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PAH and biomarker measurements in fish from condition monitoring in Norwegian waters in 2005 and 2008

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Abstract

Condition monitoring in fish from open seas are performed in Norway every third year. The objectives are to investigate whether fish from Norwegian seas contain elevated levels of components that originate from discharges from the petroleum activity. We present results from the samplings in 2005 and 2008 from four areas: The Egersund Bank (reference area North Sea), Tampen, the Halten Bank and the Barents Sea (reference area).

NPD and PAH measured in fish muscle in 2005 were found to be below LOQ for all regions. In 2008 sum NPD were measured in haddock liver and found to be low for all regions (8-34 ng/g wet weight). Bile metabolites from haddock were measured by GCMS in 2008. The main contributor to sum PAH metabolites at Tampen and at the Egersund Bank was 1-hydroxy phenanthrene with levels of 510±814 and 133±207 ng/g bile, respectively. Levels of this metabolite in haddock from the Halten Bank and the Barents Sea were 43±71 and 19±14 ng/g bile, respectively. Levels of alkylphenols were found below LOQ. Levels of Vtg in blood of male cod were generally low from all regions.

Measurements of DNA adducts in fish liver did not show changes for cod and saithe, while a significant increase were observed in haddock from Tampen compared to haddock from the Egersund Bank. Lipid content in the liver was significantly reduced in haddock from Tampen. Fatty acid profiles showed that haddock from Tampen had relatively high levels of arachidonic acid, and the ratio between omega-3 and omega-6 ((n-3)/(n-6)) poly unsaturated fatty acids were significantly lower in neutral lipids, free fatty acids and phosphotidylcholine/phosphotidylethanolamine, compared with haddock from the other regions.

Introduction

The Activity regulations require the offshore petroleum industry to perform monitoring. The condition monitoring shall document if fish from Norwegian ocean areas contain elevated values of components that originate from discharges from the petroleum activity. The major objective is to document to what extent discharges from the oil and gas installations cause contamination of fish negatively affecting the quality. For both the petroleum industry and the Norwegian fishing industry it is important that safety and quality of Norwegian seafood is documented, as well as environmental health of the marine environment.

Condition monitoring with fish from open seas in Norwegian areas are conducted every third year and shall document whether fish from Norwegian Seas are affected of pollution from oil and gas activities. The program is decided by the Norwegian Pollution Control Authority, (Klif). Sampling should be performed such that it gives a representative picture of the most important fish species in the region. In this connection knowledge of the species composition and migration pattern in each region is important.

A study reported by Klungsøyr and Johnsen (1997) on cod (*Gadus morhua* L.) and haddock (*Melanogrammus aeglefinus*) concluded that there is no general increase in levels of NPD/PAH in fish caught in the vicinity of oil and gas fields in Norwegian areas compared with remote reference areas.

In the monitoring performed in 2000, haddock were collected from ten regions: Ekofisk, Sleipner, Tampen, Møre, Trøndelag, Nordland, Troms, Finmark, the Barents Sea (reference) and the Egersund Bank (reference). The results from the analyses of 25 muscle samples from each of these regions showed that haddock only contained very low background concentrations of NPD/PAH (Klungsøyr *et al*., 2001).

In 2002, NPD/PAH were analysed in cod, haddock, saithe (*Pollachius virens*) and herring (*Clupea harengus*) from Tampen, Sleipner and the Egersund Bank (reference area). The levels of NPD/PAH in haddock muscle at Sleipner and Tampen were generally very low and at normally occurring background concentrations for fish from the North Sea. Similar results were found for fish liver samples showing that fish from Tampen and Sleipner in general contained very low background concentrations of NPD/PAH. This is in accordance with previous results and can be explained both by low exposure and/or and effective metabolic system in fish resulting in rapid excretion of aromatic hydrocarbons (Klungsøyr *et al*., 2003).

However, the analyses of biomarkers in the 2002 study revealed biological effects in haddock from Tampen and Sleipner compared with fish from the Egersund Bank. In haddock, genotoxicity was reflected in increased levels of hepatic DNA adducts probably due to exposure to NPD/PAH. Anomalies in muscle lipid composition were also detected at the Tampen and Sleipner areas compared to Egersund Bank (Klungsøyr *et al*., 2003).

Measurements of NPD and PAH in fish fillet were also conducted in several fish species after the oil discharge incident of 4400 m^3 crude oil at Statfjord in December 2007. Also in this study levels of NPD and PAH in fillets were below levels of detection (LOD) for fish sampled 6 days and one month after the discharge. However, increased levels of NPD compounds were measured in liver of haddock and pollack (*Pollachius pollachius*) sampled

in the Tampen area 6 days after the discharge (Grøsvik *et al*., 2008). For this reason it was decided to also include liver of haddock from Egersund Bank, Halten Bank and The Barents Sea in this study to learn more about background levels of NPD and PAH compounds.

The objective was to study to what extent contaminants from offshore petroleum industry bioaccumulate and cause effect in fish populations and affect food safety and quality by measurements of NPD/PAH in fish muscle and liver, metabolites of PAH and alkylphenols in bile, vitellogenin levels in blood from male fish and hepatic DNA adducts. We also wanted to perform lipid extraction and lipid class separation to analyse ratio of (n-3)/(n-6) poly unsaturated fatty acids.

Sampling

Fish were sampled from Tampen (The North Sea) which is an area with high activities of oil and gas production and with produced water discharges. This area was compared with the Egersund Bank (the North Sea) used as a reference area, the Halten Bank (The Norwegian Sea) with some oil and gas installation and the Barents Sea, a reference where produced water is not discharged (Figure 1).

Cod, saithe and haddock, were sampled during the surveys in 2005, while most focus were put on haddock in 2008 as most of the effect from 2005 were seen in haddock. Long rough dab (*Hippoglossoides platessoides*) were also sampled in the 2008 surveys.

Bottom trawl was used for collection of cod, haddock, saithe and long rough dab. From each of the regions 25 (±10%) fish of each species were sampled. After killing the fish with a blow to the head, standard IMR procedures were used for collection and storage of muscle, liver, blood and bile samples for the later chemical and biochemical analyses. Figure 1 gives the sampling locations for fish in the different areas.

Material & methods

NPD/PAH analysis of liver tissue

Wet liver tissue was boiled under reflux with 0.5N alcoholic KOH for 1.5 hours, followed by liquid/liquid extraction with hexane. Extracts were volume reduced and cleaned on silica column prior to injection on a Micromass Autospec Ultima GC/MS in SIM mode (Klungsøyr *et al*., 1988). The GC/MS system was equipped with a HP-6890 GC, a 50m x 0,25mm, 0.25µm Varian Factor Four CC VF-5ms capillary column inserted directly into the ion source. Other conditions were: injector temperature 280ºC; transfer line 275ºC; column temperature, 60ºC for 1 min, 60-100ºC at 15ºC/min, 100-280ºC at 6ºC/min, 9min at final temperature, carrier gas He at 1.5 ml/min. Electron impact ionization at 70eV was used. Samples were injected by auto sampler, 1 µl splitless injection.

The method is validated to analyse PAH in concentration of 0.2 ng/g. For some compounds the detection limit are higher, because of background problems. Levels of detection (LOD) are defined as LOD: $Y = Y\mathrm{B} + 3\mathrm{SD}_\mathrm{B}$, and levels of quantification (LOQ) is LOQ= $Y = Y\mathrm{B} + 10\mathrm{SD}_\mathrm{B}$ where Y_B is the response of blank sample signal and SD_B is the standard deviation of the blank samples.

Analysis of NPD/PAH and alkyl phenols in fish bile

Bile (100 µl) was diluted in 100 µl sodium acetate buffer (0.01 M, pH 5). 18 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 derivatized with pentafluorobenzoyl chloride as described elsewhere (Boitsov *et al*., 2007) and the samples concentrated to 0.3 ml hexane solution under a nitrogen stream (40°C). All samples were added 100 µl relative internal standard (RIS, 360 ng/ml) and analysed by GC-MS in selected ion monitoring (SIM) mode using negative chemical ionisation (NCI).

DNA adducts

Standard DNA (salmon sperm, D-1626), spermidin (S-2626), RNase A (R-4642), micrococcal endonuclease (N-3755) and spleen phosphodiesterase (P-9041) were obtained from Sigma Chemical Company, St. Louis, MO, USA. RNase T1 (109 193), proteinase K (1000144), α-amylase (102814), T_4-polynucleotidekinase (3´-phosphatase free, 838 292) and phenol (1814303) were purchased from Roche Diagnostics, Scandinavia AB, Bromma, Sweden. Nuclease P_1 (7160) was bought from Yamasa Corporation, Diagnostics Department, Chuo-Ku, Tokyo, Japan, and later Sigma-Aldrich Sweden AB, Stockholm, Sweden. Radiolabelled ATP ([γ-^{32}P]ATP) with specific activity 3000 Ci/mmol (110 TBq/mmol) were obtained from Amersham Biosciences, Uppsala, Sweden. The benzo[a]pyrene standard adduct, 7R, 8S, 9S-trihydroxy, 10R-(N^2-deoxyguanosyl-3´-phosphate)-7,8,9,10-tetrahydro-benzo(a)-pyrene (BaPDE-dG-3´p), was obtained from Midwest Research Institute, Kansas City, MO, USA. Cellulose (MN-301) was purchased from Machery-Nagel, Düren, Germany. Vinyl strips (PVC foil, 0.2 mm thickness), used for the groundwork of the polyethyleneimine cellulose sheets were obtained from Andren & Söner, Stockholm, Sweden. Scintillation fluid (Ultima gold) was purchased from CIAB, Lidingö, Sweden. All other solvents and chemicals for DNA purification and adduct analysis were purchased from common commercial sources and were of analytical purity.

DNA adduct analysis

Tissue samples were semi-thawed and the DNA extracted and purified according to Dunn *et al*., 1987; Reichert and French 1994, slightly modified as described in Ericson and Balk, 2000. DNA adducts were enriched and normal nucleotides hydrolyzed to nucleosides by the nuclease P_1 method, using 0.41 g Nuclease P1/g DNA and a 45 min incubation period at 37 °C (Reddy and Randerath 1986; Beach and Gupta 1992). The DNA adducts were radiolabelled using 5´-[γ-^{32}P]triphosphate([γ-^{32}P]ATP) and T_4 polynucleotide kinase. Separation and cleanup of adducts was performed by a modified multidirectional thin-layer chromatography (TLC) on laboratory produced polyethyleneimine cellulose sheets that serve as anionic exchanger support. After elution, adducts were then located on the sheets and quantified by storage phosphor imaging technology (PhosphorImager^{TMSI} and ImageQuant 5.0). In addition, several quality control experiments were performed in parallel to the analysis of the various fish tissue samples.

Controls used during the analytical work were: a) Pure salmon sperm as negative control, b) the standard DNA adduct B[a]PDE-dG-3´p, and c) adducted liver tissue from B[a]P exposed perch. These were processed parallel to the samples and served as quality assurance for all the analytical steps in the ^{32}P-postlabeling method. These quality assurance experiments confirm a faultless assay for the DNA adduct measurements performed in this study.

DNA for adduct analysis was quantified on the basis of its absorption at 260 nm in a GeneQuant spectrophotometer from Pharmacia Biotech, Uppsala, Sweden. Liquid scintillation spectroscopy was performed in a Packard Tri-Carb 2100TR liquid scintillation counter from Packard Instrument Company. A Desaga spreader from Desaga Heidelberg, Germany, was used to prepare the TLC-sheets. The DNA adducts were located and the levels quantified on the TLC sheets with ImageQuant, 5.0 software, Molecular Dynamics, by the storage phosphor imaging technique using a PhosphorImager™ SI instrument (Sunnyvale, CA, USA), essential according to methodology described by Reichert *et al.* 1998.

For every value that is below the detection limits, an average of zero and the background is taken in order to be able to calculate average values for groups. Because, if any adducts are present in the sample, their value is below the background value of the autoradiogram, and could range from zero up to the background value.

Lipid extraction and lipid class separation.

Total lipids were extracted from haddock samples (0.5 g) by a modified Folch method (Folch *et al*., 1957) with chloroform/methanol (2:1 v/v). An aliquot of the sample was separated into four different lipid classes: neutral lipids (NL: triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), cholesterol and cholesterol esters); free fatty acids (FFA); phosphotidylcholin (PC)/phosphotidylethanolamine (PE) and phosphotydylserine (PS)/ phosphotidylinisitol (PI).

The lipid classes separation were done using the same columns (500 mg aminopropyl SPE, Supelco) and same solvent regime as described in Perez-Palacios *et al*. (2007), but it was found that the amount of solvent had to be modified for these marine samples. The column was loaded with 0.5 ml of lipid extraction (10 mg/ml): NL were eluated with 4 ml chloroform; FFA were eluated with Diethylether:Acetic acid (98:2 v/v); PC/PE were eluated with 10 ml of methanol; PS/PI were eluted with 17 ml methanol/3N HCL in MeOH (9:1, v/v). The purity of the lipid classes fractionations were tested using thin layer chromatography (TLC) (Olsen and Henderson, 1989). All lipid classes fraction were evaporated to dryness with nitrogen (g) at 40°C.

Fatty acids analysis

Methyl esters of the fatty acids (FAME) from total lipids and the lipids classes were prepared and analysed on gas chromatography (GC-FID) as described by (Meier *et al*., 2006). The FAME was quantified using Nonadecanoic acid (19:0) as internal standard.

Statistical analyses

Data were log-transformed (base 10) and sets with homogenous variances were analysed by Dunnett's test to determine which means were significantly different. The analyses were performed with JMP, ver. 8.0, SAS Institute Inc. For the fatty acid analyses: One-way ANOVA and Tukey (HSD) test as a post-hoc test after tests for normal variation.

Results and discussion

Levels of NPD in haddock liver

Analyses of aromatic hydrocarbons (NPD and PAH) were carried out using GC/MS. NPD is the sum of naphthalene, phenanthrene, dibenzothiophene, and their C_1-C_3 alkylated homologs and are typical petrogenic compounds.

Levels of NPD and PAH in haddock were low for all regions. Sum NPD in liver of haddock ranged from 15.3±7 ng/g at the Egersund Bank, 7.8±5.9 ng/g in the Barents Sea to 10.5±13.3 ng/g at the Halten Bank. Levels found in haddock at the Egersund Bank in January 2008 (one month after the Statfjord A discharge) was 31±19 ng/g NPD, while levels found in haddock at a Tampen 6 days after the discharge were 132±123 ng/g NPD (Figure 2) and as reported in Grøsvik et al. (2008). Levels of sum PAH were low for the three regions. Sum PAHs were 2.1±3.1 ng/g (Egersund Bank), 2.6±3.5 ng/g (Barents Sea) and 1.5±2.1 ng/g (Halten Bank). Levels of sum PAH in haddock after the Statfjord incident were 26±16 at the Egersund Bank in January 2008 and 6.3±5.2 at Tampen in December 2008 (Grøsvik *et al*., 2008).

Levels of PAH metabolites in bile

The content of PAHs and alkyl phenols in bile can reflect which compounds are being metabolised in the organism in a small and concentrated volume. This has shown particularly useful for hydroxylated polycyclic aromatic hydrocarbons (PAH) (Aas *et al*., 2000). As PAHs are quickly metabolised by fish, it is more appropriate to monitor the levels of PAH metabolites (hydroxylated PAH) in fish bile than the levels of parent compounds in fish muscle or liver. PAHs are metabolised in fish in two stages, first being oxidised to hydroxylated PAHs and then conjugated into highly water-soluble conjugates of e.g. glucuronic acid. Several methods have been described for analysing PAH metabolites using solid-phase extraction, various types of derivatisation and consequent GC-MS analysis (e.g. Jonsson *et al*., 2003). Based on this, the method for analysing PAH metabolites and alkylphenols include deconjugation, derivatisation and pentafluorobenzoyl derivatization, as previously described for alkylphenol analysis (Boitsov *et al*., 2004). This allows achieving low detection limits due to the possibility of using negative chemical ionisation (NCI) mode on GC-MS.

Overall, the highest levels of sum PAH metabolites of 580 ng/g bile were measured in haddock from Tampen. Sum PAH metabolites at Egersund Bank was 231 ng/g bile, at the Halten Bank 199 ng/g bile and in haddock from the Barents Sea 35 ng/g bile (Figure 3). The main contributor to sum PAH metabolites was 1-hydroxy phenanthrene. Levels of 1-hydroxy phenanthrene in haddock bile from Tampen were 510±814 ng/g bile (Figure 4). Levels above LOQ were measured in all of haddock bile from Tampen (n=16), and 9 of the 16 samples had levels from 172 to 2934 ng/g bile. Levels of 1-hydroxy phenanthrene in haddock bile from the Egersund Bank were 133±207 ng/g, while levels from the Halten Bank and the Barents Sea were 43±71 and 19±14 ng/g, respectively (Figure 4). The increased mean and SD of 1-hydroxypyrene in haddock from the Halten Bank is influenced by one individual with levels of 546 ng/g bile, the others ranged from 9-161 ng/g bile.

This is the first study on bile metabolites in haddock. Bile metabolites were performed on cod at Tampen and the Egersund Bank approx one month after the discharge at Statfjord, December 2007, and levels of PAH metabolites were comparable with levels found in this study, except for those of 1-hydroxy phenanthrene. Mean levels of 1-hydroxy phenanthrene in cod bile in the Statfjord A study were between 6 and 14 ng/g bile (Grøsvik *et al*., 2008).

Levels of sum PAH metabolites in bile from two cod kept in cage under the oil slick after the Server accident had levels of 4026 ng/g bile (Meier et al., 2007b). This level is in the same range as reported in bile from oil exposed cod (Jonsson *et al*., 2003).

Only background levels of PAH metabolites were measured in saithe and the levels were comparable between the areas. Sum PAH metabolites ranged from 36-82 ng/g bile. PAH metabolites in long rough dab were only measured in fish from the Barents Sea, and found to be low (88 ng/g bile).

Levels of alkylphenols in bile

Metabolites of alkylphenols were analysed for a high number of metabolites in 2008. Analysed fish included haddock from Egersund Bank (n=23), Tampen (n=16), Barents Sea (n=22) and Halten Bank (n=16), in addition to saithe from Egersund Bank (n=19) and Tampen (n=19), and long rough dab from the Barents Sea (N=21). In total 143 fish were analysed and most of the alkylphenol metabolites were found to be below LOQ.

The low levels of alkylphenols measured in bile in 2008 is in accordance with the results from condition monitoring 2005 where levels of alkylphenols measured in cod liver, haddock liver and herring muscle from the Egersund Bank and Tampen regions demonstrated mostly levels below LOD for all stations (Grøsvik *et al.,* 2008), and with the results from the 2002 monitoring (Klungsøyr *et al*., 2003).

One haddock from Tampen had elevated levels of oil related alkyl phenols in the bile (4-etylphenol and dimethyl phenols. Elevated levels of 4 tert-octylphenol were measured in 10 of 22 haddock from the Barents Sea and in 2 of 23 haddock from the Halten Bank, while not in haddock from Tampen and the Egersund Bank. As this phenomenon was not observed in haddock from the North Sea, it could be due to problems with sample preparation and extraction as samples were upconcentrated 40 times. 4-tert-octylphenol and 4-tert-nonylphenol are constituents in many plastic products and it is compounds known to cause false positives in extraction analyses.

Measurements of Vtg levels in blood of male cod

Experimental studies in the laboratory have shown that alkylphenols in produced water can cause estrogenic effects in cod (Meier et al., 2010.). In this study APs in bile were analysed as well as vitellogenin in blood plasma as a biomarker of estrogenic effects.

Vitellogenin (Vtg) is a glycophospholipoprotein and the main source of yolk proteins and lipids in the growing oocyte. Vtg is synthesized in the liver in response to estrogens. Even though Vtg is a protein specific to female fish, males also possess all of the genetic system needed for VTG protein synthesis. A rise in the level of VTG is commonly used as a biomarker for estrogenic effects in vertebrates. Cod Vtg was analysed in plasma of male cod by a quantitative enzyme-linked immunosorbent assay (ELISA) (Biosense Laboratories, Bergen, Norway).

Levels of Vtg in male cod are plotted versus fish weight both on linear axis and on a logarithmic axis (Figure 6). A total of 27 fish were analysed in 2005 and 55 fish were analysed in 2008. Generally levels of Vtg in feral male cod ranged from 0 to 5000 ng/ml. A larger material should be used to be able to see regional differences. The results were in accordance with work by Scott *et al*., (2006b). Scott *et al*. reported Vtg concentrations up to 160 µg/ml in male cod, but from the open sea only in fish over 5 kg. However, in the Oslo Fjord, also many smaller male cod had induced Vtg levels (Scott *et al*., 2006b). Water column monitoring (in 2001) around oil rigs have found that cod caged closest to the platform (500 m distance) have significant, but marginal elevation of Vtg (Scott, *et al*.,

2006a). However, similar studies (2003 and 2004) did not find any differences between cod caged in differences distance from oil installations (OLF, 2005).

DNA adducts in fish liver

Aromatic hydrophobic PAH-DNA adducts were analysed in liver of cod, haddock and saithe from Tampen (oil field area), and the Egersund Bank in 2005 and from haddock and long rough dab, sampled in from Tampen and Egersund Bank (North Sea) and The Barents Sea in 2008. The DNA adducts were analysed with the ^{32}P post labelling assay, which is the most sensitive and frequently applied technique for detecting PAH-DNA adducts in marine organisms (Reichert *et al*., 1998). PAHs are readily taken up and metabolised by fish, and it is during the metabolic transformation of these compounds, that they are activated and become genotoxic. The enzymatic phase I of the biotransformation of PAHs lead to the formation of reactive electrophilic metabolites which can undergo attack and bind covalently to nucleophilic centres in large molecules such as lipids, proteins, DNA, and RNA, and form adducts. Factors that affect DNA adduct levels are exposure dose, the degree of bioactivation in phase I into reactive intermediates in relation to the phase II detoxification, DNA repair efficiency, as well as cell turnover. DNA adduct levels are thus a quantifiable measure of the biologically effective dose reaching a critical target site, and they integrate multiple toxicokinetic factors such as uptake, metabolism, detoxification, excretion and covalent binding of reactive metabolites to target tissues (Reichert *et al*., 1998). DNA adducts have shown to be predecessor of both mutagenic and carcinogenic effects, and they have shown to correlate with liver lesions in fish (Baumann, 1998; Reichert *et al*., 1998). They are widely used as, and considered to be highly relevant biomarker for PAH exposure to fish.

Twenty five individuals from each of the sampling sites were analysed for DNA adducts. The results revealed that DNA adduct levels were significantly higher in haddock from Tampen ($p = 0.05$) compared with for haddock caught at the Egersund Bank for sampling in 2005 and 2008 (Figure 5). For sampling in 2008, haddock from the Egersund Bank had DNA adduct levels of 1.66±2.10 nmol adducts/mol normal nucleotides (average ± SD) compared to haddock from Egersund bank and the Barents Sea with 0.85±1.14 and 0.82±0.93 nmol adducts/mol nucleotides, respectively. Tampen had also highest number of individuals with detectable DNA adducts or 11 (44%), compared to 7 (28%) and only 2 (8%) from Egersund Bank and the Barents Sea, respectively. The Barents Sea average includes two samples with DNA adducts (4.79 and 2.26 nmol add/mol normal nucleotides). DNA adducts from Tampen (the site with highest levels and frequency of adducts) were plotted with age of the fish, but no obvious relationship between adducts and age was apparent (not shown).

The observed DNA adduct levels in the liver of haddock from Tampen can be considered high when having in mind that the fish is caught in the open North Sea. The fact that the fish show elevated levels of DNA adducts at all is an abnormal condition, and confirms that the fish has been exposed to genotoxic pollutants beyond their DNA repair capacity and suggest PAH contamination in the area. Few studies on DNA adduct levels in fish from the North Sea or neighbouring areas, or even from open seas in general have been published. But for comparison, Aas *et al*. (2003) studied DNA adduct levels in 11 fish species from the open seas of the NE Atlantic. That study showed undetectable levels of DNA adducts in the fish, or levels just above the detection limits.

Lipid extraction and lipid class separation

Haddock have like other Gadidae large and fatty livers. These lean fish store most of its energy as fat in the liver. Analyses of lipid content demonstrated significant differences in

lipid amount in the livers of haddock from Tampen compared with haddock from the other regions (Figure 7). Haddock from Tampen had relatively smaller livers and with low lipid content. Lipid content in female and male haddock from Tampen were 26±16 % and 44±15 %, respectively, while female and male haddock from the Egersund Bank had lipid contents of 52±11 % and 53±8 %, respectively. Haddock from the Barents Sea had lipid content in liver of female and male fish of 48±14 % and 52±10 %. Haddock from the Halten Bank was not directly comparable as it were fished in beginning of December and had the largest livers and with the highest lipid content, 62±14% in females and 64±7% in males. Haddock from the Egersund Bank and Tampen were fished in July, while haddock from the Barents Sea in August. Generally, haddock from Tampen were in poorer condition than haddock from the other regions.

The neutral lipids (NL) are the energy storages of haddock and consist mainly of triacylglycerols (TAG). The NL dominated the liver lipids and constituted more than 94 % of the total amount fatty acids. 2-4 % of the total lipids are polar lipids (PL). The PL are phospholipids and found as membrane lipids. The PL is analyzed as Σ of phosphotidylcholin (PC)/phosphotidylethanolamine (PE) and Σ of phosphotydylserine (PS)/ phosphotidylinisitol (PI). PC and PE are the dominating phospholipids and contribute to more than 80 % of the PL, and PS/PI stands for the rest. The free fatty acids (FFA) contributed to around 1 % of the total fatty acids in the lipids.

We found no large differences in lipid class distribution in haddock liver caught from the four regions. In haddock with low amount of lipid (>20 %), the PL contributed up to 13 % of the total lipids.

Haddock have a typical marine fatty acid profile with high levels of polyunsaturated fatty acids (PUFA). The FA profile in the NL mainly reflects the FA profile of the diet, while the FA profile in the polar lipids (PC/PS and PS/PI) are strictly selected to fill a role in the cell membrane. The NLs are storage lipids and function as a reservoir for energy and for FAs to be incorporated during biosynthesis of phospholipids. The phospholipids are the buildings bricks in the cell membrane and the FA composition in the PL is important to obtain optimal fluidity in the membranes. Some FA like arachidonic acid (20:4 (n-6)) and eicosapentaenoic acid (20:5 (n-3)) are also the precursors of prostaglandins, leukotrienes, and related compounds, which have important roles in inflammation and in the regulation of immunity (Calder, 2008).

It has been found that hydrophobic organic pollutants can affect the lipid composition in phospholipids. Crude oil are showed to change the FA profile in bacterial membranes (Mazzella et al., (2005a and b) and exposure for alkylphenol have been shown to decrease the amount of (n-3) PUFA in the PL of Atlantic cod (Meier et al., 2007a).

The main distribution of fatty acids was similar in haddock from the different regions, but there were also some interesting differences. Fish from Tampen had relatively high levels of 20:4 (n-6) compared with the other groups and the ratio between (n-3)/(n-6) PUFA were significantly lower in NL, FFA and PC/PE, but not PS/PI (Figure 8). This agrees with earlier reports finding lower (n-3)/(n-6) ratio in fillet of haddock from Tampen compared with haddock from the Egersund Bank (Hylland et al., 2006). High levels of 20:4 (n-6) can be associated with increased inflammation and other effects in the immune system (Calder, 2008). Due to increased levels of 20:4 (n-6) in the NL of haddock from Tampen, it is possible that the difference found in the (n-3)/(n-6) ratio of the PL is caused by differences in the diet.

More work is needed to understand the mechanisms behind the effects of oil and other organic pollutants on lipid composition in fish cell membrane, and to establish whether the reduced (n-3)/(n-6) ratio at Tampen is influenced by diet or oil pollutants.

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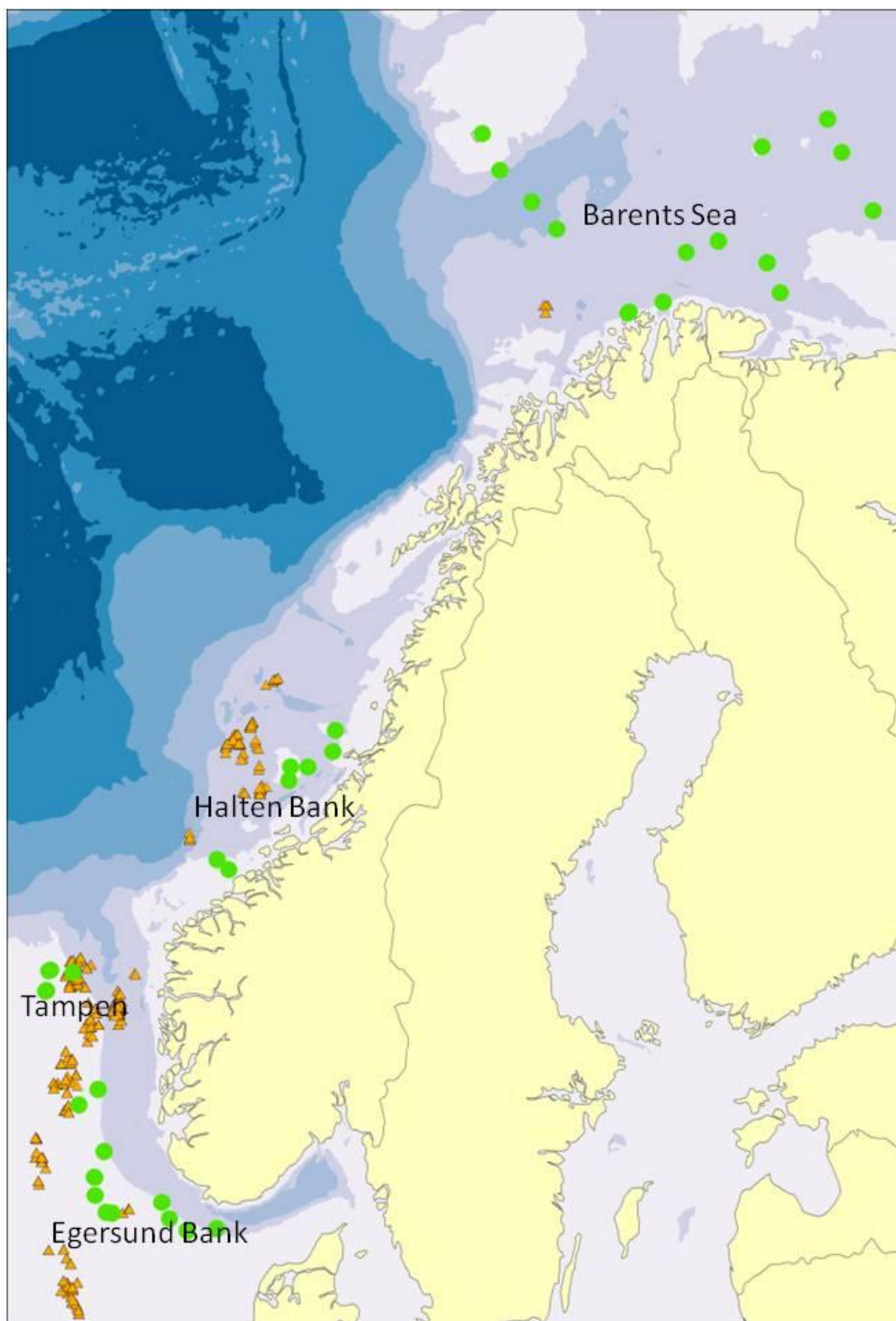


Figure 1. Stations for fish sampling 2008 shown in green circles, oil and gas installation in Norwegian sector in yellow triangles.

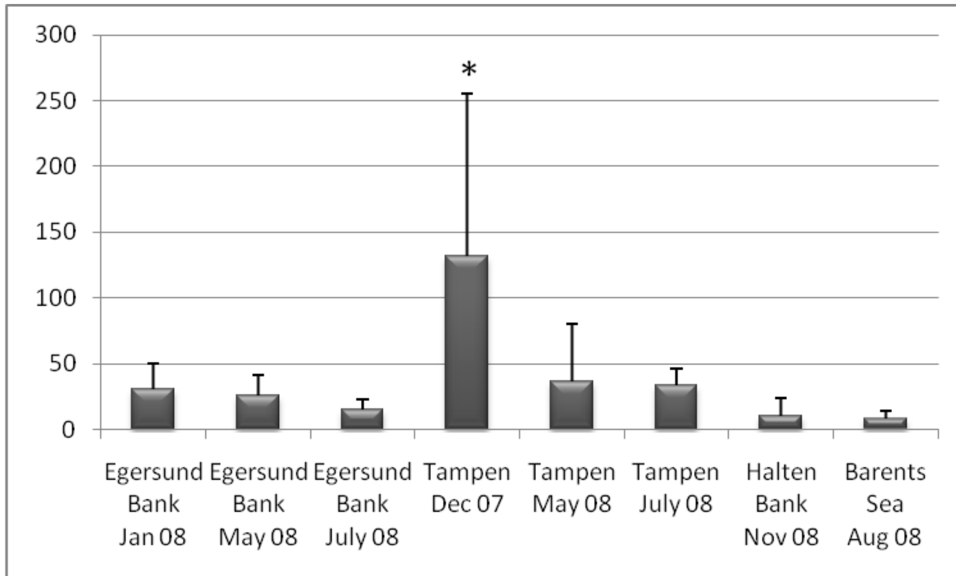


Figure 2. Sum NPD in haddock liver (ng/g wet weight) in Norwegian waters in 2007 and 2008. Haddock sampled at Tampen in December 2007 were caught 6 days after the discharge of 4400 m^3 crude oil at Statfjord. Values given as mean + SD, N=25 per sampling. Graph based on data from Grøsvik et al. (2008, 2009a,b).

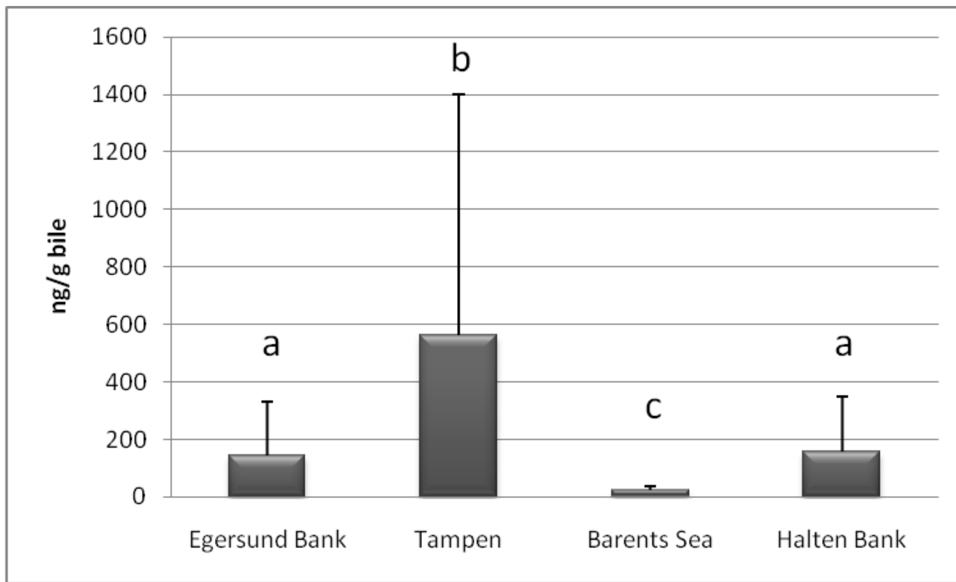


Figure 3. Bile metabolites in haddock bile (ng/g bile). Fish sampled summer and autumn 2008. Values given as mean + SD.

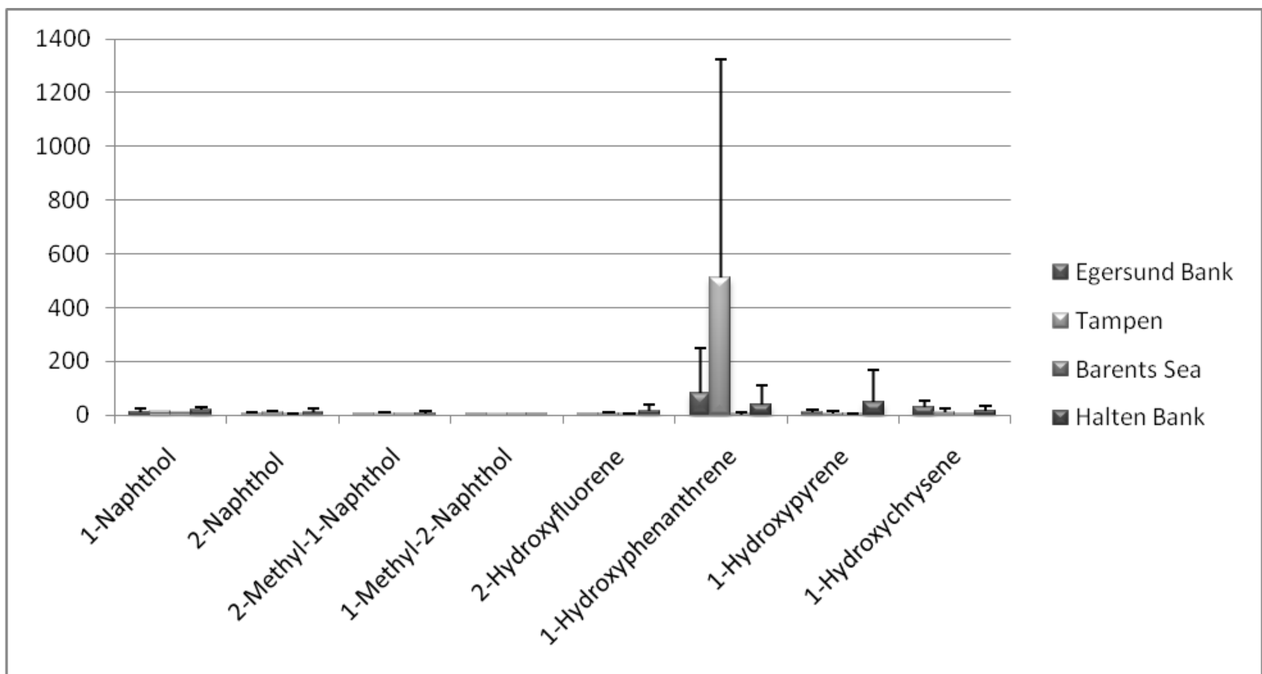
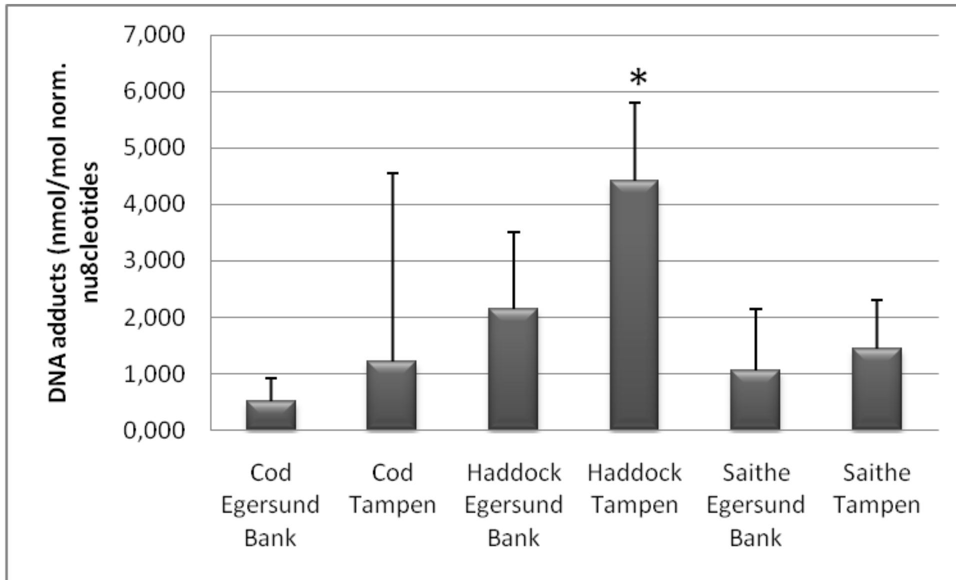
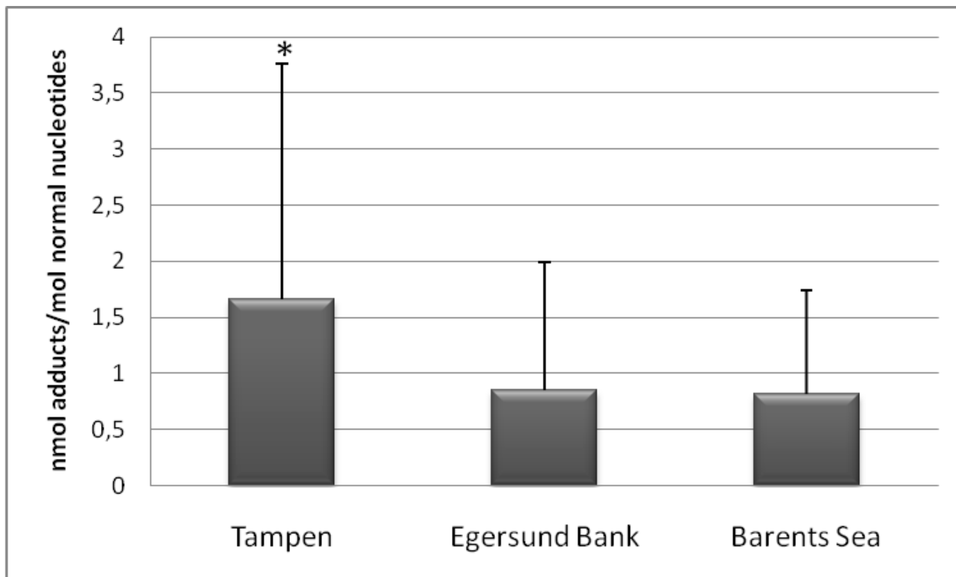


Figure 4. Bile metabolites in haddock sampled 2008.

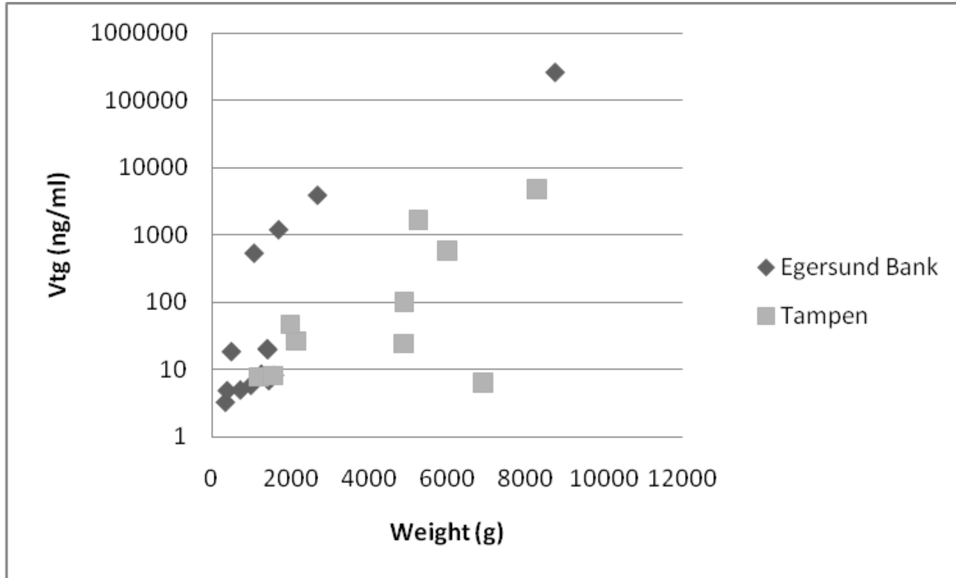


A



B

Figure 5. DNA adduct levels (nmol add/mol normal nucleotides) in liver of fish from A) Cod, haddock and saithe sampled in 2005 and B) haddock sampled in 2008. The bars represent mean + SD, n= 25. Star indicate significant difference from Egersund Bank, $p<0.05$).



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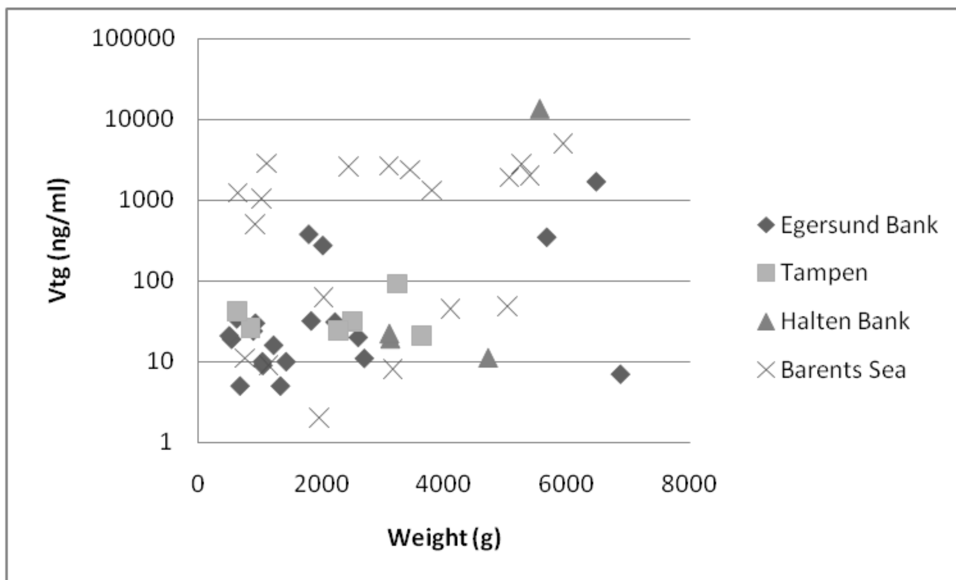

B

Figure 6. Levels of vitellogenin in plasma of male cod plotted against weight, log scale. A) Cod sampled 2005, N=27 B) ocd sampled 2008, N= 55

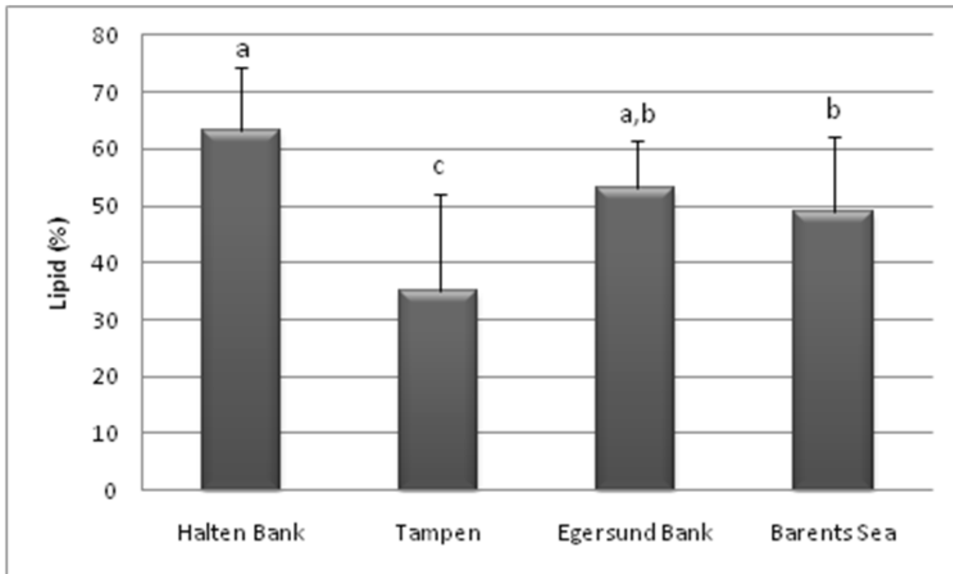

Figure 7. Lipid content of the liver (% of wet wight). Different letters indicate statistical difference p<0.05.

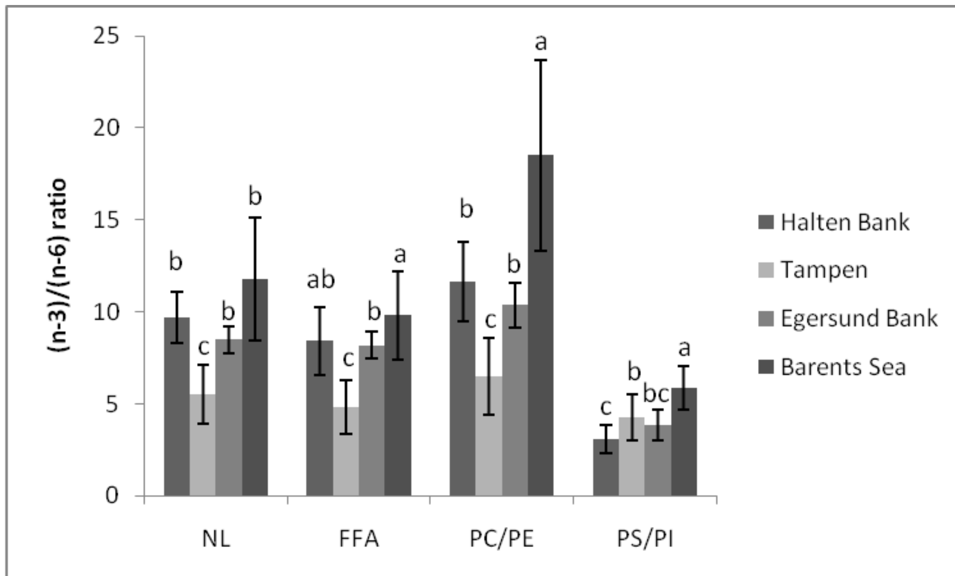

Figure 8. Ratio of (n-3)/(n-6) fatty acids in liver of haddock sampled from the four regions.