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The utility of in vitro Precision-Cut Liver Slices to investigate toxicity pathways of contaminants in polar cod (Boreogadus saida)

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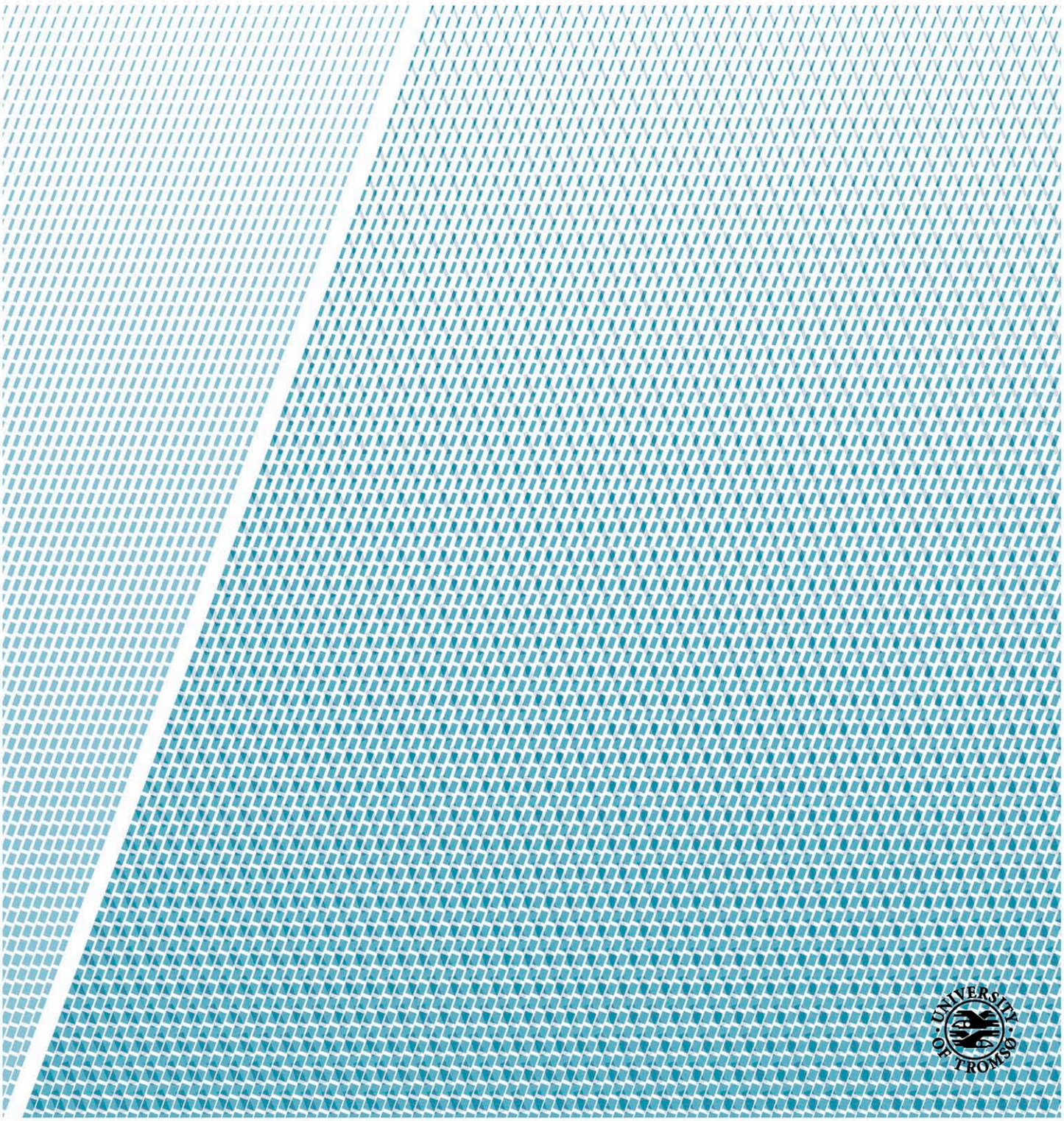

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Libe Aranguren Abadia

ABSTRACT

The development of *in vitro* methodologies has contributed significantly in elucidating mechanisms of xenobiotic toxicity in aquatic organisms. The present study aimed to develop the *in vitro* methodology Precision-Cut Liver Slices (PCLS) and to investigate the effects of model compounds (WY-14,643 [WY] and benzo(a)pyrene [BaP]) and two mixtures of 2- and 3-4-ring polycyclic aromatic hydrocarbons (PAHs), respectively, in the Arctic key species polar cod (*Boreogadus saida*). For each model compound and PAHs mixtures, PCLS (4 or 5 replicate slices per concentration) of 2 or 3 fish replicate were exposed to series of increasing concentrations (4 or 5 concentrations) during 48hr. Transcriptional responses of genes involved in biotransformation (*cyp1a1*), lipid metabolism (*pparα*, *aox*) and reproduction (*vtg*) were investigated. Viability of the slices was tested through the measurement of lactate dehydrogenase (LDH) activity in culture medium and reproducibility of the slice thickness (250µm) was assessed through histological analysis. Analysis of transcription levels only showed significant and dose-dependent up-regulation of *cyp1a1* in PCLS exposed to BaP and 3-4-ring PAHs mixture. On the other hand, transcription levels of *aox*, *pparα* and *vtg* did not significantly change for any of the model compounds and PAHs mixtures tested. In general, transcriptional levels responses of all target genes were very different between fish replicate and experiments, especially between PAHs mixtures experiments. Slice thickness, sex, PAHs physicochemical properties and species sensitivity were considered potential sources of variability on target genes transcriptional level responses. PCLS was successfully developed and the target genes responses observed show potential deleterious effects of PAHs on important metabolic pathways in polar cod. PCLS are deemed a valuable tool for studying toxicity mechanisms of different model compounds and PAHs mixtures in polar cod. However, an improvement of PCLS methodology should be considered for further studies.

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1. INTRODUCTION

1.1. Pollutants in the Arctic and risk for Arctic marine organisms

In the last decades, Arctic regions have been intensely affected by anthropogenic pollution coming from southern industrialized areas of Europe, North America and Asia (Law & Stohl, 2007). Combustion, biomass and waste burning, industry emissions, metallurgical processes, mining and agriculture are the main anthropogenic sources of pollution. Some volatile and semi-volatile contaminants from these sources, such as heavy metals, pesticides, persistent organic pollutants (POPs) and polycyclic aromatic hydrocarbons (PAHs) are transported to Arctic areas by air, oceanic currents and rivers (Figure 1) (AMAP report, 2009; Hung et al. 2010). The North Atlantic Oscillation (NAO) exerts a strong control on the atmospheric pollution transport into the Arctic, particularly in winter and spring (Shindell et al. 2008). This climatic phenomenon redistributes the atmospheric mass between the Arctic and the subtropical Atlantic (Hurrell, 1995). Long-range atmospheric transport of contaminants occurs at a faster pace than ocean transport, delivering contaminants to the Arctic in a matter of days, through a series of surface deposition and re-volatilization cycles (AMAP report, 2002; Gouin et al. 2004). River discharge is also an important pollution pathway for dissolved and particulate contaminants from land, carried and deposited into the Arctic waters (Harms et al. 2000; Carroll et al. 2008). For instance, the Ob, Yenisei and Lena rivers in Russia and the Mackenzie river in Canada significantly contribute to the inflow of freshwater containing PAHs to the Arctic shelf seas (Yunker & MacDonald, 1995; Fernandes & Sicre, 1999; Yunker et al. 2002).

Oil and gas activities, maritime shipping and tourism present an increasing risk of pollution to the Arctic. In the near future, larger areas of open water will increase the accessibility of currently ice-covered regions, allowing petroleum exploration and prospection. Longer ice-free summers have allowed for new shipping routes along the Arctic shelf seas and may also allow for transportation across the Arctic Ocean. Lastly, increasing tourism, including cruises and snowmobile excursions contribute to a local release of contaminants which may

affect Arctic habitats and their organisms (Gössling & Hall, 2006; AMAP report, 2008; Madsen et al. 2009).

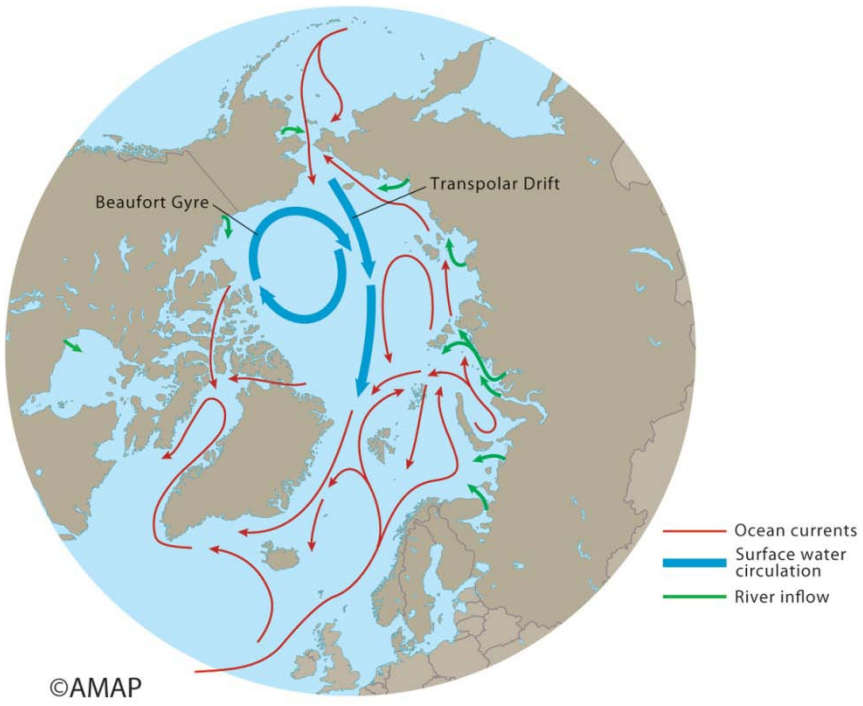

Figure 1: Different transport pollution pathways to the Arctic (AMAP report, 2002).

Arctic continental shelves are known to contain large petroleum hydrocarbon reserves and may be one of the world's largest remaining prospective region (USGS, 2003). The United States Geological Survey (USGS) estimates that the area north of the Arctic Circle could hold about 30% and 13% of the world's undiscovered gas and oil, respectively (USGS fact sheet 2008-3049, 2008). Exploration of the Barents Sea resources, a key petroleum province in the Arctic, started in 1981 and was opened for petroleum operations in 2013 (EY report, 2013; Oil and gas association report, 2014).

Operational discharge from the oil and gas industry introduces hazardous waste material (drilling mud and cuttings) and produced water to the environment. The Norwegian Environment Agency, divides these hazardous substances into four categories (color coded)

depending on their threat to the environment (Figure 2 Figures A1-2) (AMAP report, 2008; Oil and gas association report, 2014). In 1996, the Norwegian authorities set a goal for "zero-harmful discharges" during the development of oil fields, which did not include exploration drilling on the Norwegian Continental Shelf (The North Sea, The Norwegian Sea and the Barents Sea) (White paper No.58). During the following years, discharges of produced water, a mixture of different chemicals including heavy metals, alkylphenols, organic acids, phenols and PAHs, to the marine environment increased (Oil and gas association report, 2014). This situation led to more restrictive regulations which focused on the protection of the area from the Lofoten Islands to the Norwegian waters of the Barents Sea. In 2006, a "physical zero discharges" policy prohibited produced water, drill cuttings and drilling mud discharge, by petroleum operations, with a few exceptions (White Paper No. 8 2005–2006; Knol, 2011). This area is well known for being one of the most productive shelf regions in the Arctic Ocean. It holds nursery areas of major fish stocks which are vital to the fisheries industry and the diet of seabirds and marine mammals, making its protection and conservation critical (Sakshaug et al. 2009).

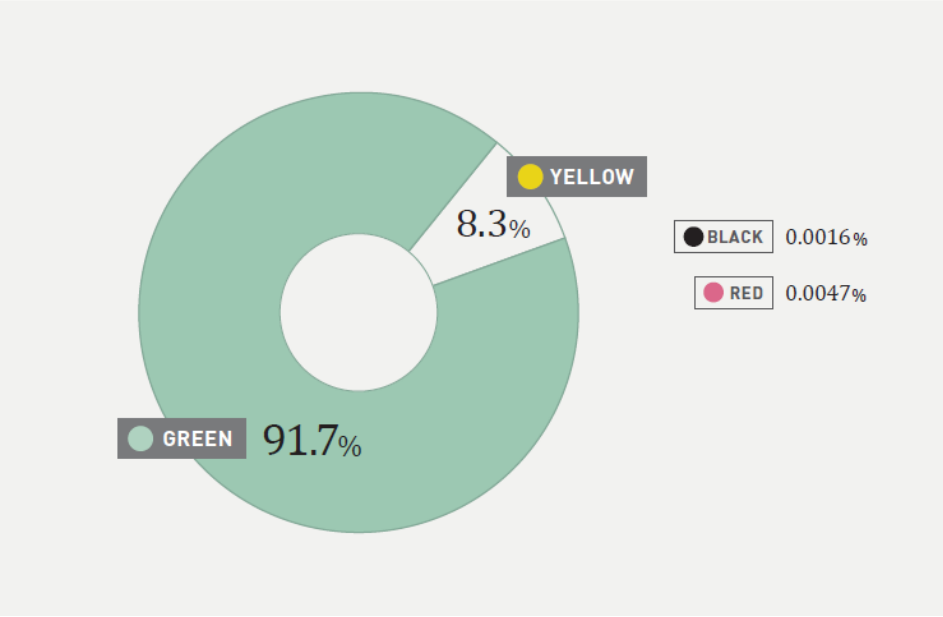

Figure 2: Hazardous substances (%) released by operational petroleum activities in Norwegian waters in 2013. Substances are divided into color categories by the Norwegian Environmental Agency depending on their threat to the environment (Figures A1-2) (Oil and gas association report, 2014).

Accidental petroleum discharge also presents a risk to Arctic ecosystems. Challenging environmental conditions such as the presence of ice, constant darkness during the polar night and the remoteness of the Arctic may enhance accidental discharge such as pipeline leaks and oil spills from transportation. Furthermore, oil spill responses in these severe environmental conditions are not yet fully developed, increasing the vulnerability of these ecosystems to oil pollution (AMAP report, 2008). Crude oil consists of thousands of different chemical compounds. The main groups of compounds are alkanes (paraffins), cycloalkanes (cycloparaffins), sulphur compounds, aromatics and PAHs. PAHs represent a small fraction of crude oil but are considered the most toxic fraction.

1.2. Polycyclic aromatic hydrocarbons (PAHs)

PAHs are an ubiquitous group of organic contaminants, composed of multiple aromatic rings arranged in planar structures. They are present as volatile, semi-volatile and particulate pollutants. This classification of compounds can be divided into PAHs of petrogenic and pyrogenic origins. Pyrogenic PAHs are formed from the incomplete combustion of organic matter, automobile exhaust, stationary matter (e.g. coal-fired), domestic matter (e.g. tobacco smoke), and area source matter (e.g. forest fires and agricultural burning) in the southern industrialized areas (Harvey, 1991; Samanta et al. 2002). Pyrogenic compounds generally include a majority of high molecular weight PAHs (HPAHs, 4- and more ring PAHs). On the other hand, petrogenic PAHs are constituents of crude oil, where low molecular weight (LPAHs, 2- and 3- ring PAHs) PAHs are usually more represented. This latter group also has a higher proportion of alkylated PAHs (i.e. with alkyl side chains) compared to the pyrogenic PAHs. Alkylated PAHs have been shown to often have an enhanced toxic potential due to an increase in hydrophobicity and potential for bioaccumulation (Meador, 2008).

PAHs have very different physicochemical properties depending on their molecular size, and alkylation. For instance, PAHs with low molecular weights are slightly more water-soluble than PAHs with high molecular weights. Thus, bioavailability of LPAHs from the water and through the gills of aquatic organisms is higher than that of HPAHs. In

opposition, HPAHs tend to bind to organic and inorganic particulates within the water column and accumulate in the sediments and are more bioavailable through food intake. Furthermore, the more hydrophobic HPAHs are more likely to be accumulated in tissues rich in lipids once taken up by organism. Therefore, the physicochemical properties of PAHs, including their water solubility and lipophilicity, are key features for understanding their bioavailability to organisms and their toxicity (Harvey, 1991; Meador, 2008).

PAHs toxicity is highly variable among species and may be strongly influenced by exposure time, bioavailability, bioaccumulation and biotransformation processes. Once these compounds are taken up by organisms, they are biotransformed into water soluble metabolites which are easily excreted (Bakke et al. 2013). Biotransformation of PAHs (and other planar organic contaminants) comprises three phases; in phase I, intra cellular PAHs bind to the nuclear aryl hydrocarbon receptor (AhR). This complex is translocated to the nucleus and by dimerization with the AhR nuclear transporter (ARNT) forms a transcriptionally active complex that bind to xenobiotic response elements (XRE) and thereby up-regulates the transcription of a battery of target genes such as the cytochrome P4501A gene (*cyp1a)* (Figure 3). CYP1A enzymes synthesized by this process convert PAHs into more water-soluble compounds through oxidation reactions. In phase II, enzymes conjugate the modified PAH compound with another molecule forming a water soluble metabolite which can be excreted by transporters (Phase III) (Klaassen, 2013).

Biotransformation of PAHs has been well studied in fish species such as thicklip grey mullet (*Chelon labrosus*) (Bilbao et al. 2006; Bilbao et al. 2010 a,b), mullet (*Mugil* cephalus) (Orbea et al. 2002), brown trout (*Salmo trutta*) (Meland et al. 2011), turbot (*Scophthalmus maximus*) (Ruiz et al. 2012) and polar cod (Nahrgang et al. 2010a). PAH metabolites formed may show a higher toxicity than their parent compounds due to a process called bioactivation (Meador, 2008). Some of these PAH metabolites, such as benzo(a)pyrene-7,8-dihydrodiol formed from benzo(a)pyrene (BaP), are carcinogenic, forming DNA adducts and causing tissue damage (Meador, 2008).

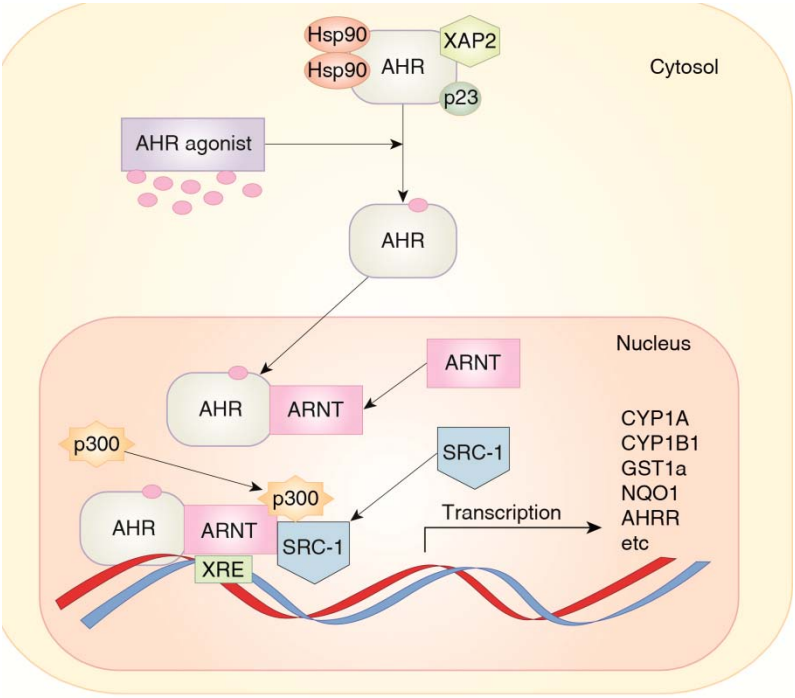

Figure 3: Initiation of biotransformation processes of PAHs. Phase I: PAH (AHR agonist) binds to the AHR nuclear receptor. AHR-PAH complex dimerizate with the AHR nuclear transporter (ARNT) that forms the transcriptionally active complex that binds to xenobiotic response elements (XRE) and thereby up regulates a number of genes, including several involved in biotransformation, as *cyp1a,* indicate here, as well as the AHR repressor (AHRR) that provides negative feedback of the system (Klaassen, 2013).

Once PAHs or their metabolites have reached their target tissues, different mechanisms of toxicity are triggered. Genotoxicity, carcinogenicity, endocrine disruption and alteration of physiological processes such as growth are the most studied (Meador et al. 2006; Bakke et al. 2013). Many researchers have focused their attention on reproductive endocrine disruption since some PAHs may have an estrogenic effect and induce vitellogenin (*vtg*) transcription (Monteverdi & Di Giulio, 2000; Goksøyr 2006; Navas & Segner, 2006). The *vtg* gene encodes the egg yolk precursor protein vitellogenin (VTG) that is normally produced in the liver of female fish and transported to the ovaries where it aids egg maturation (Schmieder et al. 2000). Production of VTG protein is mediated by the binding of 17β-estradiol hormones to the estrogen receptors (ER). Once this receptor-ligand complex is formed, its subsequent interaction with estrogen-responsive elements (ERE) on the DNA results in *vtg* transcription. The hepatic levels of VTG in male fish are commonly used as biomarker of exposure to estrogen-active compounds (Arukwe & Goksøyr, 2003;

Navas & Segner, 2006). Some PAHs have a similar structure to estrogen hormones, enabling them to bind to the ER and consequently produce estrogenic effects (Lye, 2000). However, a cross-talk between AhR and ER has been shown in different fish species, leading to negative effects on estrogenic responses (Yong et al. 2004; Navas & Segner, 2006; Kawahara et al. 2009). AhR agonists such as benzo(a)pyrene (BaP), may thus show anti-estrogenic effects on *vtg* transcription (Yong et al. 2004; Navas & Segner, 2006; Kawahara et al. 2009).

Another, arguably less well studied aspect of PAH toxicity is related to peroxisome proliferation (PP), i.e. the increase in volume and number of peroxisomes, and the potential for disruption of lipid metabolism. Peroxisomes are involved in many cellular processes, synthesizing key enzymes of the β-oxidation process, including the acyl-CoA oxidase (AOX). Peroxisome proliferation induced by xenobiotic compounds such as phthalate ester, plasticizers, PAHs, pirinixic acid WY-14643 (WY) and certain pesticides is well documented in mammals species such as rodents (Peters et al. 1997; Woods et al. 2007; Arzuaga et al. 2009) and seals (Castelli et al. 2014) and has also been linked to the development of liver carcinogenesis in rodents (Peters et al. 1997; Morimura et al. 2006; Arzuaga et al. 2009). Peroxisome proliferation has also been shown in both invertebrate species (Cajaraville et al. 2003; Bilbao et al. 2006; Cajaraville & Ortiz-Zarragoitia, 2007) and different fish species (Orbea et al. 2002; Bilbao et al. 2006; Bilbao et al. 2010 a,b; Ruiz et al. 2012) expose to xenobitocis. Some of these studies have also shown how PP can lead to an increase in AOX enzyme synthesis (Orbea et al. 2002; Bilbao et al. 2006; Cajaraville & Ortiz-Zarragoitia, 2007; Bilbao et al. 2010 a,b; Ruiz et al. 2012). However, the mechanisms implicated and the potential adverse effects to aquatic organisms are not well understood but are suggested to be linked to the peroxisome proliferator-activated receptors (PPARs) (Cajaraville et al. 2003). The PPARs are a group of nuclear receptors formed by PPARα, PPARβ and PPARγ which have different roles in fatty acid metabolism and are regulated by endogenous compounds, such as fatty acids (Maglich et al. 2003). These receptors have been described in several fish species (Andersen et al. 2000; Leaver et al. 2005). Peroxisome proliferation has been linked in particular linked to the activation of the receptor PPARα subtype.

1.3. Polar cod as a keystone and Arctic model species

Polar cod is a circumpolar species, being one of the most abundant fish species in the Arctic. In the Barents Sea, polar cod is found from Svalbard to Novaya Zemlya and overlaps with areas of oil and gas activities (Figure 4). This small gadid fish species (10-20 cm) plays an important key role in this ecosystem, transferring energy from lower to higher trophic levels (Welch et al. 1992). The diet of polar cod is varied with regard to season, area and size and is composed mainly of copepods, amphipods, or euphausiids (Bradstreet & Cross, 1982; Lønne & Gulliksen, 1989). Many marine species, such as Atlantic cod (*Gadus* morhua), harp seals (*Pagophilus groenlandicus*), white whales (*Delphinapterus leucas*) and seabirds feed on polar cod (Bradstreet & Cross, 1982; Gjøsæter, 1995). Polar cod is a bathypelagic species and occurs mainly at depths of 200-500m (Geoffroy et al. 2011) and at temperature between 0 and 4 °C, although polar cod can tolerate lower temperatures due to antifreeze proteins (Chen et al. 1997) and also higher temperatures for short periods of time (Lønne & Gulliksen, 1989; Gradinger & Bluhm, 2004). This species is characterized by its short life span (up to 7 years) (Craig et al. 1982; Lonne & Gulliksen, 1989; Rand et al. 2013) and gender specific reproductive strategy, with male specimens maturing at an earlier age than females (Nahrgang et al. 2014). Broadcast spawning occurs synchronously once a year and takes places under the ice or close to ice cover from December to March (Craig et al. 1982; Sakshaug et al. 2009; Nahrgang et al. 2014). Early life stages (buoyant eggs) of polar cod are thus associated to the ice edge and maturing specimens descend to greater depth in the first year of life (Hop &Gjøsæter, 2013).

Polar cod has been considered as a potential indicator species for environmental monitoring in Arctic ecosystems due to its pan-Arctic distribution and its importance in the Arctic food webs (Jonsson et al. 2010). Therefore, polar cod has been increasingly studied in recent years to characterize the toxic response to petroleum related compounds using biomarker responses (Christiansen & George, 1995; Nahrgang et al. (2009, 2010 a, b, c). These studies showed dose-dependent *cyp1a1* mRNA expression of specimens exposed to both BaP and crude oil, together with other genes and enzymes involved in the biotransformation

processes. However, these studies have only focused on the induction of biomarkers used in environmental monitoring, without investigating biological effects of petroleum compounds on important physiological processes such as reproduction and lipid metabolism.

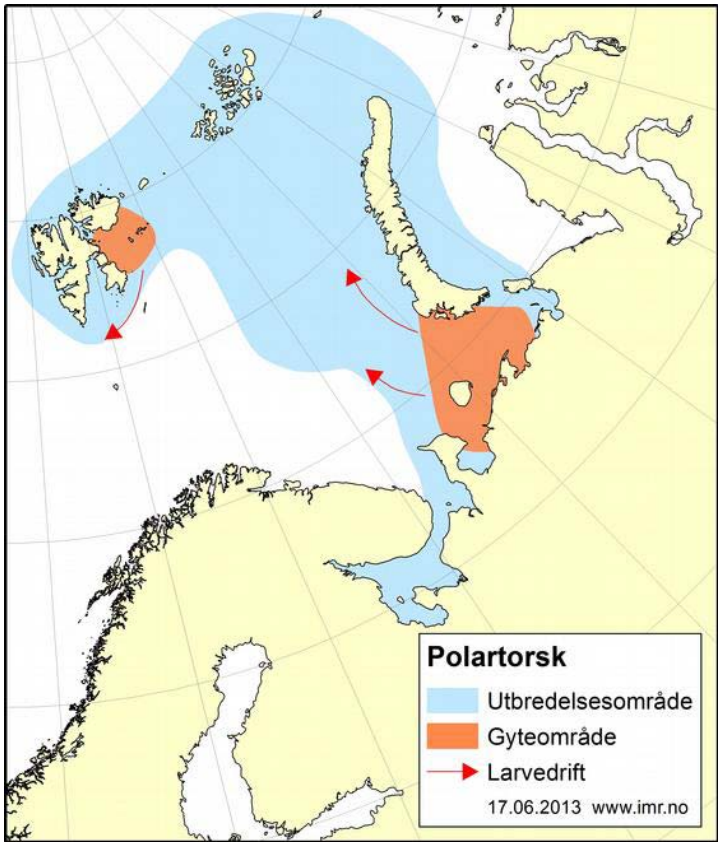

Figure 4: Polar cod distribution, spawning areas and larval drift in the Barents Sea (Sakshaug et al. 2009).

1.4. Objectives and approach

The overall objective of this project was to develop the Precision Cut Liver Slice (PCLS) *in vitro* methodology for polar cod and study some toxic effects of PAHs related to disruption of reproduction and lipid metabolism. Although the most common approach to toxicological studies is the use of *in vivo* experimentation, the difficulty to access wild specimens, their maintenance in the laboratories and the general ethical challenge of using

live organisms lead to the need for development of *in vitro* methodologies. In the present study, an *in vitro* technique was developed using a reduced number of animals. *In vitro* methods present the advantage of being cheaper, less time consuming, and more sensitive methods to detect rapid and consistent effects of chemical substances (Gad, 2000).

Over the years, the use of *in vitro* techniques have become more popular. Precision-cut liver slices (PCLS) is widely used as an *in vitro* method and has proven to be useful for the study of biochemical functions such as endogenous metabolism, biotransformation and induction and transport of drugs and other xenobiotics, as well as for toxicological studies as mentioned earlier (de Graaf et al. 2010). In the 1920's, O. Warburg and H.A. Krebs introduced the tissue slicing technique; creating hand-cut liver slices using a razor blade (Krebs, 1933; Warbug, 1923). This manual procedure ended up with thick slices which were not optimal for long time incubation. Slices incubated for a long time tended towards cell death since inner cell layers suffered a lack of nutrients and oxygen due to incomplete absorption (Smith et al. 1989). In the 70s, hepatocytes isolation culture method replaced slices and became the model of choice for toxicity studies, even though commercial slicers were available. Although hepatocytes only represent 80% of the liver volume, this method assumed that this fraction was representative of all liver functions. However for studying complex, multicellular liver functions a model containing all liver cells were needed. Furthermore, cultured hepatocytes were found to lose their differentiation (de Graaf et al. 2010). This led to the development of a manually operated microtome that produced thin slices resulting in ample oxygen and nutrient supply to the inner cell layers. This microtome increased slicing precision with regards to thickness shape and minimized tissue damage. Smith et al. (1985) started using this machine in *in vitro* hepatoxicity studies on fresh human, rat and rabbit liver. During the following years, optimization of appropriate culture media and dynamic incubation systems let the development of long-time (up to 5 days) toxicological studies (Fisher et al. 1995a,b). The implementation of this new machine enabled a broad use across many different animals' species and the maintenance of cell architecture and cell communication, which allowed microtome slices to become the preferred *in vitro* methodology (Lake & Price, 2013). Moreover, toxicity studies comparing *in vivo* and *in vitro* PCLS methods in rats proved similar xenobiotic-induced responses

(Boess et al. 2003; Jessen et al. 2003; Lake & Price, 2013), making the PCLS method a reliable and suitable alternative to *in vivo* testing.

PCLS has been commonly used in mammalian toxicological studies, especially on rats (Boess et al. 2003; Jessen et al. 2003; de Graaf et al. 2010). On the other hand, the use of PCLS on aquatic organisms such as fish is only in its infancy. Ecotoxicology studies using PCLS on fish include testing of vitellogenin expression in rainbow trout (*Oncorhynchus mykiss*) exposed to xenobiotic compounds (Schmieder et al. 2000), expression of CYP1A activity in Atlantic salmon *(Salmo salar)* and grenadier (*Coryphaenoides rupestris*) exposed to different pollutants (Lemaire et al. 2011; Lemaire et al. 2012). Most recently, mRNA expression of genes involved in biotransformation and reproduction processes were studied in Atlantic cod *(Gadus morhua*) slices exposed to two different model compounds (Eide et al. 2014).

The main objective of the present study was to 1) develop the PCLS *in vitro* method for polar cod and 2) study the effects of model pollutants (BaP, WY) and PAHs mixtures (2-ring and 3-4-ring PAHs mixtures) on selected target genes. A viability experiment was performed to test the protocol parameters selected (culture medium, temperature, etc), and assess liver slices viability, measured through LDH activity. This experiment allowed determining the optimal time for slice exposure. LDH activity analyses were also performed in exposure experiments in order to investigate how concentrations affected slices viability. In addition, fish slice thickness was measured by histological analyses in some experiments. A second objective was to investigate the mRNA expression of target genes over increasing concentrations of different model pollutants (BaP, WY) and PAHs mixtures (2-ring and 3-4-ring PAHs mixtures). Benzo(a)pyrene is known as an AhR agonist PAH and exposure of liver slices to BaP was expected to increase *cyp1a* mRNA expression and also lead to an anti-estrogenic effect, inhibiting *vtg* mRNA expression. WY is known as a PPARα agonist in mammals and has been shown to induce pparα, *aox* and *cyp1a1* mRNA expression (Woods et al. 2007; Tong et al. 2007). Exposure of liver slices to WY-14643 was thus expected to increase pparα, *aox* and *cyp1a1* mRNA expression in polar cod PCLS. Finally, the two PAHs mixtures contained compounds with different structure and toxicity

so different responses in *cyp1a, aox, pparα* and *vtg* mRNA expression were expected between experiments.

2. MATERIAL AND METHODS

2.1. Study species

Polar cod (*Boreogadus saida*) were caught in Rijpfjorden (80°N 22°E), on Svalbard, in January 2012 and brought to the biological station of UiT, The Arctic University of Norway, in Kårvika. The fish were kept in tanks under continuous, unfiltered water flow (3°C) and natural light conditions and fed until satiation twice per week with frozen *Calanus finmarchicus* (purchased from *Calanus AS*). On the day of the experiment, animals were transported from the biological station to UiT (The Arctic University of Norway) in 50L bags containing oxygenated seawater maintained at 3 °C.

2.2. Precision-cut liver slices (PCLS) procedure and slices incubation

This protocol was based on a literature review, including PCLS *in vitro* studies performed on fish (see appendix, Table A1). All consumables were autoclaved and both excision buffer and culture media were sterilized. Working surfaces were cleaned with 70% ethanol. The excision buffer and culture media were maintained in incubation cabinet at 3°C and slices were also kept in incubation cabinet at 3°C until exposure start.

Polar cod were euthanized with a solution of tricaine (Finquel®). Once immobilized, polar cod were rinsed with 70% ethanol and opened through the ventral side. The liver was cleaned with Hank's Balanced Salt Solution lacking magnesium and calcium (HBSS-, Life technologies cat no. 14175-053, Carlsbad, CA, United States) before being gently excised and transferred to a sterile petri dish containing HBSS- at 3°C. The hepatic portal vein was perfused to remove blood using HBSS- and a 10ml syringe and a 23G needle. The liver was then transferred to a new HBSS solution containing Mg and Ca (HBSS+) (Life technologies cat no. 24020-117, Carlsbad, CA, United States) at 3°C. A cylinder-shaped coring tool (diameter 8mm) was used to core out pieces from the liver. Cores were transferred to HBSS+ until slicing.

The precision-cut slicing method was performed with a Leica VT1200 S vibrating blade microtome (LEICA biosystems, Nussloch, Germany), according to procedure outlined in Lemaire et al. (2012). The core was glued to a core holder and inserted into a cutting tray containing HBSS+ and surrounded with crushed ice to maintain a low temperature. The upper part of the core (300µm) was trimmed to avoid damaged tissue and slices of 250µm thick were obtained using 0.3mm/sec cutting speed and maximum frequency. The slice thickness (250µm) and coring diameter (8mm) were selected as the most suitable and frequently used dimensions in mammalian studies (Lerche-Langrand & Toutain, 2000). Slices were immediately transferred to a Leibovitz's 15 media (Life technologies cat no. 21083-027, Carlsbad, CA, United States) and kept in an incubation cabinet at 3°C until enough slices (n=24) were generated to start an experiment. In most experiments, three to four cores were needed to obtain 24 slices of suitable size and aspect. The slicing procedure lasted for about an hour per fish.

Liver slices were transferred into 24-well plates (Thermo Scientific Nunc, Langenselborg, Germany) containing 1ml per well of incubation L15 medium supplemented with 1% Penicilin-Streptomycin and the test substances, when appropriate. The 24-well plates with slices were maintained in an incubation cabinet at 3°C, under orbital shaking during the course of the exposure experiment. Culture medium was collected and replaced every 24 hours (hr) during the experimental period.

The slicing and incubation procedure was repeated for a total of two or three replicate fish per experiment. In addition, total length (cm), total weight (g), sex, gonad weight (g) and somatic weight (g) were recorded for each fish. The gonadosomatic index (GSI, %) was determined as GSI= (gonad weight/somatic weight) x100.

2.3. PCLS experimental design

A viability experiment was used to determine the appropriate exposure time of slices. Slices were then exposed to different model compounds purchased from Sigma Aldrich, San Luis, MO, United States (BaP, cat no CRM40071 and WY, cat no C7081) and from Chiron AS, Trondheim, Norway (2- ring and 3-4-ring PAHs mixtures, see section 2.3.2).

2.3.1. Viability experiment

To determine viability, liver slices were exposed to L15 media supplemented with 1% Penicilin-Streptomycin alone for 5 days (120hr). The culture medium (n=4 per sampling time) and liver slices (n=4 per sampling time) were collected every 24hr. The medium was frozen at -80°C for further analysis of lactate dehydrogenase (LDH) activity. In addition, liver slices (n=10) sampled at 0 hr and 48 hr were fixed in 4% neutral buffered formalin for histological analysis.

2.3.2. Exposure experiments

The incubation of liver slices in test substances followed the same procedure as the viability experiment. A 48hr exposure time frame was chosen for the following exposure experiments (see Results section 3.2). Briefly, liver slices (n=4 or n=5 per concentration and per fish) were exposed for 48hr to a range of concentrations of the test substances according to Table 1. The test substances were diluted to final concentrations in L15 media containing 1% antibiotics and 0.1% acetone for exposure to BaP and PAHs mixtures. For the WY, 1% dimethyl sulfoxide (DMSO) was used. The control treatment used the same concentration of solvent as in the other treatments (0.1% acetone and 1% DMSO respectively).

Table 1: Summary of the experimental designs for each exposure experiment. Test substances, concentrations (µM), number of fish sampled and sampling times. T0, T24 and T48 are sampling points at 0, 24 and 48 hours of exposure, respectively.

Test substance	Concentration range (µM)	Fish replicates (n)	Analyses per fish replicates
Benzo(a)pyrene	0, 0.1, 1, 10, 100, 1000	3	Histology (n=4 at T0) LDH on medium (n=8 at T0 and n=8 at T24, 48) mRNA expression on slices (n=4 at T48)
WY-14643	0, 0.1, 1, 10, 100	3	Histology (n=4 at T0) LDH on medium (n=8 at T0 and n=4-5 at T24, 48) mRNA expression on slices (n=4-5 at T48)
2-ring PAHs mixture	0, 0.5, 5, 50, 500	3	LDH on medium (n=8 at T0 and n=4-5 at T24, 48) mRNA expression on slices (n=4-5 at T48)
3-4-ring PAHs mixture	0, 0.1, 1, 10, 100	2	LDH on medium (n=8 at T0 and n=4-5 at T24, 48) mRNA expression on slices (n=4-5 at T48)

2.3.3. PAH composition of the 2 and 3-4-ring mixtures

The PAH mixtures (purchased from Chiron AS, Trondheim, Norway) were created to match the proportion of 2 to 4-rings PAHs (sum of 40 Environmental Protection Agency PAHs) found in crude oil from the Barents Sea (Nahrgang J, *pers com*). Single PAHs were thus present in different amounts (Table 2). The concentrations expressed in the exposure experiments are representative of the sum of all PAHs present in the considered mixture (2-ring or 3-4-ring).

Table 2: PAH compounds and their relative (%) proportion present in the 2-ring and 3-4-ring PAHs mixtures.

2-ring mixture	% present	3-4-ring mixture	% present
Naphthalene	6.10	Acenaphthylene	0.04
2-Methyl-Naphthalene	17.56	Acenaphthene	1.39
1,2-Dimethyl-Naphthalene	6.96	Fluorene	3.79
1,6-Dimethyl-Naphthalene	6.43	Phenanthrene	10.16
2,3-Dimethyl-Naphthalene	6.49	Dibenzothiophene	1.55
2-ethylnaphthalene	6.10	9-Methylphenanthrene	20.03
1,2,3-Trimethyl-Naphthalene	12.72	4-Methyldibenzothiophene	3.67
1,3,7-Trimethyl-Naphthalene	12.59	Anthracene	0.03
1-propylnaphthalene	12.46	4-Ethyldibenzothiophene	1.71
2-ethyl-6-methylnaphthalene	12.59	1,2-Dimethyl-Phenanthrene	6.73
		9,10-dimethylphenanthrene	6.73
		3,9- dimethylphenanthrene	6.77
		9-ethylphenanthrene	6.77
		1,2,8-Trimethyl-Phenanthrene	6.85
		2,6,9- Trimethyl-phenanthrene	6.85
		4-Ethyl-6-methyldibenzothiophene	2.24
		FLuoranthene	0.16
		1-Methyl-fluoranthene	1.63
		3-Ethyl- fluoranthene	0.45
		Pyrene	0.33
		1-Methyl-pyrene	0.57
		4,5- dimethylpyrene	0.49
		1-ethyl-pyrene	0.49
		1-Propyl-pyrene	0.77
		Benzo(a)anthracene	0.16
		Chrysene	0.41
		5-Methyl-chrysene	0.61
		6-Ethyl-chrysene	0.94
		1,3,6-Trimethylchrysene	0.82
		9-propylphenanthrene	6.85

2.4. Viability analyses

2.4.1 Lactate dehydrogenase (LDH) cytotoxicity detection assay

Lactate dehydrogenase leakage into the medium was quantified using the lactate dehydrogenase (LDH) kit (Roche, cat no: 11644793001, Basel, Switzerland) according to the manufacturer's instructions. This methodology was adapted for culture medium analyses by Lemaire et al. (2011). Culture media was thawed on ice, samples (100 µl), diluted 5 times in distilled water, and were pipetted in triplicate into a 96-well plate. A reaction mixture, containing catalyst diaphorase and a dye solution, was prepared immediately prior to use. 100 µl of reaction mixture was added to each well of a 96- well plate and incubated in darkness at room temperature for 30 minutes. Absorbance was read at 490 nm and 650 nm using a Synergy H1 hybrid reader (Bio Tek Instruments Inc., Winooski, VT, USA). Relative LDH activity values were obtained by normalizing the absorbance to baseline levels ($\boldsymbol{A_{490}}$-$\boldsymbol{A_{650}}$ nm) and multiplying by the dilution factor (5x). Changes in relative LDH activity were used to assess viability of the slices during the experiments.

2.4.2 Histological analysis

Histology analyses were used on liver slices for three different experiments (viability, BaP and WY) to evaluate the slice thickness (250µm) variability and repeatability over fish replicates and between treatments.

Slices stored in 4% neutral buffered formalin were placed in histocassettes and rinsed in 70% ethanol. Cassettes were then submerged in a series of paraffin cyclic baths using the Shandon Citadel 1000 (Micron AS, Moss, Norway) for 14hr. The cassettes were submerged in wax using an embedding machine (Kunz Instruments WD-4, Stockholm, Sweden) and stored at 4ºC in a vertical position overnight before slicing. Multiple microscope slides per sample were prepared using the Leitz RM 2255 microtome and 0.5µm slices were dried at 65°C overnight. Slides were stained in hematoxylin and eosin baths using the Leica ST4020 Linear stainer (Leica biosystems, Germany) (Zimmermann et al. 2009).

For each liver slice, 6 cuts were selected to measure slice thickness at 4 points of the cut. However, some cuts were not positioned adequately in the embedding wax, preventing an accurate thickness measurement. The final number of slice measurements varied between 12 and 48, over fish specimens and between exposure experiments. Pictures of the cuts were taken with the Leica Wild M10 dissecting scope with a Leica DFC295 camera at 100 magnification (Figure 5). Slice thickness was measured using the program Image J and the Object J package. All measurements were used to calculate the distribution frequency of slice thickness for each fish replicate. Histograms showing thickness frequency percent using a range of 0 to 400µm were plotted for all samples.

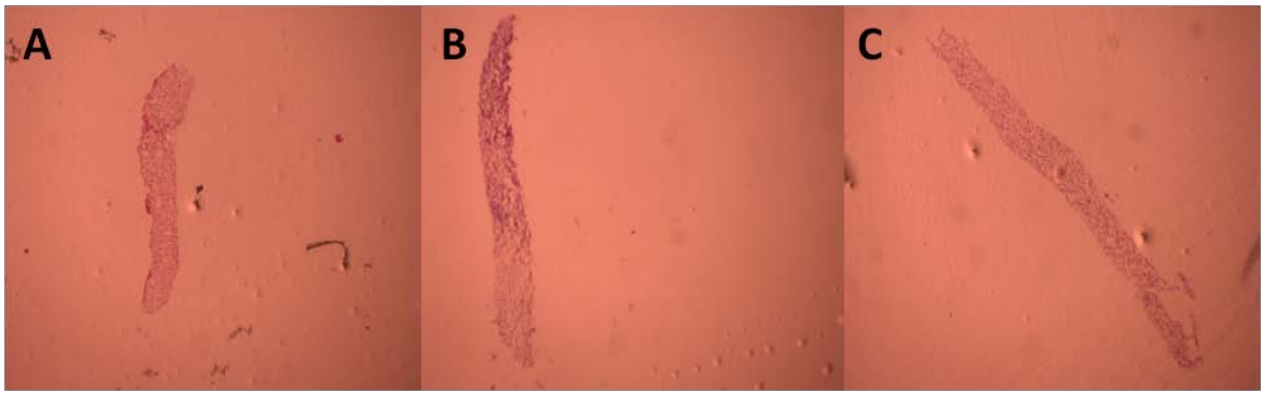

Figure 5: Photographs of polar cod PCLS. A) Fish A slice from the viability experiment at T0, B) Fish B from the viability experiment at T48 and C) Fish I slice from the WY-14643 experiment at T0.

2.5 mRNA expression

Quantitative real-time polymerase chain reaction (Q-PCR) was performed for four different target genes: *cyp1a*, *aox*, *pparα* and *vtg* (see details Table 3). In addition, three house-keeping genes (*β-actin*, *ef-1*, *18s*) were tested and of the three, only the *ef-1* was retained (See section 2.5.2).

Table 3: Target genes selected in the four different exposure experiments.

Experiment	Genes
Benzo(a)pyrene	*ef-1, cyp1a , vtg*
WY-14643	*ef-1, cyp1a , aox, pparα ,*
2-ring PAHs mixture	*ef-1, cyp1a , aox, pparα , vtg*
3/4-rings PAHs mixture	*ef-1, cyp1a , aox, pparα , vtg*

2.5.1 RNA extraction, ethanol precipitation and DNase treatment

The mRNA was extracted from the slices using the RNeasy Plus Universal Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer´s instructions. The RNA was eluted in 20µl of RNase-free water and RNA quality and quantity was determined with the NanoDrop ND-2000 UV-Vis Spectrophotometer. In order to assess purity of the samples, absorbance was measured at two different absorbance ratios: 260/280 and 260/230. 260/280 ratio was used to assess RNA purity, with an optimal ratio equal or similar to 2. All the samples had 260/280 values equal or similar to 2. 260/230 ratio was used as a secondary measure of purity with accepted values between 1.8-2.22. To clean the samples, an ethanol precipitation step was performed to obtain adequate values at 260/230 absorbance ratio. For further steps, only samples with 260/230 ratios between 1.8 and 2 were used. To remove DNA contamination from RNA samples, the Turbo DNA-free ™ Kit (Ambion, Life technologies, Carlsbad, CA, United States) was used to purify all samples. After this step, the quantity of mRNA was determined with the NanoDrop ND-2000 UV-Vis Spectrophotometer.

2.5.2 cDNA preparation and quantitative real time polymerase chain reaction (Q-PCR)

cDNA was prepared from 1µg of mRNA using the iScript ™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-rad, Hercules, CA, United States), in a 20µl reaction following the manufacturer's protocol. Some samples were lost in this step due to insufficient mRNA concentration.

Primers for Q-PCR were synthesized by Sigma-Aldrich, San Luis, MO, United States (Table 4). Before starting Q-PCR analyses, efficiency of the primers was tested. A pool of 14 samples from different experiments was made and then a dilution series with five concentrations (10, 5, 2.5, 1.25, 0.625ng/µl) was prepared in triplicate to make an efficiency standard curve for each gene. Dilution 10 ng/µl was the one chosen as best for all primers.

Table 4: Q-PCR primers for polar cod. Genes short names and primer sequences are shown (Bilbao E, *pers com*).

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	ATCGGCAACGAGAGGTTCC	CGGCGATGCCAGGGTA
ef-1	GGAGGCATTGACAGAGAACCA	ACCCAGGCGTACTTGAAGGA
18s	CGAATGTCTGCCCTATCAACTTT	CCGGAATCGAACCCRGATT
cyp1a	CCACCCCGAGATGCAGG	CGCAGGTGTCTTTGGGGA
aox	GGCATCGTGCTCTCCCAAT	TCTCCTGCGCGGATCTCT
pparα	GGCCCGGCAGATCTACGA	GTCTTCCCGGTGAGGATGGT
vtg	GCAACCCTGAAGGAAAGCAA	GGAGCGGTGTTCTTGGTCAT
cat	TGACCGGGAACGTATCCCAGAGAG	GTGTGAGAGCCGTAGCCGTTCAT

All Q-PCR reactions were perfomed using SsoAdvanced ™ Universal SYBR ® Green Supermix (Bio-rad) and the BioRad CFX Connect Fast Real-Time PCR system, following the producer's recommendations. SYBR Green is a DNA-binding dye which binds nonspecifically to double-stranded DNA. During Q-PCR, the target gene is amplified and measured by fluorescence. When enough amplified product accumulates, it yields a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle (C_T) (Bio-Rad applications guide, 2006).

First, the house keeping gene β-actin was tested as a reference gene for BaP experiment, but was not retained due to the high difference in C_T values between samples (up to eight cycles difference). The elongation factor (*ef-1)* and the 18S ribosomal RNA (*18S)* housekeeping genes were tested and *ef-1* was chosen to be the best housekeeping gene. Furthermore, a criterion of a maximum difference of three C_T cycles between samples was applied and samples which did not fit in this range were rejected.

Plates were filled with 20µl reaction mix containing 1µl primers, 10µl SYBR, 4µl H2O and 5µl cDNA and all samples were prepared in triplicate. There were two negative controls for each gene: a No Template Control (NTC) and a No Reverse Transcription Control (NRT). NRT contained reaction components (H20, primer and SYBR) and the template was mRNA. It was used to test for genomic DNA remains in the samples, which could enhance the levels of DNA amplified. NCT contained the reaction components but not template. This control was used to check for contamination of templates during plate preparation and for the presence of primers-dimers, primers binding to other fragments of DNA. These two control samples were used to assess the quality of Q-PCR by melt-curve analyses after completion of the amplification reaction (Bio-Rad applications guide, 2006).

2.5.3 mRNA data treatment

The Q-PCR results were analyzed using Bio-Rad CFX manager software. C_T values of the target genes were compared to C_T values of the reference gene, elongation factor (*ef-1*), in order to asses mRNA expression of the genes after exposure to of different pollutants (Bio-Rad applications guide, 2006). One of two methods was used to calculate the expression fold ratio: either the Livak method (Schmittgen & Livak, 2008) or the Pfaff method (Pfaffl, 2001), was used depending on the efficiency of the target genes (Table 5). These methods were used to calculate the fold ratio increase of the target gene in the test sample, relative to the calibrator sample (solvent control) and were normalized to the expression of the reference gene (*ef-1*).

To calculate the efficiency of each gene, the standard curve was made by plotting the logarithm of the serial dilutions made against the C_T values obtained during amplification (Bio-Rad applications guide, 2006). Amplification efficiency (E) was then calculated using the slope of the dilution standard curve using the following equation (Bio-Rad applications guide, 2006):

$$E = 10^{-1/slope}$$

Ideally, the amount of PCR produce is double during each cycle of exponential amplification, which would mean that there is a 2-fold increase in the number of copies with each additional cycle, 2 being the desirable efficiency (E=2). Amplification efficiency can also be presented as percentage (the percent of template amplified in each cycle) according to (Bio-Rad applications guide, 2006):

$$\% \, Efficiency = (E - 1)X \, 100$$

Table 5: Amplification efficiencies in the Q-PCR of the different target genes and housekeeping genes.

Gene	Efficiency	% Efficiency
β-actin	2.05	105.34
ef-1	2.11	111.80
18s	2.03	106.91
cyp1a	2.09	109.47
aox	1.99	99.80
pparα	2.03	103.69
vtg	2.06	106.91

The Livak method was used for those genes whose amplified efficiency was near to 100% and within 5% of *ef-1*. The Pfaff method was used for genes whose efficiency was not within 5% of *ef-1* (Table 5).

mRNA expression of target genes were calculated according to the following equations:

- The $2^{-\Delta\Delta Ct}$ (Livak) method (Livak & Schmittgen, 2001)

$$\boldsymbol{\Delta C_{T(test)} = C_{T(target,test)} - C_{T(ref,test)}}$$

$$\boldsymbol{\Delta C_{T(calibrator)} = C_{T(target,calibrator)} - C_{T(ref,calibrator)}}$$

$$\boldsymbol{\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}}$$

$$\boldsymbol{2^{-\Delta\Delta Ct} = Normalized\ expression\ ratio}$$

where:

$\boldsymbol{C_{T(target,test)}}$**:** Amplification of the target gene (e.g. *cyp1a*) at a certain concentration (e.g. 0.1µM)

$\boldsymbol{C_{T(ref,test)}}$**:** Amplification of the reference gene (*ef-1*) at a certain concentration (e.g. 0.1 µM)

$\boldsymbol{C_{T(target,calibrator)}}$**:** Amplification of the target gene (e.g. *cyp1a*) at control concentrations (0 µM). This value is the mean of all the amplification values at this concentration.

$\boldsymbol{C_{T(ref,calibrator)}}$**:** Amplification of the reference gene (*ef-1*) at control concentrations (0 µM). This value is the mean of all the amplification values at this concentration.

- The Pfaffl Method (Pfaffl, 2001)

$$Ratio = \frac{(E_{target})^{\Delta Ct,target(calibrator-test)}}{(E_{ref})^{\Delta Ct,ref(calibrator-test)}}$$

where:

$\boldsymbol{E_{target}}$**:** Amplification efficiency of the target gene (e.g. *cyp1a*).

$\boldsymbol{E_{ref}}$: Amplification efficiency of the reference gene (*ef-1*)

Calibrator: Amplification of target (e.g. *cyp1a*) or reference (*ef-1*) gene at control concentrations (0 µM). This value is the mean of all the amplification values at this concentration.

Test: Amplification of target (e.g. *cyp1a*) or reference (*ef-1*) gene at the different concentrations (e.g. 0.1 µM).

mRNA expression fold change of the target genes (mean± SE) for each fish were transformed to Log10 scale and used for both plotting and statistical analyses. For untransformed data see Appendix, Table A2-5.

2.6 Statistical analysis

IBM SPSS software was used for plotting figures and statistical analyses. Linear mixed models (LMM) were used for both LDH activity and mRNA expression analyses in each experiment. The goal of using LMM was to see the overall effect of the different factors (time or concentration) in the response variable (LDH activity or mRNA expression) in all the fish sampled. In LMM, fish replicates were set as a random factor and either concentration or time as fixed factor. If the p-value gotten by the LMM was statistically significant ($p<0.05$), a one way ANOVA was performed followed by the Tukey-Kramer *post hoc* test analysis for each fish replicate in the experiment, in order to see how each replicate responded. Tukey-Kramer *post hoc* test was not available for all results due to small sample size in some experiments. Normal distribution and homogeneity of variance was tested and when either one or both of them were not met, the Welch's test analysis was chosen instead. In some experiments, *post hoc* analyses were not available due to the small sample size.

3 RESULTS

3.1 General information on fish

For each experiment, fish information was recorded (Table 6) including total weight, total length and gender. This information was used in the interpretation of the differences observed in mRNA expression between fish slices in some target genes.

Average length (± standard deviation) of fish replicates among the viability (26.9 ±1.2 cm), BaP (23.3± 1.6 cm), WY-14643 (24.5 ± 1.5 cm), 2-ring PAHs mixture (26.3± 2.4 cm) and 3-4-ring PAHs mixture (25.1 ±3.9 cm) experiments was very similar. Similarly, average weight (± standard deviation) of fish replicates among the experiments was no significantly different (viability 110.3±22.3g; BaP 70.8±7.3g; WY-14643 89.2±19.5g; 2-ring PAHs 100.8±24.6g; 3-4-ring PAHs 89.7±21.6g). Both gender was represented in each experiment The GSI, indicating maturity stage of the gonads was higher for males in all experiments, except for the BaP where the single female showed a high GSI. Polar cod males are known to increase their gonad size several months before than females (Hop et al. 1995) in early autumn, explaining higher GSI in males in experiments performed in October (WY and PAHs mixtures).

Table 6: Date, fish code, total length (cm), total weight (g), gender and gonadosomatic index (GSI %) of the fish sampled in all the experiments (viability and exposure).

Date	Experiment	Fish	Total length (cm)	Total weight (g)	Gender	Gonadosomatic index (GSI %)
May 28th	Viability	A	28.1	136	Female	2.4
May 28th	Viability	B	25.9	95	Male	5.8
May 28th	Viability	C	26.6	100	Female	3.2
June 18th	Benzo(a)pyrene	D	24.8	78.8	Female	13.4
June 18th	Benzo(a)pyrene	E	23.6	69.5	Male	1.9
June 18th	Benzo(a)pyrene	F	21.6	64.3	Male	2.2
Oct 7th	WY-14643	G	24.5	85.5	Female	6.1
Oct 7th	WY-14643	H	23.1	71.84	Female	3.6
Oct 7th	WY-14643	I	26.1	110.4	Male	12.5
Oct 9th	2-ring PAHs mixture	J	27.5	123.2	Female	5.1
Oct 9th	2/3-4-ring PAHs mixture	K	23.1	74.4	Male	12.7
Oct 9th	2/3-4-ring PAHs mixture	L	27.2	105	Female	5.5

3.2. Viability experiment

Slice thickness was highly variable among fish and was often below the desired thickness (250 µm). Average slice thickness of fish A, B and C were 209±72, 150±24 and 201±53 µm, respectively. Fish A and C showed the highest variation in slice thickness with slices that ranged 100-410 and 100-350µm, respectively. In addition, none of the slices from fish B reached 250µm thickness. The most common slice thickness frequency range also varied among fish and was 180-260, 130-150 and 150-200 µm for fish A,B and C, respectively (Figure 6, A-C).

The increase in LDH activity was statistically significant (p=0.002) over time when the three fish were analyzed together by LMM. Individually, fish A showed a significant (p=0.000) increase in LDH activity at 96 and 120 hr of exposure compare to 24 hr. The three fish had slightly higher LDH activity values at 24 hr than at 48 hr. Thereafter, this activity stayed at a low level in fish B and C. However, LDH activity clearly increased in fish A after 48 hr (Figure 6, D). Accordingly, fish A was used as a reference to determine the exposure time of the PCLS in the following experiments in order to be sure to avoid errors associated with longer than necessary exposure time. Thus, 48 hours was considered an adequate time of exposure for the present study.

3.3. Benzo(a)pyrene experiment

Similarly to the viability experiment, the slice thickness was variable among fish replicates slices used in the BaP experiment. Average slice thickness of fish E and F were 226±39 and 190±32µm respectively. Slices from fish E showed the highest variation in thickness with slices that ranged between 140 and 300µm thick. The most common slice thickness frequency range also varied among fish sampled and was 220-260 and 175-210µm for fish E and F, respectively (Figure 7 A-B). Slices thickness was not measured on fish D due to lack of extra sampled slices for histological analysis.

LDH activity was not significantly (p=0.837) different among BaP exposure concentrations. Nonetheless, LDH activity showed a positive trend with increasing concentration for slices from fish D and F (Figure 7, C).

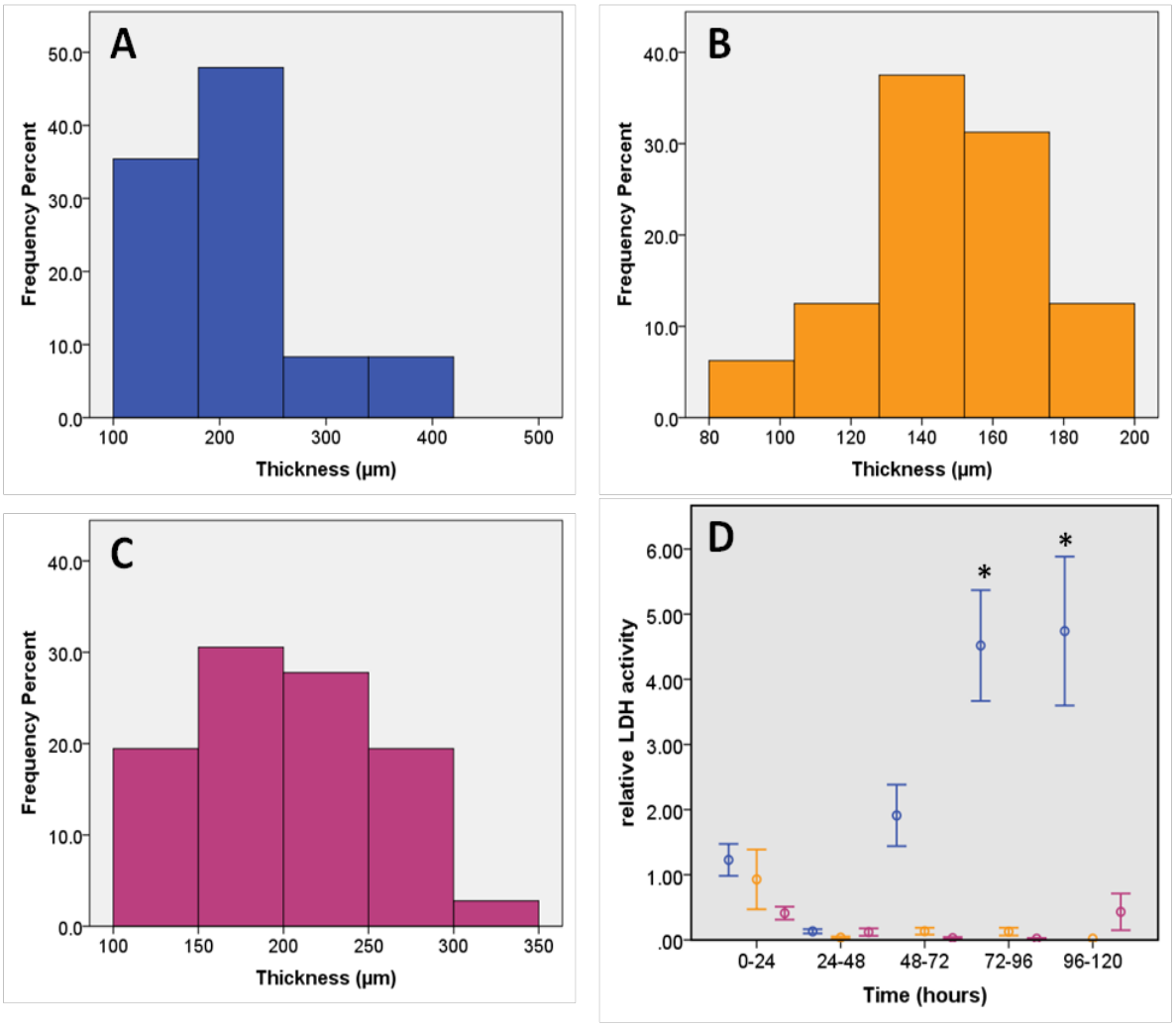

Figure 6: Viability experiment on polar cod liver slices. Panels A, B and C show slice thickness (µm) and frequency distribution (%) of Fish A, B and C slices, respectively. Panel D shows mean (circles) ± SE (lines) relative LDH activity (normalized absorbance) over time at the different sampling points for fish A (blue), B (yellow) and C (pink). Asterics show mean ± SE values which were statistically significant different than values at 0-24 hours.

cyp1a mRNA expression increased significantly with increasing BaP concentrations compared to the control for slices from fish D and fish F, except at the lowest BaP concentrations (0.1 and 1µM respectively). Fish E slices, on the contrary, did not show a

significant increase in *cyp1a* mRNA expression when concentration increased (Figure 7, D).

Similarly, *vtg* mRNA expression was significantly (p=0.011) induced with increasing BaP concentration when the three fish were analyzed together by LMM. Nevertheless, when fish were analyzed independently (one way ANOVA) there were no changes on *vtg* mRNA expression in any of them (fish D p=0.147, fish E p=0.340 and fish F p=0.471), meaning that statistically significant differences were found between fish replicates. Nonetheless, a positive trend was visible in *vtg* mRNA expression among fish when BaP concentration increased. There were no clear gender-specific trends as fish E (male) slices had higher *vtg* expression than fish D (female) and F (male) for all concentrations (Figure 7, E).

3.4. WY-14643 experiment

Similarly to the viability experiment, the slice thickness was variable among fish replicates used in the WY experiment and below the expected 250µm (147±11, 206±39 and 181±46µm for fish G, H and I respectively). Slices from fish H showed the highest variation in thickness with slices that ranged between 100 and 300µm thick. In addition, none of slices from fish G reached 250µm thickness. The most common slice thickness frequency range also varied among fish sampled and was 140-150, 170-220 and 210-250µm for fish G, H and I, respectively (Figure 8, A-C).

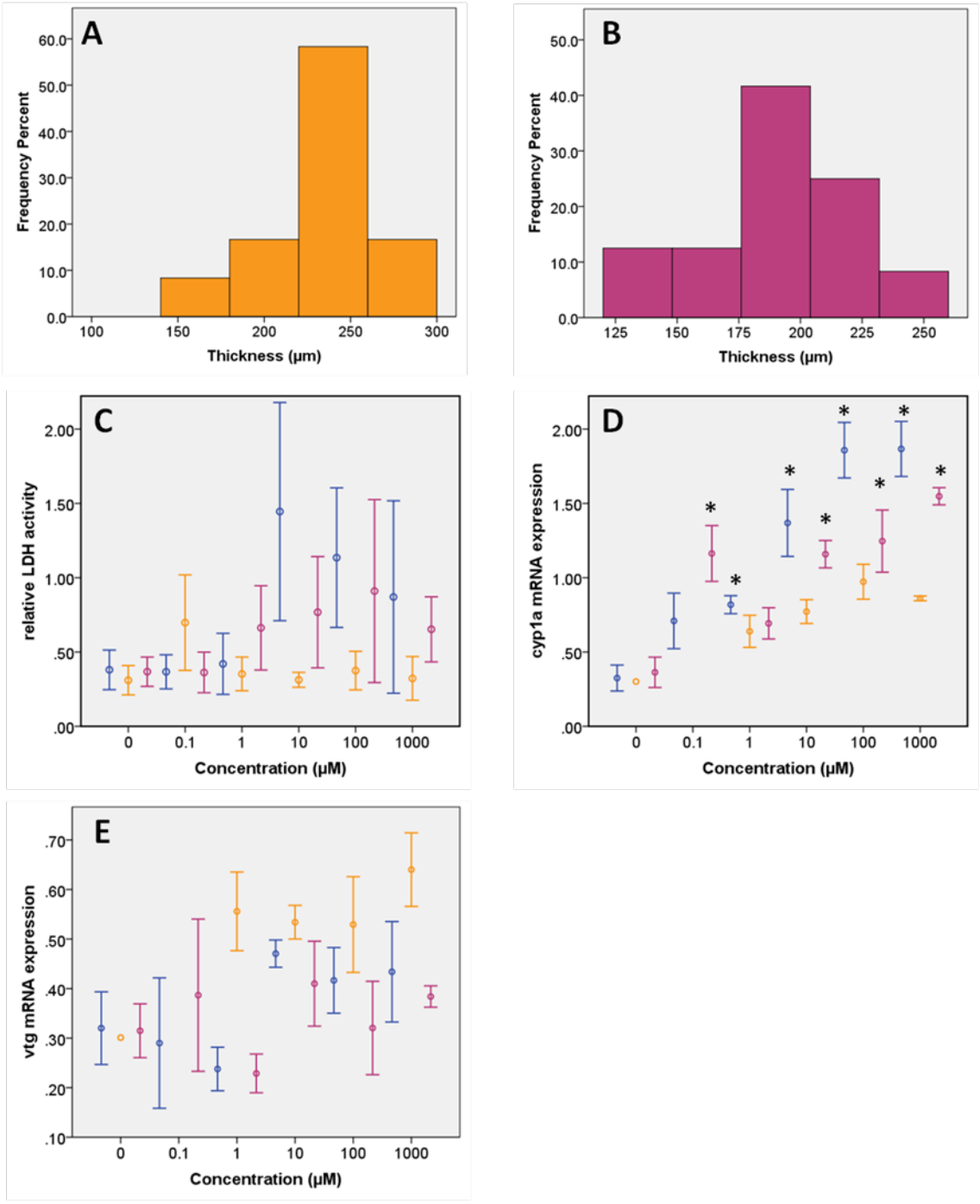

Figure 7: Benzo(a)pyrene experiment on polar cod liver slices. Panels A and B show slice thickness (µm) and frequency distribution (%) of Fish E and F slices, respectively. Panel C shows mean (circles) ± SE (lines) relative LDH activity (normalized absorbance) during the 48 hr of exposure at the different concentrations for fish D (blue), E (yellow) and F (pink). Panel D and E show mean (circles) ± SE (lines) *cyp1a* and *vtg* mRNA expression (amplification ratio), respectively, in Log10 scale in fish D (blue), E (yellow) and F (pink) after 48 hr of exposure to the different concentrations of benzo(a)pyrene. Asterics show mean ± SE values which were statistically significant different than values at 0-24 hours.

Although LDH activity did not increase significantly (p=0.301) with increasing WY, a huge variability could be observed at 100µM concentration for fish G slices (Figure 8, D).

In general, *cyp1a, aox and pparα* mRNA expressions did not change significantly (p=0.138, p=0.308 and p=0.928, respectively) with increasing exposure concentrations. However, fish H and I slices showed a positive trend of *cyp1a* mRNA expression with an increase in concentration. Furthermore, *aox* mRNA expression seemed to decrease when concentration increased in the three fish. Similarly to *aox*, fish G and H slices showed a negative trend of mRNA expression for *pparα* with increasing concentrations of WY. On the contrary, fish I slices showed an increasing trend of mRNA expression for *pparα* with increasing concentrations of WY (Figure 8, E-G).

3.5. 2-ring PAHs mixture experiment

After 48 hours LDH activity change was statistically significant (p=0.03) with increasing 2-ring PAHs mixture concentration when the three fish were analyzed together by LMM. However, when each fish was analyzed individually there was not a significant increase in LDH activity for any of them (fish J p=0.615, fish K p=0.124 and fish L p=0.421), meaning that statistically significant differences were found between fish replicates. However, an overall positive trend was shown in LDH activity with increasing concentration (Figure 9, A).

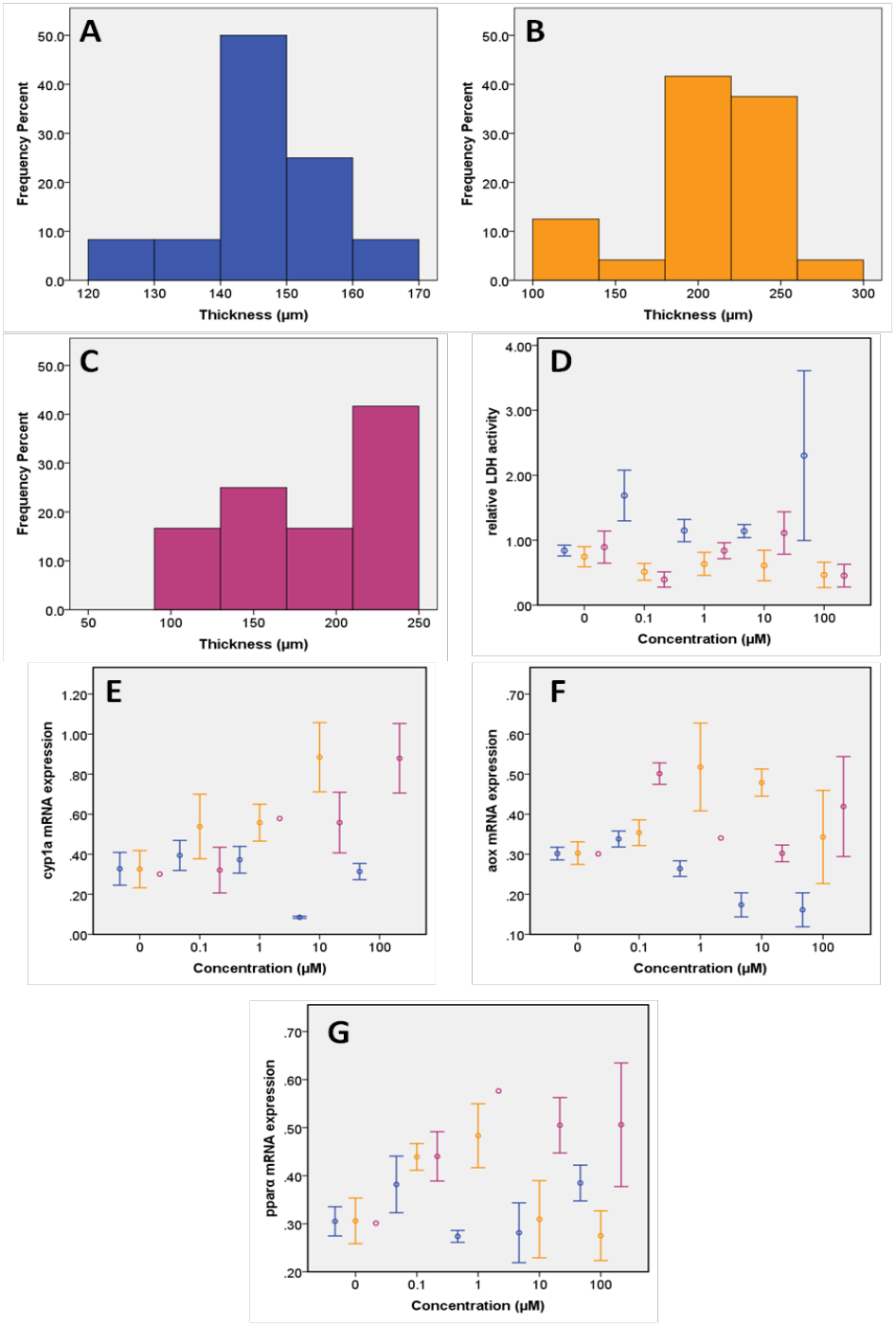

Figure 8: WY-14643 experiment on polar cod liver slices. Panels A, B and C show slice thickness (µm) and frequency distribution (%) of Fish G, H and I, respectively. Panel D shows mean (circles) ± SE (lines) relative LDH activity (normalized absorbance) during the 48 hr of exposure at the different concentrations for fish G (blue), H (yellow) and I (pink). Panel E, F and G show mean (circles) ± SE (lines) *cyp1a*, *aox* and *pparα* mRNA expression (amplification ratio), respectively, in Log10 scale in fish G (blue), H (yellow) and I (pink) after 48 hr of exposure to the different concentrations of WY-14643.

In general, *cyp1a, aox and vtg* mRNA expressions did not change significantly (p=0.130, p=0.877, p=0.301, respectively) with increasing exposure concentrations when the three fish were analyzed together by LMM. Nonetheless, a positive trend was shown in *cyp1a* mRNA expression when concentration increased in fish K and L slices (Figure 9, B). Regarding *aox* and *pparα* mRNA expression, fish J and K slices followed a decrease-increase-decrease mRNA expression trend as concentration increased (Figure 9, C-D). Fish K and L followed the same trend in *vtg* mRNA expression, while fish J slices had an increasing trend in *vtg* mRNA expression levels as concentration increased (Figure 9,E). On the other hand, *pparα* mRNA expression was almost significantly (p=0.05) induced with increasing concentration when the three fish were analyzed together by LMM. However, when each fish was analyzed individually *pparα* mRNA expression did not change significantly (fish J p=0.177, fish K p=0.157 and fish L slices (p=0.799) with increasing exposure concentrations, meaning that statistically significant differences were found between fish replicates.

3.6. 3-4-ring PAHs mixture experiment

After 48 hours LDH activity change was close to be statistically significant (p=0.041) with increasing 3-4-ring PAHs concentration when the three fish were analyzed together by LMM. Nonetheless, a negative trend was shown in LDH activity when concentration increased in fish L slices (Figure 10, A).

Increase in *cyp1a* and decrease in *vtg* mRNA expression were statistically significantly (p=0.013 and p=0.022, respectively) with increasing exposure concentrations when the three fish were analyzed together by LMM. Both fish K and L slices had significant (p=0.05 and p=0.019) increases in *cyp1a* mRNA expression but due to small sample size *post hoc* tests were not available (Figure 10, B). However, when each fish was analyzed individually there was not a statistically significant increase in *vtg* mRNA expression for any of them (fish K p=0.252 and fish L p=0.337), meaning that statistically significant differences were found between fish replicates. Nonetheless, there was a decreasing trend in *vtg* mRNA expression in fish L when concentration increased, and *vtg* expression was

higher than in fish K (Figure 10, E). In contrast, *aox* and *pparα* mRNA did not change significantly (p=0.563 and p=0.915, respectively) with increasing concentrations. Nonetheless, a positive trend was shown in *aox* and *pparα* mRNA expression when concentration increased in Fish L slices and a decreasing trend in fish K slices (Figure 10, C-D).

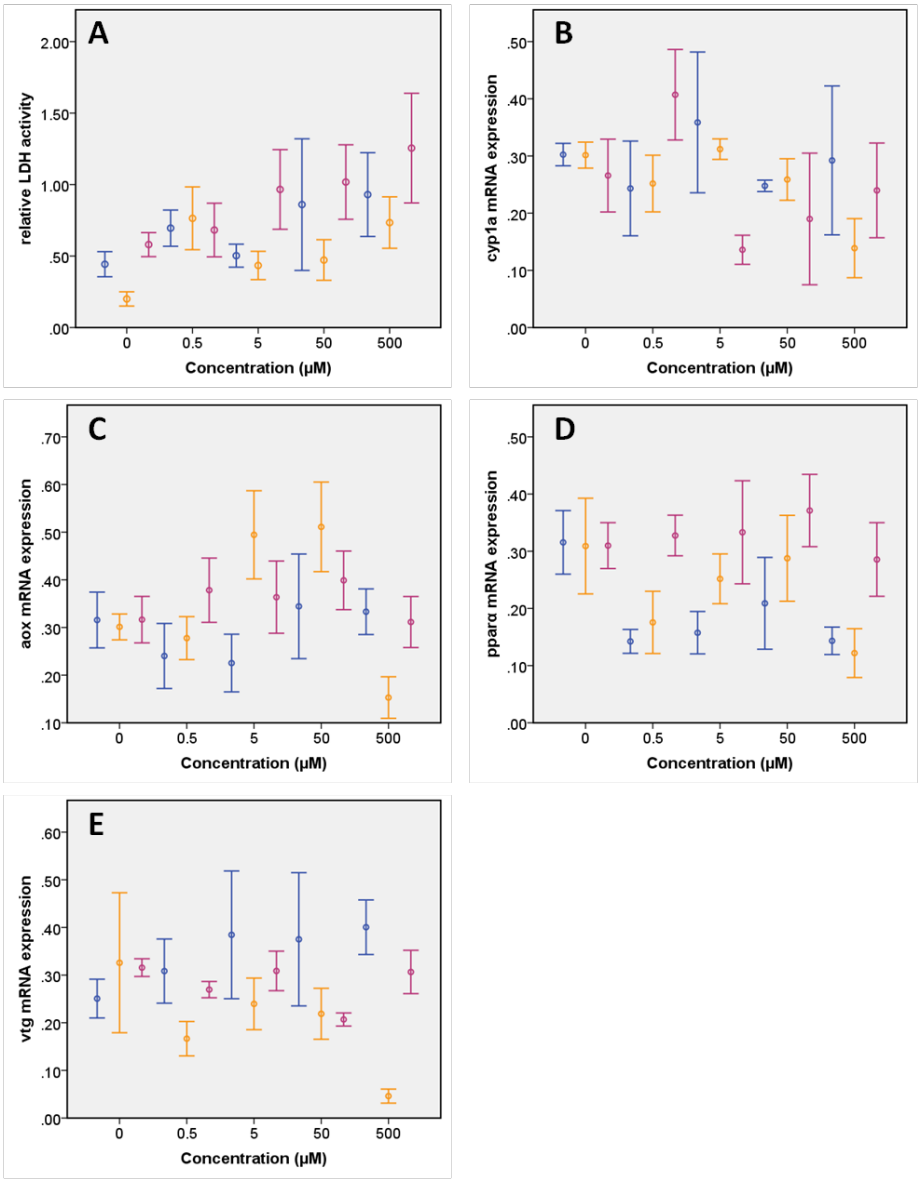

Figure 9: 2-ring PAHs mixture experiment on polar cod liver slices. Panels A shows mean (circles) ± SE (lines) relative LDH activity (normalized absorbance) during the 48 hr of exposure at the different concentrations for fish J (blue), K (yellow) and L (pink). Panel B, C, D and E show mean (circles) ± SE (lines) *cyp1a*, *aox, pparα* and vtg mRNA expression (amplification ratio), respectively, in Log10 scale in fish J (blue), K (yellow) and L (pink) after 48 hr of exposure to the different concentrations of 2-ring PAHs mixture.

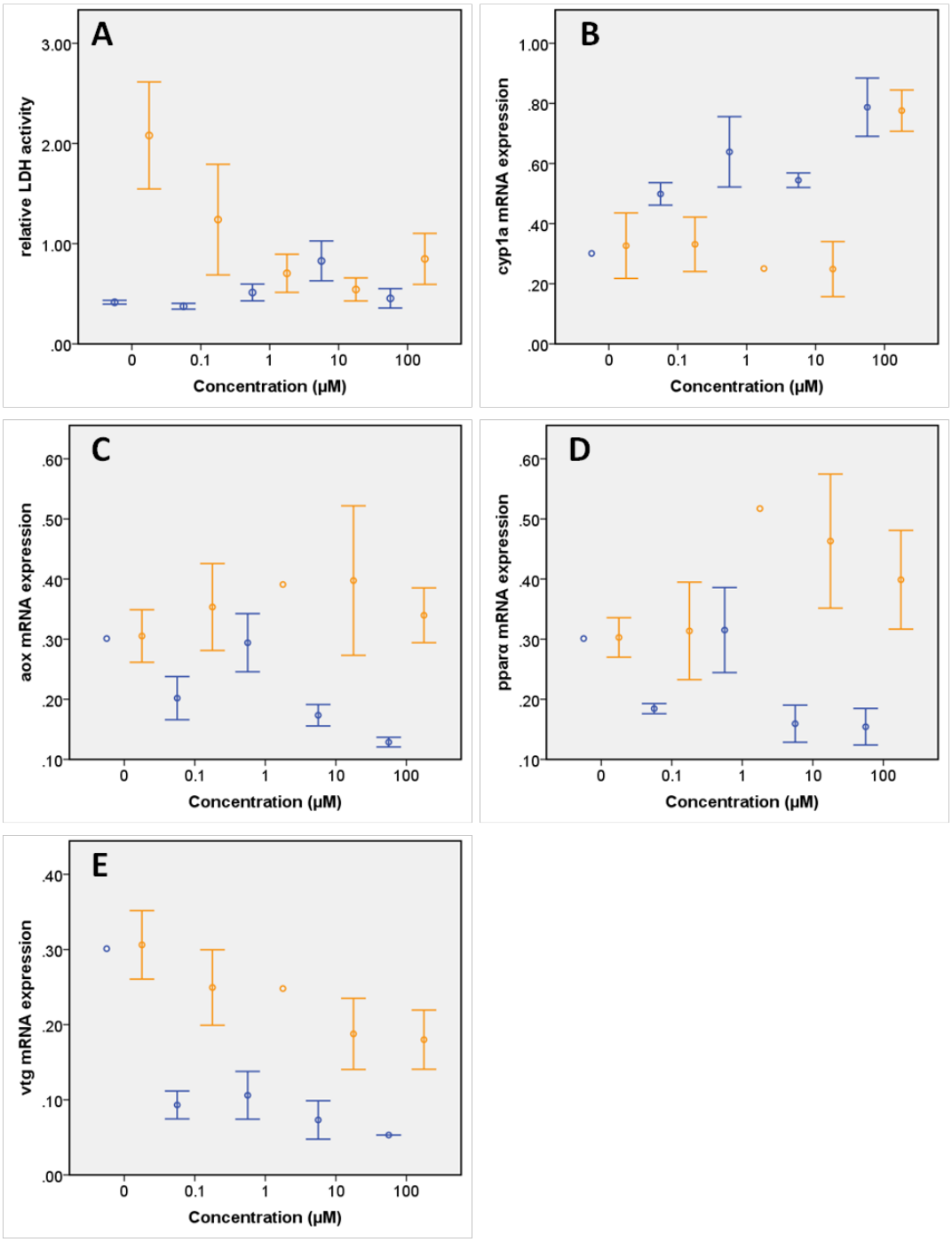

Figure 10: 3- and 4-ring PAHs mixture experiment on polar cod liver slices. Panels A shows mean (circles) ± SE (lines) relative LDH activity (normalized absorbance) during the 48 hr of exposure at the different concentrations for fish K (blue) and L (yellow). Panel B, C, D and E show mean (circles) ± SE (lines) *cyp1a*, *aox, pparα* and vtg mRNA expression (amplification ratio), respectively, in Log10 scale in fish K (blue) and L (yellow) after 48 hr of exposure to the different concentrations of 3-4-ring PAHs mixture.

4 DISCUSSION

4.1 Viability experiment

4.1.1 Lactate dehydrogenase (LDH) activity

LDH activity in the viability experiment was measured in order to determine an optimal time of exposure for the subsequent experiments. High levels of LDH enzyme activity have been linked to cell death and damage (Legrand et al. 1992) making this endpoint a valid measure of tissue viability. In the present study, the results showed that the LDH activity increased only significantly after 96 hours and only in one of the 3 fish tested (see section 3.2). Thus, 48 hours of exposure seemed to be a good compromise between time of exposure to study toxicological responses and slice viability. Exposure time is variable among PCLS toxicological studies, but the most common duration of culture is between 72 and 96 hours of exposure (Schmieder et al. 2000; de Graaf et al. 2010; Eide et al. 2014). A time-dependent increase in LDH activity has been shown in rats and murine liver *in vitro* cultures (Legrand et al. 1992; Hashemi et al. 1999).

On the other hand, Fisher et al. (1995a) found no time-dependent increase in LDH activity in Sprague-Dawley rat slices cultured over 120 hours. In addition, some PCLS toxicological studies exposing fish liver slices over 72 hours did not find a significant increase of LDH activity (Schmieder et al. 2000; Lemaire et al. 2011).

Higher levels of LDH activity in the first 24hr of exposure (0-24hr) compared to the 24-48hr exposure interval were most likely caused by tissue damage during coring and slicing processes. Due to its softness, polar cod liver tissue tended to disintegrate during the slicing process and this might have enhanced cell death and damage. Fisher et al. (1995a) suggested that tissue softness may determine the amount of damage occurring during the coring and slicing processes. Nevertheless, lower LDH activity in the 24-48hr exposure interval, indicates an apparent regeneration of liver tissue. This is supported by a study of Eide et al. (2014) showing a regeneration of Atlantic cod liver slices following 24hr of culture with healed edges and an increase in intact cells after 48hr of culture.

4.2 Histological analysis

In general, there was a large variability in slice thickness between specimens sampled. Some fish had slices that were variable in size, while other fish had lesser variations. Furthermore, slices sampled from fish B (viability experiment) and G (WY-14643) did not have a 250µm thickness nor close to it, slice thickness were between 80-200µm and 120-170µm, respectively. A study performed with the same microtome on the different mammalian species murine, rat, porcine, bovine and human showed as similar variability in slices thickness between animal species (Zimmermann et al. 2009). Some species, murine and porcine liver slices, showed a variation in slices thickness between 50-700µm and 20-700µm, respectively, while rat and human liver slices had lower variations, between 50-400µm and 10-420µm, respectively. Furthermore, this study showed that for most of the species, the average slice thickness was slightly below the set size (Zimmermann et al. 2009). Similarly, in the present study, the average thickness measured was systematically below 250µm and at about 200µm for all fish specimens. The high lipid content of polar cod liver and the relative flaccidity presented a challenge during slicing and may have played an important role in the obtained slice thickness. Furthermore, both temperature and the maturity stage of the fish sampled also affected liver slicing. The whole process of slicing should be performed under cold condition, by replacing ice frequently in the cutting tray. Maturity stage directly influences on liver lipids content, fish in a very mature stage have higher lipid content in the liver, making easier the slicing process. A previous attempt on performing PCLS on polar cod was made in January but due to small size and flaccidity of the liver it did not succeed.

The thickness of the tissue slices is an important feature in toxicological studies as this affects absorption and metabolism of contaminants and thus also the response (Lake & Price, 2013; Lerche-Langrand & Toutain, 2000). The absorption of pollutants increases with thinner slices. However, thin slices, below 100µm, are not recommended for experimental use due to the increase in the proportion of damaged cells relative to healthy ones. On the other hand, thick slices (>400µm) are more likely to suffer necrosis in the center of the slices because of reduced diffusion of oxygen and nutrients to inner cells

during the experiment (de Graaf et al. 2010). De Graaf et al. (2006) showed a strong influence between slice thickness and xenobiotic, metabolic rate, those which were thinner had higher metabolic rates. The potential effect of slice thickness on toxicological response in the present study will be discussed in the following sections.

Even though the average slice thickness was below the desired size, the method seemed reproducible among fish, which showed similar slice thickness across the different experiments. Hence, the method gave a relative good reproducibility during the slicing of polar cod livers although an improvement of the technique is needed. Further method development should consider a stabilization of the cores during slicing. Vargas et al. (2011) embedded brain tissue in a block of 2% agarose in order to remediate to the reduced tissue size. This method may be considered for polar cod PCLS to remediate to the softness of the tissue and consequently problems during slicing. In addition, as mentioned before, temperature and maturity stage should be considered as well.

4.3 Exposure experiments

4.3.1 Benzo(a)pyrene exposure

The viability of the polar cod PCLS, measured as LDH activity, did not seem to be compromised by the exposure to BaP at any concentrations (0.1 to 1000µM). Indeed, there was no significant increase in LDH activity observed with increasing BaP concentration, also corroborating previous *in vivo* studies which exposed fish to BaP. For instance, García-Tavera et al. (2013) did not find changes in LDH activity levels in plasma of adult tilapia (*Oreochromis niloticus*) exposed to BaP for 120hr. Similarly, LDH activity did not change in muscle tissues of European seabass (*Dicentrarchus labrax*) exposed to BaP concentration range for 96hr (Almeidad et al. 2012). In the present study, LDH was measured as a relative activity (increase in absorbance measured over time) similarly to Almeida et al. (2012). However, the heterogeneity of methods to evaluate viability as well as the differences in experimental designs between PCLS and other studies, make direct comparisons difficult.

The exposure to different BaP concentrations significantly induced the mRNA expression of *cyp1a1*. Exposure to BaP has previously been studied in polar cod *in vivo* and was found to be a potent inducer of *cyp1a1* (Nahrgang et al. 2009). Increase in *cyp1a1* mRNA expression by BaP has also been reported in other fish species (Levine & Oris, 1997; Levine & Oris, 1999; Yan et al. 2012; Kim et al. 2013). The significant dose-dependent induction of *cyp1a1* in polar cod PCLS can be considered as a positive control to test PCLS functionality.

Furthermore, differences in *cyp1a* mRNA expression among fish specimens might be explained by differences in slice thickness. Indeed, fish F had thinner slices (190±32µm) than fish E (226±39µm) and showed higher levels of *cyp1a* induction. As mentioned earlier (see 4.2 section), slice thickness influences pollutant absorption and metabolism (Lake & Price, 2013; Lerche-Langrand & Toutain, 2000) and slices of reduced thickness may have allowed a higher uptake of BaP in the tissue and thus an increased *cyp1a1* induction.

Unlike *cyp1a1* induction, BaP did not significantly change *vtg* mRNA expression, supporting previous *in vitro* studies. For instance, channel catfish (*Ictalurus punctatus*) hepatocytes exposed to different BaP concentrations (0.01, 10 and 100µM) did not alter *vtg* mRNA expression after 48hr of exposure (Monteverdi & Di Giulio, 1999). Furthermore, goldfish (*Carassius auratus*) exposed to BaP for 10 days also did not find an effect of it on *vtg* mRNA expression (Yan et al. 2012). Benzo(a)pyrene is well known as an AhR agonist which also has an anti-estrogenic effect on *vtg* mRNA expression (Navas & Segner, 2006; Kawahara et al. 2009; Yong et al. 2004). Nevertheless and although it was not statistically significant, the three fish showed a slight increase in *vtg* mRNA expression with increasing BaP concentration. This slight increase in *vtg* mRNA expression with increasing BaP suggests instead a possible estrogenic effect of benzo(a)pyrene on *vtg*. Monteverdi & Di Giulio (2000) showed a positive correlation between BaP metabolites accumulation in oocytes and increase in oocytes size in mummichog (*Fundulus heteroclitus*). Another fact which suggests a slight estrogenic effect of BaP is that fish E with the highest mean *vtg* values for all concentrations was a male and not a female. Finally, the female fish D had larger GSI (13.4%) compared to fish E and F (1.9% and 2.2%, respectively) suggesting that

fish D showed maturing gonads and should have had the highest *vtg* values among the three fish replicates.

4.3.2 WY-14643 experiment

The absence of an increase in LDH activity with increasing WY concentrations might have been influenced by the solvent DMSO concentration. In the original experimental design WY concentrations should have been dissolved in DMSO at 0.1% but due to a laboratory mistake, the final DMSO concentration was 1% for all WY concentrations. DMSO at 0.1% is the most frequent concentration used in toxicological *in vitro* fish liver experiments (Scholz & Segner, 1999; Schmieder et al. 2000; Lemaire et al. 2011; Gerbron et al. 2010; Eide et al. 2014) and it is suggested that DMSO at higher concentrations may have a cytotoxic effect on tissue in the culture medium (Mortensen et al. 2006). Therefore, absence of LDH activity enzyme along fish and concentrations might have been produced by high DMSO concentration in culture medium.

On the other hand, absence of LDH activity by exposure to WY has been also shown in other studies. An *in vitro* study exposing rats cerebella granule neurons to 0-100µM WY concentrations for 24hr did not find a significant increase in LDH activity at any of the concentrations (Smith et al. 2001). Another *in vitro* study exposed human chondrocytes to 0-1000µM WY concentrations for 48 hr and found a significant increase in LDH activity at 1000µM (Clockaerts et al. 2011). Similar results were obtained in PCLS with no significant increase in LDH activity in WY concentrations up to 100µM.

Increasing WY concentrations did not significantly increase *cyp1a*, *aox* and *pparα* mRNA expression. WY is known as a PPARα agonist pollutant in mammals (Woods et al. 2007). PPARα activation induces PP, increasing peroxisomal fatty acids β-oxidation enzymes synthesis, including *aox* and *cyp4a1* (Morimura et al. 2006; Tong et al. 2007). *cyp4a1* is not the only known CYP450 isoform induced by WY. Indeed, the transcription of *cyp1a* has also been documented to be activated by WY (Tong et al. 2007), but to a lesser extent than by AhR agonists, as BaP. In the present study, although it was not statistically significant, there was a positive trend in *cyp1a1* mRNA expression when WY concentration increased

for fish H and I slices. This is supported by other *in vitro* PCLS experiments performed on rats exposed to 50µM WY for 6hr (Pan et al. 2002) and 24hr (Meredith et al. 2003) where no significant raise in *cyp1a* were found, but a positive trend.

WY-14643 has been mainly used as a *pparα* agonist in toxicological studies performed in mammalian species, especially in rodents. In rodents species, an exposure to WY generally produces a significant increase in *pparα* receptor mRNA expression and consequently, an activation of genes encoding fatty acids β-oxidation enzymes, such as *aox* (Aoyama et al. 1998; Peters et al. 2000; Smith et al. 2004; Morimura et al. 2006). However, only few studies have exposed fish to WY. Leaver et al. (2005) exposed plaice (*Pleuronectes platessa*) and gilthead sea bream (*Sparus aurata*) *in vitro* larval cells to 50µM WY concentration for 20hr and showed a significant increase in *pparα* mRNA expression in both species compare to the control group. In the present study, *pparα* mRNA expression did not increase significantly, only fish I had a slight increase in *pparα* mRNA expression as concentration increased. However, fish G and H had a negative trend in *pparα* mRNA expression at high concentrations (1, 10 and 100µM) suggesting a slight inhibition of WY on *pparα* mRNA expression. In addition, *aox* mRNA expression had as well a negative trend as concentration increased in the three fish, suggesting a slight inhibition as well. Ongoing work linked to the cloning of polar cod PPAR*α* (Bilbao E. *pers com*) indicates a reduced protein homology in the ligand binding domain with other species that respond positively to WY. Thus, this may explain the lack of response in the present study.

WY-14643 may thus no represent a suitable model agonist for studying PPAR*α* mRNA expression in polar cod and other *pparα* agonist compounds should be considered.

4.3.3 PAHs mixtures experiments

No statistically significant increase in LDH activity with increasing 2-ring and 3-4-ring PAHs mixtures concentrations was seen. Previous studies have found contrasting results. For instance, Latif et al. (2014) exposed *L. rohita* to naphthalene (2-ring PAH) for 96 hr and found a significant increase in LDH activity in plasma. In addition, two studies by Tintos et al. (2006) and Tintos et al. (2007) showed in rainbow trout liver a significant

increase in lactate (µmol ml^{-1}) after 3 days of exposure to naphthalene. However, other *in vitro* (Navas & Segner, 2000) and *in vivo* (Oliveira et al. 2012) studies dealing with 3-4-ring PAHs (anthracene, 3-methylcholanthrene and pyrene) in rainbow trout liver cells and common goby, did not find a significant increase in LDH activity.

cyp1a mRNA expression was only significant induced when polar cod was exposed to 3-4-ring PAHs mixtures, but not in the 2-ring PAHs mixture. A couple of *in vivo* experiments on polar cod, have shown a dose-dependent expression of *cyp1a* when polar cod was exposed to water soluble fraction of crude oil (Nahrgang et al. 2010a) and to dietary crude oil (Nahrgang et al. 2010b). Lemaire et al. (2011) and (2012) exposed Atlantic salmon and deep-sea grenadine, respectively, to 3-methylcholanthrene concentrations similar to 10µM in PCLS for 21hr and 6hr, respectively, and observed a significant increase in *cyp1a* expression. Furthermore, Barron et al. (2004) collected data on CYP1A enzyme induction for 74 PAHs in fish and concluded that 2- and 3- ring PAHs were generally inactive in fish while 4-6 ring PAHs, containing fluoranthrene or phenanthrene structures, had the highest effect on CYP1A activity. In this study, concentration of phenanthrene related PAHs (phenanthrene, methyl phenanthrene and 9-propylphenanthrene) represented in total 40% of the entire 3-4-ring PAHs mixture.

Although there was not a statistically significant change, *cyp1a* mRNA expression had a negative trend with increasing to 2-ring PAHs mixture concentrations in all fish. Thus, this negative trend in *cyp1a* mRNA expression might be due to high concentration of PAHs in the mixture, producing baseline toxicity, also called narcosis (Meador, 2008). Baseline toxicity of PAHs is produced when the tissue concentration reaches approximately 400–1600µg g^{-1} and is considered a nonspecific, reversible and primary toxic action of PAHs. These concentration levels may disrupt cell membranes integrity and lead to death if the pollutant source is not removed (Meador, 2008). Naphthalene is known to cause baseline toxicity in fish (de Maagd et al. 1997; dos Santos et al. 2006) and may have produced narcotic effects in polar cod PCLS exposed to 500µM of naphthalene.

The *aox* and *pparα* mRNA expression did not significantly change with increasing 2 and 3-4-ring PAHs concentrations, similarly to the BaP exposure experiment. By contrast, some *in vivo* experiments have found a significant increase in *aox* and *pparα* mRNA expression when fish were exposed to crude oil (Bilbao et al. 2010b; Ruiz et al. 2012). This absence in significant increase in *aox* and *pparα* mRNA expression might be due to differences in species sensitivity to PAHs, as discussed previously (see under ligand binding domain, section 4.3.2.) or due to the absence from our mixtures of other PAHs or substance found in crude oil with the ability to induce *aox* and *pparα*.

Finally, *vtg* mRNA expression did not significantly change with increasing 2 and 3-4-ring PAHs mixtures eventhough, PAHs have been shown to exert both estrogenic and anti-estrogenic effect on *vtg* mRNA expression in other fishes species (Monteverdi & Di Giulio, 2000; Goksøyr, 2006; Navas & Segner, 2006). Weak negative trends (anti-estrogenic effect) in *vtg* mRNA expression in slices from female fish (J and L) exposed to both PAHs mixtures, might be related to a cross talk between AhR and ER as described in the introduction. Indeed, these two fish were in a gonadal maturation phase (Hop et al. 1995) with growing gonads. Supporting this anti-estrogenic theory, Navas & Segner (2000) exposed liver cells of rainbow trout to the 4-ring PAH 3-methylcholanthrene and to the 3-ring PAH anthracene (concentrations up to 6.25μM for both) together with 17 β-estradiol and found an antiestrogenic effect on VTG protein synthesis by 3-methylcholanthrene after 72hr of exposure. This latter study indicated that AhR-binding PAHs possess an antiestrogenic activity and that the antiestrogenic activity was mediated through the AhR (Navas & Segner, 2000).

These two experiments using PAHs mixtures aimed at investigating the potency of different groups of PAHs found in crude oil to polar cod PCLS. In general, slices exposed to 2-ring PAHs seemed to have a reduced response, regarding *cyp1a* mRNA expression, compared to slices exposed to 3-4-ring PAHs mixture. In addition, fish K and L slices were used for both 2-ring and 3-4-ring PAHs mixture experiments, allowing for direct comparison without the influence of inter-specimen variability. Both fish responded completely differently to these two different PAHs mixtures, indicating different toxicity properties of

the given PAHs mixtures. Several factors including the type of PAHs (alkylated or parent), and their structure and physico-chemical properties and biotransformation capacities may influence PAHs toxicity (Meador, 2008). In addition, an important aspect of the *in vitro* PCLS methodology is related to the slice thickness that may also have affected PAHs toxicity.

5 CONCLUSION

The successful development of the PCLS methodology allowed studying mechanisms of toxicity in polar cod. However, some improvement of this method on polar cod should be considered. Benzo(a)pyrene and PAH compounds showed a induction on transcriptional levels of *cyp1a1* but there were different responses between the PAHs mixtures. The lack of response of the *pparα* and *aox* exposed to a PPARα agonist model compound may be linked to a different receptor structure in polar cod compared to other species and will require further work.

6 BIBLIOGRAPHY

Almeida, J. R., Gravato, C., & Guilhermino, L. (2012). Biological parameters towards polycyclic aromatic hydrocarbons pollution: A study with dicentrarchus labrax L. exposed to the model compound benzo(a)pyrene. *Water, Air, and Soil Pollution*, *223*(8), 4709–4722. doi:10.1007/s11270-012-1227-0

AMAP report. (2002). Transport of contaminants to the Arctic and their fate. *Arctic Monitoring and Assessment Programme.*

AMAP report. (2008). Arctic Oil and Gas 2007. *Arctic Monitoring and Assessment Programme*.

AMAP report. (2009). Arctic Pollution 2009. *Arctic Monitoring and Assessment Programme*.

Andersen, O., Eijsink, V. G., & Thomassen, M. (2000). Multiple variants of the peroxisome proliferator-activated receptor (PPAR) gamma are expressed in the liver of atlantic salmon (Salmo salar). *Gene*, *255*(2), 411–418.

Aoyama, T., Peters, J. M., Iritani, N., Nakajima, T., Furihata, K., Hashimoto, T., & Gonzalez, F. J. (1998). Altered constitutive expression of fatty acid metabolizing enzymes in mice lacking the peroxisome proliferator activated receptor α (PPARα). *Journal of Biological Chemistry*, *273*(10), 5678–5684. doi:10.1074/jbc.273.10.5678

Arukwe, A., & Goksøyr, A. (2003). Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative Hepatology*, *2*, 4. doi:10.1186/1476-5926-2-4

Arzuaga, X., Ren, N., Stromberg, A., Black, E. P., Arsenescu, V., Cassis, L. A., & Hennig, B. (2009). Induction of gene pattern changes associated with dysfunctional lipid metabolism induced by dietary fat and exposure to a persistent organic pollutant. *Toxicology Letters*, *189*(2), 96–101. doi:10.1016/j.toxlet.2009.05.008

Bakke, T., Klungsøyr, J., & Sanni, S. (2013). Environmental impacts of produced water and drilling waste discharges from the Norwegian offshore petroleum industry. *Marine Environmental Research*, *92*, 154–169. doi:10.1016/j.marenvres.2013.09.012

Barron, M. G., Heintz, R., & Rice, S. D. (2004). Relative potency of PAHs and heterocycles as aryl hydrocarbon receptor agonists in fish. *Marine Environmental Research*, *58*(2-5), 95–100. doi:10.1016/j.marenvres.2004.03.001

Bilbao, E., de Cerio, O. D., Cajaraville, M. P., & Cancio, I. (2006). Cloning and expression pattern of peroxisomal enzymes in the mussel Mytilus galloprovincialis and in the

thicklip grey mullet Chelon labrosus: Generation of new tools to study peroxisome proliferation. *Marine Environmental Research*, *62*(SUPPL. 1), 109–112. doi:10.1016/j.marenvres.2006.04.004

Bilbao, E., Cajaraville, M. P., & Cancio, I. (2010a). Differential transcription of genes involved in peroxisome proliferation in thicklip grey mullets Chelon labrosus injected with benzo(a)pyrene. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *151*(3), 334–342. doi:10.1016/j.cbpc.2009.12.007

Bilbao, E., Raingeard, D., de Cerio, O. D., Ortiz-Zarragoitia, M., Ruiz, P., Izagirre, U., & Cancio, I. (2010b). Effects of exposure to Prestige-like heavy fuel oil and to perfluorooctane sulfonate on conventional biomarkers and target gene transcription in the thicklip grey mullet Chelon labrosus. *Aquatic Toxicology (Amsterdam, Netherlands)*, *98*(3), 282–96. doi:10.1016/j.aquatox.2010.02.018

Bio-Rad. (2006). Real-Time PCR Applications Guide. *Bio-Rad Laboratories.*

Boess, F., Kamber, M., Romer, S., Gasser, R., Muller, D., Albertini, S., & Suter, L. (2003). Gene expression in two hepatic cell lines, cultured primary hepatocytes, and liver slices compared to the in vivo liver gene expression in rats: Possible implications for toxicogenomics use of in vitro systems. *Toxicological Sciences*, *73*(2), 386–402. doi:10.1093/toxsci/kfg064

Bradstreet, M. S. W., & Cross, W. E. (1982). Trophic relationships at high Arctic ice edges. *Arctic*, *35*(1), 1–12.

Cajaraville, M. P., Cancio, I., Ibabe, A., & Orbea, A. (2003). Peroxisome proliferation as a biomarker in environmental pollution assessment. *Microscopy Research and Technique*, *61*(2), 191–202. doi:10.1002/jemt.10329

Cajaraville, M. P., & Ortiz-Zarragoitia, M. (2006). Erratum to “Specificity of peroxisome proliferation response in mussels exposed to environmental pollutants”. *Aquatic Toxicology*, *78S*, S111-S127. doi:10.1016/j.aquatox.2006.10.009

Carroll, J., Savinov, V., Savinova, T., Dahle, S., Mccrea, R., & Muir, D. C. G. (2008). PCBs, PBDEs and pesticides released to the Arctic Ocean by the Russian Rivers Ob and Yenisei. *Environmental Science and Technology*, *42*(1), 69–74. doi:10.1021/es071673l

Castelli, M. G., Rusten, M., Goksøyr, A., & Routti, H. (2014). MRNA expression of genes regulating lipid metabolism in ringed seals (Pusa hispida) from differently polluted areas. *Aquatic Toxicology (Amsterdam, Netherlands)*, *146*, 239–46. doi:10.1016/j.aquatox.2013.11.015

Chen, L., DeVries, a L., & Cheng, C. H. (1997). Convergent evolution of antifreeze glycoproteins in Antarctic notothenioid fish and Arctic cod. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(8), 3817–3822. doi:10.1073/pnas.94.8.3817

Christiansen, J., & George, S. (1995). Contamination of food by crude oil affects food selection and growth performance, but not appetite, in an Arctic fish, the polar cod (Boreogadus saida). *Polar Biology*, *15*(4), 277–281. doi:10.1007/BF00239848

Clockaerts, S., Bastiaansen-Jenniskens, Y. M., Feijt, C., Verhaar, J. a N., Somville, J., De Clerck, L. S., & Van Osch, G. J. V. M. (2011). Peroxisome proliferator activated receptor alpha activation decreases inflammatory and destructive responses in osteoarthritic cartilage. *Osteoarthritis and Cartilage*, *19*(7), 895–902. doi:10.1016/j.joca.2011.03.010

De Graaf, I. A. M., de Kanter, R., de Jager, M. H., Camacho, R., Langenkamp, E., van de Kerkhof, E. G., & Groothuis, G. M. M. (2006). Empirical Validation of a Rat in Vitro Organ Slice Model As a Tool for in Vivo Clearance Prediction Abstract *Drug Metabolism and Disposition*, *34*(4), 591–599. doi:10.1124/dmd.105.006726.

De Graaf, I. a M., Olinga, P., de Jager, M. H., Merema, M. T., de Kanter, R., van de Kerkhof, E. G., & Groothuis, G. M. M. (2010). Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. *Nature Protocols*, *5*(9), 1540–51. doi:10.1038/nprot.2010.111

De Maagd, P. G., van de Klundert, I. C., van Wezel, a P., Opperhuizen, a, & Sijm, D. T. (1997). Lipid content and time-to-death-dependent lethal body burdens of naphthalene and 1,2,4-trichlorobenzene in fathead minnow (Pimephales promelas). *Ecotoxicology and Environmental Safety*, *38*(3), 232–237. doi:10.1006/eesa.1997.1583

Dos Santos, T. D. C. A, Ngan, P. V., de Arruda Campos Rocha Passos, M. J., & Gomes, V. (2006). Effects of naphthalene on metabolic rate and ammonia excretion of juvenile Florida pompano, Trachinotus carolinus. *Journal of Experimental Marine Biology and Ecology*, *335*(1), 82–90. doi:10.1016/j.jembe.2006.02.019

Eide, M., Karlsen, O. A., Kryvi, H., Olsvik, P. A., & Goksøyr, A. (2014). Precision-cut liver slices of Atlantic cod (Gadus morhua): An in vitro system for studying the effects of environmental contaminants. *Aquatic Toxicology*, *153*, 110–115. doi:10.1016/j.aquatox.2013.10.027

EY report. (2013). Arctic oil and gas.

Fernandes, M. B., & Sicre, M. A. (1999). Polycyclic Aromatic Hydrocarbons in the Arctic: Ob and Yenisei Estuaries and Kara Sea Shelf. *Estuarine, Coastal and Shelf Science*, *48*(6), 725–737. doi:10.1006/ecss.1999.0472

Finlayson-Pitts, B. J., & Pitts, J. N. (1997). Tropospheric air pollution: ozone, airborne toxics, polycyclic aromatic hydrocarbons, and particles. *Science, 276*(5315), 1045–1052. doi:10.1126/science.276.5315.1045

Fisher, R. L., Hasal, S. J., Sanuik, J. T., Gandolfi, a. J., & Brendel, K. (1995a). Determination of Optimal Incubation Media and Suitable Slice Diameters in Precision-Cut Liver Slices: Optimization of Tissue Slice Culture, Part 2. *Toxicology Mechanisms and Methods, 5*(2), 115–130. doi:10.3109/15376519509045906

Fisher, R. L., Shaughnessy, R. P., Jenkins, P. M., Austin, M. L., Roth, G. L., Gandolfi, A. J., & Brendel, K. (1995b). Dynamic Organ Culture is Superior to Multiwell Plate Culture for Maintaining Precision-Cut Tissue Slices: Optimization of Tissue Slice Culture, Part 1. *Toxicology Mechanisms and Methods*, *5*(2), 99–113. doi:10.3109/15376519509045905

Gad, S. C. (2000). In vitro toxicology. *(T. & Francis, Ed.).*

García-Tavera, J. L., Valdés-Lozano, D., Poblete-Naredo, I., Albores-Medina, A., & Zapata-Pérez, O. (2013). Bile benzo[a]pyrene concentration and hepatic CYP1A induction in hypoxic adult tilapia (Oreochromis niloticus). *Chemosphere*, *92*(1), 16–23. doi:10.1016/j.chemosphere.2013.03.034

Geoffroy, M., Robert, D., Darnis, G., & Fortier, L. (2011). The aggregation of polar cod (Boreogadus saida) in the deep Atlantic layer of ice-covered Amundsen Gulf (Beaufort Sea) in winter. *Polar Biology*, *34*(12), 1959–1971. doi:10.1007/s00300-011-1019-9

Gerbron, M., Geraudie, P., Rotchell, J., & Minier, C. (2010). A new in vitro screening bioassay for the ecotoxicological evaluation of the estrogenic responses of environmental chemicals using roach (Rutilus rutilus) liver explant culture. *Environmental Toxicology*, *24*(3), 511–516. doi:10.1002/tox

Gjøsæter, H. (1995). Pelagic fish and the ecological impact of the modern fishing industry in the Barents Sea. *Arctic, 48,* 267-278.

Goksøyr, A. (2006). Endocrine disruptors in the marine environment: mechanisms of toxicity and their influence on reproductive processes in fish. *Journal of Toxicology and Environmental Health. Part A*, *69*(1-2), 175–184. doi:10.1080/15287390500259483

Gössling Stefan, H. C. M. (2006). Tourism and Global Environmental Change. *(Taylor & Francis, Ed.).*

Gouin, T., MacKay, D., Jones, K. C., Harner, T., & Meijer, S. N. (2004). Evidence for the "grasshopper" effect and fractionation during long-range atmospheric transport of

organic contaminants. *Environmental Pollution*, *128*(1-2), 139–148. doi:10.1016/j.envpol.2003.08.025

Gradinger, R. R., & Bluhm, B. a. (2004). In-situ observations on the distribution and behavior of amphipods and Arctic cod (Boreogadus saida) under the sea ice of the High Arctic Canada Basin. *Polar Biology*, *27*(10), 595–603. doi:10.1007/s00300-004-0630-4

Harms, I. H., Karcher, M. J., & Dethleff, D. (2000). Modelling Siberian river runoff - Implications for contaminant transport in the Arctic Ocean. *Journal of Marine Systems*, *27*(1-3), 95–115. doi:10.1016/S0924-7963(00)00062-2

Harvey, R. G. (1991). Polycyclic Aromatic Hydrocarbons. *Cambrige University Press.*

Hashemi, E., Dobrota, M., Till, C., & Ioannides, C. (1999). Structural and functional integrity of precision-cut liver slices in xenobiotic metabolism: a comparison of the dynamic organ and multiwell plate culture procedures. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*, *29*(1), 11–25. doi:10.1080/004982599238786

Hop, H., Graham, M., & Trudeau, V.L. (1995). Spawning energetics of Arctic cod (Boreogadus saida) in relation to seasonal development of the ovary and plasma sex steroids levels. *Canadian Journal of Fisheries and Science 52,*541-550.

Hop, H., & Gjøsæter H. (2013). Polar cod (Boreogadus saida) and capelin (Mallotus villosus) as key species in marine food webs of the Arctic and the Barents Sea. *Marine Biology Research, 9(9),*878-894. doi:10.1080/17451000.2013.775458

Hung, H., Kallenborn, R., Breivik, K., Su, Y., Brorström-Lundén, E., Olafsdottir, K., & Fellin, P. (2010). Atmospheric monitoring of organic pollutants in the Arctic under the Arctic Monitoring and Assessment Programme (AMAP): 1993-2006. *The Science of the Total Environment*, *408*(15), 2854–73. doi:10.1016/j.scitotenv.2009.10.044

Hurrell, J. W. (1995). Decadal Trends in the North Atlantic Oscillation: Regional Temperatures and Precipitation. *Science*, *269*(5224), 676–679.

Jessen, B. A., Mullins, J. S., de Peyster, A., & Stevens, G. J. (2003). Assessment of hepatocytes and liver slices as in vitro test systems to predict in vivo gene expression. *Toxicological Sciences*, *75*(1), 208–222. doi:10.1093/toxsci/kfg172

Jonsson, H., Sundt, R. C., Aas, E., & Sanni, S. (2010). The Arctic is no longer put on ice: evaluation of Polar cod (Boreogadus saida) as a monitoring species of oil pollution in cold waters. *Marine Pollution Bulletin*, *60*(3), 390–5. doi:10.1016/j.marpolbul.2009.10.014

Kawahara, S., Hrai, N., Arai, M., & Tatarazako, N. (2009). The effect of in vivo co-exposure to estrone and AhR-ligands on estrogenic effect to vitellogenin production and EROD activity. *Environmental Toxicology and Pharmacology*, *27*(1), 139–143. doi:10.1016/j.etap.2008.09.001

Kim, R. O., Kim, B. M., Hwang, D. S., Au, D. W. T., Jung, J. H., Shim, W. J., & Lee, J. S. (2013). Evaluation of biomarker potential of cytochrome P450 1A (CYP1A) gene in the marine medaka, Oryzias melastigma exposed to water-accommodated fractions (WAFs) of Iranian crude oil. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *157*(2), 172–182. doi:10.1016/j.cbpc.2012.11.003

Kim, S. G., Park, D. K., Jang, S. W., Lee, J. S., Kim, S. S., & Chung, M. H. (2008). Effects of dietary benzo[a]pyrene on growth and hematological parameters in juvenile rockfish, Sebastes schlegeli (Hilgendorf). *Bulletin of Environmental Contamination and Toxicology*, *81*(5), 470–474. doi:10.1007/s00128-008-9499-1

Klaassen, C. D. (2013). Casarett & Doull's Toxicology the Basic Science of Poisons (8th ed.). *McGraw-Hill Education*.

Knol, M. (2011). The uncertainties of precaution: Zero discharges in the Barents Sea. *Marine Policy*, *35*(3), 399–404. doi:10.1016/j.marpol.2010.10.018

Krebs, H. A. (1933). *Hoppe-Seyler's Zeitschrift*, *217,191*.

Lake, B. G., & Price, R. J. (2013). Evaluation of the metabolism and hepatotoxicity of xenobiotics utilizing precision-cut slices. *Xenobiotica*, *43*(1), 41–53. doi:10.3109/00498254.2012.734643

Latif, A., Khalid, M., & Ali, M. (2014). An Assessment of Naphthalene Stress on Renal and Hepatic Functional Integrity in Labeo Rohita. *International Journal of Current Enginering and Technology*, *4*(1), 319–324.

Law, K. S., & Stohl, A. (2007). Arctic air pollution: origins and impacts. *Science*, *315*(5818), 1537–1540. doi:10.1126/science.1137695

Leaver, M. J., Boukouvala, E., Antonopoulou, E., Diez, A., Favre-Krey, L., Tariq Ezaz, M., & Krey, G. (2005). Three peroxisome proliferator-activated receptor isotypes from each of two species of marine fish. *Endocrinology*, *146*(7), 3150–3162. doi:10.1210/en.2004-1638

Legrand, C., Bour, J. M., Jacob, C., & Martial, A. (1992). Lactate dehydrogenase (LDH) activity of the number of dead cells in the medium of cultured eukaryotic cells as marker. *Journal of Biotechnology*, *31*(2), 234. doi:10.1016/0168-1656(93)90165-J

Lemaire, B., Beck, M., Jaspart, M., Debier, C., Calderon, P. B., Thomé, J.P., & Rees, J.F. (2011). Precision-Cut Liver Slices of Salmo salar as a tool to investigate the oxidative impact of CYP1A-mediated PCB 126 and 3-methylcholanthrene metabolism. *Toxicology in Vitro: An International Journal Published in Association with BIBRA* , *25*(1), 335–42. doi:10.1016/j.tiv.2010.10.002

Lemaire, B., Debier, C., Calderon, P.B., Thomé, J.P., Stegeman, J., Mork J., & Rees, J. F. (2012). Precision-Cut Liver Slices To Investigate Responsiveness of Deep-Sea Fish to Contaminants at High Pressure. *Environmental Science & Technology*, *46*(August), 10310–10316.

Lerche-Langrand, C., & Toutain, H. J. (2000). Precision-cut liver slices : characteristics and use for in vitro. *Toxicology*, *153*, 221–253.

Levine, S. L., & Oris, J. T. (1997). Induction of CYP1A mRNA and catalytic activity in gizzard shad (Dorosoma cepedianum) after waterborne exposure to benzo[a]pyrene. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*,, *118*(3), 397–404. doi:10.1016/S0742-8413(97)00161-8

Levine, S. L., & Oris, J. T. (1999). CYP1A expression in liver and gill of rainbow trout following waterborne exposure: Implications for biomarker determination. *Aquatic Toxicology*, *46*(3-4), 279–287. doi:10.1016/S0166-445X(98)00124-6

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, *25*(4), 402–8. doi:10.1006/meth.2001.1262

Lonne, O. J., & Gulliksen, B. (1989). Size , Age and Diet of Polar Cod , Boreogadus saida (Lepechin 1773), in Ice Covered Waters. *Polar Biology,9,* 187–191.

Lye, C. M. (2000). Impact of oestrogenic substances from oil production at sea. *Toxicology Letters*, *112-113*, 265–272. doi:10.1016/S0378-4274(99)00233-7

Madsen, J., Tombre, I., & Eide, N. E. (2009). Effects of disturbance on geese in Svalbard implications for regulating increasing tourism. *Polar research 28,* 376-389. doi:10.1111/j.1751-8369.2009.00120.x

Maglich, J. M., Caravella, J. A., Lambert, M. H., Willson, T. M., Moore, J. T., & Ramamurthy, L. (2003). The first completed genome sequence from a teleost fish (Fugu rubripes) adds significant diversity to the nuclear receptor superfamily. *Nucleic Acids Research*, *31*(14), 4051–4058. doi:10.1093/nar/gkg444

Meador, J. P. (2008). Polycyclic Aromatic Hydrocarbons. Encyclopedia of Ecology. *Encyclopedia of Ecology, 4, 2881-2891. Oxford: Elsevier*

Meador, J.P., Sommers, F.C., Ylitalo, G.M., & Sloan, C.A. (2006). Altered growth and related physiological responses in juvenile Chinook salmon (Onchorhynchus tshawytscha) from dietary exposure to polycyclic aromatic hydrocarbons (PAHs). *Canadial journal of Fisheries and Aquatic Sciences,63,*2364-2376.doi: 10.1139/F06-127

Meland, S., Farmen, E., Heier, L. S., Rosseland, B. O., Salbu, B., Song, Y., & Tollefsen, K. E. (2011). Hepatic gene expression profile in brown trout (Salmo trutta) exposed to traffic related contaminants. *The Science of the Total Environment*, *409*(8), 1430–43. doi:10.1016/j.scitotenv.2011.01.013

Meredith, C., Scott, M. P., Renwick, a B., Price, R. J., & Lake, B. G. (2003). Studies on the induction of rat hepatic CYP1A, CYP2B, CYP3A and CYP4A subfamily form mRNAs in vivo and in vitro using precision-cut rat liver slices. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*, *33*(5), 511–527. doi:10.1080/0049825031000085960

Monteverdi, G. H., & Di Giulio, R. T. (1999). E nvironmental C ontamination a n d T oxicology An Enzyme-Linked Immunosorbent Assay for Estrogenicity Using Primary Hepatocyte Cultures from the Channel Catfish (Ictalurus punctatus). *New York*, *69*, 62–69.

Monteverdi, G. H., & Di Giulio, R. T. (2000). Oocytic accumulation and tissue distribution of 2,3,7,8-tetrachlorodibenzo-p-dioxin and benzo[a]pyrene in gravid Fundulus heteroclitus. *Environmental Toxicology and Chemistry*, *19*(10), 2512–2518.

Morimura, K., Cheung, C., Ward, J. M., Reddy, J. K., & Gonzalez, F. J. (2006). Differential susceptibility of mice humanized for peroxisome proliferator-activated receptor alpha to Wy-14,643-induced liver tumorigenesis. *Carcinogenesis*, *27*(5), 1074–1080. doi:10.1093/carcin/bgi329

Mortensen, A. S., Tolfsen, C. C., & Arukwe, A. (2006). Gene expression patterns in estrogen (nonylphenol) and aryl hydrocarbon receptor agonists (PCB-77) interaction using rainbow trout (Oncorhynchus Mykiss) primary hepatocyte culture. *Journal of Toxicology and Environmental Health. Part A*, *69*(1-2), 1–19. doi:10.1080/15287390500257792

Nahrgang, J., Camus, L., Gonzalez, P., Goksøyr, A., Christiansen, J. S., & Hop, H. (2009). PAH biomarker responses in polar cod (Boreogadus saida) exposed to benzo(a)pyrene. *Aquatic Toxicology*, *94*(4), 309–319. doi:10.1016/j.aquatox.2009.07.017

Nahrgang, J., Camus, L., Carls, M. G., Gonzalez, P., Jönsson, M., Taban, I. C., … Hop, H. (2010a). Biomarker responses in polar cod (Boreogadus saida) exposed to the water soluble fraction of crude oil. *Aquatic Toxicology*, *97*(3), 234–42. doi:10.1016/j.aquatox.2009.11.003

Nahrgang, J., Camus, L., Gonzalez, P., Jönsson, M., Christiansen, J. S., & Hop, H. (2010b). Biomarker responses in polar cod (Boreogadus saida) exposed to dietary crude oil. *Aquatic Toxicology*, *96*(1), 77–83. doi:10.1016/j.aquatox.2009.09.018

Nahrgang, J., Varpe, Ø., Korshunova, E., Murzina, S., Hallanger, I. G., Vieweg, I., & Berge, J. (2014). Gender specific reproductive strategies of an arctic key species (Boreogadus saida) and implications of climate change. *PLoS ONE*, *9*(5), 1–12. doi:10.1371/journal.pone.0098452

Navas, J. M., & Segner, H. (2000). Antiestrogenicity of β-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: Evidence for a role of the arylhydrocarbon receptor. *Aquatic Toxicology*, *51*(1), 79–92. doi:10.1016/S0166-445X(00)00100-4

Navas, J. M., & Segner, H. (2006). Vitellogenin synthesis in primary cultures of fish liver cells as endpoint for in vitro screening of the (anti)estrogenic activity of chemical substances. *Aquatic Toxicology*, *80*(1), 1–22. doi:10.1016/j.aquatox.2006.07.013

Oil and gas industry association. (2014). Environmental report. *Environmental Policy and Law*, *4*(4), 177–177. doi:10.1016/S0378-777X(78)80017-1

Orbea, A., Ortiz-Zarragoitia, M., Solé, M., Porte, C., & Cajaraville, M. P. (2002). Antioxidant enzymes and peroxisome proliferation in relation to contaminant body burdens of PAHs and PCBs in bivalve molluscs, crabs and fish from the Urdaibai and Plentzia estuaries (Bay of Biscay). *Aquatic Toxicology*, *58*(1-2), 75–98. doi:10.1016/S0166-445X(01)00226-0

Pan, J., Xiang, Q., Renwick, a. B., Price, R. J., Ball, S. E., Kao, J., … Lake, B. G. (2002). Use of a quantitative real-time reverse transcription-polymerase chain reaction method to study the induction of CYP1A, CYP2B and CYP4A forms in precision-cut rat liver slices. *Xenobiotica*, *32*(9), 739–747. doi:10.1080/00498250210147115

Peters, J. M., Cattley, R. C., & Gonzalez, F. J. (1997). Role of PPAR?? in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. *Carcinogenesis*, *18*(11), 2029–2033. doi:10.1093/carcin/18.11.2029

Peters, J. M., Rusyn, I., Rose, M. L., Gonzalez, F. J., & Thurman, R. G. (2000). Peroxisome proliferator-activated receptor alpha is restricted to hepatic parenchymal cells, not Kupffer cells: implications for the mechanism of action of peroxisome proliferators in hepatocarcinogenesis. *Carcinogenesis*, *21*(4), 823–826.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), e45. doi:10.1093/nar/29.9.e45

Rand, K. M., Whitehouse, A., Logerwell, E. a., Ahgeak, E., Hibpshman, R., & Parker-Stetter, S. (2013). The diets of polar cod (Boreogadus saida) from August 2008 in the US Beaufort Sea. *Polar Biology*, *36*(6), 907–912. doi:10.1007/s00300-013-1303-y

Ruiz, P., Ortiz-Zarragoitia, M., Orbea, A., Theron, M., Le Floch, S., & Cajaraville, M. P. (2012). Responses of conventional and molecular biomarkers in turbot Scophthalmus maximus exposed to heavy fuel oil no. 6 and styrene. *Aquatic Toxicology*, *116-117*, 116–28. doi:10.1016/j.aquatox.2012.02.004

Samanta, S. K., Singh, O. V., & Jain, R. K. (2002). Polycyclic aromatic hydrocarbons: Environmental pollution and bioremediation. *Trends in Biotechnology*, *20*(6), 243–248. doi:10.1016/S0167-7799(02)01943-1

Schmieder, P., Tapper, M., Linnum, a., Denny, J., Kolanczyk, R., & Johnson, R. (2000). Optimization of a precision-cut trout liver tissue slice assay as a screen for vitellogenin induction: comparison of slice incubation techniques. *Aquatic Toxicology*, *49*(4), 251–268. doi:10.1016/S0166-445X(99)00087-9

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*, *3*(6), 1101–1108. doi:10.1038/nprot.2008.73

Scholz, S., & Segner, H. (1999). Induction of CYP1A in primary cultures of rainbow trout (Oncorhynchus mykiss) liver cells: concentration-response relationships of four model substances. *Ecotoxicology and Environmental Safety*, *43*(3), 252–260. doi:10.1006/eesa.1999.1786

Shindell, D. T., Chin, M., Dentener, F., Doherty, R. M., Faluvegi, G., Fiore, A. M., & Koch, D. M. (2008). and Physics A multi-model assessment of pollution transport to the Arctic, 5353–5372.

Smith, P.F., Gandolfi, A.J., Krumdieck, C.L., Putnam, C.W., Zukoski, C.F., III, W. M., & Brendel, K. (1985). Dynamic organ culture of precision liver slices for in vitro toxicology. *Life Sciences*, *36*(c), 1367–1375.

Smith, S. A., May, F. J., Monteith, G. R., & Roberts-Thomson, S. J. (2001). Activation of the peroxisome proliferator-activated receptor-alpha enhances cell death in cultured cerebellar granule cells. *Journal of Neuroscience Research*, *66*(2), 236–241. doi:10.1002/jnr.1216

Smith, S. A., Monteith, G. R., Robinson, J. A., Venkata, N. G., May, F. J., & Roberts-Thomson, S. J. (2004). Effect of the peroxisome proliferator-activated receptor beta activator GW0742 in rat cultured cerebellar granule neurons. *Journal of Neuroscience Research*, *77*(2), 240–249. doi:10.1002/jnr.20153

Tintos, A., Gesto, M., Alvarez, R., Míguez, J. M., & Soengas, J. L. (2006). Interactive effects of naphthalene treatment and the onset of vitellogenesis on energy metabolism in liver and gonad, and plasma steroid hormones of rainbow trout Oncorhynchus mykiss. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *144*(2), 155–165. doi:10.1016/j.cbpc.2006.07.009

Tintos, A., Gesto, M., Míguez, J. M., & Soengas, J. L. (2007). Naphthalene treatment alters liver intermediary metabolism and levels of steroid hormones in plasma of rainbow trout (Oncorhynchus mykiss). *Ecotoxicology and Environmental Safety*, *66*(2), 139–147. doi:10.1016/j.ecoenv.2005.11.008

Tong, Z., Chandrasekaran, a, Jordan, R., Markiewicz, V., Li, H., Xiang, Q., … Scatina, J. (2007). Effects of ertiprotafib on hepatic cytochrome P450 and peroxisomal enzymes in rats and dogs, and in rat and human primary hepatocytes. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*, *37*(1), 1–18. doi:10.1080/00498250600965115

USGS fact sheet 2008-3049. (2008). Circum-Arctic Resource Appraisal Estimates of Undiscovered Oil and Gas North of the Arctic Circle.

Usgs, S. (2003). USGS World Petroleum Assessment 2000, *03*, 3–4.

Vargas, R., Jóhannesdóttir, I. T., Sigurgeirsson, B., Thornorsteinsson, H., & Karlsson, K. A E. (2011). The zebrafish brain in research and teaching: a simple in vivo and in vitro model for the study of spontaneous neural activity. *Advances in Physiology Education*, *35*(2), 188–196. doi:10.1152/advan.00099.2010

Warbug, O. (1923). *Biochemische Zeitschrift*, *142*,*317*.

Welch, H. E., Bergmann, M. a, Siferd, T. D., Martin, K. a, Curtis, M. F., Crawford, R. E., & Hop, H. (1992). Energy flow through the marine ecosystem of the Lancaster South Region, Arctic Canada. *Arctic*, *45*(4), 343–357.

Woods, C. G., Heuvel, J. P. Vanden, & Rusyn, I. (2007). Genomic profiling in nuclear receptor-mediated toxicity. *Toxicologic Pathology*, *35*(4), 474–494. doi:10.1080/01926230701311351

Yan, Z., Lu, G., & He, J. (2012). Reciprocal inhibiting interactive mechanism between the estrogen receptor and aryl hydrocarbon receptor signaling pathways in goldfish (Carassius auratus) exposed to 17??-estradiol and benzo[a]pyrene. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *156*(1), 17–23. doi:10.1016/j.cbpc.2012.03.001

Yong, L., Wong, C. K. C., Ying, X., & Wong, M. H. (2004). Effects of 2,3,7,8-TCDD and benzo[a]pyrene on modulating vitellogenin expression in primary culture of crucian

carp (Carassius auratus) hepatocytes. *Chinese Science Bulletin*, *49*(22), 2372–2378. doi:10.1007/BF03183425

Zimmermann, M., Lange, S., Lampe, J., Smirnow, I., Bitzer, M., Lauer, U. M., & Tübingen, M. U. (2009). Precision-Cut Slices of Normal and Tumorous Liver Tissues Generated with the Leica VT1200 S Vibrating Blade Microtome Precision-Cut Slices of Normal and Tumorous Liver Tissues Generated with the Leica VT1200 S Vibrating Blade Microtome. *Leica Biosystems*.

7 APPENDIX

Discharge	Category[1]	NEA colour category
Water		
Substances on Ospar's Plonor list		Green
Substances with no test data	0	Black
Hormone disruptors [2]	1	Black
Substances thought to be, or which are, hazardous to genes or reproduction [3]	1.1	Black
List of prioritised substances in result objective 1 (priority list), proposition no 1 (2009-2010) to the Storting	2	Black
Biodegradability < 20% and log Pow ≥ 5 [4(4)]	3	Black
Biodegradability < 20% and toxicity EC_{50} or LC_{50} ≤ 10 mg/l	4	Black
Chemicals on Ospar's taint list [5]	5	Red
Two out of three categories: biodegradability < 60%, log Pow ≥ 3, EC_{50} or LC_{50} ≤ 10 mg/l [4]	6	Red
Inorganic and EC_{50} or LC_{50} ≤ 1 mg/l	7	Red
Biodegradability < 20% [4]	8	Red

Figure A1: Hazardous substances divided into four categories (color coded) depending on their threat to the environment by The Norwegian Environment Agency.

Discharge	Category[1]	NEA colour category
Substances in yellow category:		
Substances covered by Reach annexes IV and V [6]	99	Yellow
Substances with biodegradability > 60%	100	Yellow
Substances with biodegradability 20-60%		
Sub-category 1: expected to biodegrade fully	101	Yellow
Sub-category 2: expected to biodegrade to environmentally non-hazardous substances	102	Yellow
Sub-category 3: expected to biodegrade to substances which could be environmentally hazardous	102	Yellow

[1] A description of the category is provided in the flow diagram. Category in table 5-1 has been related to category in table 6-1 to ensure correspondence with reported figures in the two tables.

[2] Removed from the black category in the activities regulations.

[3] Substances hazardous to genes or reproduction are understood to mean mutagen categories (Mut) 1 and 2 and reproduction categories (Rep) 1 and 2, see appendix 1 to the regulations on labelling, etc, of hazardous chemicals or self-classification.

[4] Data for degradability and bio-accumulation must accord with approved tests for offshore chemicals.

[5] Removed from the red category in the activities regulations.

[6] Commission regulation 987/2008. The NEA must assess whether the substance is covered by annex V.

Figure A2: Hazardous substances divided into four categories (color coded) depending on their threat to the environment by The Norwegian Environment Agency.

Table A1: Different literature used to make a protocol for PCLS in polar cod. Name of the author, species studied, excision buffer used, coring diameter (mm), silicing and incubation buffer used; time, temperature, incubation vials and shaking method used during incubation, extra comments and test used for assessing viability of the slices.

Author	**Species**	**Excision Buffer**	**Coring mm**	**Slice thickness um**	**Slicing/Incubation buffer**	**Incub time**	**Incub temp**	**Incub vials**	**shaking**	**comments**	**viability**
Marta Eide et al 2013	Cod		8	250	Slicing: Ice cod perfusion buffer in Ellesat 2011: NaCL (122mM), KCl (4.8mM), MgSo4 (1.2mM) NaHPO4 (11mM), NaHCO3 (3.7mM), pH 8.4 Incubation: 500uL L15+10% FBS, 1% penicillin/strepto/amphotericin	72h	10 C	24 well plates	No shaking	2h incub. And ½ media exchange	
Lemaire et al 2011	Salmon	Perfusion in HBSS (lacking Ca and Mg2+). when white then cut and placed in ice cold HBSS with Ca and Mg2+	8	100	Slicing: HBSS with Ca and Mg2+ Incub: 500uL Leibovitz´s 15 (without phenol red) with 10% FBS, 1% Penstrep (Penicillin 10,000U/ml, Streptomycin 10,000 ug/ml)	21h	15 C	24 wel plates	horizontal agitation (175rpm)		LDH leakage over time :50uL aliquots media (cytotoxicity detection kit from Roche) removed or replaced LDH activity in slices on homogenates (in L15+) ATP: ATP lite 1 Step kit (perkin elmer)

Hadi et al 2012	mouse	Cold Univ Wisconsin organ preservation solution (Dupont critical care IL)	5	200-300, ca 4.5-5.5 mg	Slicing: Krebs-Henseleit buffer saturated with O2/CO2 (95%/5%) For incubation : 1.3 ml of Williams´medium E with glutamax 1, 25 mM D-glucose, 50 ug /ml gentamicin (WEGG medium) under saturated carbogen	24-48h	37 C	12 well plate	90 times/min	1h Preincubation and then transfer to fresh WEGG medium and "test substance" without medium change after princub	After 24h: ATP contentb (3 replicates): 4.3 nmol/slice in control LDH leakage into media: pooled 50 uL of medium from each of the 3 replicates at 24h. Determination of LDH in the slices after 1h preincubation (3 slices) See Table 1
Gilroy et al 1996	**rainbow trout**		8	300	Cold modified Hanks medium (HM) with 5mM glucose and 10 mM Hepes (Moon et al 1985).	6hr O2/CO2 (19/1)	14 C	20ml scintillation vials (1 slice per vial) with 1% BSA	orbital shaking under		ATP production (Sigma kit): 1h intervals, 2 slices were rinsed in 3ml ice cold 0.6NHClO4 and homogenized. Levels 0.4 nmol/mg prot).
Thohan et al., 2001	rat		10	250	0.9% saline, pH 7.4	2h	37 C	23 ml vials	6rpm rotation	1 slice every 3-4sec Pre-incubation 1h selection of slice on appearance slices swept away by a stream of ice cold buffer tp a collection chamber	Release of LDH from the tissue slice into the medium and LDH activity from the tissue (Sigma kit 228-UV).

Zimmermann_Leica	Human, rat, porcine, mouse, bovine	ice cold Custodiol transplantation media	8	200	Krebs.Henseleit Buffer (with 25mM glucose, 25 mM NaHCO3, 10mM Hepes) containing 5 ml of oxygenated medium, pH 7.4.			10 cm petridish		Knife angle: 15degrees Speed: 0.4-1mm(s Oscillation amplitude: 3 recommend to start at low sectioning speed (0.4 mm/s) If the material is sufficiently stable, this parameter can be increased over time. Oscillation amplitude over 3 to avoid unwanted disruption of the material continuous strok	ATP quantification kit (promega celltiter Glo). 3 slices per timepoint frozen -80C. ATP goes down over time. Levels start at ca 100nmol/L for all species tested.
Boess et al 2003	Rats		8	200-250	Slicing: Krebs.Henseleit HEPES Buffer , pH 7.4 O2/CO2 (95%/5%) Incubation:10 ml Williams´medium E with 1.3 insulin, 2.9 mg glutamine, 0.5 gentamicin, 0.1 ampicillin	24h	37 C	25 ml flask		2h preincub. Gassed with carbogen	
Schmieder et al 2000	trout	Fish anesthetised, then liver perfusion in HBSS (lacking Ca and Mg2+), pH 7.8 for 5 min to avoid clotting	8	200	Ice cold HBSS, pH 7.8 with glucose 900mg/l Incubation:1.7 ml L15+10%Fetal bovine serum+Penicillin/Streptomicin		11 C	12 wll plate (1 slice/ well)	Orvital 125rpm	New blade for each core Normal air	LDH leakage compared to total LDH activity at T0 Cellular K+ content in slices

Shilling and William 2000	Rainbow trout		8	250	Ice cold Hanks modified salts buffer, 10mMHEPES, 8mM sodium bicarbonate, pH 7.2 Incubation: same buffer with 1%BSA and 1% gentamicin	120h	14 C	12 well plates (1/well)	90 rpm	All glassware sterilised. Medium sterilized (0.22um filter). Plates covered into sealed tupperwar e saturated with O2/CO2 (95%/5%) Refreshed after 12h-media not changed	ATP production Sigma kit
Cravedi et al 1998	trout	Krebs.Hense leit Buffer, pH 7.4 O2/CO2 (95%/5%)	8	200	Slicing:Krebs.Henselei t Buffer, pH 7.4 O2/CO2 (95%/5%) Incubation: 1ml DMEM medium, O2/CO2 (95%/5%)	24h	20 C	12 well plate	Gyratory	45 min preincub.	LDH leakage (Sigma detection kit)

Table A2: mRNA expression of *cyp1a* and *vtg* (mean fold fold change ±SE) of polar cod PCLS exposed to different concentrations (0, 0.1, 1, 10, 100 and 1000µM) of benzo(a)pyrene during 48 hr exposure.

Experiment	Gene	Fish	Treatment	Mean ± SE
benzo(a)pyrene	cyp1a	Fish D	0	1.2±0.5
			0.1	4.6±2.2
			1	5.7±0.8
			10	31.5±12.4
			100	83.8±30.3
			1000	79.2±32.2
		Fish E	0	1
			1	3.8±1.3
			10	5.2±1.3
			100	9.0±2.3
			1000	6.3±0.2
		Fish F	0	1.5±0.4
			0.1	15±6.5
			1	4.3±1
			10	14.1±3.3
			100	20.4±8
			1000	35.0±4.4
	vtg	Fish D	0	1.18±0.3
			0.1	1.0±0.6
			1	0.8±0.1
			10	2.0±0.2
			100	1.7±0.4
			1000	1.8±0.6
		Fish E	0	1
			1	2.8±0.7
			10	2.5±0.2
			100	2.5±0.7
			1000	3.4±0.7
		Fish F	0	1.1±0.2
			0.1	1.6±0.9
			1	0.7±0.2
			10	1.7±0.6
			100	1.1±0.4
			1000	1.4±0.1

Table A3: mRNA expression of *cyp1a*, *pparα* and *aox* (mean fold fold change ±SE) of polar cod PCLS exposed to different concentrations (0, 0.1, 1, 10 and 100µM) of WY-14643 during 48 hr exposure.

Experiment	Gene	Fish	Treatment	Mean ± SE
WY-14643	cyp1a	Fish G	0	1.2±0.4
			0.1	1.5±0.4
			1	1.4±0.4
			10	0.2±0
			100	1.0±0.19
		Fish H	0	1.2±0.4
			0.1	3.2±1.5
			1	2.7±0.8
			10	5.2±2.6
			100	
		Fish I	0	1
			0.1	1.3±0.6
			1	2.7
			10	3±1
			100	8.5±3.7
	aox	Fish G	0	1±0
			0.1	1.1±0
			1	0.8±0
			10	0.5±0.1
			100	0.4±0.1
		Fish H	0	1±0.1
			0.1	1.2±0.1
			1	2.5±0.8
			10	2.0±0.2
			100	2.5±1.2
		Fish I	0	1
			0.1	2.2±0.2
			1	1.19
			10	1±0
			100	2±0.8
	pparα	Fish G	0	1.0±0.1
			0.1	1.4±0.3
			1	0.8±0
			10	0.9±0.2
			100	1.4±0.2
		Fish H	0	1.0±0.2
			0.1	1.7±0.1
			1	2.1±0.5
			10	1.1±0.4
			100	0.9±0.2
		Fish I	0	1
			0.1	1.81±0.3
			1	2.7
			10	2.2±0.4
			100	2.6±1.1

Table A4: mRNA expression of *cyp1a*, *pparα, aox* and *vtg* (mean fold fold change ±SE) of polar cod PCLS exposed to different concentrations (0, 0.5, 5, 50 and 500µM) of 2-ring PAHs mixture during 48 hr exposure.

Experiment	Gene	Fish	Treatment	Mean ± SE
2-ring PAHs mixture	cyp1a	Fish J	0	1.0±0
			0.5	0.8±0.3
			5	1.6±0.8
			50	0.7±0
			500	1.2±0.7
		Fish K	0	1.0±0.1
			0.5	0.8±0.2
			5	1±0
			50	0.8±0.1
			500	0.4±0.2
		Fish L	0	1.1±0.2
			0.5	1.5±0.7
			5	1.0±0.6
			50	0.7±0.4
			500	0.7±0.3
	aox	Fish J	0	1.1±0.2
			0.5	0.8±0.2
			5	0.7±0.2
			50	1.4±0.6
			500	1.1±0.2
		Fish K	0	1±0.1
			0.5	0.9±0.1
			5	2.3±0.6
			50	2.4±0.8
			500	0.4±0.1
		Fish L	0	1.1±0.3
			0.5	1.1±0.2
			5	1.9±0.9
			50	1.6±0.3
			500	1.1±0.1
	pparα	Fish J	0	1.1±0.2
			0.5	0.3±0
			5	0.4±0.1
			50	0.7±0.3
			500	0.4±0
		Fish K	0	1.0±0.3
			0.5	0.5±0.2
			5	0.8±0.1
			50	1.0±0.4
			500	0.3±0.1
		Fish L	0	1.0±0.2
			0.5	1.0±0.1
			5	1.2±0.4
			50	1.5±0.4
			500	1.0±0.2
	vtg	Fish J	0	0.8±0.1
			0.5	1.1±0.3
			5	1.7±0.7
			50	1.6±0.7
			500	1.6±0.3
		Fish K	0	1.2±0.7
			0.5	0.5±0.1
			5	0.8±0.2

		50	0.7±0.2
		500	0.1±0
	Fish L	0	1.0±0
		0.5	1.0±0.1
		5	0.9±0
		50	0.7±0.1
		500	1.0±0.1

Table A5: mRNA expression of *cyp1a*, *pparα, aox* and *vtg* (mean fold fold change ±SE) of polar cod PCLS exposed to different concentrations (0, 0.1, 1, 10 and 100µM) of 3-4--ring PAHs mixture during 48 hr exposure.

Gene	Fish	Treatment	Mean ± SE
cyp1a	Fish K	0	1
		0.1	2.1±0.2
		1	3.6±1.0
		10	2.5±0.1
		100	5.2±1.3
	Fish L	0	1.2±0.5
		0.1	1.3±0.5
		1	0.8
		10	0.9±0.4
		100	5.2±0.8
aox	Fish K	0	1
		0.1	1.3±0.6
		1	2.7
		10	3±1
		100	8.5±3.7
	Fish L	0	1.0±0.2
		0.1	1.3±0.4
		1	1.4
		10	1.9±1.0
		100	1.2±0.2
pparα	Fish K	0	1
		0.1	0.5±0
		1	1.1±0.3
		10	0.5±0.1
		100	0.4±0.1
	Fish L	0	1.0±0.1
		0.1	1.1±0.4
		1	2.2
		10	2.2±1.0
		100	1.7±0.5
vtg	Fish K	0	1
		0.1	0.2±0
		1	0.3±0
		10	0.2±0
		100	0.1±0
	Fish L	0	1.0±0.2

	0.1	0.8±0.2
	1	0.8
	10	0.6±0.1
	100	0.5±0.1