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Biomarker Bridges – Biomarker responses to dispersed oil in four marine fish species

Report IRIS - 2011/113

Project number:	7151791
Project title:	Biomarker Bridges
Client(s):	The Research Council of Norway
Research program:	
ISBN:	978-82-490-0737-0
Distribution restriction:	Open

Stavanger 03.10.2011

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Preface

This report presents the results of four laboratory exposures carried out under the Research Council of Norway funded project "Integration of biomonitoring with risk assessment by construction of biomarker bridges for water column organisms exposed to produced water" in the program HAVKYST (PROOFNY).

RCN Project no. 178408/S40.

The principal objective with the exposures was to produce data to construct biomarker response distributions within the assessment tool "Biomarker Bridges" based on species sensitivity distributions for fitness effects and biomarker responses in marine organisms.

A secondary objective was to provide biological response data for biomonitoring of oil based discharges in lipid rich fish species abundant in Norwegian subarctic areas - Atlantic halibut, Atlantic salmon and sprat; (in Norwegian: kveite, laks og brisling) - where oil industry will be operating in the coming years. A comparison with fish of temperate marine areas is provided by exposure of turbot (in Norwegian: piggvar), commonly used as an ecotoxicological "model" fish.

The results in this report will be published in a peer reviewed journal as:

Björkblom, C., H. Jonsson, A. Ingvarsdottir, S. Sanni (*in prep.*). "Quantitative comparison of biomarker responses in different fish species in long-term (controlled laboratory) exposures to crude oil".

Stavanger, 15. December 2011

Steinar Sanni, Project Manager

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1 Materials and Methods

Exposure set-up

Exposure to crude, dispersed oil was performed using a continuous flow-through system (CFS, Sanni et al., 1998) rendering oil droplets sized 5 - 20 µm. Crude Arctic oil was diluted with seawater in a header tank to a nominal concentration of 5 mg/L (5 ppm). The header tank dispersion was then diluted by means of high precision peristaltic pumps (Watson Marlow) to obtain nominal exposure concentrations. The fish was kept in glass fibre tanks (1 x 1 m) filled with 300-320 litres of seawater in a flow-through system (2 litres/minute) with a surface overflow. Seawater was pumped from 80 m depth in Boknafjorden, South-western Norway, to the laboratory and passed through a sand filter before entering the tanks. The fish was exposed to five sub-lethal oil concentrations < 1 ppm and the results were related to unexposed control individuals. The increment of oil with increasing exposure concentrations was by a factor of 2 – 4. Fish were sampled after 0 weeks (reference, 10 individuals), 4 weeks (20 individuals/group) and 8 weeks (10 individuals/group), a total of 190 fish in each experiment. Physical parameters such as water temperature, oxygen levels, salinity and water flow rate were monitored every day. Feeding was cancelled 3-4 days prior to each sampling to allow accumulation of bile inside the gall bladder. Mortality was close to zero in all experiments and could not be related to the exposure concentration. Detailed information about the fish can be found in each individual juvenile fish report.

Sample preparations

Upon sampling, the fish were subjected to analgesic treatment for 3-5 min using tricaine methanesulphonate (MS-222, Sigma) dissolved directly in seawater to a concentration of 50-100 mg/L. This MS-222 concentration did not significantly affect water pH. Fish length and weight were measured. Blood was withdrawn from the caudal vein using a heparin-coated needle and 1 mL syringe. Blood aliquots were taken for hematocrit, haemoglobin and blood cell analyses (smears on glass slide) and plasma was separated by centrifugation at 2500 x g for 5 min at 4 °C. The plasma samples were stored at -80 °C prior to analysis. Gill biopsies were taken before sacrificing the fish by a blow to the head. Gill biopsies and gonads were fixed in 4 % phosphate-buffered formalin for histological examination. Liver and kidney were removed and weighed, followed by immediate flash-freezing on liquid nitrogen. Bile was sampled by puncturing the bile bladder with a needle and withdrawing bile fluid into a syringe. All samples were stored at -80 °C until preparation and analysis.

Water chemistry

Water samples for measurements of oil concentrations and to estimate size and distribution of oil droplets were analysed every week during the exposure experiment by means of the Multisizer™ 3 coulter counter® (Beckman coulter) and the results were treated with the Win-situ® 5 software. Water samples for analysis of PAHs were taken at least twice during the experiment. Exposure concentrations were monitored from 26 different PAH compounds analysed in water samples based on a standard

protocol (EPA 610) with modifications as previously described (Jonsson et al., 2004). Limit of quantification (LOQ) was set to approximately 0.005 µg/L for each PAH component.

Table 1. List of 26 PAHs quantified in water by GC-MS (top). The detection limit of individual compounds was 0.005 – 0.01 ppb in water samples.

PAH family	PAH compound
2-ring membered	Naphthalene
	C1-Naphthalenes
	C2-Naphthalenes
	C3-Naphthalenes
3-ring membered	Acenaphthylene
	Acenaphthene
	Fluorene
	Phenanthrene
	Anthracene
	C1-Phen/Anthr's
	C2-Phen/Anthr's
DBTs	Dibenzothiophene
	C1-Dibenzothiophenes
	C2-Dibenzothiophenes
4-ring membered	Fluoranthene
	Pyrene
	Benzo[a]anthracene
	Chrysene/Triphenylene
	C1-Chrys/Triphen's
	C2-Chrys/Triphen's
5-ring membered	Benzo[b,j]fluoranthene
	Benzo[k]fluoranthene
	Benzo[a]pyrene
6-ring membered	Indeno[1,2,3-cd]pyrene
	Benzo[g,h,i]perylene
	Dibenzo[a,h]anthracene

PAH metabolites in the bile

Method for extraction and quantitative measurement of PAH metabolites in bile was conducted with a GC-MS method as described by Jonsson et al. (2004). Semi-quantitative screening for direct fluorescence detection of PAH-metabolites in bile was performed according to Aas et al. (2000) using a quartz cuvette and a Perkin Elmer LS50B fluorometer. Bile samples were diluted 1:1600 in methanol: water (1:1). The fluorescence screening discriminated between 2, 4- and 5-ring PAH metabolites by using the different wavelength pairs 290/335 nm, 341/383 nm and 380/430 nm, respectively.

Analysis of biotransformation activities

Sub cellular liver microsomal and cytosolic fractions were prepared by differential centrifugations as described by Jonsson et al. (2010), and resulting fractions were stored at -80 °C until use. Hepatic microsomal 7-ethoxyresorufin-*O*-deethylase (EROD) activity was analysed with a Perkin Elmer LS50B fluorometer according to Nilsen et al. (1998) using excitation wavelength 535 nm and emission wavelength 585 nm. Enzyme activities were quantified from known amounts of added resorufin internal standards. A semi-quantitatively measurement of cytochrome P450 1A (CYP1A) was performed by means of an indirect enzyme-linked immunosorbent assay (ELISA) with CYP1A antibodies as described more in details in each juvenile fish report. Hepatic cytosolic glutathione S-transferase (GST) activity towards the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and excess glutathione as co-factor was measured spectrophotometrically at 340 nm (Lambda 2S, Perkin Elmer) based on the method described by Habig et al. (1974). Total protein content of the liver fractions was determined using the Quick Start Bradford Protein Assay (BioRad) as described by the manufacturer and the absorbance was measured spectrophotometrically at 595 nm (Multiskan Ascent, Labsystems). An external bovine serum albumin standard was included for calculation of total protein content. Concentrations of EROD, CYP1A and GST were normalized to the total protein content.

Analysis of reproductive markers

The vitellogenin (Vtg) content of plasma was quantified using an indirect ELISA as described in details in each individual juvenile fish report.

Analysis of genotoxicity markers

DNA adducts were analysed in liver biopsies with the ^{32}P post-labelling technique, according to the description in Aas et al. (2003) and Sundt and Bechmann (2004). Formation of micronuclei (MN) and erythrocytic nuclear abnormalities (ENAs) was analysed in erythrocytes of blood smears. After allowing the blood smears to dry on slides these were fixed with 100% methanol for 10 minutes. Fixed slides were stained with a May-Grünwald-Giemsa solution (Merck) and at least 1000 erythrocytes were analysed microscopically in each sample. The results were expressed as number of abnormalities/1000 scored cells (‰).

Histological analyses

After fixation in 4% phosphate buffered formalin, the gills were dehydrated through a series of graded ethanol solutions (50-99%), cleared in xylene and embedded in paraffin. Tissue sections of 3 µm were cut and the sections were stained with haematoxylin, eosin and saffron. Representative light micrographs were taken with a light microscope (Zeiss Axioplan2) and all micrographs were captured using a digital

colour camera (AxioCam). Analyses of histological changes were performed by visual scoring of coded slides (blind reading).

Statistical Analysis

Exposure groups were analysed for statistical difference to their corresponding control group by means of the Dunnett's test, using the JMP 5.1 software or the PASW Statistics software version 18.0. Groups rendering p-values <0.05 were considered significantly different from the control. For several parameters, raw data were log-transformed prior to running the statistical test. The basic rule was to log-transform data with a high degree of variance (intra- and/or inter-group).

2 Turbot

2.1 Materials & methods

Fish & exposure

Juvenile Turbot (*Scophthalmus maximus*) sized 52 ± 5 g were purchased from Stolt Seafarm AS, 4484 Kvinesdal, Norway. Fish were transported to the laboratory in aerated plastic bags filled with 10-12 litres of seawater and placed inside closed styropor boxes (27 x 27 x 27 cm). The seawater used for transport held 10 – 11 °C to reduce metabolic activity (temperature at fish farm 15 - 16 °C). The biomass in each box was approximately 1.3 kg, corresponding to 110 - 130 g/litre seawater. Total transport time tank-to-tank was approximately 5 h. Fish were fed pellets (same as in fish farm) at a *weekly* feeding rate corresponding to 0.8 % of the biomass in each tank and exposure (November 2007 – January 2008) started after a 15 days acclimatization period. There was no reference sampling of Turbot prior to the exposure. Fish were kept at 10:14 h day:night light cycle. Upon sampling, fish were sacrificed by a sharp blow to the head and blood samples were taken from the caudal vein. Water temperature (10.0 ± 0.6 °C), salinity (33.8 ± 0.7 ‰) and oxygen (10.8 ± 1.3 ppm) were monitored daily and did not differ between exposure tanks.

ELISA assays

CYP1A and vitellogenin (Vtg) ELISA assays were run according to IRIS SOP protocols. CYP1A protein was semi-quantified in hepatic microsomes, after scanning of samples demonstrated significant denaturation of heme protein, rendering quantification of CYP1A enzymatic activity (EROD assay) inappropriate in this experiment. Detection antibodies were from Biosense laboratories (product C10-7, monoclonal mouse-anti-fish CYP1A IgG) and diluted 1:1000 in PBS with 1 % BSA and 0.05 % Tween. Secondary antibodies were from BioRad (HRP conjugate goat-anti-mouse IgG) and diluted 1:3000 in PBS with 1 % BSA and 0.05 % Tween. Semi-quantification of plasma Vtg was made using detection antibodies from Biosense laboratories (product V01411201, polyclonal rabbit-anti-Turbot Vtg IgG) diluted 1:1000 in PBS with 1 % BSA and 0.05 % Tween. Secondary antibodies were from BioRad (HRP conjugate goat-anti-rabbit IgG) and diluted 1:3000 in PBS with 1 % BSA and 0.05 % Tween. In both assays, the colorimetric signal was developed with OPD (Sigma) and the reaction was stopped by adding excess H_2SO_4 (4 N) after a fixed incubation time. The signal was read with a plate reader using a 492 nm filter setting, and the results were reported as the mean absorbance (all samples in quadruplicate) after subtraction of the mean background signal (complete reaction minus detection antibody) of each microplate.

2.2 Results

Table 1 provides an overview of analysed parameters in Turbot. Exposure time proceeding analysis is indicated for each parameter.

Table 1. Analysed parameters in juvenile Turbot sub-divided in biomarker classes. FF = fixed fluorescence screening, GST = glutathione transferase, TBARS = thiobarbituric acid reactive substances, ENAs = erythrocytic nuclear abnormalities, Diff-counts = differential white blood cell counts.

Biomarker class	Core biomarkers (exposure time)	Additional biomarkers (exposure time)
Exposure	* Bile PAH met. by FF (4 weeks) * Bile PAH met. by GC-MS (4 weeks) * Hepatic CYP1A protein (4 weeks)	* Hepatic PAHs (4 weeks)
General stress	* Hematocrit (8 weeks) * Plasma protein (4 weeks)	* Hepatic neutral lipids (4 weeks)
Oxidative stress	* Hepatic GST activity (4 weeks)	* Hepatic lipofuscin (4 weeks) * Hepatic TBARS (4 weeks)
Genotoxicity	* Hepatic DNA adducts (8 weeks)	* Micronuclei (4 & 8 weeks) * ENAs (8 weeks)
Endocrine disruption	* Plasma vitellogenin (4 weeks)	-
Histological effect	* Gill histopathology (8 weeks)	-
Immunotoxicity	-	* Diff-counts (8 weeks)

GC/MS analyses of PAHs in water and biota

Exposure concentrations in the Turbot experiment were estimated from the sum of 26 quantified PAHs in water samples from three separate analyses (Table 2). PAHs with more than three aromatic rings were not detected in any of the tanks, and naphthalenes constituted 89 – 100 % of sum measured PAHs in all tanks receiving oil (results not shown). Table 2 also summarises accumulation of PAHs in liver, and of PAH metabolites in bile after 4 weeks exposure, demonstrating a dose-dependent bioaccumulation of PAHs in exposed fish.

Table 2. Overview of results from GC-MS analyses performed in the Turbot experiment. Mean and standard deviation of sum 26 PAHs in water (ppb) and liver (µg/g) samples, and of sum 9 PAH metabolites in bile samples (µg/g). Biota chemistry was analysed after 4 weeks exposure. n = 3 (water, liver, bile).

Exposure group	Control	Oil I	Oil II	Oil III	Oil IV	Oil V
Sum water PAHs (ppb)	0.00 ± 0.00	0.05 ± 0.05	0.31 ± 0.11	1.47 ± 0.24	4.09 ± 3.09	9.10 ± 1.35
Sum liver PAHs (µg/g)	0.13 ± 0.11	0.55 ± 0.32	1.21 ± 0.37	8.00 ± 2.22	19.05 ± 5.38	29.81 ± 4.53
Sum bile PAH met. (µg/g)	2.35 ± 1.08	6.65 ± 3.70	12.73 ± 3.36	38.28 ± 9.64	163.63 ± 76.47	237.74 ± 33.33

Biomarkers of exposure

Two different biomarkers of exposure were analysed in Turbot after 4 weeks exposure, PAH metabolites in bile (fixed fluorescence screening assay, Fig. 1) and hepatic CYP1A protein (ELISA assay, Fig. 2). A dose-dependent accumulation of bile PAH metabolites was evident and the experimental LOEC was determined to the "Oil II" exposure group, corresponding to 0.31 ppb ∑PAHs in water samples (Table 2). CYP1A protein remained unaffected in all exposure groups except the highest group (Oil V), where individuals expressed significantly higher levels than in the control group (Fig. 2).

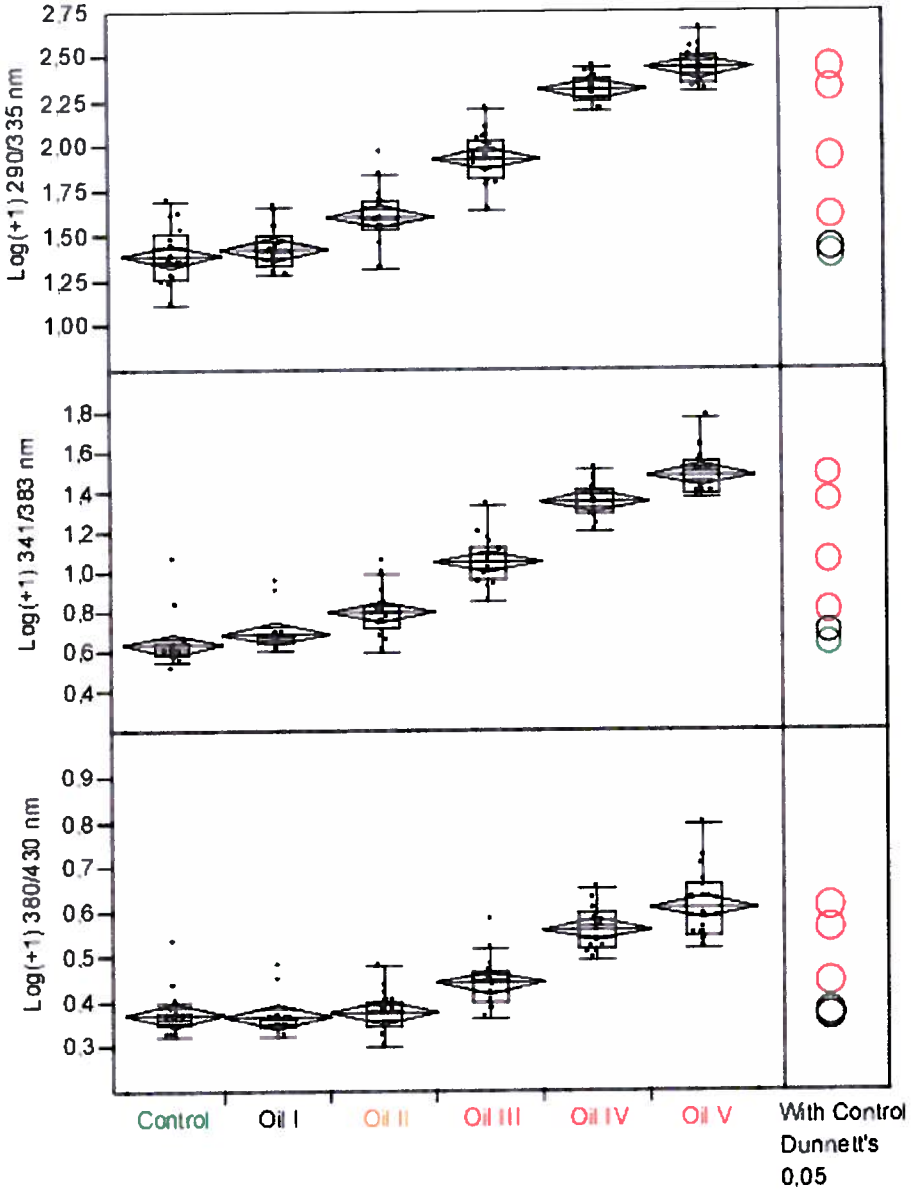

Fig. 1. Bile PAH metabolites by fixed fluorescence screening after 4 weeks exposure. 2-3 ring metabolites (top panel), 4 ring metabolites (middle panel) and 5 ring metabolites (bottom panel). Logarithmic data. n = 20.

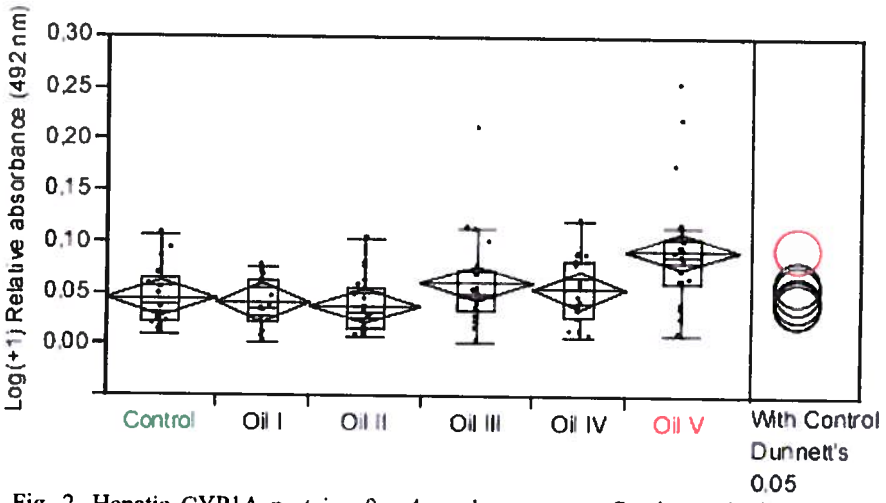

Fig. 2. Hepatic CYP1A protein after 4 weeks exposure. Semi-quantitative data from microsome subfractions. Logarithmic data. n = 20.

Biomarkers of general stress

Two different biomarkers of general stress were analysed in Turbot after 4 weeks exposure. While plasma protein dropped significantly in the three highest exposure groups (Oil III – V) and showed an overall negative trend in response to oil (Fig. 3), hepatic neutral lipid levels did not change as a result of exposure (subset of samples, Fig. 4). Furthermore, hematocrit was analysed after 8 weeks exposure and displayed insignificant increases in the three highest exposure groups (Oil III – V, Fig. 5).

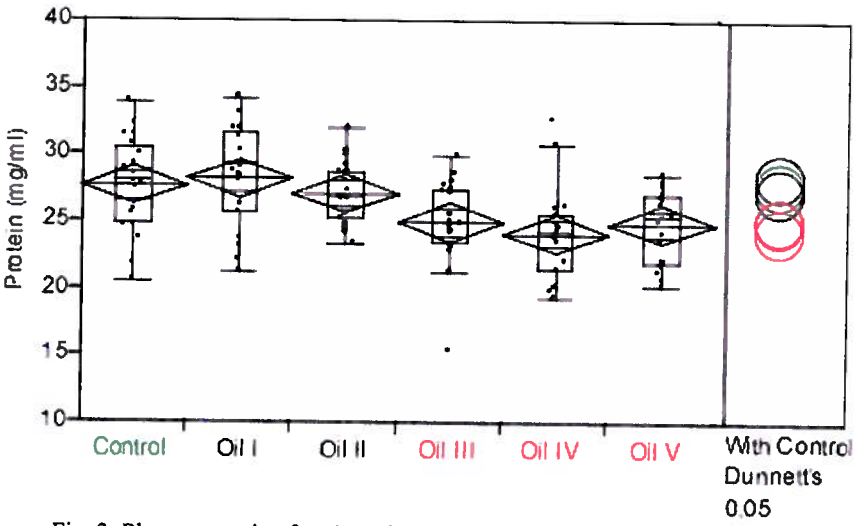

Fig. 3. Plasma protein after 4 weeks exposure. n = 20.

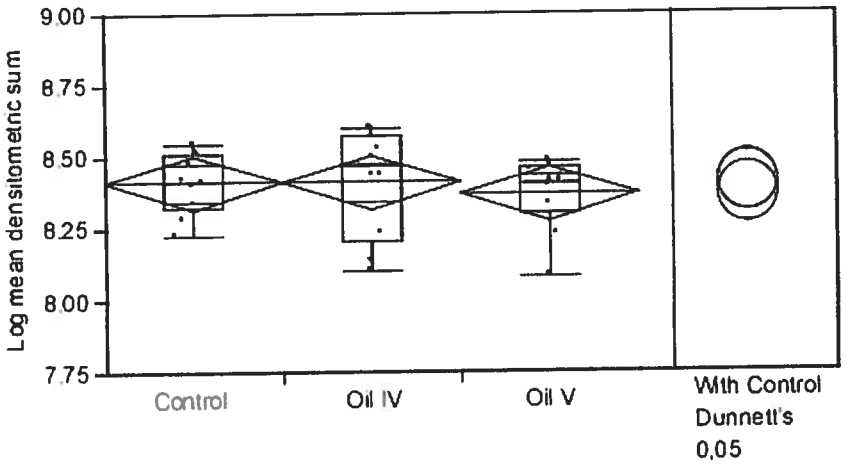

Fig. 4. Hepatic neutral lipids after 4 weeks exposure. Logarithmic data. n = 10.

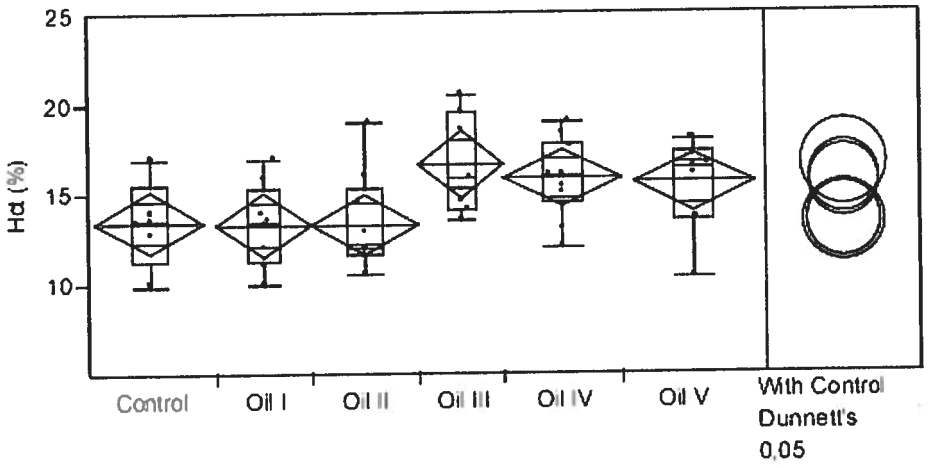

Fig. 5. Hematocrit after 8 weeks exposure. n = 10.

Biomarkers of oxidative stress

Three different parameters of oxidative stress were analysed in Turbot after 4 weeks exposure but none of the three showed significant responses to oil. Hepatic GST activity in cytosolic samples was lower than in the other experiments by a factor of 5 – 10 and did not show any trends that could be linked to the exposure (Fig. 6). Hepatic lipofuscin and thiobarbituric acid reactive substances (TBARS), such as malondialdehyde, were analysed in a subset of samples (control and the two highest exposure groups). While there was an insignificant trend of lipofuscin accumulation in hepatocytes of fish from the highest exposure group (Oil V, Fig. 7), cytosolic TBARS did not increase as a result of the exposure (Fig. 8).

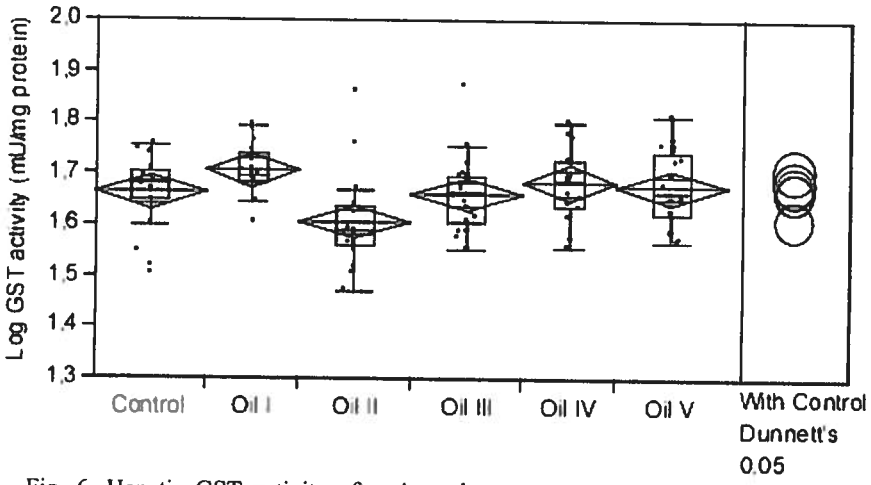

Fig. 6. Hepatic GST activity after 4 weeks exposure. Logarithmic data from cytosolic samples. n = 20.

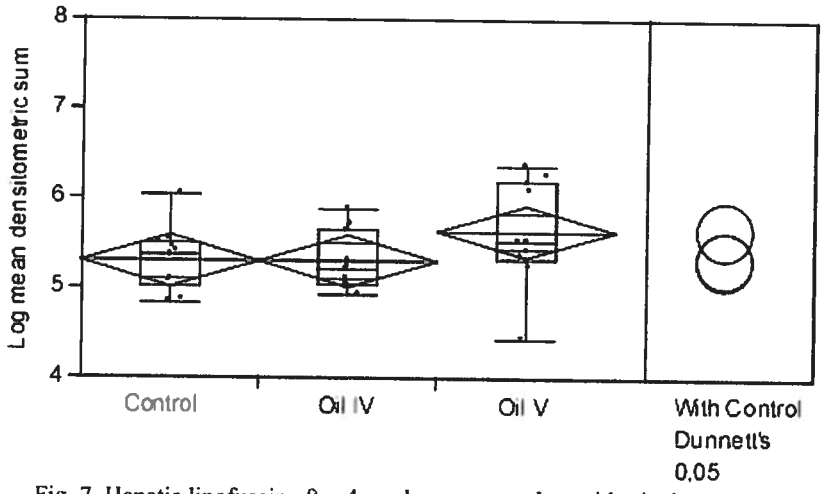

Fig. 7. Hepatic lipofuscin after 4 weeks exposure. Logarithmic data. n = 10.

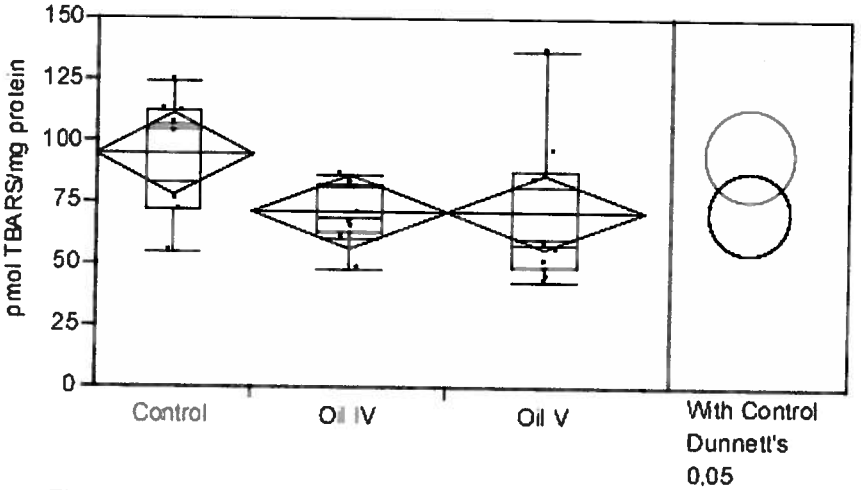

Fig. 8. Hepatic TBARS after 4 weeks exposure. Cytosolic samples. n = 10.

Biomarkers of genotoxicity

A dose-dependent increase of hepatic DNA adducts was seen after 8 weeks exposure in the three highest exposure groups (Oil III – Oil V), with the highest group significantly different from the control group (Fig. 9). The micronuclei frequency in erythrocytes was not induced in exposed groups after 4 and 8 weeks. Instead, significantly lower frequencies were occasionally scored in exposed groups after 8 weeks (Fig. 10). Erythrocytic nuclear abnormalities (ENAs) were analysed in a subset of samples after 8 weeks exposure, demonstrating a dose-dependent increase of abnormalities that was significant in the two highest exposure groups (Oil IV – V, Fig. 11).

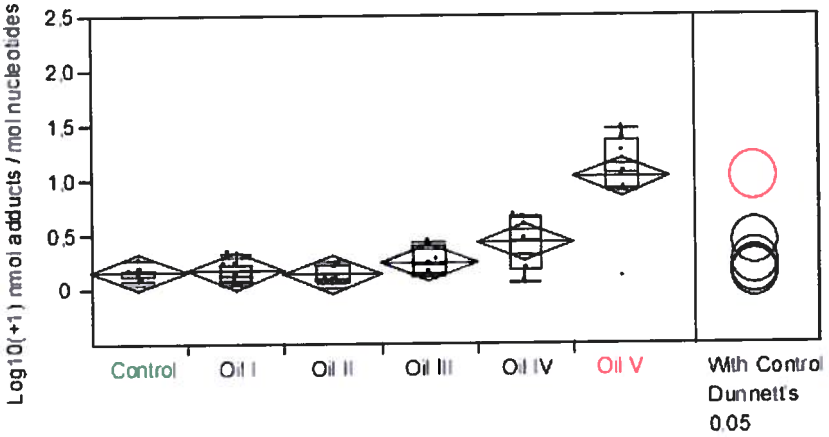

Fig. 9. Hepatic DNA adducts after 8 weeks exposure. Logarithmic data. n = 7.

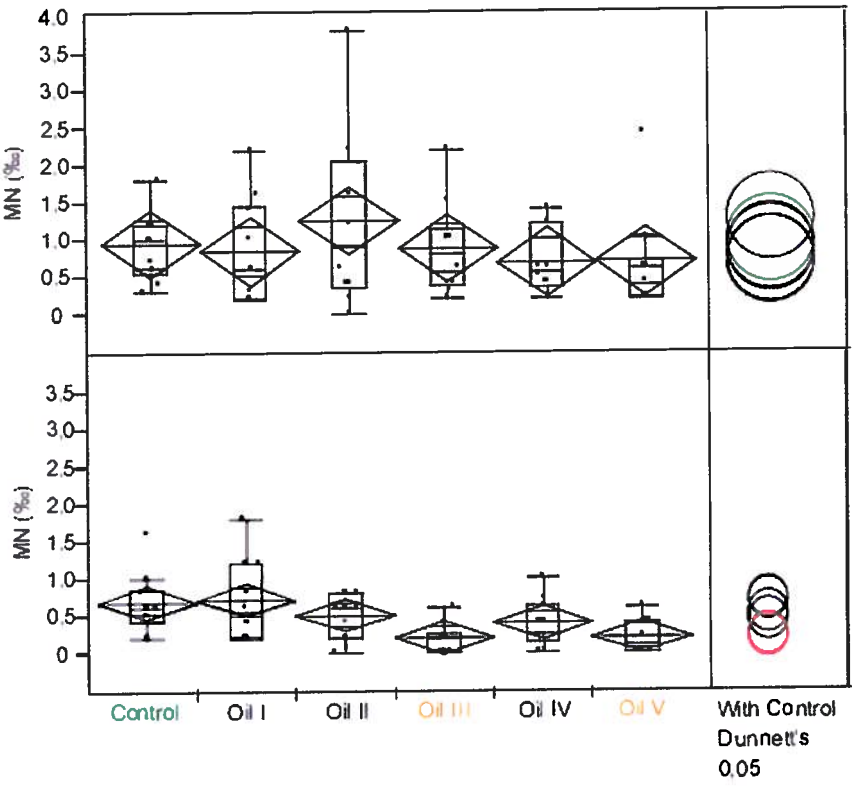

Fig. 10. Micronuclei frequency in erythrocytes. 4 weeks exposure (top panel), 8 week exposure (bottom panel). n =10.

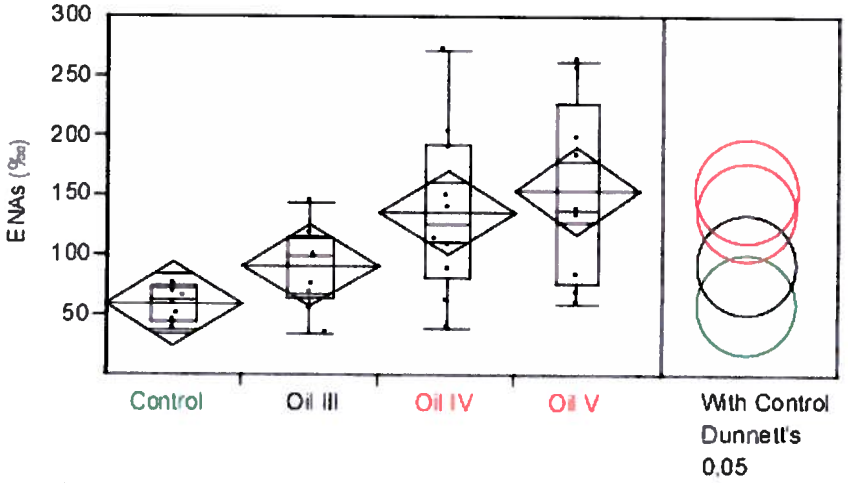

Fig. 11. Erythrocytic nuclear abnormalities after 8 weeks exposure. n =10.

Biomarkers of endocrine disruption

An insignificant trend of dropping plasma vitellogenin levels was seen in exposed groups (Fig. 12), showing that used dilutions had no measurable estrogenic effect on juvenile Turbot after 4 weeks exposure.

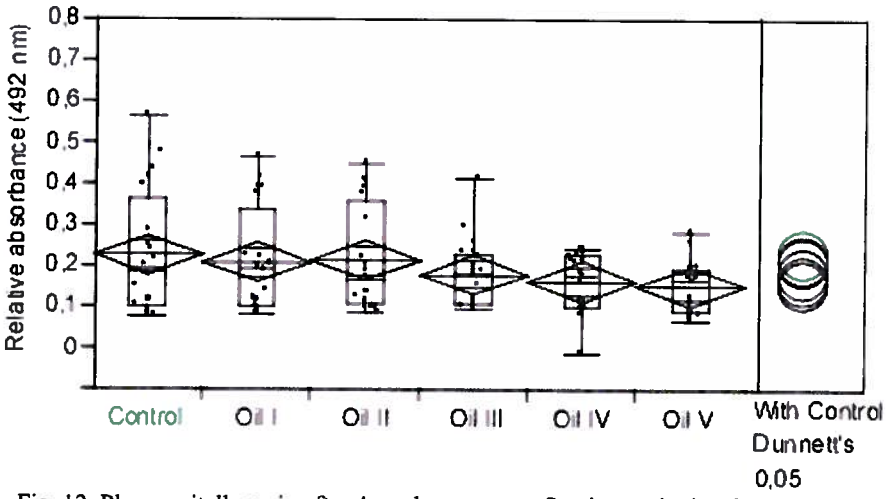

Fig. 12. Plasma vitellogenin after 4 weeks exposure. Semi-quantitative data. n = 20.

Biomarkers of immunotoxicity

Immunotoxicity was evaluated from differential white blood cell counts after 8 weeks exposure. The results showed a drop in the relative concentration of granulocytes (= phagocytic cells) in all exposed groups. In four of five exposed groups the difference was significant from the control group (Fig. 13)

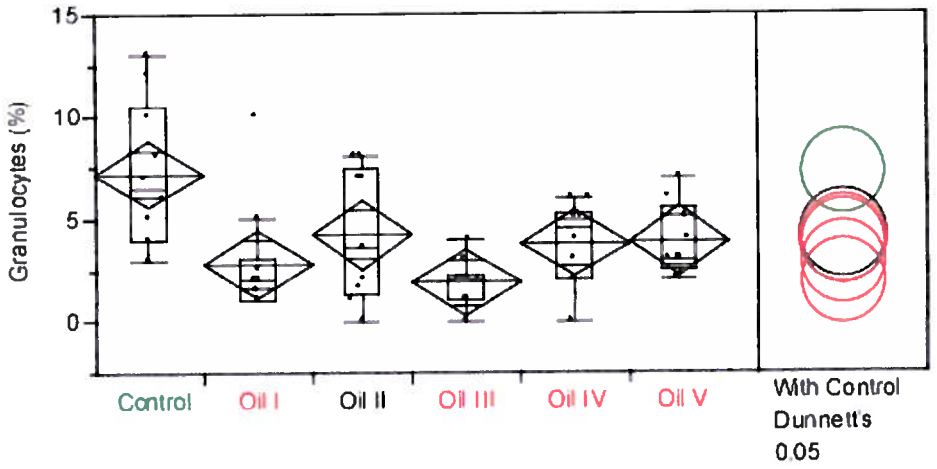

Fig. 13. Differential white blood cell counts expressed as abundance of granulocytes after 8 weeks exposure. n = 10.

Histological effects

The evaluation of histological abnormalities of Turbot gill tissue after 8 weeks exposure did not show clear effects that could be linked to oil exposure. Three abnormalities, epithelial thickening, lamellar fusion and proliferation of epithelial cells were common in all groups, including the control.

3 Halibut

3.1 Materials & methods

Fish & exposure

Juvenile Atlantic Halibut (*Hippoglossus hippoglossus*) sized 57.9 ± 11.5 g were purchased from Marine Harvest, 5583 Vikedal, Norway. Fish were transported to the laboratory in aerated plastic bags filled with 14-16 litres of seawater and placed inside closed styropor boxes (27 x 27 x 27 cm). The seawater used for transport held 7 - 8 °C to reduce metabolic activity (temperature at fish farm 11 °C). The biomass in each transport box was approximately 1.6 kg, corresponding to 100-110 g/litre seawater. Total transport time tank-to-tank was approximately 4 h. Fish were fed pellets (same as in fish farm) at a *weekly* feeding rate corresponding to 0.6 % of the biomass in each tank and exposure (April – June 2008) started after a 14 days acclimatization period. Fish were kept at a 10:14 h day:night light cycle. Fish were sampled from exposure tanks in batches of five individuals and subjected to analgesic treatment with 5 ppm metomidate for 5-10 min before withdrawing a blood sample from the caudal vein and taking biopsies from the gills. Fish was then sacrificed by a sharp blow to the head and cut open for dissection of the organs of interest. The experiment on Halibut was run on cold water to mimic Arctic/sub-Arctic conditions. Water temperature (5.4 ± 0.2 °C), salinity (34.7 ± 0.4 ‰) and oxygen (9.3 ± 0.5 ppm) were monitored daily and did not differ between exposure tanks.

ELISA assays

Vitellogenin (Vtg) ELISA assays were run according to a IRIS SOP protocol. Semi-quantification of plasma Vtg was made using detection antibodies from Biosense laboratories (product V01411201, polyclonal rabbit-anti-Turbot Vtg IgG) diluted 1:1000 in PBS with 1 % BSA and 0.05 % Tween. Secondary antibodies were from BioRad (HRP conjugate goat-anti-rabbit IgG) and diluted 1:3000 in PBS with 1 % BSA and 0.05 % Tween. The colorimetric signal was developed with OPD (Sigma) and the reaction was stopped by adding excess H_2SO_4 (4 N) after a fixed incubation time. The signal was read with a plate reader using a 492 nm filter setting, and the results were reported as the mean absorbance (all samples in quadruplicate) after subtraction of the mean background signal (complete reaction minus detection antibody) of each microplate.

3.2 Results

Table 1 provides an overview of analysed parameters in Halibut. Exposure time proceeding analysis is indicated for each parameter.

Table 1. Analysed parameters in juvenile Halibut sub-divided in biomarker classes. EROD = ethoxyresorufin-*o*-deethylase, GST = glutathione transferase, TBARS = thiobarbituric acid reactive substances, ENAs = erythrocytic nuclear abnormalities, Diff-counts = differential white blood cell counts.

Biomarker class	Core biomarkers (exposure time)	Additional biomarkers (exposure time)
Exposure	* Bile PAH met. by FF (4 weeks) * Bile PAH met. by GC-MS (4 weeks) * Hepatic EROD activity (4 weeks)	* Hepatic PAHs (4 weeks)
General stress	* Hematocrit (4 & 8 weeks) * Plasma protein (4 weeks)	* Hemoglobin (4 & 8 weeks) * Hepatic neutral lipids (4 weeks) * Hepatic LMS (4 weeks)
Oxidative stress	* Hepatic GST activity (4 weeks)	* Hepatic lipofuscin (4 weeks) * Hepatic TBARS (4 weeks)
Genotoxicity	* Hepatic DNA adducts (8 weeks)	* Micronuclei (4 & 8 weeks) * ENAs (4 & 8 weeks)
Endocrine disruption	* Plasma vitellogenin (4 weeks)	-
Histological effect	* Gill histopathology (8 weeks)	* Liver histopathology (8 weeks)
Immunotoxicity	-	* Diff-counts (4 & 8 weeks)

GC/MS analyses of PAHs in water and biota

Exposure concentrations in the Halibut experiment were estimated from 26 quantified PAH compounds in water samples from three sampling times during the experiment (Table 2). PAHs with more than three aromatic rings were not detected in any of the tanks, and naphthalenes constituted 90 – 100 % of sum measured PAHs in all tanks receiving oil (results not shown). Table 2 also summarises accumulation of PAHs in liver, and of PAH metabolites in bile after 4 weeks exposure, demonstrating a dose-dependent bioaccumulation of PAHs in exposed fish. PAH concentrations in the water of the two highest exposure tanks (Oil IV - V) were approximately 50 % of what was measured in the Turbot experiment, whereas lower PAH bioaccumulation levels (liver and bile) were seen in the three highest exposures (Oil I - III), in relation to the Turbot experiment.

Table 2. Overview of results from GC-MS analyses performed in the Halibut experiment. Mean and standard deviation of sum 26 PAHs in water (ppb) and liver (µg/g) samples, and of sum 9 PAH metabolites in bile samples (µg/g). Biota chemistry was analysed after 4 weeks exposure. n = 3 (water, liver, bile).

Exposure group	Control	Oil I	Oil II	Oil III	Oil IV	Oil V
Sum water PAHs (ppb)	0.00 ± 0.00	0.05 ± 0.05	0.33 ± 0.15	1.14 ± 0.72	1.78 ± 0.84	5.14 ± 0.87
Sum liver PAHs (µg/g)	0.00 ± 0.00	0.53 ± 0.28	1.43 ± 0.39	2.82 ± 1.13	6.68 ± 4.44	12.71 ± 3.15
Sum bile PAH met. (µg/g)	2.90 ± 1.97	4.84 ± 4.33	9.25 ± 3.08	11.17 ± 2.56	52.37 ± 28.49	68.93 ± 23.18

Biomarkers of exposure

Two different biomarkers of exposure were analysed in Halibut after 4 weeks exposure, PAH metabolites in bile (fixed fluorescence screening assay, Fig. 1) and hepatic CYP1A activity (EROD assay, Fig. 2). Both assays showed a dose-dependent induction and the experimental LOEC was determined to the "Oil II" group, corresponding to 0.41 ppb PAHs in water samples (Table 2), and underlining the mechanistic link between these two parameters.

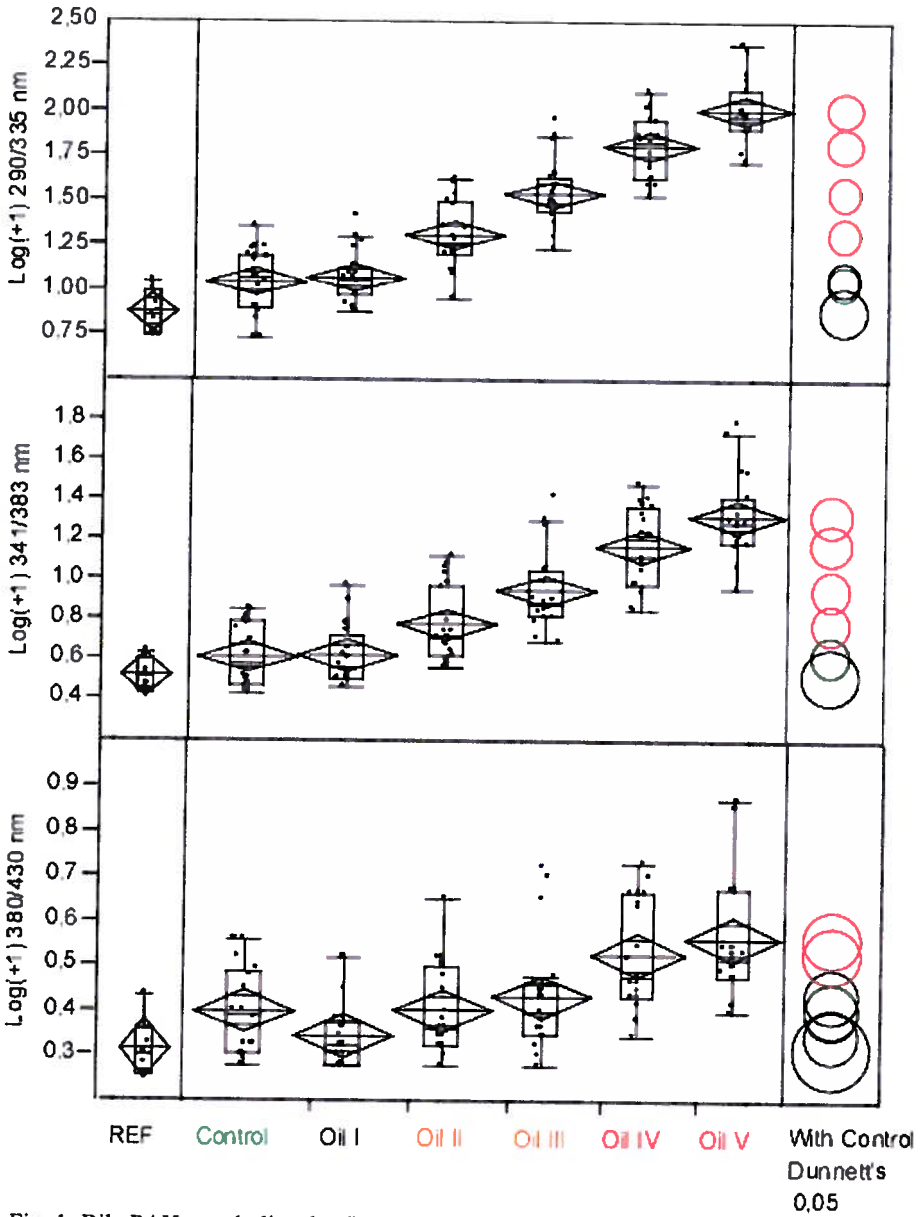

Fig. 1. Bile PAH metabolites by fixed fluorescence screening after 4 weeks exposure. 2-3 ring metabolites (top panel), 4 ring metabolites (middle panel) and 5 ring metabolites (bottom panel). Logarithmic data. n = 20 (n reference = 10).

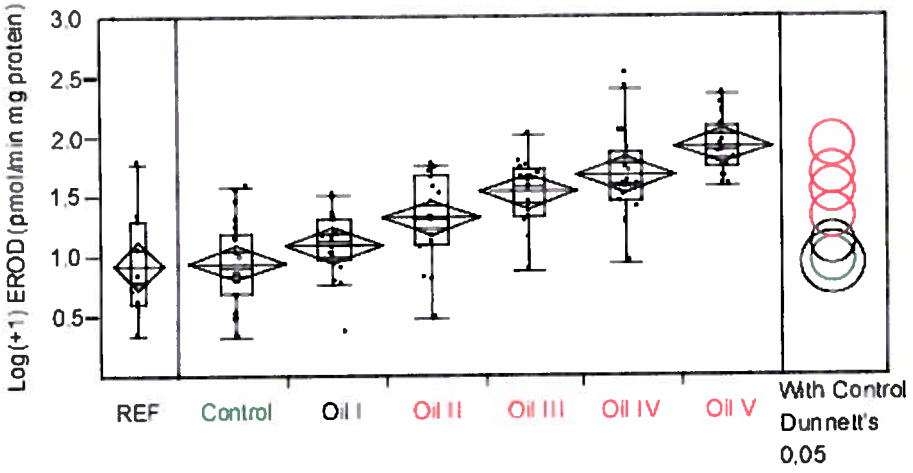

Fig. 2. Hepatic CYP1A activity by EROD after 4 weeks exposure. Data from microsome subfractions. Logarithmic data. n = 20 (n reference = 10).

Biomarkers of general stress

Five different biomarkers of general stress were analysed in Halibut; plasma protein (4 weeks exposure), hematocrit (4 & 8 weeks), haemoglobin (4 & 8 weeks), hepatic neutral lipids (4 weeks) and hepatic lysosomal membrane stability. None of the parameters showed conclusive results that could be linked to exposure. For plasma protein, significantly lower protein in the lowest exposure group ("Oil I") was paralleled by significantly higher levels in "Oil IV" (Fig. 3). Both hematocrit (Fig. 4) and haemoglobin (Fig. 5) showed a negative trend in exposed fish, but these could not be confirmed statistically. For the two hepatic parameters, negative trends were seen in the highest exposure group for neutral lipids (Fig. 6), and in the two highest exposure groups for lysosomal membrane stability (Fig. 7), but neither of these trends passed the statistical test. The results also showed that general stress did not increase during exposure, as evidenced from the reference individuals.

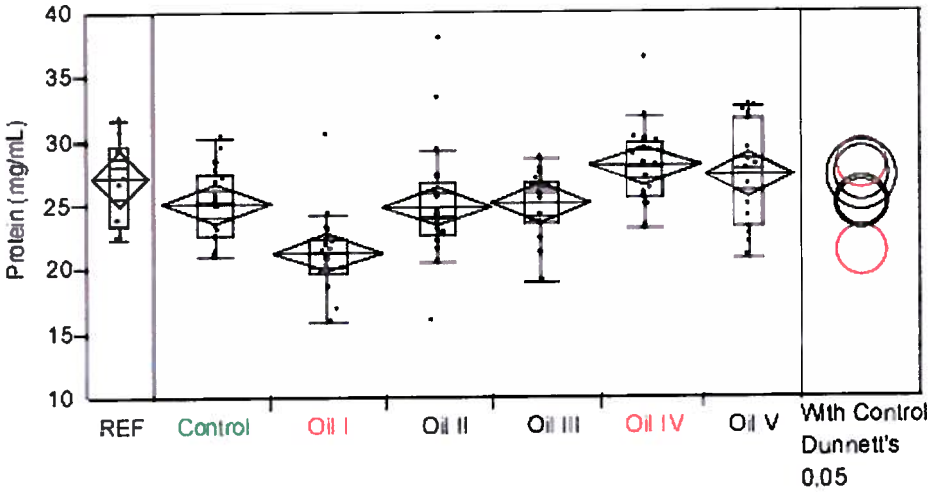

Fig. 3. Plasma protein after 4 weeks exposure. n = 20 (n reference = 10).

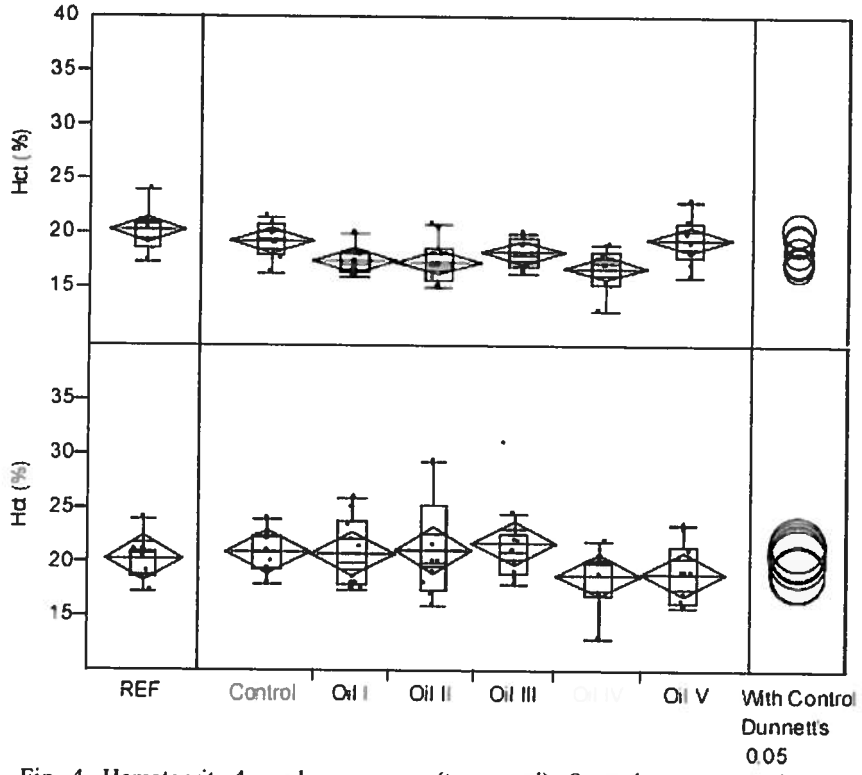

Fig. 4. Hematocrit. 4 weeks exposure (top panel), 8 weeks exposure (bottom panel). n = 20 (4 weeks), n = 10 (reference, 8 weeks).

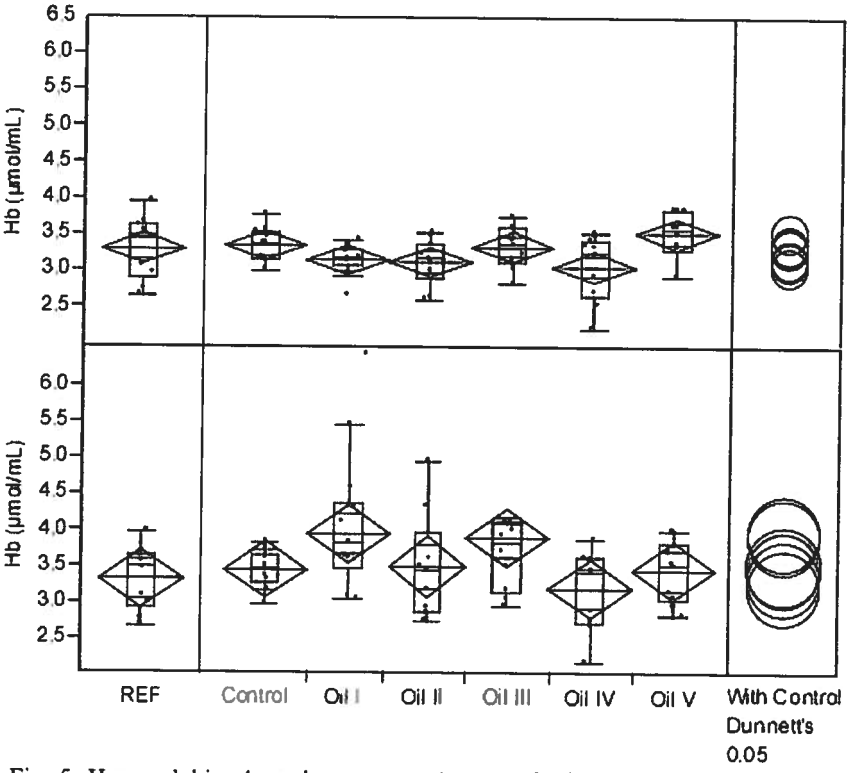

Fig. 5. Haemoglobin. 4 weeks exposure (top panel), 8 weeks exposure (bottom panel). n = 20 (4 weeks), n = 10 (reference, 8 weeks).

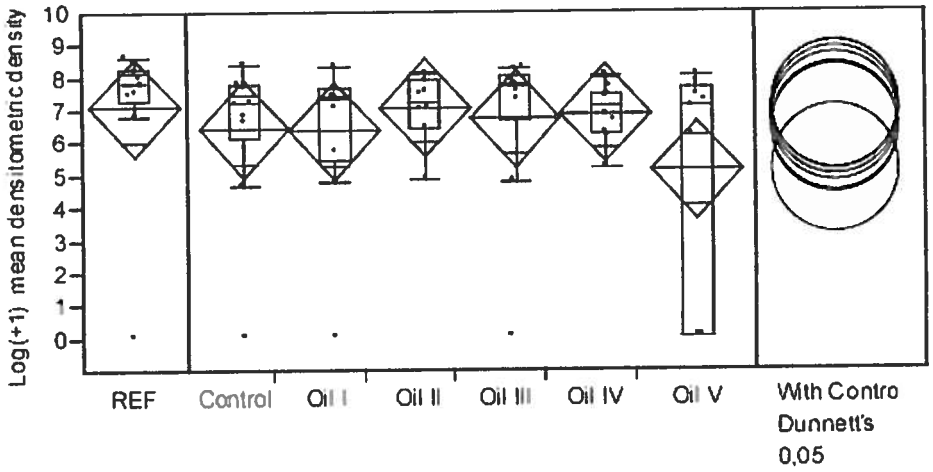

Fig. 6. Hepatic neutral lipids after 4 weeks exposure. Logarithmic data. n = 10.

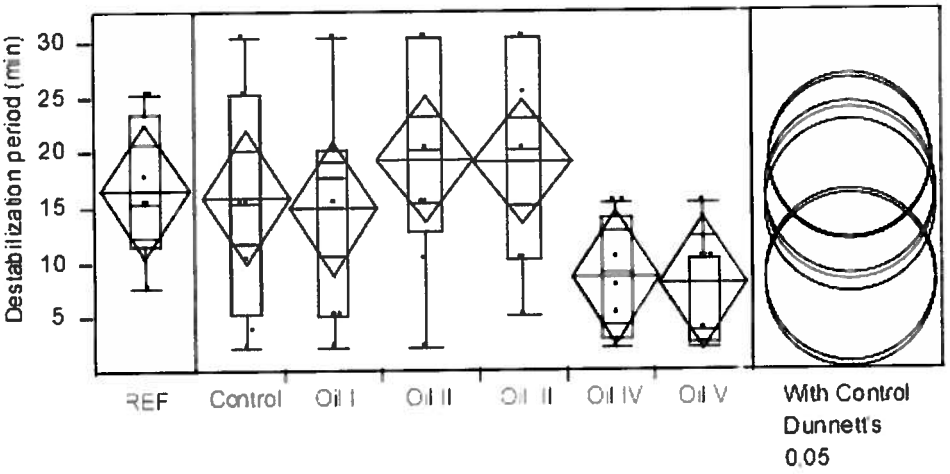

Fig. 7. Hepatic lysosomal membrane stability after 4 weeks exposure. Destabilization time in minutes. n = 10

Biomarkers of oxidative stress

Three different biomarkers of oxidative stress were analysed in Halibut after 4 weeks exposure. While hepatic GST activity increased in all exposed groups and significantly in two of them (Fig. 8), there were no signs of oxidative damage in the liver of exposed fish, as evidenced by the lipofuscin assay (Fig. 9) and the TBARS assay (Fig. 10), indicating that induction of GST (and other antioxidant enzymes) helped to prevent oxidative damage in exposed fish. The TBARS assay was performed on a subset of samples from the control group and the highest exposure group ("Oil V").

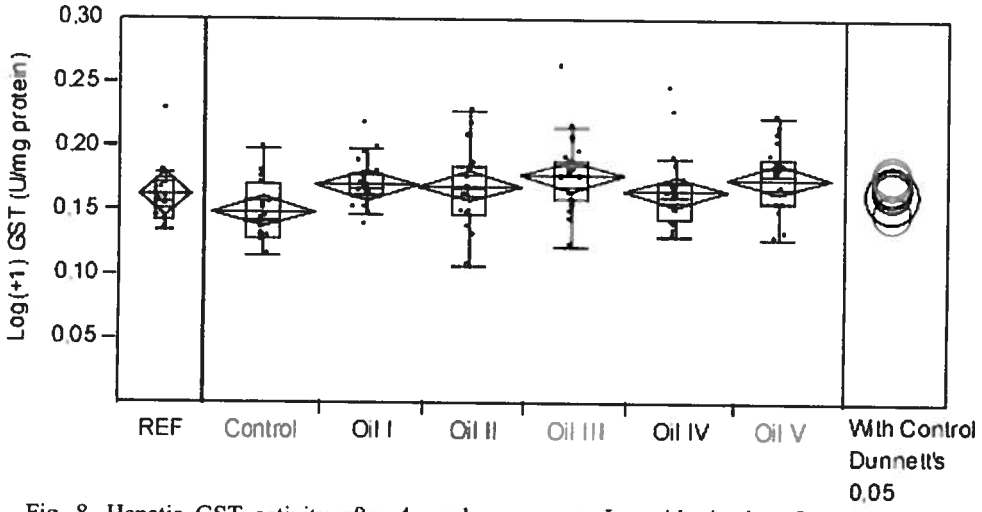

Fig. 8. Hepatic GST activity after 4 weeks exposure. Logarithmic data from cytosolic samples. n = 20 (n reference = 10).

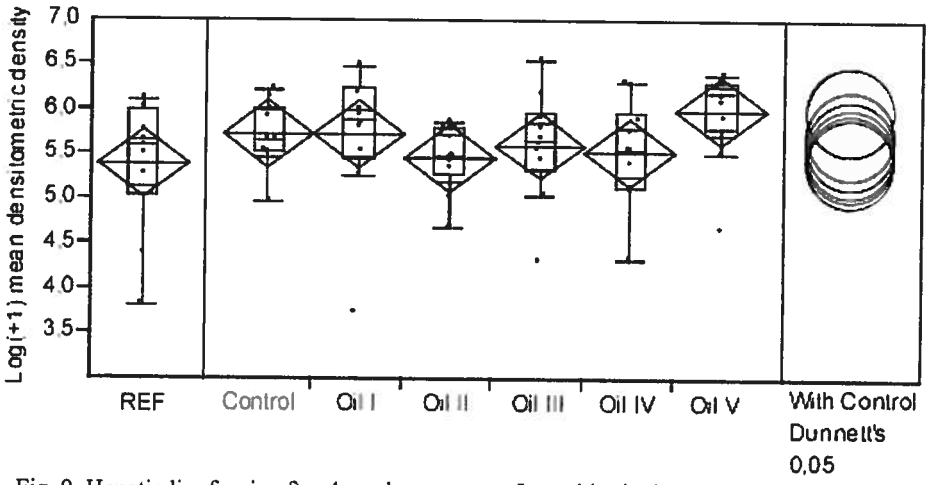

Fig. 9. Hepatic lipofuscin after 4 weeks exposure. Logarithmic data. n = 10.

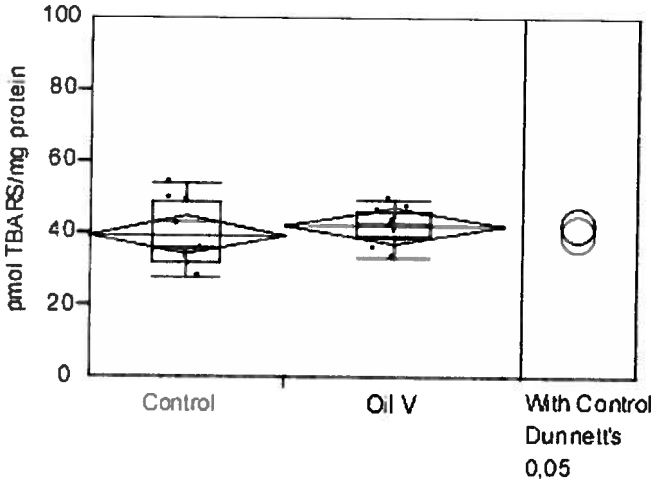

Fig. 10. Hepatic TBARS after 4 weeks exposure. Cytosolic samples. n = 10.

Biomarkers of genotoxicity

Among the three biomarkers of genotoxicity analysed in Halibut, hepatic DNA adducts showed the most conclusive results. A dose-dependent increase of DNA adducts was seen in the three highest exposure groups (Oil III – Oil V), with the two highest groups being significantly different from the control group (Fig. 11), and underlining the mechanistic link between this parameter and CYP1A activity (Fig. 2). For the two parameters analysed in erythrocytes, micronuclei and erythrocytic nuclear abnormalities (ENAs) showed increasing trends after 4 weeks exposure that were partly significant from the control group. After 8 weeks, however, the results could not be linked to the exposure, indicating that fish managed to compensate for this effect with the proceeding of the experiment (Figs 12-13).

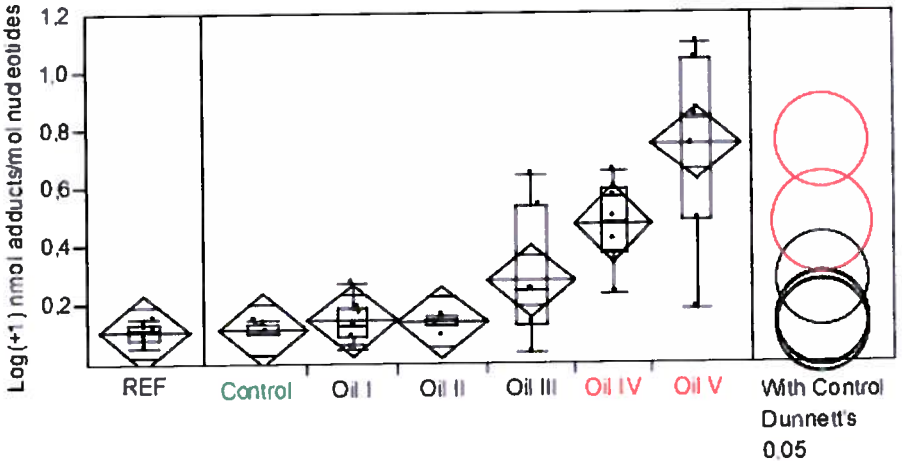

Fig. 11. Hepatic DNA adducts after 8 weeks exposure. Logarithmic data. n = 7.

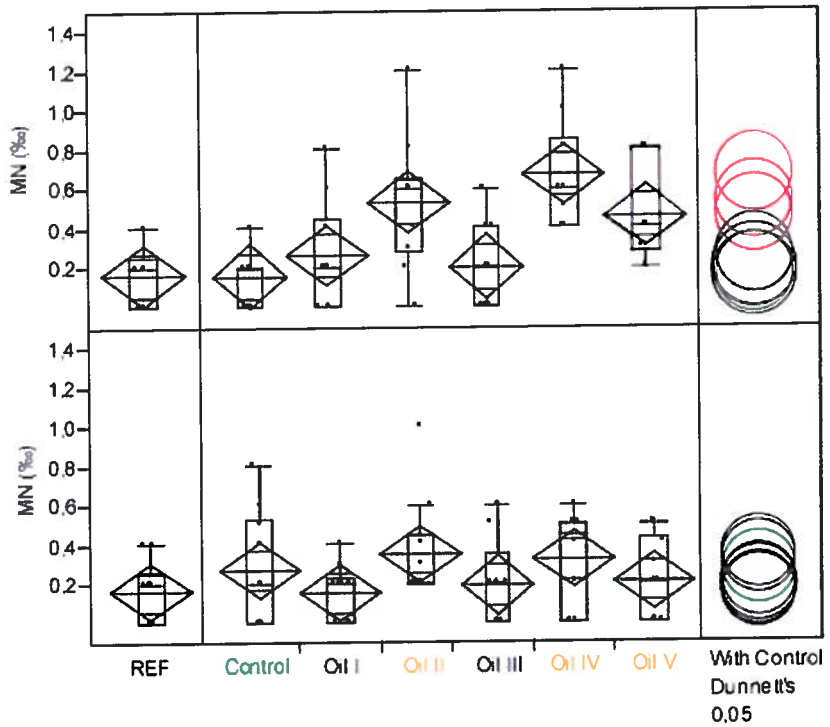

Fig. 12. Micronuclei frequency in erythrocytes. 4 weeks exposure (top panel), 8 week exposure (bottom panel). n =10.

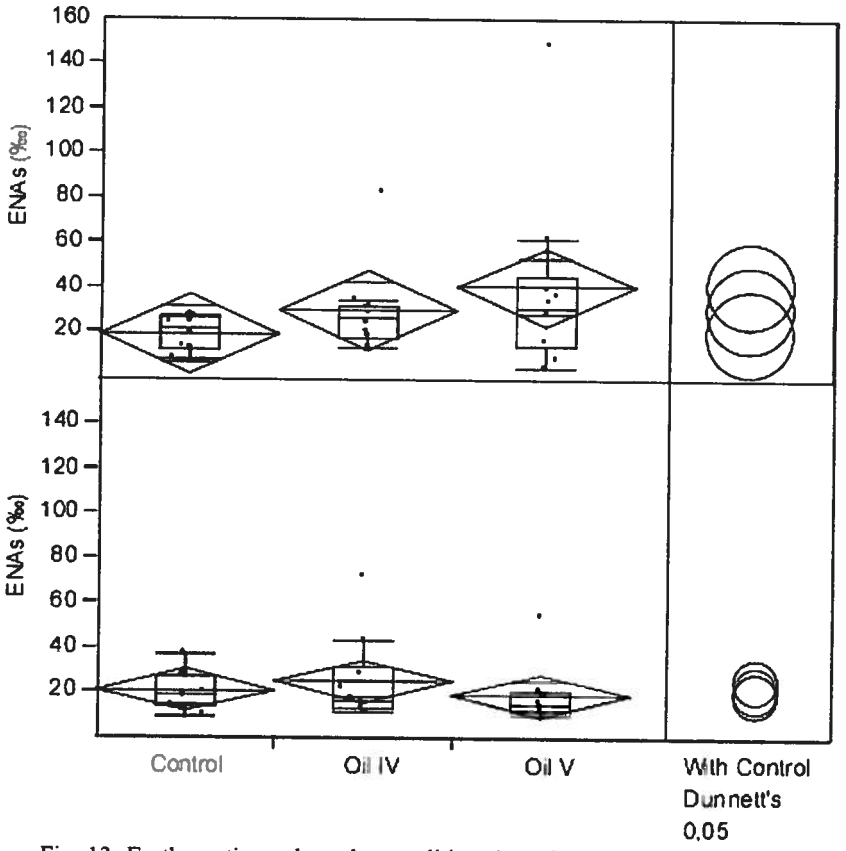

Fig. 13. Erythrocytic nuclear abnormalities. 4 weeks exposure (top panel), 8 week exposure (bottom panel). n =10.

Biomarkers of endocrine disruption

The exposure did not give rise to induction of the egg protein vitellogenin, analysed in plasma samples (Fig. 14), showing that used dilutions had no measurable estrogenic effect on juvenile Halibut after 4 weeks exposure.

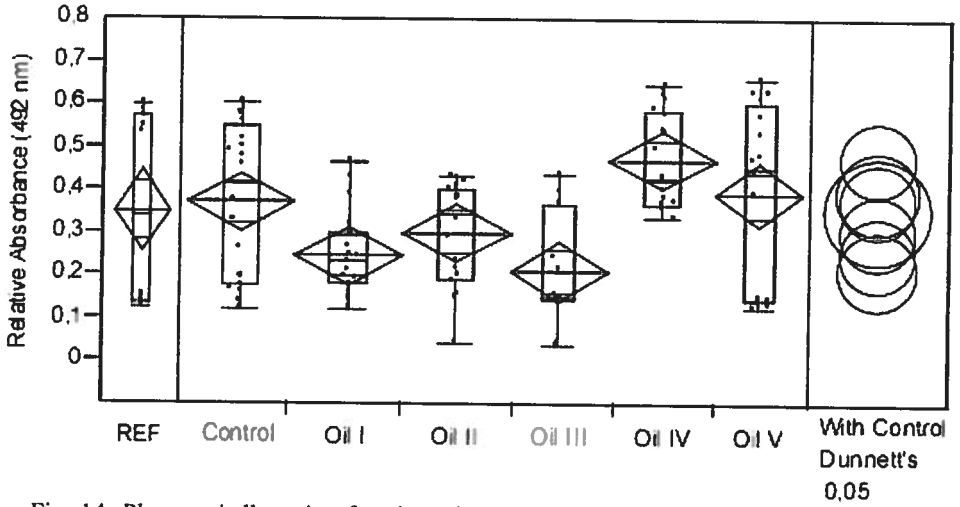

Fig. 14. Plasma vitellogenin after 4 weeks exposure. Semi-quantitative data. n = 20 (n reference = 10).

Biomarkers of immunotoxicity

Immunotoxicity was evaluated from differential white blood cell counts after 4 and 8 weeks exposure. While a dose-dependent drop in relative granulocyte levels was seen after 4 weeks, with the highest exposure group being significantly different from the control group, the results could not be linked to the exposure after 8 weeks, indicating that exposed specimens managed to compensate for this effect with the proceeding of the experiment (Fig. 15).

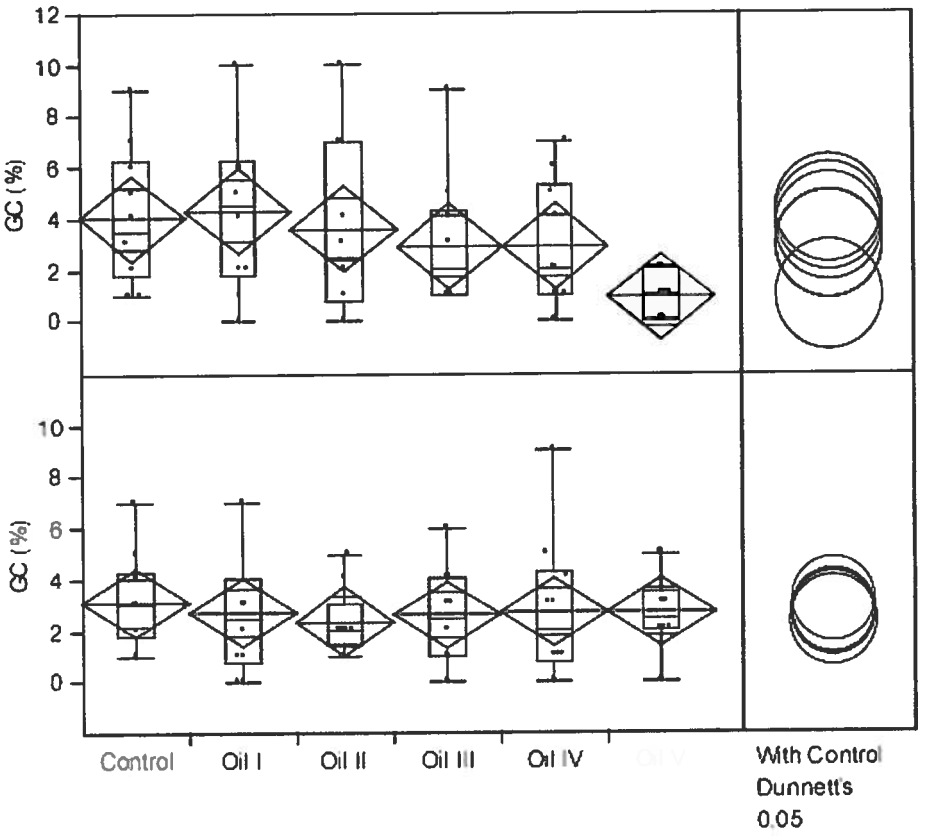

Fig. 15. Differential white blood cell counts expressed as abundance of granulocytes. 4 weeks exposure (top panel), 8 weeks exposure (bottom panel). n = 10.

Histological effects

Two Halibut tissues were evaluated for histological effects after 8 weeks exposure, gills and liver. The results are showing that more severe changes were seen in gills than in liver. The majority of the 11 evaluated parameters in gills were affected by the exposure, with epithelial thickening and proliferation of epithelial cells being particularly affected by oil. No serious detrimental effects were instead seen in liver tissue, which was generally very low in nutritional reserves (glycogen and lipids).

4 Salmon

4.1 Materials & methods

Fish & exposure

Juvenile, smoltified Atlantic Salmon (*Salmo salar*) sized 242.0 ± 40.6 g were purchased from EWOS Innovation, 4335 Dirdal, Norway. Fish were starved for 2 days prior to being transported to the laboratory in a sealed 600 litre plastic tank filled with 8 °C seawater (temperature at fish farm 9 - 11 °C). The tank was oxygenised during transport with a 25 cm ceramic diffuser device connected to a 200 bar oxygen bottle. Total transport time from tank to tank was approximately 2 h. Fish were fed pellets (same as in fish farm) at a *daily* feeding rate corresponding to 0.25 % of the biomass in each tank and exposure (February– April 2009) started after a 14 days acclimatization period. According to recommendations from EWOS, fish was kept at 24 h light/day with 50% of the tanks covered with black plastic to offer a shelter choice. Upon sampling, batches of five individuals were subjected to analgesic treatment for 5-10 min using 100 ppm tricaine (MS-222, Sigma) dissolved directly in seawater. This tricaine concentration did not significantly affect water pH. Water temperature (8.3 ± 0.5 °C), salinity (33.9 ± 0.5 ‰) and oxygen (9.0 ± 0.5 ppm) were monitored daily and did not differ between exposure tanks.

ELISA assays

CYP1A, vitellogenin (Vtg) and immunoglobulin M (IgM) ELISA assays were run according to IRIS SOP protocols. CYP1A protein was semi-quantified for comparison with the enzymatic CYP1A assay (EROD). CYP1A detection antibodies were from Biosense laboratories (product C10-7, monoclonal mouse-anti-fish CYP1A IgG) and diluted 1:1000 in PBS with 1 % BSA and 0.05 % Tween. Secondary antibodies were from BioRad (HRP conjugate goat-anti-mouse IgG) and diluted 1:3000 in PBS with 1 % BSA and 0.05 % Tween. Quantification of plasma Vtg was made using detection antibodies from Biosense laboratories (product V01402201, polyclonal rabbit-anti-Salmon Vtg IgG) diluted 1:1000 in PBS with 1 % BSA and 0.05 % Tween. Secondary antibodies were from BioRad (HRP conjugate goat-anti-rabbit IgG) and diluted 1:3000 in PBS with 1 % BSA and 0.05 % Tween. Plasma Vtg was quantified from a standard curve of known concentrations of Salmon Vtg protein (Biosense laboratories, product V01002301). Plasma IgM was semi-quantified using detection antibodies from Aquatic diagnostics (product C11, monoclonal mouse-anti-Salmon-IgM IgG) diluted 1:50 in PBS with 1 % BSA and 0.05 % Tween. Secondary antibodies were from BioRad (HRP conjugate goat-anti-mouse IgG) and diluted 1:3000 in PBS with 1 % BSA and 0.05 % Tween. In all assays, the colorimetric signal was developed with OPD (Sigma) and the reaction was stopped by adding excess H_2SO_4 (4 N) after a fixed incubation time. The signal was read with a plate reader using a 492 nm filter setting, and the results were reported as the mean absorbance (all samples in quadruplicate) after subtraction of the mean background signal (complete reaction minus detection antibody) of each microplate.

4.2 Results

Table 1 provides an overview of analysed parameters in Salmon. Exposure time proceeding analysis is indicated for each parameter.

Table 1. Analysed parameters in juvenile Salmon sub-divided in biomarker classes. EROD = ethoxyresorufin-*o*-deethylase, GST = glutathione transferase, ENAs = erythrocytic nuclear abnormalities, IgM = immunoglobulin M, Diff-counts = differential white blood cell counts.

Biomarker class	Core biomarkers (exposure time)	Additional biomarkers (exposure time)
Exposure	* Bile PAH met. by FF (4 weeks) * Bile PAH met. by GC-MS (4 weeks) * Hepatic EROD activity (4 weeks)	* Hepatic CYP1A (4 weeks)
General stress	* Hematocrit (4 & 8 weeks) * Plasma protein (4 weeks)	* Hemoglobin (4 & 8 weeks)
Oxidative stress	* Hepatic GST activity (4 weeks)	-
Genotoxicity	* Hepatic DNA adducts (8 weeks)	* Micronuclei (4 & 8 weeks) * ENAs (4 & 8 weeks)
Endocrine disruption	* Plasma vitellogenin (4 weeks)	-
Histological effect	* Gill histopathology (8 weeks)	-
Immunotoxicity	-	* Respiratory burst (4 & 8 weeks) * Plasma IgM (4 & 8 weeks) * Diff-counts (4 & 8 weeks)

GC/MS analyses of PAHs in water and biota

Exposure concentrations in the Salmon experiment were estimated from 26 quantified PAH compounds in two separate water samples (Table 2). PAHs with more than three aromatic rings were only detected in the highest exposure tank (Oil V), where they constituted 1.7 % of sum measured PAHs. Naphthalenes constituted 92 – 100 % of sum PAHs in all tanks receiving oil (results not shown). Table 2 also summarises accumulation of PAH metabolites in bile in three separate analyses after 4 weeks exposure, demonstrating a dose-dependent bioaccumulation in exposed fish. PAH concentrations in the water were comparable to what was obtained in the Halibut experiment, except in the Oil III tank where a lower concentration was measured. Generally lower PAH bioaccumulation levels were found in bile samples of Salmon than in Turbot and Halibut.

Table 2. Overview of results from GC-MS analyses performed in the Salmon experiment. Mean of sum 26 PAHs in water samples (ppb). Mean and standard deviation of sum 9 PAH metabolites in bile samples (µg/g). n = 2 (water), n = 3 (bile; "Oil II" n = 2).

Exposure group	Control	Oil I	Oil II	Oil III	Oil IV	Oil V
Sum water PAHs (ppb)	0.00 ± 0.00	0.08 ± 0.02	0.32 ± 0.01	0.59 ± 0.02	1.48 ± 0.23	5.23 ± 1.40
Sum bile PAH met. (µg/g)	0.82 ± 0.13	1.27 ± 0.22	2.74 ± 0.69	6.34 ± 1.43	10.71 ± 3.82	44.06 ± 17.37

Biomarkers of exposure

Three different biomarkers of exposure were analysed in Salmon after 4 weeks exposure, apart from semi-quantification of PAH metabolites in bile (fixed fluorescence screening assay, Fig. 1) also two different protocols of hepatic CYP1A induction, the EROD assay to assess CYP1A enzymatic activity, and a semi-quantitative ELISA assay to asses CYP1A protein. Relative levels of PAH metabolites in bile samples were similar to the other studies in terms of the experimental LOEC for each of the three wavelength pairs (Fig. 1). Among the two CYP1A protocols, the EROD assay proved the more sensitive of the two, although the dose-response relationship was less clear than in Halibut. Analogous with what was seen in Turbot, only the highest exposure group (Oil V) exhibited significantly elevated CYP1A protein after 4 weeks exposure (Fig. 2).

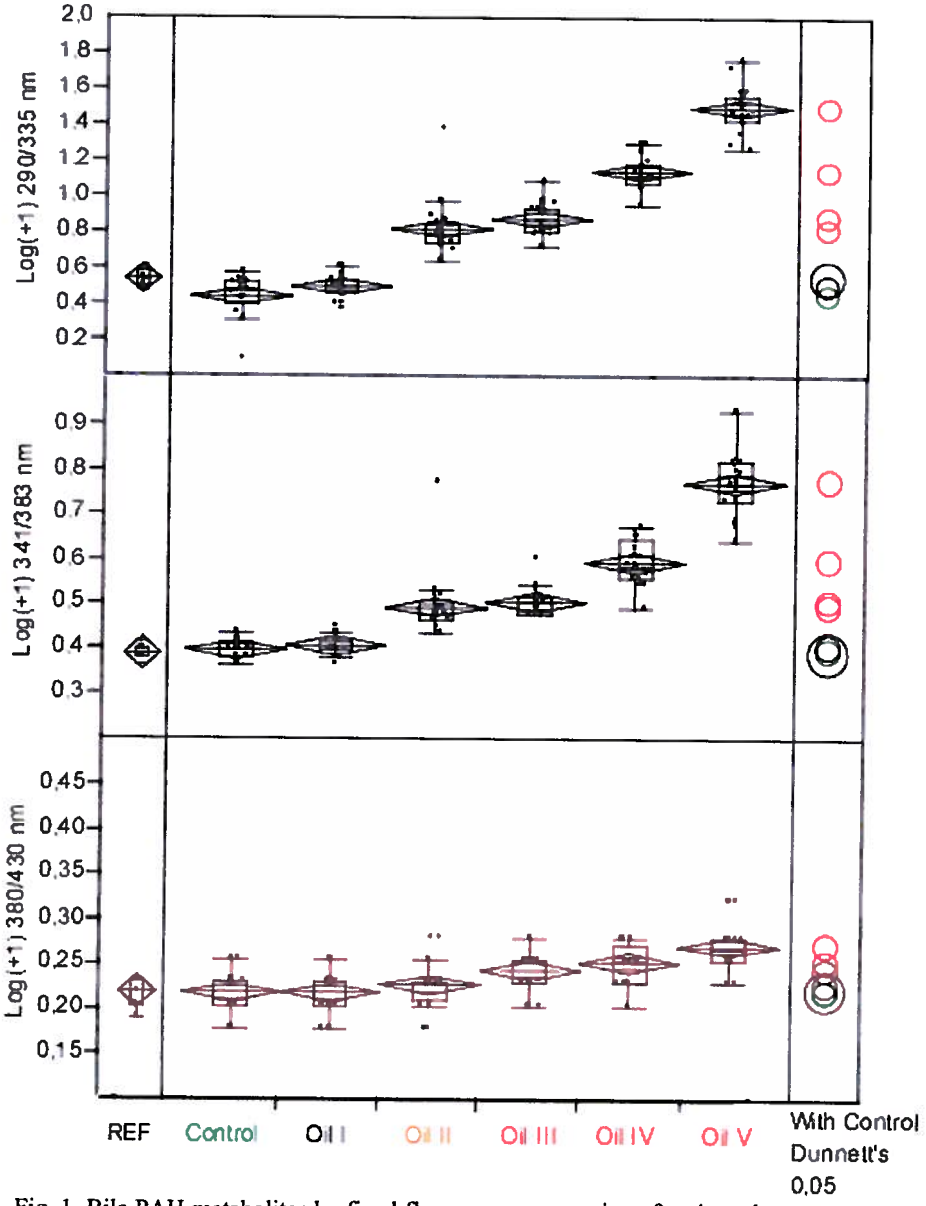

Fig. 1. Bile PAH metabolites by fixed fluorescence screening after 4 weeks exposure. 2-3 ring metabolites (top panel), 4 ring metabolites (middle panel) and 5 ring metabolites (bottom panel). Logarithmic data. n = 20 (n reference = 10).

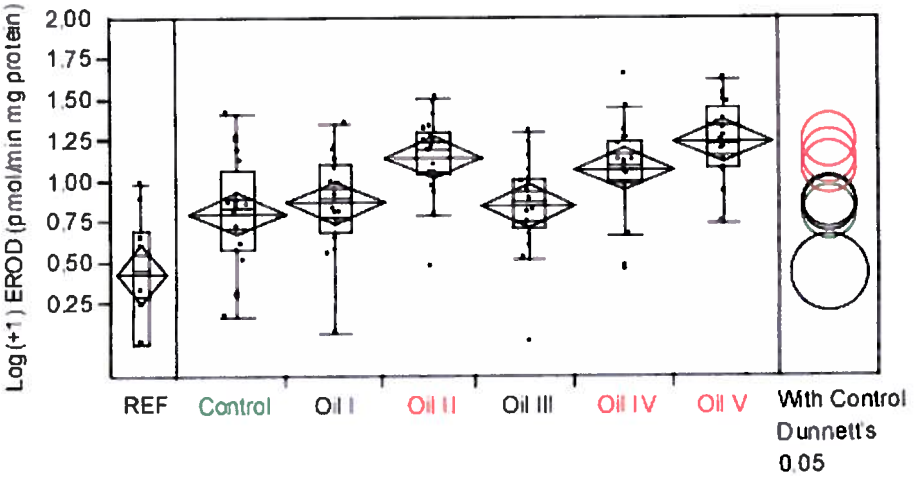

Fig. 2. Hepatic CYP1A activity by EROD after 4 weeks exposure. Data from microsome subfractions. Logarithmic data. n = 20 (n reference = 10).

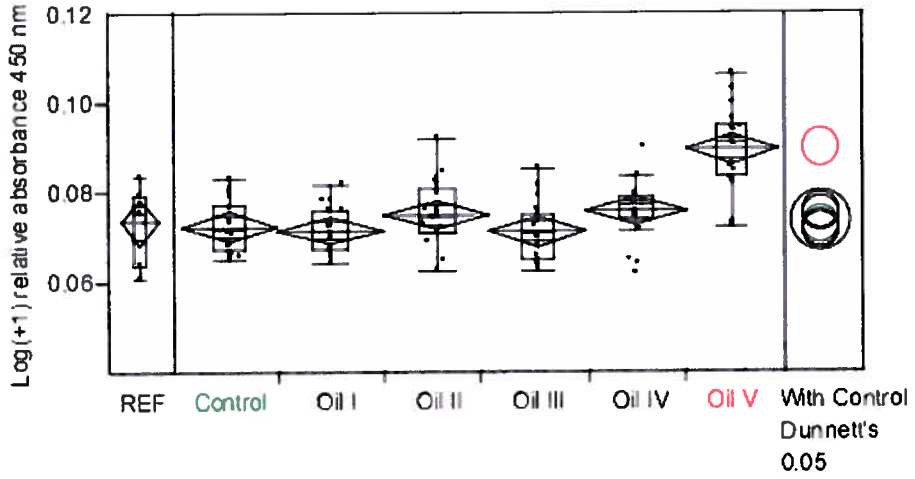

Fig. 3. Hepatic CYP1A protein after 4 weeks exposure. Semi-quantitative data from microsome subfractions. Logarithmic data. n = 20 (n reference = 10).

Biomarkers of general stress

General stress was estimated from three different biomarkers in Salmon; plasma protein (4 weeks exposure), hematocrit (4 & 8 weeks) and haemoglobin (4 & 8 weeks). Fish from the highest exposure group (Oil V) had significantly elevated plasma protein, whereas lower exposure groups were not affected (Fig. 4). Hematocrit (Fig. 5) and haemoglobin (Fig. 6) showed increasing trends in exposed fish after 4 weeks exposure, but not after 8 weeks exposure. For haemoglobin, significantly elevated levels were found in the highest exposure group after 4 weeks (Oil V). Furthermore, both assays demonstrated that the concentration of RBC increased in all groups with the continuation of the experiment, and reference fish expressed significantly lower hematocrit and haemoglobin after 8 weeks in relation to the control group sampled after 8 weeks. The reason for this development is unknown but may e.g. be linked to the lower feeding rate and the smaller tanks in relation to the fish farm.

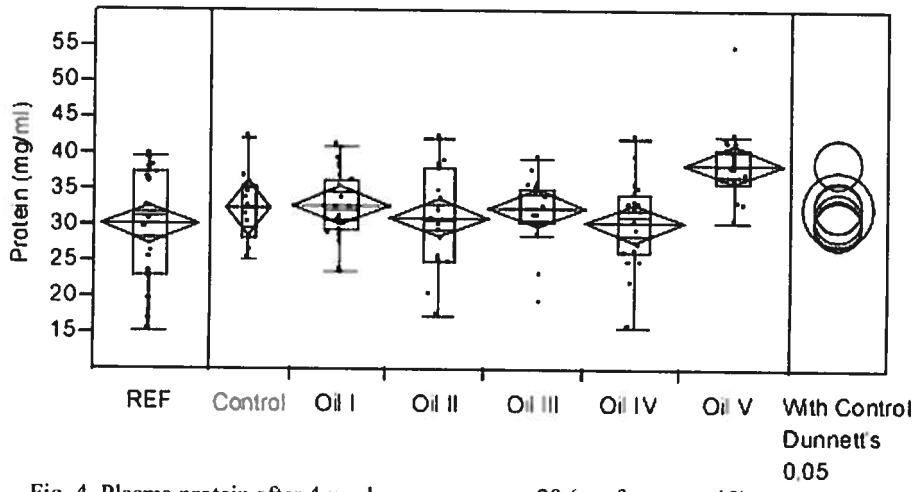

Fig. 4. Plasma protein after 4 weeks exposure. n = 20 (n reference = 10).

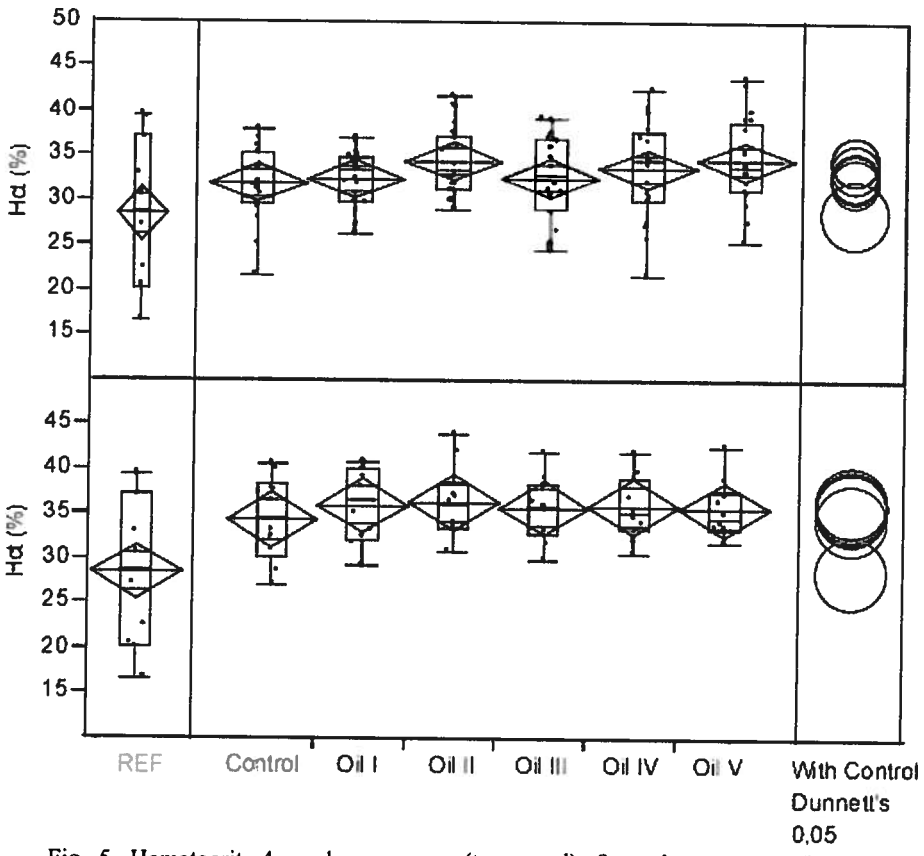

Fig. 5. Hematocrit. 4 weeks exposure (top panel), 8 weeks exposure (bottom panel). n = 20 (4 weeks), n = 10 (reference, 8 weeks).

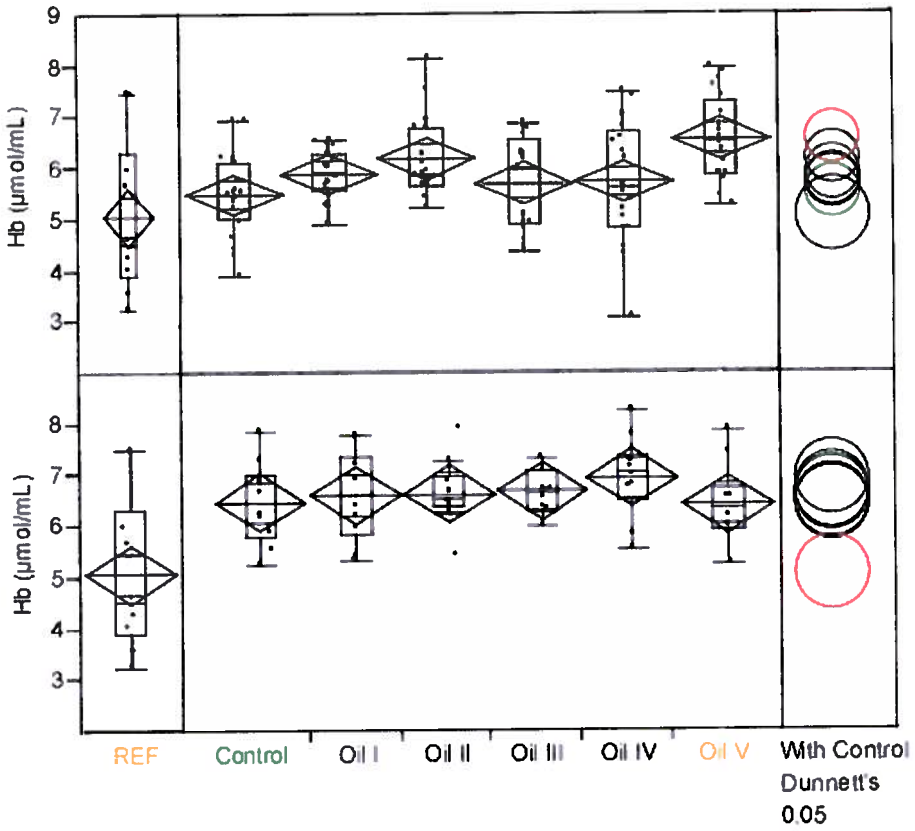

Fig. 6. Haemoglobin. 4 weeks exposure (top panel), 8 weeks exposure (bottom panel). n = 20 (4 weeks), n = 10 (reference, 8 weeks).

Biomarkers of oxidative stress

The only biomarker of oxidative stress analysed in Salmon, hepatic GST activity, was significantly elevated in three of five exposure groups (Fig. 7). However, dose-response relationships were not evident and fold-induction in induced groups was limited, rendering the results difficult to interpret.

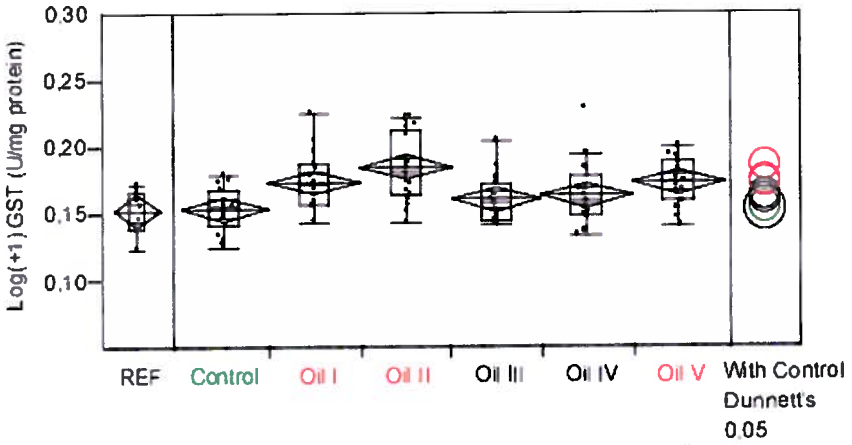

Fig. 7. Hepatic GST activity after 4 weeks exposure. Logarithmic data from cytosolic samples. n = 20 (n reference = 10).

Biomarkers of genotoxicity

Salmon exhibited a lower level of genotoxic stress than Turbot and Halibut. The four lowest exposures had no effect at all on hepatic DNA adducts after 8 weeks, whereas significantly elevated levels were detected in the highest exposure group (Oil V, Fig. 8). Furthermore, frequencies of micronuclei and erythrocytic nuclear abnormalities were quantified in ten individuals from the control and the two highest exposure groups (Oil IV – V) after both 4 and 8 weeks exposures, demonstrating zero or close to zero frequencies in all analysed groups (results not shown, refer to excel sheet for rawdata).

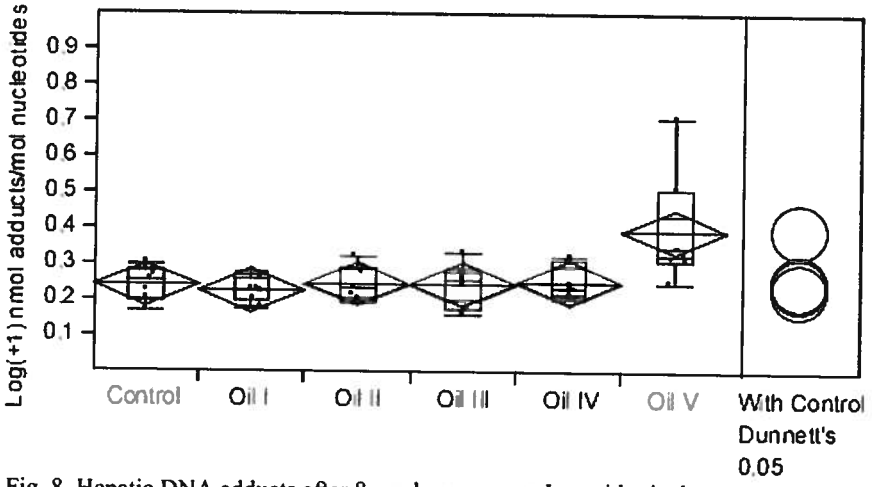

Fig. 8. Hepatic DNA adducts after 8 weeks exposure. Logarithmic data. n = 7.

Biomarkers of endocrine disruption

The exposure did not give rise to induction of the egg protein vitellogenin, analysed in plasma samples (Fig. 9), showing that used dilutions had no measurable estrogenic effect on juvenile Salmon after 4 weeks exposure.

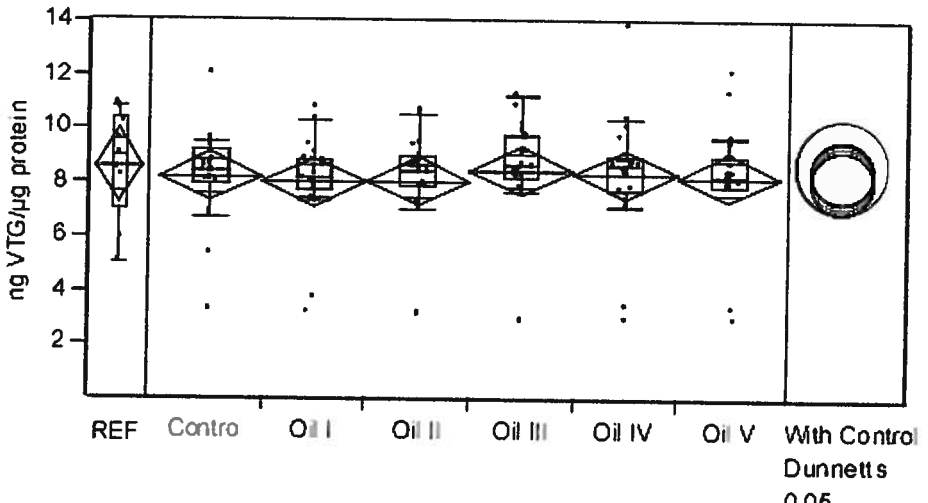

Fig. 9. Plasma vitellogenin after 4 weeks exposure. Quantitative data. n = 20 (n reference = 10).

Biomarkers of immunotoxicity

Immunotoxicity was evaluated more extensively in Salmon than in other species. A novel respiratory burst assay was set up using diluted blood samples instead of homogenised tissue as in many previous studies. The results demonstrated a dose-dependent inhibition of the respiratory burst (measured as in an increased response time) after both 4 weeks and 8 weeks exposure (Fig. 10). The dataset obtained after 8 weeks exposure exhibited less intergroup variance than what was seen after 4 weeks, yet the experimental LOEC was reduced by one exposure group in relation to fish sampled after 4 weeks exposure (Fig. 10). Semi-quantification of circulating immunoglobulins in plasma samples, an indirect measure of lymphocyte numbers, did not change between exposure groups (Fig. 11), and neither did the relative composition of white blood cells by means of differential counts (Fig. 12). In combination, these results point at an impaired function of circulating phagocytes as a result of oil exposure, whereas absolute numbers of phagocytic and non-phagocytic leucocytes were not significantly affected.

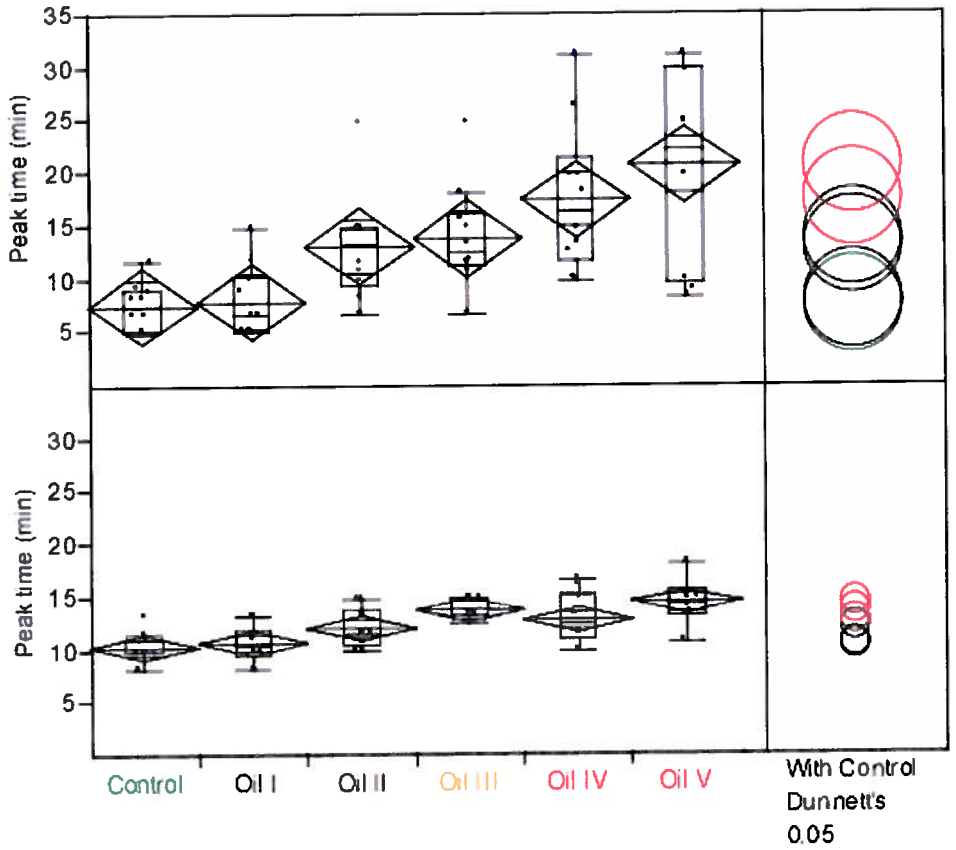

Fig. 10. Respiratory burst of diluted blood samples. 4 weeks exposure (top panel), 8 weeks exposure (bottom panel). n = 10.

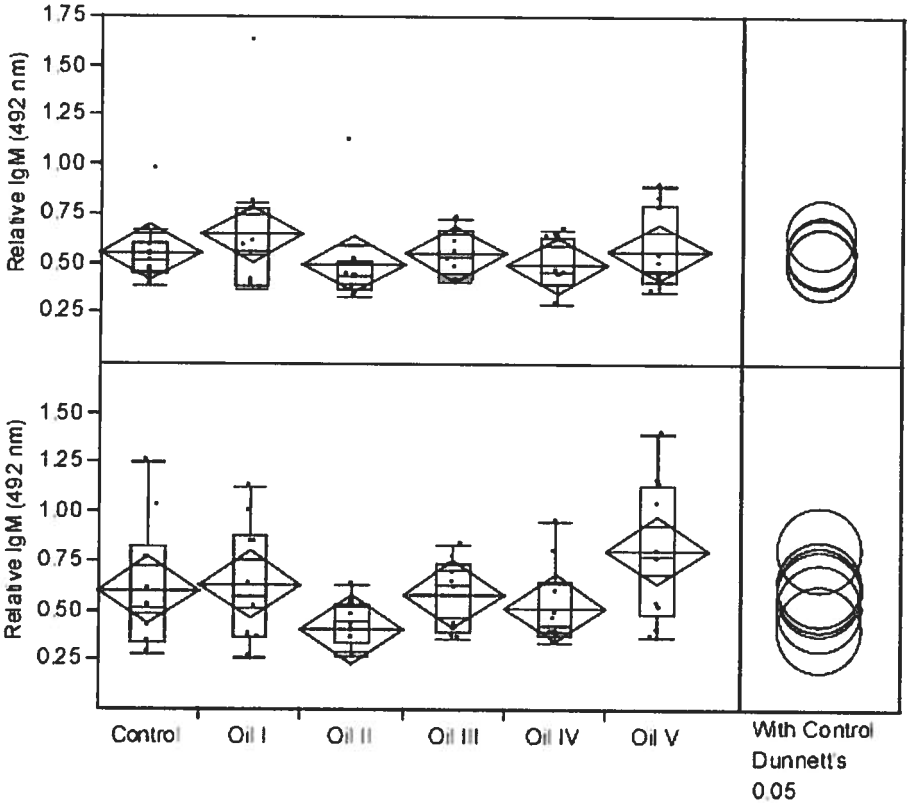

Fig. 11. Plasma immunoglobulin M. 4 weeks exposure (top panel), 8 weeks exposure (bottom panel). Semi-quantitative data. n = 10.

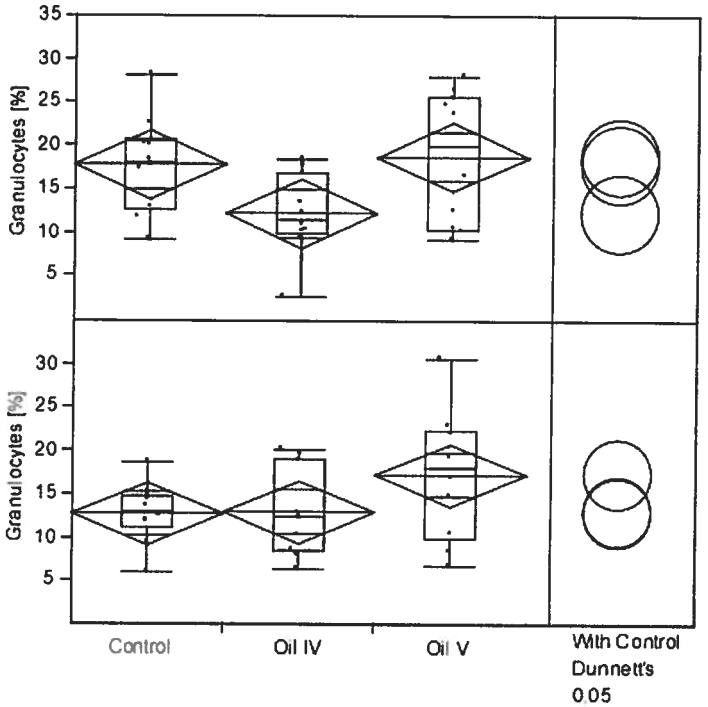

Fig. 12. Differential white blood cell counts expressed as abundance of granulocytes. 4 weeks exposure (top panel), 8 weeks exposure (bottom panel). n = 10.

Histological effects

The results of the histopathological evaluation of Salmon gill tissue were inconclusive in terms of dose-responsiveness. After 8 weeks, some abnormalities were elevated in low and medium exposure groups in relation to unexposed fish, but not in the highest exposure group (Oil V). One parameter (proliferation of epithelial cells) was consequently higher in all exposed groups. However, reference individuals displayed more abnormalities than all fish sampled after 8 weeks, including the control group, suggesting that transport of Salmon to the laboratory induced stress that affected the gills. Since control fish sampled after 8 weeks showed less abnormal gills, this would also imply that abnormalities are reversible to some extent.

5 Sprat

5.1 Materials & methods

Fish & exposure

Juvenile sprat (*Sprattus sparttus*) sized 20-30 g was caught with seine from an undisturbed environment in the south-western part of the Norwegian coast by professional fishermen. Transport to the laboratory was conducted on the fishing boat in aerated tanks. Fish were maintained in the laboratory in 1 x 1 x 0.3 (300 L) quadratic fibreglass tanks, with a natural photoperiod and salinity of 34‰. The fish were randomly separated into six groups consisting of 40-50 fish per tank, corresponding to a biomass of approximately 1,3 kg. Tanks were cleaned every day and fish were fed commercial fish pellets (Gemma Wean Diamond 0.5 and 0.8 mm, Skretting, France) supplemented with live feed (*Artemia*) at a frequency corresponding to approximately 0.3 % of the biomass each day. The fish were acclimatized to this condition five weeks prior to onset of the experiment. After an exposure of four weeks, 20 fish from each tank were removed and the rest of the fish were exposed for an additional period of four weeks, i.e. an exposure of eight weeks. Water temperature (11-12 °C over the period of the experiment) and oxygen levels (7-8 mg/L over the period of the experiment) were measured every day. Water samples for analysis of oil concentration were taken every week and water samples for analysis of PAHs were taken twice during the experiment.

ELISA assays

CYP1A and vitellogenin (Vtg) ELISA assays were run according to IRIS SOP protocols. CYP1A protein was semi-quantified in hepatic microsomes, after scanning of samples demonstrated significant denaturation of heme protein, rendering quantification of CYP1A enzymatic activity (EROD assay) inappropriate in this experiment. CYP1A detection antibodies were from Biosense laboratories (product C10-7, monoclonal mouse-anti-fish CYP1A IgG) and diluted 1:1000 in PBS with 1 % BSA. Secondary antibody was from BioRad (HRP conjugate goat-anti-mouse IgG) and diluted 1:3000 in PBS with 1 % BSA. Semi-quantification of plasma Vtg was made using detection antibodies from Biosense laboratories (product V01402201, polyclonal rabbit-anti-Salmon Vtg IgG) diluted 1:1000 in PBS with 1 % BSA. Secondary antibody was from BioRad (HRP conjugate goat-anti-rabbit IgG) and diluted 1:3000 in PBS with 1 % BSA. In the assays, the colorimetric signal was developed with OPD (Sigma) and the reaction was stopped by adding excess H_2SO_4 (4 N) after a fixed incubation time. The signal was read with a plate reader using a 492 nm filter setting, and the results were reported as the mean absorbance (all samples in triplicates) after subtraction of the mean background signal (complete reaction minus detection antibody) of each microplate.

5.2 Results

Table 1 provides an overview of analysed parameters in sprat. Exposure time proceeding analysis is indicated for each parameter.

Table 1. Analysed parameters in juvenile sprat sub-divided in biomarker classes. PAHs= polyaromatic hydrocarbons, CYP1A= Cytochrome P450 1A, GST = glutathione transferase, ENAs = erythrocytic nuclear abnormalities.

Biomarker class	Core biomarkers (exposure time)	Additional biomarkers (exposure time)
Exposure	* Bile PAH met. by FF (4 weeks) * Bile PAH met. by GC-MS (4 weeks) * Hepatic CYP1A activity (4 weeks)	
General stress	* Hematocrit (4 & 8 weeks) * Plasma protein (4 weeks)	* Hemoglobin (4 & 8 weeks)
Oxidative stress	* Hepatic GST activity (4 weeks)	
Genotoxicity	* Hepatic DNA adducts (8 weeks)	* Micronuclei (4 weeks) * ENAs (4 weeks)
Endocrine disruption	* Plasma vitellogenin (4 weeks)	
Histological effect	* Gill histopathology (8 weeks)	

GC/MS analyses of PAHs in water and biota

Exposure concentrations in the sprat experiment were estimated from 26 quantified PAH compounds in two separate water samples (Table 2). Naphthalenes constituted the majority of sum PAHs in all tanks receiving oil (results not shown). Table 2 also summarises accumulation of PAH metabolites in bile after 4 weeks exposure, demonstrating a dose-dependent bioaccumulation in exposed fish.

Table 2. Overview of results from GC-MS analyses performed in the sprat experiment. Mean of sum 26 PAHs in water samples (ppb), n = 2. Mean of sum 9 PAH metabolites in bile sample (µg/g).

Exposure group	Control	Oil I	Oil II	Oil III	Oil IV	Oil V
Sum water PAHs (ppb)	0.00 ± 0.00	0.05 ± 0.03	0.33 ± 0.14	0.85 ± 0.42	1.77 ± 0.57	5.01 ± 1.38
Sum bile PAH met. (µg/g)	3.00	6.47	14.30	40.32	45.89	148.70

Biomarkers of oil exposure

Two different biomarkers of oil exposure were analysed in sprat after 4 weeks exposure, semi-quantification of PAH metabolites in bile (fixed fluorescence screening assay, Fig. 1A-C) and induction of the hepatic CYP1A protein (ELISA assay, Fig 2). Relative levels of PAH metabolites in bile samples were similar to the other juvenile fish studies in terms of the experimental LOEC for each of the three wavelength pairs (Fig. 1). The CYP1A protein showed no dose-responsiveness to oil exposure in sprat.

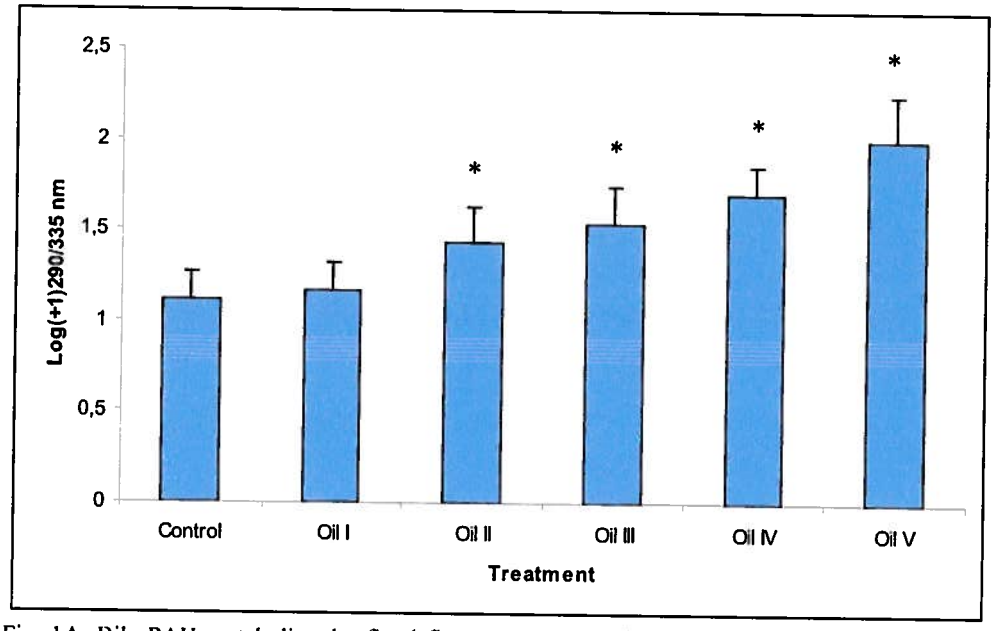

Fig. 1A. Bile PAH metabolites by fixed fluorescence screening after 4 weeks exposure, 2-3 ring metabolites. Logarithmic data. n = 20.

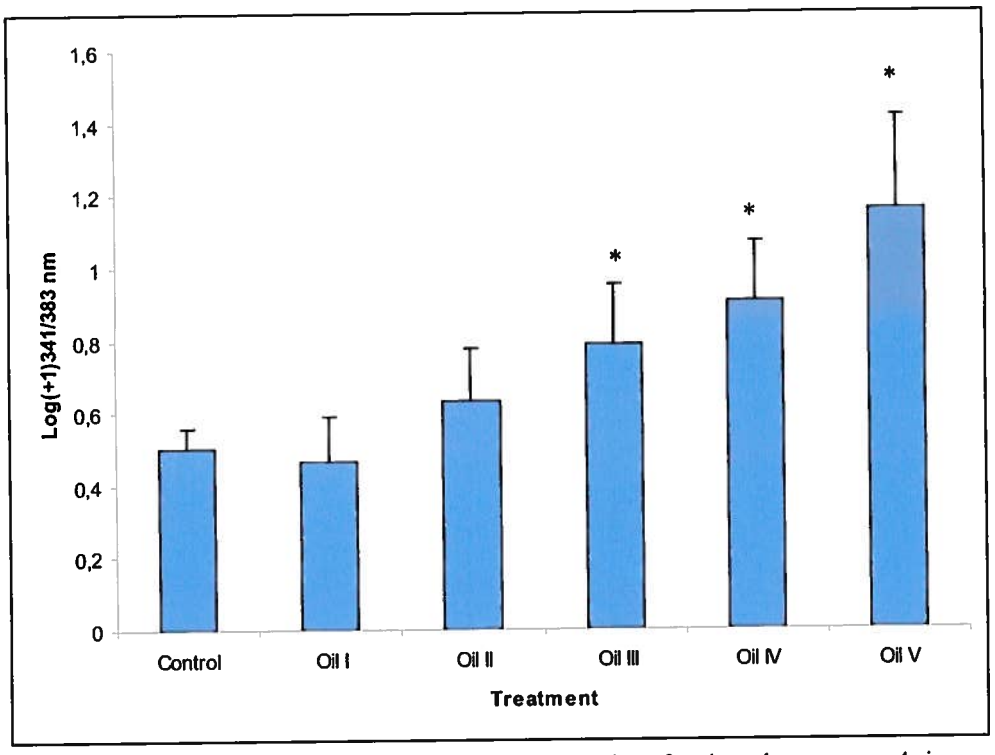

Fig. 1B. Bile PAH metabolites by fixed fluorescence screening after 4 weeks exposure, 4 ring metabolites. Logarithmic data. n = 20.

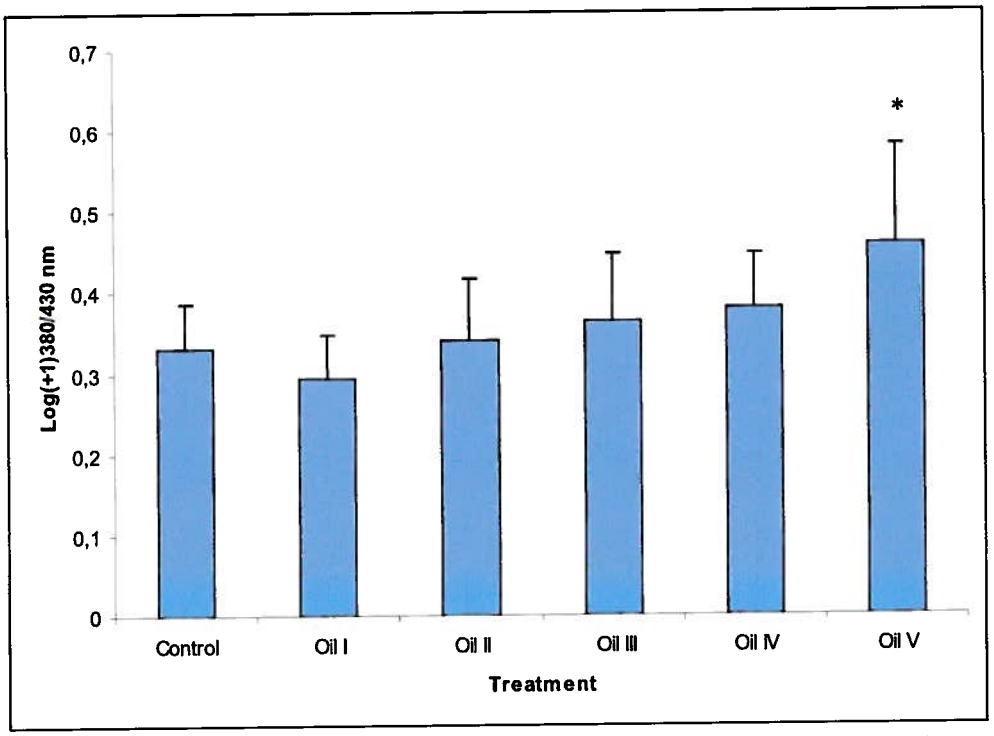

Fig. 1C. Bile PAH metabolites by fixed fluorescence screening after 4 weeks exposure, 5 ring metabolites. Logarithmic data. n = 20.

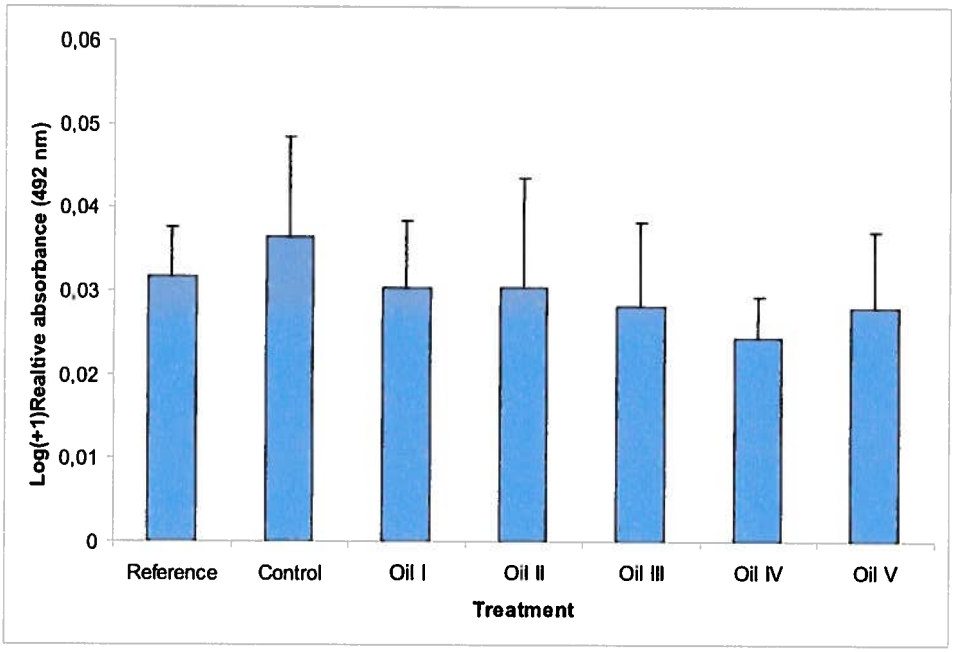

Fig. 2. Hepatic CYP1A protein levels after 4 weeks of exposure. Semi-quantitative data from microsome subfractions. n = 20 (n reference = 10).

Biomarkers of general stress

General stress was estimated from three different biomarkers in sprat; plasma protein (4 weeks exposure), hematocrit (4 & 8 weeks) and haemoglobin (4 & 8 weeks). Plasma protein levels analysed from fish from the oil exposed groups were not affected compared to the control fish (Fig. 3). Hematocrit (Fig. 4) and haemoglobin (Fig. 5) showed increasing trends in exposed fish after 8 weeks of exposure. For haemoglobin, a significantly elevated level was found in the highest exposure group after 8 weeks (Oil V).

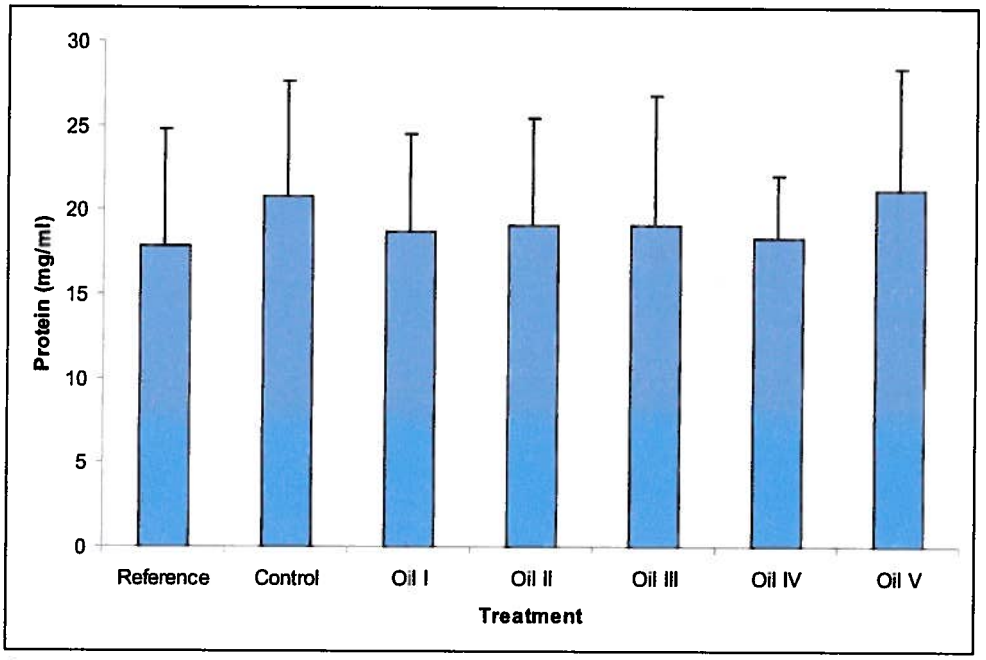

Fig. 3. Plasma protein levels after 4 weeks exposure. n = 20 (n reference = 10).

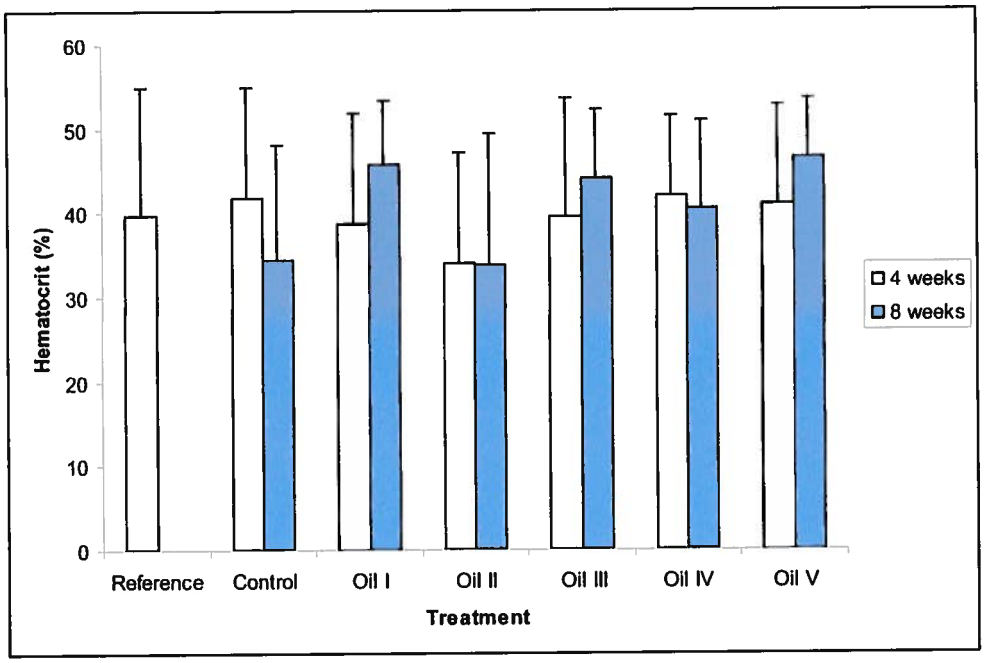

Fig. 4. Hematocrit levels after 4 and 8 weeks of exposure. n = 20 (4 weeks), n = 10 (reference, 8 weeks).

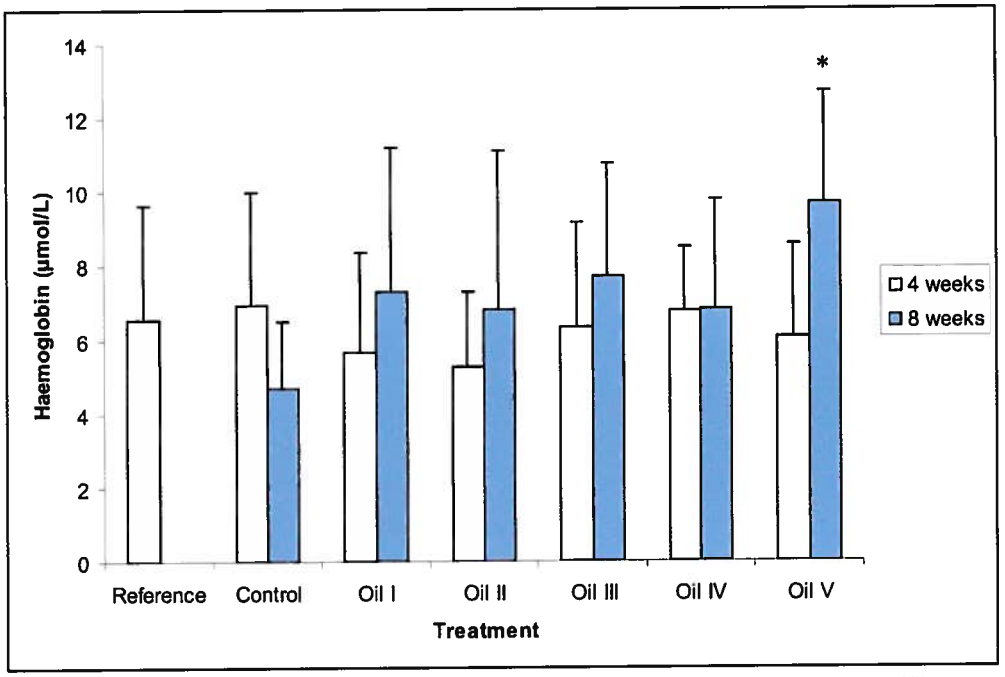

Fig. 5. Haemoglobin levels after 4 and 8 weeks of exposure. n = 20 (4 weeks), n = 10 (reference, 8 weeks).

Biomarkers of oxidative stress

The only biomarker of oxidative stress analysed in sprat, hepatic GST activity, was not significantly affected by oil exposure (Fig. 6).

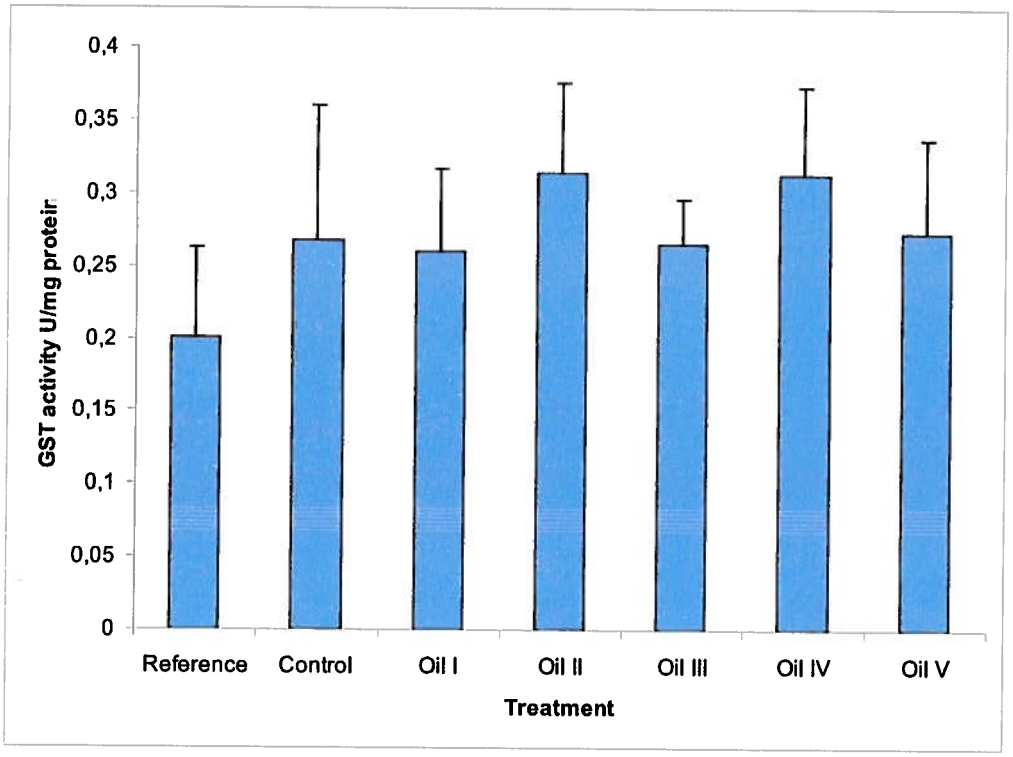

Fig. 6. Hepatic GST activity after 4 weeks exposure, data from cytosolic samples. n = 20 (n reference = 10).

Biomarkers of genotoxicity

Significantly elevated levels of DNA adducts were detected in the two highest exposure groups (Oil IV and Oil V, Fig. 7) after 8 weeks of exposure. Frequencies of micronuclei and erythrocytic nuclear abnormalities were also quantified in ten individuals from the control and the highest exposure groups (Oil V) after 4 weeks exposures. The results demonstrated, however, zero or close to zero frequencies in the analysed groups (results not shown).

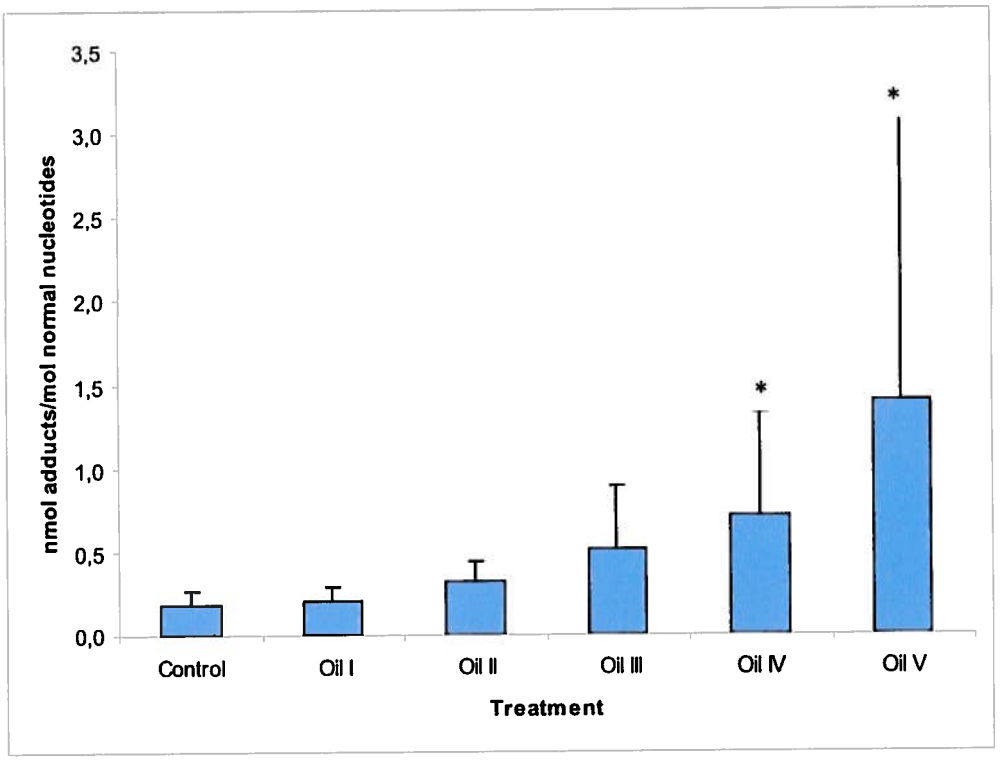

Fig. 7. Hepatic DNA adducts after 8 weeks exposure. n = 7.

Biomarkers of endocrine disruption

The exposure did not give rise to induction of the egg protein vitellogenin, analysed in plasma samples (Fig. 8), showing that the used oil concentrations had no measurable estrogenic effect on juvenile sprat after 4 weeks of exposure.

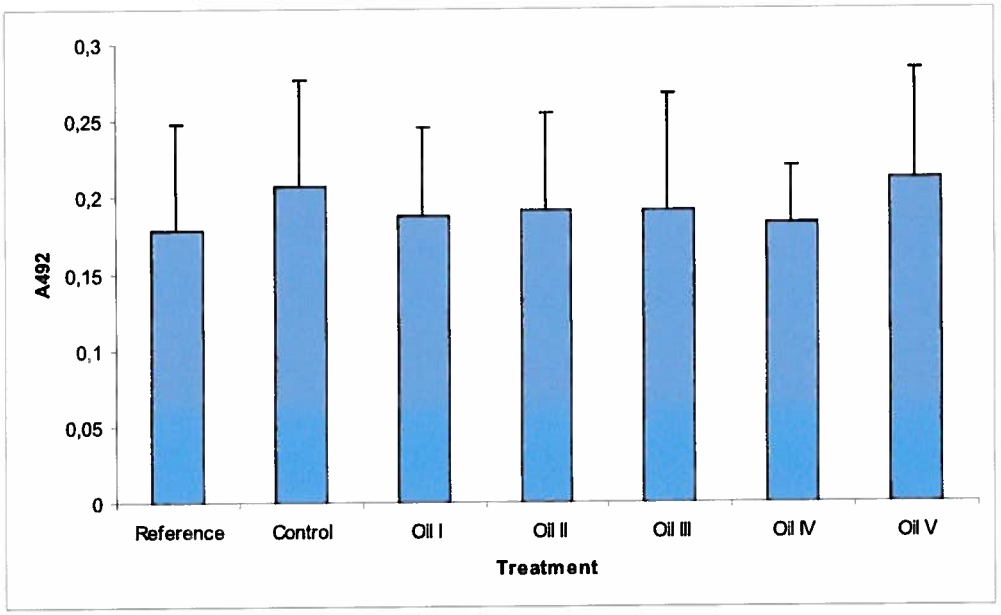

Fig. 8. Plasma vitellogenin after 4 weeks exposure, semi-quantitative data. n = 20 (n reference = 10).

Biomarkers of immunotoxicity
Immunotoxicity was not evaluated in sprat.

Histological effects
The results of the histopathological evaluation of sprat gill tissue were inconclusive in terms of dose-responsiveness. After 8 weeks, some abnormalities were increased or decreased in the oil exposed groups in relation to unexposed fish but no clear dose-response was detected. However, one parameter (proliferation of epithelial cells) was consequently and statistically significantly higher in all exposed groups compared to both the reference group and the control group.

6 References

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