Paper III

Effects of alkylphenols (4-*tert*-butyl-, 4-*n*-pentyl-, 4-*n*-hexyl- and 4-*n*-heptylphenol) on the reproductive system of Atlantic cod (*Gadus morhua*).

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1 Abstract

Offshore oil production releases large amounts of lipophilic compounds in produced water into the ocean. The discharge of produced water from the Norwegian petroleum sector are continuously increasing with the age of the oilfields and were in 2004 143 million m^3. Produced water contains significant amounts of alkylphenols, which have been reported to be estrogenic, causing endocrine disruption in fish. In year 2004, approximately 13 tons of long-chain ($\geq C_4$) alkylphenols were released on the Norwegian continental shelf in connection with discharge of produced water. Little is known about the biological effects of alkylphenols when released in the marine environment. Our objective was to clarify how alkylphenols affect the reproduction in first-time spawning Atlantic cod (*Gadus morhua*). Model compounds tested included 4-*tert*-butylphenol, 4-n-pentylphenol, 4-n-hexylphenol and 4-n-heptylphenol, all found in produced water. Two groups of cod were exposed through the feed with a mixture of these four alkylphenols from October to the end of January (14 weeks): 0.02 mg/kg low group and 2 mg/kg high group. The fish were sampled ones a month through out the experiment (4 samplings points). Variations in hormone levels (17ß-estradiol, testosterone and 11-keto-testosterone) in blood plasma and gonadal development in control groups were compared with groups of cod exposed to alkylphenols.

The study demonstrated that “estrogenic” alkylphenols induced an anti estrogenic effect in the female fish by reducing the natural estrogen levels in plasma, even at very low doses alkylphenols. For the male fish were there only minor difference between the exposed groups and the control group. In November, after 2 month exposure were both testosterone and 11-keto-testosterone significant reduced in the males from the alkylphenols groups, but this were not evident in any of the samplings points. There was an weak induction of vitellogenin in plasma of the exposed male cod, the variation in between the groups were large, but only 20 % of the male fish in the control group had detectable levels of VTG in plasma, whether the number for the low AP exposure and high AP exposure were 53% and 72% respectably.

Generally was the large variation on all variables mesured. It was evaluated that the experimental setup, where we exposed the fish as a group through the feed, could be partly responsibly for this big variation in between the group responses. There was no control of how high the doses were for each individually fish, some fish have probably eaten more than others and therefore been more exposed. The experiment was lather repeated using force-feeding of all individual fish to insure a defined dose per unit weight for all fish and the finding were confirmed.

Key words: Alkylphenols, Endocrine disruption, Cod (*Gadus morhua*), Produced water, Steroids, Ovari

2 Introduction

The estrogenic effects of alkylphenols (AP) are known from a large number of *in vitro* and *in vivo* studies (Nimrod and Benson 1996). The APs bind to, and affect the estrogen receptors in the same way as 17 β-estradiol (E_2), but the response is much weaker (Mueller and Kim 1978; Soto *et al.* 1991; Jobling and Sumpter 1993). Virtually all research in this field has dealt with the two long-chain APs: Nonylphenol (NP) and octylphenol (OP). These are degradation products of the non-ionic surfactants known as alkylphenol ethoxylates (APE). APEs are among the most widely used surfactants in the world, with an annual production of around 500,000 tonnes, and has been utilized in a large number of products, including herbicides, paints and industrial cleaning and degreasing agents (Naylor *et al.* 1992; Renner 1997). In Norway, the use of APEs has been very limited, and has fallen significantly during the 1990s, from 615 tonnes in 1995 to 113 tonnes in 2000 (www.SFT.no, 2001). The use of NP, OP and their ethoxylates has been forbidden in Norway since January 2002 (www.miljoverndepartementet.no, 2001). The European Union are also planning to ban the use of these substances (EU 2003), which are also on the Oslo-Paris Commission's (OSPAR) list of chemicals which ought to be phased out. On the other hand, APEs are still widely used in the U.S. (Renner 1997).

NP has been identified at a large number of locations in the marine environment. Analyses of coastal seawater from areas near cities have shown concentrations up to 1.2 µg/l, while values from sediment samples can be as high as 20 mg/kg in particularly exposed sites (Langston *et al.* 2005). Identification of NP- and OP in samples from offshore open waters places high demands on analytical methodology, due to the very low concentrations involved. Only three studies have to our knowledge made this kind of measurements. Kannan *et al.* (1998) found very low levels of NP in the Sea of Japan (0.002 - 0.093 ng/l), while measurement from the North Sea (German Bight) showed significantly higher values (Bester *et al.* 2001). Bester *et al*. found NP concentrations in seawater between 0.7 and 4.4 ng/l, and reported 13 µg/kg NP in sediments taken more than 100 km offshore. The water concentration of NP offshore from the Dutch coastal zone are found to be as high as 28-82 ng/l (Jonkers *et al.* 2005).

In addition to being degradation products of the APEs, alkylphenols are natural components of crude oil (Ioppolo-Armanios *et al.* 1995; Ioppolo-Armanios *et al.* 1992; Taylor *et al.* 1997; Rolfes and Andersson 2001). As a result of their solubility in water a high proportion will be found in the aqueous phase after water/oil separation and therefore be discharged into the sea with the produced water. The alkylphenols are typically found in concentrations of 0.6 - 10.0 mg/l in produced water. About 80 % of the total amount consists of the most water-soluble alkylphenols (phenol and cresol (C_1)). Of the remaining components, the higher alkylphenols from butyl- to heptylphenols occur in low concentrations of 0.07 - 237 µg/l (Grahl-Nielsen 1987; Brendehaug *et al.* 1992; Røe and Johnsen 1996; Boitsov *et al.* 2004).

In spite of the slow breakdown of long-chain alkylphenols, these substances are fully biologically degradable, and when APEs are phased out, the potential environmental problems caused by these substances will disappear in a relatively short time. On the other hand, even if the most serious environmental threat from the alkylphenols will disappear with the phasing out of APEs, any problems associated with discharges of long-chain alkylphenols from petroleum production will remain.

Very little is known about the fate of these substances in the marine offshore environment. There are no empirical data on concentrations of long-chain alkylphenols in the sea around North Sea offshore

installations. One study showed that phenol and lighter alkylphenols (C1–C4) occur at the concentrations of 486 and 140 ng/l, respectively (Riksheim and Johnsen 1994). We are therefore forced to use models when estimating the levels to which fish may be exposed. Rye *et al.* (1996) simulates the spread of AP discharges from produced water from the Halten Bank, and calculates the likely uptake by pelagic fish using a model. The model simulates the dissemination of total AP discharges from two platforms, and includes biological response estimates (Bioconcentration Factor (BCF) and constants for uptake and elimination). The calculations of a "worst case scenario" show that the body burden of AP in the fish modelled will be in the range 0 - 10 µg/kg (Rye *et al.* 1996).

This article presents the results from a project carried out during 1997-2001 where the goal was to study long-term biological effects of very low concentrations of selected C_4-C_7 AP found in production water on sex hormones and reproduction in Atlantic cod (*Gadus morhua*). The study was carried out under controlled laboratory conditions. The compounds tested were 4-tert-butylphenol, 4n-pentylphenol, 4n-hexylphenol and 4n-heptylphenol.

Given the lack of field data, we used the model values indicated in Rye *et al.*'s article as a basis for choosing the exposure regimes in our experiments. Using a mixture of four components with differing chain lengths (C4 to C7), an attempt has been made to take into account the wide range of different APs found in produced water. The intention of the tests was to dose the fish to a body burden within the range of Rye *et al.*'s estimates. Using the available information, it was concluded that 5 µg/kg of each of the four AP ought to correspond to a fairly realistic dose.

The groups exposed to AP were compared with control groups with respect to variations in the steroid hormones 17 ß-estradiol (E_2), testosterone (T) and 11-keto-testosteroner (11-KT) as well as the yolk-protein vitellogenin (VTG) in blood plasma and gonadal development. Morphological and histological methods were used to search for effects on oocyte maturation and number (potential fecundity).

3 Materials and methods

3.1 Experimental design

The experiment was undertaken for 4 months in 1997/98 (Table 1).

Table 1. *Exposure and sampling scheme.*

	Experiment
Start of exposure	1997-09-30
Sample 1	1997-10-30
Sample 2	1997-11-27
Sample 3	1997-12-16
Sample 4	1998-01-26

This experiment used 300 two-year old cod (mean weight 0.631 kg) expected to spawn for the first time in the following season. The fish came from a strain of Arcto-Norwegian cod produced at the Institute of Marine Research (IMR)'s station in Øygarden (Parisvatnet) near Bergen, Norway. The fish were transported to Bergen and divided between one control group and two experimental (exposure) groups in separate 15 m^3 outdoor tanks (100 fish per tank). The fish tanks were supplied with water from a 100 m depth and the water temperature remained stable at 8 - 10^o C throughout the experiment. The fish were fed three times a week with an amount of feed equivalent to a daily ration of 0.5% of body weight (Kjesbu *et al.* 1996). The amount of feed supplied was adjusted after each monthly sampling.

The exposed groups were given a mixture of 4-tert-butylphenol (C_4) (Aldrich, Norway), 4n-pentylphenol (C_5) (Aldrich, Norway), 4n-hexylphenol (C_6) (Aldrich, Norway) and 4n-heptylphenol (C_7) (TCI, Japan). The APs were dissolved in soya oil and mixed into the feed (wet pellets; herring:fish meal, 60:40%) in concentrations of 1 or 100 mg/kg of each compound. The intention was to achieve a daily "body burden" equivalent to a theoretical dose of 5 or 500 µg/kg of each AP per fish per day. The fish were fed three times a week with a quantity of feed equivalent to a daily ration of 0.5% of body weight, i.e. the fish received 11 or 1166 µg/kg body weight per feeding (three times a week) in the low- and high-dose groups respectively.

The low dose was intended to represent an environmentally realistic value and the high dose a positive control. Monthly samples of 15-20 fish were taken from each group.

3.1.1 Sampling

The cod were anaesthetized with benzocaine and blood samples were extracted from the sinus caudalis with a heparinised syringe. The samples were immediately centrifuged at 3000 G for 5 min. at 4^{o} C. The plasma was frozen in liquid nitrogen and kept at -80^{o} C until analysis. The fish were killed by a blow to the head and their weights and lengths measured. 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^{o} C. Tissue samples for histology were fixed as described below.

3.2 Analyses

3.2.1 Steroid analyses

The plasma steroids were analysed by enzyme-linked immunoabsorption assay (ELISA) according to a procedure described by Dahle *et al*. (2003). The female fish were analysed for E_2 and T and the males for 11-KT and T.

3.2.2 Vitellogenin

The biomarker VTG was analysed by means of a quantitative ELISA technique developed at the IMR, Austevoll Aquaculture Station. An assay was established for female fish (high VTG content) and a more sensitive assay for male fish (low VTG content, detection threshold 0.1 µg/ml). Detalies are giving in Meier et al., (2006 a,b)

3.2.3 Histology and morphology

Samples from selected groups were studied for signs of histological changes. The histological samples were collected in January (at the end of feeding) form Control group, 0.02 mg AP/kg and 2 mg AP/kg.

Fixation protocol

Tissue samples from all female fish were fixed in buffered formalin (3.6% formaldehyde based on Merck p.a.-quality formalin) in 0.0295 M sodiumdihydrogenphosphate and 0.0461 M disodiumhydrogenphosphate for estimates of fecundity and size distribution, and in modified Karnovsky fixative (2.5% formaldehyde based on Merck p.a.-quality formalin), 2.5% glutaraldehyde and 7% sucrose in 0.05M sodium dimethylarsenate (sodium cacodylate) for histological studies.

Embedding procedure

Samples for histological studies were dehydrated through a graded ethanol series, embedded in methacrylate (Technovit 7100) and sectioned on a Reichert/Jung microtome. The sections were stained with toluidine blue (1% in 2% borax solution).

Liver index, gonadosomatic index and Fulton´s K

The liver index (hepatosomatic index) was calculated as

$HSI = (LW \cdot 100)/(W)$ (%),

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

The gonadosomatic index (GSI) was defined as:

$GSI = (GW \cdot 100)/(W\text{-}GW)$ (%),

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

Fulton condition factor (Fulton K) was set equal to $(W/L^3)*100$,

where L is the length.

Follicle diameter and fecundity

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

Potential fecundity was calculated as (Thorsen and Kjesbu 2001):

(2) Potential fecundity = $2.139 \cdot 10^{11} \cdot FD^{-2.7} \cdot$ ovary weight,

Where FD is the mean follicle diameter

The potential relative fecundity was defined as: Potential fecundity / somatic weight (g), and the potential fecundity condition factor as: Potential fecundity / length^3 (cm).

Time to spawning

Time to spawning was calculated as (2). $y = 3.33 \cdot 10^6 \cdot x^{-1.817}$ (Kjesbu 1994), where y is 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).

3.2.9 Statistical analyses

One-way ANOVA and Dunnet`s test as a post-hoc test were used to analyse for statistical differences between the control group and the exposed groups for all morphological variables (size, growth, GSI, HSI, fulton K). For the vitellogenin, steroid measurements and histological data the statistical differences between the control group and the exposed groups were tested by non-parametric Kruskal-Wallis followed by two-tailed Mann-whitney U-test. The frequency distribution of VTG measurements was tested by two-tailed Chi-square test. Significance levels are given in the figure and table legends. The statistical analyses were all performed using Statview software (SAS Institute, Cary, NC, USA) or XLSTAT software (Addinsoft, US).

4 Results

4.1 Effects of alkylphenols on plasma levels of steroids and vitellogenin.

The exposure to APs had major effects on natural levels of steroids in the female fish. A highly significant down-regulation of E_2 concentrations in the exposed groups was found. In January at the end of the exposure, E2 levels were reduced in both the low-dose (0.02 mg/kg) and high-dose (2 mg/kg) AP. The trend were visible already in November (Low-dose were 68 % and high-dose 44 % of the control), became stronger in December (Low-dose were 71 % and high-dose 35 % of the control), but the difference were first significant in January (properly because that the number of fish in the analysis were to low in November and December to extract statistical significant, see table 2).

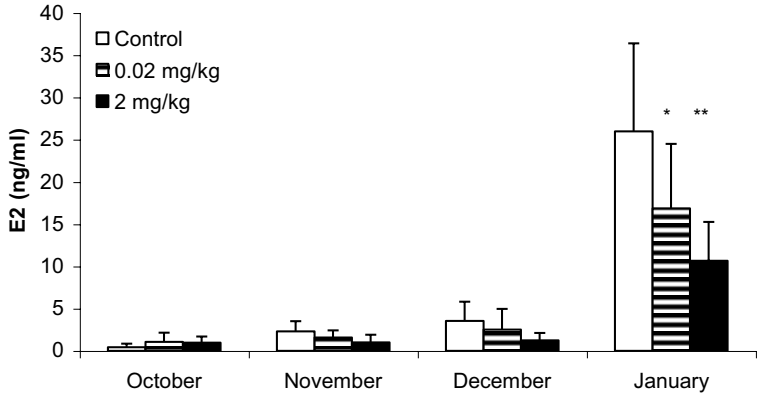

Fig1. *Experiment I. 17β-Estradiol in plasma (ng/ml) from control, low exposed (0.02 ppm) and high exposed (2 ppm) female fish. Mean values ± standard deviation. Asterisks indicate significant difference from the control group, * p≤0.05, ** p≤0.01.*

The effect of the AP exposure on the plasma T level in the female fish was less clear. Generally, T values were an order of magnitude lower than E2 values. In the early part of the experiment, the plasma concentrations of T rose significantly (October sample) in both the high and the low exposed group. Lower T value was found in the 2 mg/kg group in December, but later in the season no significant difference compared to the control could be demonstrated (Table 2).

For the male fish were there only minor difference in the steroids concentrations in plasma between the exposed groups and the control group. In November, after 2 month exposure were both testosterone and 11KT significant reduced in the males from the alkylphenols groups, but this were not evident in any of the samplings points (table 2).

There was no difference between the treatments on the VTG levels in the plasma for the females. There was a seasonal increasing in plasma VTG that followed the rice in E2 levels as one could expect. The VTG concentrations were more that 1000 times higher than for the males (table 2).

For the male fish was there weak induction of VTG in plasma of the exposed male cod. The variation in between the groups were large and when looking on average values for the groups were the differences only significant for high-doses in october, However, pooled data clearly demonstrated that a higher proportion of individuals produced VTG in the exposed groups (Fig 2) compared to control. Among the control fish, only about 20% of the individuals had more than 0.1 µg VTG/ml, whereas 53% and 72% of the fish in the 0.02 mg/kg and 2 mg/kg groups had VTG levels above 0.1 µg/ml, respectively.

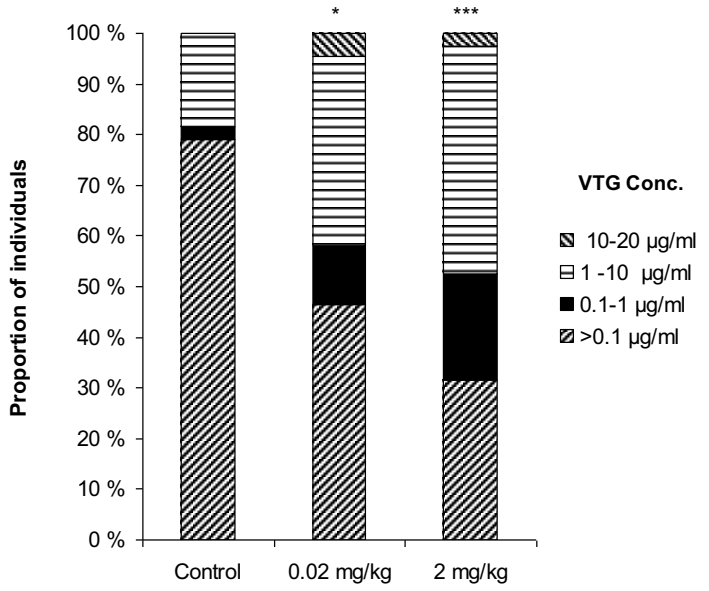

Fig. 2. *Experiment I. VTG-levels in plasma from male fish. Data from all four samplings are pooled. (Control, n=38; 0.02 mg AP/kg, n=41; 2 mg AP/kg, n=38). Asterisk indicates statistical difference from control (* P<0.05, *** P<0.001, Chi-square test)*

Table 2. *Div. Results for female and male. Plasma levels of sex hormones (T for female; 11-KT and T for male) and vitellogenin (VTG), as well as GSI, HSI and Fulton`s K in control, low dose exposed (0.02 mg /kg) and high dose exposed (2 mg /kg). AP doses are given as the sum of 4-tert-butylphenol, 4n-pentylphenol, 4n-hexylphenol and 4n-heptylphenol concentrations. Mean values ± standard deviation are tabled. Asterisk indicates statistical difference from control (* P<0.05, ** P<0.01, Dunnett`s post-hoc test for GSI, HSI and Fulton K; Mann-Whitney U-test for the 11-KT, T and VTG data).*

	Female			**Male**		
	Control	0.02 mg/kg	2 mg/kg	Control	0.02 mg /kg	2 mg /kg
	Number of fish sampled			Number of fish sampled		
October	10	9	10	11	11	11
November	7	7	7	9	7	8
December	6	6	7	6	9	9
January	11	12	11	18	16	15
	T (ng/ml)			T (ng/ml)		
October	0.5±0.2	1.5±0.4**	1.3±0.6**	0.9±0.5	1.6±0.5	1.2±0.8
November	0.7±0.3	0.5±0.2	0.5±0.1	1.9±0.9	1.0±0.3*	0.9±0.1**
December	0.9±0.4	1.1±0.5	0.5±0.1*	1.9±0.6	2.4±1.0	1.3±0.7
January	1.3±0.3	1.5±0.5	1.3±0.6	8.3±5.2	8.5±4.3	11.4±3.1
				11-KT (ng/ml)		
October				1.0±0.3	1.2±0.7	1.3±0.9
November				1.9±0.9	0.9±0.2**	0.7±0.2**
December				1.3±0.4	1.8±0.6	1.0±0.3
January				7.8±4.9	11.0±6.4	7.3±4.3
	VTG (mg/ml)			VTG (µg/ml)		
October	0.7±1.1	0.6±0.7	0.7±0.8	0.2±0.4	0.9±1.4	2.5±2.8*
November	1.1±0.7	0.9±0.6	1.2±0.5	2.0±2.7	3.9±6.8	3.6±5.7
December	1.1±0.7	2.4±1.0	1.5±0.7	0.1±0.4	5.0±7.2	1.8±2.6
January	5.6±1.4	4.9±1.6	4.8±2.4	0.2±0.7	0.8±1.6	0.7±0.7
	GSI (%)			GSI (%)		
October	2.3±0.8	2.5±0.8	2.0±0.7	2.1±1.6	1.5±1.2	2.1±2.1
November	2.8±0.5	2.7±0.8	3.0±0.7	8.6±4.2	6.9±3.0	7.9±2.3
December	4.4±2.1	4.1±1.3	3.2±1.1	7.9±2.2	10.3±2.5	7.9±2.1
January	11.5±2.3	9.8±2.8	9.7±3.2	11.9±3.6	10.3±3.3	10.1±1.9
	HSI (%)			HSI (%)		
October	9.8±2.2	9.5±1.6	9.1±2.1	9.2±1.8	10.1±2.7	8.8±2.0
November	8.3±1.8	9.3±1.5	9.0±0.6	8.0±2.7	7.1±2.0	8.3±1.6
December	8.8±2.1	11.6±2.0	8.1±1.6	6.8±2.2	9.1±1.4	7.0±1.8
January	9.8±1.8	10.3±1.5	9.2±2.8	7.4±2.2	8.7±1.4	8.2±2.0
	Condition factor (Fulton K)			Condition factor (Fulton K)		
October	1.0±0.1	1.1±0.1	1.0±0.1	0.9±0.1	1.0±0.1	1.0±0.1
November	1.0±0.1	1.1±0.1	1.0±0.1	1.0±0.2	1.1±0.1	1.0±0.1
December	1.1±0.1	1.1±0.1	1.0±0.1	1.0±0.1	1.0±0.1	1.0±0.1
January	1.1±0.1	1.1±0.1	1.1±0.2	1.0±0.1	1.0±0.1	1.0±0.2

4.2 Effects of alkylphenols on fecundity, gonadosomatic index and somatic growth

No differences in somatic growth, condition factor (Fulton's K) or hepatosomatic index were detected for nether the male or female fish. The female fish had a growth of about 30 %; from about 700 g in September to 900 g in the end of January and the males The male grow from approx. 600g in September to about 800g in January, a 25% increase in weight.

The January sample from had a lower mean GSI for the female fish in the two exposed groups compared with the controls. However, this apparent difference was not statistically significant due to high variance (Table 2). No significant effects on variables related to fecundity or oocyte size were found (Table 3). There were no significant effects in the GSI for the males.

Table 3. *Experiment I. Potential relativ fecundity, fecundity condition factor and estimated time to spawning.*

	Control	0.02 mg/kg	2 mg/kg
Relative fecundity	1062±406	995±276	901±239
Fecundity condition factor	10.6±3.7	10.1±3.3	8.7±2.7
Time to spaning (day to spawning)	26.4±3.1	29.1±3.4	28.1±3.6

5 Discussion

It was found that AP exposure brings about a considerable drop in the plasma E2 level even at very low doses of APs (0.02 mg/kg). This response was consistent and was seen at all samplings times from November to January. The plasma level of T in this female fish and T and 11-KT in the males was also affected but the results were more ambiguous than those for E2.

In this experiment, the cod were fed in groups. This resulted in some fish receiving a lower total dose of APs than others due to competition for food, and increased the variation in the results.

A second experiment was therefore carried out as a follow-up of this experiment. This was designed to obtain more control over the level of exposure for each individual fish. Cod of the same age and status were used as in this experiment. For five weeks in November 1999 five groups of cod were administered a single oral dose per week of 0.05 mg/kg, 0.5 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg total body dose of each of the four C_4-C_7 APs respectively. A control group and a positive control group dosed with 5 mg/kg E2 were also included in the experiment. The biological effect parameters examined were the same as in the experiment presented in this article.

The new experiment showed the same down regulation of E2 levels in the blood of the AP exposed females. On the contra to the present study did the gonads of exposed female cod displayed a lower gonadosomatic index (GSI) compared to controls, and their gonads developed more slowly. The steroids levels also fell in male fish given APs. There were as here seen VTG induction in the AP exposed males and there were significant changes in the maturation status of the testis. Even at the

lowest exposure to alkylphenols the amount of spermatozoa was reduced, while there were increases in spermatogonia and spermatocytes.

All the finding from this study were therefore confirmed together with a number of other responses that now gave significant differences between the control and the AP treated groups. For further discussion of the mechanism behind the effects of AP, see Meier, 2007

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