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Effects of alkylphenols on the reproductive system of Atlantic cod (*Gadus morhua*)

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Abstract

Produced water, a by-product of offshore oil production, contains significant amounts of alkylphenols (APs). Many studies have shown that APs cause endocrine disruption in marine organisms, but relatively little is currently known about their long-term effects on the biology of pelagic fish. Here, we describe in detail the effects of APs on the reproductive potential of first-time spawning Atlantic cod (*Gadus morhua*). Cod were fed with feed paste containing four APs (4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol), at a range of concentrations, for either 1 or 5 weeks. AP-exposed fish were compared to unexposed fish and to fish fed paste containing natural estrogen (17β-estradiol). Our results showed that in female fish AP exposure impaired oocyte development, reduced estrogen levels, and delayed the estimated time of spawning by 17–28 days. Similarly, in male fish, we observed that AP-exposure reduced 11-keto-testosterone concentrations, and caused a small induction of VTG levels. We also observed impaired testicular development, with an increase in the amount of spermatogonia and a reduction in the amount of spermatozoa present. Taken together these results suggest that APs released into the sea via produced water may have a negative influence on the overall reproductive fitness of cod populations.

Keywords: Alkylphenols; Endocrine disruption; Cod; *Gadus morhua*; Produced water; Steroids; Ovari; Testis

1. Introduction

Alkylphenols (APs) are natural components of crude oil (Ioppolo-Armanios et al., 1992). As a result of their solubility in water, a high proportion are present in the aqueous phase after water/oil separation. Offshore oil production, therefore, releases large amounts of APs into the seas via produced water. In 2004, it is estimated that approximately 13 metric tonnes of long-chain ($\geq C_4$) APs were released into the sea around Norway in connection with the discharge of produced water (OLF, 2005). Produced water typically contains 0.6–10.0 mg/l APs, and 80% of this amount consists of the most water-soluble APs (phenol and cresol). The remainder is made up of the higher APs (C_2–C_7) (Grahl-Nielsen, 1987; Brendehaug et al., 1992; Røe and Johnsen, 1996; Boitsov et al., 2004). Significant amounts of APs also enter the oceans from several other sources. Nonylphenol (NP) and octylphenol (OP), two relatively well studied long-chain APs, are degradation products of the non-ionic surfactants known as alkylphenol ethoxylates (APEs). APEs are commonly used in many herbicides, paints, and industrial cleaning and degreasing agents and large quantities of these chemicals enter the seas via polluted river water (Naylor et al., 1992). Historically, large quantities of APEs have also been used in offshore petroleum production, both as detergents for washing platforms and as additives in the production process. APEs are now banned in the Norwegian sector of the North Sea, and Danish, British, and European Union authorities are working to phase out their use. However, APEs are still widely used in the USA and Asia (Renner, 1997).

It has been known since the 1930s that APs exhibit oestrogenic properties, but their potential toxicity towards both humans and animals were not recognized until the early 1990s

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(Soto et al., 1991). It is now known that many long-chain *para*-substituted APs bind to piscine estrogen receptors in the same way as the natural ligand, estrogen (17β-estradiol; E2), but generate only a much weaker biological response. This in turn leads to a disruption of the natural endocrine hormone system of the fish (Nimrod and Benson, 1996). Due to these concerns attempts have been made to limit or eliminate the use of APs in many household and industrial products with varying degrees of success. However, the APs found in produced water occur naturally in crude oil and cannot be easily removed. Therefore, further research is clearly needed in order to better understand the long-term effects of APs from produced water on the biology of marine fish.

Previously numerous studies have examined the effects of individual APs (usually OP or NP) on the reproductive health of fish. However, there are only very few studies that have investigated the potential estrogenic effects of the APs found in produced water (Thomas et al., 2004). The APs in crude oil are synthesized by complex geochemical processes (Ioppolo-Armanios et al., 1995). Produced water, therefore, contains a complex mixture of many different isomers. For this reason we chose to expose cod to a mixture of four APs with differing chain lengths (C4–C7) in an attempt to better mimic the chemical composition of produced water. Importantly, all four APs used in this study have previously been shown to be present at concentrations ranging from 70 to 1070 ng/l in produced water samples from North Sea oil production platforms (Boitsov et al., 2004). In addition, 4-*tert*-butylphenol has been found in water and sediments from rivers and coastal areas (Heemken et al., 2001; Koh et al., 2006), while 4-*n*-pentylphenol, 4-*n*-hexylphenol, and 4-*n*-heptylphenol have been found in several Japanese rivers (Inoue et al., 2002) and in coastal waters around Singapore (Basheer et al., 2004). Furthermore, 4-*n*-pentylphenol has also been detected in areas of the North Sea (Heemken et al., 2001). Thus, the study described here was designed to assess the effects of exposure to APs from produced water on the reproductive potential of Atlantic cod (*Gadus morhua*).

2. Materials and methods

2.1. Chemicals

The APs used in the exposure experiment: 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol, 4-*n*-heptylphenol, and 17-β-estrodiol were all purchased from Sigma–Aldrich (Oslo, Norway). Equal proportions of each of these four APs (subsequently referred to as the AP mixture) were combined with the feed-paste as described in Section 2.3.

2.2. Fish

Two-year old cod (mean weight, 0.631 kg), expected to spawn for the first time in the following season, were used in this experiment. The fish came from a strain of Atlantic cod produced at the Institute of Marine Research's (IMRs) field station at Øygarden near Bergen, Norway. The cod were transferred from Øygarden to IMR in August. The fish were divided into seven groups of 40 fish each, and transferred to 10 m^3 indoor tanks prior to the start of the AP exposure experiment (Kjesbu et al., 1996). During the experiment, the (artificial) light was controlled to follow the natural diurnal rhythm. The fish tanks were supplied with seawater from a depth of 100 m and the water temperature remained stable at 8–10 °C throughout the experiment.

2.3. Experimental design

Given the lack of field data, we based the AP doses used in this study on the previously published model proposed by Rye et al. (1996). These authors simulated the spread of APs in produced water from two platforms on the Halten Bank and calculated the likely level of uptake by pelagic fish. The model estimated that the "worst case scenario" body burden of APs in fish could be up to 10 μg/kg (Rye et al., 1996). Many of the previous publications which have investigated the effects of APs on fish have studied only one individual AP. Therefore, in an attempt to better reflect the exposure conditions found in the natural environment in which a wide range of different APs are found in produced water we decided to use a mixture of four different APs with differing chain lengths (from C4 to C7). Based on the data presented by Rye et al. (1996), we concluded that 5 μg/kg of each of the four APs (included in the AP mixture) corresponded to a realistic level of exposure.

The cod were divided into seven groups of 40 fish each as described. Five of these groups were fed with feed-paste containing different doses of the AP mixture (subsequently referred to in the text as AP-exposed fish). These doses corresponded to 5, 500, 5000, 10,000 and 20,000 μg/kg of each of the four individual APs relative to body weight. Thus, the total AP dose that the fish received corresponded to 0.02, 2, 20, 40, or 80 mg/kg, respectively. A sixth group was fed with paste containing E2 (E2-treated fish). These fish received a dose corresponding to 5 mg E2/kg. A final group was fed with paste alone (unexposed fish).

2.4. Feeding regime

From August until November, the fish were fed dry pellets containing 10% lipids (Felleskjøpet AS, Oslo, Norway). The AP exposure regime was initiated in November and in order to accurately control the actual ingested dose of each animal the fish were fed individually as follows. The AP mixture, or E2, was dissolved in 1,2-propanediol and mixed into a paste consisting of ground dry pellets, water, and fish oil (paste composition: 50.5% dry pellets, 40.5% water, 5% fish oil and 4% 1,2-propanediol/alkylphenol solution). For feeding, the fish were first anaesthetized with benzocaine and the paste was then administered directly into the stomach using a syringe fitted with a sterile Stomach Tube (Uno Plast, Hundested, Denmark). To ensure that the fish received the correct dose both the fish and the paste were weighed immediately prior to feeding. All fish were fed once, and 7 days later twenty fish from each group were killed and analyzed. The remaining fish were fed once per week for three additional weeks, and were then killed and analyzed 5 weeks (35 days) after the original dose.

2.5. Sampling

The cod were anaesthetized with benzocaine and blood samples were extracted from the claudal blood sinus with a heparinised syringe. The samples were immediately centrifuged at 3000 × *g* for 5 min at 4 °C. The plasma was frozen in liquid nitrogen and kept at −80 °C until analysis. The fish were killed by a blow to the head, and their weights and lengths recorded. Samples of various tissues (liver, gonads, brain, and muscle) were rapidly excised with a scalpel. All samples were frozen in liquid nitrogen and stored at −80 °C. Tissue samples for histology were fixed as described below.

2.6. Analysis of steroid and vitellogenin levels in plasma

The plasma steroids E2, testosterone, and 11-keto-testosterone (11-KT) were analyzed by enzyme-linked immunoabsorption assay (ELISA) (Dahle et al., 2003).

Competitive ELISAs were developed in our laboratory to determine the plasma vitellogenin (VTG) concentration in both male and female cod. VTG was purified from a freshly thawed standard sample of cod blood plasma (immature fish, induced with estrogen) on an ion-exchange column (Silversand et al., 1993) and quantified by UV-absorption measurements at 280 nm (Norberg and Haux, 1988). Assay standards in the range of 5000–2.441 ng/ml (for the female assay) and 5000–0.038 ng/ml (for the male assay) were prepared by serial dilution of the purified VTG in assay buffer (0.02 M $NaHCO_3$, pH 9.60). The procedure to measure the VTG concentration in the serum of female cod was as follows. The wells of Maxisorp 96-well trays (Nunc) were coated overnight at 4 °C with 150 ng/well of the purified VTG standard dissolved in assay buffer. The plates were then blocked by addition of a solution containing 0.1 M $KHPO_4$, and 2% bovine serum albumin (pH 7.8) and washed with washing buffer (0.01 M $KHPO_4$; 0.5% Tween 20). Seventy five microliters of VTG standards or diluted serum samples were added to the wells followed by 75 μl of a polyclonal rabbit anti-cod VTG (final dilution 1:50,000). These were incubated simultaneously in the wells overnight at 4 °C. After washing, an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Biorad) was added (final dilution 1:3000) and incubated for 2 h at room temperature. After removing the secondary antibody by washing, 150 μl alkaline phosphate substrate (Bio-Rad) was added and the plate was incubated in the dark for 2 h before the reaction was stopped by adding 150 μl of 1 M H_2SO_4. The absorbance was then read at 405 nm on a microplate reader. Samples and standards were run in triplicate. The ELISA to measure VTG levels in male cod was preformed essentially as described above except for the inclusion of a pre-incubation step, which is necessary to achieve the required level of sensitivity (Sherry et al., 1999; Tyler, 1999). Briefly, 100 μl of VTG standards or diluted male plasma samples were added to the wells of 96-well polyetylene trays (Nunc). One hundred microliters of the polyclonal rabbit anti-cod VTG antiserum (final dilution 1:50,000) was added and the plates were incubated for 24 h at 4 °C. One hundred and fifty microliters of the pre-incubated solution was transferred to the wells of Maxisorp 96-well trays (Nunc) and incubated overnight at 4 °C prior to the addition of the alkaline phosphatase-conjugated goat anti-rabbit secondary antibody and continuation of the procedure described above. Serial dilutions of plasma from female cod were parallel to the standard curve, while weak or no cross-reactions were found in plasma dilutions from male and juvenile fish, respectively. The working range of the female ELISA was 20–625 ng/ml (20–80% binding) and the detection limit was 10 ng/ml (90% binding). The intra- and inter-assay variation were determined by analysing standard samples from estrogen induced male cod were 5% CV ($n = 5$) and 16% CV ($n = 16$), respectably. The working range of the male ELISA was 5–312 ng/ml (20–80% binding) and the detection limit was 2.4 ng/ml (90% binding). Prior to analysis all male plasma samples were diluted at least 50 times with assay buffer, thus, the detection limit of the assay was calculated to be 0.12 μg/ml plasma. The intra- and inter-assay variation were determined by analysing standard samples from estrogen induced male cod were 3% CV ($n = 5$) and 11% CV ($n = 26$), respectively.

2.7. Histology and morphology

Samples from selected groups were studied for signs of histological changes. Histological samples were collected during the final sampling, i.e. after 5 weeks (35 days) of exposure. Histological samples were collected from unexposed fish, the E2-treated fish, and from fish exposed to 0.02, 2, and 80 mg/kg of the AP mixture.

2.7.1. Fixing, embedding, and staining

Tissue samples were fixed in buffered formalin: 3.6% formaldehyde in 0.0295 M sodium di-hydrogen phosphate and 0.0461 M di-sodium hydrogen phosphate for estimates of fecundity and oocyte size distribution. For histological studies tissue samples were fixed in a modified Karnovsky fixative: 2.5% formaldehyde, 2.5% glutaraldehyde, and 7% sucrose in 0.05 M sodium dimethylarsenate (sodium cacodylate). The samples were then dehydrated through a graded ethanol series, embedded in methacrylate (Technovit 7100), and 3 μm sections were cut using a Reichert/Jung microtome. The sections were stained with toluidine blue (1 in 2% borax solution).

2.7.2. Follicle diameter

Follicle diameter and potential fecundity were estimated in all groups using a previously described method (Thorsen and Kjesbu, 2001). Background lighting was adjusted to ensure measurements as similar as possible to manual measurements made with an ocular micrometer.

2.7.3. Liver index, gonadosomatic index and Fulton's K

The liver index (hepatosomatic index) was calculated as:

$$\mathrm{HSI}\,(\%) = \left(\frac{\mathrm{LW}}{W}\right) \times 100$$

where LW is the liver weight (g) and W is the wet weight of the fish (g).

The gonadosomatic index (GSI) was defined as:

$$\text{GSI}\,(\%) = \left(\frac{\text{GW}}{W - \text{GW}}\right) \times 100$$

where GW is the gonadal weight (g) and W is the wet weight of the fish (g).

Fulton's condition factor:

$$\text{Fulton's } K = \left(\frac{W}{L^3}\right) \times 100$$

where W is the wet weight of the fish (g) and L is the length of the fish (cm).

2.7.4. Time to spawning

Time to spawning was calculated as $y = 3.33 \times 10^6\, x^{-1.817}$ (Kjesbu, 1994), where y represents days to spawning and x is the diameter of the most mature follicles (so-called G1 or leading cohort). G1 diameter was estimated as the mean diameter of the 10 largest follicles (Thorsen and Kjesbu, 2001).

2.7.5. Testis morphology

The volume fractions of spermatogonia, spermatocytes, spermatides, and spermatozoa were estimated by point counting (Howard and Reed, 1998). A raster of $3 \times 4 = 12$ points was placed in the ocular of the microscope. Counting was done under 40× objective magnification, with a 10× ocular. Several pieces of testis were embedded in a single block, and a sufficient number of fields to allow counting of approximately 240 points/tissue sample were selected on a random basis. The microscope's coordinate stage was used to locate the fields. The volume fraction of spermatogonia, spermatocytes, spermatides, spermatozoa and interstitial tissue were estimated as:

$$\text{est } V_{\text{cell type, testis}} = \frac{\sum_{i=1}^{n} P_{\text{cell type}}}{\sum_{i=1}^{n} P_{\text{testis}}}$$

where $P_{\text{cell type}}$ is the number of points falling on a particular type of cells in field i, P_{testis} the number of points falling on testis tissue in field i, and n is the total number of fields counted.

The total amount of each type of cell was then estimated as:

$$\text{total amount}_{\text{cell type}} = \text{est } V_{\text{cell type, testis}} \times \text{testis weight}$$

No distinction was made between spermatogonia A and B. No attempts were made to distinguish between spermatozoa in spermatocysts from spermatozoa in the lumen (i.e. non-spermiated and spermiated spermatozoa). Thus, the spermatozoa cell type referred to here represent the sum of spermiated and non-spermiated spermatozoa.

2.8. Statistical analyses

One-way ANOVA and Dunnet's test as a post hoc test were used to analyse for statistical differences between the unexposed, AP-exposed, and E2-treated groups for all morphological variables (size, growth, GSI, HSI, Fulton K). For the VTG, steroid measurements, and histological data the statistical differences between the unexposed, AP-exposed, and E2-treated groups were tested by non-parametric Kruskal–Wallis followed by two-tailed Mann–Whitney U-test. The frequency distribution of VTG measurements in the male fish was tested by two-tailed χ^2-test. The statistical analyses were all performed using Statview software (SAS Institute, Cary, NC, USA) or XLSTAT software (Addinsoft, US).

3. Results

3.1. Effects of APs on plasma levels of steroids

Our results showed that exposure to APs had major effects on the natural levels of steroids in female fish. A significant down-regulation of plasma E2 concentrations in AP-exposed females was observed (Fig. 1A). In females sampled 1 week after the first AP-exposure, those fed with AP concentrations of 2, 20, and 40 mg/kg had lower plasma E2 levels than unexposed females. Similarly, in females sampled at the end of the experiment, fish

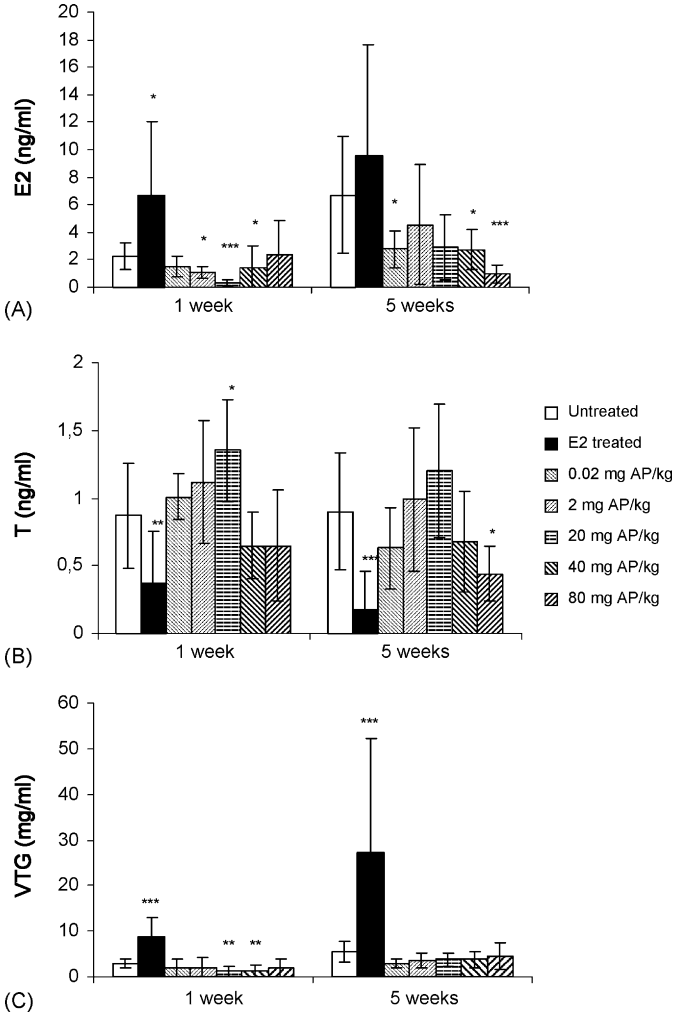

Fig. 1. Levels of 17β-estradiol (E2; panel A), testosterone (T; panel B), and vitellogenin (VTG; panel C) in the plasma of female fish from the unexposed, E2 treated and AP-exposed groups. AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. Mean values ± standard deviation, Asterisks indicate significant differences from the unexposed group, $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

Table 1
Plasma levels of testosterone (T), 11-ketotestosterone (11-KT), 17β-estradiol (E2) and vitellogenin (VTG), as well as GSI values in unexposed, AP-exposed, and E_2-treated male cod

	n	11-KT (ng/ml)	T (ng/ml)	E2* (ng/ml)	VTG (μg/ml)	GSI (%)
1 week						
Unexposed	10	3.8 ± 1.8	3.9 ± 1.6	–	2.8 ± 2.3	8.6 ± 1.9
5 mg E2/kg	9	0.9 ± 0.3**	2.2 ± 0.9	0.6 ± 0.6	8270 ± 3894**	8.4 ± 3.1
0.02 mg AP/kg	10	1.2 ± 0.5**	1.8 ± 0.7**	–	3.1 ± 2.1	8.0 ± 2.69
2 mg AP/kg	10	1.5 ± 0.6**	1.7 ± 0.5**	–	21.7 ± 13.9**	8.5 ± 2.8
20 mg AP/kg	10	1.8 ± 0.8**	2.5 ± 1.0	–	4.8 ± 5.3	8.5 ± 3.0
40 mg AP/kg	10	2.6 ± 0.6*	3.3 ± 1.6	–	4.1 ± 3.9	7.5 ± 1.6
80 mg AP/kg	10	1.8 ± 0.9**	2.5 ± 1.1	–	18.0 ± 17.3**	8.6 ± 1.9
5 week						
Unexposed	10	8.7 ± 6.2	4.7 ± 3.5	–	0.4 ± 0.7	9.9 ± 2.6
5 mg E2/kg	10	0.6 ± 0.6***	2.1 ± 2.8*	5.8 ± 5.6	91127 ± 34637**	4.9 ± 2.6**
0.02 mg AP/kg	12	4.2 ± 3.1*	6.9 ± 7.1	–	1.7 ± 2.0	8.8 ± 2.9
2 mg AP/kg	10	7.9 ± 8.6	13.0 ± 12.4**	–	10.0 ± 9.3	10.6 ± 2.8
20 mg AP/kg	10	2.0 ± 1.1***	4.6 ± 2.6	–	6.1 ± 10.3	8.9 ± 3.0
40 mg AP/kg	10	3.4 ± 3.2**	5.4 ± 5.6	–	7.4 ± 7.9	9.7 ± 1.6
80 mg AP/kg	10	5.4 ± 3.6	7.9 ± 4.9	–	23.1 ± 23.3**	9.4 ± 2.3

AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. Mean values ± standard deviations are shown. Asterisks indicate statistical difference from the unexposed fish (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Dunnett's post hoc test for GSI, Mann–Whitney *U*-test for the 11-KT, T and VTG data).

fed with 0.02, 40, and 80 mg/kg of the AP-mixture had plasma E2 concentrations lower than the unexposed fish.

The effect of the AP exposure on the plasma level of testosterone in female fish was less clear (Fig. 1B). While low doses of APs appeared not to affect or to slightly increase the plasma testosterone levels, high AP doses appeared to lower them. Females treated with 5 mg/kg E2 showed a dramatic decline in plasma testosterone levels and after 5 weeks the mean values were only one quarter of those in unexposed females.

In general, the sex steroid concentration in the plasma of male fish also appeared to be influenced by exposure to APs (Table 1). One week after the initial AP-exposure, the 11-KT levels in the male fish were significantly lower in all the AP-exposed and E2-treated groups when compared with the unexposed group (Table 1). Testosterone levels were also significantly lower in male fish that received the two lowest doses of APs when compared to the unexposed males (Table 1). In contrast, testosterone levels in the three groups of male fish that received the highest AP doses, or E2, were not statistically different from the levels in unexposed males. After 5 weeks there were significantly lower 11-KT levels in the male fish exposed to 0.02, 20 and 40 mg/kg AP, and also in the E2-treated males compared with the unexposed males. Testosterone levels were lower in E2-treated males, but were significantly elevated in the 2 mg/kg AP group when compared to the unexposed group. It should also be noted that the E2-treated males had very low 11-KT concentrations when compared with unexposed male fish. During the experiment, the 11-KT levels in the unexposed males more than doubled, whereas the levels in E2-treated males remained relatively stable. In addition, testosterone levels in the unexposed or E2-treated males did not change significantly between the two sampling times. In contrast to this, the testosterone concentration was increased in the AP-exposed males sampled after 5 weeks compared to the corresponding groups sampled after only 1 week.

E2 levels were below the detection limits for all groups of male fish, except the E2-treated group (Table 1). In the E2-treated males, the plasma E2 concentration after 1 week was 0.6 ng/ml, and this increased a further 10-fold during the next 4 weeks. After 5 weeks the E2 concentration in the E2-treated males was comparable to that found in the females at that time point (see Fig. 1 and Table 1).

3.2. Effects of APs on plasma levels of VTG

As expected, the plasma concentration of VTG was clearly increased in the E2-treated females when compared to unexposed females (Fig. 1C). After a single dose of E2 the VTG levels in the plasma of female fish were three times higher, and after four doses the levels were five times higher. The VTG levels in the females were lower in the AP-exposed groups compared to the unexposed group, and the reduction was statistically significant for the groups receiving 20 and 40 mg/kg after the first week.

Low concentrations of VTG were found in the unexposed male fish, and significantly higher VTG levels were detected in several groups of AP-exposed males (Table 1). The VTG concentrations were two to forty times higher in these AP-exposed males compared to the unexposed male fish. There was also a highly significant induction of VTG levels in the E2-treated males at both time points. The induction seen was much more pronounced than that seen after AP-exposure and was many thousand times that of the levels detected in unexposed male fish (Fig. 1 and Table 1). After 5 weeks the VTG concentration in the E2-treated males was three times higher than that of E2-treated female fish. The VTG levels were highly variable within the groups of AP-exposed males, and a statistically significant increase in the VTG concentration was only found for the 80 mg AP/kg group. However, when compared to unexposed males, the pooled data clearly demonstrated that a higher pro-

Table 2
Potential relative fecundity, fecundity condition factor, and estimated time to spawning in unexposed, AP-exposed, and E_2-treated male cod

Variable	Untreated	0.02 mg AP/kg	2 mg AP/kg	80 mg AP/kg	E2 treated
Number of individuals	9	5	9	8	6
Potential fecundity (×1000)[a]	1073 ± 238	1393 ± 295*	1015 ± 274	956 ± 224	787 ± 304
Potential relative fecundity[b]	1729 ± 340	2027 ± 507	1727 ± 437	1387 ± 335	1287 ± 464
Potential fecundity condition factor[c]	17.9 ± 4.7	20.0 ± 5.4	17.6 ± 4.7	14.1 ± 3.8	12.9 ± 5.2
Follicle diameter (μm)	423 ± 49	340 ± 41*	338 ± 53*	355 ± 47**	317 ± 47**
G1 (leading cohort) diameter (μm)[d]	549	424	415	446	378
Time to spawning (days)[e]	36.7 ± 10.5	58.2 ± 13.8*	65.3 ± 31.6**	54.1 ± 14.5**	74.8 ± 28.1**

AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. Mean values ± standard deviations are shown. Asterisks indicate statistical difference from the unexposed fish (*$p < 0.05$, **$p < 0.01$, Mann–Whitney *U*-test).

[a] Potential fecundity: number of maturing oocytes present in the ovary at the time of the sampling. Calculated according to Thorsen and Kjesbu (2001).

[b] Potential relative fecundity: potential fecundity/somatic weight (g).

[c] Potential fecundity condition factor: potential fecundity/L^3.

[d] G1 (leading cohort) diameter: mean diameter of the 10 larges follicles in the ovary G1.

[e] Time to spawning: estimated number of days from the time of the sampling to the onset of spawning using formula (given in Section 2).

portion of AP-exposed males produced increased levels of VTG (Fig. 2).

3.3. Effects of APs on somatic growth, condition factor and hepatosomatic index (HSI)

The fish were only fed once a week, and subsequently the somatic weight of the individual fish decreased (data not shown). The amount of weight lost was significantly higher for the female fish exposed to the three highest AP concentrations (3.9–4.4% decrease in weight) as compared to the unexposed females (1.5% decrease in weight). AP-exposed and unexposed males lost a similar amount of weight (2.1–3.3% decrease in weight). The male and female E2-treated fish lost the most weight (8.3% and 6.5% decrease in weight, respectively). There were no differences in conditions factor (Fulton's *K*) between the groups, but a significant increase in the HSI value of the E2-treated male fish was seen after 5 weeks (data not shown).

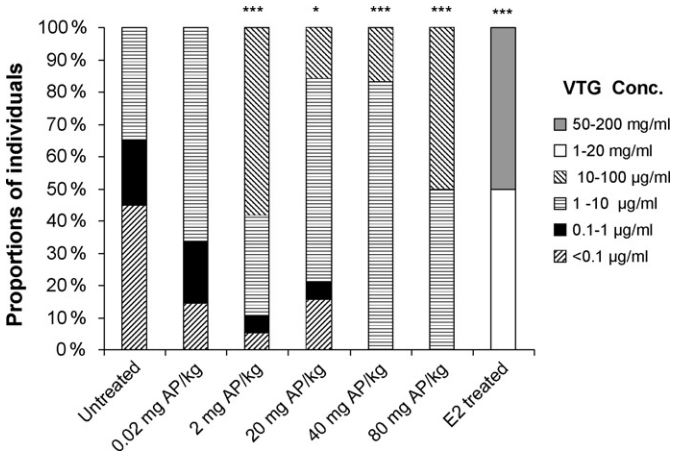

Fig. 2. VTG levels in the plasma of male fish. Data from both samplings were pooled ($n = 18$–21). AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. Asterisks indicate statistical difference from control, *$p \leq 0.05$; ***$p \leq 0.001$; χ^2-test.

3.4. Effects of APs on gonadosomatic index, potential fecundity and testis maturation status

After 5 weeks we observed a large and significant drop in the GSI value of the E2-treated female fish and in all groups of AP-exposed females compared to the unexposed female fish (Fig. 3). The E2-treated females had small, seemingly juvenile gonads, most likely in the process of resorbing. This conclusion was supported by the very high frequency of atresia also seen in the samples (from frequent to nearly total atresia) (Fig. 4). Notably, two of the females sampled did not have many atretic follicles and lacked oocytes beyond the cortical alveoli/very early vitellogenetic stage. It is therefore likely that these two fish had already resorbed their vitellogenic oocytes through atresia. The E2-treated females had few or no mature oocytes and, therefore, would not have been able to complete spawning. Importantly, exposure of the females to APs did not appear to cause an increase in the frequency of atresia, but instead slowed down oocyte development (Fig. 4). In addition, we noted that ovaries from the AP-exposed females were considerably smaller than those from the unexposed females. Samples taken

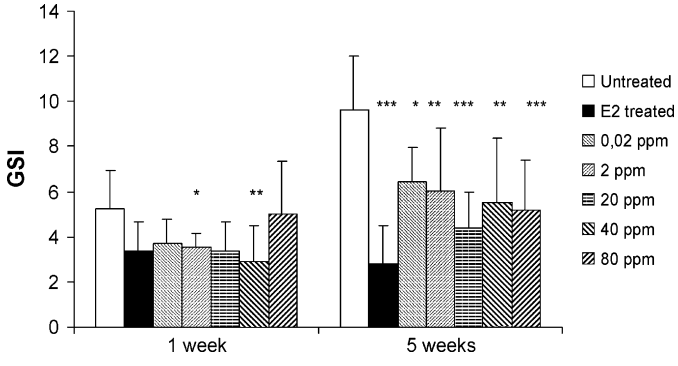

Fig. 3. Gonosomatic index (GSI) for female fish from the unexposed, E2-treated and AP-exposed groups. AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. Mean values ± standard deviation. Asterisks indicate significant differences from the unexposed group, *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

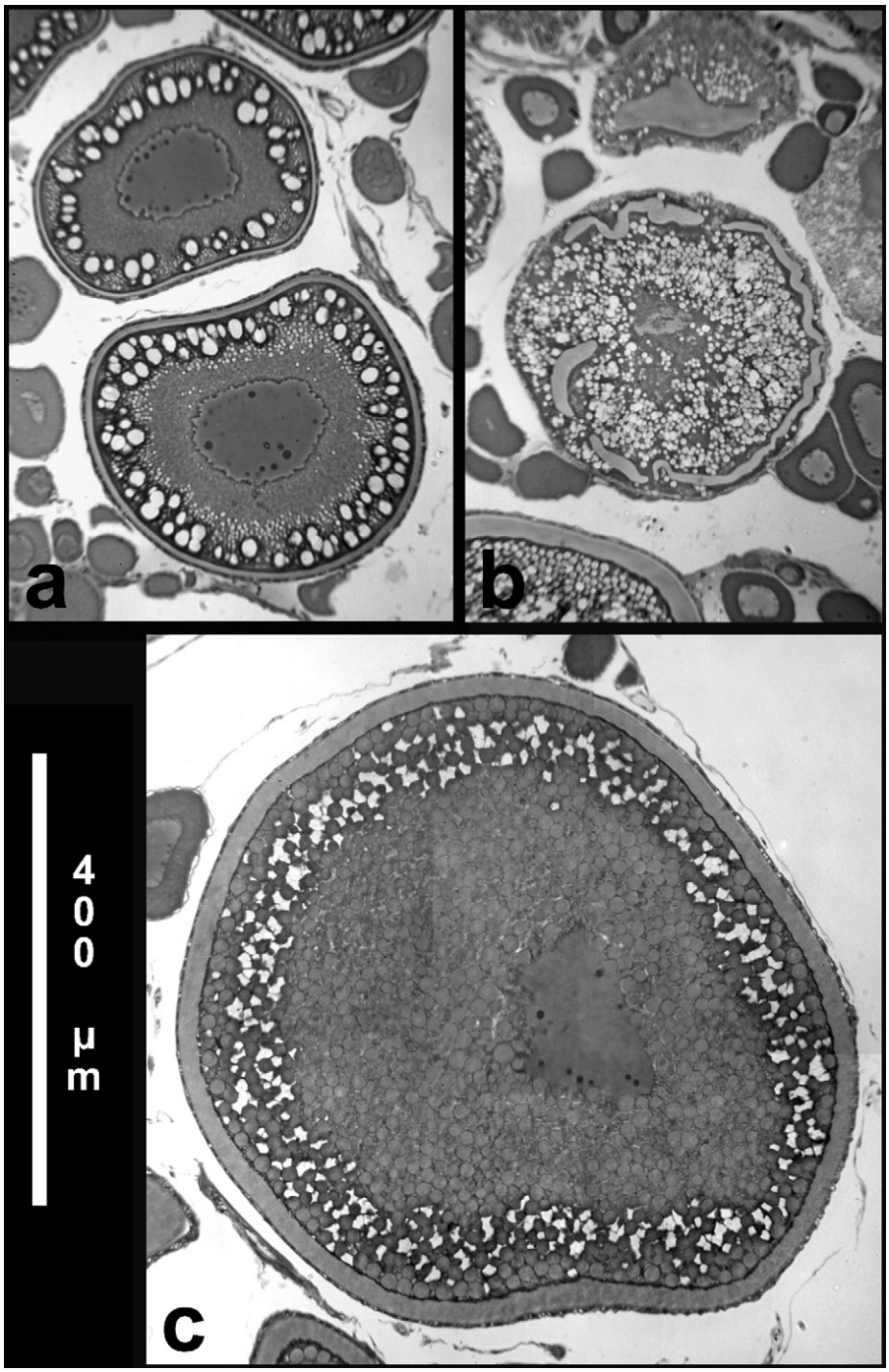

Fig. 4. Exposure to APs delays the development of oocytes in cod, while E2 causes resorbsion of the maturing oocytes. (a) 20 mg/kg AP. Early vitellogenic oocytes showing normal development. (b) 5 mg/kg E2. Atretic oocytes in the process of being resorbed. (c) Unexposed. Late vitellogenic oocytes. Representative examples from AP-exposed and untreated cod are shown. Scale bar = 400 μm.

from unexposed females showed little or no atresia, and the oocytes were judged to be in late vitellogenesis, a much more advanced stage of development (Fig. 4). No significant changes were detected in potential fecundity, potential relative fecundity, or potential fecundity condition factor in any of the AP-exposed females, with the exception of the 0.02 mg/kg group (Table 2). The 0.02 mg/kg group had a significantly higher potential fecundity than the unexposed females, but there was no comparable increase in the potential relative fecundity or the potential fecundity condition factor (Table 2). Significant differences in follicle diameter were also found in all groups of AP-exposed females. These changes in follicle diameter resulted in a significant shift in the estimated time to the onset of spawning (Table 2).

AP-exposure did not significantly affect GSI in the male fish. However, E2-treated males showed a significant decrease in GSI after 5 weeks indicating aborted maturation (Table 1).

Both AP-exposure and E2-treatment influenced testis development in males (Fig. 5). The AP-exposed males (only the

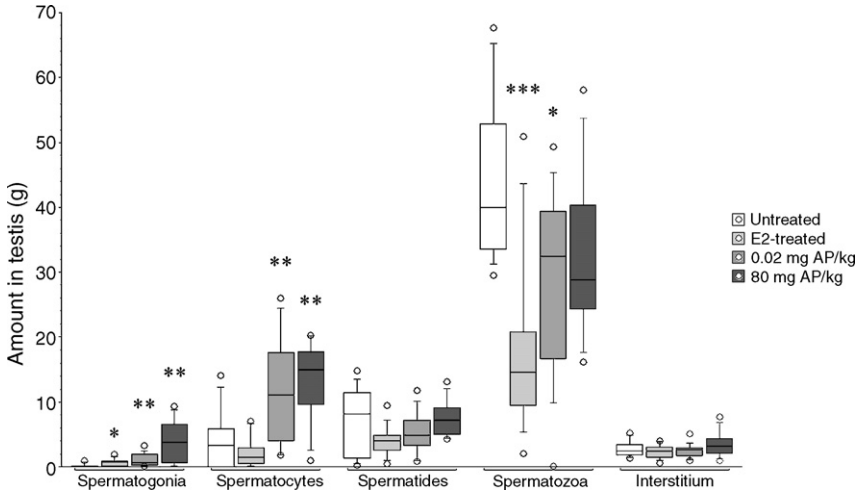

Fig. 5. The effects of APs on the amount of different cell types in testis. AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. Boxplot: indicates median (line), 25–75th percentile (box), 10–90th percentile (vertical line) and all observations beyond the 10th and 90th percentile (separate points). Asterisks indicate significant differences from the unexposed group, $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

0.02 and 80 mg/kg groups were analyzed), and the E2-treated males had a significant increase in the amount of spermatogonia when compared to the unexposed males. The two AP-exposed groups also had a significant increase in the amount of spermatocytes. However, we also observed a significant reduction in the amount of spermatozoa in both the E2-treated and the 0.02 mg/kg AP-exposed males. We did not observe any significant changes in the amount of spermatides or interstitial.

4. Discussion

Several previous studies on different fish species have shown that APs like OP and NP can affect the plasma levels of E2, but the results are not consistent. Juvenile Atlantic salmon (*Salmo salar*) injected with low doses (1 or 5 mg/kg) of APs showed a reduction in the plasma level of E2, but a similar effect was not seen with higher (25 or 125 mg/kg) doses (Arukwe et al., 1997). Female rainbow trout (*Oncorhynchus mykiss*), exposed to water contaminated with 86 μg/l NP showed a pronounced decease in the plasma E2 concentration (Harris et al., 2001). Both E2 and testosterone levels were reduced in juvenile turbot (*Scophtalmus maximus*) exposed to 30 μg/l NP in the water, while the levels of these steroids in the plasma of young cod were unaffected by the same NP concentration (Martin-Skilton et al., 2006). However, studies conducted with juvenile male flounders (*Paralichthys dentatus*) (Mills et al., 2001), and male and female fathead minnows (*Pimephales promelas*) (Giesy et al., 2000), suggest that AP-exposure can produce an increase in plasma E2 levels in some species.

One hypothesis that might explain the observed reductions in steroid concentrations suggests that AP-exposure may increase steroid catabolism. NP has been shown to be an agonist of the orphan nuclear receptors pregnane X-receptor (PXR) and constitutive androstane receptor (CAR) (Masuyama et al., 2000; Mikamo et al., 2003; Kretschmer and Baldwin, 2005). PXR and CAR are involved in the regulation of several detoxification enzymes, such as those of the CYP2B and CYP3A families, which are responsible for the metabolism of steroids. It has been shown that low doses of NP (1 mg/kg) increased progesterone metabolism in Atlantic salmon while higher doses (125 mg NP/kg) led to a decrease (Arukwe et al., 1997). In contrast, winter flounder (*Pleuronectes americanus*) injected with 100 mg/kg NP showed a 50% increase in both liver CYP3A activity (based on testosterone 6β-hydroxylation) and the amount of CYP3A protein (Baldwin et al., 2005). Samples from the present study have been used to study the possible effects of APs on the *in vivo* expression of CYP1A and CYP3A proteins (Hasselberg et al., 2004). This study demonstrated a dose-related increase in hepatic CYP1A and CYP3A protein levels in male cod, but not in females. However, the observed increase of CYP1A protein levels in the male fish was not linked to an increase in CYP1A-mediated ethoxyresorufin-*O*-deethylase (EROD) activity, implying that APs inhibit the CYP1A activity *in vivo*. In addition, *in vitro* studies of the cod hepatocytes showed a strong dose-dependent reduction in both the CYP1A and CYP3A activity (Hasselberg et al., 2004). Therefore, the increase in CYP3A levels in male cod, possibly caused by a reduction in its catabolic rate, could be one explanation for the increase in steroid catabolism. However, in the present study, a general increase in steroid catabolism does not explain the observed decrease in E2 (in the females) and 11-KT (in the males), because the testosterone levels were maintained or even increased. Therefore, another explanation for the decrease in E2 and 11-KT levels could be that AP exposure affects the steroidogenic enzymes. P450 11β-hydroxylase ($P450_{11\beta}$) is one of the enzymes involved in transforming testosterone into 11-KT. Yokota et al. (2005) showed that 4-*tert*-pentylphenol down-regulates the expression of $P450_{11\beta}$ mRNA in medaka (*Oryzias latipes*). Similar inhibition of $P450_{11\beta}$ mRNA was found in the brains of Atlantic salmon exposed to NP (Arukwe, 2005). The same study also found an induction of steroidogenic acute regulatory (StAR) mRNA and P450 cholesterol side chain clevage

($P450_{scc}$) mRNA in the brain of NP-exposed Atlantic salmon suggesting a possible stimulation of the early steps of steroid synthesis. Aromatase ($P450_{arom}$) is the key enzyme responsible for conversion of androgens to estrogens. Several studies have found that NP and OP induce $P450_{arom}$ mRNA expression in the brains of fish (Kazeto et al., 2003, 2004; Lee et al., 2006; Meucci and Arukwe, 2006), but that AP-exposure has little effect on, or in fact down regulates, $P450_{arom}$ mRNA expression and aromatase activity in the ovary (Kazeto et al., 2004; Lee et al., 2006; Martin-Skilton et al., 2006).

Steroidogenesis is controlled by pituitary hormones (the gonadotropins) via the brain-pituitary-gonadal axis. Attention is currently focusing on the role of APs in neuroendocrine disturbances in fish (Jones et al., 1998; Piva and Martini, 1998; Harris et al., 2001; van Baal et al., 2000; Zilberstein et al., 1999; Yadetie and Male, 2002). The mechanisms underlying the effects of APs on the gonadotropins are still unknown but the results of Harris et al. (2001) strongly suggest that APs disrupt the natural endocrinal feedback system of the fish somewhere in the central nervous system.

Interesting, the present study was unable to demonstrate a clear dose–response relationship between AP-exposure and the levels of E2 in the females or 11-KT in the males. Fish that received the lowest dose (0.02 mg AP/kg) showed the same decrease in E2 or 11-KT levels as those receiving higher doses. This may indicate that steroid down-regulation is a result of exceeding a certain threshold level of AP-exposure. However, a lack of a linear dose–response relationship is typical for the steroid system, and the nature of the response is often different with low and high doses of steroids. For example, low doses of E2 and testosterone stimulate the secretion of gonadotropin in fish, while high doses inhibit it (Jalabert et al., 2000). Similarly, differences in the effects of high and low doses of APs on enzymes involved in steroid-metabolism have also been observed (Arukwe et al., 1997).

The massive increase in plasma VTG levels in male and female cod following exposure to E2 confirms VTGs sensitivity as a biomarker for estrogen. Exposure to APs showed an induction of VTG levels in male cod. Unfortunately, due to the wide variation in VTG levels in the individual males a simple comparison of the mean VTG levels did not show a convincing dose–response effect. However, when the data showing the frequency distributions of the different VTG levels detected in the individual males, a clear effect of AP-exposure was seen (Fig. 2). Previous studies have shown that male cod caged close to oil installations show a similar small induction of VTG levels (CEFAS, 2005), and male flounders (*Platichtys flesus*) caught in the vicinity of the UK oil platforms were reported to have significantly elevated VTG levels (Matthiessen et al., 1998). Together, these data suggest that APs released into the sea via produced water are responsible for the observed increases in VTG levels in male fish.

Considerable effects on the pattern of maturation of the testis in males were observed. There was an increase in the amount of spermatogonia. There also appeared to be increase in the amount of spermatocytes and a reduction in spermatozoa. Previous studies have also found increases in the occurrence of spermatogonia (in addition to increased mitotic activity in the spermatogonium population) on exposure to OP, NP, and E2 (Folmar et al., 2001; Giesy et al., 2000; Jobling et al., 1996; Zaroogian et al., 2001). *In vitro* work on eels (*Anguilla anguilla*) (Miura et al., 1999, 2003) identified a protein termed "spermatogonial stem cell renewal factor" (eSRS34). This factor is synthesised by the sertoli cells, and regulated by E2. This points towards E2 as a functional hormone in the sexual maturation of males. It has also been reported that NP stimulated spermatogonial renewal in a manner similar to E2 (Miura et al., 2005). In both cases, 11-KT was required for the cells to develop (Miura et al., 2005). There are also reports of disruptions of the later stages of spermatogenesis (Gimeno et al., 1998; Lahnsteiner et al., 2005). Furthermore, NP has been shown to increase the extent of testicular apoptosis in medaka (Weber et al., 2002). These previously published results fit well with our current observations. Previous studies have also reported necrotic cells and phagocytosis in the testicular tubuli (Miles Richardson et al., 1999), suggesting necrosis of the spermatozoa. These findings may explain the decrease in the amount of spermatozoa that we observed. An alternative explanation, which is probably most relevant at high doses, is a direct cytotoxic effect of APs (Kinnberg et al., 2000; Kime and Nash, 1999). An effect of this kind could perhaps explain the observed reduction in all types of testicular cells in the E2-treated fish.

The weight of the gonads isolated from the female cod in our study increased from about 2% of the somatic body weight at the end of November to 11% in December, 5 weeks later. Normally, there is a direct relationship between E2, VTG, and gonadal growth. It is therefore not surprising that low E2 levels were accompanied by a decrease in gonad weight. E2-treated fish aborted oocyte maturation and resorbed the oocytes via atresia. It should be noted, however, that 5 mg E2/kg is far above normal physiological levels. No increase in atresia was observed in the AP-exposed groups. However, the oocytes in exposed groups showed a significant reduction on oocyte diameter. Histologically, it was clear that the oocytes were developmentally normal, but were only in the beginning of vitellogenesis at a time when the unexposed fish were in late vitellogenesis. This seems to suggest an anti-estrogenic effect of the APs, possibly by causing a down-regulation of natural E2. Moreover, the significantly smaller oocytes in the AP-exposed groups, predicted a significantly delayed spawning. Cod have a long spawning season that lasts more than 2 months, and sometimes as long as three (Brander, 1994). However, in UK waters, as many as two-thirds of the eggs are spawned during a period of 4–6 weeks (Brander, 1994). The spawning season appears to be centred on the period of plankton blooms, with *Calanus finmarchicus* as an important food source (Brander, 1994). This is important to ensure that as many eggs as possible will hatch at a time when the availability of food and the level of predation are optimal, thus ensuring good larval survival (Beaugrand et al., 2003; Platt et al., 2003). An AP-induced delay in the start of spawning, as suggested by our data, increases the chances of the eggs being spawned too late to fully exploit this rich source of food. In support of our suggestion, Noaksson et al. (2005) found a delay in the gonad maturation in wild female perch (*Perca fluviatilis*), roach (*Rutilus rutilus*), and brook trout (*Salvelinus fontinalis*)

living in lakes receiving leakage water from old refuse dumps. The compounds causing endocrine disruption were not identified in these studies. However, the observed reduction in the plasma steroid levels (testosterone and E2) in combination with a decreased GSI is similar to the findings for cod presented here.

Data from the present study were used in a risk assessment model to assess the effects of APs from produced water on the reproduction of fish in the North Sea (Myhre et al., 2005). This model includes the combined discharges from the three major Norwegian oil fields (Tampen, Ekofisk and Sleiper). The total amount of APs > C_4 discharged from all the oil installation, was estimated to be 25.6 kg/day, dissolved in 364.300 m^3/day produced water. The highest accumulated body burden in any of the fish particles was 0.09 μg/kg (Myhre et al., 2005). Therefore, this new modelling work indicate that the article of Rye et al. (1996) overestimated the body burden and that the doses used in our experiments may not be realistically expected to arise from produced water discharges alone.

The results of the present study show that exposure to AP present in produced water can influence the plasma concentration of several male and female sex hormones and the egg yolk precursor protein, vitellogenin, in Atlantic cod. This study also shows that AP-exposure interferes with the maturation of the sex organs, and that this effect is likely caused by disruption of the sex hormone system. Even though the concentrations used in our experiments are higher than may reasonably be expected as the result of oil production alone, APs also enter the sea from many land-based sources as well. Measurements of actual AP levels in areas of the North and Baltic Seas indicate that APs may still pose a significant risk to the marine environment. Bester et al. (2001) found NP concentrations in seawater from the German Bight (North Sea) between 0.7 and 4.4 ng/l. They also reported 13 μg/kg NP in sediments sampled more than 100 km offshore. Offshore from the Dutch coastal zone NP levels are reported to be as high as 1700 ng/l (Jonkers et al., 2005). In the Baltic Sea up to 21 ng NP/l and 1 ng OP/l are found in the waters along the German coast (Beck et al., 2005). Taken together, these results suggest that APs entering the sea from produced water and other sources have the potential to adversely affect the reproductive health of wild cod populations.

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