Sequential non-destructive sampling of Atlantic cod (*Gadus morhua*) in a long term exposure to produced water chemicals

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Forord

Denne masteroppgaven er skrevet ved universitet i Oslo i samarbeid med norsk institutt for vannforskning (NIVA). Under veiledning av Ketil Hylland og Thorvin Andersen.
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Abstract

A major concern related to production of oil from offshore installations is the discharge of produced water, which contains organic pollutants like PAHs (Polycyclic aromatic hydrocarbons) and APs (Alkylphenols) as well as metals and chemicals utilized in the production process. There are studies on the effect of produced water on benthic fauna, plankton and fish, but few on long term exposures under controlled conditions. The aim of this study was to investigate the long term effect of ecologically relevant dilutions of PAHs and APs found in produced water as well as how the effects change over time in individual fish. To achieve this, 90 cod (*Gadus morhua*) were exposed to these substances in three different exposure schemes for 44 weeks. Blood from each fish was sampled before starting the exposure and 7 set times throughout the study and analysed for several biomarkers.

Amounts of Vitellogenin (VTG) and zona radiata protein (ZRP) were measured to investigate estrogenic effects; oxidative stress was assessed using the total oxyradical scavenging capacity (TOSC) assay; occurrence of micronuclei was used as an indicator for genotoxicity and hematocrit values and Fulton's condition factor (CF) were used to evaluate effects on general health. In addition liver somatic index (LSI) and gonado-somatic index (GSI) was calculated for the last sample date. And the mortality of the fish was compared to fish only subject to one blood sample before starting the exposure.

The statistical analyses showed no estrogenic effects, no effects on TOSC and no indication of genotoxicity in any of the treatments at any of the sample dates. The assessment of general health showed no differences in CF and LSI but some significant differences were detected in hematocrit values between treatments and the control group but these were not consistent throughout the experiment and they are concluded to be coincidental. An apparent correlation between the change in hematocrit values and condition factor was observed.

Other studies have previously shown that exposure to produced water and as well as the chemicals we selected to have an effect on the same biomarkers that we used. However this study used a lower concentration than most other studies

The repeated blood sampling did appear to affect mortality after the 6th sample date. The mortality was 2.7 percentage points higher in the fish subject to repeated blood samples between the 6th and the 8th blood sampled taken ($p=0.05$), and 6.3 percentage points higher between the 8th and the 9th blood sampling ($p=0.03$) compared to fish only sampled once.

Index

1 Introduction

1.1 Oil as an environmental issue

Huge amounts of oil and gas are produced from offshore platforms each year. There has been an increasing awareness and worry with the effects of discharges and spills from this production. The concern is not only on ecological perspectives on smaller key species and general composition of the ecosystem, but has also been directly related to economically important fish populations. In Norway a big part of this concern has been related to the Atlantic cod (*Gadus morhua*), especially as the search for oil is moving from the North Sea towards Lofoten and the Barents Sea (OLF, 2004), which are important spawning and living habitats for cod.

There are many patterns of pollution to the marine environment related to the production of oil; drilling waste, oil spills, produced water as well as discharges from oil carriers transporting the oil to shore. This study focuses on the effects of a selection of components of produced water.

1.2 Produced Water

During production of offshore oil and gas, water trapped in the reservoir is brought up with the oil or gas extracted. This water is called produced water, it consists mainly of salt water or brine, but it also contains hydrocarbons and metals as well as chemicals added in the production process. The amount of produced water released from a platform generally increases with the age of the oil field. The exact composition of this water varies greatly between platforms, but polycyclic aromatic hydrocarbons (PAH) and alkylphenols (AP) are present at all sites and they contribute to a substantial part of the chemicals released into the environment (Brendehaug *et al.*, 1992). PAH-metabolites are known to produce free radicals that lead to oxidative stress (Flowers-Geary *et al.*, 1996;

Penning *et al.*, 1996) and some of them have been shown to be genotoxic (La Rocca *et al.*, 1996). It is well known that several APs have estrogenic properties(Soto *et al.*, 1991; Jobling *et al.*, 1993) and it has been suggested that they can contribute to oxidative stress in fish (Hasselberg *et al.*, 2004). In addition exposure to E2 have been shown to reduce CYP1A activity (Navas *et al.*, 2001) which could also apply for APs as it has estrogenic effects. This could possibly reduce the metabolism of other xenobiotica.

Although the concentrations of these chemicals in produced water is low, the amount of water is so large that it becomes an important factor. In 2006 173 million m^3 of produced water was generated on the Norwegian continental shelf, of which 83% was released into the sea (OLF, 2007). Worldwide the amount generated in 1999 was 12.2 billion m^3 (Khatib *et al.*, 2003). According to the authors this is about 3 times the worldwide production of oil.

In this study we used only lighter hydrocarbons present in produced water; a range of PAHs, alkylated PAHs and APs. The concentrations used were environmentally relevant and based upon composition of produced water from the Statfjord production area.
The two dilutions used were 200 and 2000 times dilution of produced water (The full list and concentrations are listed in appendix B), which are representative dilution factors found in situ. The concentrations at set distances from a platform obviously vary greatly with currents and the exposures of organisms will vary with their movement. To investigated potential effect of movement in areas with different exposure we employed a pulsed exposure that switched between the high concentration and no exposure at two week intervals.

1.2 Sequential and non-destructive sampling

There are many studies that focus on how contaminants affect a wide range of processes in fish, including exposure to produced water components (Hasselberg *et al.*, 2004; Sturve *et al.*, 2006; Meier *et al.*, 2007). However most exposures only last for a short period of time(Meier *et al.*, 2007); exposures lasting longer than 3 months are unusual.

The understanding of how responses to exposure change over time is limited. Most studies use only one sampling point, or at the most sampling of different individuals over the time course of the exposure. The goal of this study was not only seeing how contaminants affect processes in fish when exposed over a long period of time, but also to look for information on how such responses may change over time in individual fish. The exposure lasted for 44 weeks and blood samples were taken 8 times during this period. This required both individual marking of the fish and a gentle procedure for handling fish and sampling blood.

1.3 Biomarkers

A biomarker is a biological response than can be related to exposure to chemicals. There are many definitions of the term biomarker (Gestel *et al.*, 1996); some include behavior while others specify that it is the measure of a chemical. To integrate the term biomarker in ecotoxicology van Gestel and van Brummelen (1996) defined a biomarker as "Any biological response to an environmental chemical at the below individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism." Biomarkers can either be the measurement of an effect or it can be the concentration of a pollutant or its metabolites. Very few biomarkers are specific to just one type of chemical, since many responses can be triggered by a range of chemicals. All the biomarkers in this study are either chemical markers of a response that is known to be influenced by the chemicals used in the exposures or they are indicators of general health.

Hematocrit

Hematocrit is a measure of the amount of blood cells (white and red) in the blood. The value is given as a percentage of the total volume of blood, and can be determined by centrifuging the blood in a capillary tube. The volume of packed blood cells divided by the total volume is the hematocrit value. This is a quick and simple analysis that has been used since the 1930ies, and it have shown to be correlated with disease and stress as well as sexual and seasonal differences (Blaxhall, 1972).

Micronuclei

Micronuclei are small nuclei produced by fragments of; or entire chromosomes that lag during cell division. This can happen due to damaged or lacking centromeres or problems in the cytokinesis. The formation of micronuclei are rare, a review of several different studies (Heddle *et al.*, 1991) states that about 1-3 micronuclei pr 1000 bone marrow-erythrocyte is normal for untreated animals. The assay is simple but time consuming; cells are smeared over a surface and treated with a fluorescent dye that stains DNA. Cells and micronuclei formations are then counted manually. Since the formation of micronuclei is so rare a lot of cells needs to counted to be able to detect a possible clastogenic or aneugenic effect (Heddle *et al.*, 1991).

Total oxyradical scavenging capacity (TOSC)

The TOSC assay is used to measure the ability to handle free radicals. This assay was first described by (Winston *et al.*, 1998) measuring ability of samples from rat liver to handle peroxyl(ROO) radicals. They expanded the assay to include scavenging capacity of hydroxyl(OH) and peroxynitrite(NOO) radicals a year later (Regoli *et al.*, 1999). Most assays that address oxidative stress is measurements of certain antioxidants or formation of radicals, TOSC on the other hand is a measurement of the total oxidative stress defenses, thus TOSC could be a more biologically relevant than measurements of antioxidants. High TOSC values can in some instances be an adaptation to an environment with increased oxidative stress (Camus *et al.*, 2005); however low TOSC values can be an indication of depletion of antioxidant defenses (Camus *et al.*, 2002). Literature on TOSC measurements in fish is scarce, but as mentioned in section 1.2; components in our exposure are known to induce oxidative stress.

Vitellogenin

Vitellogenin (VTG) is an egg yolk protein precursor. It is produced in the liver and transported in the bloodstream to the ovary where it is transformed to phosvitin and lipovitellin which are among the most abundant proteins in the egg yolk of oviparous vertebrates (Wallace *et al.*, 1969; Gündel *et al.*, 2007). As ovaries mature before spawning season, the production of yolk therefore requires large quantities of VTG. The production of VTG is controlled by several hormones but 17β-estradiol (E2) and other estrogens are the most important (Carnevali *et al.*, 1992), (Sumpter *et al.*, 1995). Male fish and juvenile fish however have very low or undetectable levels of VTG (Copeland *et al.*, 1986; Scott *et al.*, 2006) but the gene can be triggered by estrogenic xenobiotica (Hylland *et al.*, 2006). This has led to the development for VTG as a biomarker for exposure to xenoestrogens, and it has become one of the most widely used and well tested biomarkers used with fish. Since we were exposing fish to alkylphenols; which are shown in several studies to have estrogenic activity (Meier *et al.*, 2007), VTG was a natural biomarker to use in this study.

Zona radiata protein.

In teleost fish zona radiata protein(ZRP) forms a protective shell around the eggs, and it is shown that just as VTG it is produced in the liver as a response to estrogens(Hyllner *et al.*, 1991; Oppen-Berntsen *et al.*, 1992) and transported to the ovary via the blood. ZRP is not as established as VTG as a biomarker for estrogens, but according to some studies (Arukwe *et al.*, 1997; Celius *et al.*, 1998) it is a more sensitive biomarker than VTG. And as this study is using low concentrations of estrogen mimicking chemicals it is an interesting biomarker to use.

1.4 Objectives

Five hypotheses were formulated for this study:

1. The general health of cod will not be affected by any of the treatments at any time during the exposure period.
2. Oxidative stress will not be affected by any of the treatments at any time during the exposure period.

3. Estrogenic activity will not be affected by any of the treatments at any time during the exposure period.
4. The amount of DNA damage will not be affected by any of the treatments at any time during the exposure period.
5. The mortality of cod will not be affected by repeated blood sampling

2 Materials and methods

2. 1 Exposure system

2.1.1 The aquarium setup

The exposure system was set up at Solbergstrand Marine Research station (NIVA), and is illustrated in figure 2.1.1. A main header tank received seawater continuously monitored for temperature and salinity from 60 meters depth. From the main header tank this water was distributed by gravity through four different plastic tubes to four secondary header tanks (one for each exposure dose) at a rate of 45 L/min (60 L/min for the control).

A stock solution of the exposure chemicals were diluted in a 30 liter glass tank in a 1:1 mixture of distilled water and acetone. From these tanks 1 mL/min was pumped to their respective secondary header tank through Teflon tubes using EW-F10FC electromagnetic metering pumps (IWAKI, Norway). Teflon coating was also used on the inside of the secondary header tanks and in the tubes leading to the aquaria to limit the adsorption of substances.

The secondary header tanks contained the desired dilution of chemicals, and using one tube per aquarium their contents was distributed to the aquaria at a rate of 15 L/m. Since the header tanks were placed about 1m above the aquaria this step did not require pumps. Through this system each of the aquariums received water and chemicals according to the exposure schemes listed in Appendix B

The thirteen 1000 liter aquaria were drained trough the bottom, and excess water was drained through the top by an overflow pipe, thus keeping the level of water at a constant level. They were covered with a net lid and transparent plastic was suspended over and between the aquariums to prevent exchange of gases from the lighter hydrocarbons. The room they were placed in is lit by sunlight and thus giving the fish a natural scheme of daylight.

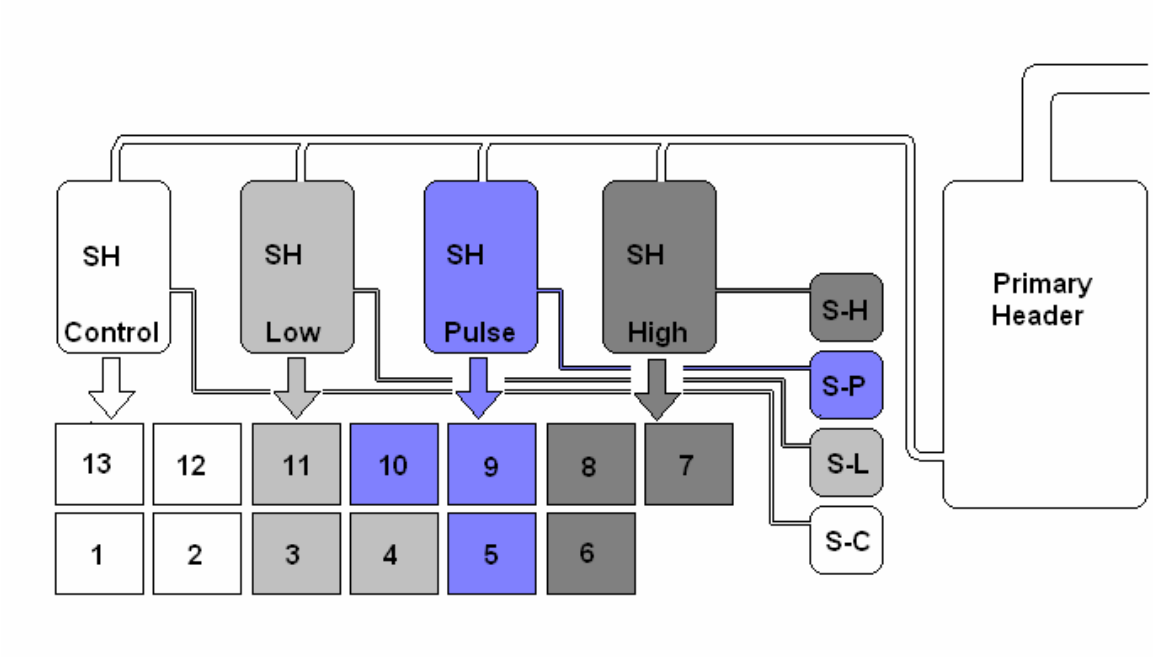

Figure 2.1.1: The aquarium setup. The aquaria are number from 1 to 13; white represents the control group; light grey the low exposure; blue the pulse exposure and dark grey the high exposure. The secondary header tanks (SH) received water from the primary header tank and exposure chemicals from the four stock solutions (S-H, S-P, S-L and S-C)

2-4 Weeks before the start of the exposure 75 fish were added to each aquarium. All the fish were taken from a group of 1 year old cod delivered by Marine Invest A/S (Norway). Blood samples were taken and weight and length was measured, and a PIT-tag (Biomark Inc.) was placed in the abdominal cavity with a MK7-implanter syringe (Biomark Inc.). These PIT-tags was used for the remainder of the experiment as means to identify each fish throughout the experiment. All the fish were now allowed at least 17 days in their final aquarium without exposure to settle into their new environment. The blood samples very analyzed for VTG (see below) and this information was used to pick out 10 assumed males in each aquarium for this study. These 130 fish were marked with fluorescent "visible implant elastomer" (VIE) (Northwest Marine Technology) which are injected underneath the skin of the fish where it solidifies and is visible under UV-light. Four

spots were defined at the root of the dorsal fin; namely at the front right side, the front left side, the rear right side and the rear left side. Using these four spots to either put a fluorescent marker or not, a binary system was employed so that the fluorescent marker as well as the PIT-tag gave a unique identifier for each of the 10 fish in each aquarium. In addition to the marking; blood samples was also taken at this point and the exposure was turned on. The remaining 65 fish in each aquarium were used for a parallel experiment following the same exposure scheme, but where the fish were terminated at each sampling to be able to measure for biomarkers in bile, kidney and liver as well as blood. These 65 were also used as comparison for mortality of the fish in this study.

2.1.2 Exposures

Three exposure schemes in addition to a control were used, and they are henceforth referred to as "control", "low", "high" and "pulse". The group "control" received only water and the acetone used to dilute the chemicals (see 2.1.1), "low" received a 2000 times dilution of PAHs and APs found in produced water (Appendix B), "high" received a 200 times dilution and "pulse" switched between "high" exposure and "control" at 2 weeks intervals. This exposure ran for a total of 44 weeks, maintenance and daily routines was performed according to the following protocol by the crew at Solbergstrand Marine Research station

Every weekday: sick and dead fish were to be removed, and identified by its PIT-tag. All hoses and pumps were checked.
Every week: feeding two times a week, the amount was modified throughout the exposure aiming to keep a condition factor (CF) at approximately 0.9-1.1. At the start of the exposure the fish were fed with 1% of the biomass of in the aquarium. The food used were frozen fish the first 18 weeks, we then change to pellets with a high fat content to assure that the fish had enough fat for gonad maturation. Oxygen concentration in each aquarium was measured and a water sample was taken from each aquarium and the primary header tank to monitor the exposure.

Every two weeks: the exposure solution was changed once every two weeks, adding the chemicals from a premixed stock solution. At the same time the pulsed exposure was also changed back and forth between control and high exposure.

2. 2 Sampling

Samples were taken 7 times during the exposure period in addition to one sample taken before starting the exposures at the 28th of April 2006. The seven times was after 2, 4, 8, 16, 24, 33 and 44 weeks of exposure. All fish were taken out of their aquarium with a net, they were then put in to a large bucket scanned and identified by their PIT tag with a FS2001F-ISO Reader (Biomark Inc.) and by the fluorescent markers at the root of the dorsal fin. Once identified each fish was weighed, measured and a small sample of about 0.7 mL blood was drawn from the caudal vain with a syringe using a 0.6 mm cannula. The syringe was precoated with aprotinin to prevent breakdown of VTG and heparin to avoid coagulation. One drop of the blood was used for the micronuclei assay, one capillary tube was filled and used for hematocrit measurement and the remaining blood was spun down at 2000 g (6200 rpm) with a PMC-060 Capsulefuge (Tomy Tech USA Inc) centrifuge and the supernatant (plasma) was divided in two small eppendorf tubes. The two tubes with plasma as well as the remaining fraction (red blood cells) were shock frozen in liquid nitrogen.

2.3 Biomarker analyses

2.3.1 Condition factor

At each sampling we measured weight and length which was calculated in to Fulton's condition factor(Nash *et al.*, 2005) which is weight (kg)*0.1 divided by the cube of the length (cm). The fish did not receive any food for 24 hours before the sampling.

2.3.2 Hematocrit

A drop of blood was drawn into a 30 mm capillary tube, these samples were then centrifuged for 5 minutes on a Bayer Compur M1100 microtube centrifuge. The

hematrocrit value was read visually by comparing the volume of the red fraction to a scale next to sample.

2.3.3 Total Oxyradical Scavenging Capacity (TOSC)

The samples were analyzed for the scavenging capacity of peroxyl (ROO), peroxynitrite (NOO) and hydroxyl radicals (OH) using a Hewlett Packard 5890 series II gas chromatograph (GC), thus 3 separate analyses were done for each sample. ROO radicals is created by thermal homolysis of 2-2'-azo-bis-(-2-methylpropionamidine)-dihydrochloride (ABAP). When these radicals react with α-keto-γ-methiolbutyric acid (KMBA), ethylene gas is created, the amount of which can easily be measured with gas chromatography. OH radicals are produced from the Fenton reaction of iron-Ethylenediaminetetraacetic (EDTA) and ascorbate, and NOO is formed by 3-morpholinosydnonimine-HCl (SIN-1) and diethylenetriaminepentaacetic acid (DTPA). KBMA reacts in the same way with the two latter radicals to create ethylene gas. All cells contain antioxidants that react with the created radicals, thus limiting the amount of radicals that can react with KMBA. The decrease in ethylene production compared to a control sample can therefore be used as a measurement of the scavenging capacity of these radicals. Glutathione (GSH) was used as a positive control for the TOSC of ROO radicals; on the other two no positive control was used.

The frozen red blood cells from the sampling where thawed and mixed with four parts homogenization buffer (0.1 M KH_2PO_4 pH 7.4 with 2.5% NaCl, adjusted with HCl), then homogenized on ice with ultrasound (Vibra-cell; Sonics & Materials. Inc.) at 4 W for 1 minute using 4 seconds intervals with 1 second breaks. The homogenized samples were centrifuged at 50.000 g for 2 hours, and the supernatant was divided in four eppendorf tubes; one for each type of radical and one for the protein quantification. All the samples where frozen again at -80°C. The prepared samples where thawed and further diluted with homogenization buffer. The dilutions chosen where decided after running samples with different dilutions to see which would be the best for each of the radicals.

The samples and chemicals where put in 10 mL rubber septasealed gastight vials in volumes indicated in tables 2.3.1 to 2.3.3. The radical producing and detection chemicals where added to the vials with 1 minute intervals, and the same intervals were used as injection times for the produced gas into the GC so that each vials gas production was measured after exactly the same amount of time. The gas production was monitored for 96 minutes with a temperature of 35°C by injecting 200 µL gas aliquots from the headspace of the vials at 12 minutes intervals

The values of the ethylene peaks for each time point both for the control and the samples were plotted and a curve by a second degree equation was fitted to match the points. The area under this curve was calculated by integration. A TOSC value is then calculated by the following equation:

$$TOSC=100-(IntSA/IntCA*100)$$

IntSA is the area under the curve defined by the sample, and intCA by the control. This calculation gives a number between 0 and 100. This value is then divided by the amount of protein (mg) in the sample. The final value is thus TOSC units per mg of protein

The protein quantification was done according the principles described by (Lowry *et al.*, 1951). The samples were diluted 50 times in a 0.1 M Tris buffer (pH 8) and 10 µL of each sample were added in triplicates in a 96 well microtiter plate. The standard series on each plate was created from a blood plasma sample from an internal NIVA standard from estrogen treated cod, which was diluted to 1.4, 1.0, 0.5, 0.25 and 0.125 mg/mL of protein. 25 µL of "Reagent A" (Alkaline copper tartrate solution) and 200 µL of "Reagent B" (diluted Folin reagent) was added to each well and the microtiter plate was shaken gently for 5 seconds. The plates were incubated for 15 minutes in room temperature and absorbance was measured on a Thermomax microplate reader (Molecular devices CA U.S.A) at 750 nm. The standard curve and protein concentrations were calculated with Softmax v5.1 (Molecular devices, CA U.S.A).

Table 2.3.1: Vial content for ROO radical analyses

Chemical/Vial	Control	GSH	Sample
Buffer	800 µL	130 µL	700 µL
GSH (15 mM)	0	70 µL	0
Sample	0	0	100 µL
KMBA (2 mM)	100 µL	100 µL	100 µL
ABAP (200 mM)	100 µL	100 µL	100 µL

Table 2.3.2: Vial content for NOO radical analyses

Chemical/Vial	Control	Sample
Buffer	700 µL	600 µL
Sample	0	100 µL
KMBA (2 mM)	100 µL	100 µL
Fe/EDTA	100 µL	100 µL
AA	100 µL	100 µL

Table 2.3.3: Vial content for OH radical analyses

Chemical/Vial	Control	Sample
Buffer	700 µL	600 µL
Sample	0	100 µL
KMBA (2 mM)	100 µL	100 µL
DTPA (1 mM)	100 µL	100 µL
SIN-1 (0.8 mM)	100 µL	100 µL

2.3.4 Vitellogenin (VTG)

Vitellogenin (VTG) was measured using a competitive ELISA assay with a primary VTG antibody from rabbit, and the secondary antibody was a HRP conjugated goat-anti-rabbit antibody. The method is based on (Hylland *et al.*, 1997)

The plates were coated with 50 ng/mL VTG in 100 µL TTBS-buffer (20 mM Tris buffer, pH 8.5, 0.5 M NaCl, 0.05% Tween-20) with 0.1% Bovine serum albumin (BSA). Except for the 4 wells dedicated non-specific binding (NSB) and blank measurements which received only TTBS-buffer with 0.1% BSA. The plates were incubated over night in 4°C, and then washed 3 times with the TTBS buffer using a Skanwasher 300 (Skatron instruments) plate washer. 300 µL of blocking solution (TTBS with 1% BSA) was added to all wells and left to incubate for 30 minutes in room temperature. The plates were again washed 3 times with TTBS.

The plasma samples were thawed on ice and kept standing in ice through the entire procedure. They were diluted 50 and 5000 times in dilution buffer (TTBS with 0.1% BSA) and 50 µL were added to the plate in quadruplicates for each of the dilutions. Standards where made from a plasma sample with a know concentration of VTG and diluted to 8 different concentrations (2.93 µg/mL, 0.97 µg/mL, 0.33 µg/mL, 0.11 µg/mL, 36 ng/mL, 12 ng/mL, 4 ng/mL and 1.3 ng/mL) and 50 µL was added in duplicates.
The reference sample was 50 µL of cod VTG from Biosense diluted to 100 ng/mL

After adding all the samples, standards and the reference, 50 µL of primary antibody was added to all wells except the blanks; which received 100 µL of dilution buffer
After the addition of the primary antibody the plates were left to incubate over night at 4°C.

The plates were washed 3 times with TTBS and 100 µL of secondary antibody where added to each well, except the blanks (which received 100µL of dilution buffer). They were then incubated for 6-7 hours at 4°C. The plates were washed 4 times with TTBS and 100 µL of TMB Plus substrate which reacts with the HRP on the secondary antibody was

added to each well, after about 10 minutes the reaction was stopped by adding 50 µL of the stopping solution. The color produced by these reactions where measured on a Thermomax microplate reader (Molecular devices CA U.S.A) at wavelength of 450 nm. The data from the plate reader was recorded and concentrations of VTG were calculated according to the standard curve with Softmax v5.1 (Molecular devices, CA U.S.A).

2.3.5 Zona radiata protein (ZRP)

The ZRP analysis was done on males only using a non competitive ELISA assay using rabbit anti-salmon ZRP as primary antibody, and the secondary antibody was a HRP conjugated goat-anti-rabbit antibody. The samples were diluted 2000 times in a coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6) and 50 µL was added in quadruplicates into 96 well Nunc immunosorp microtiter plates. Two wells were reserved as blanks, and received only coating buffer, and four wells were reserved for an internal standard. The plates were then left to incubate at 4 degrees over night. The plates were then washed 3 times in TTBS (the same as for VTG) washing buffer and each well was blocked with 300 µL of blocking solution. After washing 3 more times TTBS, 100 µL of 1:400 solution of salmon anti cod ZRP (Biosense) and dilution buffer (the same as for VTG) was added. The plates were then left to incubate over night again. The plates were washed 4 times with TTBS and 100 µL of KMB plus substrate was added and left to incubate for 20 minutes before adding 50 µL of stopping solution(1 M H_2SO_4). The color produced by these reactions where measured on a Thermomax microplate reader (Molecular devices) at wavelength of 450 nm. The values on each plate were adjusted according to the internal standard and the adjusted mean value was used in the statistical analyses.

2.3.6 Micronucleus

During each sampling a small drop of blood from each fish was put on a slide, using another slide with rounded edges the blood was smeared over the surface of the slide, yielding a thin layer of blood so that single cells could be viewed under a microscope. The slides were then dried and stored at 4°C until analysis.

The slides were gently rinsed with 0.1M Phosphate buffered saline (PBS), and excess liquid was drained. They were stained with 1 μg/mL bisbenzimide for 5 minutes and rinsed with distilled water. The cells were mounted in glycerol-McIlvaine (10 mM citric acid, 40 mM Na_2HPO_4 buffer) (1:1) and a cover slip was gently placed on the slide. The prepared slides were stored in the dark at 4°C.

The slides were analyzed with a Carl Zeiss Axioplan 2 (Carl Zeiss Inc.) fluorescent microscope 1000x magnified with an excitation filter of 365 nm and a barrier filter of 397 nm. Pictures of all the micronuclei were taken using a Carl Zeiss AxiocamHR (Carl Zeiss Inc.) camera connected directly to a computer running Axiovision 3.1(Carl Zeiss Inc.). At least 2000 cells where counted on each slide, the number counted varies a bit because the cells were counted in groups, so that all cells that could be seen in one frame of the microscope were counted and counting was stopped after thus ending at between 2000 and 2050 cells. All slides where counted without knowledge of the origin of the sample, and all samples was counted by the same person.

Only intact cells with distinct nuclear and cellular membrane were counted, to qualify as a micronucleus the following criteria were applied (see also figure 2.3.1 for an example):

- Spherical cytoplasmic inclusions with a sharp contour
- Diameter smaller than 33% of the nucleus
- Color and texture resembles that of the nucleus
- No contact with the nucleus

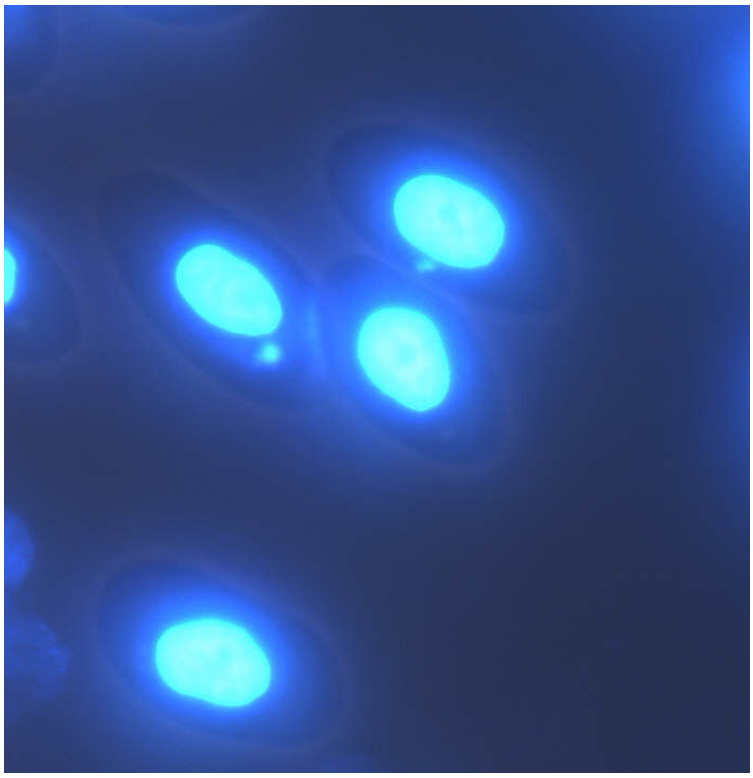

Figure 2.3.1: A micronucleus is located in the top left cell; the irregularity in the top right cell is not counted as a micronucleus as it is not spherical and lacks a sharp contour.

2.3.7 Liver somatic index

At the end of the exposure the LSI was calculated according to the formula :
liver weight / body weight.

2.3.8 Gonado somatic index

At the end of the exposure GSI was calculated according to the formula :
gonad weight / body weight.

2.3.9 Mortality

The mortality of the fish was compared to fish in the same aquaria that were not subject for blood samples. The mortality was compared for the periods 0 – 16, 16-33 and 33 – 44 weeks of exposure. The weeks 2, 4, 8 and 24 was excluded due to lack of certain knowledge of the number of dead fish. Because a number of the fish we compared

against were terminated for samples after 2, 4, and 8 weeks of exposure, a weighted average of the number of fish in the aquaria using the number of weeks and the number of fish alive between each sample date was created.

2.4 Statistical analysis

All the statistical analysis was done in R version 2.6.0 (The R foundation for statistical analysis) In addition to the standard R library, the library "lawstat" was used to perform Levene's test for homogeneity of variance, and "nlme" was used to perform the linear mixed effect models.

Analyses for condition factor, hematocrit, TOSC, VTG, ZRP, LSI and GSI was analyzed with a linear mixed effect model (lme) that incorporates treatment, time and sex as fixed effects and aquarium and repeated measurements of the same fish as random effects. For condition factor, hematocrit and LSI the model used treatment and sex as fixed effects (Lindstrom *et al.*, 1990; Pinheiro *et al.*, 2000.), for VTG and GSI males and females were analysed separately, and for the ZRP and TOSC analyses only males were analyzed.

Micronucleus analysis was only done for high exposure and control. A variable stating whether or not a sample contained any micronuclei was created for each sample. On this variable a Pearson Chi square test (Crawley, 2002) was employed for each sample date.

2.4.1 Analysis for each point in time

The data was analysed with a two way anova with sex and treatment as fixed effects and aquarium as a random effect (Lindstrom and Bates, 1990; Pinheiro and Bates, 2000.). The primary model was with interaction between sex and treatment, but the interaction was removed from the model if there were no significant interaction.

For each sample date the variance of the respons variable was tested with Levene's test for the different treatments and sex . If there was significant heterogenity in the variance, a log transformation of the data was attempted. In cases where log transformation did not produce homogenus variances, non-parametric testing was abandoned for the data in question and Kruskal-Wallis parametric test was used (Millard *et al.*, 2001).

The lme employed used treatment and sex as explanatory variables and aquarium nested in the intercept of the fixed effect as a random effect.

2.4.1 Full time-series analysis

This analysis is used to view all the sampling times at once, and incorporates the information gained by being able to follow each individual fish. Levene's test was used to test the variance of the response variable for treatment, sex and weeks of exposure. If a significant difference was found, the data was log transformed. If log transformation did not produce homogenus variances, Kruskal-Wallis parametric test was used.

The model used was a linear mixed effect model with treatment, weeks of exposure and sex as fixed effects, each individual fish was nested in aquarium and viewed as a random effect. The model also looked at interactions between the explanatory variables. When the interactions was not significant they were removed from the model and a new test was done.

3 Results

3.1 Condition factor

There were no significant differences in condition factor between exposures at any of the sample dates. At 8 weeks of exposure the males had a significantly lower condition factor than females (p=0.03), on the other sample dates there were no significant differences between males and females (Table 3.1.1 to 3.1.7)

Table 3.1.1: Two-way ANOVA of Condition factor in Atlantic cod after 2 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Weeks of exposure	Df	F-value	p-value
Treatment	2	3	0.331	0.8
Sex	2	1	0.776	0.4

Table 3.1.2: Two-way ANOVA of Condition factor in Atlantic cod after 4 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Weeks of exposure	Df	F-value	p-value
Treatment	4	3	1.39	0.3
Sex	4	1	1.69	0.2

Table 3.1.3: Two-way ANOVA of Condition factor in Atlantic cod after 8 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Weeks of exposure	Df	F-value	p-value
Treatment	8	3	0.352	0.8
Sex	8	1	3.66	0.03

Table 3.1.4: Two-way ANOVA of Condition factor in Atlantic cod after 16 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Weeks of exposure	Df	F-value	p-value
Treatment	16	3	0.288	0.8
Sex	16	1	1.35	0.3

Table 3.1.5: Two-way ANOVA of Condition factor in Atlantic cod after 24 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Weeks of exposure	Df	F-value	p-value
Treatment	24	3	0.393	0.8
Sex	24	1	2.75	0.07

Table 3.1.6: Two-way ANOVA of Condition factor in Atlantic cod after 33 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Weeks of exposure	Df	F-value	p-value
Treatment	33	3	0.333	0.8
Sex	33	1	2.27	0.1

Table 3.1.7: Two-way ANOVA of Condition factor in Atlantic cod after 44 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Weeks of exposure	Df	F-value	p-value
Treatment	33	3	0.706	0.6
Sex	33	1	0.684	0.4

The model for the entire exposure period showed no significant differences for treatment or sex. There was a significant difference between the sample dates ($p < 0.001$), and there was a significant interaction between sex and weeks of exposure ($p = 0.03$). The results of the statistical analyses are shown in table 3.1.8 and the data are graphically presented in figure 3.1.1.

Table 3.1.8: Two-way ANOVA of Condition factor in Atlantic cod in a 44 week exposure to three different doses of PAHs and APs.

Explanatory variable	Df	F-value	p-value
Treatment	3	0.259	0.9
Weeks of exposure	1	29.73	<.0001
Sex	1	0.595	0.6
Weeks of exposure x Sex	1	3.686	0.03

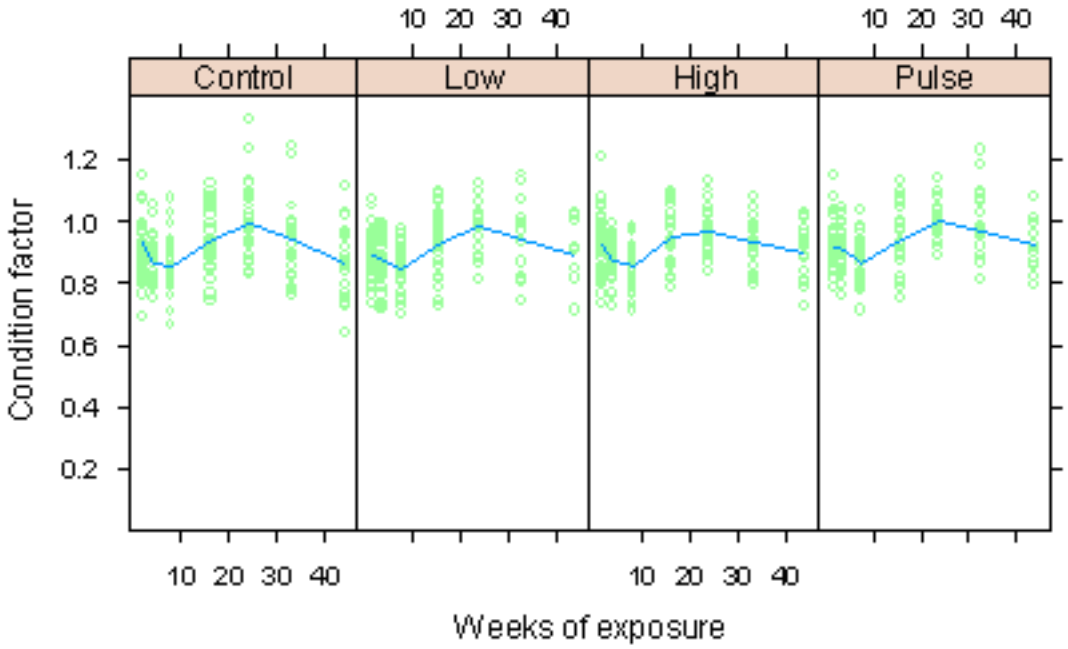

Figure 3.1.1: Condition factor for Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs. The blue line is the arithmetic mean value. The plot shows a similar change of the condition factor during the exposure for all the treatments.

3.2 Hematocrit

The males had significantly higher hematrocrit levels at 2 weeks of exposure (table 3.2.1, p=0.009). At 4 weeks of exposure there was a significant difference between the exposures (table 3.2.2, p=0.002). The median values at this point were 19 for control, 22 for low, 20 for high and 16 for pulse. There was also a significant difference between treatments at 24 weeks of exposure (table 3.2.4, p=0.02). A t-test (table 3.2.5) Shows that "high" has a higher hematrocrit level, and "pulse" has a lower level; both being close to significant (p=0.07 and 0.06). The other sample dates showed no significant differences (table 3.2.3, 3.2.5 and 3.2.6).

Table 3.2.1: A Two-way ANOVA of hematocrit levels in Atlantic cod after 2 weeks of exposure to three different doses of PAHs and APs

Explanatory variable	Analysis	Weeks of exposure	Df	F-value	p-value
Treatment	ANOVA	2	3	2.48	0.1
Sex	ANOVA	2	1	7.48	0.009

Table 3.2.2: Hematocrit levels in Atlantic cod after 4 weeks of exposure to three different doses of PAHs and APs analysed with separate models for treatment and sex.

Explanatory variable	Analysis	Weeks of exposure	Df	F-value	p-value
Treatment	KW	4	3	-	0.002
Sex	ANOVA	4	1	0.1217	0.7

Table 3.2.3: A Two-way ANOVA of hematocrit levels in Atlantic cod after 8 weeks of exposure to three different doses of PAHs and APs

Explanatory variable	Analysis	Weeks of exposure	Df	F-value	p-value
Treatment	ANOVA	8	3	1.98	0.2
Sex	ANOVA	8	1	2.74	0.1

Table 3.2.4: A Two-way ANOVA of hematocrit levels in Atlantic cod after 24 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Analysis	Weeks of exposure	Df	F-value	p-value
Treatment	ANOVA	24	3	5.89	0.02
Sex	ANOVA	24	1	1.48	0.2

Table 3.2.5: A t-test of comparing the hematrocrit values in Atlantic after 24 weeks of exposure to three different doses of PAHs and APs.

Treatment	Weeks of exposure	Value	Std.Error	Df	t-value	p-value
Low	24	0.266	1.64	8	0.162	0.9
High	24	2.86	1.34	8	2.14	0.07
Pulse	24	-3.30	1.51	8	-2.19	0.06

Table 3.2.6: A Two-way ANOVA of hematocrit levels in Atlantic cod after 24 weeks of exposure to three different doses of PAHs and APs.

Explanatory variable	Analysis	Weeks of exposure	Df	F-value	p-value
Treatment	ANOVA	24	3	0.800	0.5
Sex	ANOVA	24	1	1.85	0.2

The model for the entire duration exposure (Table 3.2.7) gives a significant difference between treatments (p=0.02) and a significant interaction between treatment and weeks of exposure (p=0.02). A t-test (Table 3.2.8) Reveals that "pulse" is different from the control (p=0.005) and that the interaction between "pulse" and weeks of exposure is significant (p=0.02)

Table 3.2.7: Two-way ANOVA of hematocrit levels in Atlantic cod in a 44 week exposure to three different doses of PAHs and APs.

Explanatory variable	**Df**	**F-value**	**p-value**
Treatment	3	5.67	0.02
Weeks of exposure	1	1.58	0.2
Treatment x Weeks of exposure	3	3.14	0.03

Table 3.2.8: A t-test of hematocrit levels in Atlantic cod in a 44 week exposure to three different doses of PAHs and APs.

Explanatory variable	**Value**	**Std.Error**	**Df**	**t-value**	**p-value**
High	-0.765	0.868	8	-0.881	0.4
Low	1.23	1.08	8	1.13	0.3
Pulse	-3.83	0.992	8	-3.86	0.005
Weeks of exposure	-0.0100	0.0273	275	-0.368	0.7
High x Weeks of exposure	0.0526	0.0394	275	1.33	0.2
Low x Weeks of exposure	-0.0446	0.0482	275	-0.923	0.3
Pulse x Weeks of exposure	0.0998	0.0441	275	2.27	0.02

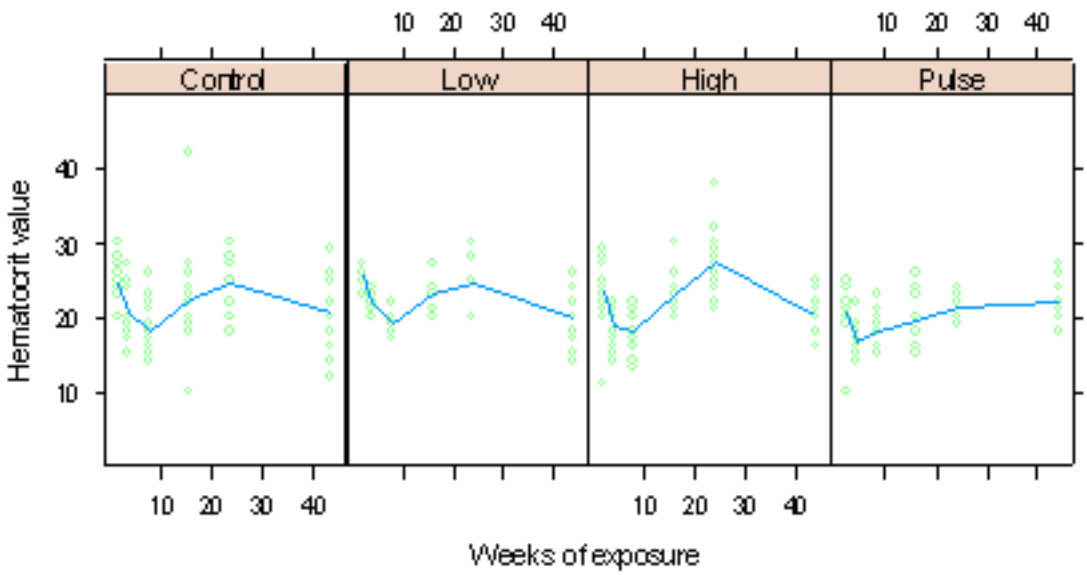

Figure 3.2.1: Hematocrit levels for Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs. The blue line is the arithmetic mean value.

3.3 TOSC

There was a significant difference on TOSC for ROO radicals between treatments at 0 weeks of exposure (p=0.004). As this is before the exposure started no further statistical tests was made on this. The other sample dates showed no significant differences (table 3.3.1). All the data are presented in figure 3.3.1 to 3.3.3.

Table 3.3.1: TOSC of ROO, NOO and OH radicals in Atlantic cod after exposed to three different doses of PAHs and APs.

Explanatory variable	Analysis	Radical	Weeks of exposure	Df	F-value	p-value
Treatment	ANOVA	ROO	0	3	13.9	0.004
Treatment	KW	NOO	0	3	-	0.1
Treatment	ANOVA	OH	0	3	4.18	0.06
Treatment	ANOVA	ROO	8	3	0.144	0.9
Treatment	KW	NOO	8	3	-	0.9
Treatment	ANOVA	OH	8	3	0.809	0.5
Treatment	ANOVA	ROO	24	3	3.00	0.1
Treatment	ANOVA	NOO	24	3	0.0744	1
Treatment	ANOVA	OH	24	3	1.02	0.4
Treatment	ANOVA	ROO	44	3	0.718	0.6
Treatment	ANOVA	NOO	44	3	1.78	0.2
Treatment	ANOVA	OH	44	3	2.31	0.2

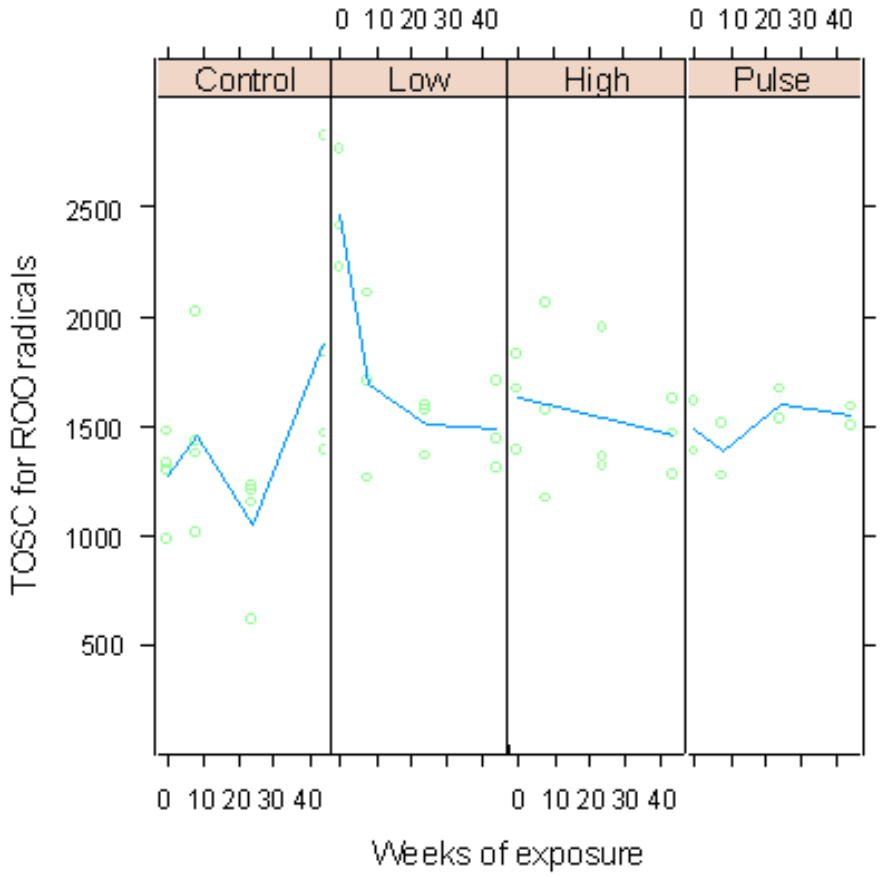

Figure 3.3.1: TOSC of ROO radicals in Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs. The scale on the y-axis is relative. The blue line is the arithmetic mean.

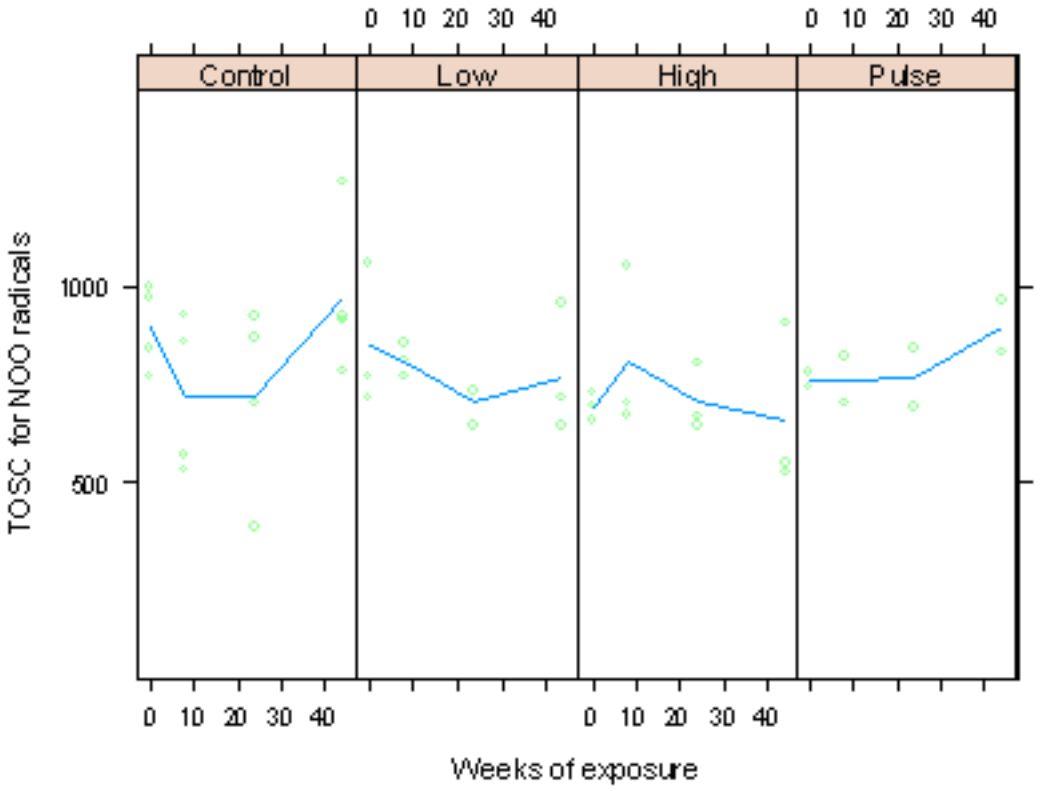

Figure 3.3.2: TOSC of NOO radicals in Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs. The scale on the y-axis is relative. The blue line is the arithmetic mean.

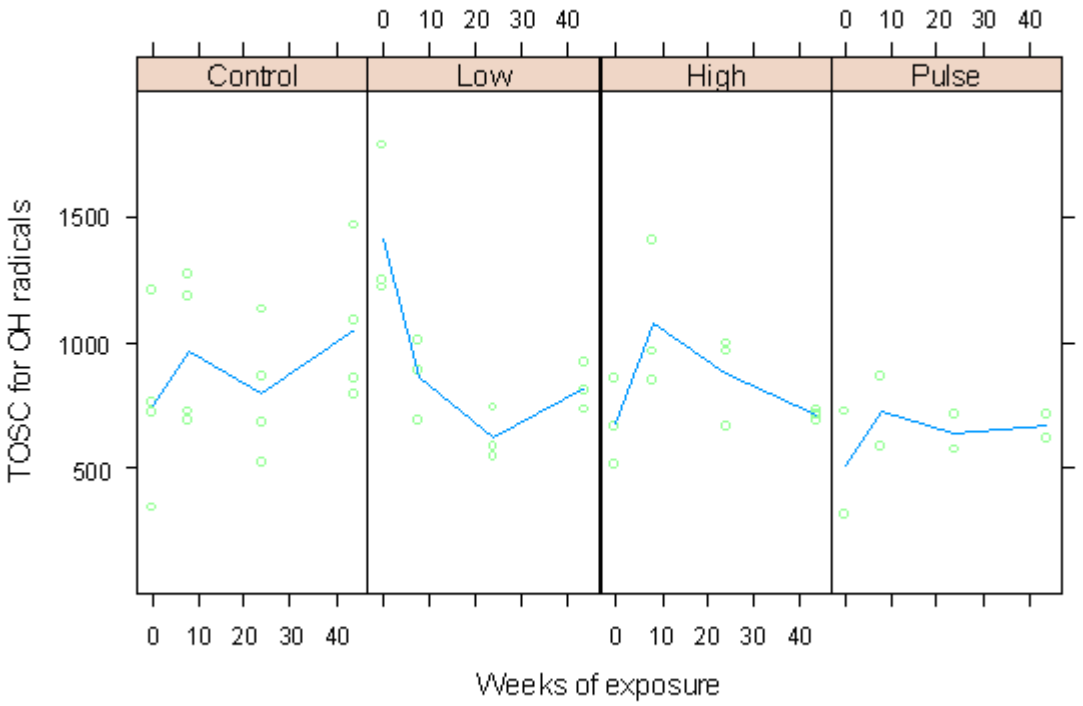

Figure 3.3.3: TOSC of OH radicals in Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs. The scale on the y-axis is relative. The blue line is the arithmetic mean.

3.4 Vitellogenin

There were no significant differences between treatments in VTG levels for any of sample dates for neither males nor females (Table 3.4.1 and 3.4.2).

Table 3.4.1: VTG levels in male Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs.

Explanatory variable	Log trans	Analysis	Weeks of exposure	Sex	Df	F-value	p-value
Treatment	NO	ANOVA	0	M	3	0.766	0.5
Treatment	YES	ANOVA	8	M	3	1.95	0.2
Treatment	NO	ANOVA	24	M	3	0.661	0.6
Treatment	NO	KW	44	M	3	-	0.6

Table 3.4.2: VTG levels in female Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs.

Explanatory variable	Log trans	Analysis	Weeks of exposure	Sex	Df	F-value	p-value
Treatment	NO	ANOVA	0	F	3	0.331	0.8
Treatment	NO	ANOVA	8	F	3	0.125	0.9
Treatment	YES	ANOVA	24	F	3	3.25	0.1
Treatment	NO	ANOVA	44	F	3	0.249	0.9

The model for the entire exposure gives no significant differences for males (Table 3.4.3). For females there is a significant difference in VTG levels at the different sample dates (p=0.0008) (Table 3.4.4).

Table 3.4.3: VTG levels in male Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs analysed with a two-way ANOVA

Explanatory variable	Sex	Df	F-value	p-value
Treatment	M	3	2.35	0.1
Weeks of exposure	M	1	0.047	0.8
Treatment x Weeks of exposure	M	3	0.641	0.6

Table 3.4.4: VTG levels in female Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs analysed with Kruskal-Wallis test.

Explanatory variable	Sex	Df	p-value
Weeks of exposure	F	3	0.0008

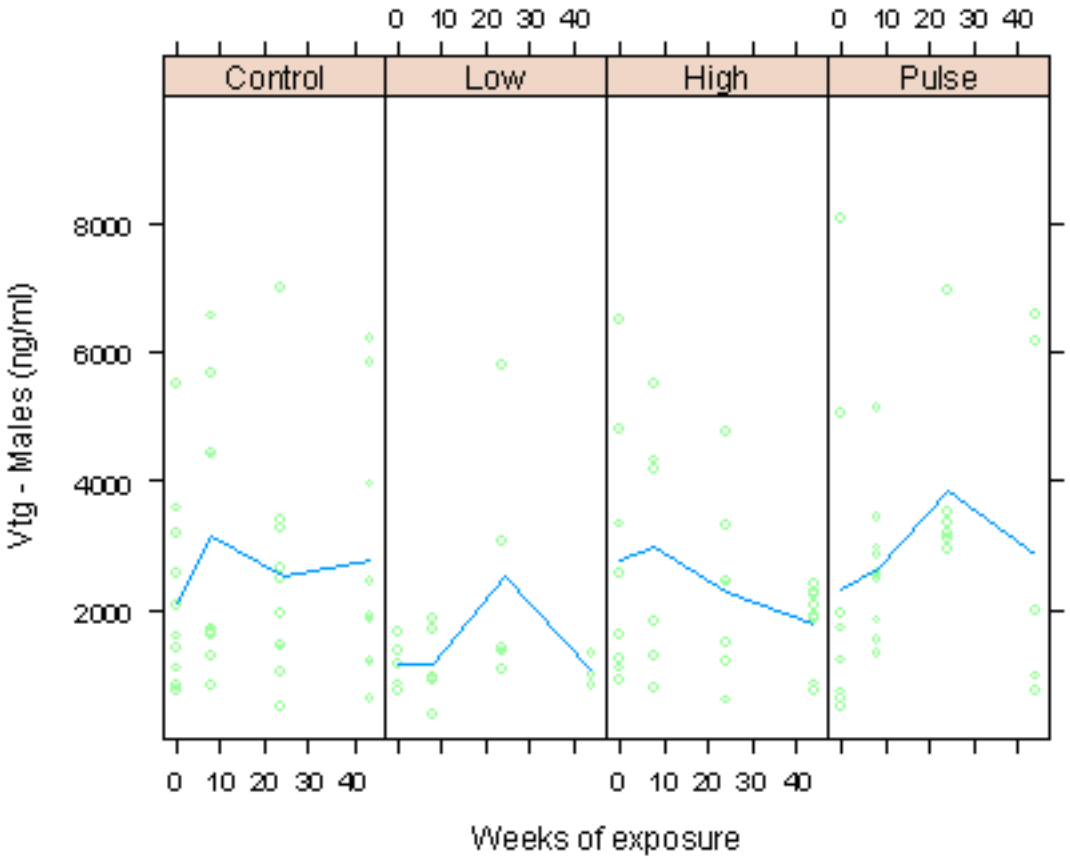

Figure 3.4.1: VTG levels for male Atlantic cod exposed for 44 weeks to thee different doses of PAHs and APs. The blue line is the arithmetic mean.

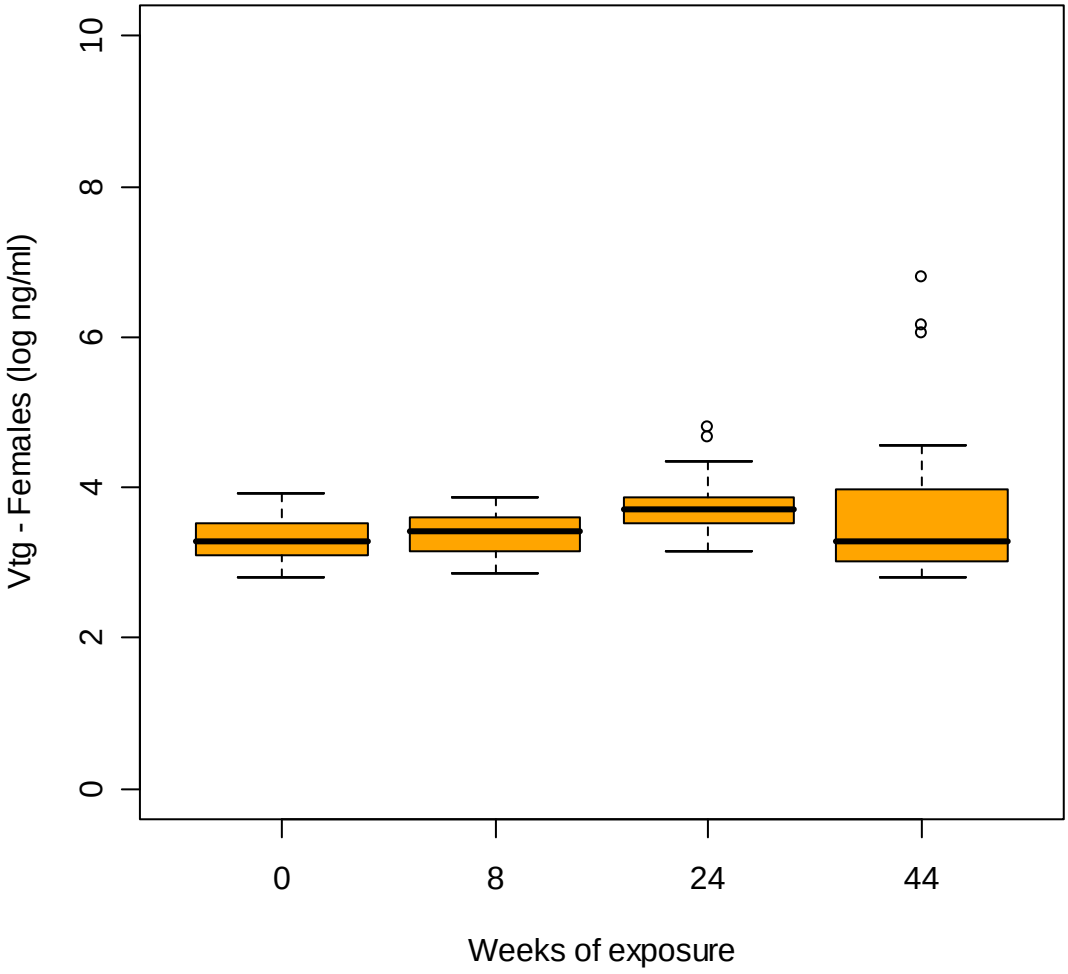

Figure 3.4.2: Log transformed VTG levels for female Atlantic cod exposed for 44 weeks to thee different doses of PAHs and APs. The blue line is the arithmetic mean; there is an enormous increase in some individuals at the end of the experiment.

3.5 Zona radiata protein

The analysis of ZRP levels in males gave no significant results for either of the sample dates (Table 3.5.1). Neither did the model for the entire exposure (Table 3.5.2).
The data are presented in figure 3.5.1.

Table 3.5.1: ZRP levels in male Atlantic cod exposed for 33 weeks to three different doses of PAHs and APs analysed with a two-way ANOVA.

Explanatory variable	Weeks of exposure	Df	F-value	p-value
Treatment	0	3	2.11	0.2
Treatment	8	3	1.83	0.2
Treatment	24	3	1.10	0.4
Treatment	33	3	0.0112	1

Table 3.5.2: ZRP levels in male Atlantic cod exposed for 33 weeks to three different doses of PAHs and APs analysed with a two-way ANOVA

Explanatory variable	Df	F-value	p-value
Treatment	3	2.12	0.2
Weeks of exposure	1	0.185	0.7
Treatment x Weeks of exposure	3	1.37	0.3

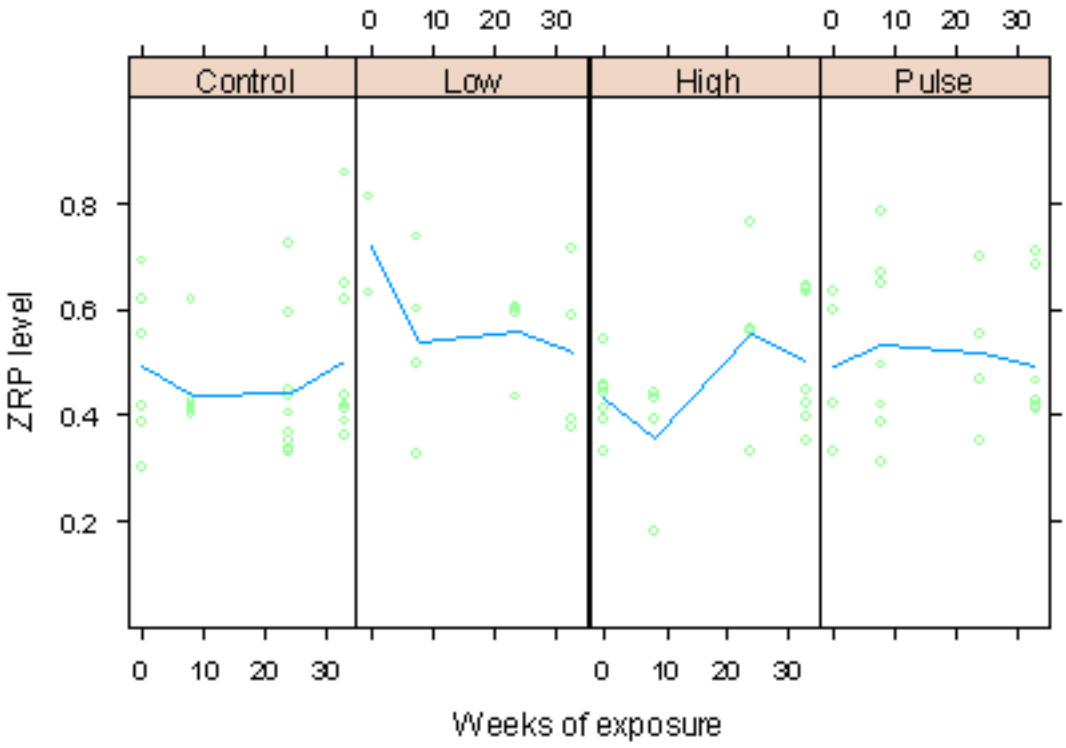

Figure 3.5.1: ZRP levels for male Atlantic cod exposed for 33 weeks to three different doses of PAHs and APs. The blue line is the arithmetic mean value. The scale on the y axis is relative.

3.6 Micronucleus

There were no significant differences in MN occurrence at any of the sample dates tested (Table 3.6.1 and 3.6.2).

Table 3.6.1: MN occurrence in erythrocytes of Atlantic cod exposed to PAHs and APs analysed with a Pearson Chi-square test.

Treatment	Weeks of exposure	MN	MN in control	P-value
High	0	6 of 8	5 of 11	0.09
High	2	3 of 9	5 of 11	0.5
High	16	2 of 9	5 of 11	0.2
High	33	4 of 9	5 of 11	0.9

Table 3.6.2: MN frequency in erythrocytes from male Atlantic cod exposed to PAHs and APs.

Treatment	Weeks of exposure	Aquarium	Fish	MN frequency
Control	0	1	1	0.00100
Control	0	1	3	0.00000
Control	0	1	4	0.00050
Control	0	2	2	0.00049
Control	0	2	4	0.00049
Control	0	2	6	0.00000
Control	0	12	1	0.00000
Control	0	12	4	0.00000
Control	0	12	7	0.00050
Control	0	13	5	0.00000
Control	0	13	8	0.00000
High	0	6	1	0.00000
High	0	6	4	0.00000
High	0	6	6	0.00050
High	0	7	8	0.00197
High	0	7	10	0.00049

High	0	8	1	0.00195
High	0	8	2	0.00295
High	0	8	3	0.00049
Control	2	1	1	0.00100
Control	2	1	3	0.00000
Control	2	2	2	0.00000
Control	2	2	4	0.00099
Control	2	2	6	0.00000
Control	2	12	1	0.00000
Control	2	12	4	0.00049
Control	2	12	7	0.00098
Control	2	13	2	0.00049
Control	2	13	5	0.00000
Control	2	13	8	0.00000
High	2	6	1	0.00000
High	2	6	4	0.00000
High	2	6	6	0.00000
High	2	7	2	0.00000
High	2	7	8	0.00000
High	2	7	10	0.00000
High	2	8	1	0.00049
High	2	8	3	0.00147
High	2	8	5	0.00589
Control	16	1	1	0.00000
Control	16	1	3	0.00000
Control	16	1	4	0.00050
Control	16	2	2	0.00000
Control	16	2	4	0.00098
Control	16	2	6	0.00050
Control	16	12	1	0.00000
Control	16	12	4	0.00000
Control	16	12	7	0.00048
Control	16	13	2	0.00000
Control	16	13	5	0.00099
High	16	6	1	0.00000
High	16	6	4	0.00000

High	16	6	6	0.00000
High	16	7	2	0.00000
High	16	7	8	0.00049
High	16	7	10	0.00000
High	16	8	1	0.00000
High	16	8	2	0.00098
High	16	8	3	0.00000
Control	33	1	1	0.00048
Control	33	1	3	0.00050
Control	33	1	4	0.00000
Control	33	2	2	0.00000
Control	33	2	4	0.00040
Control	33	2	6	0.00000
Control	33	2	8	0.00000
Control	33	12	1	0.00000
Control	33	13	2	0.00050
Control	33	13	5	0.00000
Control	33	13	8	0.00048
High	33	6	4	0.00050
High	33	6	6	0.00000
High	33	7	2	0.00000
High	33	7	8	0.00099
High	33	7	9	0.00000
High	33	7	10	0.00049
High	33	8	1	0.00000
High	33	8	2	0.00000
High	33	8	6	0.00098

3.7 Gonado somatic index

There were no significant difference for GSI for neither males (p=0.6) nor females (p=0.8) (Table 3.7.1).

Table 3.7.1: GSI in Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs analysed with one-way ANOVAs

Explanatory variable	Sex	Log trans	Df	F-value	p-value
Treatment	M	NO	3	0.597	0.6
Treatment	F	YES	3	0.402	0.8

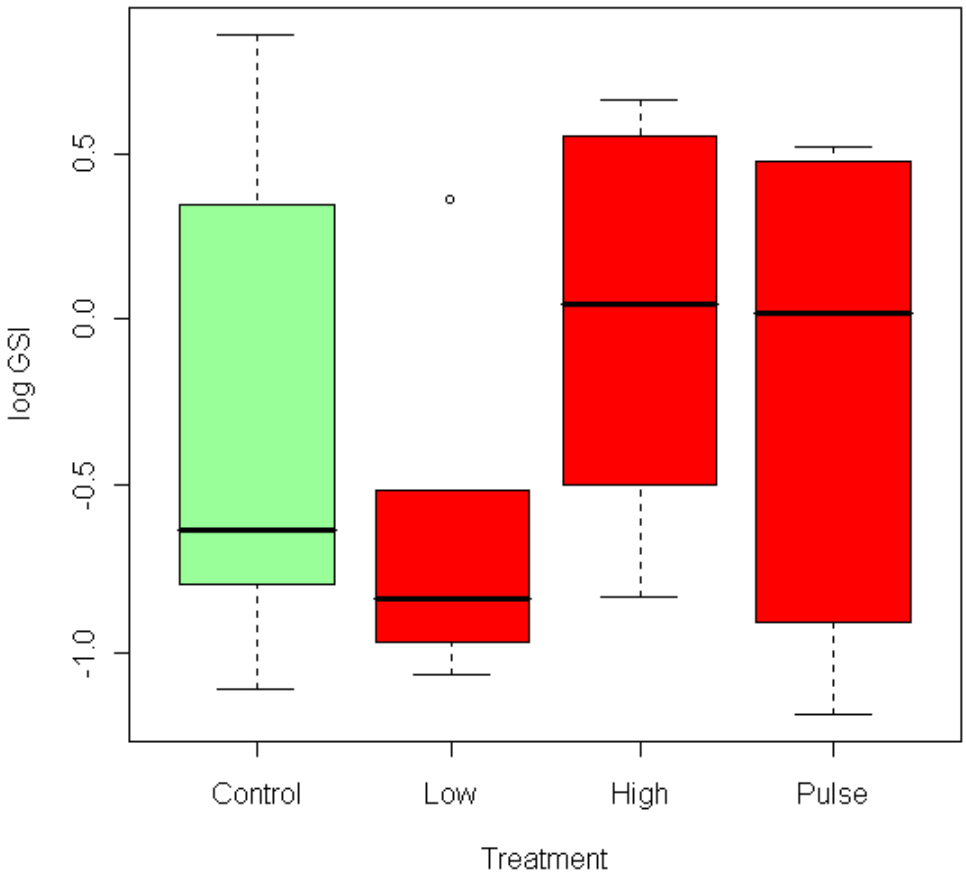

Figure 3.71: Log transformed GSI for male Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs.

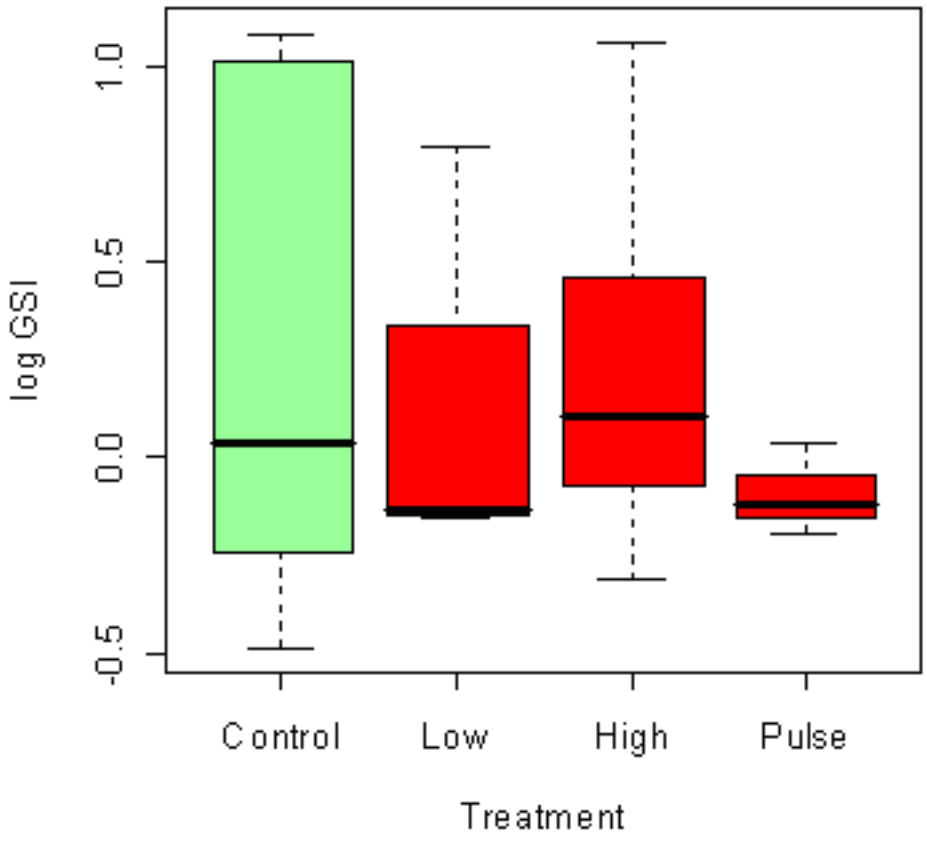

Figure 3.7.2: Log transformed GSI for female Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs.

3.8 Liver somatic index

The liver somatic index after 44 weeks of exposure showed no significant effects for neither treatment(p=0.5) nor for sex (p=0.08) (table 3.8.1 and figure 3.8.1)

Table 3.8.1: LSI in Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs.

Explanatory variable	Df	F-value	p-value
Treatment	3	0.832	0.5
Sex	1	3.31	0.08

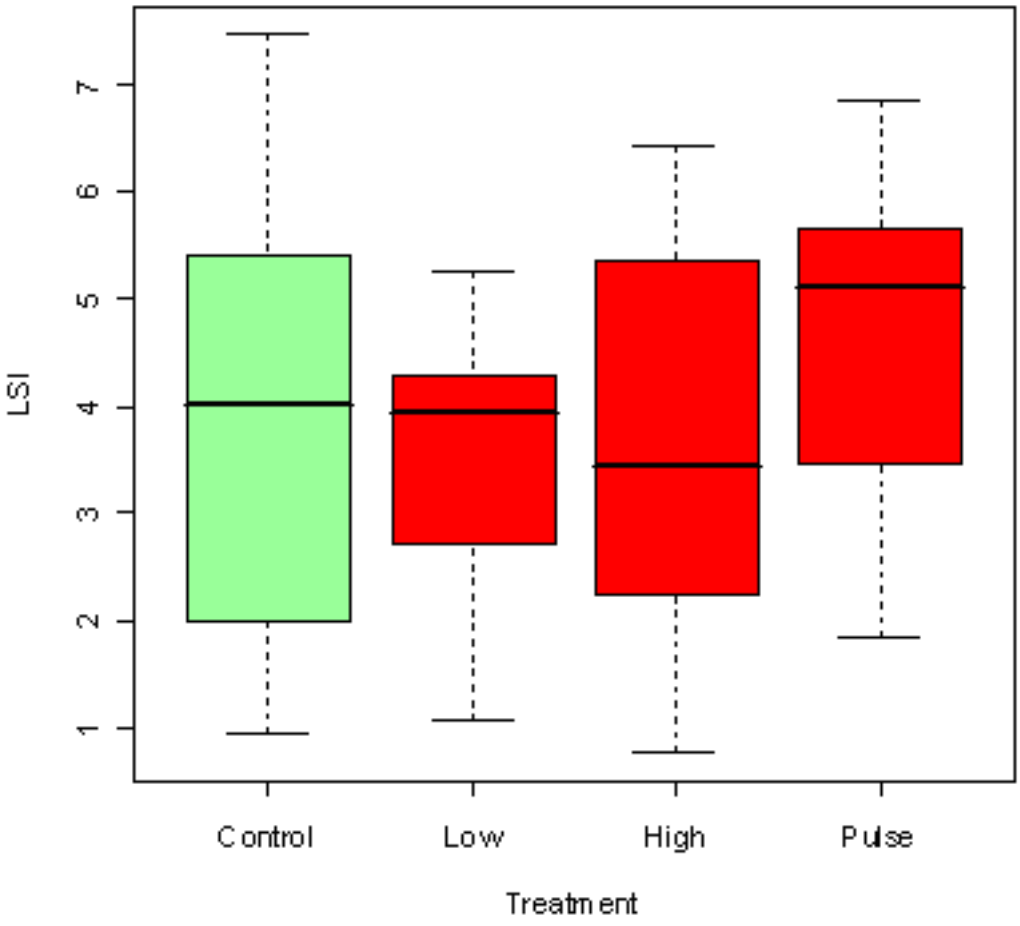

Figure 3.8.1: LSI in Atlantic cod exposed for 44 weeks to three different doses of PAHs and APs.

3.9 Mortality

There was a significant difference in mortality between the fish subject to repeated sampling compared to unsampled fish in the same aquaria (table 3.9.1) both form 16 to 33 (2.7 percentage points higher, p=0.05), and 33-44 weeks of exposure (6.3 percentage points higher, p=0.03).

Table 3.9.1: Mortality of fish subject to repeated blood sampling compared to unsampled fish analysed with a Pearson Chi-square test.

Period	No. of samples taken from repeatedly sampled fish	% mortality sampled fish	% mortality unsampled fish	p-value
0-16 weeks	2-5	16.9	18.7	0.3
16-33 weeks	6-7	17.6	14.9	0.05
33-44 weeks	8	23.6	17.3	0.03

4 Discussion

4.1 General health

There were significant differences in hematocrit levels between the treatments at different time point in this study as well as some differences between males and females but CF showed no differences. The differences found were not consistent throughout the exposure and significant numbers occuring by chance must be expected when many analyses are preformed. Although 3 out of 26 significant numbers is a bit higher than what can be expected by chance alone for a p-value of 0.05; it is not unreasonable to dismiss these as coincidences due to the lack of consistency.

What on the other hand can be seen from the condition factor is the effects on our feeding scheme. The condition factor decreased the first 8 weeks, when it was decided to increase the amount of food. A steady increase can be seen up to 24 weeks when the feeding was stabilised.

It is interesting to see that the hematocrit levels seems to follow the same trend as the condition factor throughout the exposure. No literature stating a link between condition factor and hematocrit could be found. Changes in CF takes a long time, while hematocrit values are known to respond quickly to stress (Iversen *et al.*, 1998), but there are also seasonal variances that differ between species and location (Mercaldo-Allen *et al.*, 2003), these values are usually lowest during the winter which probably is related to metabolic rate and oxygen consumption (Powers, 1980). In Atlantic cod (Magill *et al.*, 2004) found no significant differences between winter and summer although the winter values very slightly lower, while (Audet *et al.*, 1993) found significantly lower values in the winter. If explained by seasonal variations this study would contradict the two mentioned studies as the values are lowest after 8 weeks of exposure which is in June.

During the first 16 weeks of exposure we took blood samples of the fish 4 times. This did not significantly effect mortality. From 16 to 33 and from 33 to 44 the mortality was significanlty higher in the sampled fish. At these points the sampled fish had been sampled for blood 7 and 9 times, and the fish compared against only once. This suggests that the fish were affected by the repeated sampling, as were also suggested by visual inspections showing that some of the sampled fish lost pigmentation in their caudal fin. Some of these fish regained a healthy looking fin, but one was also terminated due to loss of skin on the fin. There is not much data to be found on how repeated blood sampling affects fish, but this study could suggest that repeated blood samples can increase mortality rates in fish.

4.2 Oxidative stress

This experiment showed no significant effect of the exposures on TOSC analysis for ROO, NOO and OH radicals. Oxidative stress is a phenomenon that can affect all living organisms. The radicals created can react with almost anything; including DNA, proteins and cellular membranes. The deleterious effects of this are well known, and are well studied. However it is important to consider that the pathological effects may not arise until the radicals have depleted the cells antioxidant defenses. Direct measures of antioxidants like superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glyoxalase I & II, and glutathione have been used but criticism has been raised towards this method (Regoli *et al.*, 2002; Chesman *et al.*, 2007). Their conclusion is that differences in one or a few of these might not be ecologically relevant. The TOSC assay on the other hand gives a measure of the total capacity to handle one or more specified radicals. Unfortunately, not too many studies with TOSC as a biomarker in fish have been done. (Regoli *et al.*, 2002) found increased TOSC for ROO, NOO and OH radicals at times were disposal of sediment containing PAH and other pollutants in red mullet (*Mullus barbatus)*. (Chesman *et al.*, 2007) Found that the TOSC value of cod caged in the vicinity of an oil platform had declined with distance from the platform, which indicates an increase in oxidative stress near the platform. The same study also found that this also correlates with metallothionein (MT) levels, and speculates if these two biomarkers could

have somewhat overlapping capabilities. Although no significant change in TOSC were found in this study it is important to consider that PAH levels are not well known in the study by Regoli et al. (2002), and the increase could also be related to other compounds. The effects found by Chesman et al.(2007) could also be due to oxidative stress related to Cd, Zn, Cu which increase production of MT.

4.3 Estrogenic response

The exposure gave no significant effects on VTG and ZRP levels, and the gonadosomatic index at the end of the experiment was not affected by the exposure. It is worth mentioning the many of the values were close to limit of detection, and this could produce unwanted noise in the data.

The only difference yielded by these analyses was an increased level of VTG for females. This seems to be caused by a drastic increase for a few individuals, which is also expected as maturing females are know to have enormous amounts of VTG in their plasma. The fact that some alkylphenols have an esterogenic effect and thus induce both VTG and ZRP levels are well known(Arukwe *et al.*, 1997; Meier *et al.*, 2007). However most of the literature found is regarding nonylphenol and other alkylphenols not used in this exposure. 4-tert-butylphenol (4-tBP) is probably the most estrogenic chemical in our exposure and in a cell culture study it has shown to been more estrogenic than nonylphenol(Jobling and Sumpter, 1993). Although regarded as highly estrogenic, its potency compared to the natural estrogen E2 is on the scale of 10^{-4} according to Jobling and Sumpter (1993). 4-tBP have also been shown to induce the production of VTG in the carp *Cyprinus carpio* (Barse *et al.*, 2006). The concentration on 4-tBP in this study was 0.69mg/L which is about 3000 times more than the strongest concentration in our exposure (0.18 µg/L). In Barses study the testiculo-somatic index also decreases under exposure to 4-tBP, whereas no such effects occures in our study.

The lack of litterature on fish exposed to low doses of 4-tBP and other alkylphenols through water makes it hard to compare these results. But judging by this experiment, concentrations on these scales is not enough to show an estrogenic effect.

It could also be possible that as our males share aquariums with females, a carry-over of estrogen between fish could give an induction of VTG in all aquariums. Such carriy overs have been observed (Hylland, pers.com) and could potentially cover up small estrogenic effects. It is also a possibility that some of the chemicals in this study have anti-estrogenic properties which counters the effects of 4-tBP.

4.4 Micronucleus formation

There were no significant difference in the occurence of MN between treatments in this experiment and the mean value of micronuclei was 0.42 pr 1000 cells.
Comparing results from other labs has some complications, because MN are often scored under different criteria and the interspecies variability is high. Bolognesi et al.(2006) sampled erythrocytes from 10 species in a reference area. The mean number of MN ranged from 0 to 1.75 pr 1000 cells with a median of means of 0.29 MN/1000 cells. Induction of micronuclei has been shown in studies of turbot (*Scophthalmus maximus)* exposed to 0.5 ppm North Sea oil (Bolognesi *et al.*, 2006) (Barsiene *et al.*, 2006). The study by Bolognesi et al.(2006) also gave an even larger induction when the oil was spiked with 0.1ppm APs. The study by Barsiene et al.(2006) also included erythrocytes from peripheral blood and head kidney of cod. A significant induction of MN was detected in erythrocytes from the head kidney, but not in peripheral blood cells. Other previous studies has also shown that sensitivity can be a challenge investigating MN in peripheral blood from fish(Tilbury *et al.*, 1990). In addition it is important to consider that these exposures used higher doses and include chemicals not existant in our exposure. In comparison the highest dose of APs in this study was approximately 0.011 ppm and a total of 0.023 ppm including all exposure chemicals (see appendix B)
Both the lack of significance and the rate of micronuclei seems consistant with previous experiments, and it seems that the low levels of chemicals in ecologically relevant dilutions of produced water is not enough to induce micronucleus formation

4.5 Exposure system

The exposure system worked well. There were some problems with the pumps that delivered exposure solution to secondary header tanks. They stopped from time to time, apparently because air was trapped in the pump. This was temporarily solved by removing and reattaching the hoses to get the air out. A permanent solution was achieved after about 5 weeks of exposure by adjusting the hoses between the exposure solution and the pump so that they went straight up. This prevented air bubbles from building up in the hose. In addition the exposure solution was put in flow through cold water baths to limit the amount of vaporization. Measurement of PAH metabolites in bile after 4, 8 and 33 weeks of exposure indicates values proportional with the doses in the exposure solutions (Holth T.F, unpublished material 2007)

The protocol for removing dead fish did not include determining sex of the fish; this is unfortunate because the sex of most of the fish that died before the end of the exposure remains unknown. They were believed to be males according to VTG analyses, however almost half of the fish that survived to the end turned out to be females. It is well known that sexually immature females have very low concentrations of VTG.

6 Conclusions

This study suggest that along term exposure to PAHs and Aps in low concentrations; 200 times less than what is found in produced water from the Statfjord production area does not affect Atlantic cod according to any of the biomarkers we used. That includes biomarkers for general health (hematocrit, CF and LSI), oxidative stress (TOSC), estrogenic effects (VTG, ZRP and GSI) and DNA damage (MN). The study did however show an increase in mortality when sampled 6 or more times over the period of the experiment compared to fish that were only sampled once. There was also seemed to be a correlation between hematocrit values and CF, as no known relation between these factors was found in the literature it is difficult to conclude anything about this correlation but further analyses of the data will be performed in the near future.

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Appendix A: List of chemicals

Chemical name	**supplier**
2,2'Azobis (2-methylpropionamidine) dihydrochloride(ABAP)	Aldrich
Aprotinin	Sigma
Ascorbic Acid	Sigma
bisbenzimide	Hoechst
Bovine serum albumin	Sigma
Carbonate bicarbonate buffer capsules	Sigma
Citric acid	Hoechst
Diethylenetriaminepentaacetic acid (DTPA) free acid	Sigma
DMSO	
Ethylenediaminetetraacetic (EDTA) disodium salt	Sigma
Ferric chloride hexahydrate ACS reagent ($FeCl_3*6H_2O$)	Sigma
Fetal calf serum	
Glutathion, reduced form(GSH)	Sigma
Glycerol	
Goat anti rabbit IgG conjugated with HRP	Biosense
Heparin	Sigma
H_2SO_4	
KMBA	Sigma
KH_2PO_4	Sigma
K_2HPO_4	Sigma
L-ascorbic acid	Sigma
Low melting point agarose	Sigma
NaOH	Hoechst
Na_2HPO_4	Hoechst
Normal melting point agarose	Sigma
Rabbit anti salmon ZRP	Biosense
Reagent A (Alkalinecopper tartrate solution)	BioRad
Reagent B (Diluted Folin reagent)	BioRad
Sodium Chloride	Hoechst
SIN-1 hydrochloride	Calbiochem
TMB plus substrate	Kem-en-Tech
Trizma HCl	Sigma
Trizma Base	Sigma
Tween 20(Polyoxyethylene sorbitan)	
Vitellogenin	Biosense

Appendix B: Exposure chemicals and concetrations

Chemical name	**Concentration in PW** µg/l	**2000x dilution** µg/l	**200x dilution** µg/l	**LC50 Fish** µg/l	**% of LC50** µg/l
PAHs					
Naphthalene	310.0	1.55E-01	1.55	240.00	0.646
Acenaphthylene	0.00500	2.50E-06	2.50E-05	185000	0.000
Acenaphtene	1.700	8.50E-04	8.50E-03		
Fluorene	12.37	6.19E-03	6.19E-02	5150	0.001
Anthracene	0.7239	3.62E-04	3.62E-03	2780	0.000
Phenanthrene	16.00	8.00E-03	8.00E-02	40	0.200
Pyrene	1.274	6.37E-04	6.37E-03	2.6	0.245
NPD					
Naphthalene	310.0	1.55E-01	1.55		
Phenanthrene	16.000	8.00E-03	8.00E-02		
Dibenzothiophene	3.800	1.90E-03	1.90E-02	2500	0.001
2-methylnaphthalene	340.0	1.70E-01	1.70	2000	0.085
2,6-dimethylnaphthalene	189.0	9.45E-02	9.45E-01	2250	0.042
2-ethyl-6-methylnaphtalene	113.0	5.65E-02	5.65E-01	810	0.070
2-methylanthracene	20	1.00E-02	1.00E-01	790	0.013
9,10-dimethylanthracene	20	1.00E-02	1.00E-01	280	0.036
4-methyldibenzothiophene	4.600	2.30E-03	2.30E-02		
4-ethyldibenzothiophene	6.600	3.30E-03	3.30E-02	360	0.009
9-methylphenanthrene	21.61	1.08E-02	1.08E-01		
9-ethylphenantrene	23.70	1.19E-02	1.19E-01	89	0.133
9-n-propylphenanthrene	3.510	1.76E-03	1.76E-02		
Phenols					
Phenol	1400	7.00E-01	7.00	150	4.667
p-cresol	1500	7.50E-01	7.50	4000	0.188
4-ethylphenol	630	3.15E-01	3.15	10400	0.030
4-n-propylphenol	123	6.15E-02	6.15E-01	11000	0.006
4-tert-butylphenol	35	1.75E-02	1.75E-01	5100	0.003

Appendix C: Abbreviations

4tBP	4-tert-butyl-phenol
ABAP	2,2'Azobis (2-methylpropionamidine) dihydrochloride
AP	Alkylphenols
CF	Condition factor (Fulton's)
DTPA	Diethylenetriaminepentaacetic acid (free acid)
EDTA	Ethylenediaminetetraacetic (disodium salt)
GC	Gas cromatograph
GSH	Glutathione
KMBA	α-keto-γ-methiolbutyric acid
MN	Micronucleus
MT	Metallothionein
NIVA	The norwegian institue for water research
NOO	Peroxynitrite
OH	Hydroxyl
TOSC	Total oxyradical scavenging capacity
VTG	Vitellogenin
PAH	Polycyclic aromatic hydrocarbons
ROO	Peroxyl
ZRP	Zona radiata protein
E2	17β-estradiol
ELISA	Enzyme-linked immunosorbent assay