cells (PLHC-1) and gill cells (RTgill-W1) were used.
This *in vitro* approach obliges with the 3Rs' principles (Replace, Reduce and Refine) of animal welfare in research.

Materials and methods

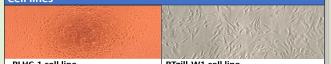
Description of sediment samples

Type	Name	Description	Extraction	Final concentration
Drill cuttings	Untreated drill cuttings	From the North Sea	6 g of samples extracted using the Soxtec system [5].	Reconstituted in 500 µL DMSO, stock concentrations equivalent to 12 g dry weight extract (eQsed)/mL
	Thermal threated drill cuttings	Cuttings treated for reduction of oil content		
Fjord	Boknafjord, Stavanger	Clean control sediment	6 g of samples extracted according to similar studies [4].	
	Vågen station 2, Fisketorget harbor Bergen	Heavily polluted sediments		

Fish cell culture:

Fish cells PLHC-1(ATCC® CRL-2406™) and RTgill-W1 (ATCC® CRL-2523™) were maintained and passaged according to methods recommended by ATCC®. For each bioassay, cell densities of 4 x 10^4 cells/mL per well were seeded in clear plate 96-well microplates. After 24h in culture, the cells were exposed to the sample extracts, at concentrations 5, 10, 20, 40, 60 and 120 mg eQsed/mL and left a further 24h with exposure medium in the cell viability and EROD assay, while in the ROS assay the cells were exposed for 60 min.

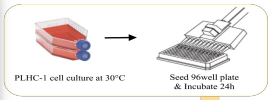
Cell lines



PLHC-1 cell line
Topminnow (*Poeciliopsis lucida*)
- MEM complete medium (5%FBS, 5%L-glutamin, Pen/strep)
- Incubated at w/CO_2 in 30ºC

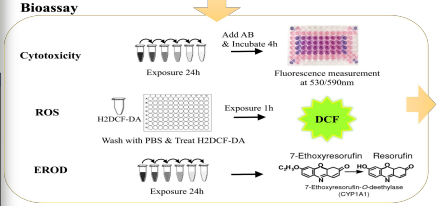
RTgill-W1 cell line
Rainbow trout (*Oncorhynchus mykiss*)
- L-15 complete medium (10%FBS, 5%L-glutamin, pen/strep)
- Incubated at wo/CO_2 in 18ºC

Bioassays



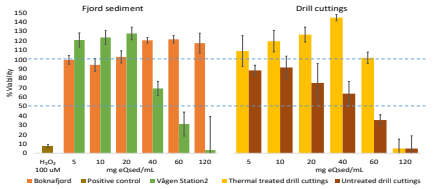
Seeding density: 40,000 cells/well

Bioassay



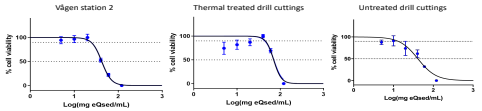
SpectraMax Paradigm Multi-Mode Microplate

Cytotoxicity



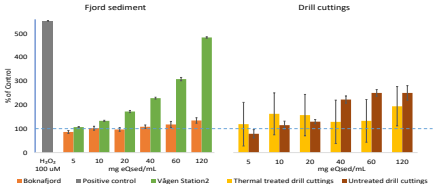
Cytotoxicity in PLHC-1 cells after 60min of exposure to different sediment extracts

Cytotoxicity dose-response



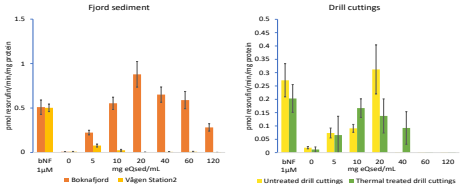
Dose-response of PLHC-1 cells of Untreated & Thermal treated drill cuttings and Vågen Fisketorget station 2

Reactive Oxygen Species (ROS)



ROS production in PLHC-1 cells after 60min of exposure to different sediment extracts

EROD



EROD activity in PLHC-1 cells after 24hr of exposure to different sediment extracts

Results & Conclusions

- Except for the sample collected at a clean location, Boknafjord, all other sediments showed cytotoxicity and had an increased ROS production
- The detected EROD activity confirmed that samples were contaminated with PAHs compounds. Ten time higher activity was detected in sediment from a contaminated fjord (Vågen), in comparison to drill cuttings.
- The *in vitro* bioassays using fish cell lines were capable of showing the expected contamination in sediment samples, making it a viable approach for early warning signal of pollution and a suitable tools for screening sediment contaminated samples.

Acknowledgements

This research was funded by the Research Council of Norway (dCOD project, grant #248840/O70) and the University of Stavanger (Master Program in Environmental Engineering).
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