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DNA damage and health effects in juvenile haddock exposed to sediment or produced water associated PAHs

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PROSJEKTRAPPORT

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DNA damage and health effects in juvenile haddock exposed to sediment or produced water associated PAHs.

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Sammendrag (norsk):
Tilstandsundersøkelsene i Nordsjøen har det siste tiår vist gentoksiske effekter (DNA-addukter) i fisk samlet inn i områder med offshore olje- og gassvirksomhet. Kilden og identiteten til de gentoksiske forbindelsene har til nå ikke blitt identifisert. Målsetningen for prosjektet har vært å studere dannelsen av DNA-skade i hyse som utsettes for ulike petrogene eller pyrogene polyaromatiske hydrokarboner (PAH). Målet har vært å identifisere kildene til forurensningen som medfører DNA-addukter observert i hyse fanget rundt oljefeltene i Nordsjøen. Dannelsen av DNA-skade over tid i hyse under kronisk eksponering for PAH og andre oljehydrokarboner fra følgende kilder er blitt studert: Ekstrakter av produsert vann (Statfjord A); destillasjonsfraksjoner av råolje fra Gullfaks (representere oljebasert borevæske); pyrogene PAH. Denne rapporten presenterer resultatene av studiene på DNA addukter i hyse og et utvalg av andre biologiske effektparametre.

Summary (English):
The Condition Monitoring in the North Sea the last ten years have documented genotoxic effects (DNA adduct) in fish collected in areas with extensive offshore oil and gas activity. However, the source and identity of genotoxic compound has not been identified. The objective of this project has been to study the formation of DNA damage in haddock exposed to petrogenic or pyrogenic polyaromatic hydrocarbons (PAHs) from different sources: Extracts of produced water (Statfjord A); distillation fractions of crude oil from Gullfaks (representing oil based drilling mud); pyrogenic PAH This report presents all the results from the study on DNA adducts and a selection of other biological effect parameters.

Emneord (norsk):	Subject heading (English):
1. Olje forurensning	**1.** Oil pollution
2. DNA addukter	**2.** DNA adducts
3. Polysykliske hydrokarboner (PAH)	**3.** Polycyclic hydrocarbon (PAH)
4. Hyse (*Melanogrammus aeglefinus)*	**4.** Haddock (*Melanogrammus aeglefinus)*

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Abbreviations

AhR	Aryl hydrogen receptor
AHRR	Aryl hydrocarbon receptor repressor
BAA	Benz(a)anthracene
BAP	Benzo(a)pyrene
BBF	Benzo(b)fluoranthene
BEP	Benzo(e)pyrene
BKF	Benzo(k)fluoranthene
BP	Benzo(ghi)perylene
BPDE-dG	benzo(a)pyrene (BP)-7,8-diol-9,10-epoxide-N(2)-deoxyguanosine
C	Chrysene
CYP1A	Cytochrome P4501A
DBA	Dibenz(a,h)anthracene
DE	Diol epoxide
DMSO	Dimethyl sulfoxide
dR	Deoxyribose
ELISA	Enzyme-linked immunosorbent assay
EROD	Ethoxyresorufin-O-deethylase
FA	Fatty acid
FF	Fixed wavelength fluorescence analysis
FL	Fluoranthene
GADD	DNA damage inducible proteins
GC-MS	Gas chromatography mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry
GST	Glutathione-S-transferase activity
IND	Indeno(1,2,3-cd)pyrene
LPO	lipid peroxidation
MNE	Mean normalized expression
MRM	Multi Reaction Monitoring
MUFA	Monounsaturated fatty acid
O&G	Oil and gas
PAH	Polyaromatic hydrocarbons
PCA	Principle Component Analysis
PCB	Polychlorinated biphenyls
PER	Perylene
Phe	Phenanthrene
PNL	Pseudo neutral loss scanning
PUFA	Polyunsaturated fatty acid
PY	Pyrene
PW	Produced water
qPCR	Quantitative real-time polymerase chain reaction
RSD	Relative standard derivation
SFA	Saturated fatty acid
TAG	Triacylglycerol
TLC	Thin layer Chromatography
UHPLC MS/MS	ultra-high performance Liquid chromatography- tandem mass spectrometry
XRE	Xenobiotic response element
8-oxo-dG	8-Oxo-2'-deoxyguanosine

I. Introduction

The objective of this project has been to study the formation of DNA damage in haddock (*Melanogrammus aeglefinus*) exposed to different petrogenic or pyrogenic polyaromatic hydrocarbons (PAHs). The main goal was to identify the sources of contaminants responsible for the DNA adducts observed in wild haddock caught around oil fields in the North Sea. This was achieved by studying the formation of DNA damage over time in haddock during chronic exposure for PAHs and other oil hydrocarbons.

Juvenile haddock were exposed for two months through the diet with two different profiles of oil hydrocarbons, one containing mainly light two ring PAHs and one containing both light (3 ring) and heavy (4 ring) PAHs. In addition, a third group was exposed to a diet containing a mixture of heavy (4-6 ring) PAHs, all treatments were compared with an unexposed control group. These groups represent exposure to either produced water, drilling mud or pyrogenic PAHs. After end of exposure, fish were followed for 2 months to measure long-term health effects or recovery.

The main endpoint was analysis of DNA adducts (^{32}P postlabelling), with a few additional samples analysed by different LC-MS methods as a pilot experiment to check the potential of this method to replace the non-specific ^{32}P-postlabelling method.

In addition, several others endpoints were analysed; bile metabolites, lipid composition, gene and protein expression and histopathology. All these measurements obtained from a controlled laboratory experiment support interpretation of the field survey results.

Several key questions were addressed:

1. Does oral exposure to PAH induce DNA-adducts in haddock?
2. What is the time effect in DNA-adduct formation during chronic exposure, and how fast will fish recover?
3. Does different PAH give different DNA adduct pattern from the ^{32}P-postlabelling method, and can the “spot position” be used to identified the source of PAH exposure?
4. How are DNA adducts correlated with other endpoints; PAH bile metabolites and CYP1A induction?

1.1. Background

Balk et al., (2011) reported that haddock caught in 2002 at the Tampen region of the North Sea had elevated levels of DNA adducts and responses in other biomarkers. This could be related to discharges from oil and gas (O&G) activity compared with haddock from a reference area (Egersund Bank, in the North Sea). This was the first time it has been reported that wild fish from the North Sea may be negatively affected by discharges from the offshore oil industry. Presence of DNA adducts in haddock from Tampen and the Viking Bank were later confirmed by the North Sea condition monitoring in 2005, 2008 and 2011 (Grøsvik *et al.* 2012). However, while the haddock from the O&G production areas have the highest levels of DNA adducts, the condition monitoring also found that haddock from the Egersund Bank had elevated levels of DNA adducts compared with more pristine

area like Iceland or the Barents Sea. This indicates that the whole North Sea has a general background contamination of PAH sufficiently high to create DNA damage in fish.

The Tampen region is holding some of the main oil fields in the Norwegian and British sector of the North Sea, and there has been oil production in this region for more than 40 years. The Tampen region has several possible sources of oil pollution, including large deposits of oil contaminated cuttings around many of the sites (Breuer *et al.* 2004), and as the oil fields mature they produce more produced water (PW). The Tampen region contributes with approx. 60 % of the total discharges of PW from the Norwegian offshore oil fields. Other sources of PAH may be pyrolytic PAHs either from incomplete combustion of flaring from the platforms during well testing or from atmospheric input. Boitsov et al. (2013) showed that the North Sea sediments contains a general background level of manly pyrogenic PAHs (Boitsov *et al.* 2013).

PAH composition is connected to the original sources. Petrogenic PAHs are dominated by 2 and 3 ring PAHs and have a large contribution of alkylated isomers, while the pyrogenic PAHs are dominated by high molecular PAHs (≥4 rings PAHs) and low levels of alkylated PAHs. PW contains mostly 2 ring PAHs (≈90 %) and only very low concentration of heavy PAH, where the oil used in oil-based drilling mud also has a small contribution of 4 rings PAHs (2%). However, during weathering and sedimentation processes the petrogenic PAHs also changes profile in the marine environment towards relatively more heavy PAHs, and the PAH profile found in old drill cuttings under platforms from the Tampen area typically contain some 4 ring PAH (9 %) and 5 ring PAH (3%) (Table 1).

Table 1. PAH composition (%) in base oil (HDF200), produced water from Gullfaks and sediments from the North Sea. Sediment has been sampled from old drill cuttings, Sediment G-16 is from the same platform area, but sampled from the periphery of the deposited drill cuttings. The sediment from Skagerrak shows the maximum background levels of PAH in the North Sea.

	HDF 200 oil	Gullfaks (PW)	Sediment (G-10)	Sediment (G16)	Sediment Skagerrak
PAH concentration	0,76 g/kg (0,08 %)	923 µg/l	40143 µg/kg	4739 µg/kg	6313 µg/kg
∑2 rings	84,0	88,4	62,4	43,7	11,6
∑3 rings	13,7	11,1	25,8	49,6	17,8
∑4 rings	2,3	0,4	8,9	4,6	12,1
∑≥5 rings	0,0	0,2	2,9	2,1	58,5

Some PAHs are known to be carcinogenic as they are metabolized into reactive intermediates that can bind covalently to DNA. The formation of PAH-DNA adducts has been connected to induction of mutations and development of tumours and cancer. The formation of PAH-DNA adducts is strongly dependent on PAH structure the and the ability to produce reactive electrophilic metabolites. There are several proposed metabolic pathways that can activate PAHs; the dihydrodiol epoxide pathway, the ortho-quinone pathway, the radical cation pathway and the arylmethyl carbocation pathway (details are given in (Bostrom *et al.* 2002; Flesher and Lehner 2016; Xue and Warshawsky 2005)).

The dihydrodiol epoxide pathway is considered as the most important, and it is generally found that only PAH with at least 4 rings and either “bay” or “fjord” like structures have the capability to be metabolized to the mutagenic diol epoxide (DE) (Figure 1). The activation of the PAH goes through ligand binding to the aryl hydrogen receptor (AhR) and induction of the cytochrome P450 detoxification system. The DE pathway for formation of DNA-adducts is well described for the highly carcinogenic, benzo(a)pyrene (BaP) (Bostrom *et al.* 2002), however, although phenanthrene (Phe)

can be metabolized into DE compounds, it is not been found to be carcinogenic (Carmella *et al.* 2004).

Flesher and Lehner (2016) are the founders of the "Unified theory" that provides a mechanistic explanation (the arylmethyl carbocation pathway) of why some methylated PAHs are much more potent carcinogens that the non-methylated parent PAHs (Flesher and Lehner 2016). Benzylic hydroxylation followed by sulphation can results in highly reactive esters which can form benzyl DNA adducts (Bendadani *et al.* 2014;Flesher *et al.* 1997)

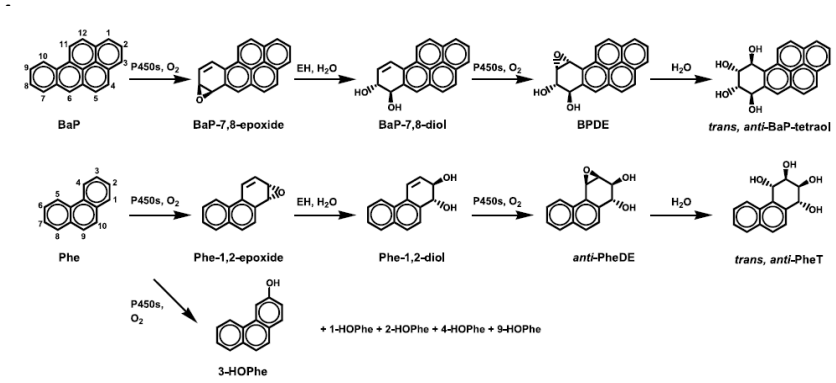


Figure 1. Metabolism of benzo[a]pyren (BaP) and phenanthrene (Phe) to bay region diol epoxides (BPDE and anti-PheDE) and tetraols (trans, anti-BaP-tetraol and trans, antiPheT), and metabolism of phenanthrene to phenanthrols (1-HOPhe, 2-HOPhe, 3-HOPhe, 4-HOPhe and 9-HOPhe (Camella et al., 2004)

There are several reports showing that exposure of crude oil and PW can induce DNA adducts in marine fish, both from laboratory studies (Aas *et al.* 2000a;Holth *et al.* 2009;Lyons *et al.* 1997;Sundt *et al.* 2012) and field observations after major oil spills (Amat *et al.* 2006;Harvey *et al.* 1999). Likewise, *in vitro* studies showed that oils and oil fractions contain genotoxic compounds that induce DNA adducts (Akkineni *et al.* 2001;Ingram *et al.* 2000;Nagy *et al.* 2004). However, the genotoxic compounds in crude oil have not been identified yet.

Alkylated three rings PAH may be candidates for oil compounds that can induce DNA adducts, as it has been shown that some dimethylphenanthrenes are potent tumor inducers in mice, while methylphenanthrene and non-methylated 2 and 3 rings PAH are not genotoxic (Lavoie *et al.* 1981;Lavoie *et al.* 1982)

Alkylated PAHs are metabolised either by hydroxylation of the aromatic ring or at the alkyl chain (Lavoie *et al.* 1981). Malmquist et al., (2013, 2015) shows that polycyclic aromatic acids are the dominating metabolites from alkylated phenanthrene, pyrene and chrysene in a marine benthic invertebrate (*Nereis diversiocolor*) (Malmquist *et al.* 2013;Malmquist *et al.* 2015). The degradation pathway suggested by Malmquist et al., goes through multi-step oxidation of the alkyl chain, starting with benzylic hydroxylation (-CH_2OH), further to aldehyde (-CHO) and to carboxylic acid (-COOH). These phase I metabolites are conjugated to amino acids (glucine), glucoronide or sulfate (Figure 2). Similar degradation pattern are also found in soil fungus (*Cunninghamella elegans*) (Boll *et al.* 2015) and bacteria (*Pseudomonas Putida*) (Mahajan *et al.* 1994). It is likely that similar metabolic pathways will also exist in fish, and together with the possibility of formation benzyl DNA adducts, one should

therefore look more into whether alkylated PAHs in crude oil are responsible for the DNA adduct formation found in wild fish in the North Sea.

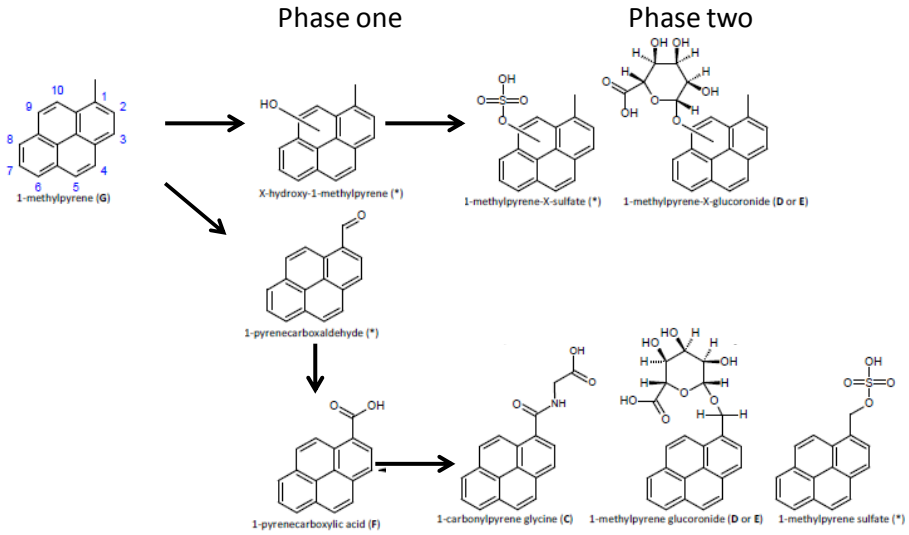


Figure 2. Biotransformation of 1-methylpyrene proposed by Malmquist et al., based by metabolites identified in *Neries diversiocolor* (letters) or found in the literature (*). The figure is modified from (Malmquist *et al.* 2013)

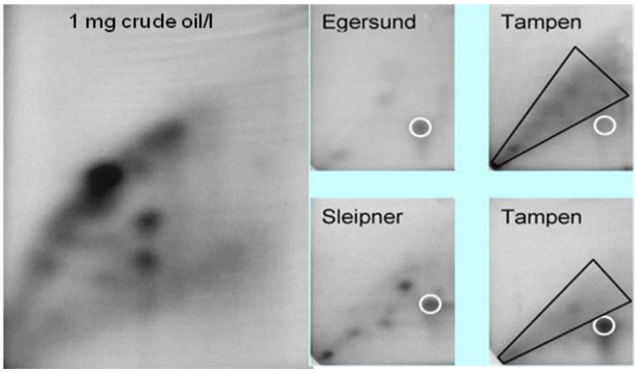


Figure 3. Autoradiogram from 32P-postlabelling analyses of Atlantic cod exposed 30 days in the laboratory for 1 mg crude oil /l (Aas *et al.*, 2000) and wild haddock caught in the North Sea (Balk *et al.* 2011). All the black dots represent different DNA adducts.

A challenge for interpretation of DNA adduct results is that the preferred analytical methods, the ^{32}P-postlabelling assay (Phillips 2013), does not provide structural information for identification of unknown adducts. It is therefore not possible to identify which PAHs (or other compounds) are responsible for the formation of DNA adducts in complex exposures like crude oil/PW (Figure 3). To overcome this challenge, a large effort has been put into the development of new mass spectrometry based methods for identification of DNA adducts (Klaene *et al.* 2013;Singh and Farmer 2006;Tretyakova *et al.* 2013). Secondary goals of this project are to generate samples with high levels of PAH DNA-adducts which can be used in a planned future project with these new methods in attempt to identify major DNA-adducts generated in haddock during PAH exposure, and to create mass spectra libraries that can be used for comparison in future field studies.

In addition to the focus on DNA adducts have we also studied different biological mechanisms/gene pathways that are known to be affected by oil pollution by characterizing genome-wide gene expression profiles (RNA-Seq). Special attention was given to genes involved in the cytochrome P450 detoxification system, the oxidative defence systems, and the DNA repair system.

Other exposure biomarkers, like liver content of PAH, PAH bile metabolites, liver lipid profiles and liver proteins (CYP1A), have also been measured to evaluate possible physiological consequences related to DNA adduct formation. After the end of the two-month exposure, some of the fish were followed for 2 months to evaluate if the PAH exposure affected long term growth and survival. These fish were examined by histopathologic methods to investigate whether DNA-adducts correlated with changes at cellular levels (neoplasia or other non-neoplastic lesions).

II. Experimental set up

The exposure experiment started 14th February and lasted till 22th April, 2014. A group of fish were kept for two months until 19th June to look for long-term effects. The exposure experiment has been conducted as planned and we have sampled 420 haddock.

2.1. Exposure regime

We exposed haddock for three different PAH profiles that represented different possible PAH sources that can be present in the North Sea; produced water (PW), oil based cutting pile (deposited before 1993) and sediments from assumed reference areas.

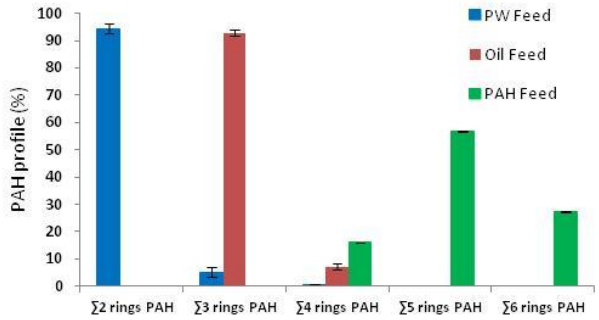
Table 2 and Table A1 give an overview of the PAH composition (%) in produced water from Statfjord A and sediments from old drill cuttings sampled either just under the platform or at short distance from the main cutting pile. The sediment from the deeper part of Skagerrak shows the maximum background levels of PAH in the North Sea.

Table 2. PAH profiles (%) and concentration (mg/L or mg/kg) in produced water and sediments from the North Sea.

PAH profile (%)	Produced water (Statfjord A)	Old drill mud (under Statfjord A)	Old drill mud (close to Statfjord A))	Marine Sediment (Skagerrak)
∑2 rings PAH	91.1	62.4	43.7	11.6
∑3 rings PAH	8.7	25.8	49.6	17.8
∑4 rings PAH	0.2	8.9	4.6	12.1
∑5 rings PAH	0.0	2.8	1.3	40.0
∑6 rings PAH	0.0	0.1	0.8	18.5
PAH concentration	3.5 mg/l	40 mg/kg	5 mg/kg	6 mg/kg

Clear differences in PAH composition have been identified, PW is dominated by 2 ringed PAHs (>90 %) with a small fraction of 3 ringed PAHs, but only very low amount of heavy PAHs (≥4 ringed PAH). The drilling mud sediments also contain high levels of 2 ringed PAHs (44-62 %), but have in addition high amount of 3 ringed PAHs (26-50 %) and some heavy PAHs (≥4 ringed PAH). The background sediments are dominated by heavy PAHs (≥4 ringed PAH).

Both PW and drilling mud sediments have high amount of 2 ringed PAH. However, we did not wish to have too large overlaps in PAH compositions between the different exposure groups. Therefore, we did not mimic the PAH profiles in the sediments exactly, but tried to make exposures that were representative for the dominating PAHs from the different sources: 2 ringed PAHs (PW), 3 ringed PAHs (oil containing sediment) and 4-6 ringed PAHs (background sediments) (Figure 4, Table 3).



PAH profile (%)	PW feed	Oil feed	PAH feed
∑2 rings PAH	94	0	
∑3 rings PAH	5	93	
∑4 rings PAH	0.3	7	16
∑5 rings PAH	0	0	57
∑6 rings PAH	0	0	27
PAH dose i feed	31±0.5 mg/kg	45±3 mg/kg	65±5 mg/Kg

Figure 4/Table 3. PAH profiles in the three exposure feeds. Analysed by GC-MS.

Table 4. Distribution of heavy PAHs in the "PAH feed". Classification of carcinogenicity from the International Agency for Research on Cancer (WHO-IARC, 2010). Group1: carcinogen to humans; Group 2A: probably carcinogen to humans; Group2B: possibly carcinogen to humans; Group3: not classifiable as to carcinogenicity to humans.

Compounds	Number of rings	MW	Distribution in the food (%)	IARC Group	DNA adducts potential
Fluoranthene	4	202	5	3	-
Pyrene	4	202	4	3	-
Benz(a)anthracene	4	228	3	2B	++
Chrysene	4	228	4	2B	++
Benzo(b)fluoranthene	5	252	13	2B	+
Benzo(k)fluoranthene	5	252	12	2B	+
Benzo(e)pyrene	5	252	5	3	+
Benzo(a)pyrene	5	252	6	1	+++
Perylene	5	252	2	3	-
Dibenz(a,h)anthracene	5	278	19	2A	+++
Indeno(1,2,3-cd)pyrene	6	276	22	2B	-
Benzo(ghi)perylene	6	276	4	3	-

2.2 Fish and exposure

The experiment was carried out on juvenile haddock (100-250 g) and the fish were exposed to different hydrocarbon mixtures through the feed. The fish were marked with electronic tags and held in five circular tanks (3 m diameter, 7 m^3). The fish were dosed with automatic feeding unit 5 times a week with a ratio corresponding 10 g pellets/kg fish/day (1 % of body mass per day). The PAH concentration in the pellets was from 31-65 mg/kg (table 3) and this correspond a daily dose of PW treatment group: 0.31 mg PAH/kg; Oil treatment group: 0.45 mg PAH/kg and PAH treatment group: 0.65 mg PAH/kg. (Detail about the exposure diet is giving in the appendix; Material and Methods).

The exposure experiment started 14th February and ended 22th April. Samples were taken one week after end of exposure and the remaining fish were transferred to one common tank and maintained for two months until 19th June in order to study long term effects.

At the start of the experiment 26 haddock were injected with single a PAH compound (2 fish for each of the 12 heavy 4, 5 and 6 ringed PAH compounds used in the PAH mixture) and sampled after two days. The PAHs were dissolved in dimethyl sulfoxide (DMSO) and fish oil to a concentration of 4 mg/ml. Each fish was injected in the abdominal cavity with 1 µl/g fish, corresponding to a dose of 4 mg/kg fish. The injected fish will be used to generate a bile metabolite and DNA adduct "library".

2.3 Sampling

Six samplings of fish were performed during the experiment (Table 5). The first sampling was done 3 days after the first oral dose or injection.

Table 5. Sampling time and number of fish.

Exposure groups	17.02.2014	24.02.2014	24.03.2014	22.04.2014	29.04.2014	19.06.2014
Control	10	10	15	20	10	36
PW	10	10	15	20	10	35
Oil	10	10	15	20	10	31
PAH	10	10	15	20	10	33
Injection (PAH)	25					

Samples were taken of several tissues (blood, bile, liver, muscle, brain, intestine, heart and whole fish) (Table M3). The samples of intestine, heart and brain will not be included in the analytical program of this application. The material will be kept at IMR for future studies.

III. Results

3.1 Exposure and appetite

The first observation after 2 and 10 days of exposure showed that the fish from the exposure groups had lower appetite than the control fish. Less food was found in the stomach of these fish and the growth was negative for many fish. This was most likely a result of reduced appetite because the fish sensed the oil compounds. To reduce the "smell" of the oil compounds, the pellets were mixed with a "paste" made of homogenized prawns before each feeding. This had an immediate positive effect on the appetite of all haddock groups and from the daily observations of feeding we registered that all groups were eating the pellets after we started to use prawn pasta.

3.1.2 Growth

All fish increased their weight at the third sampling point (five weeks after exposure). The PAH exposure group, however, had significant lower weight and growth rate (0.4 % daily growth factor) compared with the control fish (1 % daily growth factor). During recovery, some increase in the growth factor was observed in the PAH exposure group (up to 0.7 % growth a day), but there was a clearly reduced growth in the fish fed pellets contaminated with heavy PAHs. This is probably mainly

due to reduced appetite in this group, but increased metabolic cost associated with detoxification of PAH may also contribute.

After 2 months of recovery, the oil-exposure group was also found to have lower growth compared with control, while the PW fish did not differ from the control group.

Reduced growth after oral exposure of PAHs and crude oil have been reported in several fish species; Zebrafish (*Danio rerio*) had reduced growth in all three exposure groups (sediment extract=heavy PAHs), heavy oil extract and light oil extracts) receiving 9 months with 4.6-6.7 mg PAH/kg feed (Vignet *et al.* 2014), reduced growth in Rockfish (*Sebastes schlegeli*) exposed for BaP (1.5 and 2 mg BaP/kg feed) in 30 days; 6 week oral exposure for water-accommodated-fraction (WAF) of heavy oil (2.2 mg WAF/kg feed) gave growth reduction in juvenile turbot (*Scophthalmus maximus*) (Saborido-Rey *et al.* 2007); juvenile Chinook salmon (*Oncorhynchus tshawytscha*) feeded pellets that mimic PAH exposure from urban estuaries had reduced weight after 53 day at doses of 22 mg PAH/kg fish (Meador *et al.* 2006).

Table 6. Length, weight, liver weight, hepatosomatic index, condition factor and daily growth factor (weight increased relative to exposure start 14.02) for all treatment groups. Sampling under "grey" labels are during exposure and under "brown" labels are during recovery. Asterisks indicate statistical significance from the control fish (*p < 0.05).

17.02.2014		(n)	Lenght (mm)	Weight (g)	Liver weight (g)	HSI	Condition factor	Daily growth factor (%)
Kar 22	Control	10	225 ± 18	141 ± 28	22 ± 7	15 ± 2	1.23 ± 0.2	-0.2 ± 1.3
Kar 23	PW	10	228 ± 12	164 ± 37	25 ± 9	15 ± 3	1.36 ± 0.1	-0.1 ± 0.7
Kar 24	Oil	10	230 ± 8	167 ± 32	29 ± 6*	17 ± 1*	1.35 ± 0.1	-1.2 ± 1.5
Kar 25	PAH	10	226 ± 7	147 ± 15	20 ± 4	14 ± 1	1.26 ± 0.0	-0.2 ± 0.7
24.02.2014		(n)	Lenght (mm)	Weight (g)	Liver weight (g)	HSI	Condition factor	Daily growth factor (%)
Kar 22	Control	10	230 ± 19	140 ± 31	21 ± 6	15 ± 2	1.15 ± 0.2	0.6 ± 1.4
Kar 23	PW	10	228 ± 13	154 ± 33	24 ± 7	16 ± 2	1.29 ± 0.1	0.0 ± 0.2
Kar 24	Oil	10	235 ± 12	162 ± 31	28 ± 7*	17 ± 2*	1.23 ± 0.1	-0.2 ± 0.3
Kar 25	PAH	10	229 ± 15	166 ± 35	29 ± 7*	18 ± 2*	1.40 ± 0.4	-0.7 ± 0.3*
24.03.2014		(n)	Lenght (mm)	Weight (g)	Liver weight (g)	HSI	Condition factor	Daily growth factor (%)
Kar 22	Control	15	236 ± 18	180 ± 46	30 ± 9	17 ± 2	1.35 ± 0.1	0.9 ± 0.5
Kar 23	PW	15	241 ± 15	193 ± 37	35 ± 9	18 ± 3	1.37 ± 0.1	0.7 ± 0.2
Kar 24	Oil	15	239 ± 15	180 ± 34	32 ± 8	17 ± 2	1.31 ± 0.1	0.6 ± 0.1*
Kar 25	PAH	15	244 ± 19	191 ± 47	33 ± 9	17 ± 2	1.30 ± 0.1	0.4 ± 0.2*
22.04.2014		(n)	Lenght (mm)	Weight (g)	Liver weight (g)	HSI	Condition factor	Daily growth factor (%)
Kar 22	Control	20	260 ± 13	233 ± 43	41 ± 9	17 ± 2	1.32 ± 0.1	1.0 ± 0.4
Kar 23	PW	20	261 ± 16	248 ± 50	45 ± 11	18 ± 2	1.38 ± 0.1	0.8 ± 0.2
Kar 24	Oil	20	251 ± 19	206 ± 65	36 ± 17	17 ± 4	1.26 ± 0.2	0.8 ± 0.3
Kar 25	PAH	20	247 ± 18*	183 ± 46*	34 ± 9*	19 ± 4	1.19 ± 0.1*	0.4 ± 0.3*
29.04.2014		(n)	Lenght (mm)	Weight (g)	Liver weight (g)	HSI	Condition factor	Daily growth factor (%)
Kar 22	Control	10	268 ± 17	265 ± 59	48 ± 13	18 ± 3	1.36 ± 0.1	0.9 ± 0.3
Kar 23	PW	10	252 ± 16	235 ± 62	39 ± 15	16 ± 3	1.40 ± 0.1	1.0 ± 0.3
Kar 24	Oil	10	261 ± 15	254 ± 42	42 ± 7	17 ± 2	1.47 ± 0.3	0.7 ± 0.2
Kar 25	PAH	10	256 ± 13	205 ± 39*	32 ± 10*	16 ± 3	1.21 ± 0.1*	0.4 ± 0.2*
19.06.2014		(n)	Lenght (mm)	Weight (g)	Liver weight (g)	HSI	Condition factor	Daily growth factor (%)
Kar 22	Control	36	289 ± 17	336 ± 57	62 ± 14	18 ± 3	1.39 ± 0.2	1.1 ± 0.4
Kar 23	PW	35	281 ± 21	299 ± 85*	51 ± 19*	17 ± 2*	1.31 ± 0.1	1.1 ± 0.5
Kar 24	Oil	31	278 ± 20*	292 ± 75*	51 ± 15*	17 ± 3	1.33 ± 0.1	0.8 ± 0.5*
Kar 25	PAH	33	263 ± 21*	236 ± 69*	41 ± 16*	16 ± 5	1.26 ± 0.2*	0.7 ± 0.5*

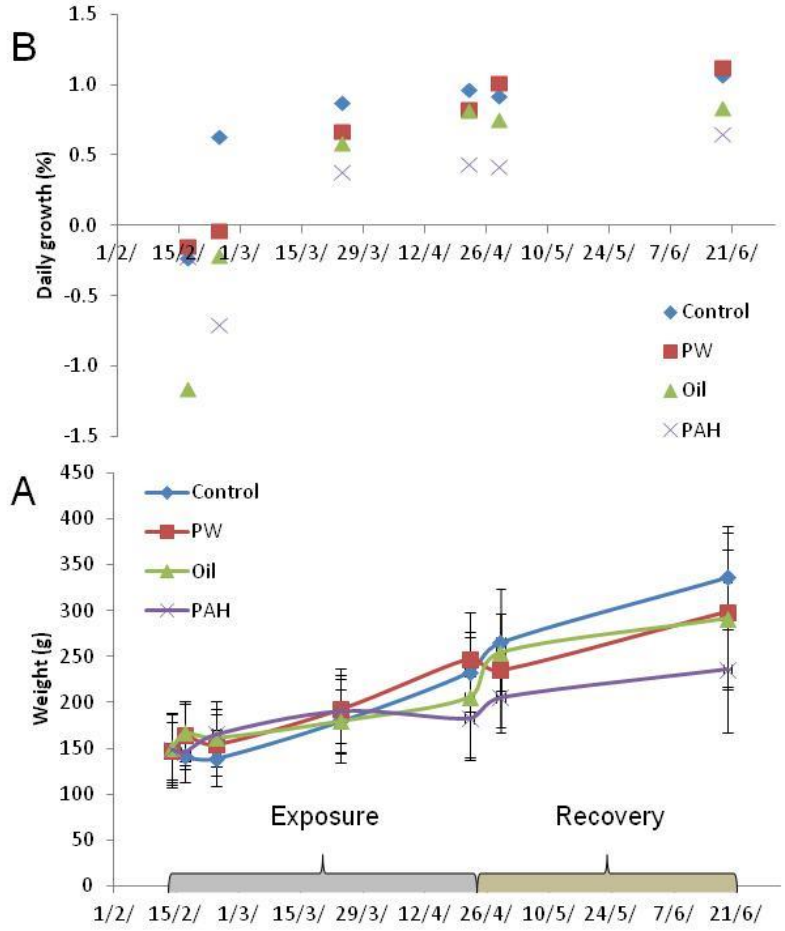


Figure 5. Weight (A) and daily growth factor (B) for all treatments groups and samplings points. Data presented as average ± stdev.

Analytical program

The main aim of this project is to study the formation of DNA damage over time in haddock during chronic exposure for different sources of PAHs. However, there are several other parameters that are relevant regarding interpretation of the results of the last years' water column monitoring survey. These include bile metabolites, lipid composition, gene and protein expression and histopathology.

3.3 DNA adducts

To study the formation of DNA adducts over time, DNA from liver samples were analyzed by the ^{32}P-postlabelling methods after 3, 35 and 67 days exposure, and again after 7 and 58 days of recovery. The analyses were done by ADn'tox laboratory in France. This is the same laboratory that has been used in the water column monitoring program, and the results can be compared with results from earlier field studies.

3.3.1 Fish injected with heavy PAHs

The ^{32}P-postlabelling analysis of fish injected with a high dose (4 mg/kg body burden) of a single compound of heavy PAHs shows that 3 compounds did not induce DNA adducts (FL, PY, IND), 5 compounds had detectable but low levels of DNA adducts (BEP, BBF, BKF, PER, BP), while 4 compounds had very high levels of DNA adduct (BAA, BAP, C, DBA). The 2 replicates showed that there was large variation in intensity for several compounds (RSD from 6-130 %), but the pattern regarding none, low or high formation of DNA adduct were stable (figure3 6 and 7).

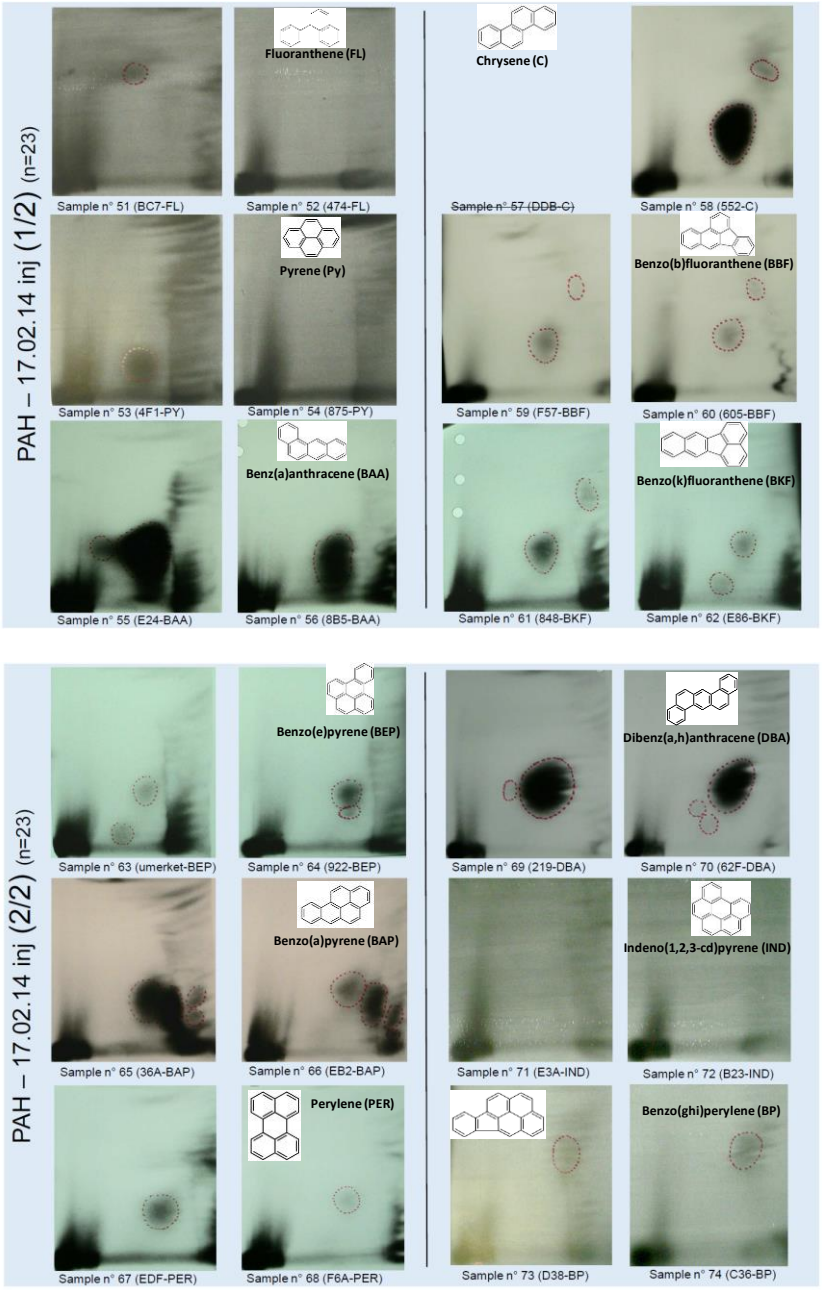


Figure 6. Individual autoradiogram of 12 heavy PAHs (4-6 rings). Fish injected with 4 mg/kg of single compounds (2 replicates for each compound) and sampled after three days.

The response in haddock fits well with the literature from *in vitro* studies, where FL, PY and IND do not induce DNA adducts and are classified as non-carcinogenic (Audebert *et al.* 2012;Tarantini *et al.* 2011) and the 4 high inducers, BAA, BAP, C and DBA are all known to be potent in inducing DNA adducts, and being carcinogenic or possible carcinogenic PAHs (WHO-IARC, 2010, Audebert *et al*., 2012). Of the 5 low inducers, several of them are suspected to be carcinogenic, but they are not very potent to induce DNA adducts (Audebert *et al.* 2012;Ericson *et al.* 1999;Platt *et al.* 2008;Tarantini *et al.* 2011)

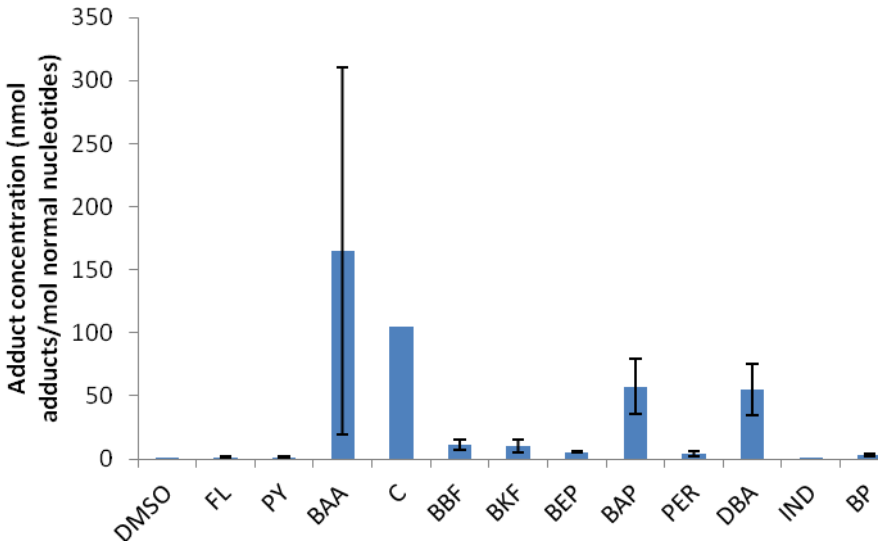


Figure 7. DNA adducts in the liver of fish injected with heavy PAH (4-6 rings) (2 replicates of each compound, exept chrysene). Data presented as average ± stdev.

The results from the injected fish showed that haddock responded quickly to intraperitoneal injection of PAHs and high amounts of DNA adducts were detected 3 days after injection.

3.3.2 Oral exposure of Haddock

Increased levels of DNA adducts compared with control fish were found in all treatment groups (control 0.15±0.07; PW 14.2±0.4; Oil 40; PAH 122±133 nmol adducts/mol normal DNA) (Figures 8 and 9). This shows that PAHs from all the different treatments groups are taken up from the food and readily metabolised to reactive metabolites that bind the DNA in the liver, and that a single oral dose is sufficent to induce DNA adducts 3 days after exposure. The exposure for heavy 4-6 rings PAHs results in a strong induction of DNA adducts that quantitative were in the same ranges as the injected fish. However, there were large variation between the two replicates (27 and 216 nmol adducts/mol normal DNA), which may reflect differences in how much food the indivudual fish consumed. However, as seen from the injected fish there can also be very large variation in the DNA adduct induction responses between fish (Figure 8 and 9). Also, the oil exposed fish had higher levels of DNA adducts compared with the PW exposed group.

Due to uncertainties with regard to how much the fish had been eating during the first feeding, we did only send two samples (as a test) for DNA adducts analyses. As shown from the presented results it was unfortunate that we did not analyze a higher number of samples per group to be able to

perform statsitical analyses. These samples are stored at –80°C freezer and may be considered to be analysed later. Likevise we did not prioitise to analyse the fish sampled after 10 days exposure. It would be interesting to analyse these to get a better understanding of the time response after oral exposures.

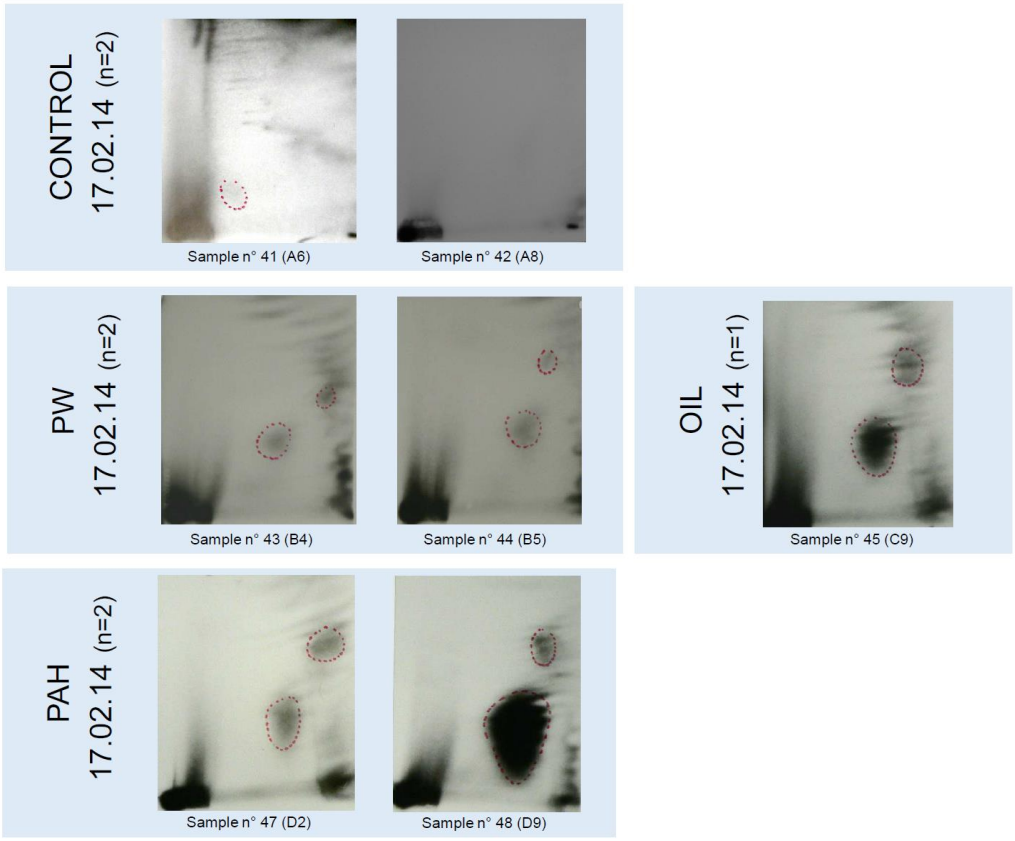


Figure 8. Autoradiograms of control, PW, Oil and PAH treated fish. Fish were sampled three days after the first oral exposure dose. Only two samples per group were selected for DNA adduct analysis. These fish were selected at "eaters" from the presence of faeces in the intestine.

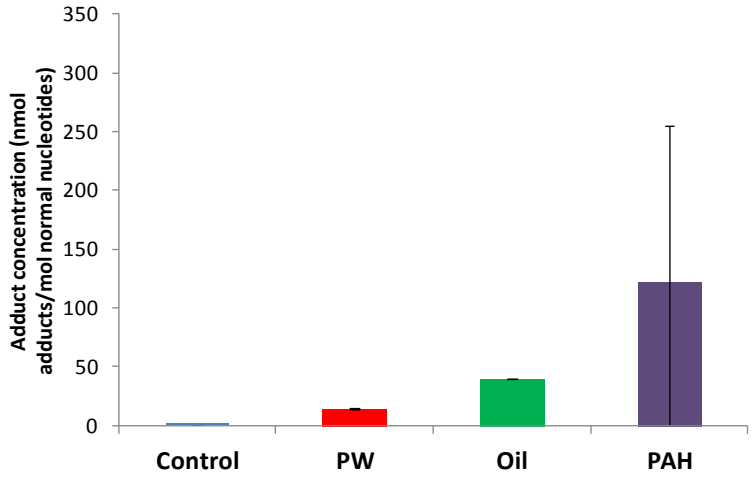


Figure 9. DNA adduct in the liver of fish from the different treatment groups three days after one oral dose. Data presented as average ± stdev.

Long-term exposure for 37 and 67 days also resulted in significant elevated levels of DNA adducts in all treatments groups compared with controls (Control: 0.3±0.5 and 1.9±2.5, PW: 3±2 and 8±9 nmol adducts/mol normal DNA nmol adducts/mol normal DNA, Oil: 4±2 and 4±4, PAH: 8±3 and 11±11 nmol adducts/mol normal DNA) (figure 11). However, the DNA adduct levels were considerably lower (nearly 10 times) compared with the single dose and 3 days' exposure. Any clear decline in the levels of DNA adducts were not seen after the recovery period, and after 58 days' recovery all three treatment groups had higher DNA adducts levels (6.7-10.5 nmol adducts/mol normal DNA) than the control group (1.2 nmol adducts/mol normal DNA).

Table 7 show a selection of exposure studies that measured DNA adduct in marine fish. Aas (2000) studied DNA adduct formation in Atlantic cod (*Gadus morhua*) exposed for crude oil through the water (0.06-1 mg oil/l = 0.33-7.8 µg PAH/l), and they found an induction of liver DNA adducts (11±4 nmol adducts/mol normal DNA) after 3 days exposure in the high exposure group, and DNA adduct levels were constantlyduring the 30 day experiment, peaking at 109±45 nmol adducts/mol normal DNA. Seven days in clean water did not show any decline and recovery in the DNA adduct levels. The low doses of oil (0.33µg PAH/l) also showed elevated DNA levels (4±2 nmol adduct/mol normal DNA) after 30 days exposure (Aas *et al.* 2000a). In a similar 14 days water exposure study with crude oil (1 mg oil/l) on cod and polar cod (*Boreogadus saida*) the same research group did only find medium induction of DNA adducts 18±11 and 12±4 nmol adduct/mol normal DNA respectely (Aas *et al.* 2003). Long-term water exposure (16-44 weeks) of cod to artificial PW (5.4 µg PAH/l + 11.4 µg alkylphenols/l) showed medium DNA adduct levels (9 nmol adduct/mol normal DNA) after 16 weeks' exposure, but high induction of DNA adducts after 44 weeks (73 nmol adduct/mol normal DNA) (Holth *et al.* 2009). Cod exposure for Ekofish PW for 28 days had low but significant induction of DNA adducts in 0.25 % PW (1.7±0.9 nmol adduct/mol normal DNA) and 0.5 % PW (4.4±2.4 nmol adduct/mol normal DNA) (Sundt *et al.* 2012).

Table 8 shows DNA adduct levels in wild haddock caught in pristine sea areas outside Island and in the Barents Sea and the North Sea (Egersund Bank and the Tampen area). The DNA adduct levels in pristine area were 0.6-0.7 nmol adducts/mol normal DNA, at the references area of the North Sea 0.6-5.5 nmol adducts/mol normal DNA and at the Tampen area 1.7-11 nmol adducts/mol normal DNA.

The levels of DNA adducts found from oral exposure in the present experiment can only be considered as highly induced at the first sampling point after 3 days of exposure. After chronical long-term exposure the DNA adduct levels were only slightly induced and in the ranges of what have been reported in wild caught haddock from the North Sea and in experimental studies with high dose of PW. These observed levels of DNA adducts after chronic exposures were surprising, and suggested an adaption during long term oral exposure, either by induced clearance rate in the intestine and thereby lower uptake to the liver, or by induction of the DNA damage repair system.

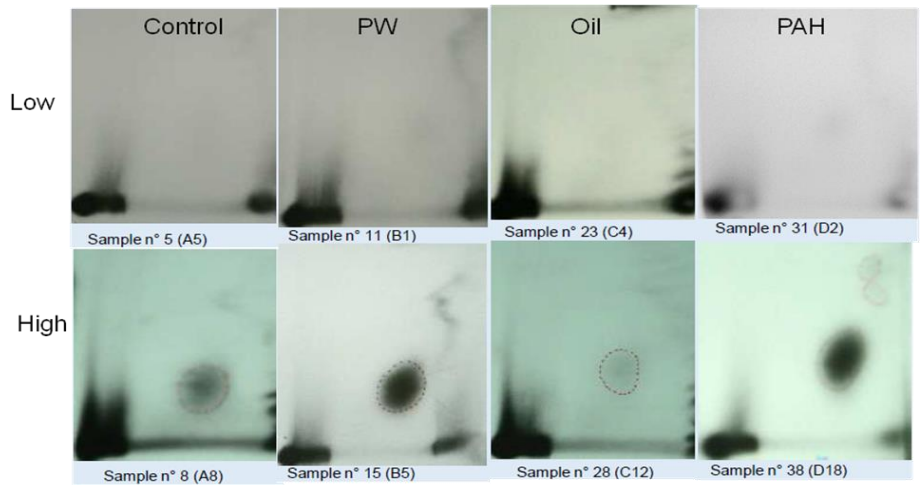


Figure 10. Individual autoradiograms of control, PW, Oil and PAH treated groups after 67 days of oral exposure (sampled 22.04.2014). Two plots from each group are shown, the highest induced fish and the lowest induced fish.

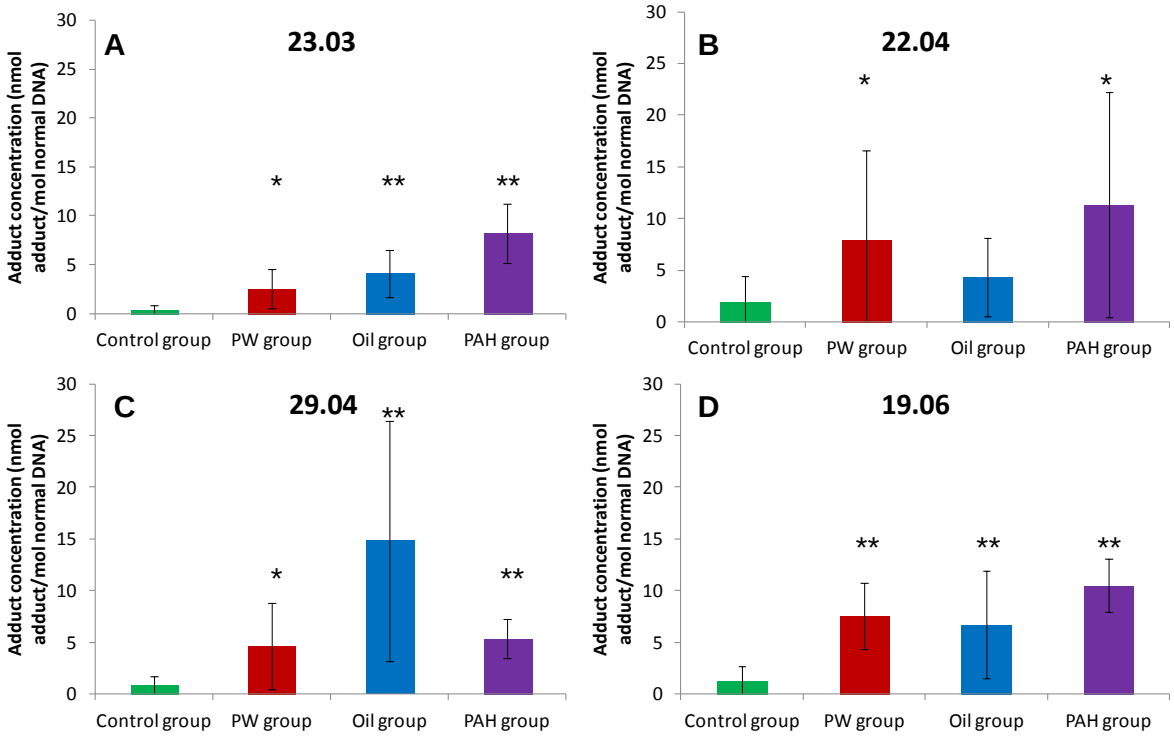


Figure 11. DNA adducts in the liver of fish from the different treatments groups after (A) 37 days and (B) 67 days of oral exposure or (C) 7 days and (D) 48 days of recovery with clean food (n=10). Data presented as average ± stdev. (*) and (**) indicate significant differences compared with control, $p< 0.05$ or $p<0.01$.

Table 7. DNA adducts data from experimental exposure (crude oil and PW) studies using water exposure.

Species	Treatment dose and exposure time	DNA adduct level (nmol/mol normal DNA)	Referances
Cod	0.04 mg oil/l (0.33 µg PAH/ l) for 30 days	4±2	(Aas *et al.* 2000a)
	0.14 mg oil/l (1.14 µg PAH/l) for 30 days	11±4	
	0.94 mg oil/l (7.8 µg PAH/ l) for 3 days	11±4	
	0.94 mg oil/l (7.8 µg PAH/ l) for 16 days	64±15	
	0.94 mg oil/l (7.8 µg PAH/ l) for 30 days	109±45	
Cod	Control	1.6±1.1	(Aas *et al.* 2003)
	1 mg oil/l for 14 days	18±11	
Polar cod	Control	1.2±0.5	(Aas *et al.* 2003)
	1 mg oil/l for 14 days	12±4	
Cod	Control	0.5±0.3	(Sundt *et al.* 2012)
	0.125 % PW for 28 days	2.0±1.6	
	0. 25 % PW for 28 days	1.7±0.9	
	0.5 % PW for 28 days	4.4±2.4	
Cod	Control, 16 and 44 weeks	<1,26-2	(Holth *et al.* 2009)
	Artificial PW; 0.54 µg PAH/l + 1.14 µg alkylphenols/l for 16-44 weeks	2-7	
	Artificial PW; 5.4 µg PAH/l + 11.4 µg alkylphenols/l for 16-44 weeks	9-73	

Table 8. DNA adducts data in haddock from field sampling 2002-2011.

Area	DNA adduct levels (nmol adduct/mol normal DNA)	Ref
Iceland 2002	0.6±0.4	(Balk *et al.* 2011)
Barents Sea 2008	0.7±0.4	(Grøsvik *et al.* 2009)
Egersund Bank 2002	4.0±3.2	(Balk *et al.* 2011)
Egersund Bank 2005	2.1±1.4	(Grøsvik *et al.* 2007)
Egersund Bank 2008	0.6±0.3	(Grøsvik *et al.* 2009)
Egersund Bank 2011	5.5±7.1	(Grøsvik *et al.* 2012)
Tampen 2002	19±11	(Balk *et al.* 2011)
Tampen 2005	4.4±4.4	(Grøsvik *et al.* 2007)
Tampen 2008	1.7±2.1	(Grøsvik *et al.* 2009)
Tampen 2011	7.3±5.6	(Grøsvik *et al.* 2012)

In all groups some individual fish had no detectable levels of DNA adducts, this may either be a result of differences in how much each fish had been eating and been exposed to, or it may be result of differences in the metabolic capacity of the individual fish (Figure 12). Typically, low but detectable levels of DNA adducts were measured in 50 % of the control fish. The PAH content of the food was

analysed by GC-MS (Figure 19) and contamination of the control food was not detected. This suggests that DNA adducts in haddock are quite common in many fish. The presence of endogenous DNA adducts in haddock have also been suggested from the water column monitoring of wild fish (Balk *et al.* 2011). The position of the DNA adduct spots in the TLC plate from the control fish are similar as for the exposed fish. It is therefore not possible to exclude false positive of endogenous DNA adducts.

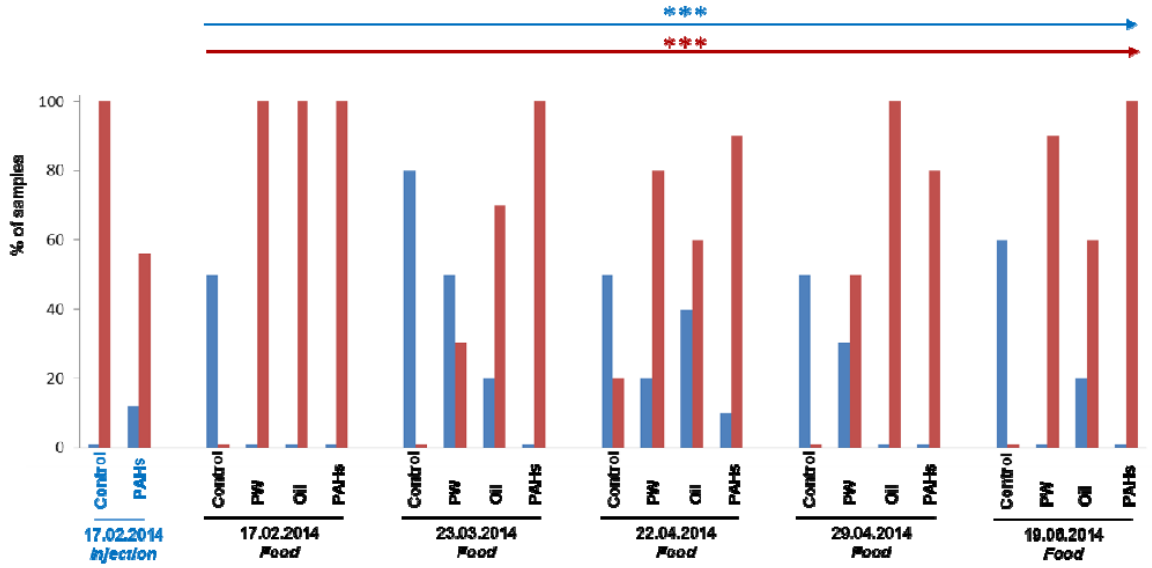
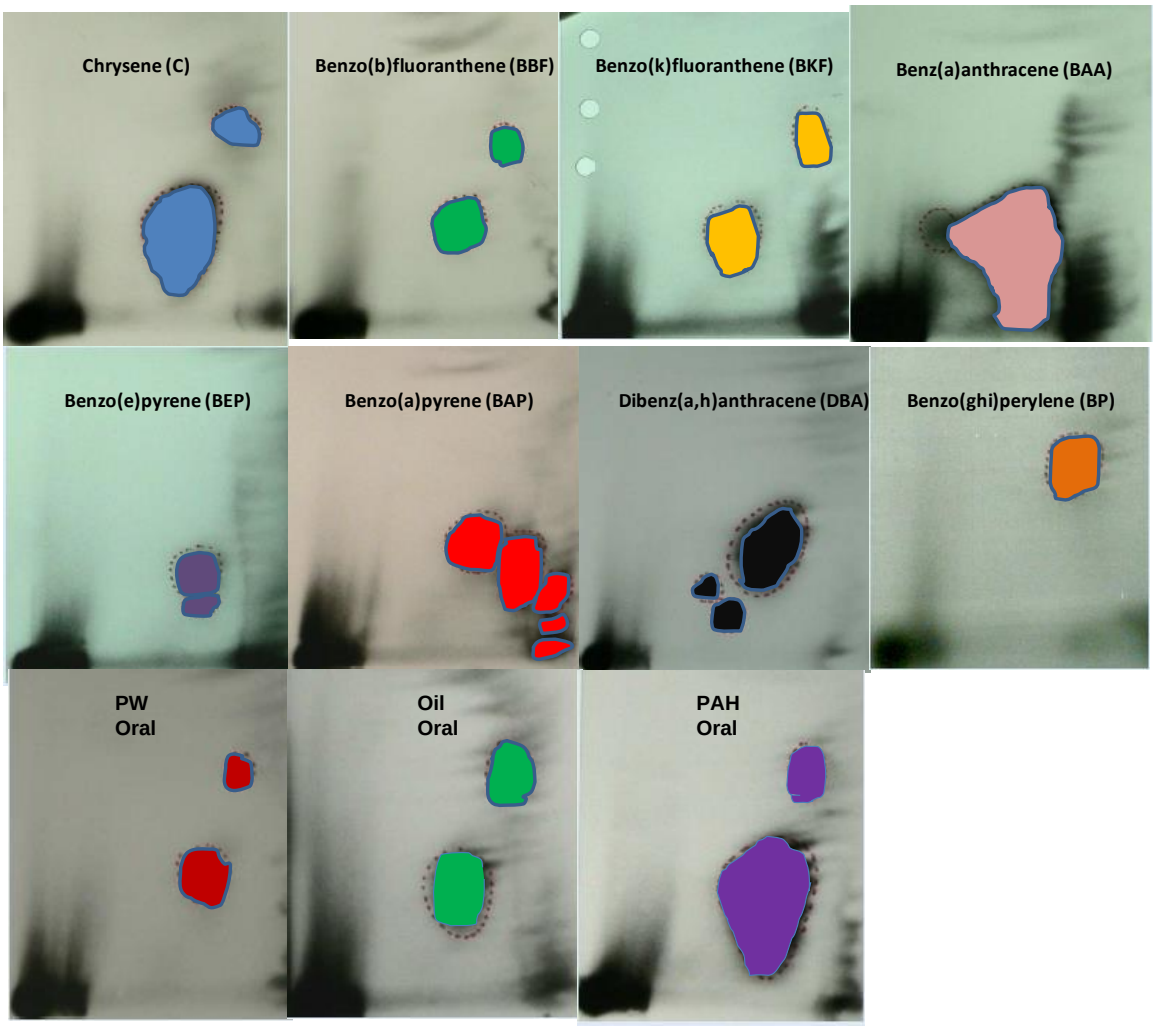


Figure 12. Frequence of samples with undetectable amount of DNA adducts (blue column >0.1 nmol adduct/mol normal DNA) and DNA adduct levels higher than 4 nmol adduct/mol normal DNA (red column). (***) indicate highly significant differences of the occurrence of samples without detectable compared with control at all sampling points, ($p<0.0001$, Cochran-Mantel-Haenszel test) and samples with elevated DNA aducts levels (>4 nmol adduct/mol normal DNA) ($P<0,0001$).

3.3.3 Autoradiogram TLC maps

One main objective of this investigation was to investigate if different PAH sources gave different DNA adduct pattern on the ^{32}P-postlabelling TLC plates, and whether the spot position could be used to identify the sources of PAH exposure. Figure 13 shows the difference spot position of the heavy PAH single compounds and the three oral mixtures.



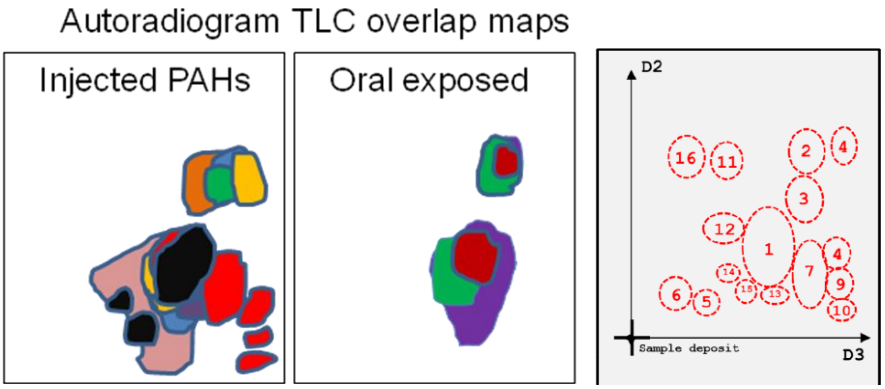


Figure 13. Autoradiogram of TLC maps in liver of haddock injected with single PAHs or exposed orally for mixtures of PAHs (PW, Oil, PAH). The lower plot show overlap maps of the TLC spots from the different injected single compounds and the oral mixtures. 16 different spots from all the 384 plates (192 samples) analyzed in this project were identified.

There are clear differences in the numbers of DNA adducts detected on the TLC plates, with BaP giving rise to at least 5 different spots while BP treatment only shows one spot. There are some differences in position of the spots from the difference PAHs, however, the main picture is that there is a strong overlap between all compounds and no unique TLC pattern was seen. Similarities in the

pattern of spots between most PAH were seen, with a big spot in the middle of the plate (spot1) and several smaller spots in the upper right corner (spot 2, 3 and 4). No major spot with high intensity appeared to be specific to one single PAH compound. Spots 7, 8 and 9 are only observed in fish exposed to BAP. Spots 12, 14 and 15 are only observed in fish exposed to DBA. Spot 13 is only observed in the liver of one fish exposed to BEP. (Figure 13).

The patterns in the oral exposed groups were not that diverse compared to the single PAH injected groups. Three spots were found in more than 10 % of all the samples, spot1, spot3 and spot5. Table 7 show that there was a clear increased presence of the DNA adducts in the exposed groups (PAH group>Oil group>PW group>>control). However, it was not possible to distinguish between the three different PAH sources from the oral exposed groups using the TLC spot pattern.

Table 7. Frequencies of the total number of samples in each treatment groups with detectable levels of the three dominating spots (1,3 and 5).

	Control group	PW group	Oil group	PAH group	Chi-square test
Spot1	21%	50%	66%	81%	P<0.0001
Spot3	0%	14%	19%	26%	P=0.007
Spot5	17%	40%	48%	49%	P=0.008

This confirms the limitation of the 32P-postlabelling assay to give structural information of the different DNA adducts and thereby the identity of the PAH metabolites that are causing the DNA damage.

The 32P-postlabelling assay is the preferred analytical method for studying DNA adducts because of its very high sensitivity (Phillips 2013). However, a major drawback of this method is the very limited capacity to identify which PAHs (or other compounds) are responsible for the formation of DNA adducts. This is especially the case for the water column survey where we are working with wild fish. During the last decade, several groups have aimed to develope mass spectrometric methods for DNA adducts analysis (Himmelstein *et al.* 2009;Singh and Farmer 2006). However, a important challenge has been to get analytical platforms that could work with the extreme low detection limits needed (1 adducts per 10^9 unmodified DNA bases). New developments in LC-MS technology have made possible the necessary demands for sensitivity, and a significant effort has been put into the development of new mass spectrometry based methods for identification of DNA adducts (Balbo *et al.* 2014;Klaene *et al.* 2013;Monien *et al.* 2015;Tretyakova *et al.* 2013). There are still several challenges in optimizations of the methods (Klaene *et al.* 2016) and there is a large need of making more standards of DNA adducts (preferably isotope marked) that can be used for confirmation of chromatography and mass spectra identity and to make quantitative assays. However, there is broad agreement in the literature that development of LC-MS methods are necessary to be able to study details in DNA-adduct formations.

In this project a small pilot study with LC-MS analysis was performed. Two samples (after month exposure) were selected for UHPLC-MS/MS, one from the PAH exposed group and one control.

The samples were analysed on UHPLC-MS/MS with several different scan methods;

Test 1: Target multiple reaction monitoring (MRM) for the known BAP DNA-adduct, dG-N-2-BPDE.
Test 2: Non target pseudo neutral loss scanning (PNL). This is a screening method looking for "unknown" DNA adducts.

The results are giving in the appendix. It suggests that the haddock do not produce the known BAP DNA-adduct, dG-N-2-BPDE, but instead are there found several other peaks in the exposed fish and the MS spectra suggests DNA adducts that contains nitrogen atom (in NH2 or NO2 functions). It remains to get a positive identification through analysing standards, but it shows that the LC-MS/MS is sensitive enough to detect DNA adducts in this samples.

3.4 Bile metabolites

Analyses of hydroxylated PAH metabolites in the bile is a well establish biomarker for exposure to oil and PAH exposure. Two different methods have been used, GC-MS of single compounds (22.04) and Fixed wavelength fluorescence analysis (23.03, 22.04 and 29.04) (FF method).

The GC-MS quantify 22 standards (2 naphthol, 10 methyl-naphthol, 2 hydroxyfluorene, 6 hydroxyphenanthrene and 1-hydroxypyrene and 2-hydroxychrysene), the results are given as the sum of all standards.

The fixed wavelength fluorescence analysis (FF) method measures the excitation:emission wavelength pairs 290:334 nm (2/3 ring-type), 341:383 nm (pyrene-type) and 380:430 nm (benzo[*a*]pyrene-type).

The GC-MS bile metabolites analysis showed suprising results (Figure 14). Highest levels of methylnaphthols, hydroxyfluorenes and hydroxyphenanthrenes were observed in the PW group, while levels of 1-hydroxypyrene were highest in the oil- and PAH treated groups. In the PW exposure dose, 94 % were 2 rings PAHs and the methylnaphthalene contributed 16 % of the total PAH, where phenanthrene only contributed 1.6 % of the total PAHs. Nevertheless, in the bile the 3 ring PAH metabolites dominated, and levels of hydroxyphenanthrene were from 24-905 ng/ml, while levels of hydroxylated methylnaphthols were only slightly elevated (11-42 ng/ml) compared with control.

Similarly, in the oil exposed groups we expected to find high levels of hydroxyphenanthrenes, but the levels were not significantly different from control group. The 1-hydroxypyrene, on the other hand, was strongly elevated in the oil and PAH exposed groups, so this metabolite seems to be a good marker for exposure.

One explanation for the results with GC-MS may be that the monohydroxylated (phenols types) metabolites are not the dominating metabolites, and more of the metabolites are dihydroxylated or even more polar (Goksoyr *et al.* 1986;Pangrekar *et al.* 2003;Sette *et al.* 2013;Wessel *et al.* 2013). Goksøyr et al., (1986) found, for example, that more than 90 % of the bile metabolites of phenanthrene in cod were dihydrodiols. The same may be the case for the alkylated PAHs, as

benzylic hydroxylation may be the dominating metabolic pathway and phenolic metabolites only will be present in small amount (Malmquist *et al.* 2015).

The FF method gave a different picture than GC-MS. This method measures fluorescence at different wavelengths and is not restricted to a single compounds, but more the structure of the aromatic rings. This measurement will therefore be able to detect several different metabolites, both mono- and polyhydroxylated and more polar compounds (Aas *et al.* 2000b).

Figure 15 shows bile metabolites analysed by the FF method sampled after one month (23.03) and two month (22.04) exposure and after one week of recovery (29.04). Significantly higher levels of the 2/3 ring metabolites (290/334 nm) were observed in the PW group after one and two months of exposure, but not after one week of recovery. The PW exposed group also had slightly elevated levels of 4 rings metabolites (341/383 nm) after one month of exposure, but this was not seen after two months of exposure. The oil exposed fish showed very high levels of 2/3 ring-type metabolites for all exposure lenghts. The 4 ring metabolites were also elevated at all exposure durations. The PAH group had elevated levels of 4 ring metabolites after both one and two month of exposure (no bile data were optained for the PAH fish after one week of recovery). Surprisingly, the PAH group did not show elevated amounts of the 5 rings PAH metabolites (380/430 nm = benzo[*a*]pyrene-type) even though these compounds were dominating the exposure regime in this treatment. Unfortunately, we did not obtain FF measurement for BaP in the injected fish that could confirm the FF method ability to detect BaP metabolites in haddock bile, but the FF methods have been validated for BaP in many others fish, including Atlantic cod (Aas *et al.* 2000b).

We compared the GC-MS and FF methods and Figure 16 shows that there was a linear correlation between the FF method and the GC-MS measurement for naphthtols ($y=0.0078x + 4.51$, $R^2 = 0.36$), the methylnaphthols ($y=0.044x + 6.32$, $R^2 = 0.67$) but not hydroxylated phenanthrene ($y=0.22x + 110$, $R^2 = 0.03$) for the PW group. In the oil group there was correlation between the FF and the GC-MS for the hydroxyphenanthrene ($y = 0.11x - 78.16$, $R^2 = 0.71$). The correlation between the 4 rings metabolites (P341/383 nm = pyrene type) and GC-MS measurement of 1-hydroxypyrene was very high for both the oil group ($y=1.18x - 122$, $R^2 = 0.96$) and the PAH group ($y=1.66x – 192$, $R^2 = 0.48$) (figure 16).

The good correlation found between the two different methods confirms that the low detection of monyhydroxylated PAH in the GC-MS measurement is most likely accurate, and not a result of method problems. The GC-MS method performance is repeatedly controlled by running standard samples for hydroxylated PAHs. This method control validates the preformance of the extraction, derivatisation and GC-MS step, however, it is not possible to have standard control for the enzymatic deconjugation process (beta-glucuronidase/aryl sulfatase, first step of the method), because it is not possible to obtain standards of the main bile conjugate gluconoids of PAH metabolites (or it is extremly expensive). IMR has participated in an intercalibration excersise for the GC-MS method and the results were validated as good (Kammann *et al.* 2013).

The relative low detection rate of PAH metabolites in the GC-MS analysis raises the question whether this is the right method to use for bile analysis in the water coloum monitoring, because this method does not target the most abundant metabolites. The FF method, on the other hand, can only be regarded as semi-quantitative as there are no standards that can cover for the complex mixture of

metabolites that can be found in bile samples (Beyer *et al.* 2010). Likewise, the wavelengths chosen for excitation and emission represent a compromise that does not cover the optimum for all metabolites of the group (Pampanin *et al.* 2016).

Several methods have been developed using LC-MS enabling analyse of intact conjugated metabolites and deconjugated products (Huang *et al.* 2014;Malmquist *et al.* 2015;Sette *et al.* 2013). We aim to re-examine samples form the present experiment using LC-MS/MS at the laboratorie of NOAA, Seattle. This will give us a better understanding of the metabolism of orally distributed PAHs in haddock.

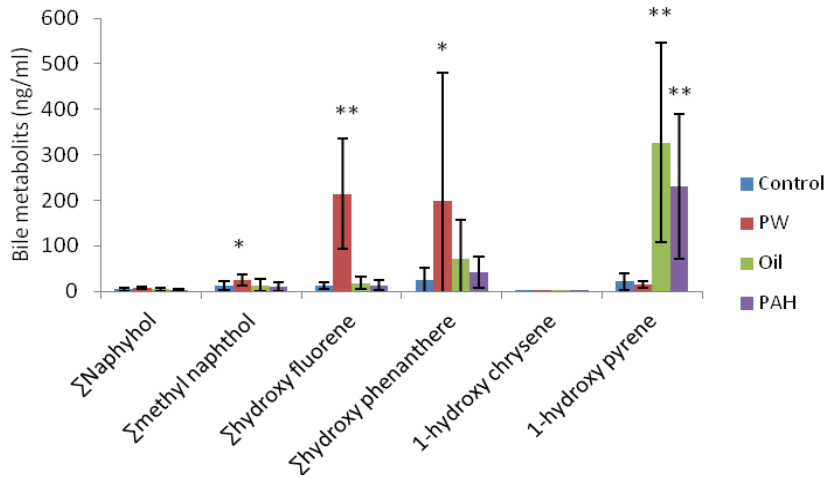


Figure 14. GC-MS analysis of monohydroxylated PAH bile metabolistes in fish from the different treatments groups after two-months of oral exposure (n=10). Data presented as average ± stdev. (* or **) indicate significant differences compared with control, p< 0.05 or p< 0.01.

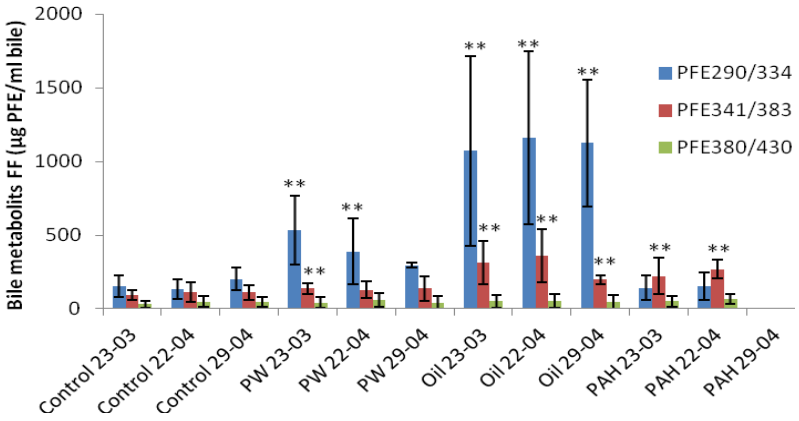


Figure 15. Fixed wavelength fluorescence (FF) analysis of PAH bile metabolites. FF were measured at the 290/334 nm (2/3 ring-type), P341/383 nm (pyrene-type) and 380/430 nm (benzo[*a*]pyrene-type). Levels are shown after 1 month exposure (23.03), two months exposure (22.04) and after one week recovery with non contaminated food (29.04). Data presented as average ± stdev. (**) indicate significant differences compared with control, p< 0.01.

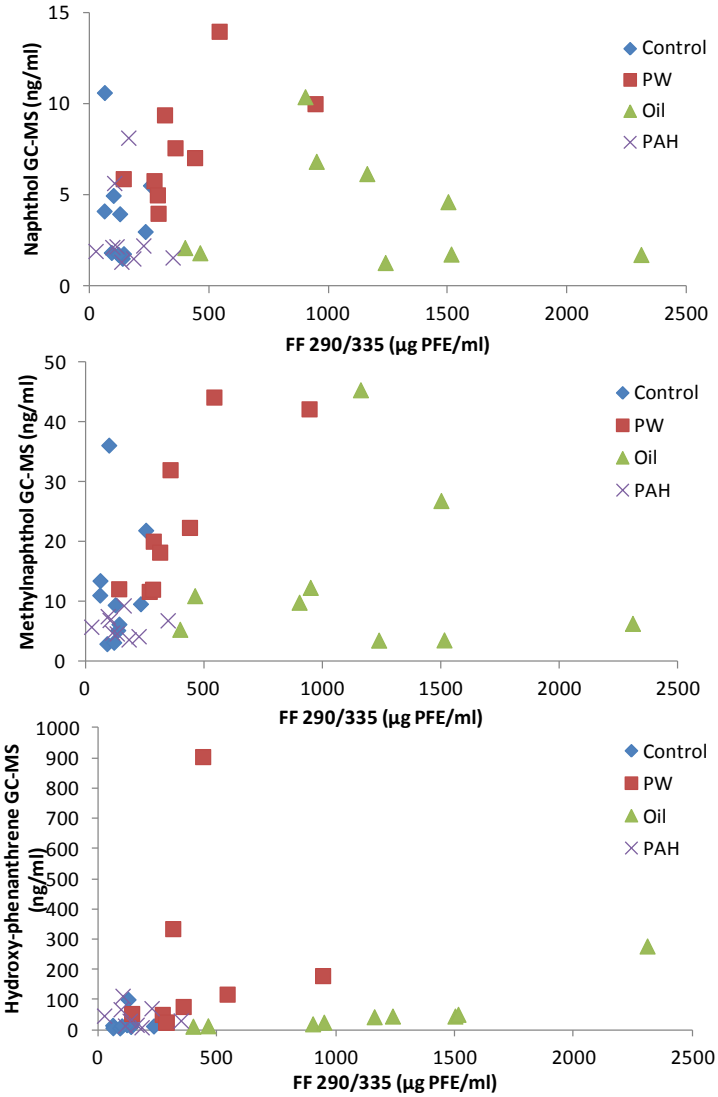


Figure 16. Comparison between the GC-MS measurements and the FF measurements for haddock after exposure for 2 months (sampled 22.04). FF 290/335 nm (2-3 rings PAH) plotted against amount of naphthols, methylnaphthols and hydroxyphenanthrene.

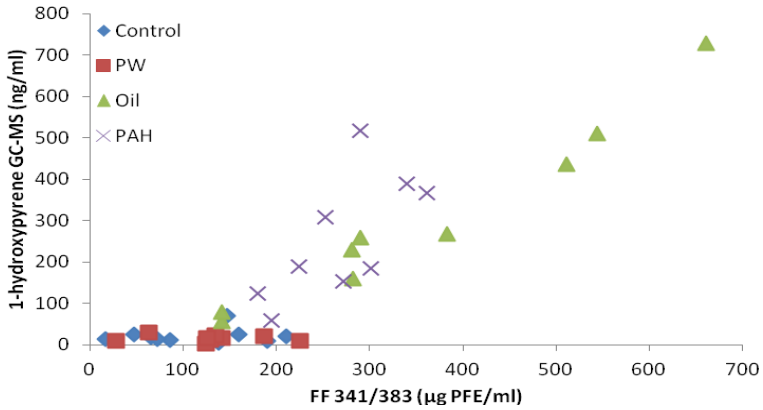


Figure 17. Comparison between the GC-MS measurement and the FF measurement for the haddock exposed for 2 months (sampled 22.04). FF 341/383 nm (pyrene) plotted against the amount of 1-hydroxypyrene.

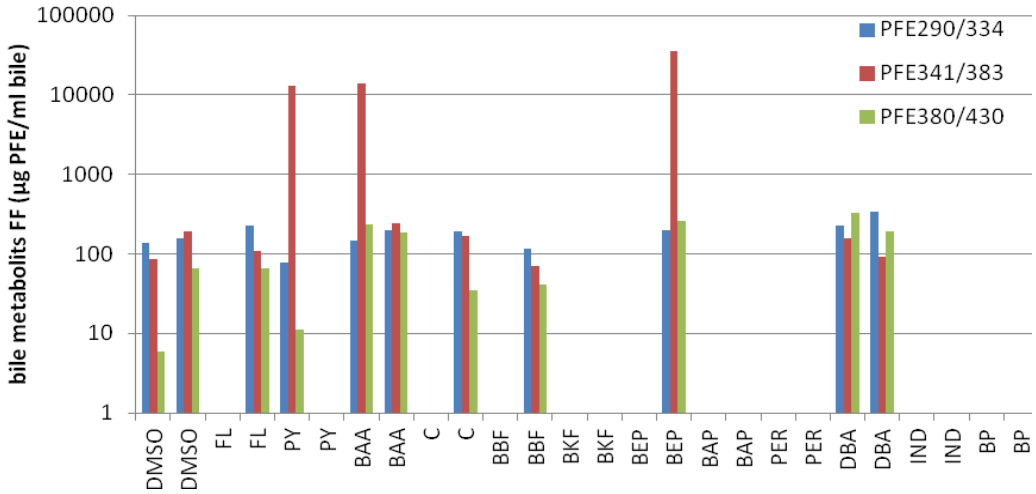


Figure 18. FF analyses of PAH bile metabolites from haddock injected with single PAH compounds or only DMSO (control). Three samples (PY, BAA, BEB) had extrem high levels of fluorescence in the bile – while most samples not were higher that the control. Unfortunately, many of the fish contained very little bile and FF analyses could not be preformed on these samples.

3.5 Liver analysis of PAH content

Because of the surprisingly low levels of bile metabolites from the GC-MS measurement, we decided to analyze levels of non-metabolized PAH in the liver to see if PAH were accumulating in the fat rich tissue. The analyses were done by DCM extraction followed by GPC cleanup and GC-MS/MS analysis (Sørensen *et al.* 2016).

The results showed large amounts of PAHs in the liver of the PW treatment groups. The profile of PAHs in the livers of PW exposed fish were dominated by 2 rings, followed by 3 rings PAHS, which reflected the profile in the feed. PAHs were also found in the oil group, however, in much lower amount. No heavy PAHs were detected in the PAH treatment group.

As a comparison, we made a theoretical calculation of how much the total liver content of PAH (concentration (ng/g) x liver size (g)) was related to a single dose with food (1 % of body weight).

In the PW group the naphthalene content in the liver corresponded 34 % of the naphthalene in one dose, for the alkylated two-ring PAHs it corresponded to 16 %, for the 3 ring PAHs it was 51 % and for the alkylated three-ring PAHs it was 12 %. The similar calculation for the oil group showed that a much lower part of the total PAH dose was found in the liver; 3 ring PAHs were 2.4%, alkylated three-ring PAHs were 0.1 % and for the 4 ring PAHs it was 0.5 %. For both the PW and the oil group it is clear that much lower amount of the alkylated PAHs were found compared with the non alkylated PAHs, suggesting a faster clearing rate for alkylated PAHs.

This is a very interesting result, suggesting that the PW exposed fish most likely accumulated PAHs in the liver because the detoxification systems were not induced as strongly as the oil and PAH groups. This fits well with what should be expected, because the small 2 ring PAHs are not agonists for the AhR, which is responsible for the induction of the metabolic enzymes (cytochrome P450, CYP) for PAHs. Many heavy PAHs, (4-6 rings) are very strong CYP inducers, likewise alkylated 3 rings PAH have been shown to bind to AhR and induce the CYP systems (Billiard *et al.* 2002). It is commonly found that oil exposure in fish results in a strong induction of CYP1A, and that this increases the metabolism of PAHs. Fish have high capacity for metabolizing PAHs and normally non-metabolized PAHs are found only in very low levels in tissues like liver and muscle (Hellou et al, 1994). CYP1A was induced in the liver of oil and the PAH treated fish (Figure 19 and 24), but not in liver of the PW treated fish. This difference in induction of the detoxicification system in the oil group may also explain the picture higher levels of bile metabolites (2/3 rings-type) found in this exposure group compared with the PW group.

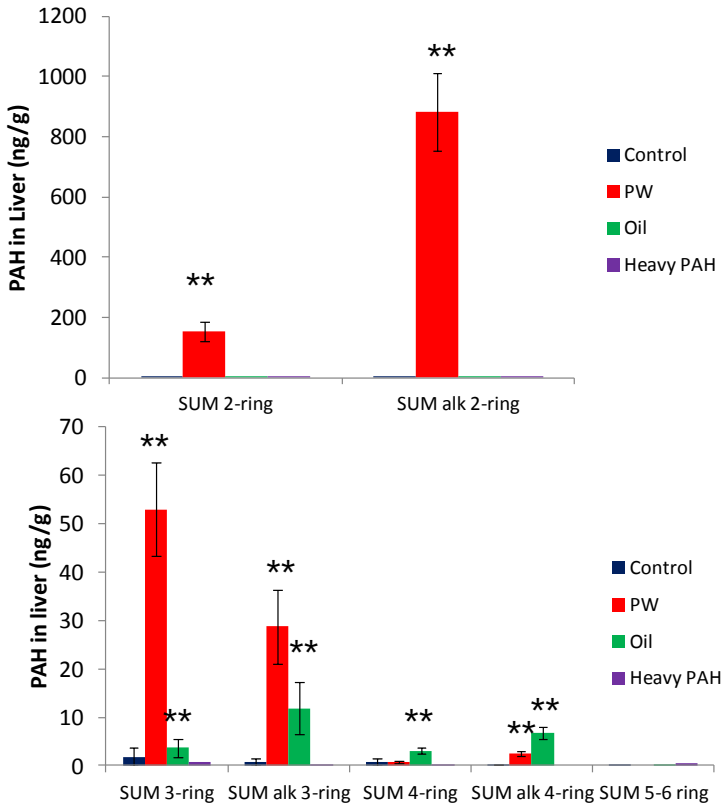


Figure 19. PAH found in the liver (ng/g) of the different treatments groups. Data presented as average ± stdev. (*) indicate significant differences compared with control, p< 0.05.

3.6 Effects on selected biomarkers

Exposure of fish to different PAH profiles may work through different mechanisms and lead to different cellular responses or different strength in responses. We have selected a few different biological effect parameters relevant for exposures to different PAHs measured at the cellular level

by mRNA levels (qPCR), protein levels (ELISA), enzymatic levels (GST activity) and membranes lipid peroxidation.

CYP1A is known to be induced by planar PAHs and dioxins, furans and planar PCBs (Goksøyr and Förlin 1992). Figure 20 shows qPCR analyses of CYP1A and aryl hydrocarbon receptor repressor (AHRR). The mean normalized expression (MNE) for CYP1A is strongest after exposure to the PAH exposure group (4-, 5-, and 6-ringed PAHs), with medium response in the oil-type exposure group (3- and 4-ringed PAHs), and less in the PW-type exposure group (2- and 3-ringed PAHs). The same pattern was observed with qPCR with primers to AHHR (Figure 20b). AHRR participates in the AhR signaling cascade, which mediates induction of the detoxification system. It functions as a feedback modulator by repressing AhR-dependent gene expression. It represses the transcription activity of AHR by competing with this transcription factor for heterodimer formation with the ARNT and subsequently binding to the xenobiotic response element (XRE) sequence present in the promoter regulatory region of variety of genes. It represses CYP1A1 by binding the XRE sequence and recruiting ANKRA2, HDAC4 and/or HDAC5. AHRR also autoregulates its expression by associating with its own XRE site (uniprot.org). To investigate responses in the cellular DNA repair system, we also included primers to transcripts involved in DNA repair processes like growth arrest and DNA damage inducible proteins (GADD) involved in the P53 signaling pathway as GADD45A, GADD45G and p53 (results not shown). However, we did not obtain a significant difference in response compared with control to these transcripts, even if we did see a clear response in DNA adduct formation in this study.

ELISA analyses of CYP1A in liver were included to provide information on expression at protein levels. Such information is important complementary information to RNA-Seq. For CYP1A we could see the same pattern on the protein levels as we could see in the qPCR analyses, although the relative differences from control to exposed groups were higher in the qPCR analyses (Figure 20A).

Measurements on glutathione-S-transferase activity (GST) were included to investigate if these different exposures affected the phase-2 detoxification proteins like GST. GST levels in the exposed groups were not significantly different from control, even if levels in the heavy PAH groups seemed to have reduced levels (Fig 25B).

Oxidative stress is also a relevant biomarker to include after exposure to PAHs. Measurements of lipid peroxidation did not reveal significant changes in the exposed groups compared with control (Fig 25C).

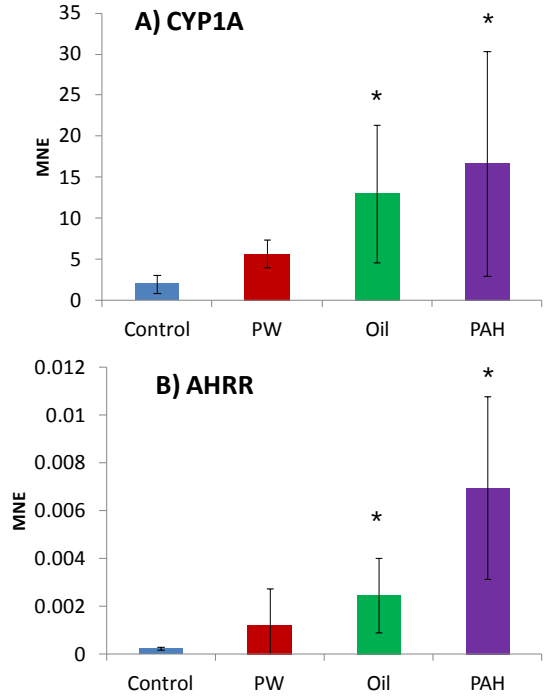


Figure 20. Mean normalized expression (MNE) of CYP1A (A) and AHRR (B) by qPCR after 2 months of exposure. Data presented as average ± stdev. (*) indicate significant differences compared with control, p< 0.05.

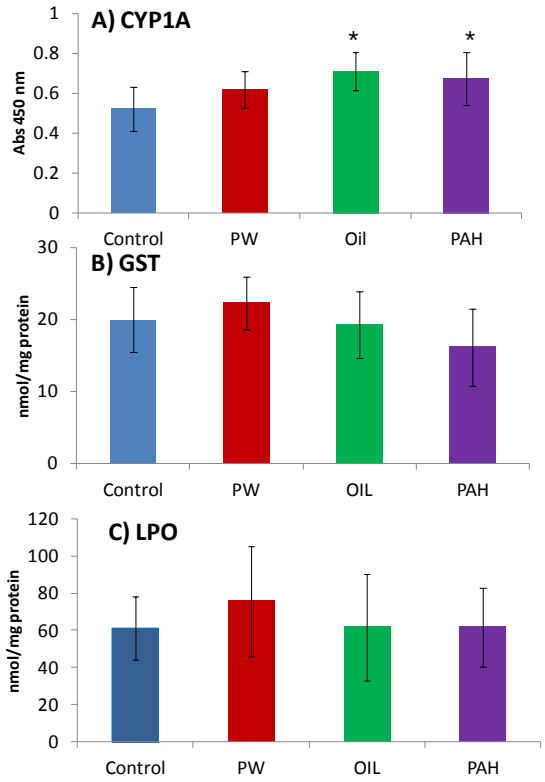


Figure 21. Responses on CYP1A by ELISA with antibody towards CYP1A (A), effects on glutathions-S-transferase activity (GST) (B) and lipid peroxidation (LPO) (C) of the different treatments groups after two months of exposure. Data presented as average ± stdev. For CYP1A detection we used polyclonal anti-trout CYP1A (CP-226, Biosense) diluted 1:1000. (*) indicate significant differences compared with control, p< 0.05. N = 10 individuals per group.

3.7 RNA-Seq

RNAseq analyses have been performed on liver from 4 individual haddock per treatment sampled after 2 months (22nd April, 2014) of oral exposure to feed spiked with different PAH profiles (resembling oil, produced water and pyrogenic PAHs) followed by two months of recovery with normal feed, sampled 19th June 2014. A total of 32 individuals were analysed. RNAseq analyses can show which metabolic or other cellular pathways that may be affected when comparing the different treatments.

A total number of 20096 transcripts were sequenced and annotated to known genes. When comparing differential expression pattern between groups, 985 RNA transcripts were significantly differentially expressed with fold changes >1.5 and Noiseq > 0.95.

The general conclusion we must draw from this analysis is that the low number of individual (n=4) is not sufficient to obtain significant differences between the treatment groups. Due to high cost and delivery time for the analyses we only analysed four individual samples per treatment. Such low number per group restricts normal distribution and statistical significance. Only a relative low number of genes were found to be differentially expressed between the different exposure and the control group; 30 genes were upregulated and 123 down regulated (Table 8).

Table 8. Differentially expressed genes by treatment sampled after two months of exposure (22nd April) and after two months of recovery (19th June). Fold changes > 1.5, p< 0.05 (Noiseq > 0.95).

Treatment	Upregulated	Downregulated
Control vs PW	**1**	**0**
Control vs Oil	**26**	**2**
Control vs PAH	**0**	**12**
Recovery control vs recovery PW	**0**	**41**
Recovery control vs recovery oil	**0**	**34**
Recovery control vs recovery PAH	**0**	**41**

The most robust responses in this data set were CYP1A (Figure 22). This biomarker gave significant changes between control and oil exposure sampled 22nd April when Noiseq was set at 1.0. When expression between all individuals were compared, CYP1A showed similar expression pattern as we observed with qPCR (Figure 20) and ELISA (Figure 21). For Ah-receptor (AHR), oil exposure gave the highest response, while transcription levels of Ah receptor repressor (AHRR) gave the same pattern for RNAseq as observed by qPCR (Figure 20). After two months of recovery, expression levels were similar as control levels for these transcripts (not shown).

The objective to perform RNAseq analyses is to investigate metabolic pathways affected by the different exposures and search for new biomarker candidates for such exposures. We did especially focus on pathways know to be involved with genotoxic stress, like the p53 signalling pathway (a protection system that either drive damages cells to cell-cycle arrest or apoptosis) and the DNA repair system. These genes are all included in pathways in cancer (Pathway ID 5200) and when we

compared control versus oil treatment transcripts, we found 13 effected gene products (Figure 23). None of the effected genes were directly involved in the p53 and gene repair system

We have identified several pathways with differential gene expression from KEGG analyses, like the Pancreatic secretion (Pathway ID 4972) and the Protein digestion and absorption (Pathway ID 4974), where 14 and 11 gene products were effected when comparing control versus oil exposed.

However, the low number of samples included in this RNA-Seq study make it difficult to make clear conclusion from this results.

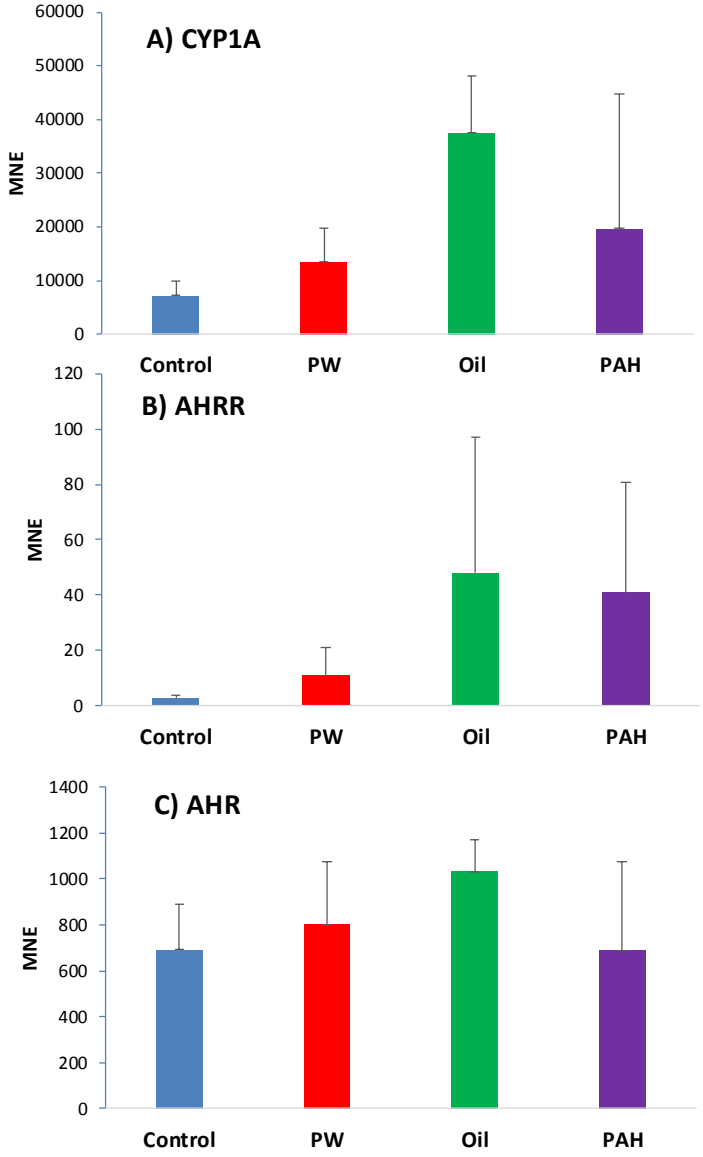


Figure 22. Transcript levels of (A) CYP1A1, (B) Ah-receptor (AHR) and (C) Ah receptor repressor (AHRR) in haddock sampled 22nd April (after two months of exposure). N=4 per group. Statistical significant differences were not obtained due to small samples size.

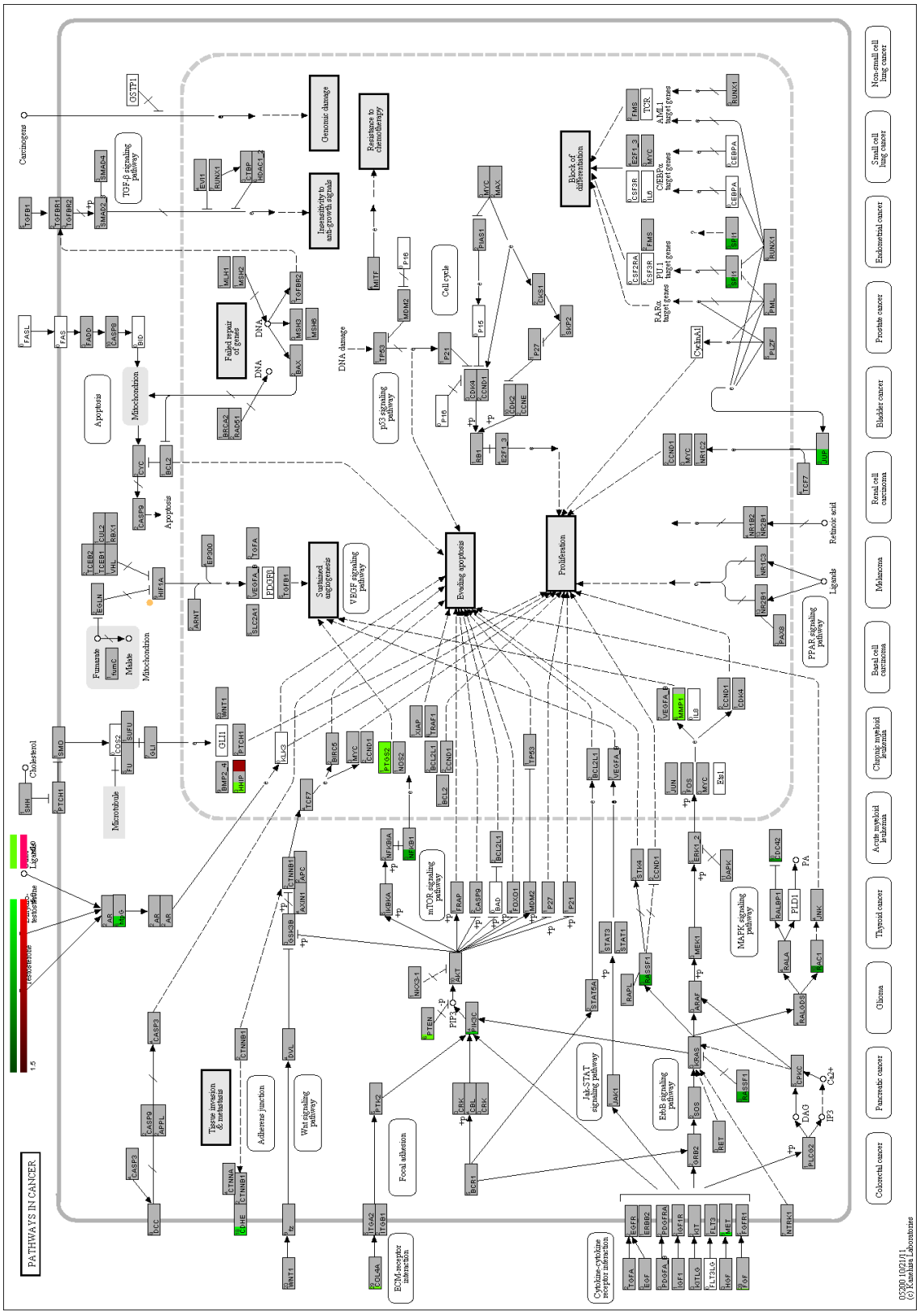


Figure 23. Effected transcripts in KEGG pathways in cancer for treatments control vs oil 2204. Green colour indicates upregulation in oil treatment, red colour indicates downregulation. Settings: 0,95 % confidence interval; fold change threshold: 1.5.

3.8 Lipid analysis.

Field studies on haddock (Balk *et al.* 2011;Grøsvik *et al.* 2012) reported differences in the fatty acid (FA) composition between haddock from Tampen and Egersund Bank that indicate a reduction of (n-3) FA and increase of (n-6) FA in haddock from Tampen.

We have investigated whether PAH exposure can affect lipid metabolism/biosynthesis in haddock and thereby change the FA composition in a similar way as observed in field samples. These analyses also provide information on energy status of the fish, which is important for the evaluation of the long-term effects on fitness. The lipid content and FA composition were analyzed from the haddock liver (storages lipid), haddock muscle (membrane lipids) and the feed.

3.8.1 Comparison of FA profiles in the diet and the fish.

The haddock liver is very lipid rich (48-61% relative to wet weight) and is totally dominated by triacylglycerols (TAG) (>95 %), which is a lipid for energy storage. The muscle is very lean (0.8-1%) and contains only phospholipids (PL) and cholesterol (membrane lipids). Figure 24 shows the FA profile in the diet and in haddock liver and muscle (control fish from 22/4 are used as example). The FA profile of the muscle shows a typical PL FA profile with very high levels of polyunsaturated (PUFA) of 52 % of the total FAs, saturated FAs (SFA) contribute with 24 % and monounsaturated FAs (MUFA) also with 24 %. The PUFA is dominated by (n-3) FAs (46 %), and 22:6 (n-3) and 20:5 (n-3) contribute with 27 % and 14 % of the total FAs. The (n-6) PUFA contribute with 6 % of the total FAs and the (n-3)/(n-6) ratio is 8. The FA profile in the liver is dominated of MUFA (47 %) followed by PUFA (27 %) and SFA (26 %), the (n-3)/(n-6) ratio in the liver is 4. The fish were given a marine diet (13 % lipid) made on fish meal and fish oil (herring). The FA profile in the food is like the FAs profile in the liver, MUFA (47 %), PUFA (28 %) and SFA (25 %), and the (n-3)/(n-6) ratio is 4 similar as for the liver (Table A10).

However, even though the FA profiles have many similarities, there were also some clear differences between the diet and the liver. Figure 25 shows a PCA plot of the feed and haddock liver and muscle from control fish from all five samples times. The PCA models explain 89 % of the total variances; Along PC1 is the muscle samples separated from the liver and diet samples. The food and the liver show little separation along PC1, but they are strongly separated along PC2. Both from the loading plot (figure 25) and Figure 24, the diet has relatively higher levels of long chain MUFAs, 22:1 (n-11), 22:1 (n-9) and 20:1 (n-9) and the short SFA, 14:0, while the haddock livers are high in short chain MUFAs 18:1 (n-9), 18:1 (n-7) and 16:1 (n-7) and middle chain length SFA (16:0 and 18:0).

This shows that the haddock modify the fatty acids from the diet both by elongation (14:0 to 16:0 and 18:0) and Δ9-desaturation (18:0 to 18:1 (n-9)). In addition, the FA profile of the liver suggests a strong selective metabolism towards high use of the long chain MUFA for catabolic energy.

In the food 22:1 (n-11) contributes to 16 % of the total FAs and 20:1 (n-9) to 10 %, while these two FA only contribute to 6 % and 7 % of the total FAs in the liver. On the other hand, the liver has 19 % of 18:1 (n-9) and the food only 8 %. This results shows that haddock have a very active metabolic modification of the diet FAs and especially selective catabolism of long chain MUFA, which is commonly seen in marine fish (Stubhaug *et al.* 2007).

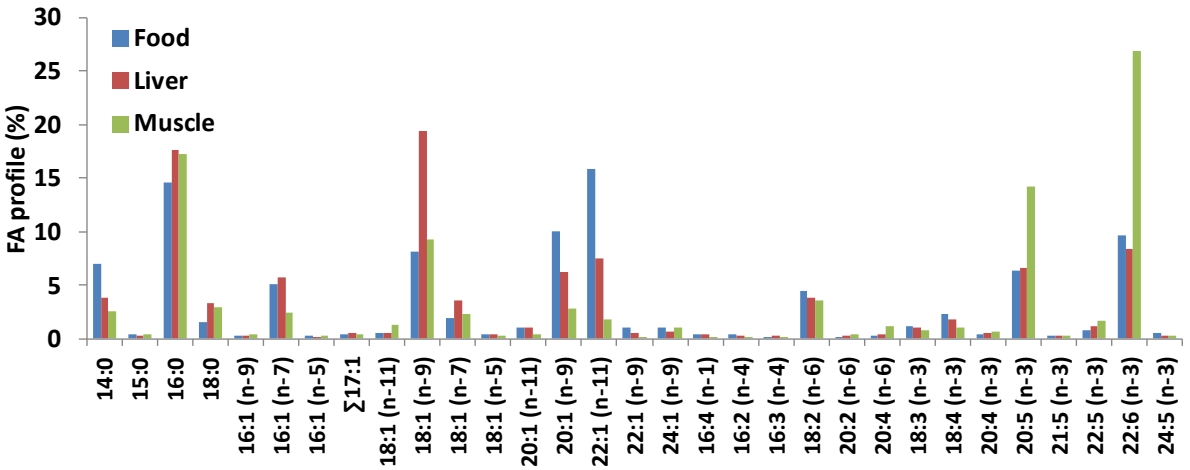


Figure 24. Fatty acid profile of the feed, haddock liver and muscle (control fish form 22/4). Only the 31 dominating FA are included in the figure (26 minor FAs that contributed less that 0.3 % has been removed).

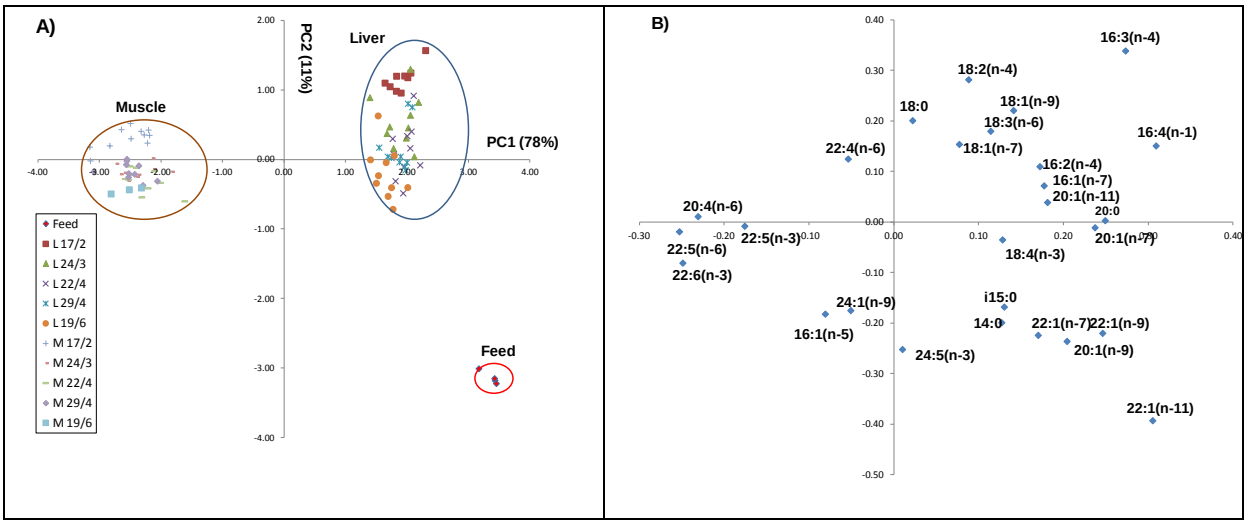


Figure 25. Principal component analysis of FA profiles from feed, haddock liver and muscle (control fish). A) shows the score plot and B) show the loading plot. The model explain 89 % of the total variance (PC1=78% and PC2=11%). Only FA that contributes to more than average to the PCA model are included in the loading plot.

3.8.2 Differences in lipid amount found in liver and muscle between the treatment groups

There were no differences in the total amount of lipids in the liver between the treatments (Figure 26). Muscle samples contained higher levels of lipids in the PW group, but lower levels of lipid in the oil and PAH treated groups compared with control after one month of exposure (24/3) (table 9). However, no differences in lipid content in the muscles were observed after two-months of exposure (22/4) and after one month of recovery (29/4) (table A8-A10).

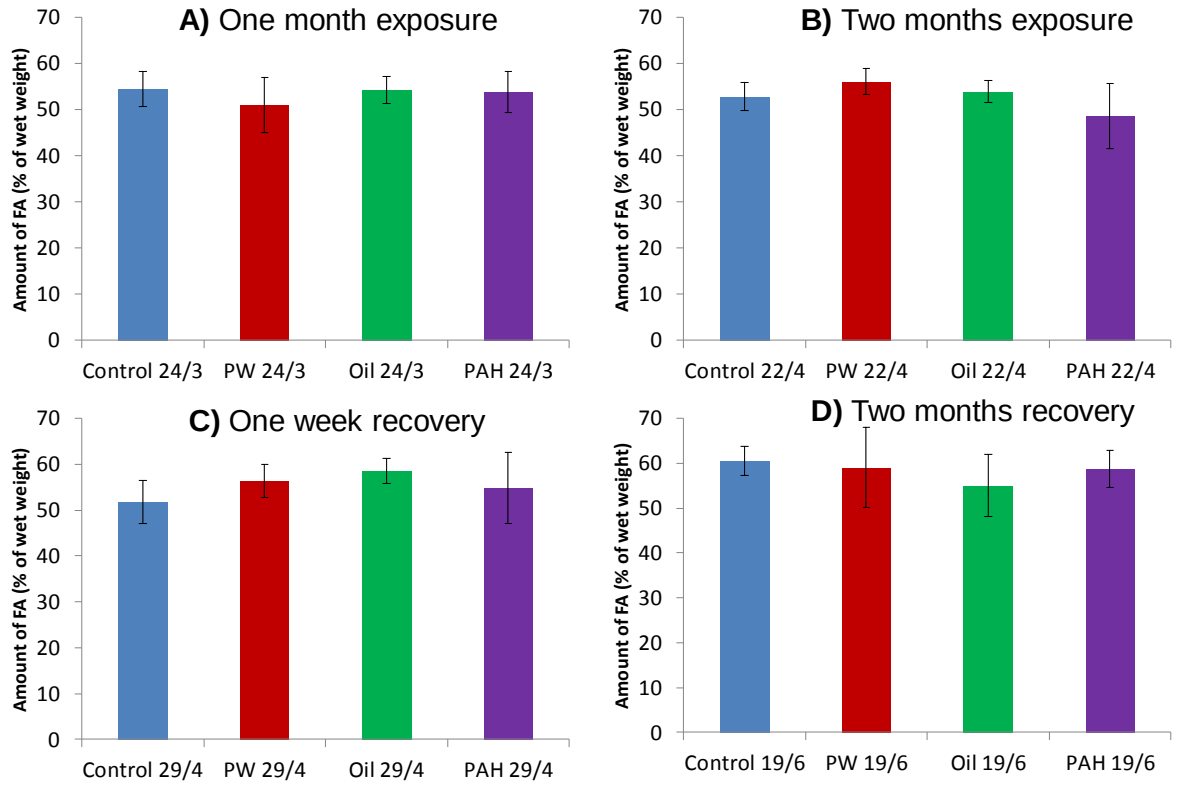


Figure 26. Lipid content in the liver of the different treatments during exposure (A and B) and recovery (C and D). Data presented as average ± stdev.

3.8.3 Differences in FA profile in the liver of different treatments groups

Small but significant differences between the FA composition in the liver of the control fish and the livers of the oil and PAH exposed fish were measured. Figure 27 shows score and loading plot of PCA for FA composition in haddock livers after one (A/B) and two-month (C/D) exposure. The FA profiles are presented in table 10 (24/3), table A4 (22/4), table A5 (29/4) and table A6 (19/6).

After one month of exposure the PAH group separates from the control group along PC1, while the PW group overlaps with the control and the oil group overlaps with both the control and the PW group (Figure 25A). After two months of exposure the oil and PAH exposed fish overlapped more in the PCA and separated from the PW and control group. There are many differences in FA between control and the PAH group, but the general picture is that the long chain MUFA (22:1 and 24:1) are relatively lower in the PAH exposed group and the middle chain length MUFA, 18:1 (n-9) and 18:1(n-7) are higher compared with control. Lower levels of the long chain PUFA, 22:6 (n-3) in the livers of the PAH and oil treated fish were also measured. This resulted in slight reduction in the (n-3)/(n-6) ratio after two-month exposure (only significant in the oil group). No differences in the (n-6) PUFAs levels were measured. This difference between the treatment groups was also seen after one week recovery, but not after 2 months of recovery.

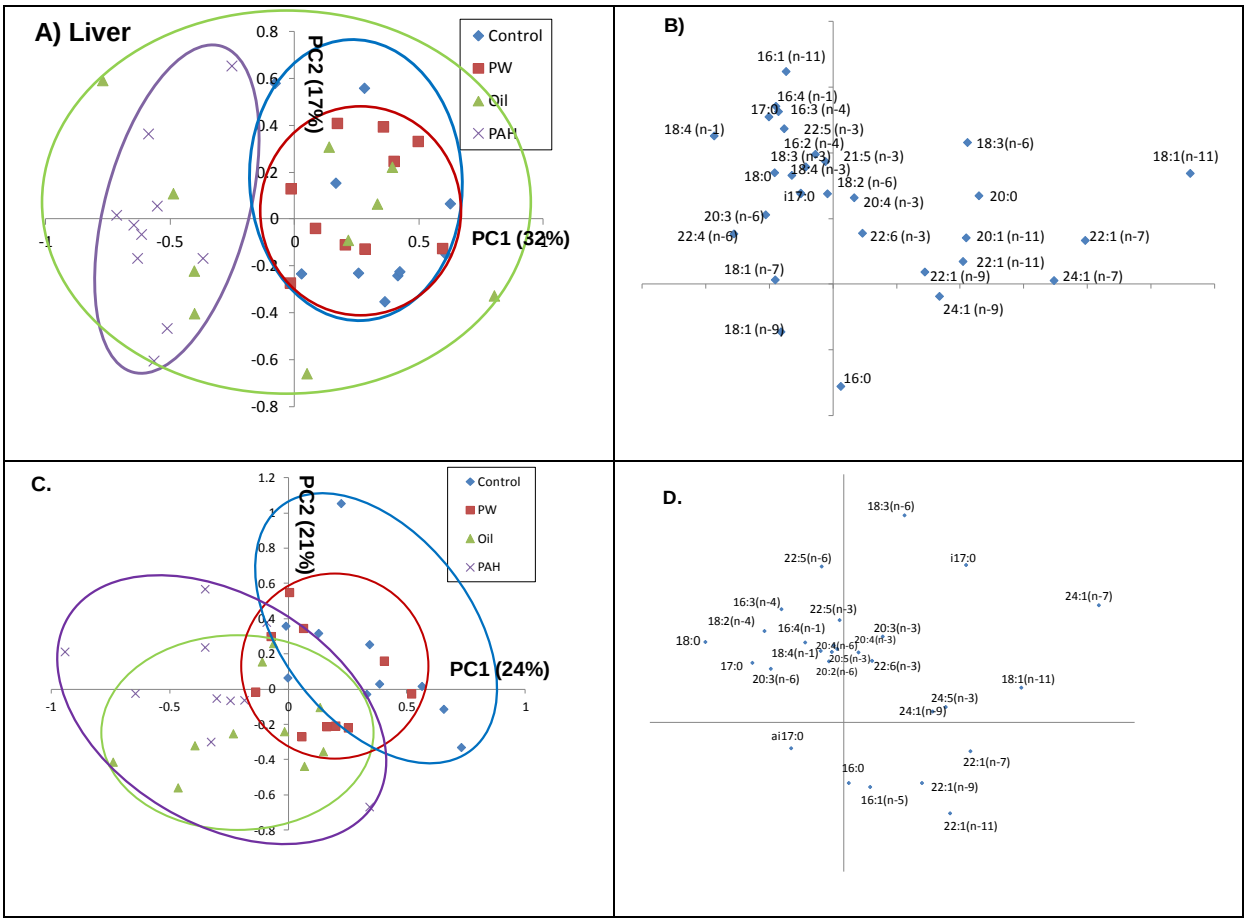


Figure 27. Principal component analysis of FA profiles in the liver from the different treatment groups after one month exposure (24/3) (A=Score plot and B=Loading plot). The model explains 49 % of the total variance (PC1=32% and PC2=17%). Score plot (C) and loading plot (D) of FA profiles in the liver after two-month exposure. The model explains 45 % of the total variance (PC1=24% and PC2=21%). Only the FA that contributes more than average to the PCA model are included in the loading plot.

Table 9. Fatty acid profile in haddock liver from the different treatments groups after one month of exposure. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs. The total amount of FAs relative to wet weight is given. Data presented as average ± stdev. Bold numbers indicate significant differences compared with control, p< 0.05.

	24/3	24/3	24/3	24/3
	Control (24/3) (n=10)	PW (24/3) (n=10)	Oil (24/3) (n=10)	PAH (24/3) (n=10)
14:0	3.50 ± 0.29	3.52 ± 0.17	3.53 ± 0.20	3.43 ± 0.26
Iso 15:0	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
15:0	0.32 ± 0.02	0.32 ± 0.02	0.31 ± 0.02	0.31 ± 0.02
16:0	17.41 ± 0.77	17.22 ± 1.08	17.78 ± 1.34	17.27 ± 1.33
Iso 17:0	0.27 ± 0.01	0.27 ± 0.01	0.28 ± 0.05	0.27 ± 0.01
Antiso 17:0	0.10 ± 0.01	0.10 ± 0.00	0.10 ± 0.01	0.10 ± 0.01
17:0	0.22 ± 0.02	0.23 ± 0.02	0.23 ± 0.03	0.23 ± 0.02
18:0	3.83 ± 0.66	4.21 ± 0.68	4.21 ± 0.60	4.14 ± 0.40
20:0	0.15 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	**0.12 ± 0.01**
∑SFA	26.34 ± 0.89	26.59 ± 1.20	27.13 ± 1.41	26.29 ± 1.43
16:1 (n-11)	0.18 ± 0.02	0.18 ± 0.02	0.17 ± 0.03	0.19 ± 0.04
16:1 (n-9)	0.34 ± 0.03	0.33 ± 0.02	0.32 ± 0.02	0.33 ± 0.03
16:1 (n-7)	5.47 ± 0.29	5.29 ± 0.38	5.35 ± 0.39	5.44 ± 0.34
16:1 (n-5)	0.16 ± 0.02	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
∑17:1	0.51 ± 0.02	0.50 ± 0.02	**0.48 ± 0.03**	**0.47 ± 0.04**
18:1 (n-11)	0.87 ± 0.14	0.89 ± 0.17	0.77 ± 0.20	**0.54 ± 0.10**
18:1 (n-9)	19.61 ± 1.34	19.44 ± 0.91	19.51 ± 1.12	***20.88 ± 1.29***
18:1 (n-7)	3.53 ± 0.22	3.54 ± 0.14	3.55 ± 0.17	***3.85 ± 0.20***
18:1 (n-5)	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.02	***0.34 ± 0.02***
20:1 (n-11)	1.15 ± 0.11	1.16 ± 0.07	1.04 ± 0.11	**0.95 ± 0.07**
20:1 (n-9)	4.75 ± 1.77	5.02 ± 1.44	5.16 ± 0.89	5.21 ± 0.41
20:1 (n-7)	0.21 ± 0.01	0.22 ± 0.01	0.21 ± 0.01	**0.20 ± 0.01**
22:1 (n-11)	6.92 ± 0.67	6.73 ± 0.50	6.41 ± 0.56	**5.60 ± 0.45**
22:1 (n-9)	0.56 ± 0.03	0.55 ± 0.03	0.54 ± 0.03	**0.48 ± 0.03**
22:1 (n-7)	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	**0.05 ± 0.01**
24:1 (n-9)	0.67 ± 0.05	0.67 ± 0.04	0.65 ± 0.06	**0.57 ± 0.04**
24:1 (n-7)	0.11 ± 0.02	0.10 ± 0.01	0.09 ± 0.03	**0.08 ± 0.03**
∑MUFA	45.46 ± 1.42	45.21 ± 0.96	44.85 ± 0.91	45.37 ± 0.82
16:4 (n-1)	0.38 ± 0.05	0.39 ± 0.03	0.40 ± 0.05	***0.41 ± 0.03***
18:4 (n-1)	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.02	***0.16 ± 0.01***
16:2 (n-4)	0.34 ± 0.04	0.35 ± 0.02	0.34 ± 0.02	0.36 ± 0.02
16:3 (n-4)	0.31 ± 0.04	0.32 ± 0.02	0.32 ± 0.03	0.35 ± 0.03
18:2 (n-4)	0.24 ± 0.03	0.24 ± 0.02	0.24 ± 0.03	0.26 ± 0.02
18:2 (n-6)	4.04 ± 0.22	4.05 ± 0.22	4.02 ± 0.24	4.08 ± 0.23
18:3 (n-6)	0.14 ± 0.02	0.13 ± 0.03	0.13 ± 0.03	0.13 ± 0.03
20:2 (n-6)	0.31 ± 0.02	0.32 ± 0.01	0.31 ± 0.02	0.31 ± 0.01
20:3 (n-6)	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.01
20:4 (n-6)	0.49 ± 0.03	0.48 ± 0.02	0.47 ± 0.02	0.47 ± 0.04
22:4 (n-6)	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.02	0.14 ± 0.02
22:5 (n-6)	0.16 ± 0.02	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
18:3 (n-3)	1.05 ± 0.08	1.06 ± 0.07	1.07 ± 0.08	1.11 ± 0.07
18:4 (n-3)	1.73 ± 0.15	1.74 ± 0.10	1.75 ± 0.14	1.82 ± 0.12
20:3 (n-3)	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
20:4 (n-3)	0.56 ± 0.03	0.56 ± 0.02	0.54 ± 0.03	**0.53 ± 0.02**
20:5 (n-3)	7.05 ± 0.48	7.06 ± 0.30	7.02 ± 0.36	7.30 ± 0.32
21:5 (n-3)	0.32 ± 0.03	0.33 ± 0.02	0.32 ± 0.03	0.33 ± 0.03
22:5 (n-3)	1.20 ± 0.19	1.31 ± 0.07	1.25 ± 0.19	1.30 ± 0.17
22:6 (n-3)	8.95 ± 0.38	8.77 ± 0.16	8.76 ± 0.40	**8.43 ± 0.59**
24:5 (n-3)	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.03
∑PUFA	28.19 ± 1.49	28.20 ± 0.95	28.02 ± 1.49	28.34 ± 1.44
∑(n-6) PUFA	5.37 ± 0.26	5.38 ± 0.23	5.33 ± 0.29	5.40 ± 0.26
∑(n-3) PUFA	21.27 ± 1.13	21.24 ± 0.66	21.10 ± 1.10	21.22 ± 1.16
(n-3)/(n-6)	3.97 ± 0.13	3.95 ± 0.06	3.96 ± 0.07	3.93 ± 0.15
Amount of Cholesterol (%)	0.22 ± 0.03	0.24 ± 0.03	0.23 ± 0.03	**0.19 ± 0.02**
Amount of FA (%)	54.52 ± 3.84	51.04 ± 5.99	54.30 ± 2.94	53.84 ± 4.45

3.8.4 Differences in FA profile in the muscle of different treatment groups

The FA profile in the muscle of the PAH treated fish shows several differences from the control, although, in an inconsistent manner between the different sampling times. After one month of exposure, higher levels of SFA and MUFA and less PUFA were found in the exposed fish (Table 10), but after two month and also after one week of recovery (Table A7 and A8), PAH treated fish had on the contrary higher levels of PUFA (mainly 20:5 (n-3)). After 2 months of recovery lower levels of the two long chain MUFAs (22:1 (n-11) and 24:1 (n-9)) were observed. No significant differences in the (n-3)/(n-6) between the treatment groups were measured at any of the sampling times. The PCA analyses (Figure 28) did not find separation along the PC1 between the different treatments groups, which shows that for the whole FA profile larger variations are found inside the groups than between the different treatments groups.

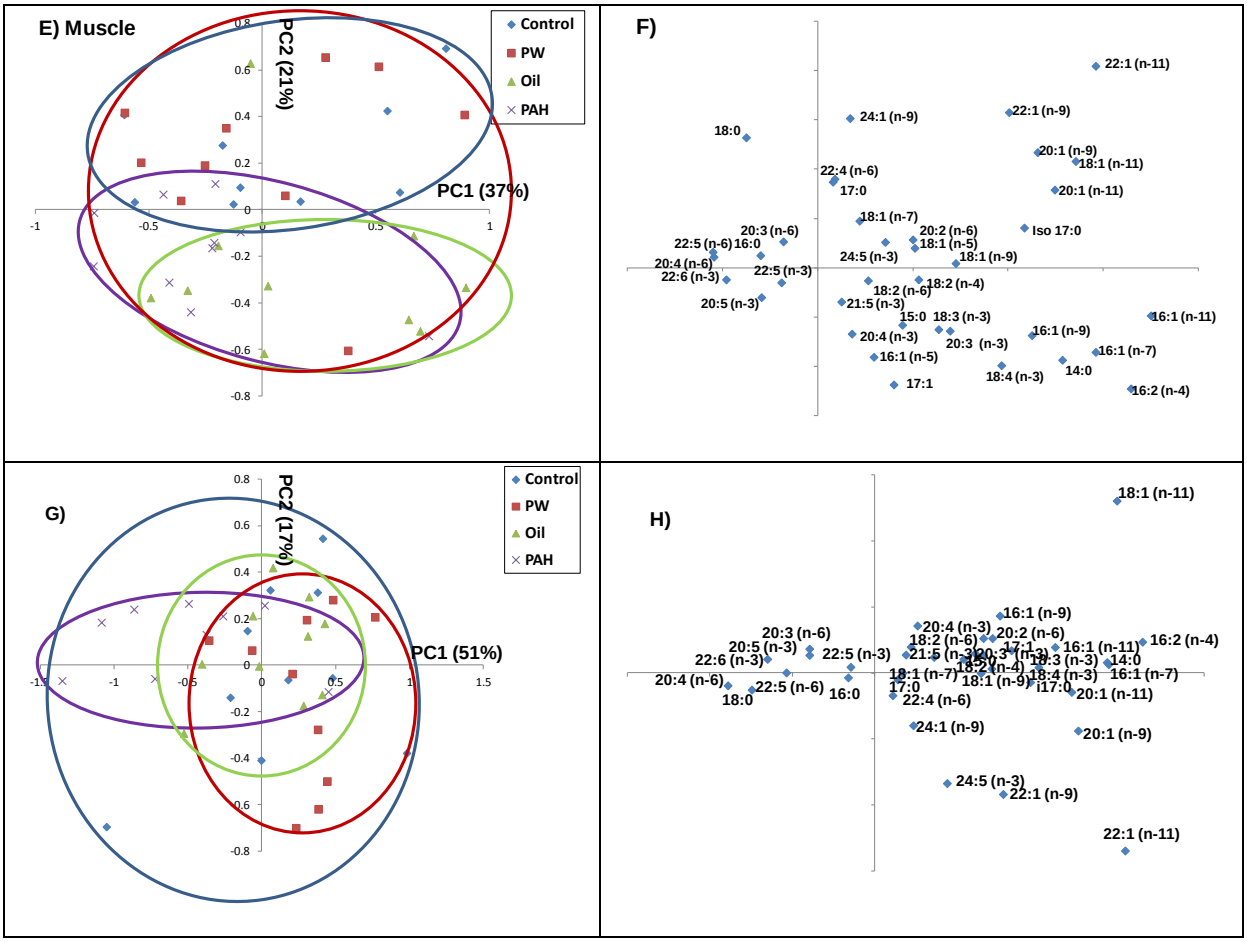


Figure 28. PCA of FA profiles in the muscle from the different treatment groups after one month of exposure (24/3) (E=Score plot and F=Loading plot). The model explains 57 % of the total variance (PC1=37% and PC2=21%). Score plot (G) and loading plot (H) of FA profiles in the muscle after two-month exposure (22/4). The model explains 68 % of the total variance (PC1=51% and PC2=17%).

Table 10. Fatty acid profile in haddock muscle from the different treatments groups after one month of exposure. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs. Data presented as average ± stdev. Bold numbers indicate significant differences compared with control, p< 0.05.

Muscle	Control (24/3) (n=10)	PW (24/3) (n=10)	Oil (24/3) (n=9)	PAH (24/3) (n=10)
14:0	2.50 ± 0.42	2.44 ± 0.35	2.29 ± 0.28	**2.11 ± 0.29**
Iso 15:0	0.09 ± 0.02	0.09 ± 0.01	0.08 ± 0.02	**0.07 ± 0.01**
15:0	0.35 ± 0.03	0.35 ± 0.02	0.33 ± 0.03	**0.32 ± 0.03**
16:0	17.44 ± 0.72	17.54 ± 0.86	17.81 ± 0.67	18.32 ± 0.87
Iso 17:0	0.19 ± 0.02	0.19 ± 0.01	***0.23 ± 0.05***	0.20 ± 0.01
Antiso 17:0	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
17:0	0.19 ± 0.00	0.19 ± 0.01	***0.22 ± 0.02***	***0.22 ± 0.02***
18:0	2.79 ± 0.25	2.74 ± 0.18	***3.40 ± 0.41***	***3.48 ± 0.25***
20:0	0.03 ± 0.00	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
∑SFA	24.01 ± 0.62	24.03 ± 0.75	***24.82 ± 0.63***	25.14 ± 0.85
16:1 (n-11)	0.16 ± 0.03	0.15 ± 0.02	0.17 ± 0.05	**0.14 ± 0.02**
16:1 (n-9)	0.33 ± 0.05	0.33 ± 0.06	0.34 ± 0.06	0.32 ± 0.04
16:1 (n-7)	2.36 ± 0.42	2.37 ± 0.40	2.27 ± 0.31	2.08 ± 0.31
16:1 (n-5)	0.26 ± 0.02	0.24 ± 0.02	**0.22 ± 0.02**	**0.22 ± 0.02**
∑17:1	0.36 ± 0.04	0.36 ± 0.04	**0.28 ± 0.07**	0.32 ± 0.05
18:1 (n-11)	0.99 ± 0.20	0.94 ± 0.16	**1.30 ± 0.25**	1.10 ± 0.25
18:1 (n-9)	8.70 ± 0.91	8.84 ± 1.05	9.20 ± 0.85	9.23 ± 0.94
18:1 (n-7)	2.24 ± 0.10	2.28 ± 0.13	***2.44 ± 0.14***	***2.49 ± 0.11***
18:1 (n-5)	0.26 ± 0.02	0.26 ± 0.02	0.27 ± 0.02	0.25 ± 0.01
20:1 (n-11)	0.43 ± 0.05	0.44 ± 0.05	0.46 ± 0.08	0.43 ± 0.09
20:1 (n-9)	2.12 ± 0.27	2.06 ± 0.29	2.38 ± 0.37	2.32 ± 0.35
20:1 (n-7)	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
22:1 (n-11)	1.27 ± 0.29	1.28 ± 0.40	1.39 ± 0.32	1.28 ± 0.32
22:1 (n-9)	0.16 ± 0.03	0.16 ± 0.04	0.17 ± 0.03	0.16 ± 0.03
22:1 (n-7)	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.02
24:1 (n-9)	0.81 ± 0.06	0.78 ± 0.16	0.90 ± 0.10	0.91 ± 0.12
24:1 (n-7)	0.18 ± 0.04	0.19 ± 0.05	**0.11 ± 0.02**	0.12 ± 0.01
∑MUFA	20.77 ± 2.02	20.83 ± 2.25	22.01 ± 2.11	21.46 ± 1.88
16:4 (n-1)	0.06 ± 0.03	0.07 ± 0.03	0.05 ± 0.02	0.04 ± 0.01
18:4 (n-1)	0.10 ± 0.01	0.10 ± 0.02	0.09 ± 0.02	0.07 ± 0.01
16:2 (n-4)	0.16 ± 0.03	0.16 ± 0.03	0.15 ± 0.02	**0.13 ± 0.03**
16:3 (n-4)	0.06 ± 0.02	0.07 ± 0.02	0.05 ± 0.02	0.05 ± 0.01
18:2 (n-4)	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
18:2 (n-6)	3.68 ± 0.16	3.68 ± 0.15	3.65 ± 0.16	3.62 ± 0.17
18:3 (n-6)	0.06 ± 0.01	*0.08 ± 0.02*	0.07 ± 0.01	0.09 ± 0.07
20:2 (n-6)	0.33 ± 0.04	0.34 ± 0.05	***0.37 ± 0.03***	***0.36 ± 0.04***
20:3 (n-6)	0.100 ± 0.004	0.104 ± 0.007	0.106 ± 0.005	***0.109 ± 0.005***
20:4 (n-6)	1.22 ± 0.08	1.25 ± 0.10	1.23 ± 0.10	1.23 ± 0.09
22:4 (n-6)	0.14 ± 0.02	0.14 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
22:5 (n-6)	0.47 ± 0.03	0.47 ± 0.03	0.47 ± 0.03	0.48 ± 0.03
18:3 (n-3)	0.76 ± 0.06	0.77 ± 0.07	0.73 ± 0.06	0.69 ± 0.07
18:4 (n-3)	1.11 ± 0.13	1.11 ± 0.12	1.00 ± 0.10	**0.92 ± 0.12**
20:3 (n-3)	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.09 ± 0.01
20:4 (n-3)	0.69 ± 0.03	0.70 ± 0.06	0.66 ± 0.04	0.62 ± 0.04
20:5 (n-3)	15.52 ± 0.75	15.46 ± 0.68	14.72 ± 0.87	14.49 ± 0.37
21:5 (n-3)	0.30 ± 0.01	0.30 ± 0.02	0.29 ± 0.01	0.29 ± 0.02
22:5 (n-3)	1.77 ± 0.04	1.75 ± 0.05	1.71 ± 0.05	1.70 ± 0.04
22:6 (n-3)	27.98 ± 1.47	27.87 ± 1.67	27.00 ± 1.44	27.72 ± 1.25
24:5 (n-3)	0.29 ± 0.02	0.28 ± 0.03	0.28 ± 0.04	0.27 ± 0.10
∑PUFA	55.22 ± 1.92	55.13 ± 1.94	53.17 ± 2.09	53.40 ± 1.19
∑(n-6) PUFA	6.06 ± 0.15	6.11 ± 0.17	6.07 ± 0.15	6.05 ± 0.21
∑(n-3) PUFA	48.61 ± 2.02	48.45 ± 2.07	46.56 ± 2.10	46.86 ± 1.28
(n-3)/(n-6)	8.02 ± 0.42	7.94 ± 0.45	7.67 ± 0.33	7.75 ± 0.37
Amount of Cholesterol (%)	0.06 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
Amount of FA (%)	0.86 ± 0.06	***1.00 ± 0.09***	**0.74 ± 0.12**	**0.72 ± 0.04**

PAH effects on lipid metabolism and membrane remodeling

In this investigation we found minor but significant differences between the FA profiles in liver and muscle between the control fish and the oil and PAH treated fish.

The lower amount of long chain MUFA in the liver of PAH treated fish suggests an increased need of energy in this fish and selective mobilization of FAs for catabolism. However, this effects is not so high that it resulted in lower lipid content in the liver or reduced liver size. The lower amount of 22:6 (n-3) found in the liver of the PAH treated fish could be a result of increased oxidative stress and lipid peroxidation (Valavanidis *et al.* 2006), but we did not observe similar effects on other highly unsaturated PUFAs (22:5 (n-3) or 20:5 (n-3)). Therefore, it seems unlikely that this observation is a result of increased degradation of PUFA. The effects seen in the muscle samples after one month with higher levels of SFA and MUFA and lower levels of PUFA in the PAH exposed fish could suggest modification of the membrane composition to compensate for increased membrane fluidity (Liland *et al.* 2014;Meier *et al.* 2007;Tekpli *et al.* 2010). But after two-month exposure (and one week recovery), we found higher levels of long chain PUFAs (20:5 (n-3), 22:5 (n-3), 22.6 (n-3)) which does not support this mechanism. It is also not likely that the muscle cells will experience high concentration of heavy PAHs, as no PAHs or PAH metabolites were detected in the liver or the bile.

The overall effects on FA profiles are small, but it shows that the PAH and oil exposure effect the lipid composition in the haddock liver and muscle. In the present project, we have only analyzed total FA profile in the whole tissue. To get a better understanding of the mechanism behind the lipid effects one would need to do more detailed lipid analysis and study the FA profiles in the different lipid classes.

However, the results of our lipid analysis do not support the hypothesis of Balk et al., (2011) that exposure for PAH and oil compounds is responsible for the increased levels of 20:4 (n-6) and reduced levels of (n-3) PUFA (=> lower (n-3)/(n-6) ratio) that were reported in muscle of cod and haddock collected at the Tampen area compared with references fish from Egersund bank. Bratberg et al., (2013) also did not find changes in the (n-3)/(n-6) ratio in liver phospholipids from cod that were exposed for five weeks with weathered crude oil (1.6 or 82 mg oil/kg fish). Investigations from the condition monitoring (Grøsvik *et al.* 2012) have found similar the differences in the FA composition of haddock as Balk et al., (2011) between Tampen and Egersund bank in 2008 and 2010, but not in 2011. The data from the condition monitoring suggest that the difference in FA profile originate from the diet and not exposure. FA analysis of stomach content showed similar differences in the (n-3)/(n-6) ratio as there were in the haddock tissue and the facts that this differences also were seen in the neutral lipids (the energy storage lipids) in the haddock liver also points to a diet effect. The haddock is an opportunistic predator that feeds on a very diverse variety of benthic invertebrates and fish (Schuckel *et al.* 2010;Tam *et al.* 2016). The large numbers of offshore platforms at the Tampen area may change the benthic communities and thereby the food availability of local haddock (Fujii 2016). We plan to publish all the lipid data from the condition monitoring surveys this year and new lipid and stomach content data from haddock collected from the whole North Sea (30 stations in 2013) are in progress.

3.9 Histopathology and liver damage

At the end of exposure we observed fish with significant grossly visible liver damage (necrosis, areas of swelling and fluid-filled cysts) mainly in the PAH groups, but also in the PW- and oil exposure groups. One should be very careful in drawing conclusions from this kind of macroscopic observation, but they suggest that the PAH treatments resulted in damage to liver physiology and tissue structure, and a histopathological evaluation was therefore prioritized for further studies. At the end of exposure 10 % (2 out of 20 fish) in the PAH group contained large areas of what appeared to be necrotic tissue, and after one week of recovery the possible necrotic tissue was also seen in the PW group (2 out of 10 fish). In the PAH group there were found fish with large fluid-filled cysts (2 of 10 fish) and one fish exhibited a significant mass or nodule/tumor which could not be confirmed by histopathological examination. Because of the low number of fish that were sampled from each time point (22.04 and 29.04) is difficult to predict if the frequencies of liver damage were representative for the whole group. After a two-month recovery on clean food 31-35 fish were sampled, and only a few fish (≈3%) were observed with clearly visible gross liver lesions. This either suggests that the number of affected fish in the previous samples were over-represented or that the exposed haddock were capable of repairing the grossly visible liver lesions.

Table 11. Numbers of fish with visible liver damages.

	22.04.2014	29.04.2014	19.06.2014
Control	0	0	0
PW	0	2 of 10	1 of 35
Oil	0	0	2 of 31
PAH	2 of 20	3 of 10	1 of 33

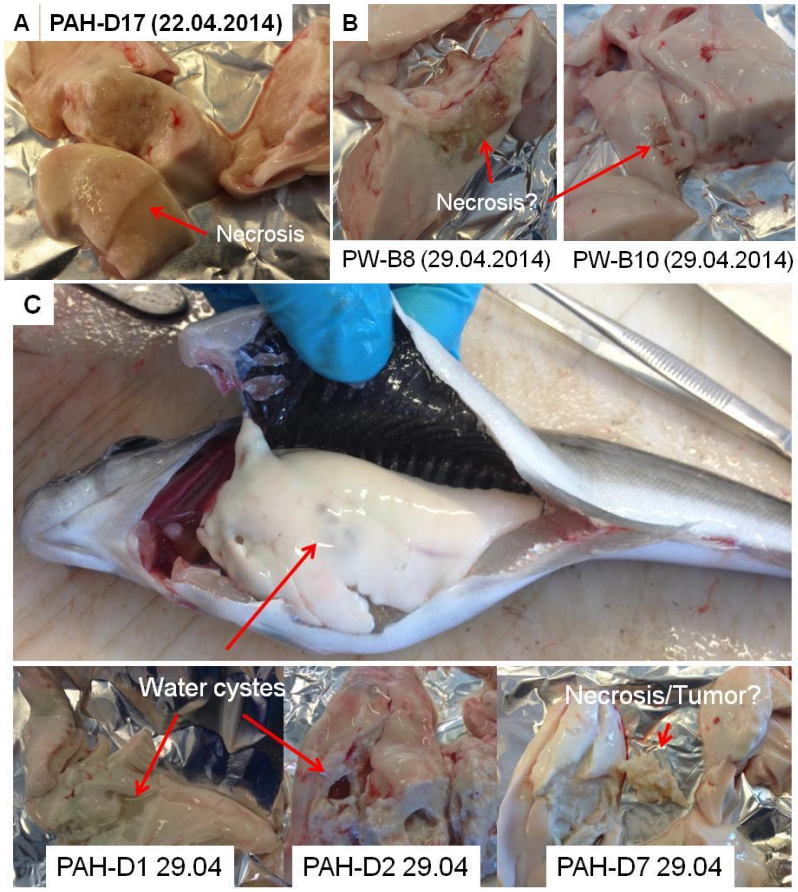


Figure 33. Examples of visible liver damages in PAH (A and C) and PW (B) treated haddock.

Samples were taken randomly from the liver and fixed for histopathological examination with the goal of detecting various potentially neoplastic or other non-neoplastic lesions. The samples were analysed at IRIS as "blind" measurement and the results were further critiqued or confirmed by a detailed histopathologic examination by Mark Myers (a world leading histopathologist formerly of NOAA Fisheries, and now of Myers Ecotoxicology Services, LLC, both in Seattle WA). In the original dataset from IRIS there were reported large amount of necrosis in all samples (>50 % in both control and exposed groups). However, the re-examination and analysis by Myers suggested that the vast majority of these observations were the result of postmortem (PM) autolysis and necropsy-related tissue trauma. Histopathological studies can be difficult and there is always a risk of misdiagnoses (Wolf *et al.* 2015), therefore a double evaluation as here performed is recommended.

The histopathological examination confirms the macroscopic observation showing increased necrosis and inflammation in the PAH treated fish at the end of the two-month exposure and also after one week recovery. After two-month recovery fish with necrosis were not found, and only a few differences in the liver histopathology were detected among the different treatment groups. These results suggest that the haddock have a high capacity to repair previously caused liver damage.

Percentage of abnormalities (% individuals affected)										
	Treatment	mmc/granulomas	granulomas	circulatory disturbances	vacuolated focus	steatosis	fibrosis/cirrhosis	necrosis	eosinophilic focus	inflammation
4/22/2014	C	100	67	11	0	0	0	0	0	0
4/22/2014	PW	100	56	0	0	0	0	0	0	0
4/22/2014	Oil	89	56	22	0	0	0	0	0	0
4/22/2014	PAH	100	67	0	0	0	11	22	0	11
4/29/2014	C	100	56	33	0	0	0	0	0	11
4/29/2014	PW	100	56	0	22	0	22	22	0	0
4/29/2014	Oil	100	67	0	11	0	0	0	0	0
4/29/2014	PAH	100	67	0	44	11	44	33	0	33
6/19/2014	C	100	78	11	0	0	0	0	0	0
6/19/2014	PW	100	67	11	0	0	0	0	0	11
6/19/2014	Oil	100	56	11	0	0	0	0	0	0
6/19/2014	PAH	100	67	22	0	0	0	0	0	11

Table 12. Histopatological examination of liver for the different treatment groups after two months of exposure (22/4), one week of recovery (29/4) and two months of recovery (19/6). Nine fish were examined from each group each time.

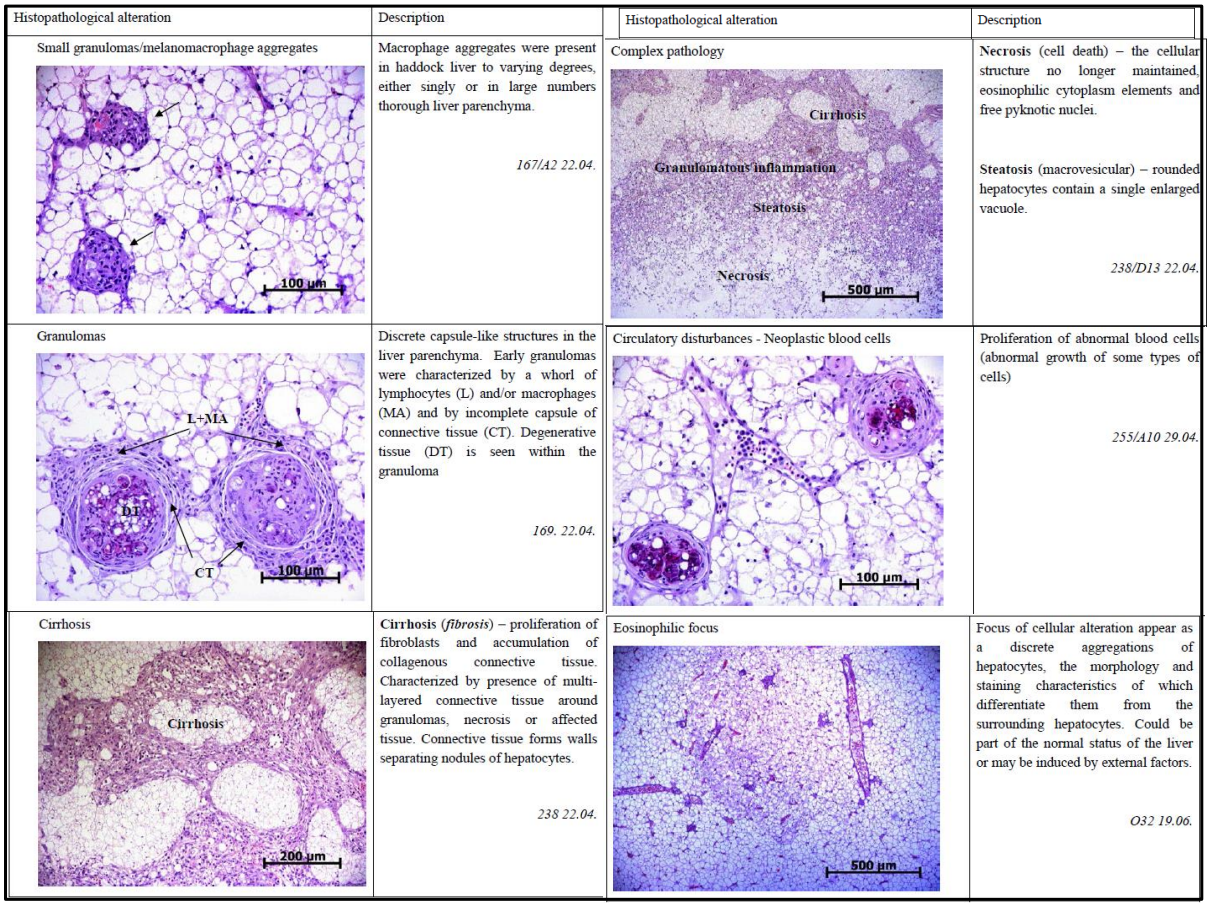


Figure 35. Examples and description of the histopathological alteration.

3.9 Vertebral malformation

As part of an ongoing VISTA/NFR project (Meier et al., 2012) we wanted to use fish from the Statoil-haddock experiment to establish background levels of vertebral malformation. These fish were sampled at the end of exposure (22.04.2014) and x-ray pictures were taken of 20 fish from each group.

To our surprise there were clear differences in the frequencies of bone malformation between the different treatment groups. It is well known that oil exposure on early life stages severely affects the bone formation (Carls *et al.* 1999), but similar effects has not been documented in adult or juvenile fish (Danion *et al.* 2011). Vertebral bone mineralization, however, has been suggested as a biomarker of PAH pollution in marine fish (Danion *et al.* 2011) and our preliminary result shows that this mechanism may be very interesting to study in future investigations.

Table13. Frequency of malformation in PAH exposed haddock. From each group20 fish were examined.

	Number of fish with vertebral malformation	(%) malformation
Control	1	5
PW	4	20
Oil	6	30
PAH	6	30

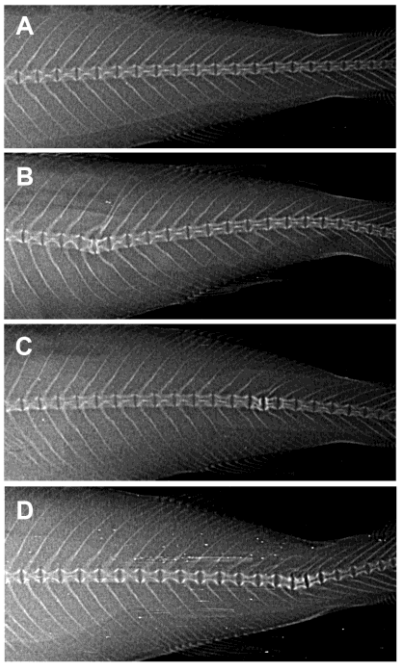


Figure 35. X-ray picture of haddock from Control (A), PAH (B) and oil (C and D). The picture shows different examples of vertebral malformations; deformities in vertebral 25-27 (B), 34-36 (C) and 36-39 (D).

IV. Discussion

There are at least two main sources of PAH that fish can be exposed for in the open sea, small 2-3 ring PAHs with a petrogenic origin and larger 4-6 ring PAHs of pyrolytic origin. The route of exposure may be uptake directly from the water, uptake from contaminated sediments or orally through the food chain. All three exposure routes may be relevant for haddock that are a bottom living fish, finding its food in or just above the sediments. In this experiment, we have focused on oral exposure, comparing three different PAH compositions that intend to represent three different sources, 2 rings PAHs extracted from PW, 3 and 4 rings PAHs obtain from crude oil distillation fractions, representing oil contaminated drill cuttings, and heavy 4-6 ring PAHs representing background in sediments contaminated from atmospheric fallout and urban runoff.

Historically, the analysis of DNA adduct profiles in fish exposed to environmental pollutants represents an important approach in environmental risk assessment since Dawe *et al.* claimed in 1964 that bottom feeding fish were "useful indicators of environmental carcinogens" (Dawe *et al.* 1964). DNA adducts are now considered as a crucial biomarker of exposure in human and sentinel organisms, especially for their early emergence after a genotoxic exposure, which may play a key role in establishing a mode of action for cancer (Pottenger *et al.* 2009). Because of its high sensibility and versatility, the method of 32P postlabelling has been applied to environmental fish studies as early as 1980s, few years after the first publication of the method (Randerath *et al.* 1981). Thus, in 1987, Dunn et al. measured significant DNA adduct levels in livers of wild Brown bullheads sampled from different sites in the Buffalo and Detroit Rivers, in association with exposure of fish to high concentrations of PAH (Dunn *et al.* 1987). Since these early works, numerous fish species were studied, in a large panel of applications (laboratory and field studies). Laboratory studies from isolated subcellular organelles (like hepatic microsomes) and cells in culture to the entire organisms allowed for better understanding of the relation between the exposure to pollutants as PAH (isolated or in mixtures), and certain biological adverse effects including genotoxicity by DNA adduct formation (Varanasi *et al.* 1989a;Wessel *et al.* 2012). In the late 1980s, hepatic DNA adducts related to BAP were for the first time detected in English sole exposed to this PAH in laboratory (Varanasi *et al.* 1989b), supporting the hypothesis of the causal relationship between fish exposure to PAH pollutants and hepatic neoplasms that are frequently described in this species at Puget Sound.

A large number of studies of oral exposure of PAHs of fish have been carried out. Bravo et al. (2011) exposed juvenile rainbow trout with feed containing a mixture of heavy PAHs (10 high molecular weight PAHS, 4-6 rings; all of this are also present in the mixture we have used in the haddock experiment). The exposure doses used in their study (Low dose=0.66 mg PAH/kg fish/day and high dose=7.8 mg PAH/kg fish/day) are related to the levels of PAH found in the stomach of wild salmon in the Puget Sound (WA, US) (Bravo *et al.* 2011). The fish were feed for 50 days and followed for 21 days of recovery period (fasting). The low dose in that investigation is therefore comparable to our haddock experiment (our doses for the PAH group were 0.65 mg PAH/kg fish/day). They analyzed bile metabolites by HPLC-fluorencence detection; using 260/380 nm (PHE-type) and 380/430 nm (BAP-type). They found a strong induction at both wavelengths after 3 days of exposure and with a maximum at day 14. The detection of PAH bile metabolites were high during the whole exposure period and remained surprisingly high during the 21 days of fasting. The authors suggested that this lack of decline in the recovery period may be explained either by the bile not being released in

fasting fish or a recirculation by intestinal reabsorption. This experiment documented that the heavy PAHs were taken up from the feed and metabolized and excreted through the bile. The presence of high levels of bile metabolites is opposite to the observation in the present haddock study. As biomarkers for AhR activation Bravo et al. analyzed hepatic microsomal ethoxyresorufin-O-deethylase (EROD) activity and CYP1A staining (antibody detection) in the liver. They found a significant increase in CYP1A protein in the liver from both doses, but only increased EROD activity in the high dose. The Comet assay was used to detect gene damage in blood cells, and a maximum amount of DNA strand breaks was found after 14 days of exposure in both low and high treatments groups. After 28 days of exposure there were still a significant increase in DNA strand breaks in the exposed groups compared with control, but in the low dose group the amount was only ¼ of that measured in the same group after 14 days exposure. At day 50 there were no significant increase in DNA damage in either of the exposure groups compared with the control. This interesting result shows a reduction in toxic response to long-term PAH-exposure in rainbow trout. This indicates that rainbow trout also can adapt through a protection system against chronically oral exposure of PAHs. Another interesting result from this study is the documentation of decreased survival in pathological challenge experiments in the PAH exposed trout (both doses) (Bravo et al., 2011). Effects on the immune system and increased disease susceptibility are parameters that could be interesting to include in the water column survey.

Ericson et al (1999,) exposed Northern pike (*Esox lucius*) to multiple oral doses (6 mg/kg fish) of single compounds or mixtures of BaP, BkF or 7H-dibenzo[c,g]carbazole (DBC) and measured DNA adduct in liver, gills, brain and intestine. The fish were exposed to 5 doses with 12-day intervals between each dose and the fish were followed 78 days for recovery after end of exposure (given at day 59) (Ericson *et al.* 1999). Rapid uptake was observed and DNA adducts were detected in the liver one day after the first dos and in the intestine after three days. The levels of DNA adducts after multiple doses was 3 times higher in the intestine (347±17 nmol adduct/mol normal DNA) that in the liver (110±9 nmol adduct/mol normal DNA). The clearances rate of DNA adduct was slow and after 78 days recovery only a 30 % reduction of DNA adducts in the intestine and no reduction in the liver were noted.

High accumulation of non-extractable BaP (=> covalent bound) has also been reported in Atlantic salmon *(Salmo salar)* (de Gelder *et al.* 2016) and polar cod (*boreogadus saida*) (Bakke *et al.* 2016) after a single oral dose of BaP and Phe. Using radioactive marked ^{14}C-BaP and ^{14}C-Phe it was found that BaP mainly accumulated in the intestine, while Phe was found in higher concentration in the liver and the bile. This shows that there may be differences in the metabolic pathways for heavy PAHs (>4 rings) and small PAHs (2-3 rings).

Several other studies have also suggested that the amount of BaP (5 ring PAH) that reach the liver is much lower in oral exposure compared with exposure through intraperitoneal injection due to high accumulation and metabolism of BaP in the intestine, Sea Bass (*Dicentrarchus-labrax*) (Lemaire *et al.* 1992), English sole (*Parophrys vetulus*), starry flounder (*Platichthys stellatus*) (Varanasi *et al.* 1986) and rainbow trout (Hendricks *et al.* 1985).

Similar comparisons between oral and waterborne exposure of BaP in Nile tilapia (*Oreochromis niloticus*) have shown that the induction of CYP1A detoxification are very different distributed though

out the body after the two different exposure routes. In the oral exposed fish, only increased EROD values in the intestine were found, while the water exposed fish has EROD induction in the gill and the liver (Costa *et al.* 2011).

Taken together there are evidences that large parts of the metabolism of 5 rings PAHs are located to the intestine and that induction of the intestinal detoxicification system are a very important protection against oral exposure of this compounds (Fang and Zhang 2010;Uno *et al.* 2004).

It is likely to believe that during chronic exposure in our experiment, the detoxification system in the digestion system have been strongly induced. High first-pass metabolism in the intestine may explain why we do not find higher effects in the liver after two months of exposure.

V. Conclusion and advice for further investigation

We have conducted a successful oral exposure experiment where juvenile haddock have been exposed for different PAH mixtures representing different pollution sources; PW, oil polluted sediments and long-transported background pollution.

A number of key questions were addressed:

1. **Does oral exposure to PAH induce DNA-adduct in haddock?**
   Strong induction of DNA adducts in the liver (PAH>>Oil>PW) three days after only one exposure dose were found. All PAH mixtures increased the levels of DNA adducts in the haddock livers. This shows that PAHs are taken up from the food and readily metabolised into reactive metabolites that bind the DNA in the liver, and that a single oral dose is sufficient to induce DNA adducts by 3 days after exposure.

2. **What is the time effect in DNA-adduct formation during chronic exposure, and how fast will fish recover?**
   The levels of DNA adduct dropped dramatically (10 fold) after one month and two months of exposure compared with the first observation after three days of exposure. The amount of DNA adducts after two months of exposure is significantly higher in all treatment groups compared with the control fish, but it can only be classified to be mildly induced. The DNA adduct levels are similar to what have been found in wild fish in the field, and fish that have been water exposed to PW and crude oil. The two-months of recovery on clean food did not result in a significant reduction of amount of DNA adduct compared with the levels at the end of exposure. The high reduction of DNA adducts after chronically exposure suggest that an induction of a protective first-pass metabolism in the intestine toward heavy PAHs. Likewise, most the DNA repair system have been active in clearances of damaged DNA in the liver after the first acute raise, but following the fish into a two months recovery period did not show any decline of DNA adduct. This show a long lifetime of DNA adducts.

3. **Does different PAH give different DNA adduct patterns from the ^{32}P-postlabelling method, and can the "spot position" be used to identified the source of PAH exposure?**

   It was not possible to distinguish between the DNA adduct spot positions from the different PAH treatments. From the fish that were injected with heavy PAHs we found 16 different spots, however, in the oral exposed fish only three spots with high frequency were found. Spot 1 is the dominating spot and this is present in all treatments groups, but also in several fish in the control group. This spot1 is either formed from a common electrophile metabolite or more probably to chemically related adducts that are not separated by the thin layer chromatography system.

   The present experiment clearly documents that the P32 postlabelling is a very sensitive detection method but it is not able to give any structure information of the DNA adduct and the PAH sources responsible for its formation. The UHPLC-MS/MS analysis, on the other hand, shows a great potential of both targeted analysis (identification with synthetic standards) or screening of unknown PAH-DNA adduct. To get any progress in the DNA adduct work on wild fish, it is our opinion that mass spectrometry methods should be used. This will require method development and more experimental work.

4. **How are DNA adducts correlated with other endpoints; PAH bile metabolites and CYP1A induction?**

   No correlations between the concentrations of PAH bile metabolites and the levels of liver were found. The study suggested that the GC-MS method used at IMR to analyse mono-hydroxylated PAH metabolites is not covering the dominating metabolites and new methods should be established.

   CYP1A was induced in liver of the oil and PAH groups, but not in the PW exposed fish. This correlated with induction of DNA adducts where the PAH and oil groups had the highest levels, but elevated DNA adducts was also found in the PW groups, so induction of CYP1A does not seem necessary for formation of DNA adducts in this fish. The relative low induction of CYP1A in the liver and the low presence of bile metabolites in the group exposed for large 4-6 rings PAHs suggest that large parts of the detoxification are happing in the ingestion systems. The presence of relatively high amount of 2 and 3-rings PAHs in the liver, suggest that small PAH is not metabolised in the intestine and that the liver will be the target tissue for these compounds.

Important questions that should be answered before scientific publication of this data:

1. The very high levels of DNA adducts found after 3 days (and a single dose) was only analyzed in 2 fish from each treatment groups and was unexpected. At least 8 more fish from each group for DNA adducts should be examined, so one can present the data with a statistical analysis.

2. Samples taken after 10 days of exposure have not been analyzed at all. It would be interesting to include this sampling point into the investigation to get a better resolution of the time-effect on DNA adduct formation observed. The parameters that should be included are; DNA adducts, bile metabolites, CYP1A qPCR.

3. The surprising results showing the strong reduction in DNA adducts over time suggest intestinal detoxicification and protection. Samples have been collected of the intestine and we suggest that DNA adduct and CYP1A induction is measured from the fish after two-months of exposure. This analysis can contribute with important knowledge of haddock capacity to protect itself against food borne PAH pollution. It will add novelty to the work and it can be important information for the water column survey, and suggest that sampling of intestine should be included in the program to investigate the route of exposure in wild haddock.

4. Bile samples should be re-examined for dihydroxylated and other more polar metabolites. This will be done by LS-MS/MS at NOAA (USA) and at IMR using preparative HPLC and GC-MS/MS. Validation of a new method for bile metabolites is very important for the field work IMR are doing and this work will be funded internally at IMR.

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VII. References

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VII. Material and Methods

Chemicals

PAHs; Fluoranthene (FL), Pyrene (PY), Benz[a]anthracene (BAA), Chrysene (C), Benzo[b]fluoranthene (BBF), Benzo[k]fluoranthene (BKF), Benzo[e]pyrene (BEP), Benzo[a]pyrene (BaP), Perylene (Per), Dibenz[a,h]anthracene(DBA), Indeno(1,2,3-cd)pyrene (IND), Benzo[ghi]perylene (BP) were all obtained from CHIRON (Trondheim, Norway).

For the injection experiment the PAHs was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oslo, Norway) to a concentration of 4 mg/ml.

For the food mixture, the PAH mixture was dissolved in fish oil (NOFIMA, Bergen, Norway)

Experimentental food

To prepare exposure mixtures that had the natural high complexity of PAH isomers found in PW and oil, we used extract of PW from Statfjord A and a distillation fraction of Gullfaks oil (delivered from Mongstad by Britt Steine). The distillation fractions were: Fr1 (240-320 °C); Fr2 (320-375°C); Fr3 (375-400 °C).

PW was collected at Gullfaks and sent with boat to IRIS Stavanger. The extractions were done in a big 1000 L polyethylene tank. Oil compounds from PW were extracted with 20 L cyclohexane. Cyclohexane was removed by evaporation and the PAH content in the PW extract analysed on GC-MS.

Table M1 shows the different compositions of the PAH sources used in the feed. The PAH profiles in the feed are given in Figure 3/Table 3 and Table A3. The PW, Oil and PAH mixtures were dissolved in fish oil mixed into the fish pellets. The fish feed (4 mm pellets) were made by NOFIMA, Feed Technology Centre, Bergen (Table M1 and Table M2).

Table M1. Composition of PW, Oil and PAH mixtures used in the feed.

	Composition	PAH content (% of weight)
PW mixture	60 g PW extract + 6 g oil Fr1	1.1
Oil mixture	30 g oil Fr2 + 36 oil Fr3 + 0.04 g pyrene (Standard)	1.5
PAH mixture	12 PAH standards (20-212 mg)	100

	Composition in feed	PAH content i feed. Measured by GC-MS
PW feed	28 g of PW mixture to 13 kg feed	31 mg PAH/ kg feed
Oil feed	27 g Oil mixture to 13 kg feed	45 mg PAH/ kg feed
PAH feed	976 mg PAH to 13 kg feed	65 mg PAH/ kg feed

Table M2. Composition of the fish pellets (made at NOFIMA, Feed Technology Centre, Bergen)

Odd Helge Romarheim Godkjent/sign: OH

DIET COMPOSITION											
Composition of diet											
Diet no	1	2	3	4	Chemical composition of ingrdients						
	%	%	%	%	water	prot	lipid	ash	CH	Prot.ford	
Fish meal 43/12	67.97	67.97	67.97	67.97	7.7	67.9	13.0	14.4	0.0	89.6	
Wheat 23/13	12.00	12.00	12.00	12.00	12.5	13.2	1.9	1.4	71.0		
Wheat gluten 36/13	10.00	10.00	10.00	10.00	5.3	80.0	4.1	0.8	9.8		
Fiskeolje O1/13	7.50	7.00	7.00	7.00			100				
PW (extract)		0.50					100				
Oil fraction			0.50				100				
Pyrogenic PAH				0.50			100				
Vitamin - mix T3/13	2.00	2.00	2.00	2.00	10	9	3	4	74		
Mineral mix T1/14	0.52	0.52	0.52	0.52	7			93			
Yttrium T20/13	0.01	0.01	0.01	0.01	5			95			

Haddock husbandry

The Juvenile haddock used in the experiment were produced at Austevoll research station from a wild broodstock population of 61 mature individuals collected February 2013 at spawning grounds in the Austevoll area, on the west coast of Norway. The broodstock were kept in two 7000 L tanks and they spawn voluntarily in capacity spawning. Fertilized eggs were collected, transferred to indoor egg incubators until hatching. The haddock larvae have been raised on natural plankton (mainly copepod of *Acartia longiremis*, harvested from the marine pod system "Svartatjern (Van der Meeren et al., 2014) until the larvae started to take dry feed and were fed marine pellets. The fish have been grown on a natural light regime and with a water temperature of 8°C.

The 17th January 2014 were 440 fish (125±35 g wet weight) marked individually with electronic tags and evenly distributed between five circular tanks (3 m diameter, 7 m^3). The fish were feed ad libitum with automatic feeding unit 5 times a week until the exposure experiment starts.

The exposure experiment started 14th February and lasted till 22th April. A group of fish from each treatment were kept for two months until 19th June to look for long-term effects. The exposure experiment has been conducted as planned and 420 haddock were sampled.

Ethics Statement

All animal experiments within the study were approved by NARA, the governmental Norwegian Animal Research Authority (http://www.fdu.no/fdu/, reference number FOTS ID 5924). All methods were performed in accordance with approved guidelines. All fish were killed before sampling with a high dose MS-222 (tricaine methanesulfonate, TS 222, Sigma-Aldrich). The animals were monitored daily, and dead fish were removed. There was low mortality during the experiment, totally 19 fish died (<5%) and without any correlation to the exposure.

The Austevoll Aquaculture Research station has the following permission for catch and maintenance of Atlantic haddock: H-AV 77, H-AV 78 and H-AV 79. These are permits given by the Norwegian Directorate of Fisheries. Furthermore, the Austevoll Aquaculture Research station has a permit to run as a Research Animal facility using fish (all developmental stages), with code 93 from the National IACUC; NARA.

Experimental design

The exposure experiment was started 14th February and lasted till 22th April. Approximated 45 fish from each treatment group were keep for a recovery study and feed an uncontaminated diet until the 19th June. The control fish weights at start of experiment were 147±31 g and at experiment end 336±57 g. The fish were feed four different diets with automatic feeding unit 5 times a week with a ratio corresponding 10 g pellets/kg fish/day (1 % of body mass per day). This correspond a daily dose of PW treatment group: 0.31 mg PAH/kg; Oil treatment group: 0.45 mg PAH/kg and PAH treatment group: 0.65 mg PAH/kg.

Samples one week after end of exposure and the remaining fish were transferred to one common tank and maintained for one month until 19th June and sampled to study long term effects.

The experiment was carried out on juvenile haddock (100-250 g) and the fish were exposed to different hydrocarbon mixtures through the feed. The fish were marked with electronic tags and held in five circular tanks (3 m diameter, 7 m^3). The fish were feed with automatic feeding unit 5 times a week with a ratio corresponding 10 g pellets/kg fish/day (1 % of body mass per day). This correspond a daily dose of PW treatment group: 0.31 mg PAH/kg; Oil treatment group: 0.45 mg PAH/kg and PAH treatment group: 0.65 mg PAH/kg.

Injection experiment

At the start of the experiment (14.02.2014), 26 haddock were injected with single PAH compounds (2 fish for each of the 12 heavy 4, 5 and 6 ringed PAH compounds used in the PAH mixture) and sampled after two days. The PAHs were dissolved in DMSO and fish oil to a concentration of 4 mg/ml and each fish were injected in the abdominal cavity with 1 µl/g fish corresponding to a dose of 4 mg/kg fish. The injected fish will be used to generate bile metabolite and DNA adduct "library".

Sampling.

Six samplings of fish were preformed during the experiment (Table M3).

We sampled for two projects: Statoil-haddock and iNEXT (a PhD project at IRIS, Phd candidate; Karianne Skogland, supervisor; Daniela Pampanin).

All samples were frozen down on liquid nitrogen and stored at – 80° C. Samples to IRIS have been sent to Stavanger and the Statoil-Haddock material are at IMR in Bergen.

Table M3. Samplings time and number of fish.

Exposure groups	17.02.2014	24.02.2014	24.03.2014	22.04.2014	29.04.2014	19.06.2014
Control	10	10	15	20	10	36
PW	10	10	15	20	10	35
Oil	10	10	15	20	10	31
PAH	10	10	15	20	10	33
Injection (PAH)	25					

Samples of several tissues were taken (Blood, bile, liver, muscle, brain, intestine, heart and whole fish) (Table M4). The samples of intestine, heart and brain will not be included in the analytical program of this application. The material will be kept at IMR for future studies.

Table M4. Overview of samples collected from each sampling time.

Code and sample	Analysis parameters	Statoil haddock	IRIS
Bile samples			
B1	PAH Metabolits	minimum 50 µl	
B2	protein-adducts		50-100 µl
Liver samples			
L1	DNA addukt (32P-postlabering)	500 mg	
L2	DNA addukt (LC-MS/MS)	500 mg	
L3	RNA-Seq	500 mg	
L4	Lipid -FA	500 mg	
L5	ELISA protein	500 mg	
L6	Protein-PAH adduct analysis		500 mg
L7	Histology		Sample on fix
Blood samples			
P1	protein-adducts		200 µl
Muscle samples			
M1	Lipid	1 g	
Brain samples			
H1	Lipid	whole	
Intestine samples			
I1	RNA-Seq	Inner intestine	
I2		Middle intestine	
I3		Back intestine	
Heart samples (only from 22.04.2014)			
HA	RNA-Seq	Whole heart	
Whole fish for x-ray (only from 22.04.2014)			
X-ray	Vertebral body malformations	Whole fish	

DNA adduct analyses (^{32}P- postlabelling)

Detail about the ^{32}P- postlabelling methods are given in report from ADn'tox, Caen, France.

DNA adduct analyses (LC-MS/MS)

An ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC MS/MS) method for the detection of DNA adducts, currently under development, was first applied to a sample exposed for PAH mixture for 2 months (n°38). This sample contain high level in DNA adducts as revealed by its 32P postlabelling analysis (40 nmol adducts/mol normal DNA). The method that has been developed by the ADn'tox partner PRISMM (an academic platform of the University of Caen, France) is suitable for detection of the major BaP adduct: benzo(a)pyrene (BP)-7,8-diol-9,10-epoxide-N(2)-deoxyguanosine (BPDE-dG) in the MRM (Multi Reaction Monitoring) mode, but can also be used for the pre-screening of multi-adduct detection in the PNL (Pseudo Neutral Loss) mode. The later mode is focused on the loss of the deoxyribose (dR) from the protonated DNA adducts ([M+H-116]+), a particular fragmentation common to all nucleosides in MS, including the parent dG DNA adducts.

As for postlabelling, the sampled DNA content is firstly extracted by phenol-chloroform and then hydrolyzed with a five enzyme cocktail in individual nucleosides, before the UHPLC separation and MS/MS detection.

Analysis of PAH metabolites in fish bile (GC-MS)

Bile (100 µl) was diluted in 200 µl sodium acetate buffer (0.01 M, pH 5). 36 µl β-glucuronidase (115600 units/ml) were added, and samples were incubated at 37°C for 2 hours. Surrogate internal standard (SIS) including two deuterated hydroxyl PAH, 1-naphthol-d7 and 1 hydroxypyrene-d9, were added to the solution which was then further diluted with 2 ml acetic acid (0.1 %). The mixture was then loaded onto Oasis (HLB) SPE column (4 cc volume), previously preconditioned with 1 ml methanol and 1 ml acetic acid (0.1 %), successively. The column was rinsed with 3 ml acetic acid (0.1 %) and dried for ½ hour under vacuum. The analytes were extracted by 4 ml of methanol. The extract was then evaporated to ca. 0.2 ml under a nitrogen stream (40°C). The eluate was derivatizated with pentafluorobenzoyl chloride as described elsewhere (Boitsov *et al.* 2004) and the samples concentrated to 0.5 ml hexane solution under a nitrogen stream (40°C). All samples were analysed by GC-MS in selected ion monitoring (SIM) mode using negative chemical ionization (NCI).

Table M5. Mass spectrometric analysis, the quantifier ion (m/z) and retention time of the different PAH-OH (as pentafluorobenzoate derivatives) that were scanned for in SIM mode (methyl-naphthols in cursive are coeluating on the GC)

	RT	Quantifier ion (m/z)
1-Naphthol	18,23	338
2-Naphthol	18,80	338
7-Methyl-1-naphthol	*19,55*	352
8-Methyl-2-naphthol	*19,55*	352
2-Methyl-1-naphthol	19,74	352
3-Methyl-1-naphthol	19,82	352
6-Methyl-1-naphthol	20,14	352
3-Methyl-2-naphthol	20,30	352
7-Methyl-2-naphthol	20,73	352
6-Methyl-2-naphthol	20,85	352
4-Methyl-1-naphthol	20,97	352
5-Methyl-1-naphthol	*21,03*	352
1-Methyl-2-naphthol	*21,06*	352
4-Methyl-2-naphthol	21,23	352
5-Methyl-2-naphthol	21,38	352
2-Hydroxyfluorene	24,82	376
9-Hydroxyfluorene	28,32	167
4-Hydroxyphenanthrene	29,51	388
3-Hydroxyphenanthrene	31,79	388
1-Hydroxyphenanthrene	31,85	388
9-Hydroxyphenanthrene	32,27	388
2-Hydroxyphenanthrene	32,66	388
1-Hydroxypyrene	38,61	348
2-Hydroxychrysene	45,58	438
SIS		
1-Naphthol-d7	18,13	345
1-Hydroxypyrene-d9	38,49	356

Analysis of PAH metabolites in bile (fix wavelength fluorescence method)

Analysis of bile samples were diluted 1:1600 in methanol: water (1:1). Slit widths were set at 2.5 nm for both excitation and emission wavelengths, and samples were analysed in a quartz cuvette. All bile samples were analysed by FF at the wavelength pairs 290/335, 341/383 and 380/430 nm, optimised for the detection of 2-3 ring, 4-ring and 5-ring PAH metabolites, respectively. The fluorescence signal was transformed into pyrene fluorescence equivalents through a standard curve made by pyrene (Sigma St Louis, USA). Pyrene was measured at the same fluorimeter, with the same cuvette, same solvent, and with the same slit settings as the bile samples. It was, however, measured at the optimal wavelength pair of pyrene, 332/374 nm (excitation/emission). The concentration of PAH metabolites in bile samples was expressed as µg pyrene fluorescence equivalents (PFE)/ mL bile.

Analysis of PAH in haddock liver (GC-MS/MS)

Extraction of haddock liver samples (0.4-0.5 g) was performed as described in Sørensen et al (2016). After addition of surrogate standards (naphthalene-*d*8, biphenyl-*d*8, acenaphtylene-*d*8, anthracene-*d*10, pyrene-*d*10, perylene-*d*12 and indeno[1,2,3-*cd*]pyrene-*d*12; 20 ng/g sample), the samples were homogenized in *n*-hexane-DCM (1:1 v/v, 3 mL), followed by addition of sodium sulphate (150 mg), vortex extraction (30 s) and centrifugation (2000 rpm, 2 min). The supernatant was collected and the extraction repeated twice. The combined organic extract was concentrated to ~0.5 mL, filtrated and volume adjusted to 1 mL.

The samples were further subjected to purification to remove co-extracted lipids. For the removal of triacylglycerids and phospholipids, gel permeation chromatography was applied. An Agilent 1220 Infinity series LC was used coupled to a diode array detector (DAD) for retention time monitoring. GPC columns were supplied by Waters (Envirogel GPC cleanup, 300×19 mm). 500 µL samples were injected and eluted using dichloromethane at a flow rate of 5 mL/min. The PAH fraction was collected from 10-14 minutes. The volume of the eluent was adjusted to 1 mL prior to clean-up by normal phase SPE (Agilent Bond Elut SI, 500 mg) to remove remaining polar lipids (cholesterol, free fatty acids). The extract was eluted with dichloromethane in *n*-hexane (1:9, v/v, 6 mL). Immediately prior the analysis, the volume of the purified extract was reduced to 100 µL under a gentle stream of N_2.

An Agilent 7890 GC coupled with an Agilent 7010 triple quadrupole MS fitted with an EI source and collision cell was used for the analysis. Two DB-5MS UI GC-columns (15 m × 0.25 mm x 0.25 µm) were coupled in series through a purged ultimate union (PUU). The carrier gas was helium at constant flow (1.2 mL/min). Samples (1µL) were injected at 280 °C splitless. The oven temperature was held at 60 °C for 1 min, then ramped to 120 °C by 40 °C/min and finally ramped to 310 °C by 5 °C/min. The temperature was held at 310 °C for 5 minutes, while the first column was back-flushed. The transfer line temperature was 280 °C, the ion source temperature was 230 °C and the quadrupole temperatures were 150 °C. The EI source was operated at 70 eV. N_2 was used as collision gas at a flow of 1.5 mL/min and helium was used as a quench gas at a flow of 4 mL/min. An 11-level calibration curve was prepared (0.1-250 ng/mL) and fitted with quadratic regression for the quantification of analytes after normalization to the response of internal standards.

qPCR

Quantitative PCR assay was carried out starting from homogenate liver samples through RNase-Free DNase Set by Qiagen. Before RNA cleanup as described by RNeasy Mini Handbook (Qiagen, 2012), total RNA was extracted from all the samples using a specific method for liver tissue using trizol (1 ml for each sample), 200 µl chloroform, 1.2 M sodium citrate, isopropanol and ethanol 75% (35 ml absolute ethanol added to 50 ml DEPC water).

Primers selected were CYP1A, AHR (Aryl hydrocarbon receptor repressor), AHRR (Aryl hydrocarbon receptor repressor), GADD45A (growth arrest and DNA-damage-inducible, alpha), GADD45G, p53 (part of p53 pathway, selected to understand whether they may be activated due to DNA damage after PAHs exposure). Real time qPCR was performed referring to Fast SYBR Green Master Mix Protocol (Thermo Fisher Scientific). The plate was read using ABI 7900HT Fast Real Time PCR System at 95° for cDNA denaturation and at 60° for primers attachment for 40 cycles.

Table M6. PCR primers, contig names and amplicon sizes.

Gene ID	Gene name	Marker for	Contig name	Forward primer	Reverse primer	Amplicon size (bp)
CYP1A1	Cytochrome P450, family 1, subfamily A	Detoxification	>Soerhus-mRNA-2-dpf-1_CGATGT_L001_R1_001_(paired)_contig_3768	CTGCGCCACAAAAGACACAT	TTGAAGGTGGACGGTTCCTT	120
AHRR	Aryl-hydrocarbon receptor repressor	Detoxification	>Soerhus-mRNA-2-dpf-1_CGATGT_L001_R1_001_(paired)_contig_92712	AGCCAGACGCTGAACCTCAT	ATGCCGTGACCCTTGAACTC	122
p53	Tumor protein p53	DNA damage	>Soerhus-mRNA-2-dpf-1_CGATGT_L001_R1_001_(paired)_contig_2079	CCTGCTGAACTTCATGTGCAA	CCGAGAACATGCCCTTCAGA	102
GADD45A	growth arrest and DNA-damage-inducible, alpha	DNA damage	>Soerhus-mRNA-2-dpf-1_CGATGT_L001_R1_001_(paired)_contig_13955	ACGGTGTCAAAGGCAATCG	CTGGGTCCGCATTGAGAGAT	103
GADD45G	growth arrest and DNA-damage-inducible, gamma	DNA damage	>Soerhus-mRNA-2-dpf-1_CGATGT_L001_R1_001_(paired)_contig_5185	GTGCGCGTCAACGATATTGA	AAGGGTCTTTCCATGGGTTTG	121
EEF1A	Eukaryotic translation elongation factor 1 alpha 1	RefGen	>Soerhus-mRNA-2-dpf-1_CGATGT_L001_R1_001_(paired)_contig_221	CACATCGCCTGCAAGTTCAA	GGCTTGCTTGGGATCATGTT	128
UBA52	Ubiquitin A-52 residue ribosomal protein fusion product 1	RefGen	>Soerhus-mRNA-2-dpf-1_CGATGT_L001_R1_001_(paired)_contig_5918	TGAGGTCGAACCCAGTGACA	CTGCTTGCCAGCGAAGATC	103
ACTB	Beta actin	RefGen	>Soerhus-mRNA-2-dpf-1_CGATGT_L001_R1_001_(paired)_contig_877	ACAGCCGAGCGTGAGATTGT	TCGGGAAGCTCGTAGCTCTTC	125

RNAseq

RNAseq analyses have been performed on liver from 4 individual haddock per treatment sampled after 2 months (22nd April, 2014) of oral exposure to feed spiked with different PAH profiles (resembling oil, produced water and pyrogenic PAHs) followed by two months of recovery with normal feed, sampled 19th June 2014. A total of 32 individuals were analysed.

cDNA library preparation and sequencing was performed by the Norwegian Sequencing Centre (NSC, Oslo, Norway) using the Illumina TruSeq RNA Sample Preparation Kit. Using the multiplexing strategy of the TruSeq protocol paired end libraries were performed and ran on the Illumina HiSeq 2500.

High sequence similarity between cod and haddock justified use of the cod gene model as a template; the average sequence similarity between mapped haddock reads and the cod reference was 98.4%. Further, we chose to use a verified gene model over a reference-free de novo transcriptome approach to avoid noise from fragmentation and redundancy from un-collapsed genes resulting in a high number of false positives. Thus, the RNA sequencing data was mapped to the coding sequences of the cod gene models (Star et al., 2011) using the Burrows-Wheeler aligner (Li and Durbin, 2009). The cod gene models were annotated with various resources, including Swiss-Prot, Uniref90, GeneOntology and KEGG (Kyoto Encyclopaedia of Genes and Genomes). Samtools idxstat (Li et al., 2009) was used to extract number of mapped reads. The reads were normalised by the total number of mapped sequences. NOISeqBIO (Tarazona et al., 2011) was used to obtain DEGs between the developmental stages (threshold of 0.95). Only genes with 10 reads or more in at least one of the samples were included for further analysis. KEGG pathways analysis (Kanehisa et al., 2012) was performed by mapping the KEGG annotated DEGs from NOISeqBIO to KEGG pathways as described in the KEGG Mapper tool.

ELISA analyses of CYP1A content in liver

Buffer for homogenising

0,1 M sodiumphosphate ($NaH_2PO4 \cdot H_2O$), 0,15 M potassiumchloride, 1 mM EDTA, 1 mM DTT, 10% v/v glycerol, pH 7,4.

Homogenising of liver and preparation of postmitochondrial supernatant (PMS)

Approx. 0,5 g liver vas added homogenising buffer (2 ml pr 0.5 g liver) and homogenised with use of Potter Elvehjem homogeniser (7 strokes). The homogenate was transferred to Eppendorf vials and centrifuged for 20 min at 12.000xg, 4˚C. Samples were stored at -80˚C.

Measurements of protein content

Performed according to Bradford (1976). PMS-fraction of fish liver was diluted 1:1000 in dH2O. 50 µl of sample (in triplicate) was added ELISA-plate (Nunc 96 wells, flat bottom). 300 µl Coomassie G-250 / 17% phosphoric acid (1:1) was added the samples and incubated for 5 min. Absorbance was measured at 595 nm by plate reader (Tecan SPECTRA Fluor). Protein concentration determined by standard curve with bovine serum albumin.

ELISA

Performed as described in Nilsen et al. (1998). 1 μg total protein added per well, 4 parallels per sample, divided on two plates. For measurements of CYP1A1 in cod liver we used monoclonal mouse anti-cod CYP1A (NP-7, Biosense, Norway), diluted 1:1000. For CYP1A measurements in haddock, we used polyclonal rabbit anti-trout CYP1A (CP-226, Biosense, Norway), diluted 1:1000. For secondary antibodies, we used polyclonal goat anti-mouse/rabbit from DacoCytomation, Denmark, diluted 1:2000. Plates were incubated with TMB substrate for 22.5 minutes before addition of 0.5 M H_2SO_4 and absorbance read at 450 nm.

Preparation of post-mitochondrial supernatant (PMS) fraction for Glutathione S-transferase (GST) activity measurement and estimation of lipid peroxidation (LPO)

Post mitochondrial supernatant (PMS) was obtained according to the method of Ahmad et al. (2000). The liver was homogenized, using a Potter homogenizer, in phosphate buffer (0.1 M, pH 7.4). This homogenate was divided in two aliquots for LPO and PMS preparation. PMS preparation was accomplished by centrifugation in a refrigerated centrifuge at 13,400 g for 20 min (4°C).

GST activity measurement

GST activity was determined in PMS fraction, following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) by the method of Habig et al. (1974) with some modifications. The reaction mixture consisted of 1.85 ml sodium phosphate buffer (0.1 M, pH 7.4), 0.050 ml reduced glutathione (1 mM), 0.050 ml CDNB (1 mM) and 0.050 ml PMS. Absorbance was recorded at 340 nm (25 °C) and expressed as nmol CDNB conjugate formed/min/mg protein ($\varepsilon = 9.6 \times 10^3$ mM^{-1} cm^{-1}).

Estimation of LPO

LPO levels were determined in the liver homogenate by the procedure of Ohkawa et al. (1979) and Bird and Draper (1984) with some modifications. Briefly, to 150 µl homogenate, 5 µl of 4% butylated hydroxytoluene (BHT) in methanol, was added and mixed well. To this aliquot, 1 ml of 12% trichloroacetic acid (TCA) in aqueous solution, 0.90 ml Tris–HCl (60 mM, pH 7.4 and 0.1 mM DTPA) and 1 ml 0.73% 2-thiobarbituric acid (TBA) were added and mixed well. The mixture was heated for 1 h in a water bath set at boiling temperature and then cooled to room temperature and centrifuged at 13,400 g for 5 min. Absorbance was measured at 535 nm and LPO expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed/mg protein ($\varepsilon = 1.56 \times 10^5$ M^{-1} cm^{-1}).

Fatty acids analysis

Collection of small subsamples (20-50 mg) of haddock liver was done while the liver was still frozen to avoid “lipid bleading”. Similar small samples (50-80 mg) were taken of the haddock muscle.

All samples were methylated and the respective fatty acid methyl esters (FAME) were analyzed on a HP-7890A gas chromatograph (Agilent, USA) with a flame ionization detector (GC-FID) according to a method described in (Meier *et al.* 2006) with the fatty acid 19:0 as an internal standard. As a methylation reagent 2.5 M dry HCl in methanolwas used. The FAMEs were extracted using 2x2 ml of hexane. The extracted hexane was diluted or concentrated to obtain a suitable chromatographic response. One microliter was injected splitless (the split was open after 2 min), the injection temperature was set to 270°C. The column was a 25 m × 0.25 mm fused silica capillary, coated with polyethylene-glycol of 0.25 µm film thickness, CP-Wax 52 CB (Varian-Chrompack, Middelburg, The Netherlands). Helium (99.9999 %) was used as mobile phase at 1 ml/min in 45 min, followed by a 3

ml/min in 25 min. The temperature of the flame ionization detector was set at 300 °C. The oven temperature was programmed to hold at 90 °C for 2 minutes, then from 90 °C to 150 °C at 30 °C/min and then to 240 °C at 2.5 °C/min and held there for 30 minutes total analysis time was 70 min. Seventy well-defined peaks in the chromatogram were selected, and identified by comparing retention times with a FAME standard (GLC-463 from Nu-Chek Prep. Elysian, MN, USA) and retention index maps and mass spectral libraries (GC-MS) (http://www.chrombox.org/index.html) performed under the same chromatographic conditions as the GC-FID (Mjos 2004). Chromatographic peak areas were corrected by empirical response factors calculated from the areas of the GLC-463 mixture. The chromatograms were integrated using the EZChrom Elite software (Agilent Technologies). Only those 42 FA that contribute with more that 0.1 % of the total fatty acids amount was used included in the data calculation. The total amount of fatty acids and cholesterol was calculated using the internal standard 19:0, and the results is giving as mg FA/chol/100 mg wet weight sample. The fatty acids profile is precent FA relative to total FA.

Statistics

Statistical analyses of were performed using XLSTAT software (Addinsoft, US). One-way ANOVA and Dunnett's post-hoc test were used to analyse statistical differences in most variables (except DNA adducts). The statistical analysis of the DNA adduct results presented in the report is based on the use of SAS® software by Mr. Didier Pottier, engineer biostatistician at the University of Caen (EA 4651 ABTE-TOXEMAC, France). Predictably, the DNA adduct levels measured in the overall samples, considered by condition, do not respect the classical Normal distribution, even after the logarithmic with base 10 transformation (Shapiro-Wilk test, results not shown). Therefore, the following statistical analyses are above all based on some nonparametric tests, Kruskal-Wallis Test. Parametric tests are used in order to complete (or reinforce) the statistical results.

The Principle Component Analysis (PCA) of the FA profiles was carried out using Sirius (Version 8.1, Bergen, Norway). Before PCA the relative values (i.e. percent of the sum) were scaled by dividing each value by the mean of the values of all samples for that particular FA, with the intention to level out the quantitative difference among the FAs, leaving them all to vary around one. Score and loading plots from PCA analysis were generated in Sirius.

Appendix

Table A1. PAH levels in PW, base oil (HDF200) and sediments form the North Sea

	Concentration Number of rings	(µg/L) Statfjord A (PW)	(µg/kg) HDF 200 oil	(µg/kg) Kaks (G-10)	(µg/kg) Kaks (G16)	(µg/kg) Sediment Skagerrak
Naphthalene	2	913	9627	3500	88	83
∑C1-Naphthalene	2	1689	51640	10100	410	187
∑C2-Naphthalene	2	470	214890	1230	621	252
∑C3-Naphthalene	2	104	360121	10200	950	210
Phenanthrene	3	58	1312	1580	146	184
∑C1-Phenantrene	3	82	1471	1740	349	248
∑C2-Phenantrene	3	15	195	3090	494	184
∑C3-Phenantrene	3	9		822	364	60
Dibenzothiofen	3	24	2251	299	31	17
C1-Dibenzothiophene	3	102	23970	838	199	20
C2-Dibenzothiophene	3	2.0	11532	1130	364	320
C3-Dibenzothiophene	3	1.5		418	341	42
Acenaphthylene	3	5.2	4453	49	20	9
Acenaphthene	3	4.2	58198	253	20	5.6
Anthracene	3	0.9	428	137	23	31
Fluoranthene	4	1.1	641	1040	77	238
Pyrene	4	1.4	16446	979	88	172
Benz(a)anthracene	4	0.6	297	905	23	161
Chrysene	4	2.9	242	652	31	193
Benzo(b)fluoranthene	5	0.4		0	0	
Benzo(k)fluoranthene	5	0.4		745	20	1087
Benzo(e)pyrene	5	0.5		0	0	283
Benzo(a)pyrene	5	0.0		376	20	250
Perylene	5	0.0		0	0	90
Dibenz(a,h)anthracene	5	0.0		20	20	817
Indeno(1,2,3-cd)pyrene	6	0.0		20	20	982
Benzo(ghi)perylene	6	0.0		20	20	187
Sum		3487	757714	40143	4739	6313

Table A2. PAH levels in PW extract (Gullfaks) and destilation fraction of Gullfaks oil used to make the experimental feed

	mg/kg PW ekstract	mg/kg Oil Fr1 (240-320 °C)	mg/kg Oil Fr2 (320-375 °C)	mg/kg Oil Fr3 (375-400 °C)	mg/kg Oil Fr4 (420-525 °C)
Naphthalene	13	115	7	0.8	0.1
∑C1-Naphthalene	409	6000	10	1.2	0.9
∑C2-Naphthalene	1974	25995	64	15	9.1
∑C3-Naphthalene	1653	24311	42	5.2	1.0
∑C4-Naphthalene	86	17	10	0.3	0.1
Phenanthrene	139	1168	520	0.7	0.1
∑C1-Phenantrene	396	469	4918	10.7	1.1
∑C2-Phenantrene	521	7	4538	37.5	1.0
∑C3-Phenantrene	555	0	6630	1115	121.9
∑C4-Phenantrene	4	0	9	65	9.9
Dibenzothiofen	6	103	39	0.0	0.0
C1-Dibenzothiophene	145	1285	3622	1.2	0.2
C2-Dibenzothiophene	287	88	4375	1.3	0.2
C3-Dibenzothiophene	353	2	4182	0.0	2.5
Acenaphthylene	36	22	0.3	0.0	0.0
Acenaphthene	13	6	0.2	0.0	0.0
Anthracene	7	48	48.8	2.5	0.5
Fluoranthene	4	0.0	75.1	1	0.0
Pyrene	5.1	0.2	92.3	6	0
Benz(a)anthracene	7.0	0.0	1.5	34	8
Chrysene	4.0	0.0	1.1	66	12
1-methylchrysene	3.7	0.1	0.2	54	24.4
6-ethylchrysene	1.4	0.1	0.1	15.3	0.0
6-propylcrysene	1.5	0.1	0.0	1	0
Benzo(b)fluoranthene	1.9	0.6	0.1	12	13
Benzo(k)fluoranthene	0.0	0.0	0.0	11	5.8
Benz(e)pyrene	8.9	0.0	0.0	86	0
Benzo(a)pyrene	1	0.2	0.1	10	11
Perylene	41.5	0.1	0.2	148.4	163
Dibenz(ah)anthracene	0.0	0.0	0.1	1.2	6
Indeno(1,2,3-cd)pyrene	0.0	0.3	0.3	2.0	11
Benzo(ghi)perylene	3	0	0	4	11
∑2 rings	4135	56439	133	23	11
∑3 rings	2462	3198.0	28883	1234	137
∑4 rings	27	0.4	170.3	178	44
∑5 rings	53.2	1.0	0.5	269	199
∑6 rings	2.63	0.33	0.39	6.52	22.22
Sum	6680	59639	29186	1710	414
%	0.67	5.96	2.92	0.17	0.04

Table A3. PAH profile (%) in the PW, oil and PAH mixture used in the feed

	PW mixture	Oil mixture	PAH mixture
Naphthalene	0.19	0.02	0
∑C1-Naphthalene	7.98	0.04	0
∑C2-Naphthalene	36.18	0.25	0
∑C3-Naphthalene	32.31	0.15	0
∑C4-Naphthalene	0.70	0.03	0
Phenanthrene	2.02	1.60	0
∑C1-Phenantrene	3.50	15.14	0
∑C2-Phenantrene	4.13	14.07	0
∑C3-Phenantrene	4.39	24.47	0
∑C4-Phenantrene	0.03	0.27	0
Dibenzothiofen	0.13	0.12	0
C1-Dibenzothiophene	2.16	11.12	0
C2-Dibenzothiophene	2.34	13.44	0
C3-Dibenzothiophene	2.79	12.84	0
Acenaphthylene	0.30	0.00	0
Acenaphthene	0.10	0.00	0
Anthracene	0.09	0.16	0
Fluoranthene	0.00	0.00	5
Pyrene	0.03	4.33	4
Benz(a)anthracene	0.04	0.31	3
Chrysene	0.06	0.13	4
1-methylchrysene	0.03	0.25	0
6-ethylchrysene	0.03	0.20	0
6-propylcrysene	0.01	0.06	0
Benzo(b)fluoranthene	0.01	0.01	13
Benzo(k)fluoranthene	0.02	0.04	12
Benz(e)pyrene	0.00	0.04	5
Benzo(a)pyrene	0.07	0.32	6
Perylene	0.01	0.04	2
Dibenz(ah)anthracene	0.33	0.55	19
Indeno(1,2,3-cd)pyrene	0.00	0.01	22
Benzo(ghi)perylene	0.00	0.01	4
∑2 rings	77.36	0.49	0
∑3 rings	22.01	93.23	0
∑4 rings	0.20	5.27	17
∑5 rings	0.43	1.00	57
∑6 rings	0.00	0.01	26

Table A4. Fatty acid profile in haddock liver from the different treatments groups after two one month exposure. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs. The total amounts of FAs relatively too wet weight is giving in bottom of table. Data presented as average + stdev. Bold numbers indicate significant differences compared with control, p< 0.05.

	Control (22/4) (n=10)	PW (22/4) (n=10)	Oil (22/4) (n=10)	PAH (22/4) (n=10)
14:0	3.80 ± 0.23	3.76 ± 0.25	3.68 ± 0.14	3.76 ± 0.25
Iso 15:0	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
15:0	0.32 ± 0.01	0.32 ± 0.02	0.31 ± 0.02	0.33 ± 0.02
16:0	17.58 ± 1.82	18.68 ± 1.26	18.66 ± 0.42	18.05 ± 0.76
Iso 17:0	0.259 ± 0.013	0.252 ± 0.011	0.248 ± 0.004	0.234 ± 0.024
Antiso 17:0	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
17:0	0.20 ± 0.03	0.19 ± 0.02	0.20 ± 0.01	0.22 ± 0.02
18:0	3.35 ± 0.53	3.50 ± 0.57	4.07 ± 0.62	3.88 ± 0.72
20:0	0.128 ± 0.008	0.126 ± 0.006	0.124 ± 0.006	**0.119 ± 0.003**
∑SFA	26.12 ± 1.82	27.29 ± 1.28	27.78 ± 0.35	27.15 ± 0.78
16:1 (n-11)	0.20 ± 0.04	0.17 ± 0.03	0.17 ± 0.02	0.21 ± 0.03
16:1 (n-9)	0.301 ± 0.021	0.294 ± 0.013	0.307 ± 0.004	***0.337 ± 0.022***
16:1 (n-7)	5.74 ± 0.48	5.87 ± 0.41	5.63 ± 0.13	5.74 ± 0.38
16:1 (n-5)	0.16 ± 0.01	0.16 ± 0.02	0.16 ± 0.01	0.16 ± 0.02
∑17:1	0.49 ± 0.03	0.49 ± 0.03	0.48 ± 0.01	0.51 ± 0.03
18:1 (n-11)	0.50 ± 0.12	0.40 ± 0.08	0.35 ± 0.04	0.44 ± 0.15
18:1 (n-9)	19.45 ± 1.13	19.88 ± 1.17	20.16 ± 1.31	20.46 ± 1.50
18:1 (n-7)	3.53 ± 0.21	3.48 ± 0.13	3.44 ± 0.15	***3.73 ± 0.22***
18:1 (n-5)	0.36 ± 0.02	0.35 ± 0.02	0.35 ± 0.01	0.36 ± 0.02
20:1 (n-11)	1.09 ± 0.12	1.08 ± 0.10	1.05 ± 0.04	1.08 ± 0.10
20:1 (n-9)	6.17 ± 0.40	5.82 ± 0.53	5.82 ± 0.44	5.87 ± 0.62
20:1 (n-7)	0.21 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
22:1 (n-11)	7.44 ± 0.78	7.04 ± 0.51	6.79 ± 0.61	**6.29 ± 0.93**
22:1 (n-9)	0.58 ± 0.04	0.56 ± 0.03	0.55 ± 0.05	**0.52 ± 0.06**
22:1 (n-7)	0.09 ± 0.01	**0.08 ± 0.01**	**0.07 ± 0.01**	**0.07 ± 0.01**
24:1 (n-9)	0.66 ± 0.04	0.63 ± 0.03	**0.60 ± 0.03**	**0.56 ± 0.03**
24:1 (n-7)	0.110 ± 0.005	0.101 ± 0.007	**0.101 ± 0.006**	**0.068 ± 0.021**
∑MUFA	47.13 ± 0.95	46.67 ± 0.75	46.28 ± 0.99	46.67 ± 1.04
16:4 (n-1)	0.350 ± 0.042	0.360 ± 0.033	0.362 ± 0.018	0.348 ± 0.026
18:4 (n-1)	0.144 ± 0.013	0.139 ± 0.013	0.137 ± 0.006	0.140 ± 0.009
16:2 (n-4)	0.33 ± 0.03	0.32 ± 0.03	0.33 ± 0.01	0.34 ± 0.02
16:3 (n-4)	0.284 ± 0.042	0.284 ± 0.028	0.289 ± 0.005	0.298 ± 0.031
18:2 (n-4)	0.21 ± 0.03	0.21 ± 0.02	0.20 ± 0.01	0.23 ± 0.02
18:2 (n-6)	3.88 ± 0.28	3.79 ± 0.28	3.85 ± 0.12	3.92 ± 0.33
18:3 (n-6)	0.11 ± 0.02	0.11 ± 0.03	0.12 ± 0.03	0.10 ± 0.02
20:2 (n-6)	0.30 ± 0.02	0.28 ± 0.02	0.28 ± 0.01	0.29 ± 0.02
20:3 (n-6)	0.065 ± 0.008	0.062 ± 0.004	0.063 ± 0.003	0.068 ± 0.005
20:4 (n-6)	0.41 ± 0.02	0.40 ± 0.02	0.39 ± 0.01	0.40 ± 0.04
22:4 (n-6)	0.14 ± 0.02	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.02
22:5 (n-6)	0.14 ± 0.02	0.13 ± 0.01	**0.13 ± 0.02**	0.13 ± 0.02
18:3 (n-3)	1.03 ± 0.08	1.00 ± 0.07	1.01 ± 0.01	1.08 ± 0.04
18:4 (n-3)	1.77 ± 0.12	1.74 ± 0.14	1.77 ± 0.08	1.76 ± 0.07
20:3 (n-3)	0.118 ± 0.007	0.112 ± 0.005	0.114 ± 0.002	**0.107 ± 0.009**
20:4 (n-3)	0.512 ± 0.042	0.487 ± 0.031	0.495 ± 0.003	0.480 ± 0.040
20:5 (n-3)	6.60 ± 0.50	6.47 ± 0.36	6.39 ± 0.18	6.60 ± 0.35
21:5 (n-3)	0.31 ± 0.03	0.29 ± 0.02	0.28 ± 0.02	0.29 ± 0.02
22:5 (n-3)	1.12 ± 0.10	1.06 ± 0.07	1.06 ± 0.03	1.07 ± 0.10
22:6 (n-3)	8.39 ± 0.42	8.17 ± 0.31	8.05 ± 0.44	**7.91 ± 0.31**
24:5 (n-3)	0.26 ± 0.02	0.24 ± 0.02	**0.24 ± 0.02**	**0.22 ± 0.02**
∑PUFA	26.75 ± 1.76	26.04 ± 1.32	25.94 ± 0.72	26.19 ± 1.02
∑(n-6) PUFA	5.09 ± 0.37	4.94 ± 0.32	4.99 ± 0.10	5.10 ± 0.34
∑(n-3) PUFA	20.19 ± 1.27	19.63 ± 0.92	**19.46 ± 0.68**	19.58 ± 0.73
(n-3)/(n-6)	3.97 ± 0.07	3.98 ± 0.11	**3.90 ± 0.13**	3.85 ± 0.26
Amount of Cholesterol (%)	0.15 ± 0.02	0.15 ± 0.01	0.16 ± 0.02	***0.19 ± 0.04***
Amount of FA (%)	52.79 ± 3.04	56.07 ± 2.79	53.93 ± 2.30	48.70 ± 7.05

Table A5. Fatty acid profile in haddock liver from the different treatments groups after one week recovery. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs. Data presented as average ± stdev. Bold numbers indicate significant differences compared with control, $p < 0.05$.

	Control (29/4) (n=10)	PW (29/4) (n=10)	Oil (29/4) (n=10)	PAH (29/4) (n=10)
14:0	4.03 ± 0.23	3.91 ± 0.14	**3.74 ± 0.22**	**3.48 ± 0.20**
Iso 15:0	0.15 ± 0.01	0.14 ± 0.01	**0.14 ± 0.01**	**0.14 ± 0.01**
15:0	0.34 ± 0.02	0.32 ± 0.02	**0.31 ± 0.02**	**0.31 ± 0.01**
16:0	18.39 ± 1.43	17.75 ± 1.53	17.78 ± 1.06	**16.25 ± 1.41**
Iso 17:0	0.23 ± 0.01	0.21 ± 0.01	**0.19 ± 0.02**	**0.17 ± 0.01**
Antiso 17:0	0.111 ± 0.004	0.108 ± 0.006	0.109 ± 0.003	0.111 ± 0.004
17:0	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.02	***0.25 ± 0.03***
18:0	3.61 ± 0.82	3.25 ± 0.59	3.49 ± 0.41	4.26 ± 0.76
20:0	0.122 ± 0.008	**0.106 ± 0.004**	**0.108 ± 0.006**	0.121 ± 0.016
∑SFA	27.53 ± 1.50	26.43 ± 1.44	26.47 ± 1.04	**25.46 ± 1.57**
16:1 (n-11)	0.20 ± 0.03	0.18 ± 0.04	0.18 ± 0.02	0.21 ± 0.04
16:1 (n-9)	0.32 ± 0.02	0.29 ± 0.03	0.30 ± 0.02	0.32 ± 0.03
16:1 (n-7)	5.86 ± 0.47	5.82 ± 0.44	5.73 ± 0.29	**5.35 ± 0.36**
16:1 (n-5)	0.17 ± 0.02	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
∑17:1	0.51 ± 0.04	**0.46 ± 0.03**	0.49 ± 0.03	0.51 ± 0.03
18:1 (n-11)	0.54 ± 0.07	**0.28 ± 0.04**	**0.33 ± 0.09**	**0.40 ± 0.08**
18:1 (n-9)	19.77 ± 0.72	19.18 ± 0.81	19.84 ± 1.28	20.08 ± 1.21
18:1 (n-7)	3.57 ± 0.14	3.52 ± 0.14	3.57 ± 0.21	***3.75 ± 0.16***
18:1 (n-5)	0.36 ± 0.01	0.35 ± 0.01	0.36 ± 0.01	0.34 ± 0.02
20:1 (n-11)	1.11 ± 0.07	**0.90 ± 0.08**	**0.86 ± 0.11**	**0.89 ± 0.10**
20:1 (n-9)	6.22 ± 0.38	6.42 ± 0.41	6.27 ± 0.19	5.79 ± 0.63
20:1 (n-7)	0.20 ± 0.01	0.22 ± 0.01	0.21 ± 0.01	0.22 ± 0.01
22:1 (n-11)	6.95 ± 0.64	7.47 ± 0.82	7.04 ± 0.47	6.11 ± 0.90
22:1 (n-9)	0.56 ± 0.03	0.58 ± 0.04	0.56 ± 0.03	0.53 ± 0.05
22:1 (n-7)	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	**0.04 ± 0.02**
24:1 (n-9)	0.59 ± 0.04	***0.70 ± 0.04***	***0.67 ± 0.06***	***0.66 ± 0.06***
24:1 (n-7)	0.053 ± 0.012	0.074 ± 0.004	0.065 ± 0.007	***0.092 ± 0.039***
∑MUFA	47.11 ± 0.67	46.77 ± 1.01	46.78 ± 1.31	**45.49 ± 1.79**
16:4 (n-1)	0.33 ± 0.04	0.34 ± 0.04	0.32 ± 0.04	0.36 ± 0.05
18:4 (n-1)	0.13 ± 0.01	0.15 ± 0.02	0.14 ± 0.01	***0.16 ± 0.01***
16:2 (n-4)	0.33 ± 0.03	0.32 ± 0.05	0.32 ± 0.03	0.35 ± 0.03
16:3 (n-4)	0.28 ± 0.04	0.28 ± 0.05	0.27 ± 0.04	***0.32 ± 0.05***
18:2 (n-4)	0.21 ± 0.02	0.21 ± 0.03	0.21 ± 0.02	***0.25 ± 0.03***
18:2 (n-6)	3.88 ± 0.28	3.91 ± 0.37	3.93 ± 0.25	***4.17 ± 0.23***
18:3 (n-6)	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	***0.11 ± 0.01***
20:2 (n-6)	0.29 ± 0.02	0.29 ± 0.02	0.30 ± 0.01	***0.31 ± 0.02***
20:3 (n-6)	0.065 ± 0.008	0.070 ± 0.010	0.071 ± 0.005	***0.081 ± 0.008***
20:4 (n-6)	0.37 ± 0.03	0.41 ± 0.04	0.41 ± 0.02	***0.45 ± 0.06***
22:4 (n-6)	0.12 ± 0.03	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.02
22:5 (n-6)	0.12 ± 0.02	0.14 ± 0.02	***0.15 ± 0.01***	***0.17 ± 0.02***
18:3 (n-3)	1.07 ± 0.09	1.10 ± 0.13	1.07 ± 0.07	1.15 ± 0.09
18:4 (n-3)	1.75 ± 0.12	1.84 ± 0.14	1.79 ± 0.12	1.83 ± 0.13
20:3 (n-3)	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
20:4 (n-3)	0.49 ± 0.04	0.54 ± 0.06	0.52 ± 0.03	***0.56 ± 0.05***
20:5 (n-3)	6.32 ± 0.42	6.75 ± 0.75	6.72 ± 0.35	***7.52 ± 0.65***
21:5 (n-3)	0.28 ± 0.03	0.31 ± 0.04	0.30 ± 0.02	***0.34 ± 0.04***
22:5 (n-3)	1.00 ± 0.09	1.09 ± 0.13	1.12 ± 0.08	***1.30 ± 0.18***
22:6 (n-3)	7.67 ± 0.31	8.29 ± 0.29	***8.36 ± 0.33***	***8.94 ± 0.69***
24:5 (n-3)	0.22 ± 0.02	0.21 ± 0.02	0.22 ± 0.01	**0.20 ± 0.02**
∑PUFA	25.37 ± 1.49	26.80 ± 2.13	26.75 ± 1.37	29.05 ± 2.14
∑(n-6) PUFA	4.98 ± 0.36	5.09 ± 0.48	5.12 ± 0.31	5.47 ± 0.32
∑(n-3) PUFA	18.95 ± 1.01	20.28 ± 1.48	20.24 ± 0.95	21.99 ± 1.68
(n-3)/(n-6)	3.81 ± 0.10	3.99 ± 0.13	3.96 ± 0.09	4.02 ± 0.12
Amount of Cholesterol (%)	0.17 ± 0.02	0.16 ± 0.01	0.17 ± 0.01	0.24 ± 0.20
Amount of FA (%)	51.85 ± 4.72	56.38 ± 3.64	58.53 ± 2.66	54.96 ± 7.73

Table A6. Fatty acid profile in haddock liver from the different treatments groups after two months of recovery. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs. Data presented as average ± stdev. Bold numbers indicate significant differences compared with control, p< 0.05.

	Control (19/6) (n=10)	PW (19/6) (n=10)	Oil (19/6) (n=9)	PAH (19/6) (n=10)
14:0	3.90 ± 0.22	3.91 ± 0.37	3.59 ± 0.23	**3.46 ± 0.15**
Iso 15:0	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
15:0	0.31 ± 0.01	0.32 ± 0.02	0.30 ± 0.01	0.30 ± 0.01
16:0	17.97 ± 1.20	17.29 ± 1.33	17.83 ± 0.89	16.69 ± 1.27
Iso 17:0	0.19 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.23 ± 0.01
Antiso 17:0	0.108 ± 0.004	0.109 ± 0.004	0.107 ± 0.005	0.110 ± 0.005
17:0	0.19 ± 0.02	0.20 ± 0.03	0.20 ± 0.02	***0.23 ± 0.03***
18:0	3.28 ± 0.51	3.25 ± 0.51	3.71 ± 0.43	***4.00 ± 0.42***
20:0	0.096 ± 0.007	0.101 ± 0.006	0.100 ± 0.005	0.104 ± 0.005
∑SFA	26.46 ± 1.13	25.87 ± 1.39	26.49 ± 0.89	25.58 ± 1.21
16:1 (n-11)	0.19 ± 0.01	0.20 ± 0.03	0.18 ± 0.03	0.21 ± 0.05
16:1 (n-9)	0.30 ± 0.02	0.31 ± 0.04	0.30 ± 0.02	0.32 ± 0.03
16:1 (n-7)	5.77 ± 0.42	5.63 ± 0.49	5.52 ± 0.26	5.41 ± 0.31
16:1 (n-5)	0.17 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0.16 ± 0.01
∑17:1	0.49 ± 0.02	0.49 ± 0.02	0.50 ± 0.02	0.51 ± 0.02
18:1 (n-11)	0.65 ± 0.13	0.79 ± 0.13	0.77 ± 0.17	***0.86 ± 0.20***
18:1 (n-9)	19.63 ± 1.52	18.78 ± 1.93	20.74 ± 0.91	20.70 ± 1.10
18:1 (n-7)	3.55 ± 0.23	3.51 ± 0.33	3.65 ± 0.15	3.76 ± 0.21
18:1 (n-5)	0.37 ± 0.02	0.37 ± 0.03	0.37 ± 0.02	0.36 ± 0.03
20:1 (n-11)	0.98 ± 0.09	1.02 ± 0.06	0.99 ± 0.06	0.96 ± 0.14
20:1 (n-9)	6.62 ± 0.47	6.87 ± 0.71	6.55 ± 0.34	6.60 ± 0.52
20:1 (n-7)	0.21 ± 0.01	0.21 ± 0.01	0.20 ± 0.02	0.21 ± 0.01
22:1 (n-11)	7.74 ± 0.94	7.81 ± 1.53	7.18 ± 0.45	6.74 ± 0.79
22:1 (n-9)	0.61 ± 0.06	0.62 ± 0.08	0.58 ± 0.03	0.55 ± 0.05
22:1 (n-7)	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
24:1 (n-9)	0.70 ± 0.06	0.70 ± 0.07	0.64 ± 0.05	0.64 ± 0.04
24:1 (n-7)	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
∑MUFA	48.18 ± 0.74	47.71 ± 0.75	48.54 ± 0.73	48.20 ± 0.82
16:4 (n-1)	0.29 ± 0.03	0.29 ± 0.04	0.28 ± 0.03	0.30 ± 0.03
18:4 (n-1)	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
16:2 (n-4)	0.29 ± 0.03	0.30 ± 0.02	0.29 ± 0.02	0.31 ± 0.03
16:3 (n-4)	0.23 ± 0.03	0.24 ± 0.04	0.23 ± 0.02	0.27 ± 0.04
18:2 (n-4)	0.19 ± 0.02	0.19 ± 0.03	0.19 ± 0.02	0.21 ± 0.02
18:2 (n-6)	3.76 ± 0.20	3.93 ± 0.19	3.77 ± 0.14	4.01 ± 0.21
18:3 (n-6)	0.087 ± 0.007	0.089 ± 0.006	0.085 ± 0.003	0.090 ± 0.006
20:2 (n-6)	0.28 ± 0.02	0.30 ± 0.02	0.29 ± 0.01	**0.30 ± 0.02**
20:3 (n-6)	0.065 ± 0.007	0.066 ± 0.008	0.063 ± 0.004	0.069 ± 0.006
20:4 (n-6)	0.37 ± 0.01	0.38 ± 0.04	0.36 ± 0.02	0.39 ± 0.03
22:4 (n-6)	0.13 ± 0.01	0.12 ± 0.01	**0.11 ± 0.01**	**0.11 ± 0.01**
22:5 (n-6)	0.12 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.13 ± 0.01
18:3 (n-3)	1.05 ± 0.07	1.09 ± 0.06	1.04 ± 0.04	1.11 ± 0.06
18:4 (n-3)	1.77 ± 0.13	1.83 ± 0.11	1.74 ± 0.07	1.80 ± 0.08
20:3 (n-3)	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
20:4 (n-3)	0.50 ± 0.03	0.53 ± 0.03	0.50 ± 0.02	0.52 ± 0.03
20:5 (n-3)	6.18 ± 0.33	6.45 ± 0.54	6.09 ± 0.18	6.49 ± 0.43
21:5 (n-3)	0.28 ± 0.02	0.30 ± 0.03	0.27 ± 0.02	0.29 ± 0.02
22:5 (n-3)	0.94 ± 0.07	0.99 ± 0.13	0.93 ± 0.06	0.99 ± 0.09
22:6 (n-3)	8.16 ± 0.26	***8.51 ± 0.41***	7.97 ± 0.28	8.17 ± 0.45
24:5 (n-3)	0.21 ± 0.02	0.22 ± 0.03	0.19 ± 0.02	0.20 ± 0.02
∑PUFA	25.36 ± 1.15	26.42 ± 1.54	24.97 ± 0.79	26.22 ± 1.37
∑(n-6) PUFA	4.85 ± 0.25	5.06 ± 0.26	4.85 ± 0.16	***5.15 ± 0.29***
∑(n-3) PUFA	19.24 ± 0.82	20.08 ± 1.19	18.87 ± 0.60	19.72 ± 1.03
(n-3)/(n-6)	3.97 ± 0.09	3.97 ± 0.10	3.89 ± 0.06	**3.83 ± 0.11**
Amount of Cholesterol (%)	0.16 ± 0.02	0.15 ± 0.03	0.14 ± 0.03	0.15 ± 0.02
Amount of FA (%)	60.45 ± 3.24	59.05 ± 8.98	58.36 ± 6.94	58.88 ± 4.15

Table A7. Fatty acid profile in haddock muscle from the different treatments groups after two months ofexposure. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs. Data presented as average ± stdev. Bold numbers indicate significant differences compared with control, p< 0.05.

Muscle	Control (22/4) (n=10)	PW (22/4) (n=10)	Oil (22/4) (n=10)	PAH (22/4) (n=9)
14:0	2.44 ± 0.43	2.56 ± 0.12	2.30 ± 0.25	**1.97 ± 0.31**
Iso 15:0	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	**0.07 ± 0.01**
15:0	0.34 ± 0.03	0.34 ± 0.01	0.33 ± 0.02	**0.31 ± 0.03**
16:0	17.31 ± 0.75	17.50 ± 0.78	17.44 ± 0.57	17.48 ± 0.48
Iso 17:0	0.16 ± 0.01	0.16 ± 0.00	0.16 ± 0.01	**0.14 ± 0.03**
Antiso 17:0	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.07 ± 0.01
17:0	0.20 ± 0.01	0.20 ± 0.01	0.21 ± 0.03	0.20 ± 0.01
18:0	2.99 ± 0.39	2.94 ± 0.20	3.34 ± 0.43	3.29 ± 0.38
20:0	0.046 ± 0.007	0.048 ± 0.006	0.045 ± 0.011	**0.037 ± 0.005**
∑SFA	24.05 ± 0.72	24.34 ± 0.90	24.43 ± 0.97	24.00 ± 0.57
16:1 (n-11)	0.18 ± 0.03	0.17 ± 0.02	0.17 ± 0.01	0.16 ± 0.03
16:1 (n-9)	0.32 ± 0.04	0.32 ± 0.02	0.34 ± 0.04	0.31 ± 0.04
16:1 (n-7)	2.35 ± 0.41	2.51 ± 0.14	2.27 ± 0.32	**1.94 ± 0.31**
16:1 (n-5)	0.25 ± 0.02	0.25 ± 0.01	0.25 ± 0.01	0.23 ± 0.02
∑17:1	0.40 ± 0.04	0.42 ± 0.01	0.42 ± 0.04	0.38 ± 0.05
18:1 (n-11)	1.13 ± 0.33	0.87 ± 0.26	1.05 ± 0.17	1.02 ± 0.22
18:1 (n-9)	9.13 ± 0.73	***10.02 ± 0.71***	9.61 ± 1.13	8.85 ± 0.63
18:1 (n-7)	2.33 ± 0.10	2.38 ± 0.12	2.37 ± 0.10	2.32 ± 0.08
18:1 (n-5)	0.31 ± 0.03	0.31 ± 0.02	0.30 ± 0.01	**0.28 ± 0.03**
20:1 (n-11)	0.45 ± 0.06	0.46 ± 0.09	0.42 ± 0.04	0.39 ± 0.06
20:1 (n-9)	2.79 ± 0.37	2.90 ± 0.28	2.62 ± 0.28	**2.30 ± 0.43**
20:1 (n-7)	0.07 ± 0.02	0.08 ± 0.01	0.07 ± 0.02	0.06 ± 0.01
22:1 (n-11)	1.93 ± 0.37	2.06 ± 0.40	1.70 ± 0.27	**1.35 ± 0.36**
22:1 (n-9)	0.20 ± 0.03	0.23 ± 0.04	0.21 ± 0.01	0.18 ± 0.03
22:1 (n-7)	0.05 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.04 ± 0.01
24:1 (n-9)	1.01 ± 0.08	1.11 ± 0.15	1.04 ± 0.04	0.99 ± 0.08
24:1 (n-7)	0.15 ± 0.06	0.14 ± 0.02	0.15 ± 0.03	0.18 ± 0.04
∑MUFA	23.09 ± 2.07	24.29 ± 1.36	23.07 ± 1.86	20.99 ± 2.17
16:4 (n-1)	0.041 ± 0.021	0.062 ± 0.029	0.052 ± 0.018	0.031 ± 0.007
18:4 (n-1)	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	**0.08 ± 0.01**
16:2 (n-4)	0.14 ± 0.02	0.15 ± 0.01	0.13 ± 0.02	**0.11 ± 0.02**
16:3 (n-4)	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	**0.04 ± 0.01**
18:2 (n-4)	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.02	0.12 ± 0.01
18:2 (n-6)	3.51 ± 0.32	3.62 ± 0.11	3.57 ± 0.18	3.53 ± 0.25
18:3 (n-6)	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
20:2 (n-6)	0.35 ± 0.04	0.37 ± 0.03	0.38 ± 0.09	0.34 ± 0.03
20:3 (n-6)	0.102 ± 0.006	0.101 ± 0.006	0.100 ± 0.006	***0.110 ± 0.006***
20:4 (n-6)	1.16 ± 0.10	1.14 ± 0.08	1.19 ± 0.08	***1.29 ± 0.13***
22:4 (n-6)	0.16 ± 0.02	***0.18 ± 0.02***	0.16 ± 0.01	0.17 ± 0.01
22:5 (n-6)	0.48 ± 0.03	0.47 ± 0.02	0.48 ± 0.03	***0.52 ± 0.04***
18:3 (n-3)	0.75 ± 0.08	0.79 ± 0.03	0.73 ± 0.06	**0.67 ± 0.08**
18:4 (n-3)	1.00 ± 0.10	1.06 ± 0.04	0.98 ± 0.10	**0.87 ± 0.10**
20:3 (n-3)	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.03	0.10 ± 0.01
20:4 (n-3)	0.65 ± 0.07	0.64 ± 0.03	0.66 ± 0.05	0.65 ± 0.05
20:5 (n-3)	14.23 ± 0.48	13.82 ± 0.68	14.04 ± 0.96	***15.11 ± 0.79***
21:5 (n-3)	0.29 ± 0.02	0.30 ± 0.01	0.29 ± 0.02	0.29 ± 0.02
22:5 (n-3)	1.68 ± 0.06	1.67 ± 0.07	1.73 ± 0.09	***1.75 ± 0.06***
22:6 (n-3)	27.38 ± 1.61	26.09 ± 1.10	27.11 ± 2.28	28.72 ± 1.73
24:5 (n-3)	0.27 ± 0.04	0.27 ± 0.06	0.25 ± 0.03	0.25 ± 0.03
∑PUFA	52.85 ± 1.83	51.38 ± 1.71	52.50 ± 2.68	**55.01 ± 1.96**
∑(n-6) PUFA	5.90 ± 0.35	6.00 ± 0.17	6.00 ± 0.19	6.09 ± 0.19
∑(n-3) PUFA	46.44 ± 1.75	44.83 ± 1.67	45.98 ± 2.71	**48.48 ± 2.03**
(n-3)/(n-6)	7.89 ± 0.48	7.47 ± 0.27	7.67 ± 0.49	7.97 ± 0.46
Amount of Cholesterol (%)	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.01	0.06 ± 0.00
Amount of FA (%)	0.74 ± 0.06	0.76 ± 0.06	0.76 ± 0.05	0.73 ± 0.02

Table A8. Fatty acid profile in haddock muscle from the different treatments groups after one week recovery. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs. Data presented as average ± stdev. Bold numbers indicate significant differences compared with control, $p < 0.05$.

	29/4	29/4	29/4	29/4
Muscle	Control (29/4) (n=10)	PW (29/4) (n=10)	Oil (29/4) (n=10)	PAH (29/4) (n=9)
14:0	2.22 ± 0.47	2.50 ± 0.48	2.17 ± 0.47	1.69 ± 0.53
Iso 15:0	0.08 ± 0.02	0.09 ± 0.02	0.08 ± 0.02	0.06 ± 0.02
15:0	0.33 ± 0.03	0.34 ± 0.03	0.32 ± 0.03	**0.28 ± 0.05**
16:0	17.78 ± 0.88	17.46 ± 0.78	17.42 ± 0.89	17.42 ± 0.69
Iso 17:0	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
Antiso 17:0	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
17:0	0.20 ± 0.01	0.20 ± 0.02	0.20 ± 0.01	0.20 ± 0.01
18:0	3.08 ± 0.52	2.98 ± 0.33	3.05 ± 0.36	3.51 ± 0.68
20:0	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
∑SFA	24.38 ± 0.81	24.30 ± 0.69	23.93 ± 0.84	23.91 ± 0.79
16:1 (n-11)	0.16 ± 0.03	0.18 ± 0.04	0.16 ± 0.03	0.15 ± 0.02
16:1 (n-9)	0.32 ± 0.04	0.34 ± 0.06	0.32 ± 0.06	0.28 ± 0.05
16:1 (n-7)	2.16 ± 0.36	2.36 ± 0.50	2.24 ± 0.48	**1.66 ± 0.43**
16:1 (n-5)	0.26 ± 0.03	0.27 ± 0.02	0.25 ± 0.03	**0.21 ± 0.03**
∑17:1	0.41 ± 0.04	0.43 ± 0.05	0.44 ± 0.04	0.41 ± 0.05
18:1 (n-11)	1.07 ± 0.26	1.19 ± 0.32	1.05 ± 0.28	0.97 ± 0.20
18:1 (n-9)	9.11 ± 0.84	9.15 ± 1.22	9.35 ± 1.01	8.26 ± 0.97
18:1 (n-7)	2.32 ± 0.07	2.34 ± 0.10	2.36 ± 0.13	2.29 ± 0.10
18:1 (n-5)	0.31 ± 0.03	0.31 ± 0.03	0.30 ± 0.03	**0.25 ± 0.03**
20:1 (n-11)	0.44 ± 0.06	0.51 ± 0.10	0.43 ± 0.07	0.37 ± 0.06
20:1 (n-9)	2.41 ± 0.36	2.79 ± 0.56	2.54 ± 0.51	2.09 ± 0.34
20:1 (n-7)	0.06 ± 0.01	0.07 ± 0.02	0.07 ± 0.02	0.06 ± 0.01
22:1 (n-11)	1.58 ± 0.30	1.80 ± 0.46	1.79 ± 0.77	1.39 ± 0.37
22:1 (n-9)	0.19 ± 0.02	0.21 ± 0.04	0.22 ± 0.07	0.20 ± 0.05
22:1 (n-7)	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
24:1 (n-9)	1.00 ± 0.09	1.01 ± 0.05	1.09 ± 0.22	1.18 ± 0.29
24:1 (n-7)	0.15 ± 0.05	0.17 ± 0.04	0.19 ± 0.04	0.15 ± 0.05
∑MUFA	22.01 ± 1.96	23.18 ± 2.94	22.86 ± 2.93	19.97 ± 2.38
16:4 (n-1)	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.03 ± 0.01
18:4 (n-1)	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.02	0.07 ± 0.02
16:2 (n-4)	0.12 ± 0.03	0.14 ± 0.03	0.13 ± 0.04	0.09 ± 0.03
16:3 (n-4)	0.04 ± 0.01	0.05 ± 0.02	0.05 ± 0.02	0.03 ± 0.01
18:2 (n-4)	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
18:2 (n-6)	3.57 ± 0.22	3.59 ± 0.21	3.60 ± 0.24	3.30 ± 0.31
18:3 (n-6)	0.06 ± 0.02	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.02
20:2 (n-6)	0.35 ± 0.06	0.37 ± 0.05	0.35 ± 0.04	0.31 ± 0.05
20:3 (n-6)	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
20:4 (n-6)	1.17 ± 0.12	1.13 ± 0.14	1.20 ± 0.10	***1.45 ± 0.19***
22:4 (n-6)	0.17 ± 0.02	0.18 ± 0.02	0.17 ± 0.02	0.16 ± 0.03
22:5 (n-6)	0.48 ± 0.03	0.47 ± 0.05	0.48 ± 0.04	***0.53 ± 0.05***
18:3 (n-3)	0.70 ± 0.07	0.71 ± 0.09	0.70 ± 0.11	**0.58 ± 0.12**
18:4 (n-3)	0.95 ± 0.12	1.00 ± 0.16	0.97 ± 0.17	0.79 ± 0.19
20:3 (n-3)	0.10 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.02
20:4 (n-3)	0.65 ± 0.07	0.66 ± 0.06	0.66 ± 0.05	0.61 ± 0.09
20:5 (n-3)	14.46 ± 0.46	13.90 ± 0.73	14.56 ± 0.77	***15.37 ± 0.68***
21:5 (n-3)	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.27 ± 0.03
22:5 (n-3)	1.69 ± 0.06	1.67 ± 0.10	1.70 ± 0.06	1.71 ± 0.07
22:6 (n-3)	28.04 ± 1.49	27.40 ± 2.42	27.39 ± 2.49	29.99 ± 2.65
24:5 (n-3)	0.24 ± 0.03	0.26 ± 0.04	0.25 ± 0.04	0.25 ± 0.03
∑PUFA	53.62 ± 1.39	52.52 ± 2.63	53.21 ± 2.50	56.12 ± 2.28
∑(n-6) PUFA	5.96 ± 0.22	5.96 ± 0.13	6.02 ± 0.19	6.00 ± 0.19
∑(n-3) PUFA	47.18 ± 1.53	46.07 ± 2.73	46.69 ± 2.67	49.73 ± 2.40
(n-3)/(n-6)	7.93 ± 0.47	7.74 ± 0.55	7.77 ± 0.64	8.31 ± 0.56
Amount of Cholesterol (%)	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
Amount of FA (%)	0.74 ± 0.05	0.78 ± 0.14	0.83 ± 0.10	0.84 ± 0.07

Table A9.

Fatty acid profile in haddock muscle from the different treatments groups after one week recovery. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs. Data presented as average ± stdev. Bold numbers indicate significant differences compared with control, p< 0.05.

	19/6	19/6	19/6	19/6
Muscle	Control (n=10)	PW (n=9)	Oil (n=11)	PAH (n=9)
14:0	2.35 ± 0.21	2.44 ± 0.35	2.02 ± 0.41	2.19 ± 0.54
Iso 15:0	0.09 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	0.08 ± 0.02
15:0	0.33 ± 0.01	0.34 ± 0.03	0.30 ± 0.03	0.31 ± 0.05
16:0	17.09 ± 0.87	17.26 ± 0.64	17.60 ± 0.64	17.68 ± 0.71
Iso 17:0	0.24 ± 0.04	0.26 ± 0.04	0.23 ± 0.03	0.24 ± 0.04
Antiso 17:0	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.00
17:0	0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.02
18:0	2.86 ± 0.30	2.88 ± 0.30	3.19 ± 0.48	3.10 ± 0.62
20:0	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
∑SFA	23.60 ± 0.87	23.89 ± 0.60	24.04 ± 0.66	24.19 ± 0.70
16:1 (n-11)	0.18 ± 0.03	0.18 ± 0.02	0.16 ± 0.03	0.16 ± 0.03
16:1 (n-9)	0.34 ± 0.05	0.35 ± 0.04	0.31 ± 0.05	0.32 ± 0.06
16:1 (n-7)	2.11 ± 0.28	2.15 ± 0.30	1.87 ± 0.40	1.97 ± 0.44
16:1 (n-5)	0.27 ± 0.01	0.27 ± 0.02	0.24 ± 0.03	0.25 ± 0.04
∑17:1	0.61 ± 0.18	0.61 ± 0.18	0.51 ± 0.10	0.66 ± 0.23
18:1 (n-11)	1.70 ± 0.40	1.66 ± 0.34	1.39 ± 0.46	1.37 ± 0.55
18:1 (n-9)	9.29 ± 0.93	9.05 ± 0.80	8.85 ± 1.07	8.92 ± 0.90
18:1 (n-7)	2.37 ± 0.09	2.35 ± 0.14	2.36 ± 0.13	2.35 ± 0.09
18:1 (n-5)	0.34 ± 0.03	0.33 ± 0.02	0.31 ± 0.05	0.31 ± 0.04
20:1 (n-11)	0.46 ± 0.06	0.52 ± 0.07	0.48 ± 0.07	0.43 ± 0.09
20:1 (n-9)	2.40 ± 0.28	2.34 ± 0.28	2.31 ± 0.40	2.13 ± 0.40
20:1 (n-7)	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
22:1 (n-11)	1.45 ± 0.30	1.27 ± 0.29	1.30 ± 0.32	**1.08 ± 0.26**
22:1 (n-9)	0.17 ± 0.03	0.16 ± 0.03	0.16 ± 0.03	0.14 ± 0.03
22:1 (n-7)	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
24:1 (n-9)	0.94 ± 0.12	0.98 ± 0.15	1.04 ± 0.11	**0.87 ± 0.07**
24:1 (n-7)	0.12 ± 0.04	0.15 ± 0.06	0.14 ± 0.06	0.12 ± 0.04
∑MUFA	22.87 ± 1.98	22.48 ± 1.27	21.55 ± 2.47	21.19 ± 2.41
16:4 (n-1)	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
18:4 (n-1)	0.07 ± 0.01	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
16:2 (n-4)	0.11 ± 0.02	0.12 ± 0.02	0.09 ± 0.02	0.10 ± 0.03
16:3 (n-4)	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
18:2 (n-4)	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
18:2 (n-6)	3.62 ± 0.12	3.61 ± 0.18	3.44 ± 0.25	3.46 ± 0.25
18:3 (n-6)	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.01
20:2 (n-6)	0.34 ± 0.03	0.39 ± 0.04	0.35 ± 0.07	0.35 ± 0.06
20:3 (n-6)	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
20:4 (n-6)	1.13 ± 0.06	1.11 ± 0.08	1.22 ± 0.17	1.22 ± 0.16
22:4 (n-6)	0.16 ± 0.02	0.15 ± 0.03	0.16 ± 0.02	0.16 ± 0.02
22:5 (n-6)	0.41 ± 0.02	0.40 ± 0.02	0.44 ± 0.05	0.43 ± 0.05
18:3 (n-3)	0.70 ± 0.05	0.72 ± 0.06	0.63 ± 0.09	0.65 ± 0.10
18:4 (n-3)	0.99 ± 0.10	1.05 ± 0.11	0.89 ± 0.14	0.94 ± 0.16
20:3 (n-3)	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.02	0.09 ± 0.02
20:4 (n-3)	0.67 ± 0.03	0.67 ± 0.03	0.63 ± 0.09	0.62 ± 0.09
20:5 (n-3)	14.91 ± 0.62	14.81 ± 0.43	14.34 ± 0.55	14.81 ± 0.56
21:5 (n-3)	0.29 ± 0.01	0.25 ± 0.09	0.27 ± 0.02	0.27 ± 0.03
22:5 (n-3)	1.67 ± 0.07	1.68 ± 0.04	1.70 ± 0.07	1.68 ± 0.06
22:6 (n-3)	27.73 ± 1.27	27.75 ± 1.20	29.50 ± 2.55	29.05 ± 2.95
24:5 (n-3)	0.17 ± 0.06	**0.28 ± 0.05**	0.15 ± 0.07	**0.24 ± 0.04**
∑PUFA	53.54 ± 1.67	53.63 ± 1.16	54.41 ± 2.10	54.62 ± 2.11
∑(n-6) PUFA	5.83 ± 0.14	5.83 ± 0.21	5.78 ± 0.22	5.79 ± 0.14
∑(n-3) PUFA	47.30 ± 1.66	47.38 ± 1.26	48.26 ± 2.19	48.43 ± 2.27
(n-3)/(n-6)	8.12 ± 0.30	8.14 ± 0.41	8.37 ± 0.57	8.38 ± 0.59
Amount of Cholesterol (%)	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.06 ± 0.01
Amount of FA (%)	0.75 ± 0.11	0.77 ± 0.10	0.74 ± 0.13	0.76 ± 0.12

Table A10. Fatty acid profile in the pellets use in the experiment. FA that represents less than 0.1 % of the total FAs are removed from the table, but is included in the total sum of FAs.

	Food (n=4)
14:0	6.97 ± 0.17
Iso 15:0	0.25 ± 0.01
15:0	0.45 ± 0.00
16:0	14.64 ± 0.31
Iso 17:0	0.26 ± 0.00
Antiso 17:0	0.08 ± 0.00
17:0	0.19 ± 0.01
18:0	1.48 ± 0.04
20:0	0.18 ± 0.00
∑SFA	24.95 ± 0.22
16:1 (n-11)	0.10 ± 0.00
16:1 (n-9)	0.24 ± 0.00
16:1 (n-7)	5.06 ± 0.01
16:1 (n-5)	0.30 ± 0.00
∑17:1	0.44 ± 0.01
18:1 (n-11)	0.59 ± 0.01
18:1 (n-9)	8.18 ± 0.22
18:1 (n-7)	1.94 ± 0.06
18:1 (n-5)	0.43 ± 0.02
20:1 (n-11)	0.98 ± 0.00
20:1 (n-9)	9.99 ± 0.07
20:1 (n-7)	0.26 ± 0.01
22:1 (n-11)	15.79 ± 0.59
22:1 (n-9)	0.99 ± 0.03
22:1 (n-7)	0.16 ± 0.02
24:1 (n-9)	1.10 ± 0.06
24:1 (n-7)	0.05 ± 0.01
∑MUFA	46.65 ± 0.41
16:4 (n-1)	0.44 ± 0.04
18:4 (n-1)	0.12 ± 0.01
16:2 (n-4)	0.36 ± 0.01
16:3 (n-4)	0.21 ± 0.01
18:2 (n-4)	0.09 ± 0.01
18:2 (n-6)	4.51 ± 0.14
18:3 (n-6)	0.08 ± 0.01
20:2 (n-6)	0.17 ± 0.00
20:3 (n-6)	0.04 ± 0.00
20:4 (n-6)	0.32 ± 0.02
22:4 (n-6)	0.03 ± 0.00
22:5 (n-6)	0.11 ± 0.01
18:3 (n-3)	1.17 ± 0.00
18:4 (n-3)	2.36 ± 0.05
20:3 (n-3)	0.09 ± 0.00
20:4 (n-3)	0.44 ± 0.02
20:5 (n-3)	6.39 ± 0.08
21:5 (n-3)	0.25 ± 0.00
22:5 (n-3)	0.78 ± 0.03
22:6 (n-3)	9.64 ± 0.15
24:5 (n-3)	0.50 ± 0.03
∑PUFA	28.40 ± 0.28
∑(n-6) PUFA	5.30 ± 0.15
∑(n-3) PUFA	21.77 ± 0.23
(n-3)/(n-6)	4.11 ± 0.10
Amount of Cholesterol (%)	0.34 ± 0.01
Amount of FA (%)	12.90 ± 0.45

Pilot study of mass spectrometry identification of DNA adducts.

One aim for this project was to generate samples with very high levels of DNA adducts from PAH exposure that could be used into future method development of mass spectrometric analysis of PAH derived DNA adducts in haddock livers. The goal would be to identify the major DNA-adducts generated in haddock during PAH exposure and to create a mass spectra library that could be used for comparison in future field studies.

An ultra-high performance Liquid chromatography coupled to tandem mass spectrometry (UHPLC MS/MS) method for the detection of DNA adducts, is currently under development by the ADn'tox partner PRISMM (an academic platform of the University of Caen, France). The method is now suitable for detection of the major BAP adduct: benzo(a)pyrene (BP)-7,8-diol-9,10-epoxide-N(2)-deoxyguanosine (BPDE-dG) in the MRM (Multi Reaction Monitoring) mode, but can also be used for the pre-screening of multi-adduct detection in the PNL (Pseudo Neutral Loss) mode. The later mode is focused on the loss of the deoxyribose (dR) from the protonated DNA adducts ([M+H-116]+), a particular fragmentation common to all nucleosides in MS, including the parent dG DNA adducts.

In this project a small pilot study with LC-MS analysis was performed. Two samples were selected for UHPLC-MS/MS, one from the PAH exposed group, n◦38 (D18) (DNA adduct 40 nmol adduct/mol normal DNA), and one control n◦5 (DNA adduct not detected) (Figure A1). Both fish were sampled 2 months after start of the experiment. The autoradiograms of these two samples can be seen in figure 10.

The samples were analysed on UHPLC-MS/MS with several different scan methods;

Test 1: Target multiple reaction monitoring (MRM) for the known BAP DNA-adduct, dG-N-2-BPDE.

Test 2: Non target pseudo neutral loss scanning (PNL). This is a screening method looking for "unknown" DNA adducts.

Test 3. Target MRM scanning for 8-Oxo-2'-deoxyguanosine (8-oxo-dG). 8-oxo-dG is an oxidized derivative of deoxyguanosine and is one of the major products of DNA oxidation. Concentrations of 8-oxo-dG within a cell are a measurement of oxidative stress.

Preliminary results of the 3 UHPLC-MS/MS tests

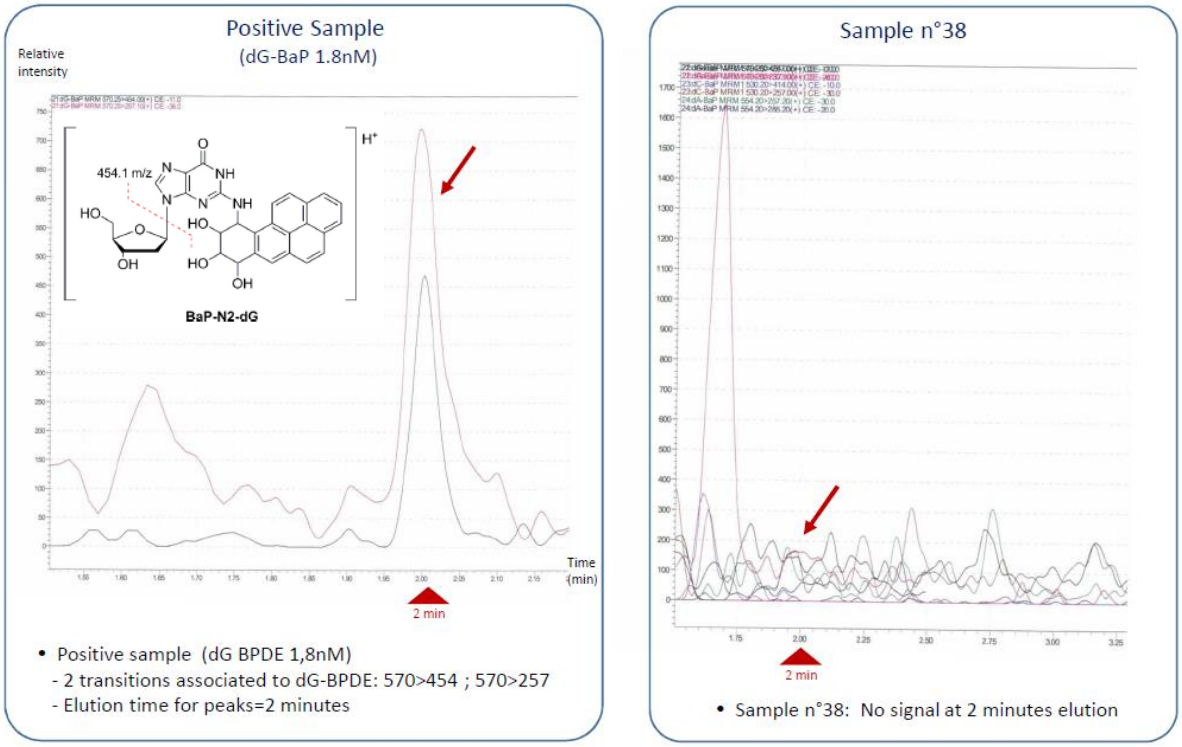

Figure A1. HPLC-MS/MS analysis (MRM scanning for dG BPDE) of a selected haddock sample (PAH exposed for 2 months, sample no38) and a positive control sample with a known amount of dG BPDE. The MRM scan shows that dG BPDE was not detected in the haddock sample.

Test 1 showed no presence of the known BaP-DNA adduct found in the oral exposed haddock. In the MRM mode, the detection of BPDE-dG is associated to the matching of signals (peaks) in both transition patterns 570>454; 570>257. Peaks associated with this adduct appeared after 2 minutes elution (see positive control, Figure A1). On this basis, the patterns obtained with sample n°38 show the absence of dG-BPDE adduct at the limit of detection of 0.6 nM. The autoradiogram of this sample (Figure 10, sample no38)) showed a large “spot 1” and two minor peaks in the “spot 2/3” area. This suggest that BaP produce other DNA adducts in haddock that what have been identified inn mammals.

Test 2 in the PNL mode, interesting signals are simultaneously observed in both transition patterns 538>422 (2 peaks) and 537>421 (3 peaks). These signals would be associated to 2 and 3 isomers of 2 different DNA adducts. The comparison of the PNL patterns obtained from both samples n°5 (unexposed control haddock) and n°38 (PAH exposed haddock) reveals that this peaks are only found in the PAH exposed fish (Figure A2). On the basis of the “Nitrogen rule”, some potential chemical structures are proposed. One of both supposed DNA adducts probably contains nitrogen atom (in NH2 or NO2 functions) (Figure A4). It remains to get a positive identification through analysing standards, but it shows that the LC-MS/MS is sensitive enough to detect DNA adducts in this samples.

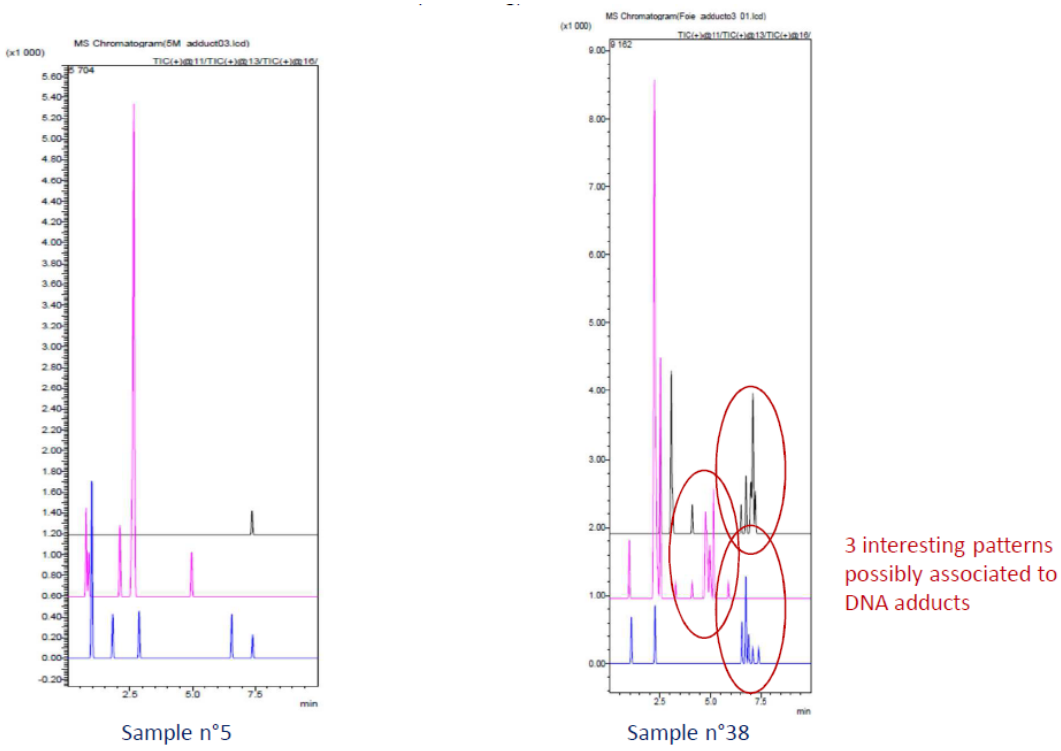

Figure A2. HPLC-MS/MS analyses (PNL scanning) of selected haddock sample (PAH exposed in 2 months, sample no38) and negative control sample (sample no5).

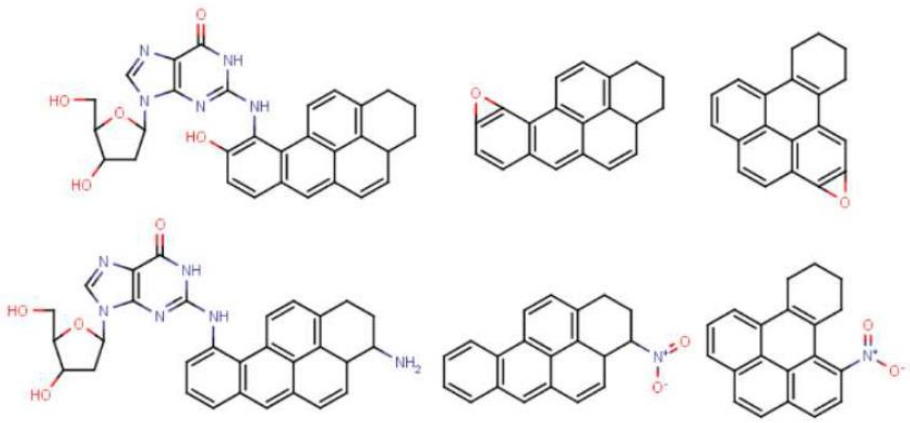

Figure A3. Suggestion of potential DNA adducts of BaP or BeP that can correspond to the scanning signals found in Figure A3. These results need to be confirmed by analyzing standards of the different DNA adducts.

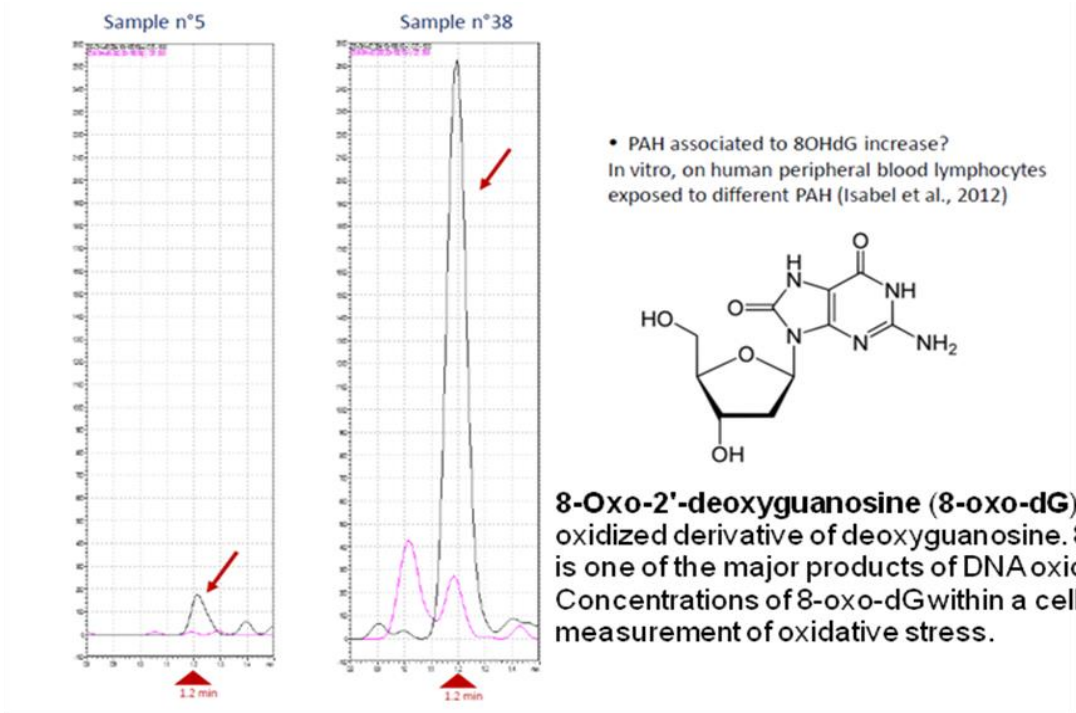

Figure A4. HPLC-MS/MS analyses (MRM scanning for 8-oxo-dG) of selected haddock sample (PAH exposed in 2 month) and negative control sample.

Test 3, the 8-hydroxy-2'-deoxyguanosine (8-OH-dG), a well-known DNA adduct used as marker of oxidative stress of various origins, is detected with higher level by the MRM mode in sample n°38 compared to n°5. Analyses of 8-oxo-dG have been suggested as measurement of oxidative stress from PAH exposure (Isabel *et al.* 2012;Penning 2014) and it may be an interesting parameter to consider to include in the water column survey.