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Aquatic ecotoxicological assessment of sediments collected in Bergen using *in vitro* bioassays based on fish cell lines

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Abstract

Bergen is located along the coast in western Norway and is the second biggest city in the country, acting as a gateway to oceans and fjords in the region featuring extensive fish populations. As such, environmental evaluation has been a major focus of aquatic ecosystem research, with various sources of pollutants caused by human activity, including petroleum, shipping, tourism and domestic sewage. This thesis, using *in vitro* bioassays with fish cell lines, was carried out to conduct an environmental assessment of sediments collected along the coastline and around Bergen. By using fish cell lines (i.e., PLHC-1 and RTgill-W1), the toxicity and quality of sediments were assessed by *in vitro* bioassays, including general cytotoxicity, reactive oxygen species production assay (ROS) and metabolism of cytochrome P-4501A enzyme (EROD activity). Sediment samples were collected from seven sites in total and prepared with mechanical extraction at the University of Bergen.

The obtained results were compared to controls at different doses of each sediment sample extract. The cell viability, ROS production, and EROD activity showed an adverse outcome data with both cell lines. The results with PLHC-1 cells show a more sensitive response in cytotoxicity tests than RTgill-W1 cells, and are capable of distinguishing the most toxic sediment samples in comparison to the other sites. ROS assay results showed higher sensitivity with RTgill-W1 cells, and it revealed more samples possessing toxicity. The overall data, after the principal component analysis (PCA) with the concentration of marked chemicals from the *in vitro* bioassays selected for this study, was capable of assessing the environmental quality of sediments and identifying a higher anthropogenic impact.

Acknowledgement

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Stavanger, 15.12.2019

Ji-ae Park

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List of Acronyms

AhR	Aryl hydrocarbon receptor
B(a)P	Benzo[a]pyrene
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
CYP1A	Cytochrome P1A
DCF	2',7'- dichlorofluorescein
EC_{50}	Half maximal effective concentration
EROD	7-ethoxy-resorufin-O-deethylase
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
L-15	Leibovitz's L-15 Medium
MEM	Minimum Essential Medium
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic aromatic hydrocarbons
PCA	Principal Component Analysis
PHHs	Planar halogenated hydrocarbons
PLHC-1	Poeciliopsis lucida hepatocellular carcinoma
ROS	Reactive oxygen species
RTgill-W1	Rainbow trout gill-Waterloo1
SOP	Standard operation procedure
UiB	Universitiy of Bergen, Norway
UiS	University of Stavanger, Norway
UPV/EHU	University of the Basque Country, Bilbao, Spain
USEPA	United States Environmental Protection Agency

1. Introduction

1.1. Aim of the Study

This thesis work aimed to monitor and evaluate the toxicity of sediment samples collected in Bergen, using cell line-based assays.

The research work was focused on the use of *in vitro* tests, which are the best foot forward to be the animal-alternative methods supporting the 3Rs' principle (Replace, Reduce and Refine), and forecasting the toxicity of sediments and the way of how it could affect fish in the marine environment. By using fish cell lines (i.e., PLHC-1 and RTgill-W1), the quality of these sediment from different sites was assessed through assays for general cytotoxicity, reactive oxygen species production (ROS) and EROD activity (an enzyme related to polycyclic aromatic hydrocarbon(PAH).

The methodology of *in vitro* bioassays applied in this study is related to the cell-based bioassays by Cinta Porte *et al.* [1] and aim to assess the environmental quality of sediments by evaluating different biomarker endpoints.

1.2. Importance of Environmental Monitoring

The borderless environmental issues of soil, water, air, and biota resulted in the implication of pollution on a planetary level by the toxicants. Some of the significant contaminants have come to be remained and detected in various ecological habitats far from industrial activity, demonstrating diffusion and bioaccumulation after only a relatively brief period of widespread use [2].

By environmental awareness rising as an essential issue socially and internationally, environmental monitoring is carried out for testing and investigations about hazardous substances. Moreover, as most toxicants confirmed presently are magnified through the food chain in the ecosystem, environmental monitoring is designed to able to understand the effects on the natural ecosystem and to protect it from any adverse outcomes of human activity [3].

1.3. Source of contaminants to Marine sediment

1.3.1. Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are ubiquitous environmental contaminants of concern based on their potential effects on human and ecological health. With non-polar planar molecules, PAHs compose two or more aromatic rings fused by alternating double and single bonds sharing of carbon pair atoms. Above all, sixteen structure of the priority PAHs is reported as toxic pollutants by U.S. Environmental Protection (USEPA) as in Figure 1.3.1. They are highly hydrophobic and tend to adsorb onto the surface of soil or sediments in a marine environment [4].

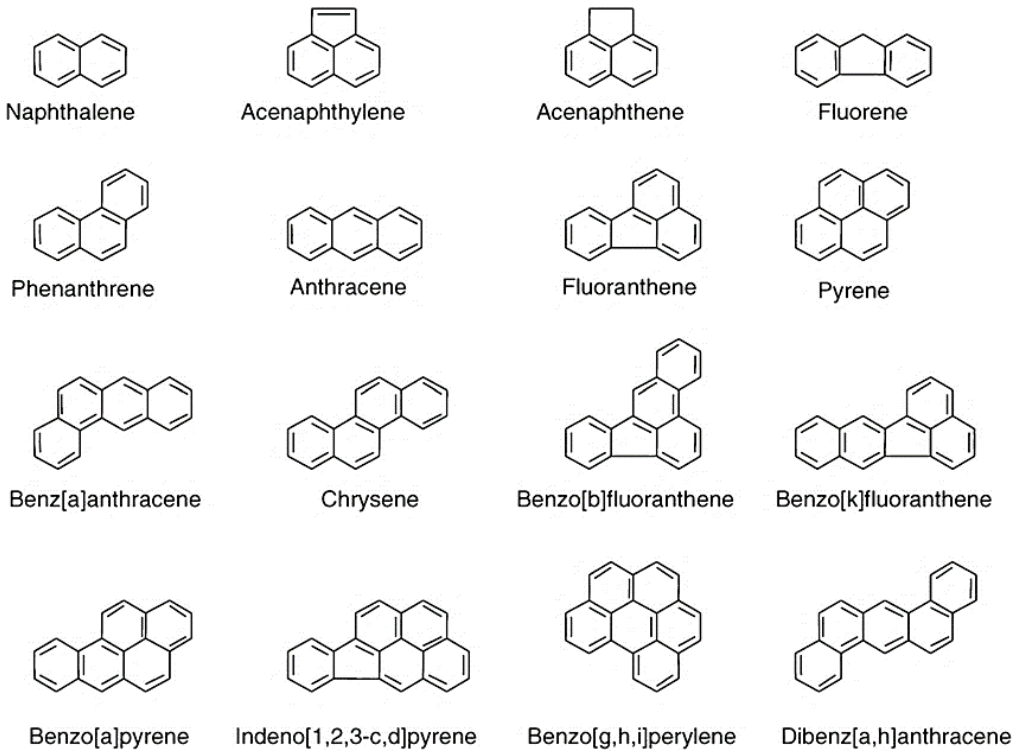


Figure 1.3.1 Chemical structure of the USEPA listed 16 PAHs (Source: USEPA)

PAHs can be abundant as fossil molecules naturally by the fact that their source is from ancient sedimentary rocks, coal, and petroleum. Therefore, PAHs can be found in the sediment with higher concentration compared to the biota and water column. However, when the residues in marine sediments, such as estuaries, shows a significant level of PAHs, the oxidation products are formed. Then PAHs can be more easily excreted to the marine environment due to the oxidation process of making the compounds more water-soluble. There has been plenty of great research effort in order to unravel the harm caused by PAHs on biota.

Therefore, PAHs can be persisted and buried in the sediment for a long time, and may also enter the food chain via biomagnification from benthic biota to higher organisms [5-8].

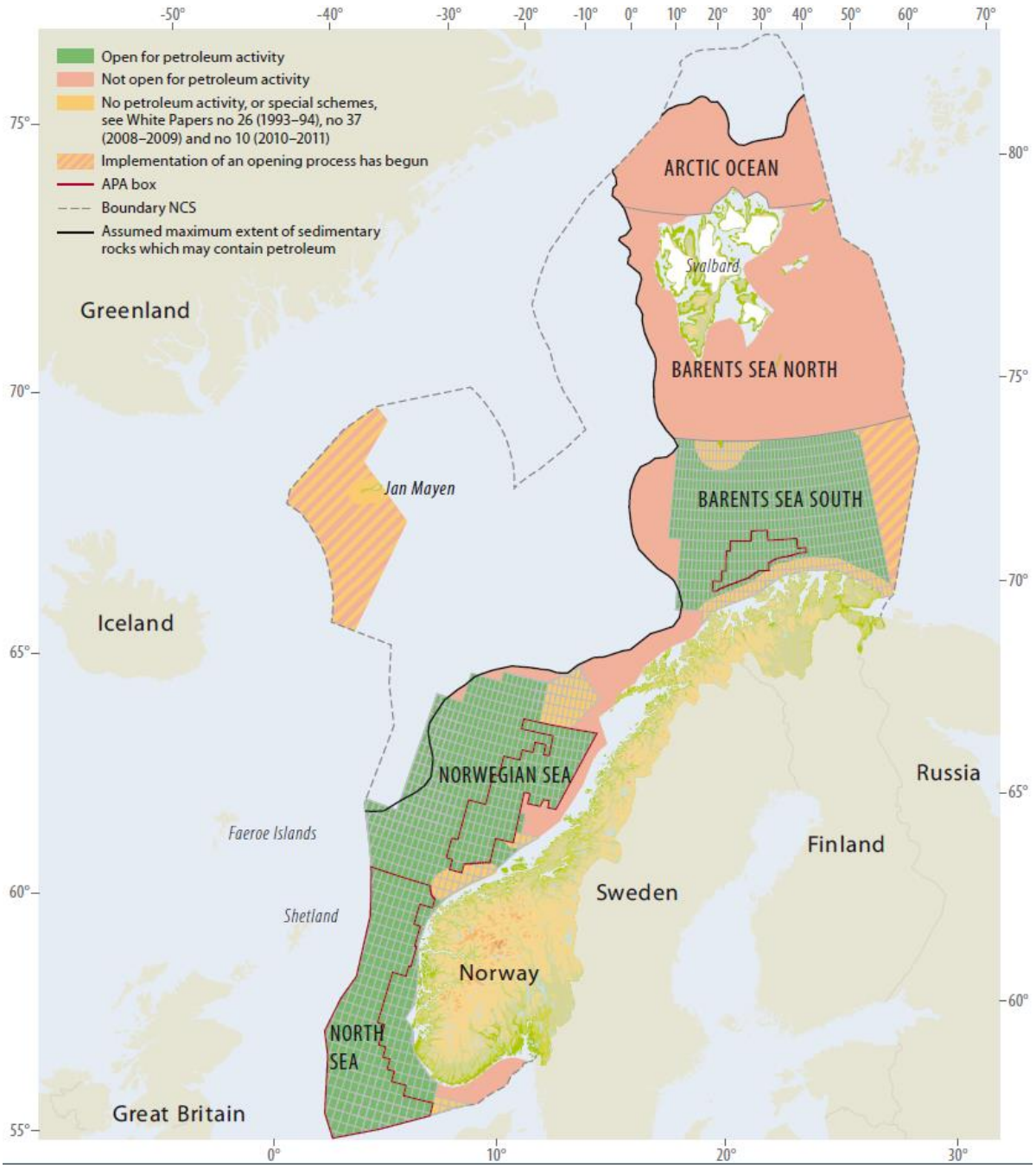


Figure 1.3.2 Area status on the Norwegian continental shelf per March 2012 (Source: Norwegian Petroleum Directorate)

Norwegian marine areas, including parts of the western Barents Sea, the Norwegian Sea, the eastern North Sea, and the northern part of the Skagerrak, where nearly half of the seabed in this area consists of sedimentary source rocks, do contain petroleum resources. As

described in Figure 1.3.2, this extensive open sea area may possess contamination of various degrees by petroleum activity activities. PAHs and many other organic contaminants could be released into the marine environment from different sources as part of the natural sink into the sediment [9].

Besides, a high concentration of PAHs, caused by human activity and effluents, tends to be found in urban areas, which has a long habitation history according to urban soil studies. Through the previous studies, the Bergen city, which is the largest city in western Norway, the chemical history and various organic compounds have been actively studied in the central port and around the city, and PAHs contamination has been confirmed by the historical flow and the demonstration of the causes. [10, 11].

1.3.2. Metallic Ions

Metallic ions, particularly heavy metals, are a significant concern in the environment. Because the levels of heavy metals, in particular in the marine environment, have been increased and causing the anthropogenic impacts. Sources of heavy metals are predominantly from industries, agricultural activities, scrap metal recycling, commercial ports and sewage (Figure 1.3.3). Then, because of the physio chemical characteristics of heavy metals, they tend to accumulation and in marine organisms [12].

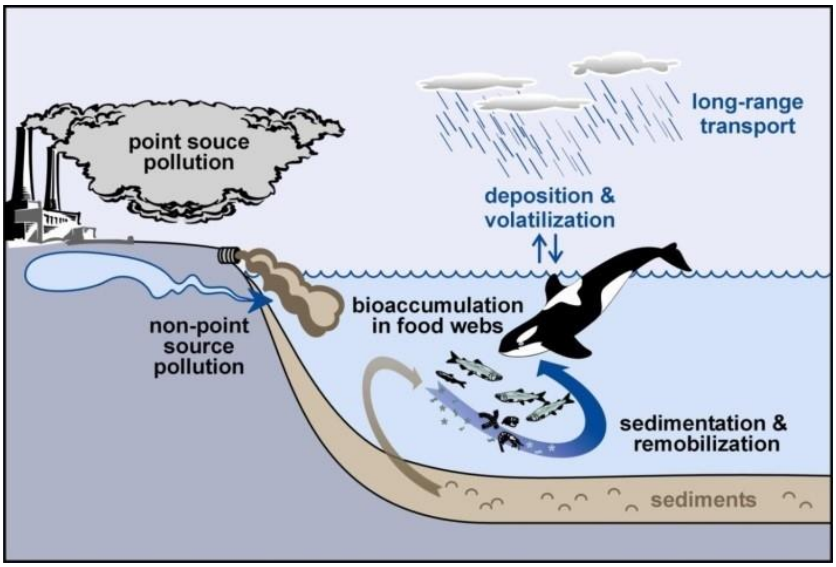


Figure 1.3.3 Bioaccumulation of contaminants in the food web (Source: Peter S. Ross)[13]

Metallic ions, such as Arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), and zinc (Zn), are primary heavy metals because of their toxicity, non-

biodegradability, and bioaccumulation. The toxicity of heavy metals may affect adversely to marine organisms as detected in fish. Eventually, the contamination by heavy metals can impact human health due to their behaviours of persistence and biomagnification along the food chain [14].

Consequently, ecosystems such as harbours or industrialized coastal regions usually have the chronic accumulation of metals and contaminated sediments. This phenomenon is predicted to observe the toxic effects and potential bioaccumulation of metals into the organisms exposed to sediment. Therefore, the area where it is highly expected to be contaminated by heavy metals, is essential to be studied for the impact of metal contamination on ecosystems [12].

Bergen is the second biggest city of Norway, which is a centre of aquaculture, shipping, the offshore petroleum industry and subsea technology. The port of Bergen is Norway's busiest harbour in terms of both shipping and passengers and it is recorded that nearly a half-million passengers and tourists are visiting Bergen with over 300 cruise ship calls a year. Furthermore, it has been shown that all the cruise ships consume nearly 7,000 metric tons of fuel while docked in the port of Bergen. It is an equal amount of emissions to 13,000 diesel cars when the largest cruise ship burns up to 30 tons of fuel per day at berth. Furthermore, there have been research about recent sediment in the Bergen port, which was subjected to injection of untreated sewage from around 15,000 person equivalent [15-18].

Therefore, it is important to monitor the levels of pollutants, including PAHs and heavy metals, and establish the background levels of the area, and detect "critical spots".

2. Background

Sediments represent reservoirs that contain artificial pollutants, which can adversely affect inhabited bento and other aquatic organisms. Therefore, there are many approaches to monitor sediment quality, which is essential to characterize the health of the aquatic environment, ultimately to minimize threats, and to prevent adverse effects on aquatic wildlife [19]. This chapter introduces the principles of bioassays, using biological models and their biological reaction principles of contaminants used in sediment monitoring.

2.1. Bioassay

Bioassay means in terms of a procedure for determining the concentration or biological activity by substances. In level of an organism or tissue, a bioassay measures the toxic effects in comparison with a standard preparation. When there is exposure to chemicals that operate a toxic mechanism, the reporter genes, contained in an organism, mediate a characteristic response when there is exposure to chemicals that operate a toxic mechanism. For these reasons, bioassay plays an essential role in evaluating water quality and safety since the biological response is a useful method to measure the level of contaminants [20].

2.1.1. *In vitro* toxicity tests

Chemicals can be so selective that it is challenging to extrapolate toxicity data from one species to another. However, *in vitro* test aids the development of models to predict toxicity to individual species. Furthermore, in environmental toxicology, *in vitro* studies are used as useful methods to replace, reduce, and refine (3R) the *in vivo* tests. Thus, in the field of environmental toxicology, it has been focused on the use of *in vitro* assays with fish cells as a non-animal alternative, to replace the *in vivo* test with fish. Additionally, it can be a part of an alternative testing strategy for bioaccumulation testing with fish. Therefore, *in-vitro* assay is the potential technology to enhance the environmental assessment of chemicals, reducing the need of animal testing [20, 21].

2.1.2. Fish cell lines

Poeciliopsis lucida hepatocellular carcinoma (PLHC-1 cell), the hematoma of fish cell line from *Poeciliopsis lucida*, was used to assess the cytotoxicity, ROS and EROD activity assay. The PLHC-1 cell line was created in order to be extracted from hepatocyte and widely used in toxicological studies. The PLHC-1 cell line is polygonal in shape with more regular

dimensions and grows attached to a substrate in discrete patches, epithelial-like cells, and has preserved some of the original liver function for cytochrome P4501A activity [22]. Several recent studies of environmental monitoring, in particular with sediment extraction exposure, have also used this cell line to assess the toxicity of different sites of coastal sediment contamination [1, 22-24].

Rainbow trout gill-Waterloo1 (RTgill-W1 cell), the cell line derived from *Oncorhynchus mykiss,* was also used for three different bioassays in this thesis. Rainbow trout is known as one of the most sensitive species in cytotoxicity research by the organisation for economic co-operation and development (OECD) [25]. It has been tested to be a reliable model to use in cytotoxicity and many studies have been approaching to screen the environmental pollutants using bioassays such as EROD activity [26-28].

Therefore, in this study, PLHC-1 and RTgill-1 cell lines were selected to compare the effects in cells from the hepatic and non-hepatic origin and due to their reported functional correlations with in vivo experiments [29]. These two organs undoubtedly concentrate more on toxicants than other organs, and active transport or binding to the mechanisms is expected to be involved.

2.2. Biomarker

The biomarker is any biological response to a chemical at the individual level or below demonstrating a departure from normal status. They are usually restricted to responses at the level of the organization of the whole organism or below defined as cellular, biochemical, a molecular indicator of exposure. Environmental toxicology has developed and evaluated biomarkers for use in the assessment of exposure to hazardous toxicants. In particular, cell-based toxicity tests are used widely in environmental monitoring by multiple biomarkers and their endpoints [24][30].

2.2.1. Cytotoxicity

Cytotoxicity assays evaluate the cell viability by categorizing as methods 1; assessing loss of membrane integrity, 2; membrane metabolic activity, 3; loss of monolayer adherence and arrest of cells in various stages of cell cycle, etc. [31].

Measuring cell viability with Alamar blue is based on metabolic activity assessing the resazurin, which is defined as a cell health indicator. Alive cells maintain resazurin within the cytosol of the cell and it is reduced to resorufin by aerobic respiration of metabolically active

cells [7]. When cells are damaged by xenobiotic or toxicants, resazurin is converted to the reduced form by mitochondrial enzyme activity, like in Figure 2.2.1. Using the fact that resazurin does not produce the fluorescence but resorufin does, it is able to measure the cell viability with the absorbance of resorufin at an excitation/emission of 530nm/590nm [32].

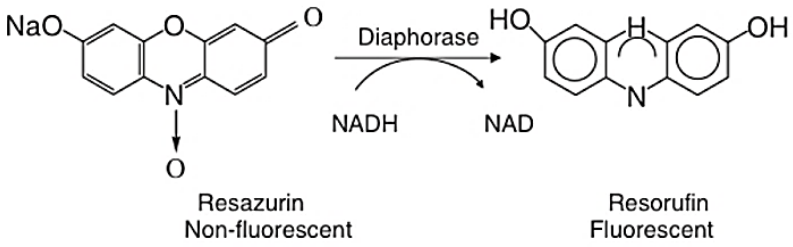


Figure 2.2.1 Resazurin assay of radiation response in cultured cells(Source: Frank A Gomez)[33]

2.2.2. Reactive Oxygen Species

The reduction of O_2 to active O_2 metabolites occurs typically as a by-product of cellular metabolism during both microsomal and mitochondrial electrons transfer the reactions. The cytotoxic oxygen species may mediate or induce the actions of various neurotoxicants. To measure this amount of oxidation stress, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is used as cell-permeant. Like in Figure 2.2.2, H_2DCFDA passively diffuses into cells, and it is retained in the intracellular level after cleavage by intracellular esterases. Upon oxidation by ROS assay, the non-fluorescent H_2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Therefore, it is able to measure the oxidation stress with the DCF at 485/528 nm excitation/emission [34].

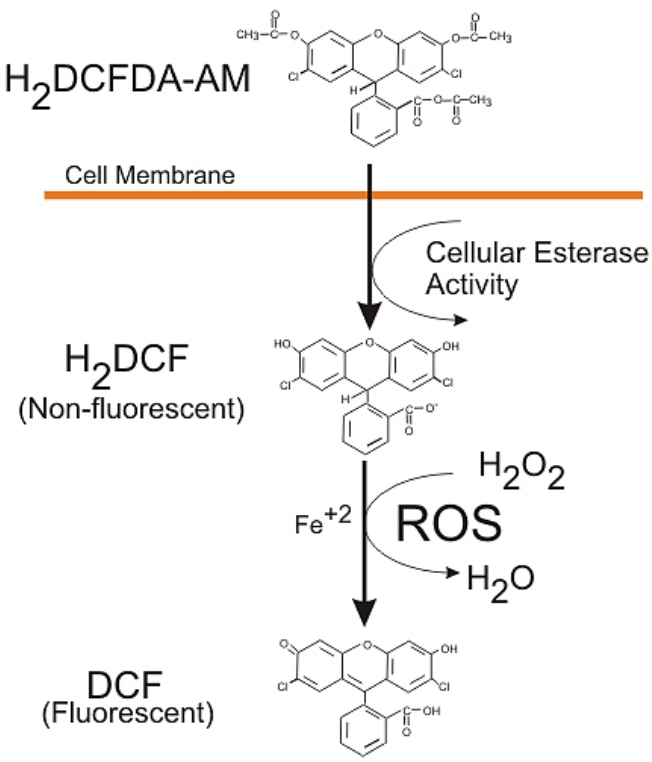


Figure 2.2.2 Formation of fluorescent Compound DCF by ROS(Source: Biotek)[35]

2.2.3. EROD activity

The activity of the enzyme 7-ethoxy-resorufin-O-deethylase (EROD) is one of the most sensitive biomarkers of uptake toxicity in fish. In fish, the cytochrome P450(CYP450) is mainly found in the liver, kidneys, intestines, and gill tissues. EROD is the activated enzyme by planar halogenated hydrocarbons (PHHs), PAHs, persistent organic pollutants (POPs), and other structurally similar chemicals. EROD activity may be affected by water temperature, age, and reproductive cycle, as well [36].

Figure 2.2.3 describes the EROD activity with the rate of the CYP1A mediated deethylation of the substrate 7-ethoxyresorufin to form the product resorufin. For instance, 2,3,7,8-Tetrachlorodibenzodioxin(TCDD), which is reported as a POPs, can lead to the induction of EROD activity. When 2,3,7,8-TCDD is introduced as an inducer, it is attached to the AhR receptor. In this process, a molecular event occurs consecutively, and several genes, including Cytochrome P1A(CYP1A) induction, are also expressed. CYP1A, which is a type subfamily in CYP450, is induced to mediate a heterologous substance by aryl hydrocarbon receptor(AhR) of cytosolic. Therefore, by detecting the produced resorufin, EROD activity is used as a methodology to predict the toxicity of chemicals in biological models. In particular, the measurement of EROD activities using the immortalized cell lines is a tool of bioassay in

many studies in the laboratory due to reproducible simultaneous determination of protein and resorufin concentrations [37, 38].

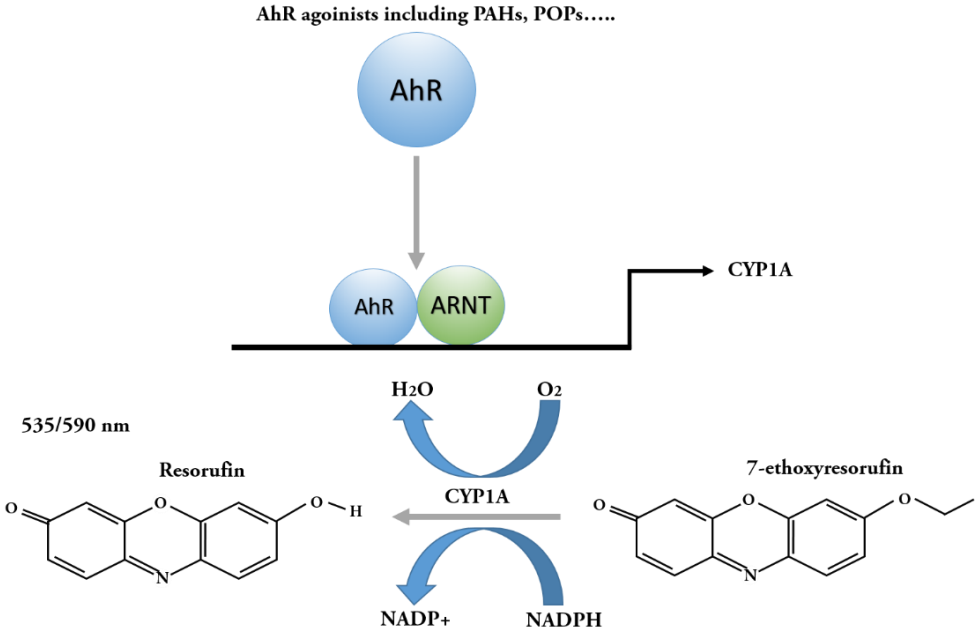


Figure 2.2.3. EROD activity by substrate 7-ethoxyresorufin to form the product resorufin (Source: private)

2.3. Thesis design

The thesis work was divided into three main parts: 1) the participation in the Master course on *In-vitro* Toxicity Testing, organized by Prof. Miren P. Cajaraville at the University of the Basque Country (UPV/EHU), 2) The application of the acquired knowledge for *in vitro* toxicity tests using fish cell lines, and 3) Data analysis and discussion of the obtained results.

In December 2018, I was attending the "Master course on In-vitro Toxicity Testing" organized at the University of the Basque Country, Bilbao, Spain (UPV/EHU). The methodology applied in this thesis using cells of non-mammalian, vertebrates and invertebrates, in particular with PLHC-1 cell line, was taught in the course [39].

PLHC-1 cells from topminnow (*Poeciliopsis lucida*) and RTgill-W1 gill cells from rainbow trout (*Oncorhynchus mykiss*) were used in this thesis. Methodology and standard operation procedures(SOP's), based on toxicity testing course and papers, were amended for each cell line type. Sediment samples and PLHC-1 cells were provided by the dCod.1 project in University of Bergen (UIB) [40] and RTgill-W1 gill cells were provided by the PhD fellow Eystein Opsahl from University of Stavanger (UiS). Seven extracted sediment samples were transferred from UiB to the cell laboratory in UiS and were stored in -20℃. To find the ideal confluence of cells in each well of the microplate, cell counting and seeding with different densities of both cell lines were initiated and 50,000 cells/well was chosen for both cell lines. PLHC-1 and RT gill W1 cell lines were tested to evaluate the toxicity of the 7 samples, using the amended SOP and methodology of bio-assays.

Finally, the data was statistically analysed and discussed based on the chemistry results of PAHs and metals.

3. Materials and Methods

3.1. Sediment sample

3.1.1. Sampling sites

Information on sampling sites and the depths are reported in Table 3.1.1 and Figure 3.1.1. The location of each sampling station in Figure 3.1.1 were marked after that the original GPS data was converted in DMS as given in Appendix B.

Sediment samples were collected from 7 different stations around Bergen, from the city on the western coast of Norway by R/V Hans Brattstrøm. The sample from station 2 Fisketorget was collected from the port of Bergen where is a crowded port with many cruise ships and ferries along the Askøyruten and Beffen. Samples from station 4, and 5 were collected from Puddefjorden. While the samples from station 2, 4, and 5 were relatively close to Bergen city center, the station 1 was collected in Byfjorden which is the northern side along the Askøyruten. The samples from station 9, 8, and 7 were sampled along the cruise route of Bergen, Norway – Stavanger, Norway - Hirtshals, Denmark. The station 9 was sampled from the North Sea closed to Skutevika, and the station 8 was collected in proximity to Molvika. Lastly, the sample from Station 7 was collected from the open sea area close to Hysteinen.

In chapter 3 and 4, all the sediment samples were summarized with the corresponding station number, but without the name of the location, for easier readability.

Table 3.1.1 Coordinates of sediment sampling stations

No	Station	Latitude N	Longitude E	Depth (m)
1	Helleneset	60°25.725'	5°16.275'	332
2	Fisketorget	60°23.782'	5°19.381'	9
4	Nordnes	60°23.277'	5°18.382'	90
5	Puddefjord	60°23.046'	5°18.908'	38
7	Korsfjorden	60°08.377'	4°57.371'	614
8	Skogsvåg	60°15.771'	5°08.071'	220
9	Askøy S	60°22.851'	5°10.321'	226

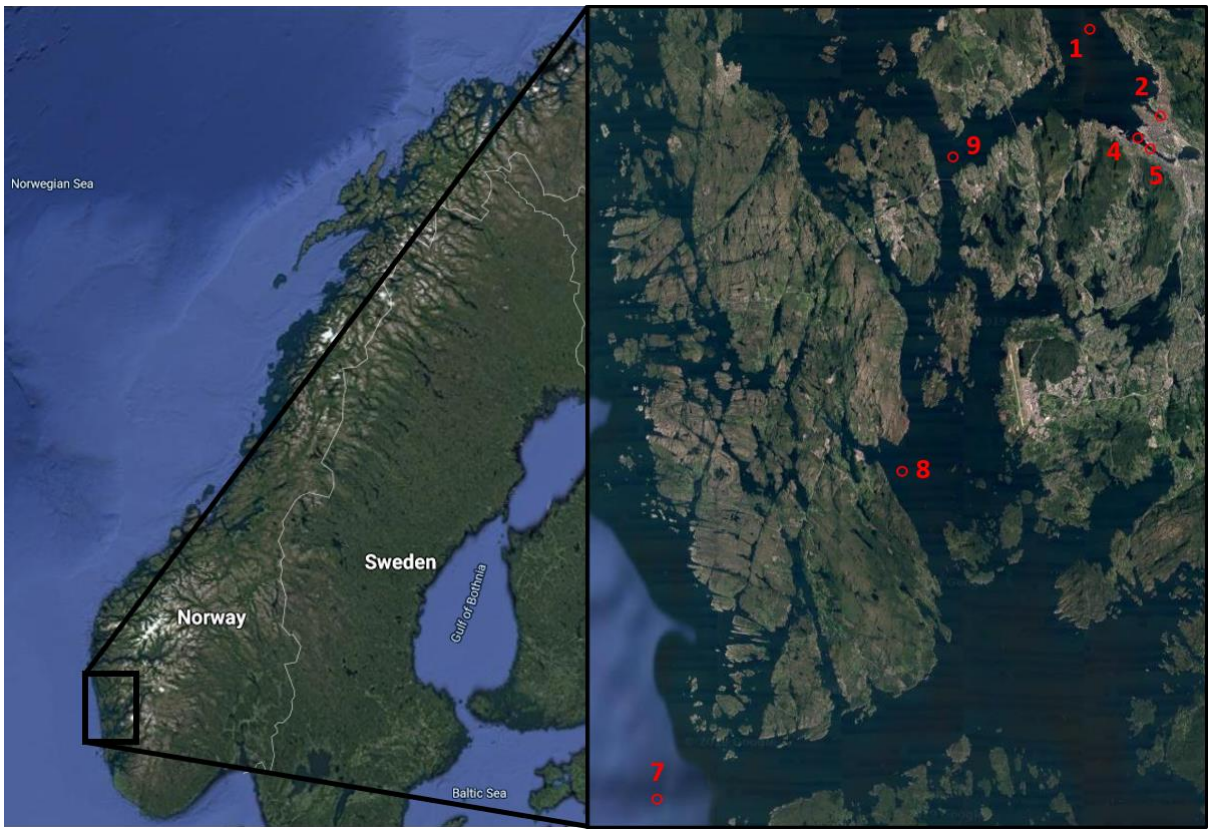


Figure 3.1.1 Map of South West Norway and the location of sediment sampling stations around Bergen, Norway (Source: © Google, Maps.co)

3.1.2. Chemical analysis of sediment samples

Sediment samples were extracted and prepared with mechanical extraction at UiB. Each site's stock concentrations were equivalent to 12 g dry weight sediment extract (mg eQsed/mL). For the toxicity tests, extracts were serially diluted in the adequate media to desired concentrations. Chemical analysis of sediment extraction was done by Havforskingsinstituttet, Bergen, and all raw data of concentration of PAHs, and metallic ions are given in Appendix A.

3.2. Materials

Table 3.2.1 Cell growth medium

Solutions	Catalogue number	Manufacturer
Eagle's Minimum Essential Medium (MEM)	L0430-500	Biowest
Leibovitz's L-15 Medium (L-15)	21083027	Gibco
Fetal bovine serum(FBS)	S181H-500	Biowest
L-glutamine	G8540	Sigma Aldrich
Penicillin/Streptomycin	15140122	Biochrom

Table 3.2.2 Bioassay chemicals, reagents, and buffers

Chemicals	Catalogue number	Manufacturer
7-Ethoxyresorufin	16122-NOR	Cayman Chemical
7-hydroxyresorufin sodium salt	B21187.06	Alfa Aesar
β -Naphtoflavone (bNF)	A18543.03	Alfa Aesar
2′7′-dichlorodihydrofluorescein diacetate (H2DCF-DA)	D399	Invitrogen™
Resazurin	V23110	Life Technologies

Solutions	Catalogue number	Manufacturer
Phosphate buffered saline (PBS)	-	Life Technologies
Trypsin–EDTA	T4049-500ML	Millipore Sigma
Dimethyl sulfoxide (DMSO)	276855	Life Technologies

Table 3.2.3 Commercial kit

KIT	Catalogue number	Manufacturer
Pierce(R) BCA Protein Assay	23221	Thermo Fisher Scientific/ Pierce Biotechnology

Table 3.2.4 Instruments

Instrument	Catalogue number	Manufacturer
Muse® Cell Analyzer	0500-3115	Luminex™
Maxisafe 2020 Class II Biological Safety Cabinets	51026651	Thermo Scientific™
SpectraMax Paradigm Multi-Mode Microplate Reader	-	Molecular Devices

Table 3.2.5 Software programs

Software	Description	Supplier
Excel 2016	Data export and calculation	Microsoft
SPSS Statistics 25	Statistical analysis	IBM Corporation
GraphPad Prism 8.3.0	Plotting Dose-response curve	GraphPad Software, Inc
Minitab 19	PCA analysis	Minitab, LLC

3.3. Cell preparation

3.3.1. Cell culture

PLHC-1 cells and RTgill-1 cells were stored in liquid nitrogen. On 08/08/2019, PLHC-1 cells from passage 7 were retrieved from liquid nitrogen, thawed and subsequently transferred to a 75 cm2 cell cultivating flask, containing 10 mL preheated completed Eagle's minimum essential medium (MEM). The completed MEM was prepared with 5% FBS, 10,000 U/mL penicillin / 10,0000 μg/mL streptomycin and 2 mM L-glutamine. The growth medium was changed after 24 hours. Cells were grown in a humidified atmosphere (30℃, 5% CO2) and the growth medium was changed 3 times a week. Cells were split 1-2 times per week, depending on the experimental setup. Subculturing was performed when the cells were confluent. The growth medium was then removed from the culture flask and rinsed with 20 mL phosphate-buffered saline (PBS). The PBS was then removed and replaced with 2 mL trypsin, which was left for 2-3 minutes. Afterwards, 4 mL of MEM was added to the cell suspension and the flask was rinsed a few times with the mixture. Suspended cells were then transferred to a new cell cultivating flask. To store the different passages of PLHC-1 cells aliquots, cells were suspended in freeze medium (95% MEM, 5% of DMSO) after having been centrifuged for 5 minutes at 900 rpm. The cryotube was stored in the freezer at -80℃ for 24 hours and then transferred to liquid nitrogen(-196℃).

The same subculture procedure was used and prepared for freezing down with the RTgill-1 cells, but with different incubation temperatures, growth medium and volume of regents. On 18/10/2019, RTgill-1 cells from passage 4 were retrieved from the liquid nitrogen and subsequently transferred to a 75 cm2 cell cultivating flask, containing 13 mL preheated completed Leibovitz's L-15 Medium (L-15). The completed medium was prepared with 10% FBS and Pen/Strep and L-glutamine. The growth medium was changed after 24 hours. RTgill-W1 cells were grown in a normal atmosphere (18℃, without CO_2, in the dark) and the growth medium was changed 3 times a week. Cells were split 1-2 times per week, depending on the experimental setup. Subculturing was performed when the cells were confluent, keeping the total 13 ml of medium volume in the cultivating flask. The method of freezing down for gill cell aliquots was also prepared in the same way with the L-15 medium.

3.3.2. Cell seeding

Flasks at 90% of confluence were used to seed 96-well cell culture microplates for the sediment extract exposure. Prior to the sediment extracts exposure, both cell lines were counted and seeded with the same density of 50,000 cells/mL. Cell counting was done by Muse® Cell Analyzer and both cell lines were incubated at 30°C/18°C after seeding into the microplates. Cells were then allowed to attach for 24 hours and form monolayer as observed in Figure 3.3.1.

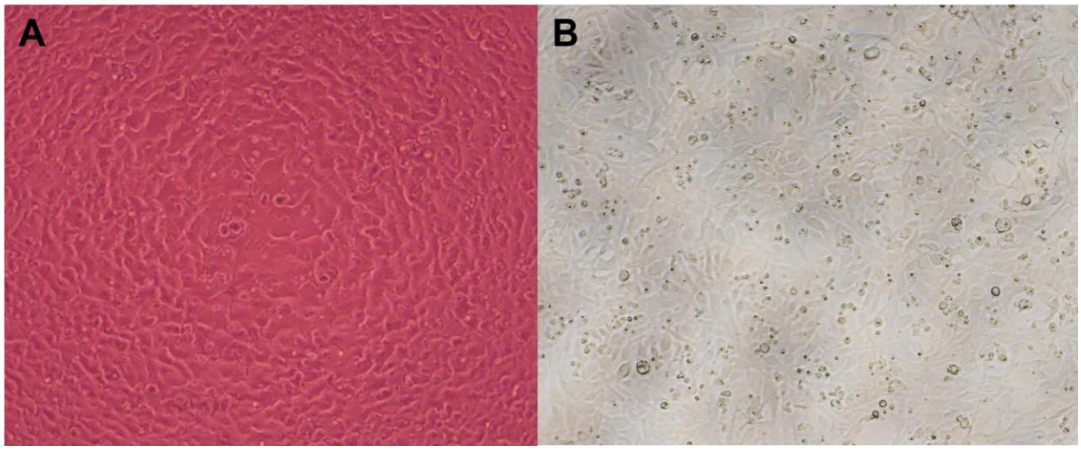


Figure 3.3.1 Micrographs of A: PLHC-1 cells and B: RTgill-W1 cells after seeding for 24 hours (20x magnification, Source: private)

After 24 hours of incubation, the culture medium was replaced with an appropriate medium containing sediment extracts. Exposure medium was prepared on the day and serially diluted to 5, 10, 20, 40, 60, and 120 mg eQsed/mL. As the positive controls, 100 μM H_2O_2 was used in the cell viability and ROS assays, 1 μM β-naphtholflavone (bNF) was for EROD assay. Four replicates were done for each concentration of sediment extract and for each control (0 mg eQsed/mL) and positive control (n = 56).

3.4. Bio-assay

3.4.1. Cytotoxicity

The general cytotoxicity was tested in order to monitor the metabolic activity, based on the conversion of resazurin to fluorescence compound resorufin using Alamar blue assay. The assay was performed after cells were seeded, as explained in 3.3.2.

Stock solutions of resazurin fluorescence dye was prepared in a mixture of 4.86 mg resazurin powder with 40 ml PBS then and sterilized by filtration through 0.25 µm. This stock solution was stored in 50 ml centrifuge tube covered with aluminum foil and maintained at 4°C unless changes in color were observed.

Under dark working conditions, 20 µL of resazurin solution was added to wells with cells and suspended to mix well. The wells with only culture medium was used to correct for background fluorescence and wells with cells was exposed to 100 µM H2O2 as a positive control. The dye was mixed thoroughly using pipette to allow it to be mixed well and reach all cells, and the plates were covered with an aluminum foil to protect against light. Afterwards, they were incubated for 4 hours at 30°C/18°C respectively in accordance to culture conditions. Using the microplate reader (SpectraMax Paradigm Multi-Mode), the fluorescence was detected at 570/585 nm emission/excitation. The relative fluorescence unit (RFU) was converted to the percentage of cell viability relative to the control cells without exposure medium.

3.4.2. Reactive oxygen species (ROS) assay

The generation of reactive oxygen species (ROS) measured in exposed fish cells, was achieved by measuring the highly fluorescent 2',7'-dichlorofluorescein (DCF), using a slightly modified version described by LeBel et al.[41]. Cells were seeded as explained in 3.3.2 and exposed to the sediment extracts as explained below.

A stock solution of 10 M 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) 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 20 µM.

For the exposure experiment, the culture medium was removed, cells were rinsed with 200 µL PBS and 100 µL of 20 µM H_2DCF-DA probe was added. With the probe, cells were

incubated for 30 minutes at 30℃ (PLHC-1) and 18℃ (RTgill-W1), followed by two additional steps of washing with PBS. The exposure media was then prepared in PBS instead of medium. The cells with exposure media were incubated for 60 minutes. The fluorescence emitted from the oxidation of H_2DCFDA was detected by the microplate reader at 485/528 nm excitation/emission. Results were expressed as the percentage difference in fluorescence relative to the control cells.

3.4.3. 7-Ethoxyresorufin-O-deethylase(EROD) activity

The induction of EROD activity was measured as the fluorescence molecule resorufin derived from deethylation of 7-Ethoxyresorufine as the endpoint. The assay was performed as indicated in Perez-Albaladejo et al.[42], with slight modifications. The cells were seeded as explained in 3.3.2.

The 2 μM 7-ethoxyresorufin probe was made by diluting one tube of 40 μL of 2000 μM 7- ethoxyresorufin into the 40 mL of 50 mM Na-phosphate buffer with pH 8.0.

After 24 hours of incubation, cell culture medium was aspirated, followed by washing with 200 μL PBS. Cells were then exposed to the 200 μL different concentrations of sediment extracts and 1 μM β-naphthoflavone (bNF) as positive control and then incubated for a further 24 hours. Immediately after the 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 blank row containing the probe to correct for background fluorescence. After incubation at 30℃/18℃ for 15 minutes, the fluorescence was read at 537/583 nm emission/excitation. Quantification of the activity was performed by calibration of 7-hydroxyresorufin. The fluorescence signal from the assay was used to calculate the conversion of pmol resorufin formed per minute.

For the total protein determination, cell lysing was performed by removing all the fluids from the plates and freezing them down for 48 hours with one thawing in between. After re-thawing in room temperature, 50 μL of 50 mM Na-phosphate buffer was aliquoted into each well, followed by scraping with a pipette tip to mix the supernatant. Twenty-five μL of lysed cells were used to determine total cellular proteins based on bicinchoninic acid protein assay (BCA Kit) [43] with bovine serum albumin as a standard. Results were expressed as pmol of resorufin formed per minute and per milligram of protein (pmol/min/mg protein).

Both calibration curves for 7-hydroxyresorufin and BSA serum can be seen in Appendix C.

A summary of all bioassays is given in Figure 3.4.1.

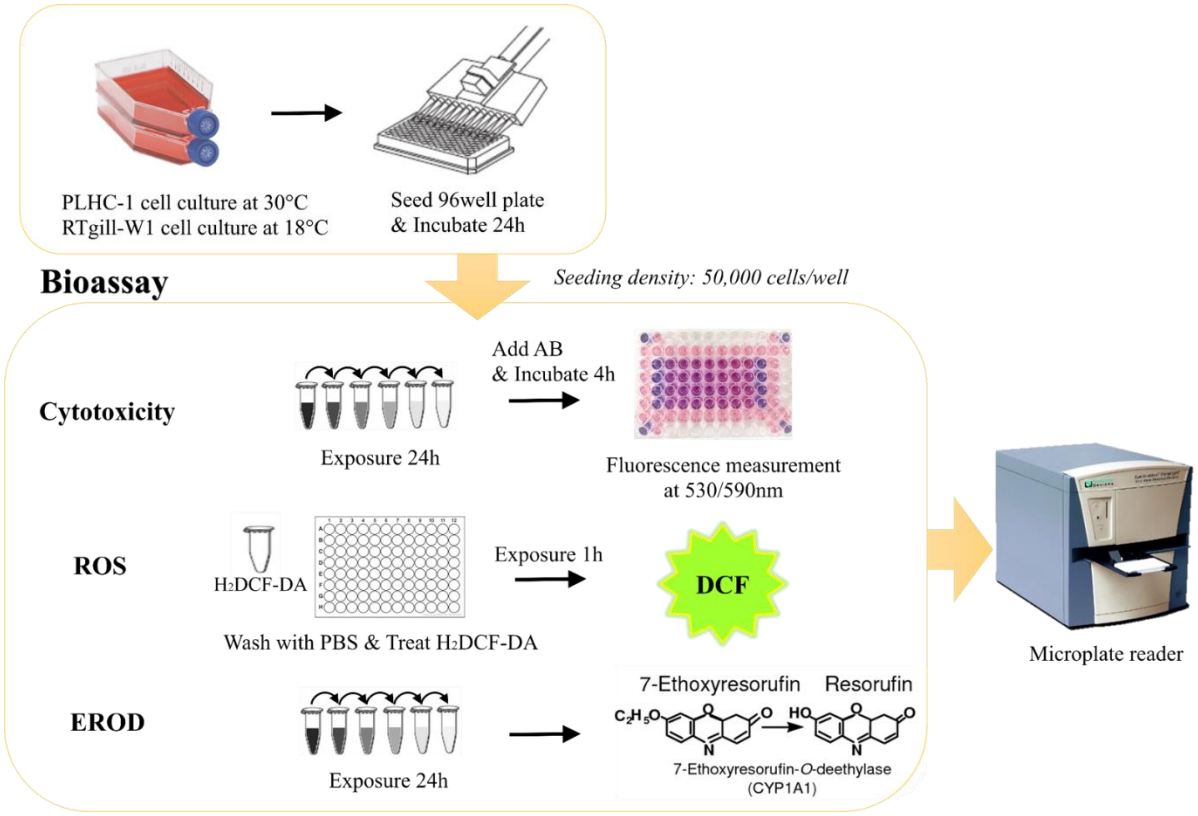


Figure 3.4.1 Summary schematic of bioassay methodology (Source: private)

3.5. Statistical Analysis

Comparisons between contaminated sediments and the natural sediment reference sample were made using one-way ANOVA followed by multiple independent group comparisons (Tukey's and Dunnett test). All the statistical analyses were performed with the software package SPSS (IBM SPSS Statistics 25, SPSS Inc., Chicago, USA), and p values lower than 0.05 were considered statistically significant. When it was possible, the dose-response curve was reported, and the EC_{50} was determined and calculated by GraphPad Prism (GraphPad Software 8.3.0, La Jolla, CA, USA). In order to assess the degree of anthropogenic impact, a principal Component Analysis (PCA) was performed using the Minitab 19 (LLC, PA, USA).

4. Results and discussion

The toxicity assessment of marine sediments was performed by using three different bioassays, in order to understand the contamination levels/toxicity of the sediment samples. In this chapter, the data from each bioassay are presented and discussed with features observed from the chemical analysis of sediment extracts. Results are arranged by cell types, PLHC-1 and RTgill-W1.

4.1. Cell viability

4.1.1. PLHC-1 cell

Results of cytotoxicity with PLHC-1 cells are presented in Figure 4.1.1. The graph describes the cell viability as the percentage of fluorescence response from each dose compared to control cells, which is defined as 100% cell viability.

In regard to the station 2 sample, statistically significant cytotoxicity was observed from 40, 60, and 120 mg eQsed/mL after 24 hours of exposure ($p<0.05$). The highest toxicity was recorded at 120 mg eQsed/mL, which caused an average of 5 % cell viability. The dose-response curve is shown in Figure 4.1.2, revealing EC_{50} of 43.8 ± 4 mg eQsed/mL.

There was no significant lethal effect observed in cell viability in the other station samples. However, samples from station 7, 8, and 9 showed a significant increased cell viability from 40 to 120 mg eQsed/mL. In spite of the fact that the cell viability decreases when the hazardous toxicant concentration increases, this suggests that the cause could be a positive effect on cell viability by the sediment extract.

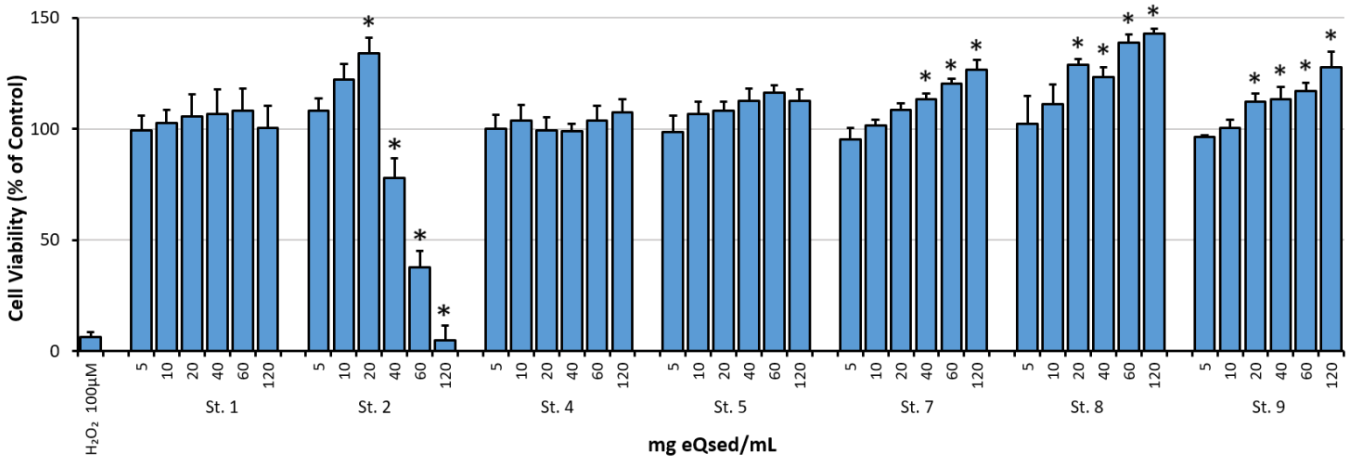


Figure 4.1.1 Cell viability in PLHC-1 cell after 24 hours of exposure to the sediment extracts from different sites(St). Values are expressed as mean ± SD (n=4). The significance level observed is * (p-value < 0.05) in comparison to control cells (0 mg eQsed/mL).

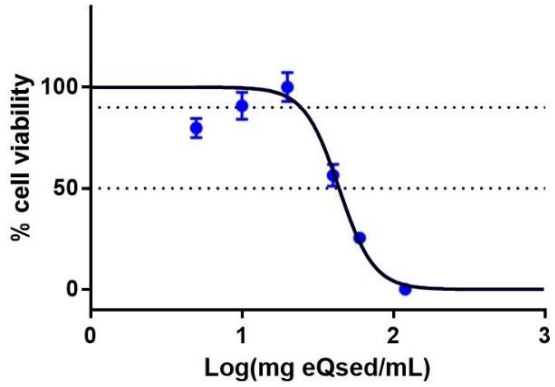


Figure 4.1.2 After a 24 hours exposure of sediment extract from Station 2 to PLHC-1 cell, a dose-response curve was observed.

4.1.2. RTgill-W1 cell

Results of cytotoxicity using RTgill-W1 cells for sediment extracts are presented in Figure 4.1.3.

Statistically significant cytotoxic effects were observed from station 2, 5, 7, and 8. The highest cytotoxicity was observed for station 2 sample at 5 to 120 mg eQsed/mL. The dose-response curve that describes the station 2 sample is shown in Figure 4.1.4, revealing an EC_{50}s of 155.7 ± 42 mg eQsed/mL respectively.

With the station 2 sample, a dose-response curve could be described with the results of both cell lines. Major differences among the graphs in Figure 4.1.2 and Figure 4.1.4, is due to the slope of the dose-response curve. There is a steeper slope and lower threshold with PLHC-1 cells than RTgill-W1 cells. This suggested that the station 2 sample affects higher toxicity in lower dose with PLHC-1 cells. Therefore, it could be explained that the toxic effect in hepatocyte started to occur earlier to detoxify a toxicant or repair injury when it has been exceeded [44].

Several samples had significant cytotoxicity effects, as shown in Figure 4.1.3 respectively, station 4, 5, 7, and 8. Those values were significantly different from the control using the t-test. However, using the GraphPad, none of them was able to describe the dose-response curve in order to calculate EC_{50}, which is used as a measurement of toxicants effective concentration. The station 4 sample also showed significant differences in comparison to the control, and the the cell viability increased from 5 to 60 mg eQsed/mL. However, it was not able to explain the dose-response relationship with any tendency. Therefore, further study and testing is suggested to expose the higher dose to find out the correlation between cell viability and the samples from 4, 5, 7 and 8.

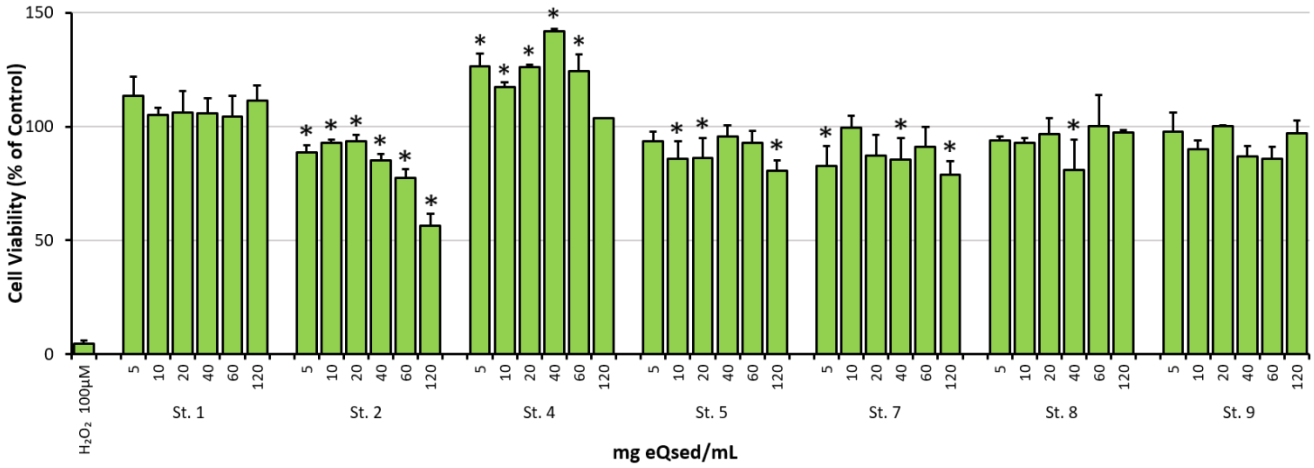


Figure 4.1.3 Cell viability in RTgill-1 cell after 24 hours of exposure to the sediment extracts from different sites(St). Values are expressed as mean ± SD (n=4). *Statistically significant ($p < 0.05$) difference in cell viability relative to control cell (0 mg eQsed/mL).

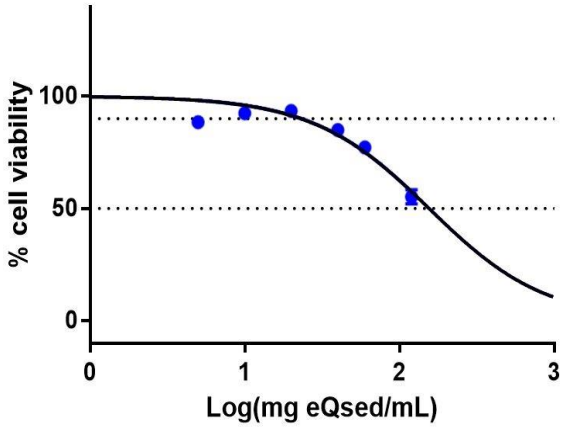


Figure 4.1.4 After a 24 hours exposure of sediment extract from Station 2 to RT-gill W1 cell, a dose-response curve was observed.

4.2. Oxidative stress

4.2.1. PLHC-1 cell

ROS production in PLHC-1 cells was described by the percentage of response by dose compared to the control cells, which was incubated with growth medium only. All results were recorded after 60 minutes of exposure.

As Figure 4.2.1 shows, the significant level of ROS production was noted at the highest dose, 120 mg eQsed/mL, for all the analysed samples. While the samples from station 4 recorded significant levels from 60 to 120 mg eQsed/mL, the station 5 recorded it at 20 to 120 eQsed/mL. This could be explained by the station 5 sample initiated producing the oxidative stress significantly from 20 mg eQsed/mL, compared to controls exposed only to growth media.

The station 2 was showed that the ROS production was increased noticeably from 10 to 120 eQsed/mL with greater range. The highest concentration of the station 2 reached 500% of the control value, while the positive control resulted around 700%. This can be shown by the station 2 did not produce the critical amount of ROS to the positive control, however, it was able to clearly notice higher oxidation stress production than other station samples. With the cytotoxicity result in 4.1.1, this could be read as a corresponding significant toxicity of the station 2 sample to PLHC-1 cells.

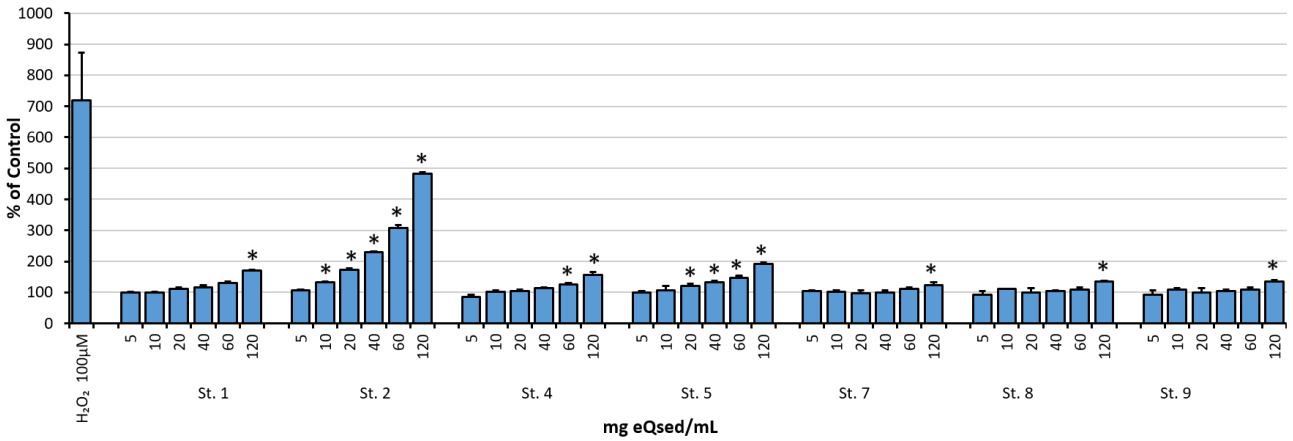


Figure 4.2.1 ROS production in PLHC-1 cells after 60 minutes of exposure to different sites(St). Values are expressed as mean ± SD (n=4). *Statistically significant ($p < 0.05$) difference in cell viability relative to control cell (0 mg eQsed/mL).

4.2.2. RTgill-W1 cell

Results of ROS assay using RTgill-W1 cells for sediment extracts are presented in Figure 4.2.1.

Results had very small variations between the four replicates and a significant induction of ROS was recorded in 5 out of 7 samples. The samples from station 1 and 9 had significant levels at concentrations from 40 to 120 mg eQsed/mL and station 4 had it from 20 to 120 mg eQsed/mL, while the station 2 and 5 showed more efficiency in oxidizing H_2DCF than the rest of the samples. A significant induction was detected in the station 5 sample at 5 to 120 mg eQsed/mL compared to the control cells. In the station 2, the ROS production was remarkably increased 1.5-fold compared to the positive control of H_2O_2 100 µM at the highest exposure concentration, 120 eQsed/mL respectively. It could be discussed as the highest concentration, which is the station 2 produced the highest oxidation stress, had big differentials than any other samples and even positive control in 60 minutes exposure.

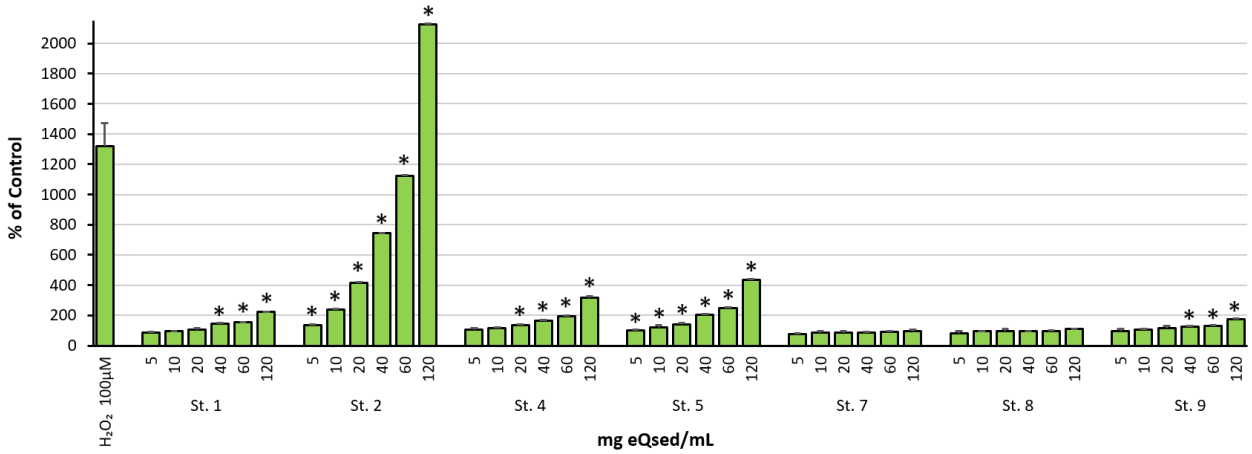


Figure 4.2.1 ROS production in RTgill-W1 cells after 60 minutes of exposure to different sites(St). Values are expressed expressed as mean ± SD (n=4). *Statistically significant ($p < 0.05$) difference in cell viability relative to control cell (0 mg eQsed/mL).

4.3. EROD activity

4.3.1. PLHC-1 cell

EROD activity results with PLHC-1 cells are summarized in Figure 4.3.1. Results are reported in pmol/min/mg protein, from 0 to 120 mg eQsed/mL. One µM of bNF was used as a positive control and the value was used as a reference for all the results.

In regard to the station 2 sample, statistically significant EROD activity was observed from 40, 60, and 120 mg eQsed/mL after 24 hours of exposure ($p<0.05$). The highest toxicity was recorded at 120 mg eQsed/mL and the value of 40 mg eQsed /mL was the same as for one of the positive control. It is known that the CYP1A gene gets higher expression due to protection metabolism by xenobiotic, usually PAHs [45, 46]. Therefore, above 40 mg eQsed/mL of the station 2 sample, the CYP1A metabolism required higher inducing than what bNF was recorded at. Thus, it shows that the station 2 sample had toxicity effect on over 40 mg eQsed/mL than the positive controls by exposure of toxicants.

While comparing all the station samples, a significant increased activity was observed in the station 1 sample. From 0 to 5 mg eQsed/mL, it showed that the activity was increased after exposure with the lowest dose. Following values were decreased by degree of the doses and it was observed that the activities from 5 to 40 mg eQsed/mL were higher than positive control.

In addition to sample 1, the trends, recording the greatest value at the lowest concentration, were also found in samples 4, 5, 7, 8 and 9. At 5 mg eQsed/mL exposure, which is the least kinetic dose, all of the station samples were recorded with greater values than positive control. Therefore, further experimentation at concentrations below 5 mg eQsed/mL seems to be required. This may help to identify the point where the CYP1A metabolism starts and leading to toxicity effect with lower values than bNF.

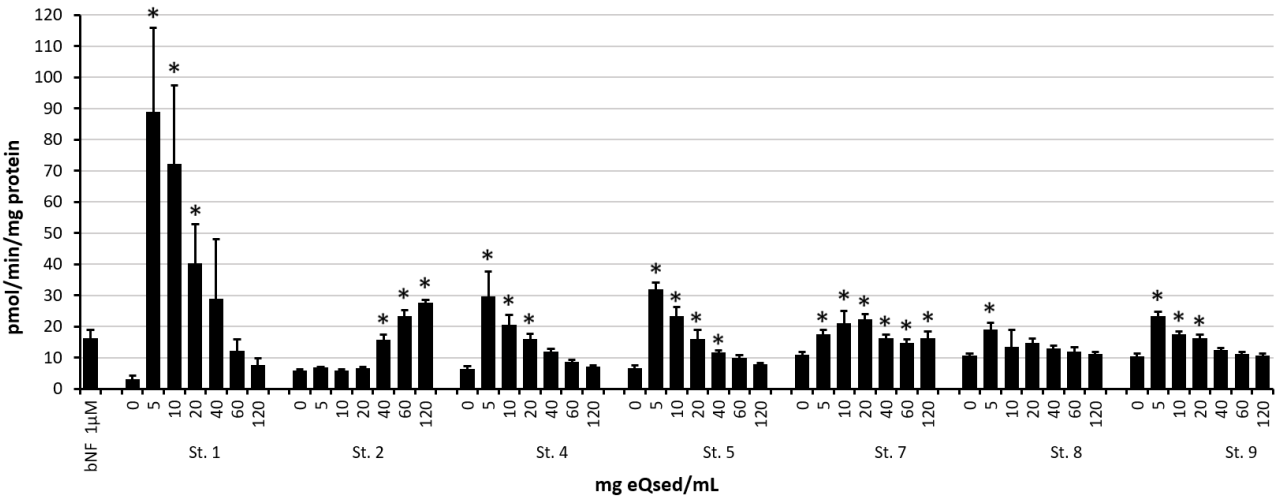


Figure 4.3.1 EROD activity in PLHC-1 cells exposed for 24 hours to the sediment extracts from different sites(St) and positive control β-Naphthoflavone (1 µM). Values are expressed in pmol/min/mg protein, as mean ± SD (n = 4). *Statistically significant ($p < 0.05$) difference relative to the control cells (0 mg eQsed/mL).

4.3.2. RTgill-W1 cell

EROD activity results with RTgill-W1 cells are summarized in Figure 4.3.2.

All the samples of stations except the station 2 sample, had distinct changes in EROD activity values compared to the cells exposed only to growth media. However, in most cases, the value was lower than the positive control and there was no trend of the activity increasing or decreasing by the degrees of concentration.

According to the results of the earlier bioassays in this thesis, the station 2 sample, which could be concluded to be fatal, had the smallest activity value and no statistically correlated concentration.

RTgill-W1 cells are good biota for toxicological studies and have been used in many research and experiments for metals, waste water and chemical contaminants [47]. On the other hand, the RTgill-W1 cell line was recommended just for the cytotoxicity research by Schirmer, because the measurement of cytochrome P4501A, which is used for EROD activity, is undetectable with this cell line [48, 49]. Therefore, despite of bNF exposure, EROD activity from the rest of the samples with the RTgill-W1 cell line has shown to have very low correlation between EROD activity induction and sediment samples.

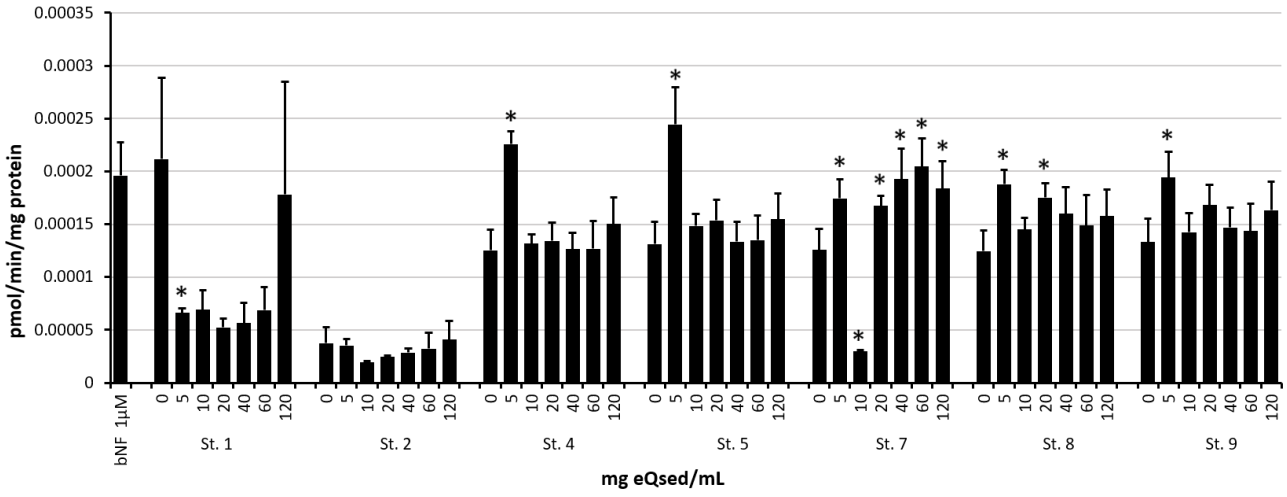


Figure 4.3.2 EROD activity in RTgill-W1 cells exposed for 24 hours to the sediment extracts from different sites(St) and positive control β-Naphthoflavone (1 µM). Values are expressed in pmol/min/mg protein, as mean ± SD (n = 4). *Statistically significant ($p < 0.05$) difference relative to the control cells (0 mg eQsed/mL).

4.4. Multivariate Analysis

4.4.1. Selection of Chemical Markers in the Sediment Samples

Prior to the principal component analysis (PCA), all the sediment samples were rated and chosen for further analysis by quality comparing with the selected elements such as metals and PAHs. The standard quality values were from a table scored by Norwegian classification of environmental quality in sediments. In Table 4.4.1, it is classified by five quality classes based on the statistical distribution of levels of the contaminants in sediments along the Norwegian coast [50]. The classification data was modified to a table by J. Søndergaard to compare with the sediment sample [51].

To decide the marker used in PCA, the data of PAH concentration in the sediment samples used in this thesis(Appendix A-PAHs) and the PAHs reference in Table 4.4.1 were compared. The station 2 sample was given a “Very bad” level with a sum of 16 PAHs and the samples from station 1, 4, 5 and 9 were given a “Bad” level. In addition, when the Benzo(a)pyrene was compared, the samples from station 2 and 4 received a “Bad” level. Therefore, recording “Bad” or “Very bad” levels in two or more correlated sediment samples, the sum of 16 PAHs and Benzo(a)pyrene were selected for the PAHs marker used in PCA analysis.

For the markers of metals, the data of metals concentration in the sediment samples used in this thesis(Appendix A-Metals) and the Metals reference in Table 4.4.1 were compared. The samples from station 1 and 2 were given a “Very bad” level with Copper(Cu) and “Bad” to stations 4 and 5. In addition, when comparing with Lead(Pb), the samples from station 1, 2 and 5 were given “Bad” levels. Thus, recording “Bad” or “Very bad” levels in two or more correlated sediment samples, Cu and Pb were selected for the markers of metals used in PCA analysis.

Table 4.4.1 Classification of metals and organic contaminants in sediments (mg/kg dry weight,)

	I	II	III	IV	V
	Background	**Good**	**Moderate**	**Bad**	**Very bad**
Cr	<70	70-560	560-5900	5900-59000	>59000
Ni	<30	30-46	46-120	120-840	>840
Cu	<35	35-51	51-55	55-220	>220
Zn	<150	150-360	360-590	590-4500	>4500
As	<20	20-52	52-76	76-580	>580
Cd	<0.25	0.25-2.6	2.6-15	15-140	>140
Hg	<0.15	0.15-0.63	0.63-0.86	0.86-2	>2
Pb	>30	30-83	83-100	100-720	>720

	I	II	III	IV	V
	Background	**Good**	**Moderate**	**Bad**	**Very bad**
Naphthalene	<2	2- 290	290 - 1000	1000 - 2000	>2000
Acenaphthylene	<1.6	1.6 - 33	33 - 85	85 - 850	>850
Acenaphthene	<4.8	2.4 - 160	160 - 360	360 - 3600	>3600
Fluorene	<6.8	6.8 - 260	260 - 510	510 - 5100	>5100
Phenanthrene	<6.8	6.8 - 500	500 - 1200	1200 - 2300	>2300
Anthracene	<1.2	1.2 - 31	31 - 100	100 - 1000	>1000
Fluoranthene	<8	8 - 170	170 - 1300	1300 - 2600	>2600
Pyrene	<5.2	5.2 - 280	280 - 2800	2800 - 5600	>5600
Benzo(a)anthracene	<3.6	3.6 - 60	60 - 90	90 - 900	>900
Chrysene	<4.4	4.4 - 280	280 - 280	280 - 560	>560
Benzo(b)fluoranthene	<46	46-240	240 - 490	490 - 4900	>4900
Benzo(k)fluoranthene	-	<210	210 - 480	480 - 4800	>4800
Benzo(a)pyrene	<6	6 - 420	420 - 830	830 - 4200	>4200
Indeno(1.2.3-cd)pyrene	<20	20 - 47	47 - 70	70 - 700	>700
Benzo(ghi)perylene	<18	18 - 21	21 - 31	31 - 310	>310
Dibenzo(ah)anthracene	<12	12 - 590	590 - 1200	1200 - 12000	>12000
Sum 16 PAHs	<300	300 - 2000	2000 - 6000	6000 - 20000	>20000

In PCA, all the bioassay data was correlated with each contaminant's concentration in the sediment samples and each of them were calculated:

$$\text{Conc. of contaminant in the sediment extract (mg/L)} = \left(\frac{\text{mg equivalent dry sediment weight}}{\text{mL sediment extracts}}\right) \times \left(\frac{\text{mg contaminant weight}}{\text{kg dry sediment weight}}\right)$$

4.4.2. Principal Component Analysis (PCA)

According to the observed responses in the bioassays, PCA analysis is a method of classifying the sediment samples by each markers of contaminants [42].

PCA scores were plotted according to the bioassay data from different station samples and summarized in Figure 4.4.1 and Figure 4.4.2. It was used as a graphical method of separating individual stations based on each bioassay data, which was calculated in the percentage of controls. This can show the identification of each station sample and how clearly each of them is distinguished using the variance of the bioassay data.

In Figure 4.4.1, PCA score plot was arranged by a set of marked contaminants(16 PAHs, B(a)P, Cu, Pb) and bioassays with PLHC-1 cells. Total variances explained was 16 PAHs: 99.5%, B(a)P: 93.5%, Cu: 94.6%, Pb: 98.1%. The results group of the station 2 sample with brown box shape and shown in a red line, was a comprehensive level of contamination, which is corresponding to the sediment sample concentrations 40 to 120 mg eQsed/mL. Those three concentration groups of bioassay were indicated as showing the higher anthropogenic impact. It was also able to estimate that the three groups of station 2 samples were clearly distinguished from other station groups, particularly, in the plot with B(a)P and Cu.

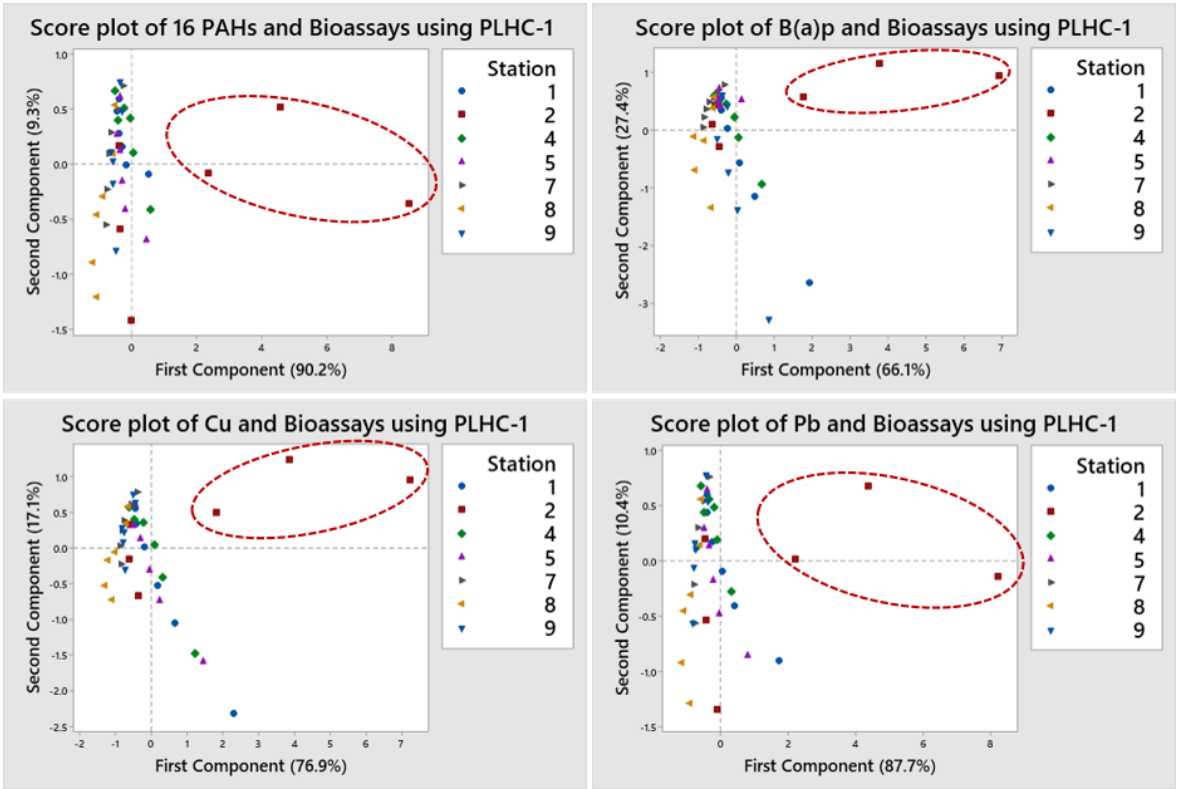


Figure 4.4.1 PCA of marked contaminants and bioassays with PLHC-1 cells. Total variances explained in the bioassays and 16 PAHs: 99.5%, B(a)P: 93.5%, Cu: 94.6%, Pb: 98.1%

In Figure 4.4.2, the PCA score plot was arranged by a set of similar marked contaminants but with RTgill-W1 cells. Total variances explained was 16 PAHs: 99.8%, B(a)P: 86.3%, Cu: 91.5%, Pb: 97.3%. Likewise, the group of station 2 samples also showed a comprehensive level of contamination, which is corresponding to the sediment sample concentrations from 20 to 120 mg eQsed/mL. Those four concentration groups of bioassay results were indicated as showing the higher anthropogenic impact. With RTgill-W1 cells, most of the bioassay results were to shown that it had higher anthropogenic impact from even lower concentrations of station 2 sample that with PLHC-1 cells. This shows that the selected bioassays can show the different affects between the sites of marine sediments due to differences in contamination levels.

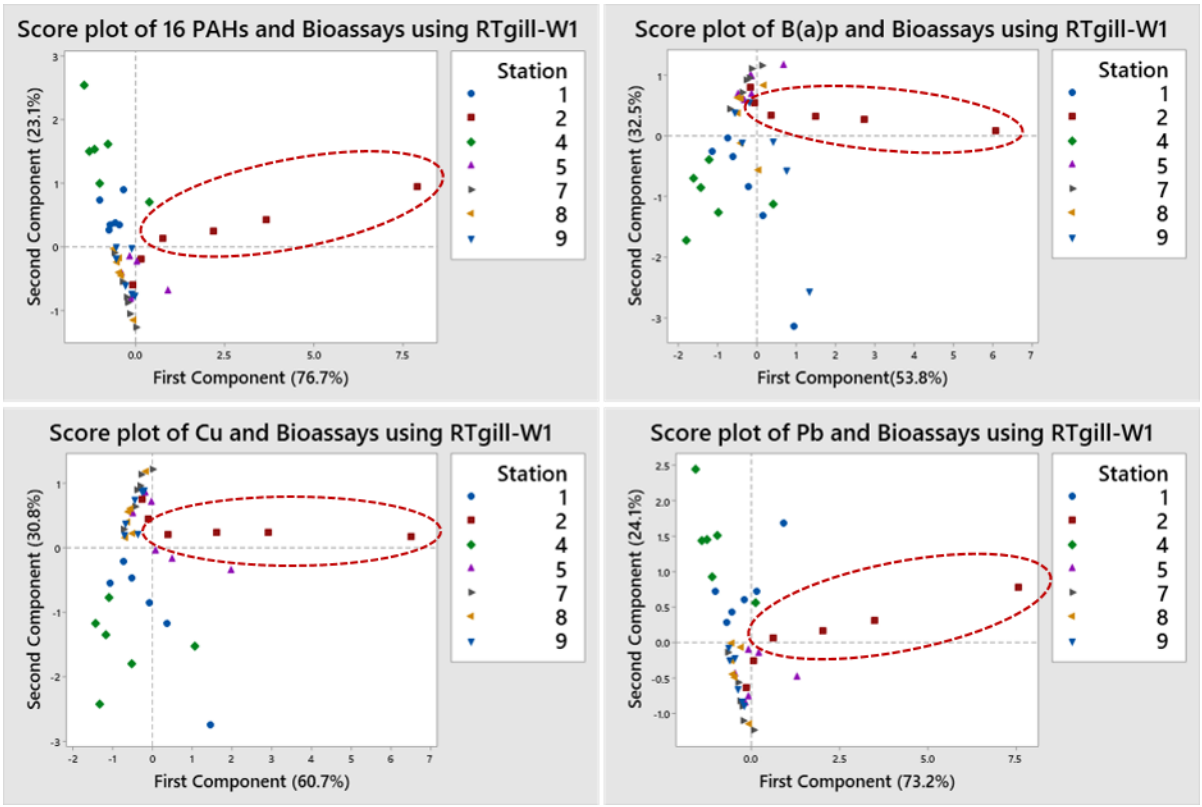


Figure 4.4.2 PCA of marked contaminants and bioassays with RT-gill cells. Total variances explained in the bioassays and 16 PAHs: 99.8%, B(a)P: 86.3%, Cu: 91.5%, Pb: 97.3%

5. Conclusion

The overall results from bioassays performed in this study were able to show the different toxicity levels of the selected sediment samples by using fish cell lines.

Cell viability assay results demonstrated that both PLHC-1 cells and RTgill-W1 cells were capable of showing the level of effect on metabolic activity by sampling sites. The station 2: Fisketorget, was the only sample showing a significant dose-depended sub-lethal effect in both cell lines. According to the graded dose-response curve, higher toxicity was observed with PLHC-1 cells than RTgill-W1 cells. Therefore, it could be possible to conclude that the PLHC-1 cells from hepatocyte may start to react and detoxify faster and with more sensitivity than RTgill-W1 cells.

ROS assay results obtained from both fish cell lines revealed a significant difference in ROS production between control and exposed to sediments cells. Comparing by cell types, there were more samples with significant values on RTgill-W1 cells, which also discovered toxic effects with the samples from station 1, 4, 5, 8, and 9, even though they did not reveal significant cytotoxicity. Therefore, ROS was able to show more samples in significant levels detecting the cell respiratory sensitively. To conclude, the results revealed that the ROS assay detects more effectively than the cytotoxicity due to the sensitivity.

The EROD activity assay, showed significant activity in the station 1: Helleneset and 2: Fisketorget with PLHC-1 cells. However, the following values were decreased by the doses were increased, and there was no significant toxicity observed in the other samples. Likewise, RTgill-W1 cells showed way too low EROD activity values and the least correlation to the significant toxicity. Therefore, further experiments with different doses of sediment samples for PLHC-1 cells but not RTgill-W1 cells, is suggested as in the previous section.

The PCA was able to separate the most toxic sediment samples from the others and showed significant correlation between the bioassay results and the concentration of pollutants, which was selected as a sum of 16 PAHs, B(a)P, Cu, Pb.

In conclusion, in the sample from station 2: Fisketorget had a significant toxic effect on all of the bioassays and was indicated to possess higher anthropogenic impact with the contaminants, which was marked by comparing the quality of chemicals, than the other sites by PCA. On the other hand, the sample from station 7: Korsfjorden, which was collected in the furthest area away from Bergen, had the least toxicity according to the overall results.

Furthermore, the sediments from stations 1: Helleneset, 4: Nordnes, 5: Puddefjord, 8: Skogsvåg, and 9: Askøy S, which showed significant oxidation stress, should continue to be monior . The selected bioassays were a suitable tool to grade the environmental quality of the marine sediments and provided valuable information for future environmental monitoring.

Reference

[1] M. Blanco, E. Perez-Albaladejo, B. Pina, G. Kuspilic, V. Milun, R. Lille-Langoy, *et al.*, "Assessing the environmental quality of sediments from Split coastal area (Croatia) with a battery of cell-based bioassays," *Science of the Total Environment,* vol. 624, pp. 1640-1648, May 15 2018.

[2] F. Duodu and N. Nasiritousi, "Global Environmental Problems and Politics."

[3] H. Ali, E. Khan, and I. Ilahi, "Environmental Chemistry and Ecotoxicology of Hazardous Heavy Metals: Environmental Persistence, Toxicity, and Bioaccumulation," *Journal of Chemistry,* vol. 2019, p. 14, 2019.

[4] S. K. Shukla, N. Mangwani, T. S. Rao, and S. Das, "8 - Biofilm-Mediated Bioremediation of Polycyclic Aromatic Hydrocarbons," in *Microbial Biodegradation and Bioremediation*, S. Das, Ed., ed Oxford: Elsevier, 2014, pp. 203-232.

[5] A. Makishima, "Chapter 7 - Life on Mars From the Martian Meteorite?," in *Origins of the Earth, Moon, and Life*, A. Makishima, Ed., ed: Elsevier, 2017, pp. 139-162.

[6] R. J. Wenning and L. Martello, "Chapter 8 - POPs in Marine and Freshwater Environments," in *Environmental Forensics for Persistent Organic Pollutants*, G. O'Sullivan and C. Sandau, Eds., ed Amsterdam: Elsevier, 2014, pp. 357-390.

[7] D. M. Pampanin, *Introduction to Petrogenic Polycyclic Aromatic Hydrocarbons (PAHs) in the Aquatic Environment* vol. 1: Bentham Science Publishers Ltd., 2017.

[8] R. van der Oost, J. Beyer, and N. P. Vermeulen, "Fish bioaccumulation and biomarkers in environmental risk assessment: a review," *Environ Toxicol Pharmacol,* vol. 13, pp. 57-149, Feb 2003.

[9] M. o. P. a. E. Norwegian Petroleum Directorate. (2012, read 01/12/2019). *Facts 2012* [Online]. Available: https://www.regjeringen.no/globalassets/upload/oed/pdf_filer_2/faktaheftet/og_facts_2012_web.pdf

[10] T. Haugland, R. T. Ottesen, and T. Volden, "Lead and polycyclic aromatic hydrocarbons (PAHs) in surface soil from day care centres in the city of Bergen, Norway," *Environmental Pollution,* vol. 153, pp. 266-272, 2008/05/01/ 2008.

[11] M. Andersson, M. Klug, O. A. Eggen, and R. T. Ottesen, "Polycyclic aromatic hydrocarbons (PAHs) in sediments from lake Lille Lungegårdsvannet in Bergen, western Norway; appraising pollution sources from the urban history," *Science of The Total Environment,* vol. 470-471, pp. 1160-1172, 2014/02/01/ 2014.

[12] T. M. Ansari, I. L. Marr, and N. Tariq, "Heavy Metals in Marine Pollution Perspective-A Mini Review," *Journal of Applied Sciences,* vol. 4, pp. 1-20, January 01, 2004 2004.

[13] (read 01/12/2019). *Orcas are living in toxic waters* [Online]. Available: https://georgiastrait.org/work/species-at-risk/orca-protection/southern-resident-orcas/orcas-are-living-in-toxic-waters/

[14] S. Sharifuzzaman, D. M. H. Rahman, S. M. Ashekuzzaman, M. Islam, S. Chowdhury, and M. S. Hossain, "Heavy Metals Accumulation in Coastal Sediments," ed, 2016, pp. 21-42.

[15] N. Heggemsnes. (2012, read 03/12/2019). *Bergen havn* [Online]. Available: https://snl.no/Bergen_havn

[16] G. P. WIJNEN. (2017). *Cruise ships consume 7,000 tons of fuel in Bergen harbour* [Online]. Available: https://norwaytoday.info/finance/cruise-ships-consume-7000-tons-fuel-bergen-harbour/

[17] K. Senthil Kumar, K. S. Sajwan, J. P. Richardson, and K. Kannan, "Contamination profiles of heavy metals, organochlorine pesticides, polycyclic aromatic hydrocarbons and alkylphenols in sediment and oyster collected from marsh/estuarine Savannah GA, USA," *Marine Pollution Bulletin,* vol. 56, pp. 136-149, 2008/01/01/ 2008.

[18] M. Paetzel, G. Nes, L. Leifsen, and H. Schrader, "Sediment pollution in the Vågen, Bergen harbour, Norway," *Environmental Geology,* vol. 43, pp. 476-483, 02/01 2003.

[19] D. Fernandes, S. Pujol, E. Pérez-Albaladejo, R. Tauler, M. J. Bebianno, and C. Porte, "Characterization of the environmental quality of sediments from two estuarine systems based on different in-vitro bioassays," *Marine Environmental Research,* vol. 96, pp. 127-135, 2014/05/01/ 2014.

[20] C. H. Walker, *Principles of ecotoxicology*, 3rd ed. ed. Boca Raton, Fla: CRC Taylor & Francis, 2006.

[21] K. Rehberger, C. Kropf, and H. Segner, "In vitro or not in vitro: a short journey through a long history," *Environmental Sciences Europe,* vol. 30, p. 23, 2018/06/26 2018.

[22] M. L. Fernandez-Cruz, T. Lammel, M. Connolly, E. Conde, A. I. Barrado, S. Derick, *et al.*, "Comparative cytotoxicity induced by bulk and nanoparticulated ZnO in the fish and human hepatoma cell lines PLHC-1 and Hep G2," *Nanotoxicology,* vol. 7, pp. 935-52, Aug 2013.

[23] R. Thibaut and C. Porte, "Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: cytotoxicity and interactions with cytochrome P450 1A," *Toxicol In Vitro,* vol. 22, pp. 1128-35, Aug 2008.

[24] D. Caminada, C. Escher, and K. Fent, "Cytotoxicity of pharmaceuticals found in aquatic systems: Comparison of PLHC-1 and RTG-2 fish cell lines," *Aquatic Toxicology,* vol. 79, pp. 114-123, 2006/08/23/ 2006.

[25] OECD, *Fish Toxicity Testing Framework*, 2014.

[26] A. Abrahamson, C. Andersson, M. E. Jonsson, O. Fogelberg, J. Orberg, B. Brunstrom, *et al.*, "Gill EROD in monitoring of CYP1A inducers in fish: a study in rainbow trout (Oncorhynchus mykiss) caged in Stockholm and Uppsala waters," *Aquat Toxicol,* vol. 85, pp. 1-8, Nov 15 2007.

[27] R. Fernandez, M. Yoshimizu, Y. Ezura, and T. Kimura, "Comparative Growth Response of Fish Cell Lines in Different Media, Temperatures, and Sodium Chloride Concentrations," *Fish Pathol.,* vol. 28, 01/01 1993.

[28] A. Kienzler, X. Tronchère, A. Devaux, and S. Bony, "Assessment of RTG-W1, RTL-W1, and PLHC-1 fish cell lines for genotoxicity testing of environmental pollutants by means of a Fpg-modified comet assay," *Toxicology in Vitro,* vol. 26, pp. 500-510, 2012/04/01/ 2012.

[29] K. Fent, "Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples," *Toxicol In Vitro,* vol. 15, pp. 477-88, Aug-Oct 2001.

[30] M. R. Stephen, C. J. Robert, and L. W. Phillip, *Principles of Toxicology: Environmental and Industrial Applications*, Third edition. ed.: United States: John Wiley & Sons Inc, 2014.

[31] V. Patravale, P. Dandekar, and R. Jain, "4 - Nanotoxicology: evaluating toxicity potential of drug-nanoparticles," in *Nanoparticulate Drug Delivery*, V. Patravale, P. Dandekar, and R. Jain, Eds., ed: Woodhead Publishing, 2012, pp. 123-155.

[32] J. O'Brien, I. Wilson, T. Orton, and F. Pognan, "Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity," *European Journal of Biochemistry,* vol. 267, pp. 5421-5426, 2000.

[33] X. Liu, R. Lo, and F. Gomez, "Facile Fabrication of an Enzyme Microreactor Using Magnetic Microbeads," 12/13 2019.

[34] S. M. Roberts, R. C. James, and P. L. Williams. (2015). *Principles of toxicology : environmental and industrial applications (Third edition. ed.)*.

[35] (2014, 11/12/2019). *An Introduction to Reactive Oxygen Species - Measurement of ROS in Cells* [Online]. Available: https://www.biotek.com/resources/white-papers/an-introduction-to-reactive-oxygen-species-measurement-of-ros-in-cells/

[36] J. R. Petrulis, G. Chen, S. Benn, J. LaMarre, and N. J. Bunce, "Application of the ethoxyresorufin-O-deethylase (EROD) assay to mixtures of halogenated aromatic compounds," *Environ Toxicol,* vol. 16, pp. 177-84, 2001.

[37] A. Mohammadi Bardbori, *Assay for quantitative determination of CYP1A1 enzyme activity using 7-Ethoxyresorufin as standard substrate (EROD assay)*, 2014.

[38] J. J. Whyte, R. E. Jung, C. J. Schmitt, and D. E. Tillitt, "Ethoxyresorufin-O-deethylase (EROD) Activity in Fish as a Biomarker of Chemical Exposure," *Critical Reviews in Toxicology,* vol. 30, pp. 347-570, 2000/01/01 2000.

[39] A. Katsumiti, *We are pleased to announce the first edition of our course on "In Vitro Toxicity Testing" from 10 to 14 December 2018. If you're interested, please contact us: Alberto Katsumiti (alberto.katsumiti@ehu.eus)*, 2018.

[40] U. o. Bergen. (2016, read 08/12/2019). *dCod 1.0: Decoding the systems toxicology of Atlantic cod* [Online]. Available: https://www.uib.no/en/dcod

[41] C. P. LeBel, H. Ischiropoulos, and S. C. Bondy, "Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress," *Chem Res Toxicol,* vol. 5, pp. 227-31, Mar-Apr 1992.

[42] E. Perez-Albaladejo, J. Rizzi, D. Fernandes, R. Lille-Langoy, O. A. Karlsen, A. Goksoyr, *et al.*, "Assessment of the environmental quality of coastal sediments by using a combination of in vitro bioassays," *Marine Pollution Bulletin,* vol. 108, pp. 53-61, Jul 15 2016.

[43] "Pierce™ BCA Protein Assay Kit," in *INSTRUCTIONS*, ed. Thermofisher Scientific.

[44] L. J. Casarett, *Casarett and Doull's Toxicology : the basic science of poisons*, 8th ed. ed. New York: McGraw-Hill Medical, 2013.

[45] D. R. Livingstone, C. L. Mitchelmore, L. D. Peters, S. C. M. O'Hara, J. P. Shaw, B. S. Chesman, *et al.*, "Development of hepatic CYP1A and blood vitellogenin in eel (Anguilla anguilla) for use as biomarkers in the Thames Estuary, UK," *Marine Environmental Research,* vol. 50, pp. 367-371, 2000/07/01/ 2000.

[46] S. M. Billiard, A. R. Timme-Laragy, D. M. Wassenberg, C. Cockman, and R. T. Di Giulio, "The Role of the Aryl Hydrocarbon Receptor Pathway in Mediating Synergistic Developmental Toxicity of Polycyclic Aromatic Hydrocarbons to Zebrafish," *Toxicological Sciences,* vol. 92, pp. 526-536, 2006.

[47] L. E. Lee, V. R. Dayeh, K. Schirmer, and N. C. Bols, "Applications and potential uses of fish gill cell lines: examples with RTgill-W1," *In Vitro Cell Dev Biol Anim,* vol. 45, pp. 127-34, Mar-Apr 2009.

[48] K. Schirmer, A. G. J. Chan, B. M. Greenberg, D. G. Dixon, and N. C. Bols, "Ability of 16 priority PAHs to be photocytotoxic to a cell line from the rainbow trout gill," *Toxicology,* vol. 127, pp. 143-155, 1998/05/15/ 1998.

[49] K. Schirmer, D. G. Dixon, B. M. Greenberg, and N. C. Bols, "Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill," *Toxicology,* vol. 127, pp. 129-141, 1998/05/15/ 1998.

[50] T. Bakke, T. Källqvist, A. Ruus, G. D. Breedveld, and K. Hylland, "Development of sediment quality criteria in Norway," *Journal of Soils and Sediments,* vol. 10, pp. 172-178, 2010/03/01 2010.

[51] J. Søndergaard, S. Wegeberg, D. Boertmann, L. Bach, P. Lassen, D. Clausen, *et al.*, *A chemical environmental screening study at the former Qullissat coal mine, West Greenland in 2015*, 2017.

Appendix

A. Raw data of chemical analysis of Sediment extracts from Bergen area

- PAHs

Beregning av PAH i sediment for MSD

Skjema Ref.id: .KJEM.ORG.SKJ.ARBSKJ-16

Dok.id.:	Versjon:	Forfatter:	Godkjent av:	Sist endret:
D02825	13.00	STB/ANE		21.08.2018

Mottaksjournalnr.:	2094			
ID PAH standardkurve:	Kal. PAH 19.09.17			
Kvantiteringsmetode:	PAH_DB17TWS_2018_3.M			
Komponent navn:	*(engelsk)*	RIS Phenanthrene-d10	**IS Naphthalene-d8**	Naphthalene
Komponent navn:	*(norsk)*	RIS Fenantren-d10	**IS Naftalen-d8**	Naftalen
Nivåer, ng/g tørrvekt				
LRM sediment 11.10.18				170.14
St.1 Helleneset sediment 11.10.18				168.84
St.4 Nordnes sediment 11.10.18				262.66
St.5 Puddefjord sediment 11.10.18				160.01
St.7 Korsfjorden sediment 11.10.18				20.35
St.8 Skogsvåg sediment 11.10.18				21.44
St.9 Mellom Sotra og Askøybroa sediment 11.10.18				88.75
St.2 Fisketorget sediment 11.10.18 (fort.: 1:9)				1651.67

Benzothiophene	**IS Biphenyl-d10**	2-Methylnaphthalene	1-methylnaphtalene	Biphenyl	2,6 - Dimethylnaphthalene	1,3-Dimethylnaphthalene
Benzotiofen	**IS Bifenyl-d10**	2-Metylnaftalen	1-metylnaftalen	Bifenyl	2,6 - Dimetylnaftalen	1,3-Dimetylnaftalen
0.00		349.74	374.15	43.39	99.37	381.51
8.76		73.92	56.33	29.00	31.82	64.33
8.37		208.61	199.03	65.94	65.01	281.03
6.86		64.99	53.15	22.51	27.53	58.30
0.44		24.34	23.42	8.57	5.16	25.74
0.86		14.02	11.83	5.83	9.35	13.36
4.78		25.87	23.07	10.51	12.90	34.48
82.80		708.52	720.52	299.47	298.19	879.33
2,3 Dimethylnaph	1,4 Dimethylnap	**IS Acenaphthylene-d8**	Acenaphthylene	Acenapht	Dibenzofuran	
2,3-Dimetylnaftaler	1,4 Dimetylnafta	**IS Acenaftylen-d8**	Acenaftylen	Acenafter	Dibenzofuran	
98.26	45.14		1.57	3.34	64.00	
21.58	8.31		48.87	29.23	62.97	
102.73	41.08		40.31	96.97	130.85	
18.60	8.28		36.60	75.10	69.09	
8.66	2.88		1.28	1.89	11.18	
4.11	1.46		3.05	2.74	10.76	
11.11	5.11		37.69	40.54	45.52	
294.79	124.44		329.26	1016.10	1620.57	

1,3,7-Trimethylnaphthalen	2,3,5-Trimethylnaphtalene	1,2,3-Trimethylnaphtha	1,4,6,7-Tetramethylnaphthalene			
1,3,7-Trimetylnaftalen	2,3,5-Trimetylnaftalen	1,2,3-Trimetylnaftalen	1,4,6,7-Tetrametylnaftalen			
93.22	95.33	22.08	10.38			
20.41	26.77	5.57	4.66			
121.41	249.51	51.95	33.72			
20.56	25.79	5.83	4.33			
5.82	7.38	1.62	0.84			
3.36	4.07	0.80	0.58			
13.13	18.46	3.70	3.22			
337.31	368.78	83.41	60.21			
1,2,5,6-Tetramethylnaphth	**IS Anthracene-d10**	Fluorene	1-Methylfluorene	9-Ethylfluorene	Dibenzothiophene	
1,2,5,6-Tetrametylnaftalen	**IS Antracen-d10**	Fluoren	1-Metylfluoren	9-Etylfluoren	Dibenzotiofen	
65.80		51.58	76.77	1.38	25.94	
18.83		60.52	20.65	0.37	29.28	
209.58		117.79	93.78	1.17	75.55	
19.16		79.76	22.50	0.30	38.79	
6.53		6.12	6.12	0.00	4.15	
2.96		5.96	3.44	0.00	3.85	
12.22		63.50	14.35	0.40	35.87	
314.34		2150.55	341.50	7.14	640.69	

Phenanthrene	Anthracene	4-methyldibenzothiophene	3-Methylphenanthrene	2-Methylphenanthrene	
Fenantren	Antracen	4-metyldibenzotiofen	3-Metylfenantren	2-Metylfenantren	
299.43	3.11	25.67	122.98	142.79	
515.28	186.94	17.41	85.64	109.60	
1528.90	312.81	42.48	321.43	385.96	
732.58	209.32	19.34	95.93	128.57	
40.96	4.41	3.10	11.72	16.10	
47.51	13.07	2.50	9.58	12.27	
711.32	261.36	11.48	76.04	95.76	
13061.80	4343.50	230.78	1412.98	1785.43	
9-Methylphenai	1-Methylphe	4-ethyldibenzothiophene	3,6-Dimethylphenantren	4-propyldibenzothiophene	
9-Metylfenantre	1-Metylfenai	4-etyldibenzotiofen	3,6-Dimetylfenantren	4-propyldibenzotiofen	
171.89	119.95	3.87	15.86	3.33	
66.58	73.74	2.80	13.01	1.31	
285.65	316.10	4.93	40.09	2.77	
67.43	74.53	3.10	14.28	2.10	
12.20	13.45	0.42	1.30	0.44	
8.45	8.09	0.36	1.06	0.39	
48.04	54.31	0.92	7.11	0.56	
779.36	1298.48	26.61	139.57	17.11	

1,7-Dimethylphenanthrene	1,2-Dimethylphenanthrene	2,6,9-Trimethylphenanthrene	1,2,6-Trimethylphenanthrene	
1,7-Dimetylfenantren	1,2-Dimetylfenantren	2,6,9-Trimetylfenantren	1,2,6-Trimetylfenantren	
97.83	24.93	27.65	17.47	
88.44	13.73	23.39	17.58	
272.28	53.56	62.87	54.15	
90.35	14.81	31.80	21.40	
20.83	2.28	2.97	2.26	
8.59	1.72	3.01	1.70	
47.77	10.05	13.93	9.97	
2628.06	207.31	315.36	314.12	
1,2,7-Trimethylphenanthrene	1,2,6,9-Tetramethylphenanthrene	**IS Pyrene-d10**	Fluoranthene	Pyrene
1,2,7-Trimetylfenantren	1,2,6,9-Tetrametylfenantren	**IS Pyren-d10**	Fluoranten	Pyren
9.80	3.18		44.08	66.33
10.17	2.67		1196.10	1085.31
29.31	5.66		2395.80	2291.14
11.40	4.35		1319.21	1349.70
1.33	0.35		27.16	26.37
1.01	0.28		73.68	58.60
5.31	1.03		1283.10	1074.92
196.48	53.72		17433.48	16320.28

Benz(a)anthracene	Chrysene	**IS Perylene-d1**	Benzo(b)fluoranthene	Benzo(k)fluoranthene	Benzo(j)fluoranthene	Benzo(e)pyrene	
Benz[*a*]antracen	Krysen	**IS Perylen-d12**	Benzo[*b*]fluoranten	Benzo[*k*]fluoranten	Benzo[*j*]fluoranten	Benzo[*e*]pyren	
16.73	48.90		77.01	14.03	15.73	110.27	
812.26	580.41		576.21	312.25	284.52	412.04	
1796.73	1108.92		984.05	526.73	494.93	758.68	
843.71	723.90		654.94	321.31	288.19	492.45	
16.42	19.94		85.85	31.70	29.86	55.70	
50.22	46.78		124.62	51.60	47.31	77.99	
648.11	515.06		444.21	223.65	208.39	326.53	
8890.29	7117.64		1251.54	661.09	619.11	911.57	
Benzo(a)pyrene	Perylene	**IS Indeno[1,2,3**	Indeno(1,2,3-cd)pyrene	Dibenz(a,h)anthracene	Benzo(ghi)perylene	**ng/g dry weight**	
Benzo[*a*]pyren	Perylen	**IS Indeno[1,2,3**	Indeno[1,2,3-*cd*]pyren	Dibenz[*a,h*]antracen	Benzo[*ghi*]perylen		
						Sum alle	
25.98	94.76		25.43	6.68	98.91	170.14	
631.65	219.46		952.79	205.97	1012.07	1462.81	
1019.41	355.06		1275.34	369.06	1354.17	7499.74	
669.40	235.49		817.44	219.18	877.64	18314.03	
29.08	77.91		55.71	9.02	53.26	24367.80	
58.44	31.47		179.71	30.75	171.52	24746.06	
515.85	195.30		431.32	101.07	487.44	25496.97	
1469.24	502.98		5709.65	1643.35	5923.80	31621.23	

- Metals

			Tørrstoff	Kobber (Cu)	Krom (Cr)	Nikkel (Ni)	Sink (Zn)	Arsen (As) Premium LOQ	Bly (Pb) Premium LOQ	Kadmium (Cd) Premium LOQ	Kvikksølv (Hg) Premium LOQ
			Tørrstoff	Kobber (Cu)	Krom (Cr)	Nikkel (Ni)	Sink (Zn)	Arsen (As)	Bly (Pb)	Kadmium (Cd)	Kvikksølv (Hg)
Prøveserie	Prøvekode	Prøvereferanse	%	mg/kg TS	mg/kg TS	mg/kg TS	mg/kg TS	mg/kg TS	mg/kg TS	mg/kg TS	mg/kg TS
EUNOBE-00031379	441-2018-1211-144	Stasjon 1 Helleneset	33,2	320	94	54	1200	20	290	< 0,010	0,672
EUNOBE-00031379	441-2018-1211-145	Stasjon 2 Fisketorget	49,0	290	43	18	870	27	570	1,8	6,58
EUNOBE-00031379	441-2018-1211-146	Stasjon 4 Nordnes	67,6	210	23	8,9	120	15	85	0,028	0,444
EUNOBE-00031379	441-2018-1211-147	Stasjon 5 Puddefjord	60,3	210	57	19	370	12	140	0,39	1,22
EUNOBE-00031379	441-2018-1211-148	Stasjon 7 Korsfjord	56,2	18	44	31	83	9,7	25	0,073	0,014
EUNOBE-00031379	441-2018-1211-149	Stasjon 8 Skogsvåg	51,7	23	36	24	96	7,1	49	0,075	0,022
EUNOBE-00031379	441-2018-1211-150	Stasjon 9 Mellom Sotra- og Askøybrua	62,5	23	24	11	85	9,1	43	0,082	0,062

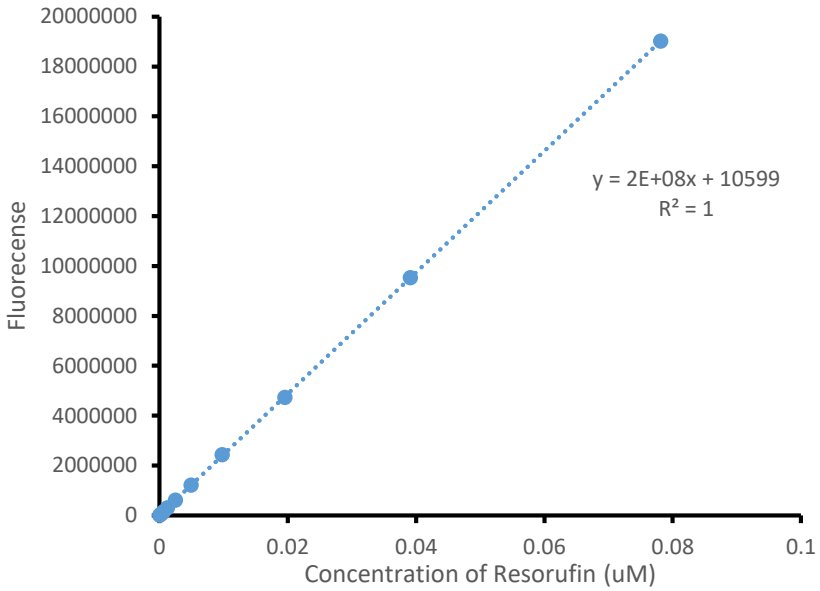
B. Sample sites map Converted in DMS

					Converted in DMS (degrees, minutes)[1]	
					Latitude N ___ ° ___ '	Longitude E ___ ° ___ '
No	Station	Latitude N	Longitude E	Depth (m)	Latitude N	Longitude E
2	Fisketorget	60 23.782	5 19.381	9	60.3963667	5.323016667
4	Nordnes	60 23.277	5 18.382	90	60.38795	5.306366667
5	Puddefjord	60 23.046	5 18.908	38	60.3841	5.315133333
1	Helleneset	60 25.725	5 16.275	332	60.42875	5.27125
9	Askøy S	60 22.851	5 10.321	226	60.38085	5.172016667
8	Skogsvåg	60 15.771	5 08.071	220	60.26285	5.134516667
7	Korsfjorden	60 08.377	4 57.371	614	60.1396167	4.956183333

[11] GPS data was converted in DMS with the original information of degrees and minutes but seconds.

C. Prepared Calibration curve for EROD activity

a. Quantitative calibration curve of 7-hydroxyresorufin



b. Quantitative BSA serum calibration curve by BCA kit