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Development of Atlantic cod (*Gadus morhua*) exposed to produced water during early life stages: Effects on embryos, larvae, and juvenile fish

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ABSTRACT

Produced water (PW) contains numerous toxic compounds of natural origin, such as dispersed oil, metals, alkylphenols (APs), and polycyclic aromatic hydrocarbons (PAHs). In addition, PW also contains many different chemicals which have been added during the oil production process. In the study described here, cod were exposed to real PW collected from an oil production platform in the North Sea. This was done in order to best recreate the most realistic field-exposure regime in which fish will be affected by a wide range of chemicals. The biological effects found in this study therefore cannot be assigned to one group of chemicals alone, but are the result of exposure to the complex chemical mixture found in real PW. Since APs are well known to cause endocrine disruption in marine organisms, we focused our chemical analysis on APs in an attempt to better understand the long-term effects of APs from PW on the biology of fish. In this study, cod were exposed to several concentrations of real PW and 17β-oestradiol (E_2), a natural oestrogen, at different developmental stages. Cod were exposed to PW either during the embryo and early larvae stage (up to 3 months of age) or during the early juvenile stage (from 3 to 6 months of age). Results showed that, in general, APs bioconcentrate in fish tissue in a dose and developmental stage dependent manner during PW exposure. However, juveniles appeared able to effectively metabolise the short chain APs. Importantly, PW exposure had no effect on embryo survival or hatching success. However, 1% PW clearly interfered with the development of normal larval pigmentation. After hatching most of the larvae exposed to 1% PW failed to begin feeding and died of starvation. This inability to feed may be linked to the increased incidence of jaw deformities seen in these larvae. In addition, cod exposed to 1% PW, had significantly higher levels of the biomarkers vitellogenin and CYP1A in plasma and liver, respectively. No similar effects were seen in cod exposed to either 0.1% or 0.01% PW.

1. Introduction

Produced water (PW) is a combination of formation water, condensation water, injection water and water used for desalting oil. Therefore, PW is a complex mixture of seawater, dispersed oil, PAHs and other dissolved hydrocarbons, organic acids, alkylphenols (APs), metals, and traces of production chemicals (Neff, 2002). As oil fields age, the amount of water injected into the reservoir to help maintain reservoir pressure increases. Since many oil fields in the North Sea are approaching the end of their productive lives the amount of water injected into the reservoirs has increased rapidly, resulting in a huge increase in the amount of PW discharged into the sea (Durell et al., 2006). From 1990 to 2006 the annual discharge of PW from the Norwegian sector increased from 10 mill m^3 to 162 mill m^3, and together with PW from the British, Danish and Dutch sectors, the total amount of PW released into the North Sea is estimated to be more than 500 mill m^3 per year.

APs are natural components of crude oil (Ioppolo-Armanios et al., 1992), and as a result of their solubility in water high concentrations are still present in the aqueous phase after oil/water separation (Boitsov et al., 2007). Oil production platforms, therefore, release large amounts of APs into the seas via PW. APs have been shown to mimic the effects of the natural female sex hormone

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oestrogen, resulting in disruption of the endocrine and reproductive systems (Meier et al., 2007; Tollefsen and Nilsen, 2008). Previously, the main focus of research has been on the effects of long-chain APs (octyl-, and nonylphenols) which are degradation products of non-ionic surfactants known as AP-ethoxylates. More recently, it has been shown that APs with shorter chain lengths can also bind to the oestrogen receptor and produce mild oestrogenic effects (Tollefsen and Nilsen, 2008).

There is a huge amount of literature available describing the effects of APs on the endocrine system of vertebrates, and on gonadal development in fish in particular (Arukwe and Goksøyr, 1998; Servos, 1999). APs affect a number of reproductive parameters in fish, including induction of plasma vitellogenin (Vtg) in male and juvenile fish (Jobling and Sumpter, 1993; White et al., 1994), inhibition of spermatogenesis (Jobling and Sumpter, 1993; Gimeno et al., 1998; Miles-Richardson et al., 1999; Weber et al., 2002), and oogenesis (Tanaka and Grizzle, 2002; Weber et al., 2003). In addition to binding to the oestrogen receptor, APs have also been shown to affect the brain-pituitary-gonad axis via induction/inhibition of the gonadotropins (Harris et al., 2001; Yadetie and Male, 2002) and to have direct effects on steroidogenesis (Yokota et al., 2005; Arukwe, 2005).

In many fish species sexual differentiation appears to be a relatively plastic process that has proved to be especially vulnerable to the endocrine-disrupting effects of APs (Gimeno et al., 1996; Seki et al., 2003; Gronen et al., 1999; Kang et al., 2003; Knorr and Braunbeck, 2002). Very little is known about the developmental processes that govern sexual differentiation in fish and especially in marine fish like Atlantic cod (*Gadus morhua*). Like several other fish species, cod likely exhibit specific "developmental windows" during larval development in which they are especially vulnerable to the effects of endocrine-disrupting chemicals and other environmental toxins (Gimeno et al., 1996; Devlin and Nagahama, 2002; van Aerle et al., 2002). In salmonids like rainbow trout (*Oncorhynchus mykiss*), a sensitive developmental window affecting sexual differentiation is known to occur early in the larval stage (Billard, 1992). However, in several marine fish the developmental window important for sexual differentiation occurs later, at the juvenile stage after metamorphosis (Blazquez et al., 1998; Hendry et al., 2002; Chiasson et al., 2008).

Recently, Chiasson et al. (2008), have studied gonadal differentiation in Atlantic cod and haddock (*Melanogrammus aeglefinus*). They found that female cod appear before males, with ovarian cavities first observed at 102 days post-hatch (dph) when the fish were 27 mm long (total length, TL). This is similar to what is seen in other marine species like Atlantic halibut (*Hippoglossus hippoglossus*) (Hendry et al., 2002). Male cod were first positively identified (by the appearance of testis containing primary spermatogonia) at 221 dph (TL = 94 mm).

Although a large amount of literature exists concerning the short-term toxic effects of exposure to high concentrations of APs and PW, only a few studies have examined more long-term effects. This study was specifically designed to examine the long-term effects on cod exposed to real PW during specific stages in their early development. Our chemical analysis focused mainly on the AP fraction of PW due to their known oestrogenic properties. The aim of this study was to investigate whether cod exposed to realistic concentrations of PW during early life suffer from decreased fitness. As measures of fitness, survival, growth, and frequency of malformations were studied. In addition, expression of the protein biomarkers vitellogenin (Vtg) and cytochrome P450 (CYP1A) were analysed by ELISA.

The fish described in this study were monitored until sexual maturation and the long-term effects of PW on gonad development and reproductive success analysed. The results of that study will be published elsewhere.

2. Materials and methods

2.1. Rationale for exposure regimens

The aim of this study was to investigate the effects of realistic doses of PW on the sexual differentiation and fitness of cod exposed to PW during early life. PW, released from an oil production platform into the sea is quickly diluted. Previous computer-modelling studies have concluded that PW is diluted approximately 1:30 at 10 m, 1:100 at 100 m, and 1:1000 at 1 km from the outlet pipe (Neff, 2002). Additional results from modelling and field measurements have shown that the concentration of dispersed oil present in the North Sea in an area of 50–100 km surrounding the largest oil fields is approximately 1–3 ppb, which roughly corresponds to a dilution factor of 1:10,000 (Rye et al., 1998). In the study described herein, fish were exposed to three different concentrations of PW that reflects the estimated concentrations found in the North Sea (Table 1). Cod were exposed to PW diluted 1:100 (1% v/v, high dose group, H-PW), 1:1000 (0.1% v/v, medium dose group, M-PW), and 1:10,000 (0.01% v/v, low dose group, L-PW). In addition to the three PW exposed groups, another group of fish were exposed to 10 $\mu g\ l^{-1}$ 17β-oestradiol (E_2) to study the effects of high concentrations of oestrogenic compounds. A further group of fish were maintained in clean seawater throughout the experiment (unexposed fish, U).

2.2. Cod eggs

The eggs used in this study were obtained from wild cod caught in Tysfjorden in Lofoten, Norway. For spawning, one male and one female fish were placed in a spawning tank and the resultant eggs collected from a filter placed under the runoff outlet. To ensure a realistic level of biological variation in this study, eggs collected from five separate pairs of spawning cod were mixed. The average egg diameter (D) from each spawning pair was measured, and the number of eggs calculated according to the following formula: N (number of eggs per mL) = $1222 \times D^{-2.71}$, (Kjesbu, 1989). Using this formula, 60,000 eggs (12,000 from each of the five pairs) were added to 100 L of water in each exposure tank.

2.3. Description of the PW exposure experiments

The experiments were conducted from March to December 2004. Fertilized eggs were collected over 2 days in March 2004 (25–26/03). Fifty percent hatching occurred on 13/04. Three separate exposure experiments were performed as described below (see also Fig. 1).

2.3.1. Experiment 1: PW exposure of cod eggs and yolk sac larvae in multi-well trays

Fertilized cod eggs (1–2 days old) from each of the 5 spawning pairs were transferred to the IMR laboratory in Bergen and incubated in 24-well plates (NUNC) (one egg per well). Five parallel

Table 1
Theoretical dilution factors and estimated environmental relevance of the PW concentrations used in this study.

Groups	Dilution factor	Estimated distance from platform (m)
High (H-PW)	1:100 (1%)	0–50
Medium (M-PW)	1:1000 (0.1%)	50–1000
Low (L-PW)	1:10000 (0.01%)	>2000
Oestrogen (E)	10 μg/l 17β-estradiol	–

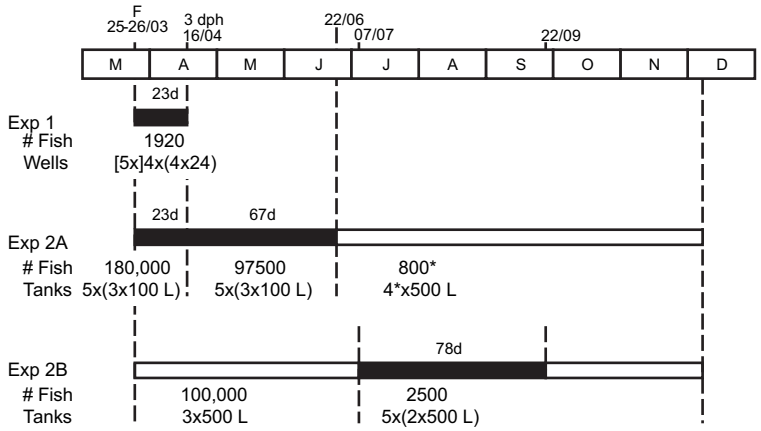

Fig. 1. Schematic representation of Experiments 1, 2A and 2B. Experiment 1 was conducted in 24-well trays and ended on 16/04 (3 dph). Fish in Experiment 2A were maintained in 100 L tanks during the PW exposure phase. At the end of the exposure, 200 randomly selected fish from the M-PW, L-PW, U and E_2 treatment groups were transferred to four 500 L tanks containing normal seawater, where they were maintained till December 2004. The fish in the H-PW group died during the exposure, so we were unable to study these fish further (*). In Experiment 2B, the fish were maintained in 500 L tanks throughout the experiment. The fish were exposed to PW or E_2 for 78 days, after which they were maintained in normal seawater until December 2004. In the schematic diagram above filled boxes indicate exposure to PW or E_2, while open boxes indicate normal seawater. The time frame of the experiment is shown at the top, where the months of the year are indicated by capital letters. Fertilization (F) and three days post-hatch (3 dph) are marked above the relevant dates. Dashed lines indicate the beginning and end of experiments and exposure periods (see Materials and Methods for a detailed description). The total number of fish used at each stage of the experiments is shown by the first line of numbers under the exposure regimen. The second line of text indicates the number of parallel tanks and their volume (or wells for Experiment 1) within the brackets, and the number of groups is indicated outside the brackets.

exposure experiments were conducted: one for each individual family (spawning pair). For each family 16 plates containing 384 eggs in total were set up. The plates were divided into four groups and exposed to H-PW, M-PW, L-PW and normal seawater. Therefore, each treatment group consisted of 480 eggs divided into 20 plates (four plates from each of the 5 families per treatment group). The plates were incubated at 5 °C, and the water was changed every second day. The eggs, and subsequent larvae, were examined microscopically every second day. Survival rate, hatching success, and frequency of lethal deformities were recorded until 3 days post-hatch (dph).

2.3.2. Experiment 2A: exposure of cod eggs and early larvae to PW

Three parallel 100 L tanks, each containing 60,000 fertilized cod eggs (12,000 eggs from each family; 1–2 days post-fertilization), were subjected to one of the five different exposure regimens described in Section 2.1 for 23 days (in March/April 2004), until 3 dph. The larvae were counted and 6500 from each tank were transferred to a fresh 100 L tank and the exposure continued for 67 days (473 degree-days), through the whole start-feeding phase. At the end of this 90 day period (in June 2004) the fish were approximately 2 cm in length and 10 mg in weight (dry weight). The surviving fish from each exposure regimen were pooled and 200 randomly selected early juveniles transferred into 500 L tanks containing normal seawater. All fish in the H-PW group died during this 90 day exposure regimen so no longer-term studies could be performed on this group. The fish (from the remaining four treatment groups) were maintained in these 500 L tanks for approximately 7 months (until December 2004).

2.3.3. Experiment 2B: exposure of cod to PW from 3 to 6 months of age

For this experiment 100,000 cod eggs (20,000 eggs from each of the 5 spawning pairs) were used. The eggs and larvae were maintained in an identical manner to those in Experiment 2A except that they were incubated in clean seawater for 3 months until the early juvenile stage (average length 3.1 ± 0.5 cm). After 3 months, 250 fish were randomly transferred into each of ten 500 L tanks. Separate groups of fish were then exposed to one of the five treatments as described in Section 2.1 (2 parallel tanks per treatment). The exposure lasted for 78 days from the early juvenile to juvenile phase (from July to September 2004), at which time the fish were approximately 11 cm in length and around 13 g in weight. The fish were maintained in these tanks for approximately 3 months (until December 2004).

2.4. Tank set up and dilution of PW in Experiments 2A and 2B

The fish in Experiments 2A and 2B were maintained in 100 L and 500 L tanks during the PW exposure as described. Clean seawater was added to all treatment tanks from a shared header tank by gravity flow. Water from all PW header tanks was diluted 1:100 before being added to the exposure tanks by adjusting the flow rates of the feeder pumps. In Experiment 2A the flow rate during the embryo phase was 25 ml/min from the seawater header tank and 0.25 ml/min from the PW or 17β-oestradiol (E_2) header tanks. During the larval phase the initial flow rate started at 50 ml/min clean seawater and 0.5 ml/min diluted PW or E_2, and was gradually raised to 500 ml/min seawater and 5 ml/min diluted PW or E_2 as the fish increased in size. One PW header tank was set up for each exposure group (three PW header tanks for Experiment 2A and three for Experiment 2B). The header tank feeding the H-PW exposure tanks contained undiluted PW; the header tank feeding the M-PW exposure tanks contained 100 ml PW per L seawater; and the header tank feeding the L-PW exposure tanks contained 10 ml PW per L seawater. Header tanks containing 1 mg l^{-1} E_2 were also set up to feed the E_2 exposure tank (E). Ten mg E_2 was first dissolved in one ml ethanol and 100 μL of this stock solution was added per L of seawater. The nominal concentration of E_2 was therefore 10 μg l^{-1}. The flow rate from all header tanks was monitored daily.

2.5. Collection and treatment of PW

The exposure regimen in this study was carried out with PW obtained from the Oseberg C oil production platform located in the North Sea off the west coast of Norway. The PW was collected in four 1000 L tanks and transferred to land by boat. The PW was then

aliquoted into 25 L containers and frozen at −30 °C until needed. Frozen PW was allowed to thaw over a period of 24 h, bubbled with air for 5 min using an aquarium pump to remove accumulated hydrogen sulphide (H_2S), before dilution and addition to the exposure tanks.

2.6. Feeding

Newly hatched larvae were fed with natural zooplankton, collected by filtering seawater through a Hydrotech drum filter. The collected zooplankton consisted predominantly of copepods at various developmental stages. The zooplankton concentration was measured daily and kept at approximately 1000 zooplankton per L. For the first four weeks the larvae were fed with zooplankton retained between 80 and 250 μm mesh size. As the fish increased in size they were eventually fed with zooplankton retained between 350 μm and 1000 μm. Two types of cultivated plankton algae (*Isochrysis galbana* and *Rhodomonas* sp.) were also added to the tanks daily. Fish were subsequently weaned onto marine fish feed pellets (Dana Feed, Horsens, Denmark), and fed eight times every day from automatic feeders placed over the tanks. The fish were fed with successively larger pellets as they grew.

In Experiment 2A, 40 eggs from each treatment group were incubated in a 1 L beaker. After hatching these larvae were not fed, but were monitored daily until all the larvae died of hunger (unfed group).

2.7. Temperature and light regimen

Experiment 1 was performed at a constant 5 °C in a climate room. In Experiments 2A and 2B, the average temperature in the tanks during the embryo phase was 4.9 °C. The temperature was then gradually increased to 8.6 °C throughout the larval phase and to 12 °C during the early juvenile stage. The fish in the tanks were maintained under day length conditions for Bergen, Norway.

2.8. Chemical analysis of water and fish

The concentration of APs present in the diluted PW header tanks and in the exposure tanks was monitored regularly. Using these data the empirical dilution factor of APs in the exposure tanks relative to undiluted PW was calculated. The uptake of APs into fish tissue was measured at three different time points: yolk sack larvae from Experiment 2A were analysed at 3 dph (after 22 days of exposure to PW, E_2, or clean seawater); early juveniles from Experiment 2A were analysed at 71 dph (after 90 days of exposure); and late juveniles from Experiment 2B were analysed directly following the 78 day exposure period detailed above. The concentrations of 52 APs were measured in the water and in the fish tissue according to previously published methods (Boitsov et al., 2004; Meier et al., 2005). Concentrations of PAHs present in the PW and in the exposure tanks were also measured according to previously published methods (Boitsov et al., 2004), except that PAH standards labelled with stable isotopes were used in addition to the internal AP standard.

2.9. Sampling

Fish were sampled multiple times during the course of Experiments 2A and 2B as follows. In Experiment 2A, larvae were sampled 7 times between March and June (3 dph–67 dph) (see Fig. 1). At each sampling, 15 larvae from each tank were randomly selected, euthanized, and their length and dry weight measured. Whether the larvae had begun feeding was assessed by microscopic examination of the stomach. The fish in Experiment 2A were sampled 5 times (roughly once a month) between June and December (between 3 and 9 months post-hatch). In Experiment 2B fish were sampled 6 times between June and December. During these samplings, 50 fish were randomly selected, removed from the tank, individually weighed and returned to the tank. During the last sampling (in December) all the fish in each tank (approx. 200–250) were removed and individually weighed and measured. Finally, 125 fish from each exposure group were transferred to sea cages at the Austevoll research station as part of a longer-term study (*manuscript in preparation*). The remaining fish in each group were slaughtered, and their sex was determined by morphological examination.

2.10. Sampling for biomarker analysis

At the end of the exposure period in Experiment 2A, whole larvae were sampled for proteomic studies and the results are presented elsewhere (Bohne-Kjersem et al., 2010). At the end of the exposure period in Experiment 2B, liver samples were taken for proteomic analysis (Bohne-Kjersem et al., *in preparation*). Blood and liver samples from Experiments 2B were also analysed for the presence of the biomarkers Vtg and CYP1A, respectively (Nilsen et al., 1998).

2.11. ELISA of CYP1A and Vtg

A quantitative ELISA kit (Biosense, Bergen, Norway) was used to measure the Vtg levels in plasma of juvenile cod (Scott et al., 2006a). A semi-quantitative ELISA using a monoclonal anti-cod CYP1A antibody (clone NP7; Biosense, Bergen, Norway) diluted 1:1000 was used to measure CYP1A levels in the livers of juvenile cod (Nilsen et al., 1998).

2.12. Statistical analyses

The normality of each data sample and homogeneity of group variances were examined and when necessary the data were log transformed prior to testing. Differences between groups were analyzed by one-way ANOVA followed by a Dunnett's post-test for normally distributed data or Kruskal–Wallis non-parametric test followed by Dunn's post-test when data failed tests of normality. The statistical analyses were all performed using XLSTAT software (Addinsoft, US).

3. Results

3.1. Chemical analysis of the PW

Continual analysis of the PW obtained from Osberg C throughout the exposure phase demonstrated that the AP profile and concentration remained constant over time (Fig. 2). Our data also showed that freezing and thawing the PW had no effect on the AP profile or concentration (Fig. 2). The average concentration of all APs of each chain length (from phenol to C9) was also measured (Table 2). The AP profile and relative concentrations in the PW obtained from Oseberg C for this study are similar to those measured previously in PW from the same platform (Boitsov et al., 2004, 2007). The AP data also showed how the AP concentration in PW falls as the solubility in water decreased (Table 2). The PW contained high concentrations of the most water soluble APs, phenol and cresol (C0, C1), but the relatively insoluble long-chain APs ($\geq$C6) were present at much lower concentrations (down to the ng l^{-1} range) (Table 2).

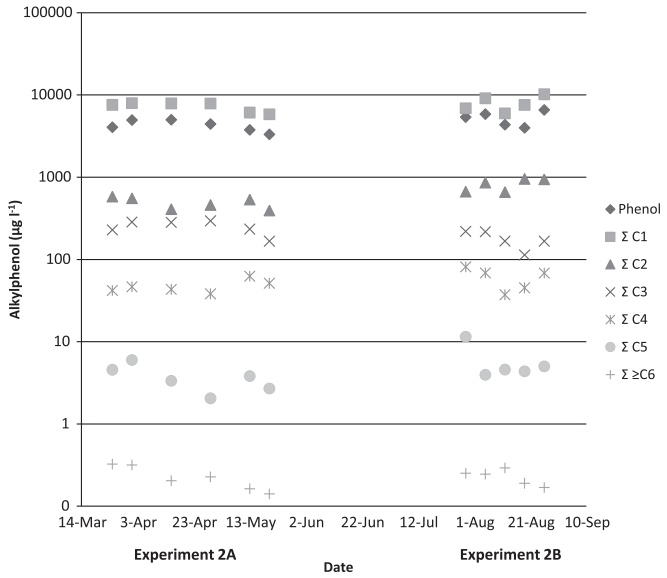

Fig. 2. Stability of the AP concentrations (µg l^{-1}) in the undiluted PW over time. The results are given as the sum of all isomers at each molecular size.

Table 2
Average AP concentrations (µg/L) in the undiluted PW throughout the whole exposure period. The values shown are the average concentrations of 11 separate measurements ($n = 11$) taken over a period of 154 days. The results are given as the sum of all isomers at each molecular size.

	Average ± SD	RSD (%)
Phenol	4696 ± 967	21
Σ C1	7542 ± 1334	18
Σ C2	638 ± 202	32
Σ C3	216 ± 58	27
Σ C4	53 ± 15	28
Σ C5	4.7 ± 2.5	53
Σ C6	0.10 ± 0.04	35
Σ C7	0.02 ± 0.01	70
Σ C8	0.02 ± 0.02	96
Σ C9	0.09 ± 0.05	61

3.2. Chemical analysis of the water in the PW exposure tanks

A clear concentration gradient of APs between the different treatment groups (H-PW > M-PW > L-PW ≈ U) was observed. In addition, the dilution factor of the most volatile components, phenol and cresol, was markedly lower than the nominal concentration. This can most likely be explained by the fact that these compounds are extremely volatile and evaporate faster than they can be replenished despite the continual flow of PW into the exposure tanks. For the long-chain APs (C2–C5), the actual measured concentration was similar to the nominal concentration, at least in the H-PW and M-PW tanks. The dilution factor of the L-PW was so great that the AP concentrations measured were similar to the background levels present in the normal seawater added to the tanks containing unexposed fish (U) (Tables 3 and 4).

3.3. PAH measurements

Typically, for PW originating from Oseberg C, approximately 30% of the PAHs present consisted of naphthalene and methyl-naphthalene (Durell et al., 2006). The relative absence of the highly volatile light PAHs was most likely due to the "airing" of the PW to remove H_2S gas. The highly volatile nature of naphthalene also likely explains why the measured dilution factor in the exposure tanks was only roughly one third of the nominal concentration (Table 5).

Table 3
Concentration of APs in each exposure group in Experiments 2A and 2B. Individual measurements were made on two separate days in Experiment 2A ($n = 2$) and on four separate days in Experiment 2B ($n = 4$). The results are given as the sum of all isomers at each molecular size.

Concentration (µg l^{-1})	U	L-PW	M-PW	H-PW
Experiment 2A				
Phenol	1.04 ± 0.00	3.60 ± 3.83	3.41 ± 3.60	7.33 ± 3.33
Σ C1	0.81 ± 0.98	0.75 ± 0.70	0.89 ± 0.79	7.41 ± 1.63
Σ C2	0.04 ± 0.00	0.13 ± 0.04	0.42 ± 0.08	3.90 ± 0.20
Σ C3	0.04 ± 0.02	0.09 ± 0.01	0.34 ± 0.20	2.58 ± 1.84
Σ C4	0.015 ± 0.007	0.027 ± 0.002	0.067 ± 0.008	0.399 ± 0.109
Σ C5	0.001 ± 0.001	0.003 ± 0.002	0.004 ± 0.001	0.041 ± 0.013
Σ ≥C6	ND	ND	ND	ND
Experiment 2B				
Phenol	2.96 ± 0.66	5.33 ± 1.84	10.29 ± 6.68	29.74 ± 11.33
Σ C1	0.46 ± 0.10	0.81 ± 0.49	11.03 ± 12.16	51.09 ± 24.64
Σ C2	0.04 ± 0.00	0.08 ± 0.03	1.67 ± 1.93	9.22 ± 3.76
Σ C3	0.01 ± 0.00	0.02 ± 0.01	0.27 ± 0.18	2.24 ± 0.46
Σ C4	0.014 ± 0.002	0.018 ± 0.005	0.060 ± 0.033	0.401 ± 0.089
Σ C5	0.006 ± 0.004	0.007 ± 0.005	0.010 ± 0.005	0.034 ± 0.007
Σ ≥C6	ND	ND	ND	ND

Table 4
Empirical dilution factors of the APs in each exposure group in Experiments 2A and 2B. The results are given as the sum of all isomers at each molecular size.

Dilution factor (%)	U	L-PW	M-PW	H-PW
Experiment 2A				
Phenol	0.02 ± 0.02	0.08 ± 0.07	0.07 ± 0.07	0.17 ± 0.03
Σ C1	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.11 ± 0.05
Σ C2	0.01 ± 0.00	0.03 ± 0.01	0.11 ± 0.02	0.97 ± 0.08
Σ C3	0.02 ± 0.00	0.04 ± 0.01	0.14 ± 0.04	1.07 ± 0.43
Σ C4	0.03 ± 0.02	0.06 ± 0.01	0.14 ± 0.03	0.86 ± 0.33
Σ C5	0.02 ± 0.02	0.09 ± 0.08	0.12 ± 0.05	1.41 ± 0.65
Σ ≥C6	–	–	–	–
Experiment 2B				
Phenol	0.06 ± 0.01	0.11 ± 0.05	0.20 ± 0.10	0.60 ± 0.24
Σ C1	0.01 ± 0.00	0.01 ± 0.00	0.13 ± 0.11	0.66 ± 0.27
Σ C2	0.00 ± 0.00	0.01 ± 0.00	0.19 ± 0.20	1.12 ± 0.32
Σ C3	0.01 ± 0.00	0.01 ± 0.01	0.16 ± 0.11	1.47 ± 0.66
Σ C4	0.03 ± 0.01	0.04 ± 0.02	0.12 ± 0.05	0.83 ± 0.31
Σ C5	0.13 ± 0.08	0.15 ± 0.10	0.21 ± 0.11	0.73 ± 0.17
Σ ≥C6	–	–	–	–

Table 5
Actual concentrations of PAHs (µg l^{-1}) measured in the undiluted PW and in the H-PW, M-PW, and L-PW exposure tanks. The background concentration of PAHs in the normal seawater used in this study was also measured (U). The results shown are from one single measurement ($n = 1$) in August 2004.

	PW	H-PW	M-PW	L-PW	U
Naphthalene	242.9	0.791	0.173	0.046	0.020
C1-naphthalene	237.5	0.584	0.288	0.121	0.231
C2-naphthalene	97.5	0.303	0.072	ND	ND
Acenaphthylene	2.7	ND	ND	ND	ND
Acenaphthene	4.4	ND	ND	ND	ND
Fluorene	8.1	0.027	0.017	ND	ND
Anthracene	1.3	ND	ND	ND	ND
Phenanthrene	17.8	0.050	0.028	ND	0.032
C1-Phenanthrene	16.6	ND	ND	ND	ND
Pyrene	0.5	ND	ND	ND	ND
Benz(a)anthracene	0.8	ND	ND	ND	ND
Perylene	ND	ND	ND	ND	ND
Dibenz(a,h)anthracene	ND	ND	ND	ND	ND
Total PAHs	630.1	1.8	0.6	0.2	0.3

3.4. Uptake of APs into the tissue of PW exposed fish

Analysis of the AP levels in cod tissue showed a dose related uptake. For yolk sac larvae the AP concentration was measured in a pooled sample made up of approximately 2000 individual larvae (approx. wet weight 1 g) from all the parallel exposure tanks and sampled 3 dph. For early juveniles (sampled 78 dph) the AP analysis was performed on a pooled sample consisting of 10 individuals (approx. wet weight 1 g) from each of the 3 parallel exposure tanks. For late juveniles (sampled in September 2004) the AP analysis was performed on liver samples from 5 individual fish from each of the two parallel exposure tanks. Our analysis clearly showed that the fish in the H-PW group had taken up the highest levels of APs. Detectable levels of APs were also seen on the M-PW group, but the levels present of the L-PW group were not significantly different from the background levels found in the fish maintained in normal seawater (Fig. 3A–C).

In addition, there were clear differences in the levels of APs found in the different developmental stages. The average bio-concentration factor (BCF) for each species of AP (C_2–C_5) at each developmental stage (where BCF = AP concentration (μg kg^{-1} wet weight) in tissue/AP concentration (μg l^{-1}) in seawater) was also calculated. There was a notably higher BCF in the yolk sac larvae compared to the early and late juveniles for the short chain APs. The average BCF for C_2 phenols (9 different isomers) was 12 for the yolk sac larvae, 2 for the early juveniles, and 0.6 for late juveniles. A similar pattern was also seen for the C3 phenols (10 different isomers), but not for the C4 (10 different isomers) or for the C5 phenols (6 different isomers) (Table 6).

3.5. Effects of PW on cod embryos and yolk sac larvae

The effects of PW on cod eggs and embryos were studied both in multi-well dishes (Experiment 1) and in tanks (Experiments 2A). None of the PW concentrations had any apparent effect on survival or hatching success (an average of between 30 and 35% hatched in all treatment groups) of cod eggs in Experiments 1 and Experiment 2A (Fig. 4 and data not shown). However, 100% of the embryos and newly hatched larvae exposed to H-PW (1% PW) in the Nunc trays (Experiment 1) lacked pigmentation (Fig. 5), and a similar lack of pigmentation was also observed in the larvae exposed to H-PW in the tanks (Experiment 2A). This effect on pigment development appeared to be only transient, as fish sampled at later time points had apparently normal pigmentation.

Experiment 1 also revealed a large difference in egg quality between the 5 different spawning pairs (SP). Each individual egg was inspected microscopically a total of 5 times up to 3 dph and the percentage of visible deformities resulting in death were recorded. While one pair (SP5) demonstrated an average deformity rate of only 10%, one pair (SP3) had an average deformity rate of approximately 60%. However, there was no increase in the rate of embryo mortality as a result of the PW treatment (Fig. 4).

3.6. Effects of PW on larvae and early juveniles

The larvae exposed to the highest concentration of PW (H-PW) showed clear effects: just after start-feeding the larvae were still noticeably less pigmented and the stomach was often empty or contained only a small amount of food (Fig. 6). Interestingly, many larvae in the H-PW group appeared to have deformed jaws (Figs. 5 and 6), and this may partly explain why these larvae are unable to feed efficiently.

At 19 dph large numbers of the larvae in the H-PW group died. This corresponds with the time of death observed in all the unfed groups. Therefore, exposure to 1% PW adversely affected the ability of cod larvae to begin feeding, and most larvae died of starvation. At the end of the larval exposure regimen described in Experiment 2A (in June 2004) the surviving fish in each of the three parallel tanks of the H-PW group were counted. In one tank no fish survived and in the other two tanks only 12 and 15 fish survived, respectively. In the unexposed control group (U) the number of surviving fish in each of the three parallel tanks was 693, 561, and 440 respectively. The percentage survival of the unexposed fish (U) was, therefore, slightly less than 10% (Fig. 7). This enormous level of mortality and relatively low percentage survival is a normal feature of development in batch spawners such as cod, that release millions of eggs every 3–4 days during the spawning period (Kristiansen et al., 1997). The percentage survival in the L-PW, M-PW, and E_2 treatment groups was slightly lower than the U group, but the differences observed were not significant (Fig. 7).

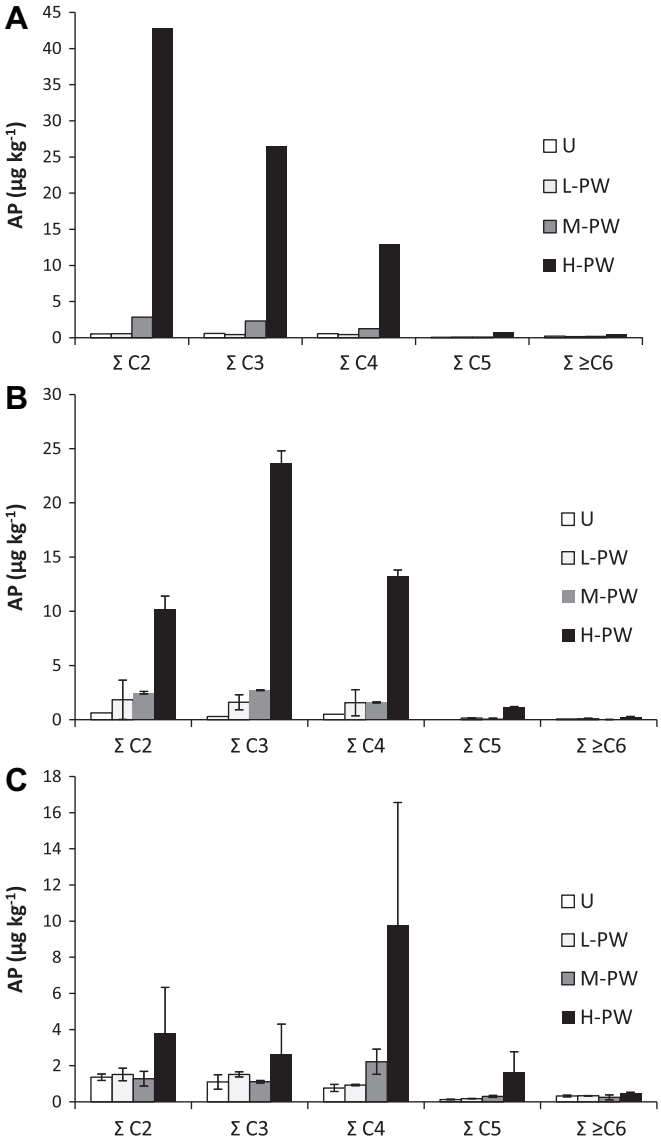

Fig. 3. Tissue concentration of APs (μg kg^{-1} wet weight) in yolk sac larvae (A), early juveniles (B), and juveniles (C). The results are given as the sum of all isomers at each molecular size. For yolk sac larvae measurements were performed on only one sample ($n = 1$) pooled from each of the three parallel tanks corresponding to approximately 2000 larvae (1 g of tissue, wet weight). For early juveniles, measurements were performed on one sample from each of the three parallel tanks ($n = 3$). Each sample was made up of ten individual fish (1 g of tissue, wet weight). Results are shown as the average ± SD. For juveniles the measurements were performed on approximately 0.5 g (wet weight) of liver from 5 individual fish from each of 2 parallel tanks ($n = 10$).

Table 6
Average bioconcentration factor (BCF = concentration in wet weight tissue/concentration in seawater) at each molecular size for the H-PW group. For C_2, the concentrations of 8 isomers were measured and the average is shown in the figure. Six isomers were measured for C_3, seven for C_4 and two for C_5. For yolk sac larvae and early juveniles the measurements were performed on pooled samples of whole individuals. One gram of tissue was used for analysis which corresponded to approximately 2000 yolk sac larvae and 10 early juveniles. For juveniles the measurements were performed on approximately 0.5 g of liver from individual fish.

	Yolk sac larvae	Early juveniles	Juveniles
C2-phenol	12 ± 7	2 ± 2	0.6 ± 0.4
C3-phenol	14 ± 5	10 ± 7	3 ± 3
C4-phenol	31 ± 15	30 ± 23	30 ± 40
C5-phenol	68 ± 15	74 ± 46	175 ± 169

At the start of Experiment 2A (in April) the standard length of all fish was approximately 5 mm. At the end of the experiment (in June) the standard length ranged from 11 to 28 mm. Visually, the few surviving H-PW exposed fish were larger than the fish in the other groups at the end of Experiment 2A (*data not shown*). However, due to the low number of survivors in the H-PW group, statistical significance could not be shown. This suggests that there has been a selective mortality and density dependent growth in the H-PW exposed group. No differences in growth were found in any of the other groups.

After the exposure ended (in June 2004) the surviving fish were transferred to clean seawater and growth and survival was monitored until December 2004. There were no significant differences in mortality throughout the experiment (which was less than 10% in all groups from June to December 2004). But the fish in the M-PW (19.2 ± 1.9 mm) and L-PW (19.4 ± 1.9 mm) groups were slightly smaller than the E_2 (20.0 ± 1.9 mm) and U (19.9 ± 2.0 mm) groups at the end of the experiment (in December 2004). However, we feel that this small difference in growth (which is statistically significant) has little if any biological relevance, and the statistical significance is partly due to the large number of observations ($n = 200$ in each group).

3.7. Effects on cod juveniles exposed to PW between 3 and 6 months of age

The percentage survival during Experiment 2B was between 86 and 91%, and there was no significant difference among any of the groups (data not shown). There was also no significant difference in the growth rate of the unexposed fish (U) and any of the PW exposed groups (Fig. 8). E_2 on the other hand, had a huge effect on growth. At the end of the exposure regimen (in September 2004) the average weight of the E_2 exposed fish was only 43% of the average weight of the unexposed fish. This difference in weight was still evident in December after the fish had been kept in normal seawater for nine weeks (Fig. 8).

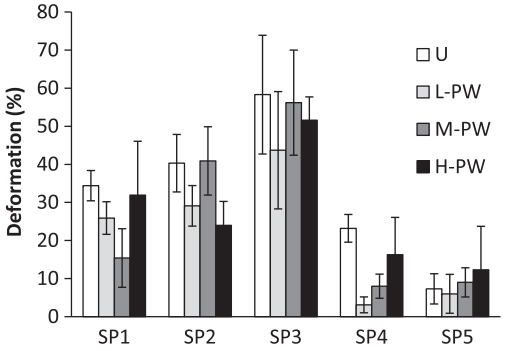

Fig. 4. Percentage of lethal deformities seen throughout the entire embryo stage in Experiment 1. Eggs from each of the 5 spawning pairs (SP1-5) were incubated individually in the wells of 24-well plates (4 parallel plates were analysed for each treatment) filled with the same PW concentrations used in Experiments 2A and 2B.

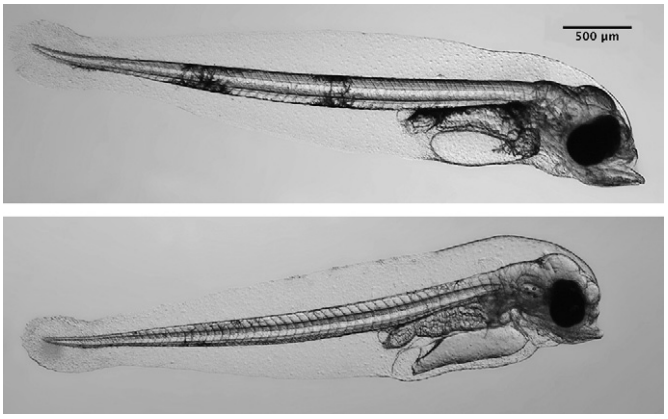

Fig. 5. Representative larvae (4 dph) from the U (A) and H-PW (B) groups following exposure to PW at the embryo stage. The average length of the larvae at 4 dph was 4.5 mm. The lack of pigment and the jaw deformation can clearly be seen on the larvae from the H-PW group. Pictures were retouched in Adobe Photoshop CS4. The clone stamp brush was used to remove foreign particles and contrast was increased using the levels function. As a final retouch sharpening was performed using the unsharp mask filter.

3.8. Expression of Vtg and CYP1A

A significant induction of plasma Vtg levels in cod treated with H-PW and E_2 was observed (Fig. 9A). In the unexposed fish (U) and the L-PW, and M-PW groups Vtg concentrations ranged from 8 to 14 ng ml^{-1}. In the H-PW group the Vtg concentration was 5 mg ml^{-1}, and in the E_2 treated group 150 mg ml^{-1}. Therefore, the Vtg concentration in the plasma of H-PW and E_2 treated fish is 0.5×10^6–1×10^6 times greater than the levels in the plasma of unexposed fish. However, although the Vtg induction in the H-PW group is clearly significant, the levels in plasma are still only 3% of the levels present in the plasma of E_2 treated fish.

CYP1A levels were significantly increased in the H-PW group probably due to the PAHs in the PW. Levels of CYP1A in cod liver were significantly down-regulated following E_2 exposure (Fig. 9B).

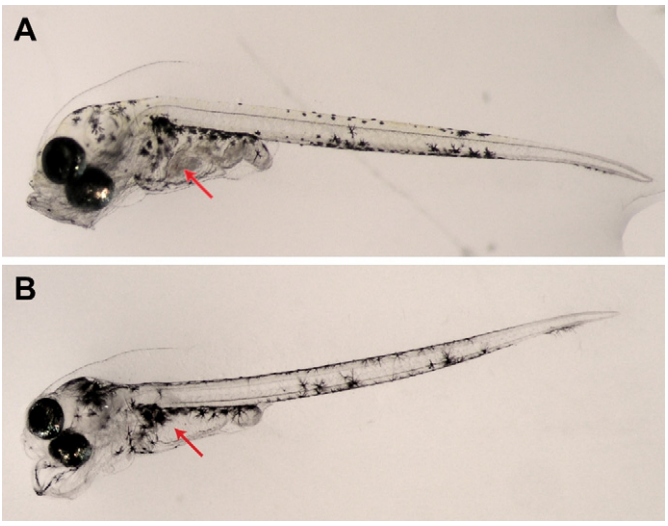

Fig. 6. Representative larvae (14 dph) from the U (A) and H-PW (B) groups. The average length of larvae at 14 dph was 5 mm. An arrow shows the stomach. Zooplankton can clearly be seen in the stomach of the larvae from the U group (A), but not in the stomach of the larvae from the H-PW group (B). The larvae from the H-PW group also have deformed jaws.

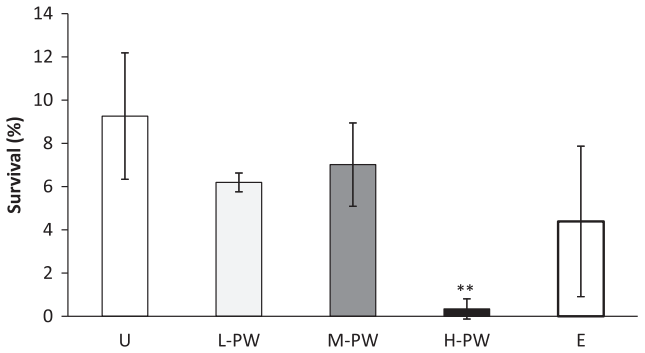

Fig. 7. Survival and growth in Experiment 2A. Survival after 90 days of exposure during the egg and larval stage.

4. Discussion

4.1. PW exposure and rationale behind the experimental design

The exposure experiments described here were performed using real produced water, collected immediately prior to discharge, from the Oseberg C oil production platform (StatoilHydro, Norway) located in the Norwegian sector of the North Sea. The advantage of using real produced water is that it more closely represents the complex mixture of chemical components (both known and unknown) that are discharged into the sea from oil platforms. The PW was transported to shore in 1000 L polyethylene (PE) containers and was then aliquoted into 25 L PE containers and frozen at −30 °C. The PW was flushed with air for 5 min before freezing and after thawing in order to remove the highly toxic hydrogen sulphide gas (H_2S). A similar procedure has recently been used to study the effects of PW on adult cod (Sundt et al., 2009). However, the chemical makeup of PW is not stable and our treatment would be expected to alter the composition. Flushing with air will likely remove the most volatile components like benzene, toluene, ethylbenzene, and xylenes (BTEX). These compounds will also likely evaporate very quickly after the PW is released into the sea. Importantly, our analysis showed that the concentration of the AP fraction remains stable over time and throughout at least one freezing and thawing cycle (Fig. 2). Furthermore, our analysis of the water chemistry during the actual exposure experiments showed the presence of a clear gradient in the AP concentration among the H-PW, M-PW and L-PW groups. Moreover, the measured AP concentrations were very close to the nominal concentrations specified in the experimental design (1%, 0.1%, and 0.01% PW, respectively) (Table 5).

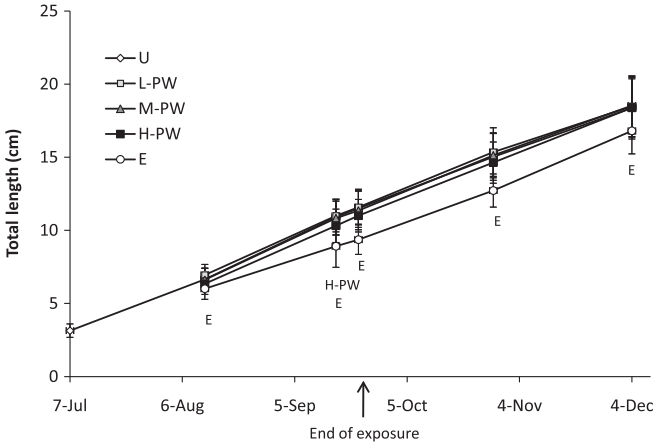

Fig. 8. Growth in Experiment 2B. Growth of juveniles, measured as total length (cm), both during and after exposure (July–December).

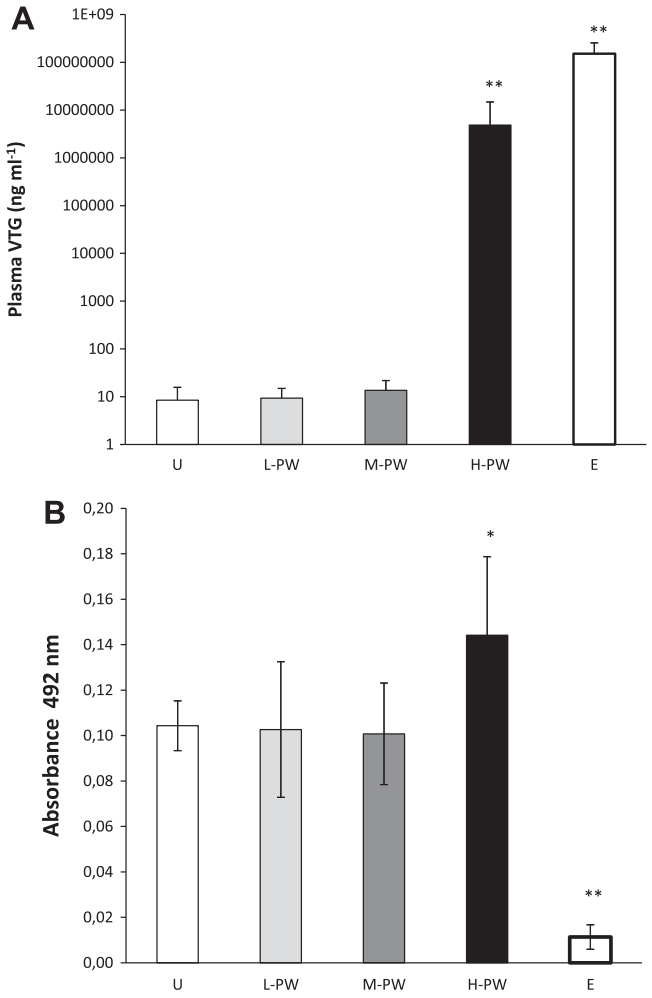

Fig. 9. A. Concentration of Vtg in serum as measured by ELISA. B. Levels of CYP1A in liver as measured by ELISA.

4.2. Uptake of APs into fish tissues

The range of bioconcentration factors (BCF) observed in this study was similar to those that have been reported previously (Servos, 1999). In addition, our results showed that bioaccumulation was dependent on the developmental stage of the fish. Newly hatched yolk sac larvae had a higher BCF of short chain APs (C_2 AP, C_3 AP) compared with early and late juveniles. This may be partly explained by the fact that yolk sac larvae have a higher surface area to weight ratio than later developmental stages (Petersen and Kristensen, 1998). However, it is also likely that the detoxification enzyme system is less well developed in newly hatched cod, as has been shown to be the case for the cytochrome P450 system (Goksøyr et al., 1988). The relative lack of short chain APs (C_2 AP and C_3 AP) in the liver tissue of late juveniles supports the theory that the detoxification enzyme system is much more developed at this later stage. Generally, fish are quite efficient at metabolizing APs, mainly by phase II enzymes that conjugate intact APs to their corresponding glucuronides (Ferreira-Leach and Hill, 2000).

4.3. Effects of PW on cod embryos

The concentrations of PW used in this study (up to 1% in the H-PW group) were not acutely toxic to cod embryos. Although there was no increase in lethal malformations after PW exposure, several sub-lethal effects were noted. Embryos exposed to PW failed to develop pigmentation at the same time as embryos incubated in clean seawater. This effect on pigmentation has been observed previously in marine embryos exposed to oil-related hydrocarbons (Falk-Petersen et al., 1985; Paine et al., 1992). A similar effect is seen in zebrafish (*Danio rerio*) embryos treated with 1-phenyl-2-thiourea (PTU). PTU is used routinely by zebrafish researchers to inhibit pigmentation (Karlsson et al., 2001). This compound inhibits melanogenesis by reducing the activity of the tyrosinase enzyme that converts tyrosine into melanin. Interestingly, it has also been shown that PTU is a weak activator of the aryl hydrocarbon receptor signaling pathway and induces weak transcription of CYP1A1 in zebrafish embryos (Wang et al., 2004). PTU has also been suggested to block production of thyroid hormone in developing zebrafish (Elsalini and Rohr, 2003). These effects have also been seen after exposure of fish embryos to oil hydrocarbons (Alkindi et al., 1996; Stephens et al., 1997; Billiard et al., 2008).

During vertebrate embryogenesis melanoblasts migrate from the neural crest and out into the developing tissue where they ultimately develop into mature melanocytes. This migration is normally a tightly controlled process, but the cellular signals that direct this migration are not well understood (Sulaimon and Kitchell, 2003). It has been suggested that abnormal development and migration of neural crest derived melanocytes may be a highly sensitive indicator of exposure to oestrogenic contaminants in the environment (Bevan et al., 2003, 2006). Clearly, more research on the effects of PW on the development of pigmentation and other developmental process is needed.

4.4. Effects of PW on cod larvae and early juveniles

Our findings strongly suggest that exposure to 1% PW prevents the larvae from beginning to feed on their own, leading to death by starvation. The results presented here are also in agreement with our own earlier studies that showed that yolk sac larvae exposed to 1.5% PW for five days also failed to begin feeding on their own and died of starvation (*unpublished observations*). No apparent effects on survival and growth were seen in either the M-PW or L-PW exposed fish.

Larvae exposed to 1.5% PW show reduced swimming ability and at PW concentrations higher than 4% they go into narcosis and became unresponsive to stimuli (*unpublished observations*). This narcotic effect may explain why the larvae are not able to start feed. Alternatively, the inability to start feed could also be explained by the increased incidence of lower jaw deformities seen in larvae exposed to 1% PW. Lower jaw deformities are a well characterised effect of exposure to oil compounds (Tilseth et al., 1984; Pollino and Holdway, 2002; Carls et al., 1999; Heintz et al., 1999; Debruyn et al., 2007).

Our own unpublished observations, suggesting a narcotic effect, are in agreement with earlier work on the effects of PW exposure. In one such study, turbot (*Scopthalmus maximus*) larvae were exposed to concentrations of PW ranging from 0.001% to 1% for a 6 week period early in development (from 53 dph) (Stephens et al., 2000). The authors reported no increases in mortality in any of the exposure groups but found a reduction in the swimming activity of larvae exposed to 1% PW. Larvae exposed to 0.1% and 1% PW showed changes in the ultrastructure of the cell membranes of the gills which could result in a reduced ability to take up oxygen. In addition, numerous other signs of chronic stress were observed, including increased levels of cortisol and cytochrome P450, and increased activities of CYP1A and 7-ethoxyresorufin-O-deethylase (EROD) (Stephens et al., 2000).

Similarly, a reduction on growth was also observed in cod and herring (*Clupea harengus*) cultivated in a mesocosmos system and exposed to PW diluted 400–800 times (Gamble et al., 1987). A tenfold increase in the cytochrome P450 activity in herring larvae exposed to PW was also reported, indicating an increase in the metabolism of hydrocarbons. In addition, cod larvae exposed to the water soluble fraction (WSF) of crude oil displayed a decreased ability to begin feeding. This effect was observed at a total hydrocarbon concentration (THC) of 250 $\mu g\ l^{-1}$, a THC that corresponds to approximately 1% PW, assuming a typical THC of 20 $mg\ l^{-1}$ (Tilseth et al., 1984).

Concentrations of PW above 1% prevent cod larvae from beginning to feed on their own. However, although the presence of APs in the water during the PW exposure and bioaccumulation of APs in the fish was clearly shown, PW contains many other compounds which likely also contribute to the toxicity. The total PAH concentration in the H-PW tanks was approximately 2 $\mu g\ l^{-1}$. From the literature the lowest observed effect concentrations (LOEC) of PAHs on fish larvae are reported to range from 1 to 23 $\mu g\ l^{-1}$ (reviewed in (Carls et al., 2008)). In addition to APs and PAHs, PW also contains a large "hump" of unresolved complex mixture (UCM), which is composed of a large number of unknown compounds that likely also contribute to the toxicity (Neff et al., 2000; Rowland et al., 2001; Booth et al., 2007; Melbye et al., 2009).

4.5. Effects of PW on cod juveniles

Cod at later developmental stages (after metamorphosis) appear to be more robust and their survival and growth are not affected by exposure to PW (up to 1%). Plasma and liver tissue from cod juveniles were investigated for expression of two well known biomarkers, Vtg and CYP1A, respectively. These biomarker studies showed a significant induction of CYP1A in the livers of cod in the H-PW group (no effect was found in the M-PW or L-PW groups). Up-regulation of CYP1A is a marker for an increase in PAH metabolism (Billiard et al., 2002; Whyte et al., 2000). Vtg was also up-regulated in the fish exposed to H-PW. Vtg is a biomarker that is up-regulated after exposure to oestrogen and other oestrogenic chemicals (Rotchell and Ostrander, 2003). Although the Vtg up-regulation in the H-PW group was only 3% of that observed in the E_2 treated fish (Fig. 9), it is still a clear indication that PW is a potent source of oestrogenic compounds.

Livers from the cod juveniles contain approximately 20 $\mu g\ kg^{-1}$ of C_2–C_5 APs (Fig. 3C). Until recently, it was the release of long-chain APs (octylphenol and nonylphenol) into the environment that was considered to be of most concern, as they were thought to be the most potent agonists of the oestrogen receptor (ER). However, Tollefsen and Nilsen (2008) have now shown that a large spectrum of AP isomers, including short chain APs, can act as ER agonists. Previous *in vitro* studies have also shown that PW contains oestrogenic compounds, and most studies have focussed on the well known oestrogenic effects of APs (Thomas et al., 2004a,b; Tollefsen et al., 2007). However, PW contains many unknown compounds that might have oestrogenic effects. For example, napthenic acids present in PW can function as xeno-oestrogens (Thomas et al., 2009). Many laboratories, including our own, are currently working towards a better understanding of the mechanisms behind the effects of PW in fish. For example, material from this study has been analysed using proteomics in order to search for new biomarkers, and changes in the larval protein profile were observed even at the lowest PW concentration (0.001%) (Bohne-Kjersem et al., 2010). Several reports have also been published

which have used microarray analysis to study the effects of PW at the mRNA level (Olsvik et al., 2007; Holth et al., 2008).

4.6. Effects of E_2 exposure on the early life stages of cod

Severe effects were observed in the cod that had been exposed to E_2, but different effects were seen depending on the developmental stage of the fish at the time of exposure. In late juveniles, E_2 inhibited growth by more than 50% (Experiment. 2B), but no effect on growth was seen in fish that had been exposed to E_2 during the embryo and larval stages (Experiment 2A). Previous studies with tilapia (*Oreochromis niloticus*) have shown similar growth-inhibiting effects of oestrogens (E_2 and 17α-ethinylestradiol). Oestrogen exposure produced significant changes in the expression of insulin-like growth factor I (IGF-I) mRNA in the liver, and also of IGF-I and growth hormone (GH) mRNA in the brain (Shved et al., 2007, 2008; Davis et al., 2008).

The biomarker analysis of cod plasma (from Experiment 2B) showed an enormous induction of Vtg in the plasma of E_2 treated fish. These observations are in agreement with our own previously published work, which showed that Vtg levels were induced to extreme levels in adult male cod following E_2 treatment (Meier et al., 2007). E_2 also strongly down-regulated expression of CYP1A via a mechanism that likely involves crosstalk between the ER and the AhR (Safe et al., 2000; Navas and Segner, 2001).

4.7. Environmental implications of our results compared to the situation in the field

Field studies conducted with fish, mussels, and passive samplers in cages have shown that it is possible to detect an increased uptake of PAHs and APs up to several kilometres from the platform (Hylland et al., 2008; Durell et al., 2006; Johnsen and Røe, 1998; Tollefsen et al., 2005; Harman et al., 2009). Biomarker analyses have also shown that fish held in cages close to the PW discharge outlet show a small but detectable induction of Vtg and CYP1A (King et al., 2005; Scott et al., 2006a; Zhu et al., 2008). In addition, a size dependent increase in the levels of Vtg in plasma has been found in wild male cod from the North Sea (Scott et al., 2006b). Scott et al. (2006b) suggest that oestrogenic exposure may originate from compounds that are biomagnified up through the food chain, but their results also showed that cod with increased Vtg levels were not only found in areas with high oil production activity.

It is important to point out that no definite proof that wild fish caught between North Sea oil fields are adversely affected by PW has been found (Grøsvik et al., 2007; Hylland et al., 2008). On the other hand, wild haddock caught in the Tampen region (an area with high oil production activity) showed increased levels of PAH metabolites in the bile and an increase in the occurrence of DNA adducts in the liver when compared with haddock caught in the Egersund Bank (an area with no oil activity). No similar adverse effects were found in cod or saithe (*Pollachius virens*) caught in the Tampen region (Hylland et al., 2006; Grøsvik et al., 2007).

Results presented here show that the cod yolk sac larvae stage was the most sensitive to the harmful effects of PW. Our data demonstrate that the lowest observable effect concentration (LOEC) on yolk sac larvae is between 0.1% and 1% PW. But due to the dilution factor this concentration can only be expected to be found very close to oil platforms. After PW is discharged into the sea it is quickly diluted and the bioactive compounds will most likely be diluted to a concentration that does not produce any large scale harmful biological effects (Durell et al., 2006; Neff et al., 2006).

However, due to the sheer volume discharged into the North Sea (and discharges are forecast to continue rising until at least 2012–2014), PW is still considered to be a major source of environmental pollution. The concerns about PW have led the Norwegian government to enforce a strict "zero-discharge" policy for all oil exploration activities in the Norwegian Arctic areas.

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