Hormonal effects of C_4-C_7 alkylphenols on cod (*Gadus morhua*)

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Foreword

There is a great need for more knowledge of the effects on the marine environment of discharges to the sea from the offshore oil and gas industry. The long-term effects in particular are of great interest, as we know very little about them. More knowledge is thus essential if the authorities are to be able to control the development of this sector and to coordinate the exploitation of our oil and gas reserves with other uses of the marine environment. A central aspect of this is that the total impact on the marine environment must not bring about changes in biological diversity or in the marine ecosystem.

A review of the problem was prepared by a working group led by the Ministry of Petroleum and Energy (OED) in the wake of the Storting's discussion of White Paper no. 26 (1993 - 94) about biological monitoring, long-term effects of oil and chemicals, and combat system for oil spills. The report of the working group was published in December 1996. Since then, a new review has been carried out by the Research Council of Norway (NFR); this was submitted to OED in spring 2001: Long term effects of discharges to sea from the offshore sector. To date, these reviews have not led to significant public-sector support for concrete projects.

In 1997, the Institute of Marine Research made a start on one topic with its proposal for a project entitled "The hormonal effects of alkylphenols on cod". The project was based on the fact that significant quantities of alkylphenols are released into the sea by petroleum installations as a result of discharges of produced water. It has been shown that alkylphenols may have oestrogenic (feminising) effects on e.g. rainbow trout, human beings and other mammals, with reproductive disturbances as the end result (Jobling & Sumpter, 1993); Colnborn & Clement, 1992). The question was whether cod, Norway's most important commercially fished species, might be similarly affected.

The project proposal was submitted to the Norwegian Oil Industry Association (OLF) and OED. OLF was positive to the project proposal. OED sent it to NFR for evaluation arguing that the Ministry lacked the necessary expertise. However, some additional support from OLF enabled the project to be started in autumn 1997 according to original plan. We want to express our gratitude to OLF for their financial support. We are also very grateful to NFR who contributed to funding for the period 1998-2000.

Contents

Summary

Produced water discharged by the offshore oil and gas industry contains small amounts of alkylphenols, some of which have the potential to disrupt endocrine processes. This report presents the results of a project carried out during 1997-2001 by the Institute of Marine Research. The aim of the project was to study the biological effects on hormones and reproduction in cod (*Gadus morhua*) of selected C_4-C_7 alkylphenols that are found in produced water. The study was carried out under controlled laboratory conditions. Model compounds tested included 4-tert-butylphenol, 4n-pentylphenol, 4n-hexylphenol and 4n-heptylphenol. Variations in hormone levels (17ß-oestradiol, testosterone and 11-ketotestosterone) in blood plasma and gonadal development in control groups were compared with groups of cod exposed to alkylphenols. Effects on phase-1 and phase-2 enzymatic systems for the detoxification and excretion of xenobiotics were also analysed.

In the first experiment, two-year-old cod spawning for the first time were fed pellets containing alkylphenols. One group (n=100) were given a high dose of 500 ppb (based on total fish weight) per day of each of the four alkyl phenols (total dose 2 ppm). A second group (n=100) were given a low dose of 5 ppb of each of the same four alkylphenols (total dose 20 ppb). The control group (n=100 cod) was fed uncontaminated fish pellets. The exposure took place from October 1997 – January 1998 (14 weeks) by feeding the cod three times per week. Spawning took place during February-April 1998.

In experiment 1, the cod were fed in groups. This resulted in some fish receiving a lower total dose of alkylphenols 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 the first 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 the first experiment. For four weeks in November 1999 five groups of cod were administered a single oral dose per week of 5 ppb, 500 ppb, 5 ppm, 10 ppm and 20 ppm total body dose of each of the four C_4-C_7 alkylphenols respectively. A control group and a positive control group dosed with 5 ppb 17ß-oestradiol were also included in the experiment. Each group consisted of 40 fish. The biological effect parameters examined were the same as in Experiment 1. A total body burden in cod of 20 ppb (four compounds) is theoretically equivalent to a 0.032 ppb concentration in seawater, using a bioconcentration factor of 600 for the four selected alkylphenols.

Levels of 17ß-oestradiol in blood plasma were reduced in female cod given the lowest dose of C_4-C_7 alkylphenols. The gonads of exposed female cod displayed a lower gonadosomatic index (GSI) compared to controls, and their gonads developed more slowly. It was calculated that in cod given the lowest dose, spawning would start approximately 21 days later than in controls. If this also occurs in wild populations of cod, it may severely influence their reproductive potential.

Testosterone levels also fell in male fish given the lowest dose. The male fish started to produce vitellogenin, which is not normal. There were significant changes in the maturation status of the testis. At the lowest exposure to alkylphenols the amount of spermatozoa was reduced, while there were increases in spermatogonia and spermatocytes. This may influence the ability to fertilize eggs.

Analyses of the cytochrome P-450 system (Phase-1), glutathione and glutathione reductase (Phase-2) in cod liver showed only slight effects of exposure to alkylphenols. Glutathione S-transferase and glucose-6-phosphate dehydrogenase activities were not significantly affected.

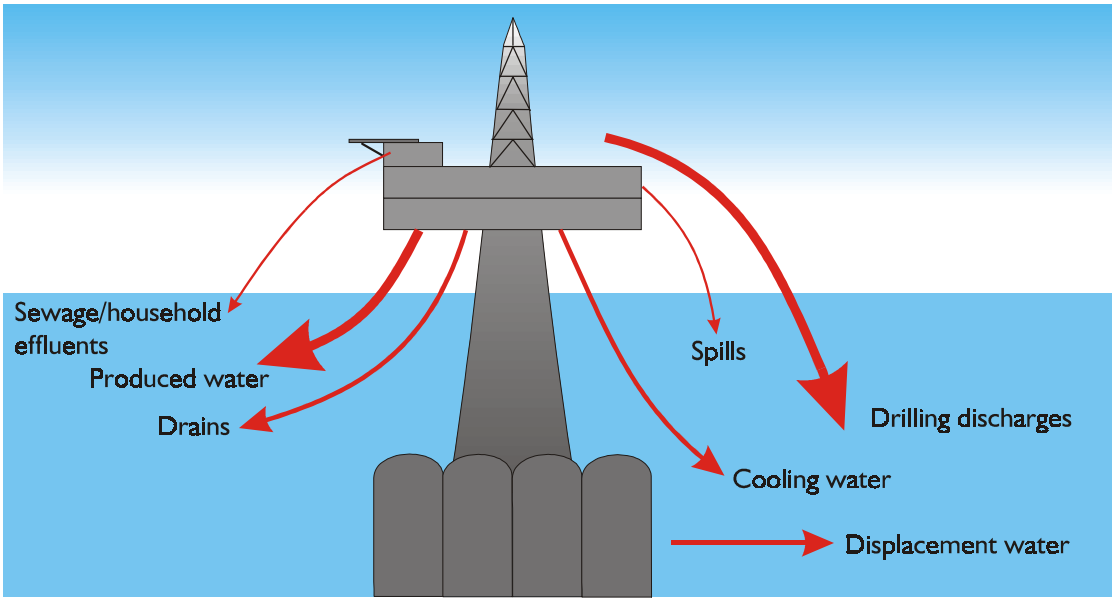


Fig. 1. Discharges to water from an oil production platform (OLF, 1997)

1 Introduction to the problem of the offshore sector's discharges to the sea

1.1 Produced water - composition and effects on the marine environment

Petroleum activities on the Norwegian continental shelf discharge large quantities of produced water. The older the field, the more produced water it discharges, and it is not unusual to find that towards the end of the useful life of a field, as much of 98% of what is pumped up from its wells is produced water. In the course of the economic lifetime of a field, the volume of produced water may be twice as great as the volume of oil brought to the surface. The potential consequences of such large discharges for the marine environment are causing anxiety to the oil companies, the authorities and the man in the street, not to mention a number of scientific groups.

1.2 Produced water - what is it and how much is discharged?

Produced water is water that follows with oil and gas from the reservoir and which is separated from the oil/gas flow on the platform or production vessel. Produced water consists of formation water, i.e. water that occurs naturally in the geological structure, and water that has been injected into the reservoir in order to maintain pressure within the formation. The produced water is cleansed of oil to a maximum content of 40 mg/l, and most of it is then discharged to the sea. A small proportion is re-injected into the reservoir. The upper discharge limit refers to dispersed oil. There is no equivalent limit for dissolved components. In addition to the remaining oil the water contains chemicals and other substances that naturally occur in the well, such as metals, alkylphenols, and aromatic hydrocarbons (including PAH). In 2000, approximately 44 tonnes of alkylphenols were released on the Norwegian continental shelf in connection with discharges of produced water.

Platforms with oil storage cells also discharge displacement water with the produced water. There is also drainage water from the platform decks, which may also contain oil. In 2000, 2,817 tonnes of the oil discharged to the Norwegian continental shelf came from produced water, while 342 tonnes were from displacement and drainage water. Most of the remainder of the total discharges of 3,196 tonnes was due to acute discharges in connection with

operation of the installations. Fig. 2 shows developments in the quantity of produced water and discharges of oil to the Norwegian continental shelf (Sources: SFT; OLF).

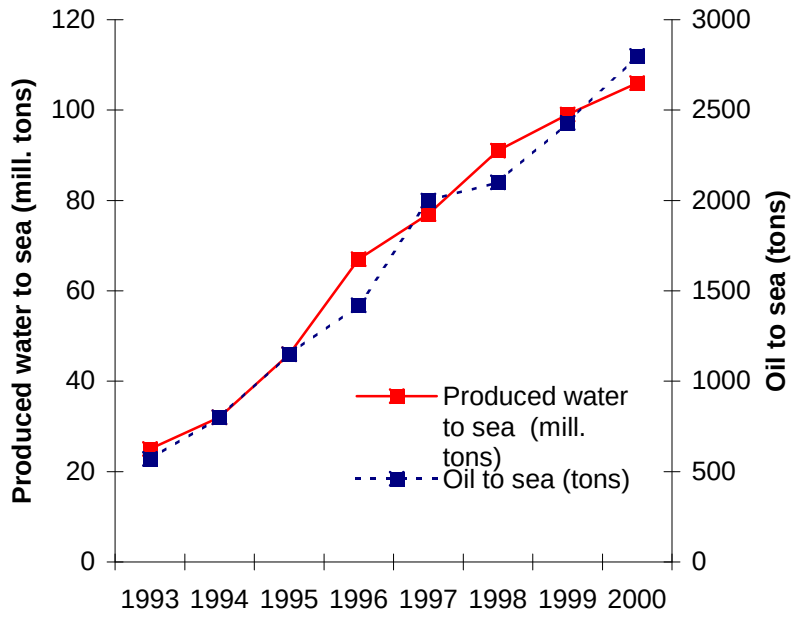


Fig. 2. Amount of produced water and discharged oil in Norwegian Sector during the period 1993-2000 (Source: SFT, OLF)

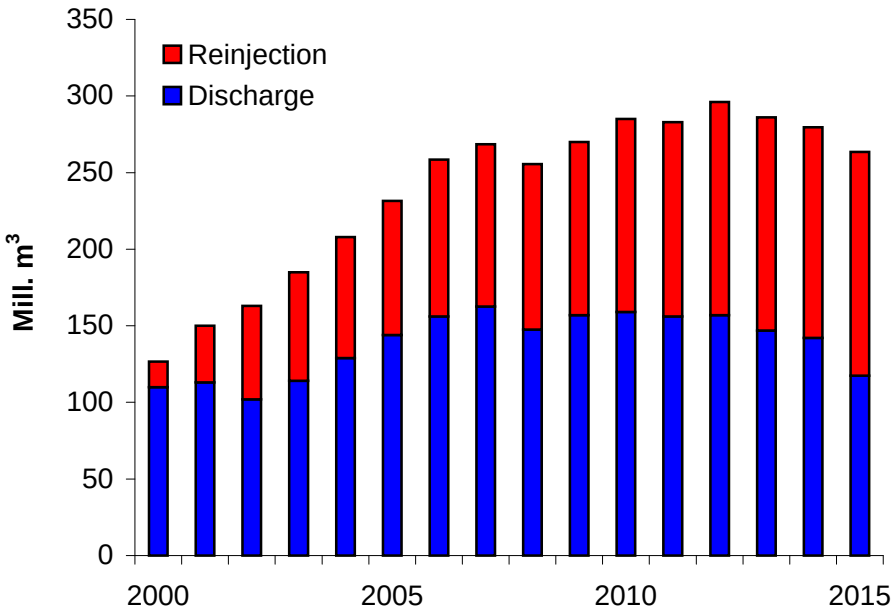


Fig. 3. Prognosis for water production from Norwegian oil and gas production platforms (Miljøsok, 2000).

1.3 Periods of increasing discharges

Norway is currently in a period during which discharges of produced water from the petroleum sector are increasing rapidly (Fig. 3). From discharges of 26 million m^3 in 1993, it is expected that discharges in 2001 will have risen to 120 million m^3. This is due to the fact that a number of oil and gas fields are coming to the end of their useful life. There are also significant discharges from e.g. the UK zone.

1.4 What does produced water consist of?

The components of produced water can be roughly grouped into the following categories:

Dispersed oil

Dissolved oil components:
- BTEX (Benzene, Toluene, Ethyl benzene, Xylene)
- NPD (Naphthalene, Phenantrene, Dibenzothiophene and their C1-C3 alkyl homologues)
- PAH (Polycyclic Aromatic Hydrocarbons)
- Alkylphenols
- Organic acids

Heavy metals:
- Barium (Ba)
- Cadmium (Cd)
- Copper (Cu)
- Iron (Fe)
- Mercury (Hg)
- Lead (Pb)
- Zinc (Zn)

Radionuclides:
- Radium (^{226}Ra)

Chemical additives:
- Scale inhibitors
- Corrosion inhibitors
- Biocides
- Emulsion breakers
- Foam-damping agents
- Paraffin/asphaltene treatment agents

Salt:
- Mainly NaCl

Table 1. Chemical composition of produced water from oil production fields in the Norwegian Sector of the North Sea compared to observed background levels (Røe, 1998).

Chemical compound	**Produced water**			**Seawater**	
	Range	Mean	Units	Range	Units
THC (IR)	15-60	44	mg/l	NA	NA
BTEX	1-67	6	mg/l	NA	NA
NPD	0,06-2,3	1,2	mg/l	9-185	ng/l
PAH	130-575	468	µg/l	1-45	ng/l
Organic acids (‹ C6)	55-761	368	mg/l	NA	NA
Phenols (C0-C4)	0,1-43	8	mg/l	NA	NA
Ba	0,2-228	87	mg/l	22-80	µg/l
Cd	0,4-5	2	µg/l	4-23	ng/l
Cu	22-82	10	µg/l	20-500	ng/l
Fe	0,1-15	4,3	mg/l	1,8	µg/l
Hg	‹ 0,1-26	1,9	µg/l	1-3	ng/l
Pb	0,4-8,3	0,7	µg/l	20-81	ng/l
Zn	0,5-13	7	mg/l	0,3-1,4	µg/l
^{226}Ra	NA	NA	Bq/l	NA	Bq/l

NA: Not available

THC: Total hydrocarbon; BTEX: Benzene, Toluen, Ethylbenzene, Xylene; NPD: Naphtalene, Phenantrene, Dibenzothiophene and their C1-C3 alkyl homologues; PAH: Polycyclic Aromatic Hydrocarbons

Some of the produced water has been in contact with the geological formations for millions of years. Its composition is field-dependent and may also change in the course of the production life of the reservoir, as ever more water has to be injected into the structure in order to maintain its pressure.

Table 1 provides an overview of the chemical composition of produced water in the Norwegian sector of the North Sea, as well as the results of field studies of background levels of some components of produced water.

1.5 Effects on the marine environment

The above paragraphs make it clear that produced water consists of a large number of different chemical components, some of which have been identified, while others have not. Discharges of produced water above a certain level may locally bring about changes in ecological systems. The degree of change will be dependent on a number of factors, such as the level of discharge and the capacity of the environment to absorb, utilise or render harmless the contaminants involved. Certain components of pollution are more easily made harmless than others, and some are readily brought into the food chain while others are not.

Individual components of the produced water may act as direct nutritional components for marine microorganisms, and these are possibly primarily found in the organic acid fraction. Discharges of short-chain fatty acids are high (Table 1), and given that these are a source of nutrition for a number of different microorganisms it is easy to imagine that this could influence the composition and quantity of the local natural fauna. Certain microorganisms benefit from the extra good growth conditions, which may lead to others being forced out. The result may be a distortion of the natural species composition in the area. At present, there is very little knowledge of this aspect.

There is a great deal of interest in the potentially negative effects of various chemicals in produced water on the marine environment, and in the course of time a great deal of testing has been done in this field (see, e.g. Ray and F.R. Engelhardt, 1992; Reed and Johnsen, 1996). Various test organisms from different links in the food chain have been used, and “screening protocols” have been developed to identify the environmental factors involved. This usually involves making simplifications of the various biological systems of which the food chains consist, and the results of such tests are often of limited value. Life in the sea is extremely complex, and it may even be difficult to transfer results from one species to another. In order to be completely certain of the specific effects that given chemicals have on a species of fish, that particular species needs to be studied. This may be difficult to manage in practice, and studies of effects must then be done on a representative selection.

The following paragraphs focus on the toxic effects of the components that are found in the oil fraction of produced water, and these have been divided into acute and chronic toxicity.

Acute toxicity

Generally speaking, there are few acute toxic effects of produced water on marine organisms, although there are variations from one field to another. Acute toxicity data from produced water in the North Sea show that the level at which effects can be demonstrated lies above 0.3 mg/l for all organisms that have been tested - algae, shellfish, copepods, amphipods, shrimps and fish (Røe, 1998). Acute effects are not expected to be found further than 50 m from the point of discharge. Studies of which fractions of produced water are the cause of acute toxicity have been performed. The tests utilised were Microtox ® (*Photobacterium phosphoreum*) and biodegradation tests (OECD 301E), and these have shown that the most

important contributors to the acute toxicity of produced water were the aromatics and phenol fractions (Røe, 1998).

Chronic toxicity
Chronic toxicity, or long-term effect, is defined as an effect that is long-lasting or that continues for a long period of time (weeks to years, depending on the life-cycle of the organism concerned). The expression "chronic" can be used to define either the exposure or the response to exposure (the effect). A typical characteristic of chronic exposure is that its biological effects develop slowly and may continue for a long time. Long-term effects can be summarised as shown in Table 2.

Table 2. Overview of responses and effects at different levels of the ecosystem

Level	Type of response	Effect on the next level
Biochemical	Metabolic disturbances Effect on detoxification systems	Reduced energy production Depletion of energy reserves Adaptation of the organism
Organism	Metabolic changes Behavioural changes Reduced growth and reproductive ability Reduced motility Reduced resistance to diseases	Reduced population performance
Population	Changes in population dynamics Population adaptations to stress	Effects on occurrence of other organisms and communities
Community	Changes in species composition Reduced energy transfer Adaptation of ecosystem	Weakening of community Reduced secondary production

The long-term effects of the PAH fraction of oil have been studied very closely for a number of years. There is now considerable evidence to suggest that exposure to various PAH compounds may lead to cancer, weakening of the immune response and effects on reproduction and development in fish (Aas, 2000). Alkylphenols are suspected of having similar effects as the female hormone oestrogen (i.e. they are hormone mimics). PAHs and alkylphenols are thus important contributors to both acute and chronic toxicity (Røe, 1998).

The potential for causing chronic toxicity is closely related to the potential for bioaccumulation and biomagnification. It has been shown that PAH compounds can accumulate in organisms further down the food chain, but to a much lesser extent in organisms higher in the chain (e.g. fish). This is related to the fact that fish have a much greater capacity for chemical transformation (i.e. biotransformation, leading to detoxification) of foreign matter than lower organisms. A concrete example of this can serve to illustrate the situation. The PAH compound phenantrene is easily accumulated in plankton (*Calanus finnmarchicus*) and an important part of the amount absorbed remains in exposed individuals for a long period of time (months) (Røe, 1998). If fish are exposed to the same substance, it will be relatively rapidly excreted after the end of exposure. Plankton are thus more liable to suffer damage from phenantrene than fish because basic biological defence mechanisms are much less fully developed in plankton. We currently know little about this phenomenon.

2 Scientific background

2.1 Introduction

The endocrine system is the key to successful reproduction, and every disturbance of this system is liable to bring about a reduction in fertility or incomplete or abnormal sexual

development. In fish, such effects may hinder gonadal development, changes in vitellogenin production (an important component of the egg-yolk), a fall in the production of gonadal and hypophysal hormones, reduced sperm motility and a fall in hatching percentage and larval survival, in addition to larval deformities.

As well as controlling the seasonal reproduction cycle, hormones also play an important role in sexual differentiation. Sex is less definitely differentiated in fish than in mammals, and can even be reversed by treatment with androgens or oestrogens at an early stage in the life cycle. A lack of hormones at a critical stage may have consequences that are just as serious as the wrong hormone. The uptake of pollutants by eggs and larvae through direct exposure may affect hormonal balance and thus the sexual differentiation and fertility of individuals later in their life cycle.

2.2 General background to reproduction

Reproductive development and functions in fish are very similar to those in mammals, including human beings. External stimuli act via the brain-hypothamalus-hypophysis axis, stimulating the secretion of gonadotropins in the hypophysis (follicle-stimulating hormone (FSH) and luteinising hormone (LH)), which in turn stimulate the maturation of the gonads and causes these to produce steroid hormones (oestradiol and testosterone in female fish; testosterone and 11-ketotestosterone in males). The target organ for oestradiol is the liver, which it stimulates to produce vitellogenin which is incorporated in the egg-yolk of the developed oocyte. 11-ketotestosterone is probably involved in spermatogenesis. Steroids from the gonads also regulate secretions from the hypophysis via feedback mechanisms, and are eventually metabolised in the liver (for details, see Baroiller *et al*., 1999; Nagahama, 2000).

When the ovulatory system and the sperm are mature, external stimuli such as an adequate spawning substrate, appropriate temperature, the presence of a mate and its display of courting behaviour will send signals to the hypophysis, causing it to liberate large quantities of gonadotrophins, which in turn stimulate the gonads to produce a maturation-inducing steroid (17α, 20β,-dihydroxy-4-pregnen-3-one; 17,20-βP), which leads to the breakdown of germinative vesicles, maturation of the sperm and ovulation. Ovulated oocytes are fertilised by mature sperm in the water, the eggs hatch, and the larvae develop and mature to produce the next generation. This process, which takes place in most marine and freshwater fish with relatively small variations from one species to another, is summarised in Figure 4.

2.3 Effects of pollution on reproduction

Aquatic pollution may have severe effects at several different levels in this cycle (Kime, 1995). Since the beginning of the 90s there has been a sharp focus on hormone-disrupting substances. A large number of chemical compounds have been shown to “resemble” hormones or in other ways to affect the hormonal balance, thus disturbing natural reproductive processes. Most anxiety has concerned chemicals with “oestrogen mimicking” effects (surveyed by Arukwe and Goksøyr, 1998), while a growing attention is now also being paid to other classes of hormone such as the androgen system and the thyroxine hormones (Oberdorster & Cheek, 2001). Among the substances that have been shown to have oestrogen-disrupting effects (whether agonistic or antagonistic), we find alkylphenols, phthalates, bisphenol A, chlorinated hydrocarbons such as polychlorinated biphenyls (PCBs), dioxins and pesticides such as chlordane, dieldrin, DDT and its metabolite DDE (Arukwe & Goksøyr,1998).

The fact that the endocrine apparatus is a system with many mechanisms and is thus liable to suffer disturbances at many levels is described by the general definition of Kavlock *et al.* (1996) of hormone-disrupting substances, i.e. that they are “exogenous agents that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones”.

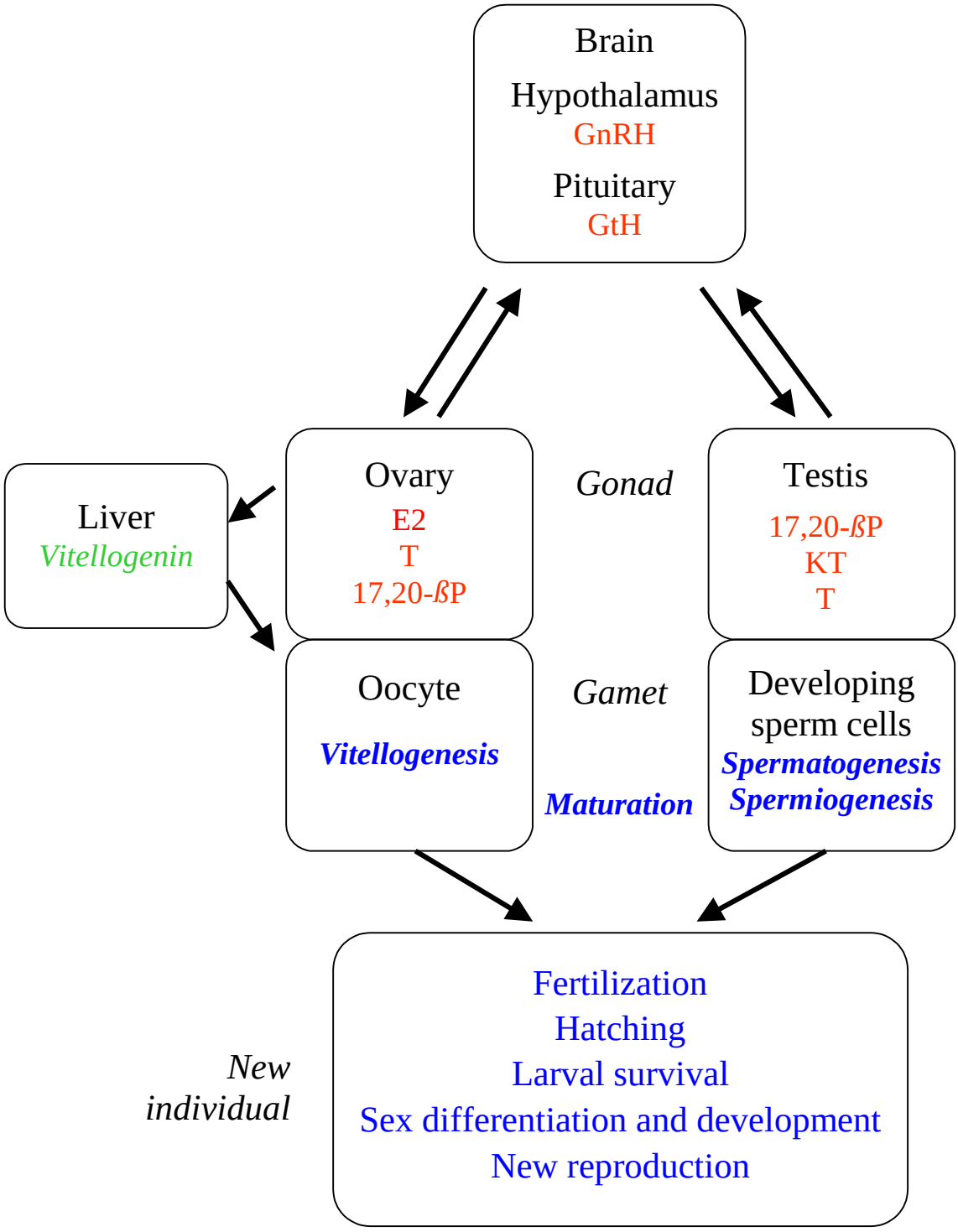


Figure 4. The reproductive system of fish and possible sites of action of contaminants. The hormonal system is regulated by a series of complex feed back mechanisms between the organs involved. (GnRH = gonadotropin-releasing hormone; GtH = gonadotropin; E2 = 17ß-Oestradiol; T = testosterone; KT = 11-ketotestosterone; 17,20-ßP = 17α, 20ß-dihydroxy-4-pregnen-3-one). Black – structures, red – hormones, green – lipoprotein, blue – processes.

The high degree of "plasticity" in the sexual development of fish results in the existence of "critical windows" in early life stages, during which fish are particularly sensitive to hormonal effects, and when even brief exposures or exposures to low concentrations may have important and irreversible consequences. This phenomenon is actively exploited in aquaculture in order to produce monosex fish cultures. Hormonal treatment of fish in aquaculture is forbidden in Norway, although it is widely used in many other countries. Hormonally controlled feminisation of a number of different species of fish is widely used. This is primarily carried out by treating eggs and/or larvae with oestrogens (Piferrer, 2001).

The sensitivity of early life stages to the effects of oestrogen is also reflected in findings from the field. The clearest evidence of hormonal disturbance in wild fish comes from reports of the feminisation of male fish, with findings of intersex/ovo-testis gonads (testis that contain morphological characteristics of female fish; i.e. hermaphroditism), in both a number of freshwater fish species (Harshbarger *et al.*, 2000; Jobling *et al.*, 1998; van Aerle *et al*., 2000; Vigano *et al.*, 2001) and saltwater fish (Allen *et al.*, 1999a and b; Hashimoto *et al.*, 2000; Minier *et al.*, 2000; Simpson *et al.*, 2000).

The yolk protein vitellogenin (VTG) is a sensitive biomarker which is widely used in studies of the effects of oestrogen mimics in fish. Even though VTG is a protein specific to female fish, males also possess all of the genetic system needed for VTG protein synthesis. Oestrogen induces VTG synthesis in the liver of both males and females, and a rise in the level of VTG can therefore be used as an indication of oestrogen influence. Several studies have found increased VTG levels in wild male fish and in fish kept in cages in polluted areas. Most of these studies have been done on freshwater fish: carp (*Cyprinus carpio*) caught in the Hudson River near New York (Folmar *et al.*, 1996); walleye (*Stizostedion vitreum*) caught in the Mississippi (Folmar *et al.*, 2001). In the UK, fish from a large number of rivers and estuaries have been studied (for review, see Tyler & Routledge, 1999), and elevated VTG levels have been found in several species from different locations: rainbow trout (*Oncorhynchus mykiss*) (Harries *et al.*, 1996, 1997) and roach (*Rutilis rutilis*) (Jobling *et al.*, 1998; Routledge *et al.*, 1998). The livers of immature powan (*Coregonus lavaretus*) kept in cages outside a wastewater outlet from a paper mill (Lake Saimaa in Finland) displayed induced VTG mRNA (Mellanen *et al.*, 1999).

Abnormally high levels of VTG have also been found in saltwater fish: flounder (*Platichthys flesus*) caught off the British coast (Allen *et al.*, 1999 a and b; Lye *et al.*, 1997) and near offshore installations in the UK sector of the North Sea (Mathiessen *et al.*, 1998) and in flounder (*Pleuronectes yokohamae*) caught in the Bay of Tokyo (Hashimoto *et al.*, 2000).

Unlike the great deal of interest that has been shown in oestrogenic effects and feminisation of male fish, there are only a few reports of masculinising effects on females. It is known that eels (*Anguilla anguilla*) are particularly sensitive to early exposure to environmental hormones, and it has been suggested that the high proportion of male eels that are found in European rivers is due to environmental factors (Beulens *et al.*, 1997). It has been shown that discharges of wastewater from papers mills can also contain substances with androgenic or anti-oestrogenic effects (Bortone *et al*., 1989, 1994, 1999; Karels *et al.*, 1999; Hegrenes, 1999; Larsson *et al*., 2000).

A few laboratory studies have shown that certain environmental toxins may interact with receptors for maturation-stimulating hormones, but there are no data from field studies that confirm this (Thomas *et al.*, 1998; Das & Thomas, 1999; Thomas, 2000). Similarly, there is little in the literature regarding disruptions of the thyroxine hormones in fish (Oberdorster & Cheek, 2001; Zhou *et al.*, 2000).

In spite of the above relative comprehensive list of field studies, most of the evidence for hormonal disturbances caused by hormone mimics is the result of laboratory studies. There

has been some criticism of the fact that many of these studies have been carried out using unrealistically high concentrations in comparison with the concentrations that are actually found in nature (Cooper & Kavlock, 1997; Oberdorster & Cheek, 2001; Tyler *et al.*, 1998). More field studies and lower, more realistic concentrations in the laboratory studies, as well as a sharper focus on long-term effects, have been called for.

2.4 Alkylphenols and their potential effects on fish reproduction

The oestrogenic effects of alkylphenols are known from a large number of *in vitro* and *in vivo* studies (Nimrod & Benson, 1996). Virtually all research in this field has dealt with the two long-chain alkylphenols nonylphenol (C_9) and octylphenol (C_8). These are derivatives of degradation products of the non-ionic surfactants known as alkylphenol ethoxylates (APE), which consist of an alkylphenol group, principally nonylphenol (82%), but which also contain octylphenol or dodecylphenol (C_{12}) which are coupled to long ethylene oxide chains (see Nimrod & Benson, 1996). APE is and has been utilised in a large number of products, including herbicides, paints and industrial cleaning and degreasing agents (Naylor *et al.*, 1992). APE is one of the most widely used surfactants in the world, with an annual production of around 500,000 tonnes (Renner, 1997). In Norway, the use of APE has been very limited, and has fallen significantly during the 90s, from 615 tonnes in 1995 to 113 tonnes in 2000 (www.SFT.no, 2001). The use of nonylphenol, octylphenol and their ethoxylates will be forbidden in Norway from January 2002 (www.miljoverndepartementet.no, 2001). A number of other European countries are also planning to forbid the use of these substances, which are also on the Oslo-Paris Commission's (OSPAR) list of chemicals which ought to be phased out.

The long-chain alkylphenol ethoxylates are not toxic and have no hormone-mimicking effects, but they are broken down gradually and relatively rapidly in waste-treatment plants into the more resistant alkylphenol mono- and di-ethoxylates AP1EO and AP2EO and the short-chain carboxylic acid derivatives (the alkylphenol carboxylates AP1EC and AP2EC). These in turn break down into pure alkylphenols (Nimrod & Benson, 1996). A large proportion of these degradation products finally end up in the aquatic environment, and nonylphenol and octylphenol have thus been found in a large number of freshwater systems all over the world, in concentrations of up to 180 µg/l in particularly highly polluted areas, but with typical values in the low ppb (µg/l) range (Giger *et al.*, 1984; Kvestak *et al.*, 1994; Blackburn & Waldock, 1995; Franke *et al.*, 1995; Bennie *et al.*, 1997; Espadler *et al.*, 1997; Bennett & Metcalfe, 1998; Dachs *et al.*, 1999; Khim *et al.*, 1999a; Tanghe *et al.*, 1999; Ahel *et al.*, 2000; Maruyama *et al.*, 2000; Valsecchi *et al.*, 2001).

Nonylphenol has been identified in several locations in the marine environment. Measurements of seawater from coastal areas near cities have shown concentrations of up to 1.2 µg/l, while values from sediment samples can be as high as 30 ppm (mg/kg) at exposed sites (Marcomini *et al.*, 1990; Valls *et al.*, 1990; Kvestak & Ahel, 1994; Blackburn *et al.*, 1999; Khim *et al.*, 1999b; Lye *et al.*, 1999; Shang *et al.*, 1999; Hale *et al.*, 2000; Yamashita *et al.*, 2000; Bester *et al.*, 2001; Ferguson *et al.*, 2001). The determination of nonyl- and octylphenols in open waters offshore makes extremely high demands of analytical methodology, due to the extremely low concentrations involved. There exist only two studies in which such measurements have been made. Kannan *et al.* (1998) traced very low levels of nonylphenol in the sea off Japan (0.002 - 0.093 ng/l), while a measurement from the North Sea (German Bight) produced significantly higher values (Bester *et al.*, 2001). These authors found nonylphenol concentrations in seawater of between 0.7 and 4.4 ng/l, and they have also found values of nonylphenol as high as 13 µg/kg in offshore sediments sampled more than 100 km from land.

Nonylphenol and octylphenol are both bioconcentrated and have been identified in aquatic organisms in nature. Ahel *et al.* (1993) found concentrations of nonylphenol of up to 1600

µg/kg (dry weight) in various freshwater fish taken in Swiss rivers. Nonylphenol (up to 184 µg/kg) has been found in carp caught in Lake Mead in the USA (Snyder *et al.*, 2001). Molluscs (squid and shellfish) from Italian harbours in the Adriatic contained 67 - 566 µg/kg nonylphenol and 3.6 - 3.8 µg/kg octylphenol (Ferrara *et al.*, 2001). Fish caught in various lakes in Michigan, USA had tissue concentrations of <3.3 to 29.1 µg/kg nonylphenol (Keith *et al.*, 2001). Fish from Japanese rivers have been shown to contain from 1 - 110 µg/kg nonylphenol (Tsuda *et al.*, 2001). Wahlberg *et al.* (1990) found between 200 and 400 µg/kg nonylphenol in mussels gathered from the sea near the wastewater outlet of a plant that produced APE (Sweden). Flounders (*Platichthys flesus*) caught in brackish water outside the rivers Tyne and Tees in England have been shown to contain 5 - 118 µg/kg nonylphenol (Lye *et al.*, 1999). Nonylphenol was not demonstrated (above a detection threshold of 100 µg/kg) in fish caught in the British sector of the North Sea (Blackburn *et al.*, 1999). Apart from one special case in the Detroit River in the USA, where large amounts of 2,4 di-tert-pentylphenol were found (Shiraishi *et al.*, 1989), all the studies of alkylphenols of which we are aware concerned octylphenol and nonylphenol. We have found no field studies that have analysed petroleum-related alkylphenols.

Alkylphenols are some of the most intensively studied substances that have hormone-disrupting effects. The alkylphenols bind to and affect the oestrogen receptors in the same way as 17 ß-oestradiol, but the response is much weaker (Mueller & Kim, 1978; Soto *et al.*, 1991; Jobling & Sumter, 1993; White *et al.*, 1994; Soto *et al.*, 1995; Shelby *et al.*, 1996; Yadetie *et al.*, 1999a). An *in vitro* study showed that the oestrogenic effects of alkylphenols depend on both the position (para>meta>ortho) and branching (tertiary>secondary=primary) of the alkyl group. Maximum activity (1000 - 6000 times less potent than oestradiol) has been found for C_6 - C_8 parasubstituted tertiary alkylphenols, but C_5, C_4 and C_3 phenols are also "oestrogenic" (10^5 - 10^7 times less potent than oestradiol) (Routledge & Sumpter, 1997). It is important to note that *in vitro* effects cannot be directly transferred to an *in vivo* situation. Most plasma oestrogen is bound to plasma proteins (globulins and albumin), and the affinity of these proteins to alkylphenols is much lower. For this reason, the bioavailability and effects of phenols may be considerably greater than studies based on receptor affinity might suggest. *In vivo* studies also suggest that the oestrogenicity of branched alkylphenols is higher than that of linear isomers (Pedersen *et al.*, 1999). Table 3 shows a selection of studies of the effects of alkylphenols on fish, and provides an overview of various measurement variables that have turned out to be affected.

Table 3: Selected references to biological experiments with alkylphenols (where concentrations are quoted per kg, the substance was administered by injection; when quoted per litre, the substance was provided in the water. BP - butylphenol; PP - pentylphenol; OP - octylphenol; NP - nonylphenol)

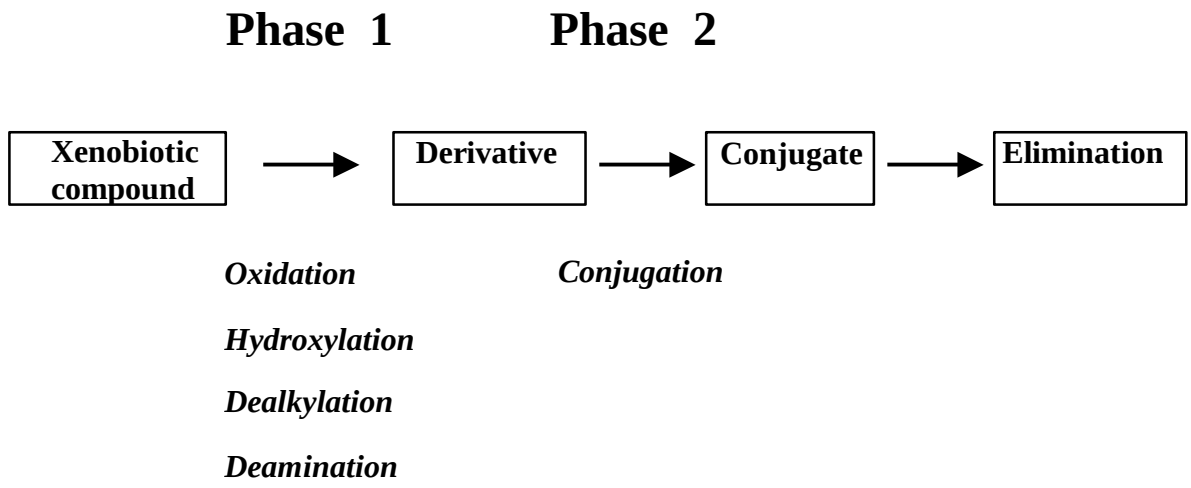
Reference	**Species and concentration**	**Feminisation (intersex, secondary sexual character-istics)**	**Vitellogenin (VTG), zona radiata protein (ZRP) and steroid**	**Gametogenesis**	**Histopathology**	**Other effects**
Arukwe *et al*., 1997	Immature salmon (*Salmo salar*) 1 - 125 mg/kg NP		Reduction in E2 at 1 and 5 mg/kg. No effects at higher doses			Dose-related reduction in EROD. Higher activity of steroid-hydrogenase at 1 and 5 mg/kg, lower at higher doses
Bayley *et al*. 1999	Guppy (*Poecilia reticulata*) Nominally 150 µg/l OP	Males displayed reduced aggressiveness to competitors				
Dreze & Monod, 2000	"Mosquitofish" (*Gambusia holbrooki*). 0.5 - 50 µg/l NP	100% female secondary sex characteristics at 50 µg/l. At 0.5 and 5 µg/l, occurrence of individuals with partially developed gonopodia		Female or undeveloped gonads at 50µg/l, none with testis	Effect on liver at 50 µg/l (reduced lipids, perivascular necrosis, hepatocytes with pyknotic or hypertrophic cores	
Gimeno *et al.* 1997	Carp (*Cyprinus carpio*). 140 µg/l PP	Oviduct formed by exposure during sexual differentiation		Reduction in number of primary sex cells		
Gimeno *et al.* 1998	Carp (*Cyprinus carpio*). Mature fish 32 µg/l PP			Reduced number of spermatogenetic cysts	Reduced GSI after three months of exposure	
Gray *et al.* 1999	Medaka (*Oryzias latipes*). 10 - 100 µg/l OP	Ovotestis in two fish (50 and 100 µg/l. One of them was fertile, while the other did not induce spawning				Reduced pairing activity (50 µg/l. Poor hatching or deformities in offspring of exposed fish (10 - 100 µg/l)

Gronen *et al.* 1999	Medaka (*Oryzias latipes*). 10 - 100 µg/l OP	Ovotestis in two fish (one at 74 and one at 230 µg/l)	Dose-dependent and reversible. Larval survival correlated with VTG serum levels	Unexposed female fish together with exposed males spawned fewer eggs (50%) than controls. Abnormal embryonic development	More spermatogonia (A and B) at concentrations above 41 µg/l OP	
Hemmer *et al.* 2001	Sheepshead minnow (*Cyprinodon variegatus*). 0.64 - 42.7 µg/l NP		Induction of VTG above 5.4 µg/l			
Jobling *et al.*, 1996	Rainbow trout (*Oncorhynchus mykiss*) males. 0.6 - 44 µg/l OP; 1.0 - 54.3 µg/l NP		Induction of VTG at 4.8 µg/l OP and 20.3 µg/l NP	Decrease in GSI (4.8 µg/l OP; 54.3 µg/l NP). Spermatogonia accumulation. Decrease in spermatocyte fraction		
Kinnberg *et al.*, 20000b	Platy (*Xiphophorus maculatus*). 80 - 1280 µg/l NP			Reduced number of cysts in testis; hypertrophy of Sertoli cells (dose-related)	Free sperm in enlarged ductus spermaticus (dose-related)	
Kwak *et al.*, 2001	Swordtail (*Xiphophorus helleri*). 4 - 100 µg/l NP		VTG-mRNA expression from lowest dose		Increased apoptosis and necrosis. Lesions in testis at 100 µg/l	
Miles-Richardson *et al.*, 1999	Fathead minnow (*Pimephales promelas*). 1.6 - 3.4 µg/l NP			Tendency to increase in ”severity score” based i.a. on Sertoli cell hypertrophy and lesions. This study had weaknesses		

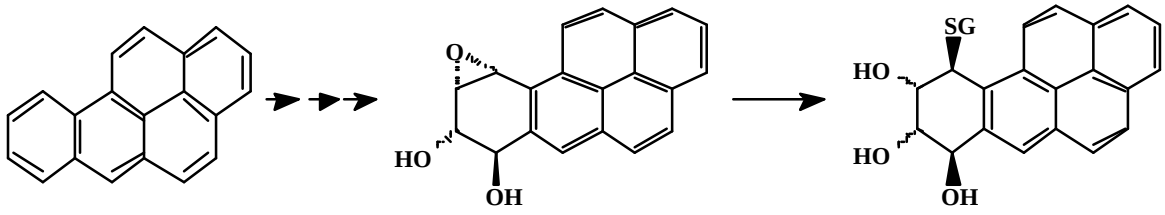
Schwaiger *et al.*, 2000	Carp (*Cyprinus carpio*). 1 - 15 µg/l NP					Effects on blood parameters. Enlarged erythrocytes, and possible anaemia from 10 µg/l
Shioda & Wakabayashi, 2000	Medaka (*Oryzias latipes*). 150 - 1500 µg/l BB; 6.6 - 66 µg/l NP					Reduced hatching fraction is pairs in which the male had been exposed to BP. Reduced successful fecundity at 6.6 µg/l NP
Tabata *et al.*, 2001	Medaka (*Oryzias latipes*). 0.1 - 100 µg/l NP		FSP (VTG + ZR + ?) induced at 0.1 µg/l (immunodetection). Measurable concentration at 100 µg/l		Abnormal gonadal development in two (of seven) individuals at 100 µg/l	Reduced survival at more than 50 µg/l. LC50 for larvae 130 µg/l, and 860 µg/l for mature fish
Thorpe *et al.*, 2001	Rainbow trout (*Oncorhynchus mykiss*). Immature fish. 2.4 - 24 µg/l NP		Induction of VTG above 6.1 µg/l			
Toft & Baatrup, 2001	Guppy (*Poecilia reticulata*). Nominally 100 - 900 µg/l OP	Reduced size and intensity of sexually attractive orange marks (from 100 µg/l)		Increased quantity of spermatozoa in ejaculate (from 100 µg/l). Variable results among groups.	Inhibition of testis growth (measured as GSI) from 100 µg/l	
Yadetie *et al.*, 1999a	Salmon (*Salmo salar*). 5 - 125 mg/kg NP		Induction of VTG and ZR at 25 mg/kg			Up-regulation of oestrogen receptor mRNA is liver at 25 mg/kg

2.5 Metabolism of foreign compounds

Many polar water-soluble foreign compounds (xenobiotics) are eliminated unchanged from the organism. Fat-soluble (hydrophobic) compounds, on the other hand, have to be transformed into more polar metabolites before the organism is capable of eliminating them. Fat-soluble substances will either simply remain in the fat or be resorbed by the kidney tubules or equivalent organs and will continue to recirculate for a very long time. The formation of polar metabolites can thus be regarded as the organism's defence mechanism against fat-soluble, and potentially damaging, foreign substances. A few exceptions, in which active (reactive) metabolites are created, are of interest from a toxicological and, in medicine, therapeutic, point of view (Rang *et al.*, 1999). The metabolism of foreign compounds takes place in two phases, as shown in Fig. 5 below.



Example:



Benzo[a]pyrene

(+) Anti-benzo[a]pyrene-7,8-diol-9,10-oxide

Glutathione conjugate of (+) Anti-benzo[a]pyrene-7,8-diol-9,10-oxide

Fig. 5. The two phases in the metabolism of foreign compounds

During the first phase, small polar groups are revealed or created by oxidative, hydrolytic and reductive processes. These phase 1 metabolites are often sufficiently polar to be directly eliminated in the urine or other aqueous phase, but a phase 2 reaction usually takes place prior to excretion. Most of the phase 2 metabolites, such as the glucuronides and the sulphate and glutathione conjugates, are more polar and water-soluble (exceptions: methylated and acetylated metabolites), and in fish these are readily excreted via the bile, kidneys or gills (Hayes, 1994). Even though foreign substances can be metabolised in many different tissues, the liver is undoubtedly the most important organ for the

metabolic transformation of foreign substances (Rang *et al.*, 1999). Many aquatic organisms are fully equipped with phase 2 enzymes, although these are not as active as in mammalian systems.

2.5.1 Phase 1 metabolism

In fish, phase 1 oxidation is primarily catalysed by cytochrome P450, which is a large family of isoenzymes. In fish, P450-dependent mono-oxygenase (MO) is similar in many ways to equivalent enzyme systems in mammals. However, there are certain unique features in aquatic organisms, for example that MO has a lower optimal temperature than its equivalent in mammals (Buchelli & Fent, 1995)

Nomenclature

The nomenclature of P450 is made up of an Arabic number that indicates the family, a letter for the sub-family, and a further Arabic number that signifies the gene (Nelson *et al.*, 1996).

Cytochrome P450

Cytochrome P450, a family of closely related haemoproteins with a mole weight of 50 kD, is mainly to be found in the endoplasmic reticulum of the liver, but is also found in other organs. These isoenzymes catalyse the general reaction:

$$NADPH + H^+ + O_2 + R\text{-}H \rightarrow NADP^+ + H_2O + R\text{-}OH,$$

where R-H represents the substrate, which may consist of alkanes, alkenes, aromatic rings or heterocyclic rings, and which acts as a site for the oxidation process. R-OH is the hydroxylised product. This reaction is called mono-oxygenation, and the P450 isoenzymes are therefore mono-oxygenases, since only one of the two atoms of oxygen is incorporated into the substrate (Rang *et al.*, 1999).

P450 induction

In fish and mammals, P450 can be induced by several different substances. This is to say that exposure to these substances leads to increased transcription of P450 mRNA, which is translated into protein. The protein is incorporated into the endoplasmic reticulum, where it performs its activity of increasing the metabolism of the foreign matter. The different P450 isoenzymes can be detected at three different levels in the induction response (Table 4):

Table 4. Detection of P450 isoenzymes at different levels of the induction response (Goksøyr,1995)

Level of detection	Probe	Assay
P450 mRNA	cDNA oligonucleotide	Northern Blotting; RT-PCR; *in situ* hybridisation
P450 protein	Antibody	Western Blotting; ELISA; Immunohistochemistry
P450 enzyme	Catalytic assay	Specific assay for each individual isoenzyme (e.g. EROD, AHH for CYP1A)

EROD: 7-ethoxyresorufin O-deethylase; AHH: aryl hydrocarbon hydroxylase; ELISA: enzyme-linked immunosorbent assay; RT-PCR: reverse transcriptase-polymerase chain reaction

It is important to note that at high concentrations, some inductors may inhibit the activity of P450. In such cases, the measurement of the specific enzyme activity may show less activity, while the amount of protein has not changed (Stegeman & Hahn, 1994). The induction response in fish may be modulated by any of a number of factors, such as sex, water temperature and stage in the reproductive cycle (Andersson & Førlin, 1992).

P450 as a biomarker

Various hepatic isoenzymes in the P450 family respond to different extents to foreign substances. CYP1A, for example, is strongly induced by exposure to organic contaminants such as PAHs and PCBs, while other groups of these isoenzymes are not induced (Foureman, 1989). It has also been shown that there is a high correlation between CYP1A induction and the level of PAH/PCB pollution, e.g. in wild fish (Stegeman & Hahn, 1994). P450 is also a very sensitive biological response parameter, and its response time to exposure is very short (Haux & Førlin, 1988). These factors have led to cytochrome P450-dependent mono-oxygenase becoming the most widely used biomarker for a large number of organic environmental toxins.

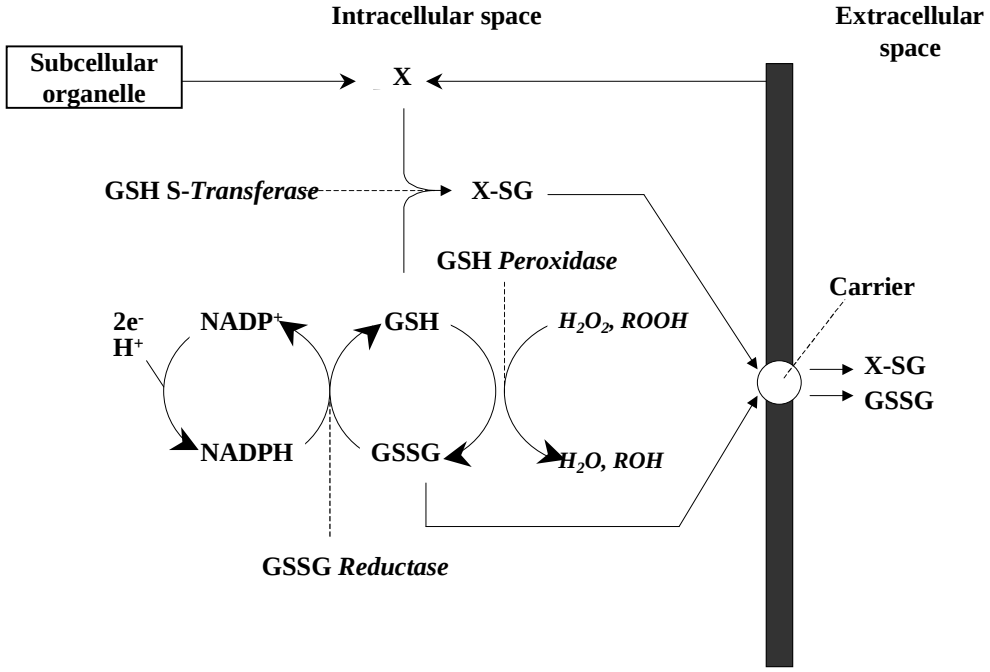


Fig. 6. Illustration of two important functions of glutathione – conjugate formation with foreign compounds and removal of reactive oxygen-intermediates. X, electrophile as substrate for glutathione S-transferase; X-SG, glutathione S-conjugate; ROOH, organic hydroperoxide.

2.5.2 Phase 2 reactions

If a molecule has a suitable "handle" (e.g. a hydroxyl, thiol or amino group) which it either already possessed or was given by a phase 1 reaction, it is receptive to conjugation, i.e. the addition of a substituent. The conjugate, which may be regarded as a harmless form of the foreign compound, is much more water-soluble than the original substance, and can be eliminated via the urine or bile. The groups that are most frequently involved in the formation of conjugates are glutathione, glucuronyl, sulphate, methyl, acetyl, glycyl and glutamyl (Rang *et al.*, 1999).

The following sections discuss only glutathione (GSH) and various enzymes involved in the synthesis and metabolism of glutathione. This detoxifying compound is emphasised because of its general importance in all cells and tissues. In addition to its importance in the metabolism of foreign substances, it has well-documented functions in a range of apparently unrelated biological processes such as protein and DNA synthesis, transport, enzyme activity, metabolism and cell protection. The

multifunctional properties of GSH are reflected in the growing interest in this little molecule by researchers in such diverse areas as enzyme mechanisms, macromolecule biosynthesis, intermediate metabolism, metabolism of foreign substances, radiation, cancer, oxygen toxicity, transport, immunological phenomena, endocrinology, environmental contamination and aging.

Two of the functions of GSH are of particular importance in the detoxification of exogenous and endogenous compounds:

Conjugation of glutathione: glutathione S-transferase
GSH reacts with a large number of foreign compounds of widely different chemical structures, forming what are known as GSH conjugates (Fig. 6). Substances with an electrophilic centre can often react directly with GSH. In other cases electrophilic centres are introduced via other reactions such as the P-450 system in the endoplasmic reticulum of the cells. The epoxide formed reacts with GSH. The interaction of foreign compounds with GSH may be spontaneous or catalysed by GSH S-transferases (GST). The GSH conjugates are often transformed into mercapturic acids via a series of enzyme reactions (Sies & Ketterer, 1988). The GSH conjugates and their breakdown products are non-toxic and polar, and can therefore be eliminated from cells. GST is inducible by pro-oxidants and/or electrophilic substances. These activate gene transcription via an antioxidant-respondent element (Hayes & McLellan, 1999).

Glutathione - the cells' antioxidant
Superoxide (O_2^{2-}) and hydrogen peroxide (H_2O_2) are formed naturally in large quantities in biological systems. Such reactive oxygen compounds are extremely toxic, as they react with the membrane lipid of the cells. In order to keep the level of such reactive oxygen intermediates at a minimum, the cells have developed enzyme systems (GSH peroxidase, Fig. 6), that use GSH as a substrate and transform the intermediates into water. As a consequence of the effects of GSH peroxidase, GSH is oxidised to glutathione disulphide (GSSG), which in turn is reduced back to GSH with the aid of glutathione disulphide reductase (GSSG reductase, Fig. 6).

Glucose-6-phosphate dehydrogenase - production of NADPH
Glucose-6-phosphate dehydrogenase (G6PDH) catalyses the reaction:
D-Glucose-6-phosphate + $NADP^+$ ⇆ D-glucono-δ-lactone-6-phosphate + NADPH

G6PDH catalyses one of the reactions in the "pentose phosphate pathway" that produces NADPH, which is a carrier of chemical energy in the form of reducing agents. NADPH is used in a large number of biochemical reactions, e.g. of GSSG reductase (Fig. 6). In oxidative stress, which may be caused by the metabolism of foreign compounds, the consumption of NADPH may increase, which may induce G6PDH to increase the synthetic capacity of NADPH. Elevated G6PDH activity can thus be a bio-indicator of increased oxidative stress (Winzer *et al.*, 2001; van Noorden *et al.*, 1997).

2.6 Alkylphenols and offshore oil and gas production

Historically, large quantities of alkylphenol ethoxylates (APE) have been used in offshore petroleum production, both as detergents for platform washing purposes and as additives in the production process. Blackburn *et al.* (1999) suggest that discharges on the British continental shelf may have risen to as much as 100 tonnes a year per platform. The use of APE is now forbidden in the Norwegian sector of the North Sea (letter from SFT to all operators on the Norwegian shelf, dated 31.08.98). The Danish and UK authorities are also working on phasing out APE in their own sectors of the North Sea (Lye, 2000).

In spite of the slow breakdown of long-chain alkylphenols, these substances are fully biologically degradable, and when APE is phased out the potential environmental problems created by these substances will disappear within a relatively short period. However, even though the most serious environmental threat from the alkylphenols will disappear with the phasing out of APE, the problems associated with discharges of long-chain alkylphenols from petroleum production will remain.

Alkylphenols are a natural component of crude oil, and as a result of their solubility in water a high proportion will be found in the aqueous phase after water/oil separation (see Introduction). The

alkylphenols are typically found in concentrations of 0.6 - 10.0 ppm (mg/l) in produced water. Some 80% of the total consists of the most water-soluble alkylphenols, phenol and cresol (C_1). Of the remaining components, the higher alkylphenols from C_4 to C_7 occur in low concentrations of 2 - 237 ppb (µg/l) (Brendehaug *et al.*, 1992). We know very little about the fate of these substances in the marine offshore environment. There are no empirical data on concentrations in the sea around North Sea offshore installations, and we are therefore forced to use models when assessing the levels to which fish may be exposed. Rye *et al.*, (1996) simulated the spread of alkylphenol discharges from produced water from the Halten Bank, and calculated the likely uptake by pelagic fish using a model. The point of departure of the model was the dissemination of total alkylphenol discharges from two platforms, and it included biological response estimates (BCF and constants for uptake and elimination). The calculations showed that the body burden of alkylphenols in the fish modelled would lie in the area of 0 - 10 ppb (Rye *et al.*, 1996).

Given the lack of real field data, we have used the model values indicated in Rye's article as a point of departure for the dose regimes in our exposure experiments. We have also tried to take into account the fact that produced water contains a wide range of different alkylphenols, by using a mixture of four components with different chain lengths, from butylphenol to heptylphenol. The intention of the tests was to dose the fish to a body burden within the range of Rye's estimates. We thus found that 5 ppb of each of the four alkylphenols ought to correspond to a fairly realistic dose.

2.6.1 Bioaccumulation of alkylphenols

Alkylphenols are a highly diverse group of substances in terms of their physico-chemical properties. The water-solubility of phenol and the short-chain alkylphenols are high, but falls drastically with increasing chain length and thus increasing hydrophobicity. Table 5 presents an overview of three important physico-chemical properties of importance for the behaviour of these substances in the environment. The data described in Table 5 closely match general evaluations of the bioconcentration ability of these chemicals, as described by Mackay & Fraser (2000), who write that chemicals with a log K_{ow} = 4 will probably have a BCF of around 500. Butyl- to heptylphenols have been shown experimentally to have a log K_{ow} 3.04 to 4.0, and BCFs from 118 to 578 have been found (Table 5). A number of studies on rainbow trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*) have shown that octyl- and nonylphenol are taken up rapidly by the organism, via exposure both in the water (Lewis & Lech, 1996); Meldahl *et al.*, 1996; Arukwe *et al.*, 2000; Ferreira-Leach & Hill, 2001) and in food (Thibaut *et al.*, 1998; Arukwe *et al.*, 2000). The phenol group (aromatic hydroxyl group) makes the alkylphenols directly available to phase 2 enzymes (see section 3.2.2) which means that the alkylphenols are relatively rapidly metabolised (their half-life in trout and salmon is about 20 hours). This is confirmed by the above studies, which show that the majority (>50%) of the metabolites are excreted via the bile and the faeces in the form of glucuronides of intact alkylphenols. Some phase 1 metabolism also occurs, particularly ω and ω-1-hydroxylation of the alkyl chain. It is not yet known which enzymes are responsible for this metabolism, but P450 CYP3A has been proposed as a candidate by several authors (Thibaut *et al.*, 1998; Arukwe *et al.*, 2000). Uptake studies show that alkylphenols accumulate preferentially in the bile, digestive system and liver, but they also show that alkylphenols can pass the blood-brain barrier and be taken up in the brain.

Arukwe *et al.* (2000) have compared tissue distributions of nonylphenol in salmon following two different exposure regimes, via the water and via food. They found that dosing in the water results in a more regular distribution throughout the body than oral dosing, where nonylphenols are concentrated around the digestive system to an even greater extent.

An important reference for our studies is Tollefsen *et al.* (1998), who studied the uptake and excretion kinetics of heptylphenol in cod (*Gadus morhua*). They found that with exposure via the water the alkylphenols followed a single-compartment first-order kinetics, with a half-life of around 13 hours. This agrees very well with our studies of the uptake and excretion of heptylphenol in cod that had been dosed via oral exposure. We found that heptylphenol followed a two-compartment model, with an α-phase half-life of 11 hours and a ß-phase half-life of 57 hours (Meier *et al.*, in prep.).

In fish, the biological uptake of chemicals with log K_{ow} = 4 mainly takes place via the water (theoretically 20 times as fast as uptake via food), while for more hydrophobic substances with log

K_{ow} = 6, the situation is the opposite, with uptake via food being more important (Mackay & Fraser, 2000). As far as the alkylphenols are concerned, this means that bioaccumulation takes place primarily via uptake through the gills and skin. The rapid metabolism of nonylphenol that has been demonstrated suggests that these substances will probably not be biomagnified through the food chain. However, we know little about the metabolism of these compounds in organisms that belong to the lower end of the food chain. It is possible that they, as is the case with PAH components, have a poorer capacity to metabolise alkylphenols than fish, and that biomagnification may thus occur at these lower steps. Food may therefore be an important source of exposure for fish.

Our decision to expose fish via their food in our experiments is due to the fact that long-term exposure via the water would cause practical problems. We use large fish and large enclosures. The fish require a continuous supply of large quantities of water. A large-scale exposure experiment would thus involve a large consumption and major discharges of alkylphenols. Long-term exposure in the water is thus not practical.

On the basis of the literature and our own uptake studies, we have found that alkylphenols are rapidly taken up via the stomach and the gut. It can be discussed whether oral exposure will mean that alkylphenols will be distributed in the body to a lesser extent and thus possibly cause fewer effects, but nothing suggests that oral exposure will lead to a rise in bioaccumulation and thus an overestimation of the effects compared to exposure via water; if anything, probably the opposite will be the case.

Table 5. Selection of physicochemical properties of alkylphenols which may have relevance for their environmental fate. Aqueous solubility, the logarithm of the water/octanol partitition coefficient (K_{ow}) and the bioconcentration factor (BCF). The overview is from Shui *et al*. (1994) and Servos (1999)

	Water solubility (mg/l)	**Log K_{ow}**	**BCF in fish**		
Phenol	67000-93325	1,46-1,6	17-158	Div. fish	Servos, 1999
p-Cresol	1800-53000	1,62-2,06	-		
4-Ethylphenol	5000	2,39-2,58	-		
4-n-Propylphenol	1278	3,18-3,20	-		
4-sec-Butylphenol	-	2,1	37	Salmon	McLeese *et al.*, 1981
4-tert-Butylphenol	580-1848	3,04-3,31	118	Golden Ide	Freitag *et al.*, 1985
4-Hexylphenol	-	3,60	346	Salmon	McLeese *et al*., 1981
4-Heptylphenol	-	4,00	578	Cod	Tollefsen *et al*., 1998
4-Octylphenol	12,6	4,12	261	Killifish	Tsuda *et al*., 2001
4-Nonylphenol	5,4-7	4,20-6,36	75-1250	Div. fish	Servos, 1999

Definitions of important concepts (Mackay & Fraser, 2000)

The **Bioconcentration Factor (BCF)** is the relationship between the concentration in the fish and the concentration in the water; and describes only uptake via gills and skin.
The **Bioaccumulation Factor (BAF)** also describes the relationship between the concentration in the fish and the concentration in the water, but this concept includes all potential exposure routes, and BAF may be regarded as the sum of BCF and uptake via food.
The **Biomagnification Factor (BMF)** is the relationship between the concentration in an organism and the organism's diet. Biomagnification can be regarded as a special form of bioaccumulation and it describes the ability of a chemical to rise in concentration up through the food chain.

3 Materials and Methods

3.1 Experimental design

Experiment 1.
This experiment used 300 two-year-old first-time spawning cod. The fish came from a strain of Norwegian-Arctic cod produced at the Institute of Marine Research's station in Øygarden (Parisvatnet) near Bergen. The fish were transported from Tveit (Tysnes) to the Institute of Marine Research in Bergen in September 1997 and were allocated to a control group and two experimental (exposure) groups in separate 15 m^3 outdoor tanks (100 fish per tank). The experiments started on October 10 1997 and lasted until spawning had come to an end on April 7 1998. The fish tanks were supplied with water from a great depth (100 m) and the water temperature remained stable at 8 – 10 ^oC 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.

The exposed groups were administered a mixture of 4-tert-butylphenol (C_4), 4n-pentylphenol (C_5), 4n-hexylphenol (C_6) and 4n-heptylphenol (C_7). The alkylphenols were dissolved in soya oil and mixed into the feed (wet pellets; herring:fishmeal, 60:40%) to concentrations of 1 or 100 ppm of each compound. This was equivalent to theoretical doses of 5 or 500 ppb of each alkylphenol per fish per day; 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 a realistic value and the high dose a positive control. Monthly samples of 20 fish from each group were taken. Feeding stopped on January 20 and the fish that remained were not fed during the spawning season. The fish spawned naturally in the three 15 m^3 tanks, and the eggs were collected every day from strainers mounted on the water outlets. During spawning seven female fish and five to seven males were left in each tank (the other fish had been sacrificed in the course of the monthly sampling).

Experiment 2.
This was carried out in autumn 1999 as a follow-up of experiment 1. The exposure regime was repeated using the same mixture of the four alkylphenols, but was increased to five different doses: 5 ppb, 0.5 ppm, 5 ppm, 10 ppm and 20 ppm of each alkylphenol relative to body weight. An oestrogen-exposed positive control group was also included (dose: 5 ppm 17ß-oestradiol). Unlike experiment 1, the fish were exposed to the chemicals only once a week, i.e. the fish were administered 5, 500, 5 000, 10 000 or 20 000 µg/kg body weight once a week. This experiment was carried out on two-year-old first-time spawning cod from the same strain as used in experiment 1. The cod were fetched in August 1999 from Austevoll Aquaculture Research Station to Bergen, where they were kept in large outdoor tanks until the experiment started in November.

The fish were divided into seven groups of 40 and transferred to 10 m^3 indoor tanks. During the experiment, the lighting was controlled to follow the natural light regime. From August until the experiment started, the fish were fed commercial fish feed (dry pellets from Felleskjøpet AS, 10% lipid).

In order to achieve better control of actual individual exposure than in experiment 1 (when the fish were fed in groups), the fish were fed individually via a probe inserted directly into the stomach. The alkylphenols and oestradiol were dissolved in 1,2-propandiol 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; the lipid content and fatty acid profile of the paste were identical that of the dry pellets' original composition). The paste was sucked up into a plastic tube with the aid of a piston and then put directly into the stomach of the anaesthetized fish. Both the fish and the paste were weighed immediately before the dose was administered. The fish were exposed once a week for four weeks. One week after the first exposure, 20 fish from each group were removed for analysis. The remaining fish were given three further doses and were sacrificed a week after the final dose.

The fish in experiment 2 thus received exactly the stated dose, and a week passed before they were given the following dose. The real concentration in the body will therefore probably be lower than in the first experiment as a result of the metabolism and excretion of the substances between doses. In experiment 1 the intention was to achieve a daily "body burden" equivalent to the stated doses. The fish in experiment 1 were fed three times a week with a quantity of feed equivalent to a daily ration of 0.5% of body weight, and thus 5 or 500 ppb alkylphenol.

3.1.1 Sampling

The cod were anaesthetised 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 separated, 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 removed by scalpel, while urine and bile (for another metabolism study) were collected by syringe. All samples were frozen in liquid nitrogen and stored at -80 ^{o}C. The liver samples for enzyme analysis were stored in liquid nitrogen until analysis. Tissue samples for histology were fixed as described in section 4.2.7.

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 Nash *et al.*, (2000). The female fish were analysed for 17ß-oestradiol (E2) and testosterone (T), and the males for testosterone and 11-ketotestosterone (11KT) (and the male positive controls for E2).

3.2.2 Vitellogenin

The biomarker vitellogenin (VTG) was analysed by a means of a quantitative ELISA technique developed at the Institute of Marine Research, Austevoll Aquaculture Station (Meier *et al.*, in prep.). An assay was performed for female fish (high VTG content, >10 mg/ml) and a more sensitive assay for male fish (low VTG content, detection threshold 0.1 µg/ml).

Brief description of procedure: for each analysis, VTG from a freshly thawed standard sample of cod blood plasma (immature fish, induced with oestrogen) was purified in an ion-exchange column (Silversand *et al.*, 1993) and quantified by UV-absorption measurements at 280 nm (Norberg & Haux, 1988). Nunc Maxisorp 96-cell trays were coated with 150 ng VTG standard and incubated with primary VTG antibody (polyclonal rabbit anti-cod VTG) and plasma. After rinsing, the plates were incubated with secondary antibody and developed using an alkaline phosphatase assay. Plasma for female fish was diluted (100 to 20 000 times, depending on the VTG content), and incubated directly in the wells together with VTG standard and anti-VTG. Plasma from males was diluted 1:50 and pre-incubated with anti-BVTG for 24 h before being incubated together with VTG standard. Pre-incubation significantly increased the sensitivity, as has already been pointed out in several studies of VTG (Sherry *et al.*, 1999; Tyler *et al.*, 1999), but this process is also more labour intensive. The method was therefore only used for male fish, which have lower levels of VTG and thus require a more sensitive analysis.

3.2.3 Enzyme analyses

Preparation of post-mitochondrial fraction

Pieces of frozen liver were weighed, buffer was added (1:5 w/v) and the mixture was homogenised by a Potter-Elvehjem Teflon-glass homogeniser. The homogenate was centrifuged at 12 000 g for 20 min at 4 ^{o}C. The supernatant was stored at –80 ^{o}C until protein and enzyme activity were analysed. For the determination of EROD activity, glutathione S-transferase and glucose-6-phosphate dehydrogenase, the following homogenisation buffer was used: 0.1 M sodium phosphate, pH 7.4; 0.15 M KCl; 1 mM ethylenediamine tetraacetic acid (EDTA); 1 mM dithiothreitol (DTT) and 10% glycerol. For the determination of glutathione reductase activity, 0.1 M sodium phosphate, pH 7.4, was used as the homogenisation buffer.

Measurement of enzyme activity in postmitochondrial supernatant
Glutathione reductase was determined according to a method described by Cribb *et al.* (1989), using 0.1 mM DTNB, 12 mM NADPH and 3.25 mM GSSG. Absorbance at 405 nm with reference 750 nm was measured every 30 seconds for three min. at room temperature.

Glutathione-S-transferase was determined according to a method of Habig *et al.* (1974), in which conjugate formation is measured as a function of time. This assay used 2 mM CDNB dissolved in DMSO and 1 mM GSH, and measurements were made at 340 nm.

Reducing capacity was measured as the enzyme activity of G6PDH and 6PGDH, in U/mg protein (Deutsch, 1974). The rate of rise of absorbance at 340 nm (in the presence of glucose-6-phosphate and NADP) was a measure of the enzyme activity.

EROD assay
The catalytic activity of cytochrome P450 1A (CYP1A) was measured by 7-ethoxyresorufin-*O*-deethylase (EROD) assay (Nielsen *et al.*, 2000). CYP1A catalyses the *O*-deethylation of 7-ethoxyresorufin, with resorufin as the product. Resorufin is measured fluorometrically (E_x = 535 nm, E_m = 585 nm). Quantification was obtained by adding a known amount of resorufin as an internal standard.

Protein determination
Protein was measured by the Coomassie ® Plus Protein Assay Reagent from Pierce (Rockford, IL, USA) with bovine gammaglobulin (BGG) as standard (Bradford, 1976).

3.2.4 Immune blotting (Western Blotting)

CYP1A and CYP3A were separated on polyacrylamide gel and transferred to a nitrocellulose membrane by electrophoresis (Towbin & Staehelin, 1979). Non-specific binding was blocked with 5% dried milk and the membrane was incubated with primary antibody for 1 h. CYP1A was detected using 1-12-3 mouse monoclonal antibody (1:500) (Park & Miller, 1986), while for CYP3A polyclonal rabbit anti-CYP3A (1:1000) was used (Celander *et al.*, 1996). After rinsing the membrane was incubated with peroxidase-coupled secondary antibody against anti-mouse IgG or anti-rabbit IgG respectively (1:1000). The signal was detected using ECL detection reagent from Amersham Pharmacia Biotech.

3.2.5 Quantification of total free and reduced glutathione

Total free and reduced glutathione were determined according to a method of Svardal *et al.* (1990), whereby 50 - 100 mg aliquots of frozen liver were homogenised in ice-cold 5% sulphosalicylic acid containing 50 µM DTE (1:40 w/v) and centrifuged at 10 000 g for 5 min at 5 ^{o}C. Samples were withdrawn from the supernatant for determination of total free glutathione (reduced form + disulphide + dissolved mixed disulphide) and reduced glutathione (GSH). $NaBH_4$ was used to reduce oxidised glutathione and monobromobimane for the derivatization of free -SH groups. After derivatization, the thiol-bimane adducts were quantified using reverse-phase ion-pair liquid chromatography and fluorescence detection.

3.2.6 Liver index and gonadosomatic index

The liver index (hepatosomatic index) was calculated as

$$HSI = \frac{(LW \cdot 100)}{(W)}$$

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

The gonadosomatic index (GSI) was defined as:

$$GSI = \frac{(GW \cdot 100)}{(W\text{-}GW)}$$

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

3.2.7 Histology and morphology

Samples from selected groups were studied for signs of histological changes. From experiment 1: female fish from the final sample (January) from the control group, 0.02 ppm and 2 ppm. From experiment 2: female fish from the control group, 0.02 ppm, 2 ppm and 80 ppm, and males from the control group, 0.02 ppm and 80 ppm. Also in the second sampling, histological samples were taken only from the final samples (after five weeks exposure).

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 sodium dihydrogen phosphate and 0.0461 M disodium hydrogen phosphate 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 dimethylarsonate (sodium cacodylate) for histological studies. Tissue samples from male fish were only fixed in modified Karnovsky fixative.

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).

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

Potential fecundity was measured as (Thorsen & Kjesbu, 2001):

Potential fecundity = $2.139 \cdot 10^{11} \cdot X^{-2.7} \cdot$ ovary weight, where X is the mean follicle diameter

Time to spawning
Time to spawning was calculated as $y = 3.33 \cdot 10^{6} \cdot x^{-1.817}$ (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 & Kjesbu, 2001).

Table 6. Variables for female fish

Variable	Description
Potential fecundity	Number of maturing oocytes present in the ovary at the time of the sampling
Potential relative fecundity	Potential fecundity / somatic weight (in grams)
Potential fecundity condition factor	Potential fecundity / $(\text{length})^3$
G1 (”leading cohort”)	The 10 largest oocytes in a sample from the ovary
G1-diameter	Mean diameter of the 10 largest follicles
Time to spawning	Estimated number of days from the time of the sampling to the onset of spawning

The volume fraction of spermatogonia, spermatocytes, spermatides and spermatozoa was estimated by simple point counting (Howard & Reed, 1998, describe the principles of this method). A raster of 3x4=12 points was placed in the ocular of the microscope. Counting was done with a 40x objective, and a 10x ocular. Several pieces of testis were embedded in a single block, and a suitable number of fields to ensure counting of a total of about 240 points per tissue sample were selected on a systematic random basis. The microscope’s coordinate stage was used to place the selected fields. The volume fraction of spermatogonia, spermatocytes, spermatides, spermatozoa and interstitial tissue were estimated as:

$$estV_{cell\,type,testis} = \frac{\sum_{i=1}^{n} P_{cell\,type}}{\sum_{i=1}^{n} P_{testis}}$$, where $P_{cell\ type}$ is the number of points falling on a particular type of cell in field *i*, P_{testis} is the no. of points falling on testis tissue in field *i*, and *n* is the number of fields counted.

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

$$Total\ amount_{cell\ type} = estV_{cell\ type,\ testis} \ x\ \ testis\ weight$$

No distinction was made between spermatocytes A and B. It was not possible to distinguish between spermatozoa in spermatocysts and spermatozoa in the lumen (i.e. not spermiated and spermiated spermatozoa) by our techniques. The cell type “spermatozoa” is therefore the sum of spermiated and unspermiated spermatozoa.

3.2.8 Statistical analyses

All the data (except vitellogenin in male fish (which contained zero values) and the histological data) were log-transformed before analysis in order to obtain homogeneous variance. Statistical differences between the control group and the two exposed groups were tested by unpaired t-test. Significance levels are given in the figure texts. The statistical analyses were all performed using the Statview software (SAS Institute, Cary, NC, USA).

4 Results

Dosages in all the following figures are shown in terms of total dose of alkylphenol, i.e. the sum of butylphenol, pentylphenol, hexylphenol and heptylphenol.

4.1 Experiment 1: Female fish

4.1.1 Oestrogen

17ß-oestradiol was measure in blood plasma in all the female fish from all samples in the experiment. Fig. 7 shows the 17ß-oestradiol levels of each group. As we can see, the oestradiol level falls significantly in the exposed groups and the fall begins as early as November, when the gonadal maturation process (vitellogenesis) gets well under way.

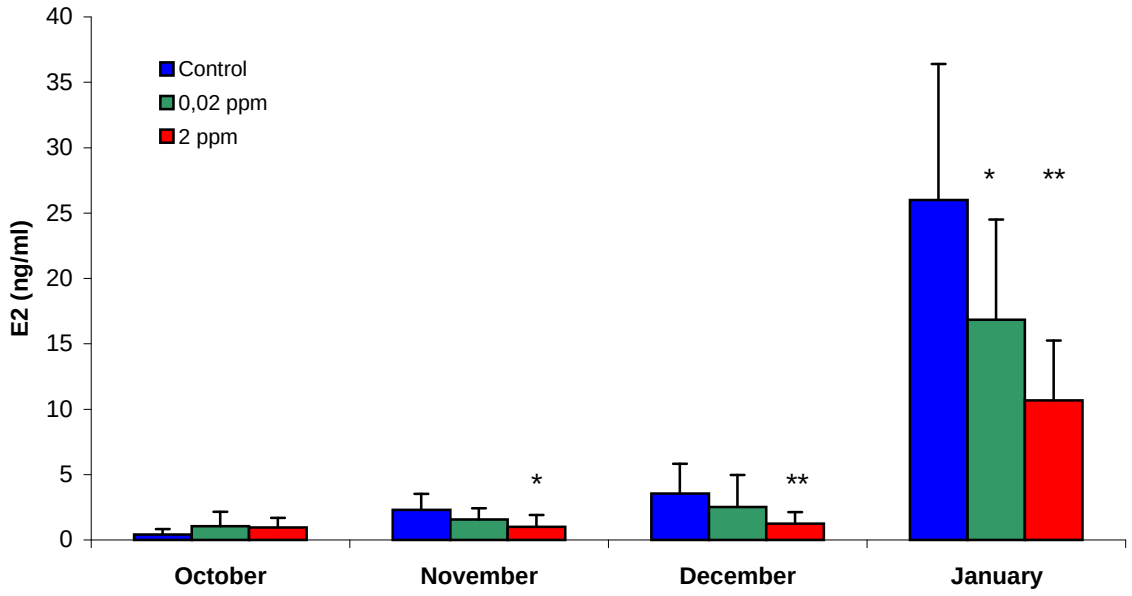


Fig. 7. 17ß-Oestradiol in plasma (ng/ml) from control, low exposed (0,02 ppm) and high exposed (2 ppm) female fish. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), * p≤0,05, ** p≤0,01.

4.1.2 Vitellogenin

The liver is the site of synthesis of the proteins that make up most of the egg-yolk mass (vitellogenin, VTG) and the eggshell (zona radiata proteins, ZRP). This process of protein synthesis is induced when 17ß-oestradiol is transported in the blood from the ovaries to the liver and binds to receptors in the cytoplasm of the hepatocytes. The liver almost exclusively synthesises vitellogenin and egg-shell proteins in this period. These are secreted into the blood and transported to the ovaries, where they are taken up by, or deposited around, the growing oocyte. Vitellogenesis can be artificially induced in males and young fish by injecting 17ß-oestradiol. Vitellogenesis can also be influenced by hormone mimics (Jones *et al.*, 2000). In females, no clear relationship between group VTG levels and alkylphenol dose could be observed (Fig. 8). In the January sample the exposed groups showed a certain tendency to fall, but the drop was not statistically significant.

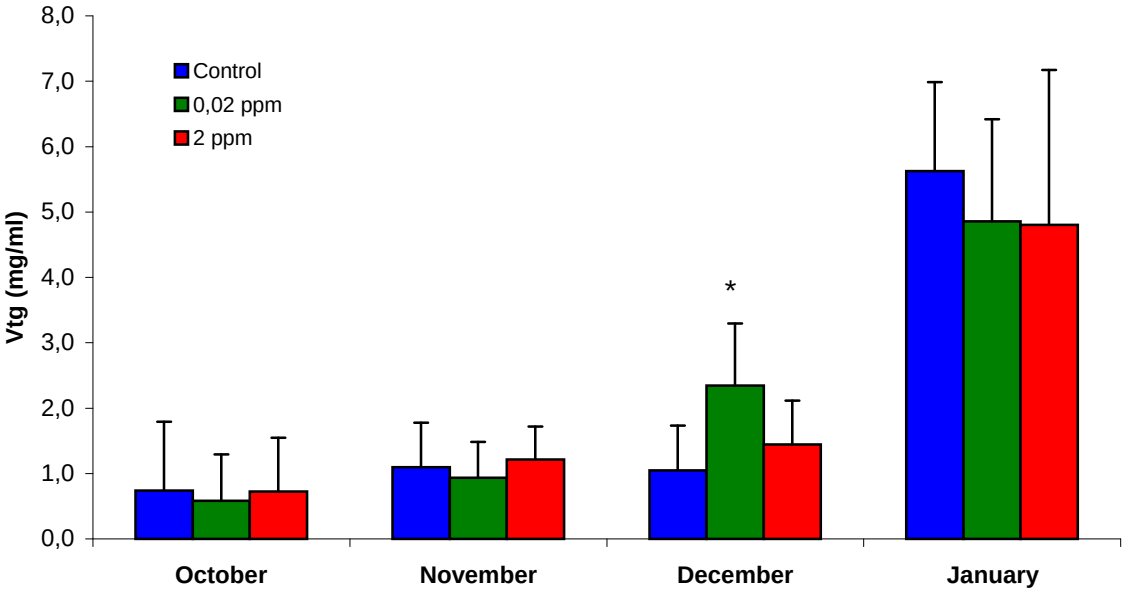


Fig. 8. Vitellogenin in plasma (mg/ml) from control, low exposed (0,02 ppm) and high exposed female fish. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), *p≤0,05.

4.1.3 Testosterone

The level of testosterone in female fish rose significantly at first (October sampling) in the two exposed groups, before becoming more similar to that of the controls later in the season (Fig. 9).

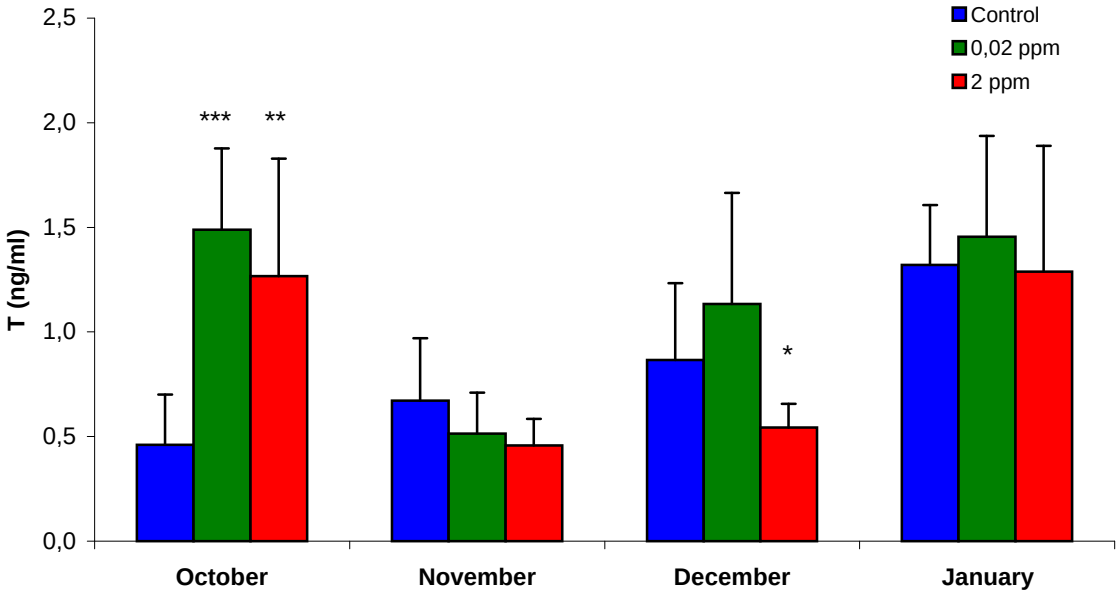


Fig. 9. Testosterone in plasma (ng/ml) from control, low exposed (0,02 ppm) and high exposed (2 ppm) female fish. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), *p≤0,05, **p≤0,01, ***p≤0,001.

4.1.4 Gonadosomatic index

The gonadosomatic index was calculated for all groups from all samples (Fig. 10). In the December and January samples the index showed a tendency to fall, but the decrease was not significant due to high variance.

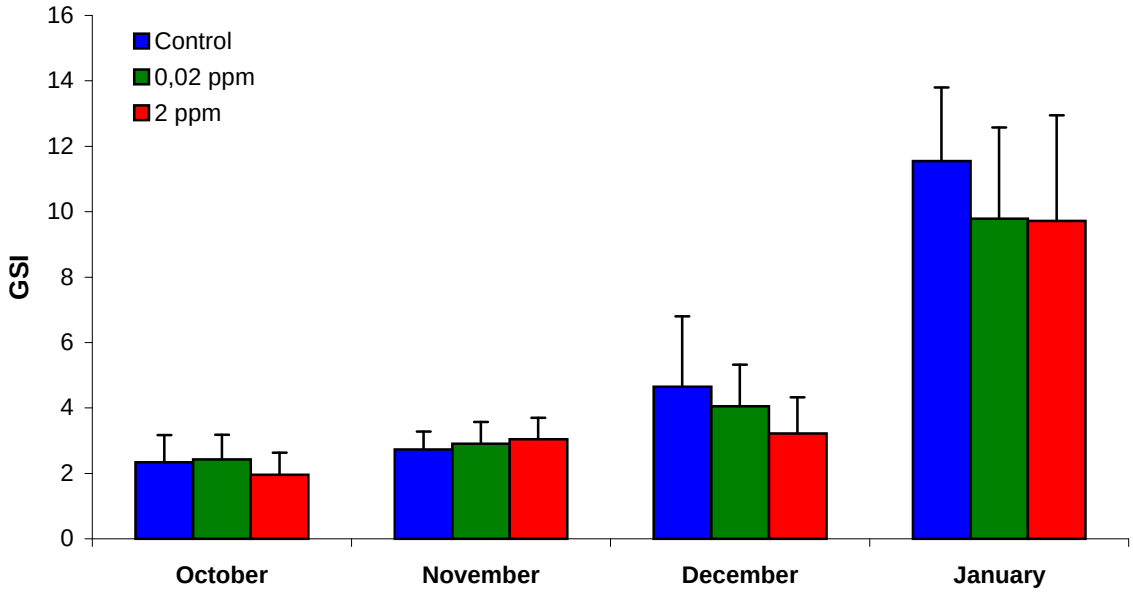


Fig. 10. Gonadosomatic index (GSI) for female fish: control, low exposed (0,02 ppm) and high exposed (2 ppm). Mean values ± standard deviation.

4.1.5 Potential fecundity and oocyte size

In the first experiment no significant effects on variables related to potential fecundity or oocyte size were found. Fig. 11 and 12 show potential relative fecundity and time to spawning. Differences between groups are not significant. Note, however, the trends in the material, which are identical to the trends in experiment 2, in which significant differences were found.

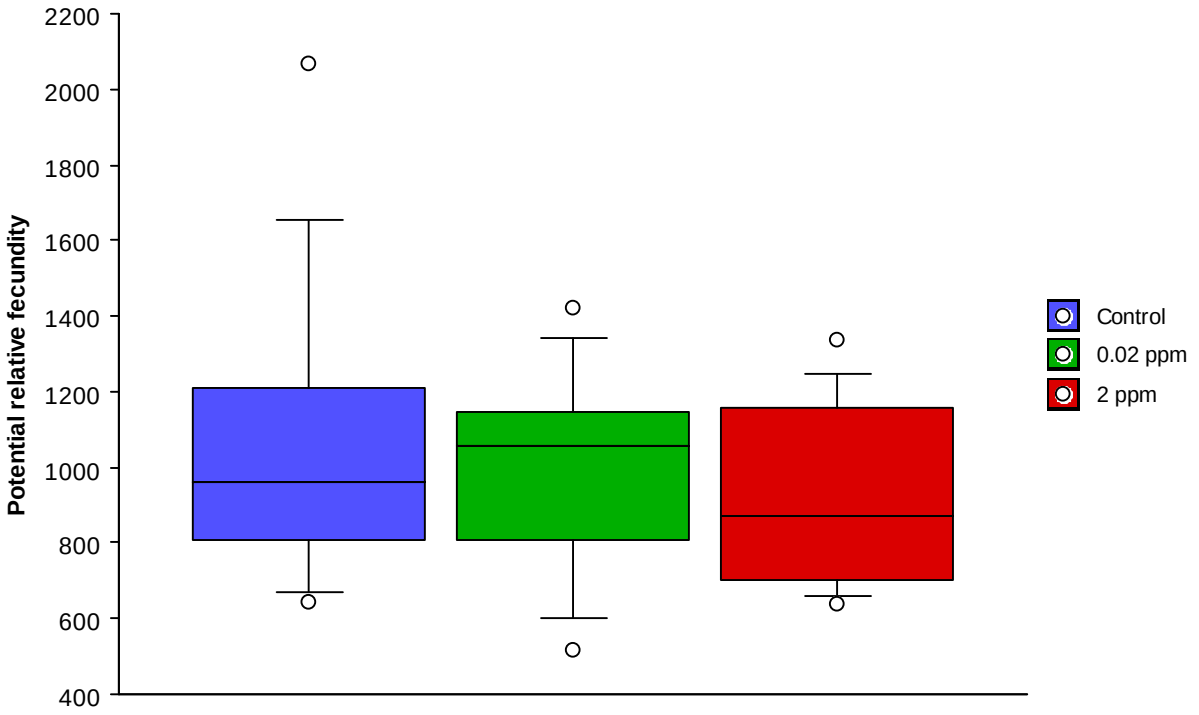


Fig. 11. Potential relative fecundity in the first experiment. There are no significant differences between the groups. Boxplot: median (line), 25-75 percentile (box), 10-90 percentile (vertical line) and all observations beyond 10 and 90 percentile as points.

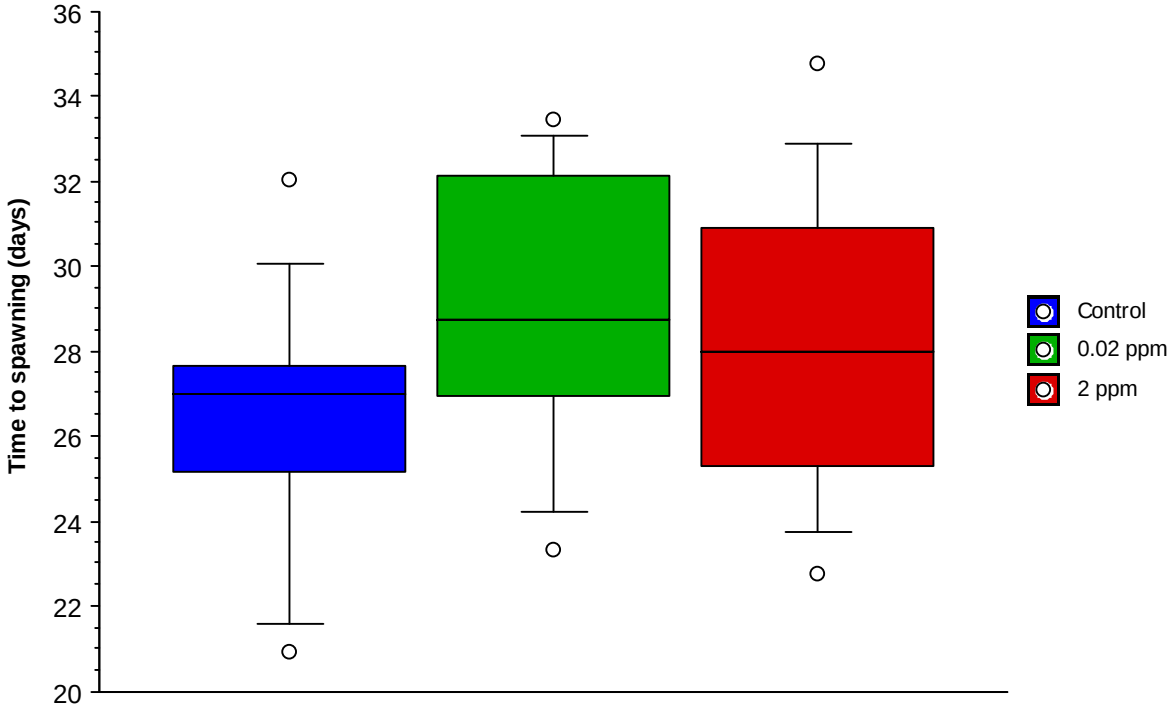


Fig. 12. Time from sampling to spawning (days). There are no significant differences between the groups. Boxplot: median (line), 25-75 percentile (box), 10-90 percentile (vertical line) and all observations beyond 10 and 90 percentile as points.

4.2 Experiment 1: Males

4.2.1 Testosterone

Like the females, the males tended to display higher testosterone levels at the beginning of exposure, but this was not statistically different in either exposure group (Fig. 13). In November there was a clear drop in testosterone levels. In December and January there were no statistically significant differences between the exposed groups and the controls.

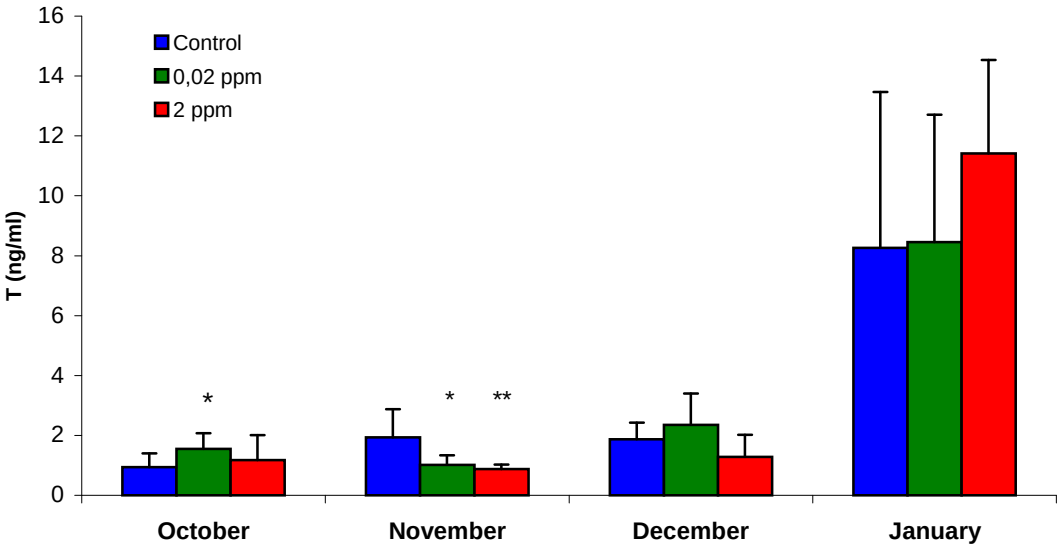


Fig. 13. Testosterone in plasma (ng/ml) from control, low exposed (0,02 ppm) and high exposed (2 ppm) male fish. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), *p≤0,05, **p≤0,01.

4.2.2 11-Ketotestosterone

11-Ketotestosterone is involved in spermatogenesis. Early in the season there was a fall in this hormone (November sample), which was most marked with the highest dose (Fig. 14). Further into the experiment, no significant differences between the exposed groups and the controls could be observed.

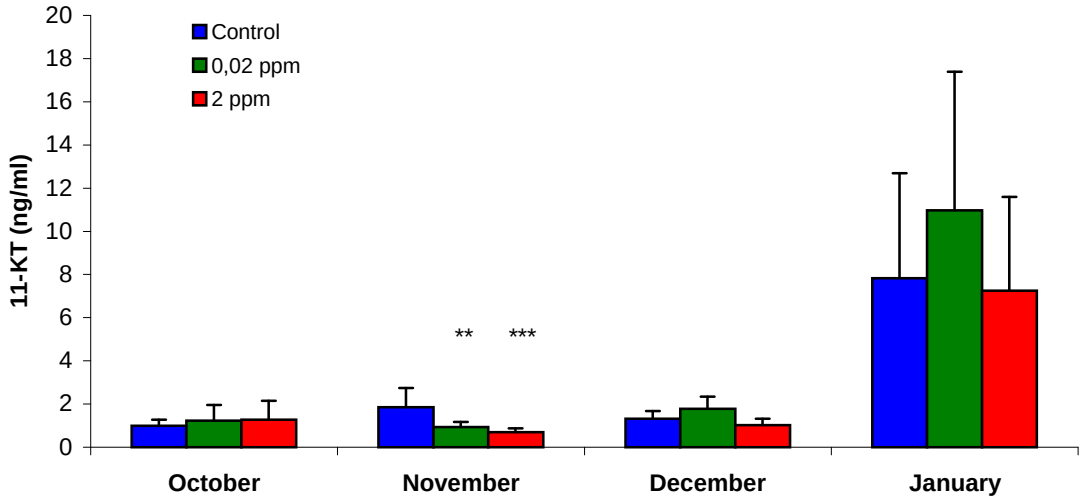


Fig. 14. 11-Ketotestosterone in plasma (ng/ml) from control, low exposed (0,02 ppm) and high exposed (2 ppm) male fish. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), , **p≤0,01, ***p≤0,001.

4.2.3 Vitellogenin

Under normal conditions the level of vitellogenin in male fish is very low, but males have the ability to synthesise VTG when they are exposed to oestrogen or oestrogen mimics. Fig. 15 shows that VTG in the two exposed groups increased in all samples, but as a result of wide individual variation in the response this effect was only statistically significant in the October sample.

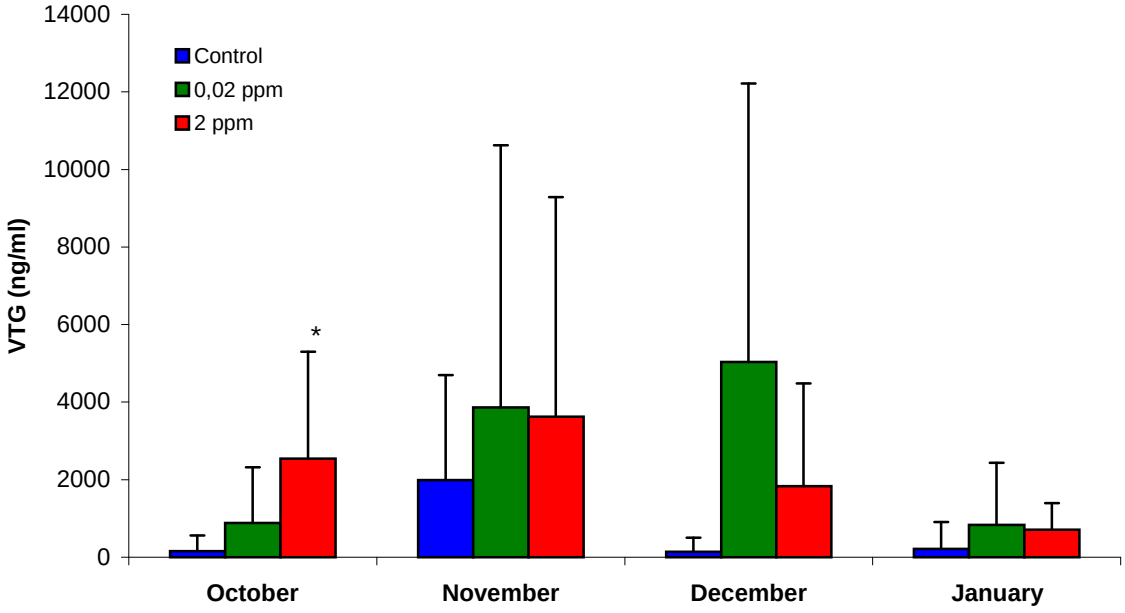


Fig. 15. Vitellogenin in plasma (ng/ml) from control, low exposed (0,02 ppm) and high exposed male fish. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), *p≤0,05.

If we consolidate the corresponding groups from all samplings and distribute the individuals by interval on the basis of the VTG level response, we see that the number of individuals with high VTG increased in the exposed groups relative to controls, and that the response was greatest in the group given the highest dose of alkylphenols.

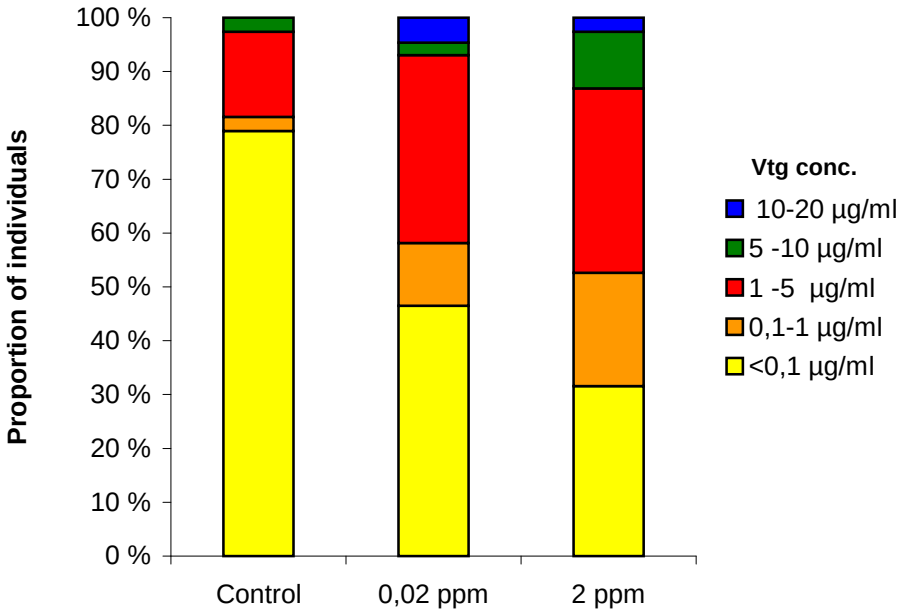


Fig. 16. Interval division of VTG-levels in plasma from male fish from all samplings. (Control, n=38. Low dose, n=41. High dose, n=38).

4.2.4 Gonadosomatic index

The GSI in the exposed groups from individual samplings is shown in Fig. 17. No significant differences were found.

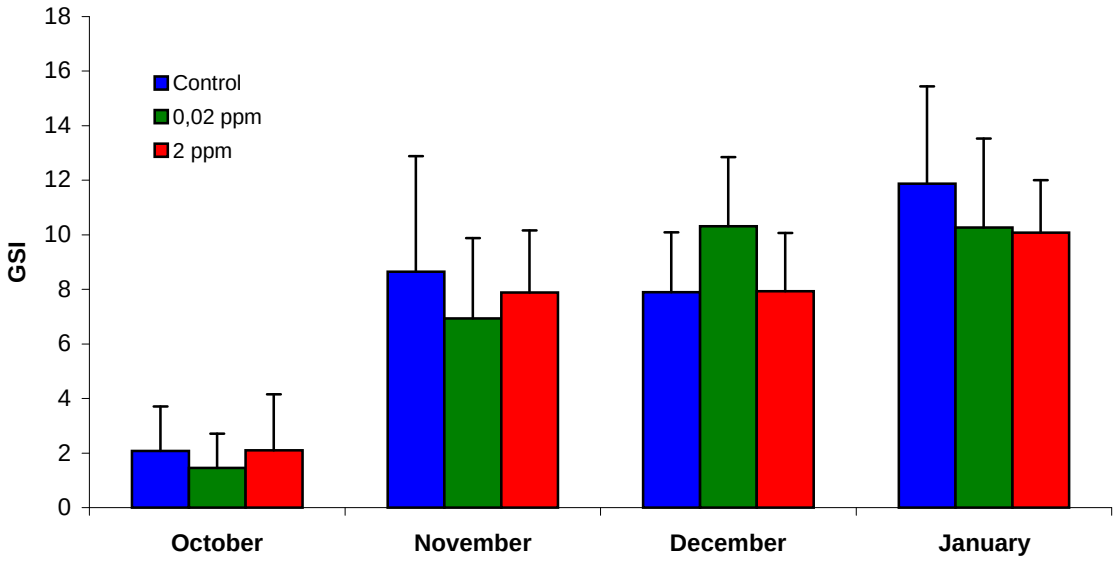


Fig. 17. Gonadosomatic index (GSI) for male fish: control, low exposed (0,02 ppm) and high exposed (2 ppm). Mean values ± standard deviation.

Experiment 1 produced a number of interesting results which we felt needed to be confirmed in a new experiment, e.g. the fall in oestrogen levels in female fish even at low dosage levels, and the induction of VTG in males. In order to achieve better control of actual individual exposure than in experiment 1 (in which the fish were fed as a group), the fish in this new experiment were fed individually via a feeding tube into their stomach. We also extended the experiment by adding more exposed groups and a positive oestrogen control, in order to determine the response to pure oestrogen exposure.

4.3 Experiment 2: Female fish

4.3.1 Oestrogen

As in experiment 1 we found that the level of 17ß-oestradiol fell in the exposed groups, most markedly in those that had been administered the highest dose of alkylphenols and had been exposed for the longest period of time. The drop relative to the controls was statistically significant in most groups (Fig. 18).

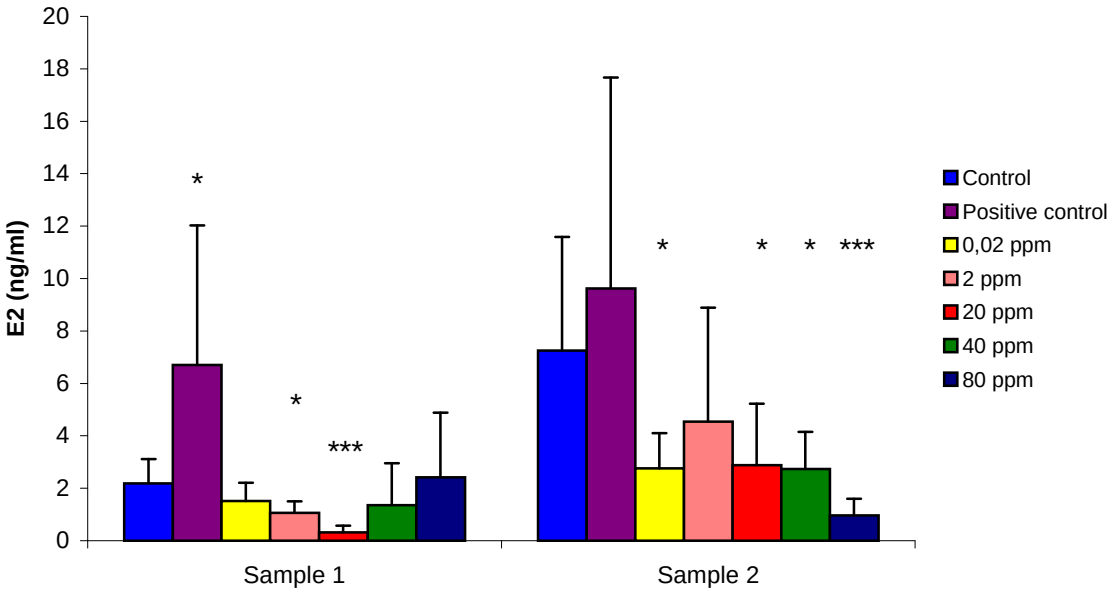


Fig. 18. 17ß-Oestradiol in female fish plasma (ng/ml) from control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$.

4.3.2 Vitellogenin

The level of VTG tended to be somewhat lower in the exposed females than in the controls, but the difference was statistically significant only in some of the groups (Fig. 19). In the second sampling, i.e. after a lengthy period of exposure, there was a consistently smaller increase in VTG level as the dose of alkylphenols increased relative to the group that had been exposed to the lowest dose.

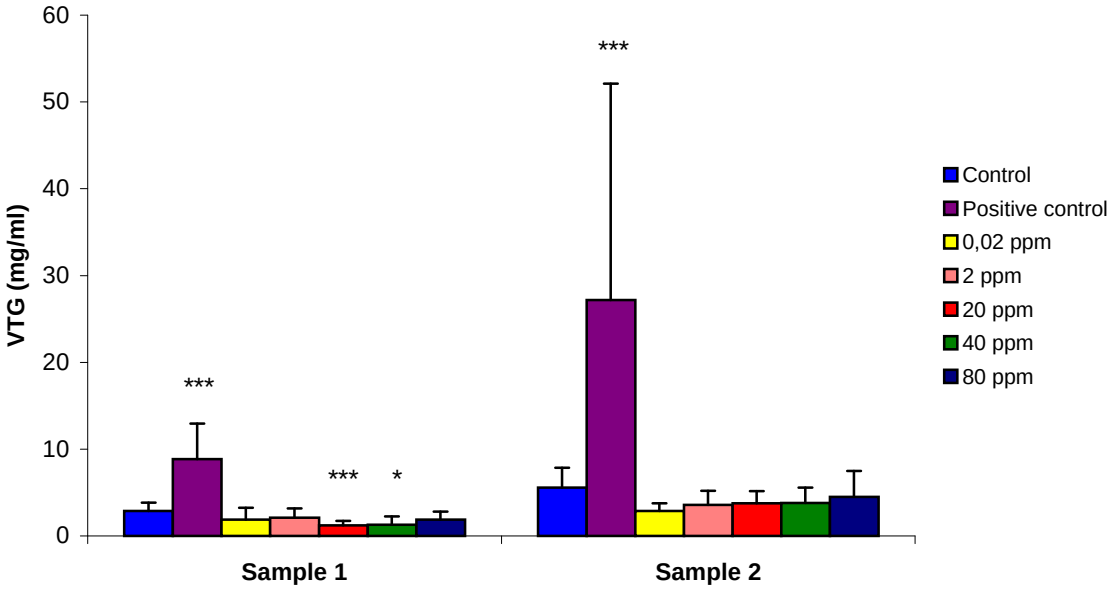


Fig. 19. Vitellogenin in female fish plasma (mg/ml) from control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$.

4.3.3 Testosterone

The testosterone response to exposure to alkylphenols appeared to be dose-dependent. There was a tendency for lower doses to raise testosterone levels in females, while higher doses caused levels to fall, as was the case with 17ß-oestradiol (Fig. 20).

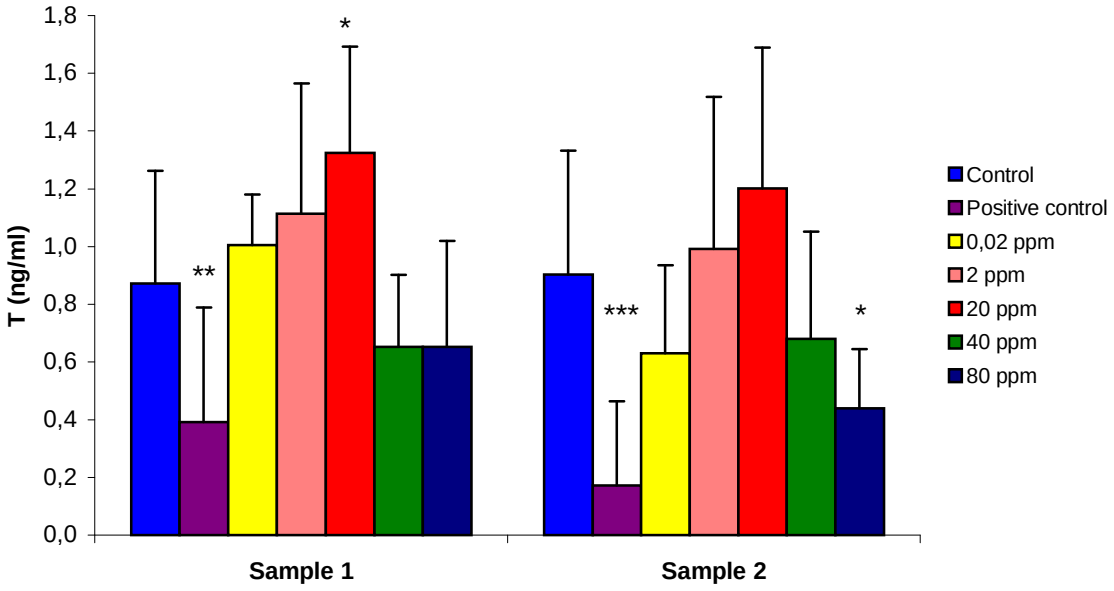


Fig. 20. Testosterone in female fish plasma (ng/ml) from control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$.

4.3.4 Gonadosomatic index

Exposure to alkylphenols had a significant effect on the GSI in this experiment (Fig. 21). In the second sampling, the fall was great in all the exposed groups.

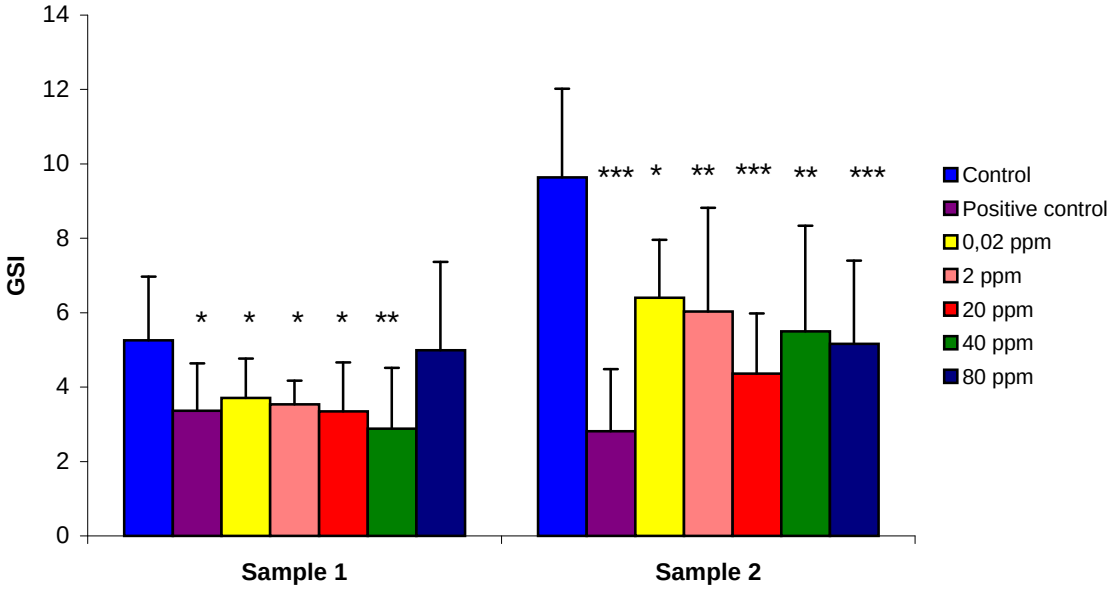


Fig. 21. Gonadosomatic index (GSI) for female fish: control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), * $p \leq 0,05$, ** $p \leq 0,01$, *** $p \leq 0,001$.

4.3.5 Potential fecundity

With the exception of the group exposed to 0.02 ppm alkylphenols, no significant changes were observed in potential fecundity, potential relative fecundity or potential fecundity condition factor as a result of the exposure (Table 7). The 0.02 ppm group had a significantly higher potential fecundity than the controls. However, this was not the case for potential relative fecundity or the potential relative fecundity condition factor.

Table 7. Effects on female fish (experiment 2).

Variable		**Control**	**0.02 ppm**	**2 ppm**	**80 ppm**	**Positive control**
Number of individuals	n	9	5	9	8	6
Potential fecundity (x 1000)	mean ±SD	1073±238	1393±295	1015±274	956±224	787±304
	p-value *	-	*0.046*	0.638	0.315	0.062
Potential relative fecundity	mean ±SD	1729±340	2027±507	1727±437	1387±335	1287±464
	p- value *	-	0.210	0.992	0.055	0.052
Potential fecundity condition factor	mean ±SD	17.9±4.7	20.0±5.4	17.57±4.86	14.10±3.76	12.91±5.21
	p- value *	-	0.463	0.894	0.090	0.0767
Follicle diameter (µm)	mean ±SD	423±49	340±41	338±53	355±47	317±47
	p-value *	-	*0.007*	*0.003*	*0.010*	*0.001*

Variable		Control	0.02 ppm	2 ppm	80 ppm	Positive control
G1 ("leading cohort") diameter (µm)	mean	549	424	415	446	378
Time to spawning (days)	mean ±SD	36.7±10.5	58.2±13.8	65.3±31.6	54.1±14.5	74.8±28.1
	p- value *	-	*0.007*	*0.021*	*0.012*	*0.002*

** Comparison between the exposed groups and the control group. Significant values in italic.*

4.3.6 Oocyte size

Significant differences in follicle diameter were found in all exposed groups (Fig. 22; Table 7). Changes in follicle diameter resulted in a significant displacement of the time to spawning (Fig. 23; Table 7).

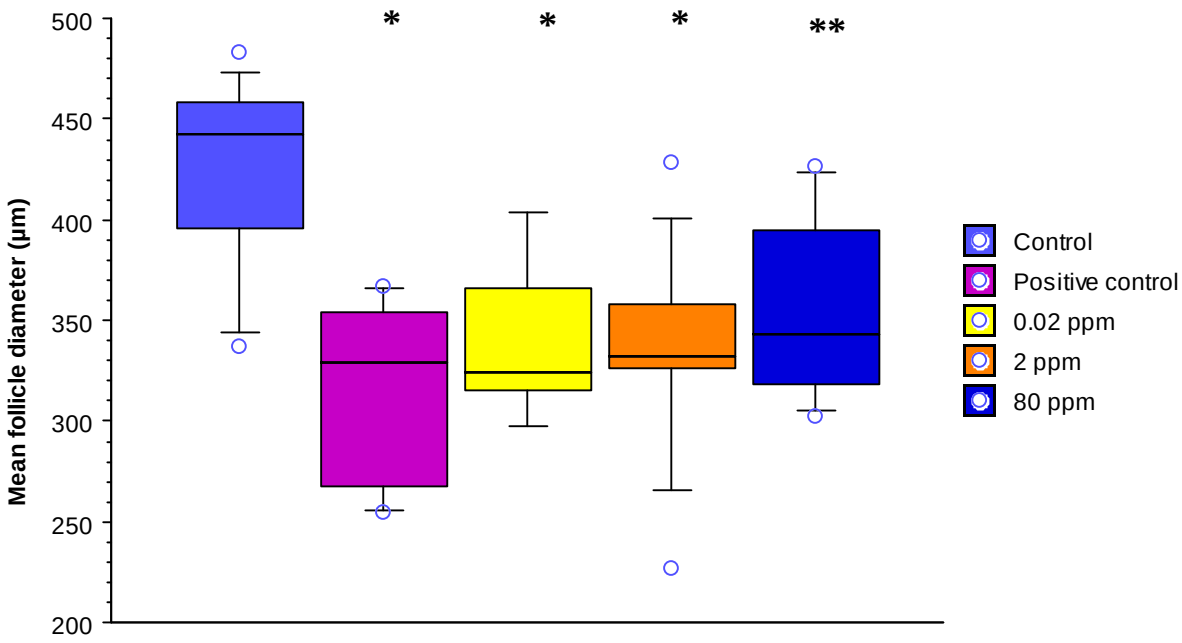


Fig. 22. Mean follicle diameter after alkylphenol exposure. Boxplot: median (line), 25-75 percentile (box), 10-90 percentile (vertical line) and all observations beyond 10 and 90 percentile as points. * $p \leq 0.05$, ** $p \leq 0.01$.

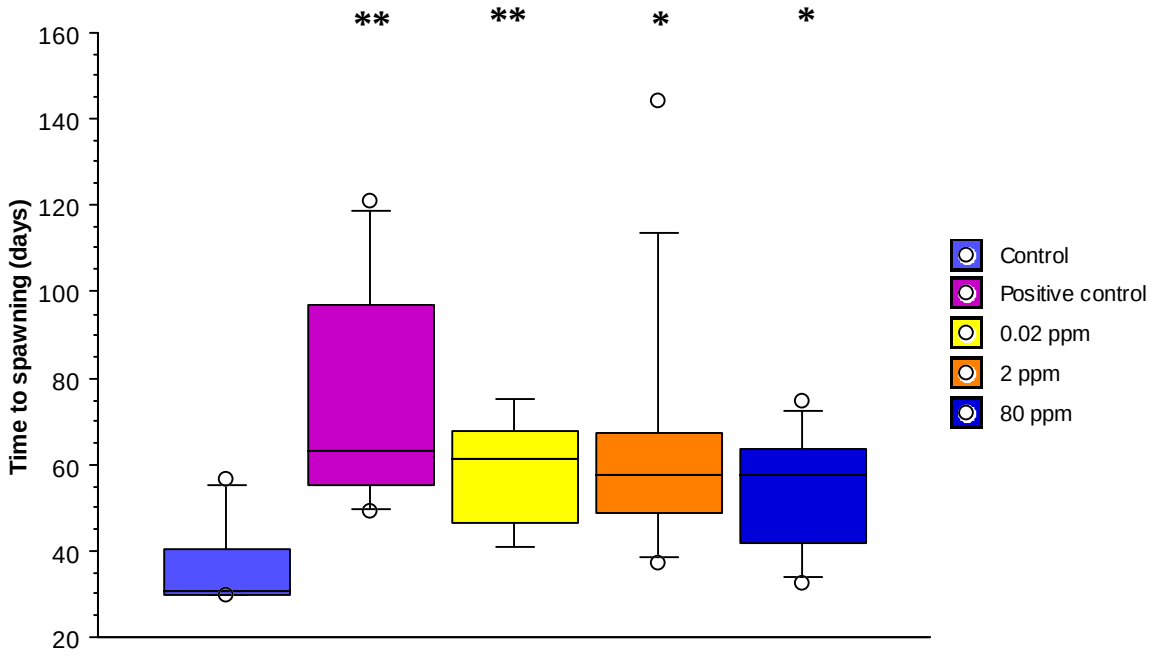


Fig. 23. Time to spawning (days). Time to spawning is a function of follicle size. Boxplot: median (line), 25-75 percentile (box), 10-90 percentile (vertical line) and all observations beyond 10 and 90 percentile as points. * $p \leq 0.05$, ** $p \leq 0.01$.

4.4 Experiment 2. Male fish

4.4.1 Testosterone

Testosterone levels fell significantly in most groups of males exposed to alkylphenols. The size and duration of the dose appears to have had an influence on the effects, but there was no clear dose-response relationship. The positive control group shows that a strong oestrogen effect may reduce testosterone levels in male fish to a minimum.

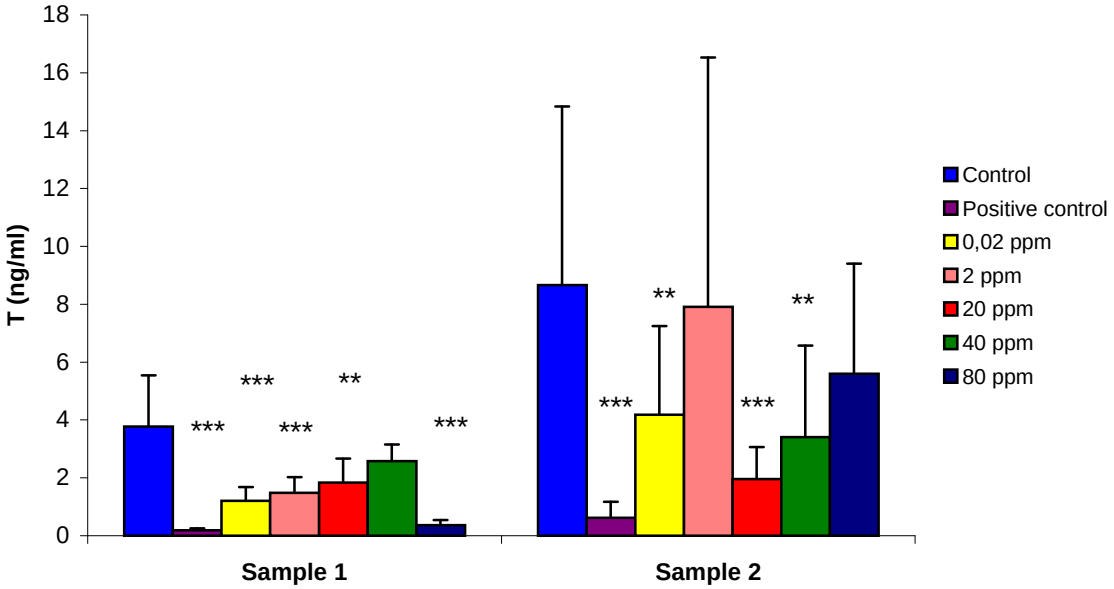


Fig. 24. Testosterone in male fish plasma (ng/ml) from control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), ** p≤0,01, *** p≤0,001.

4.4.2 Oestrogen

As was to be expected, there was a very high level of 17ß-oestradiol in the positive control group of males, of the same order of magnitude as in the positive control females (Fig. 25)

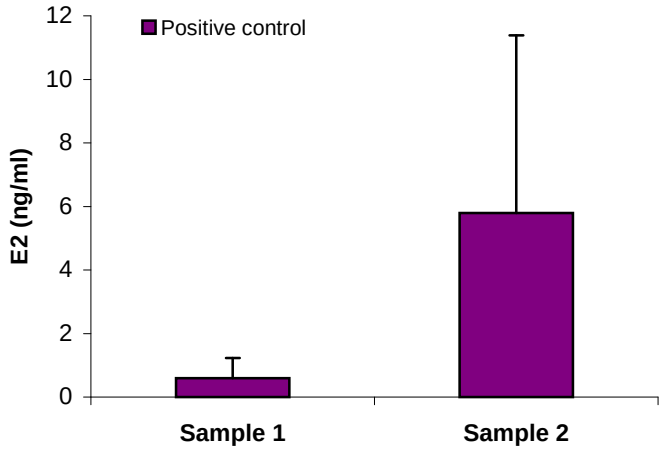


Fig. 25. 17ß-Oestradiol in male fish plasma (ng/ml) from positive control (5 ppm E2).

4.4.3 11-Ketotestosterone

There was a clear fall in 11-ketotestosterone in most groups after one dose. In the second sampling, the situation appeared to be the opposite in that there was either a similar or somewhat higher level of 11-ketotestosterone than in the controls, depending on dose (Fig. 26).

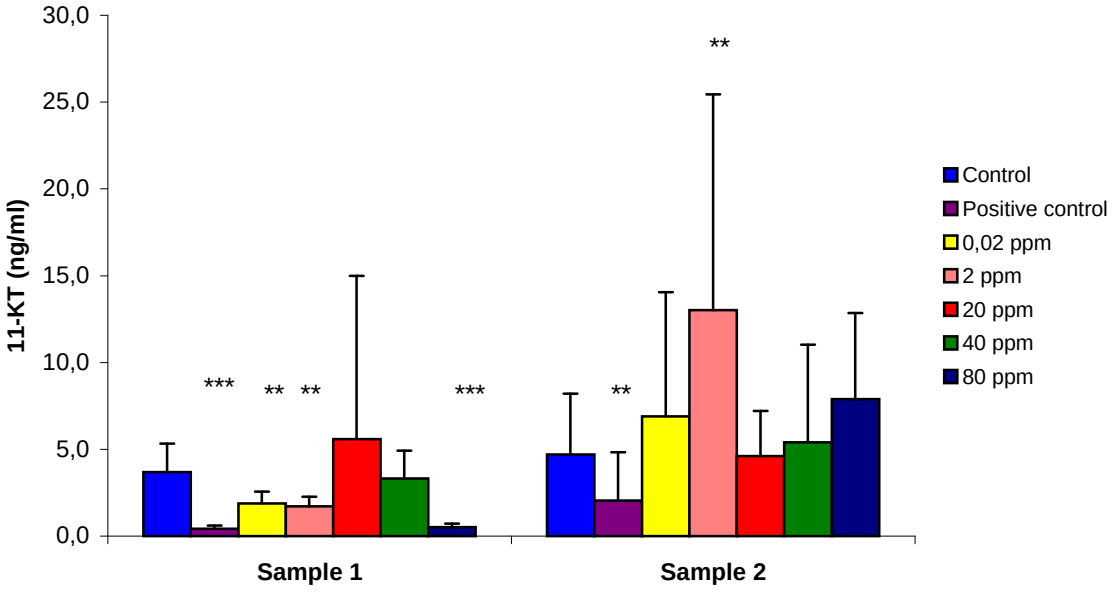


Fig. 26. 11-Ketotestosterone in male fish plasma (ng/ml) from control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), **p≤0,01, ***p≤0,001.

4.4.4 Vitellogenin

Exposure to 17ß-oestradiol (positive controls) resulted in an very big production of VTG in male fish (Fig. 27). Alkylphenols also induced VTG production in males (Fig. 28; statistically significant in most groups from second sampling), but to a much lesser extent than 17ß-oestradiol. The latter aspect

can be seen clearly from Fig. 27, where the levels of VTG in the groups exposed to alkylphenols did not emerge at all (note change in y-axis scale in Fig. 27 and 28!)

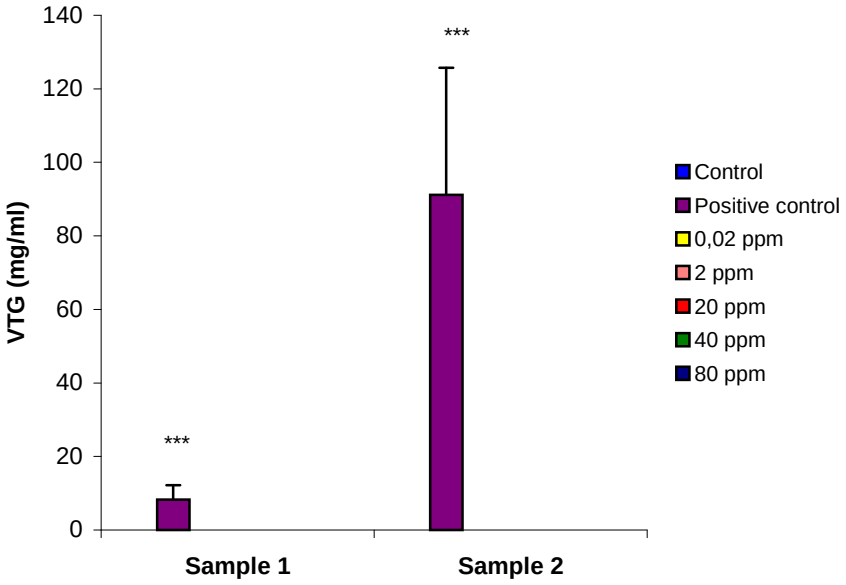


Fig. 27. Vitellogenin in male fish plasma (mg/ml). Positive control has obtained a strong induction of VTG. Several tousand times higher values than those for the alkylphenol exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), *** p≤0,001.

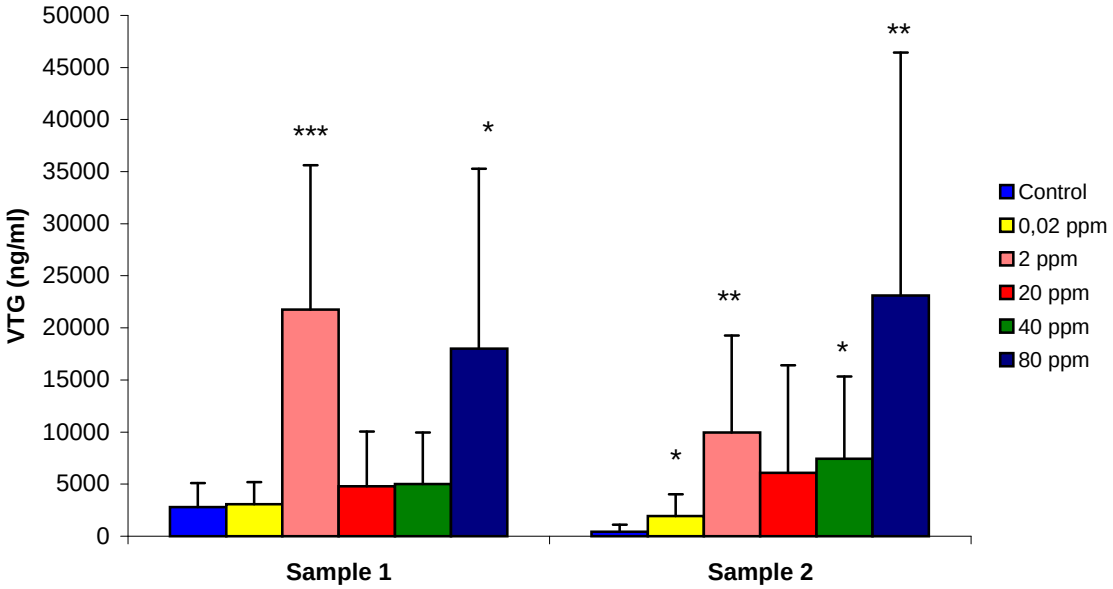


Fig. 28. Vitellogenin in male fish plasma (ng/ml): control, 0,02 ppm, 2 ppm, 20 ppm, 40 ppm og 80 ppm alkylphenol exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), *p≤0,05, **p≤0,01, ***p≤0,001.

The interval distribution of VTG shows that the number of individuals with high levels of VTG was greater in the exposed groups than in the controls. This happened as early as in the first sampling (Figures 29 and 30).

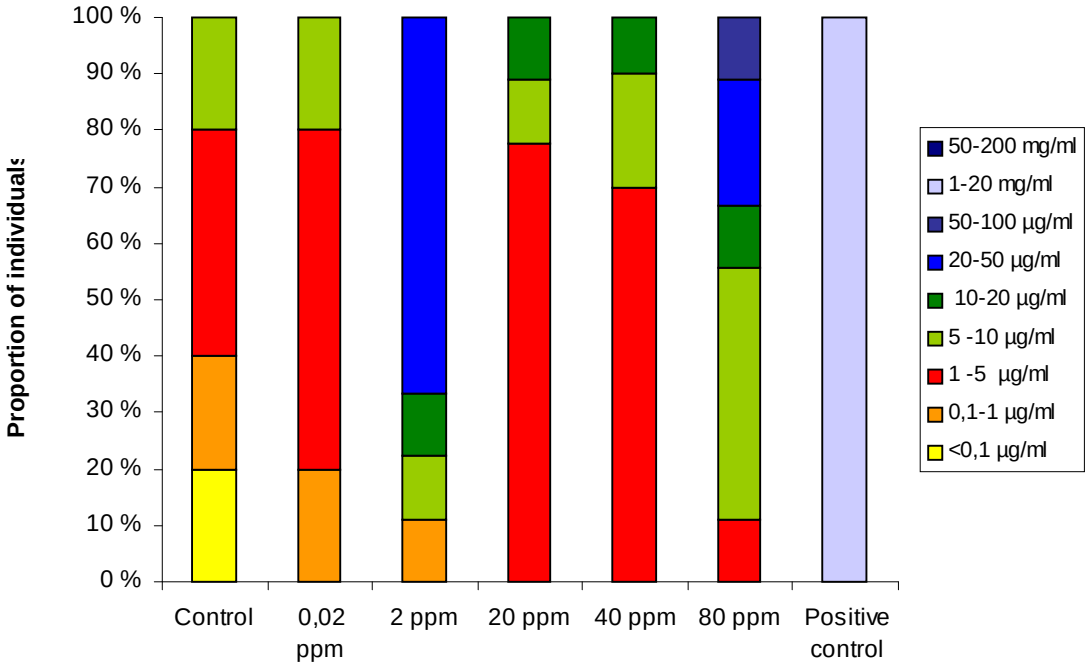


Fig. 29. Interval division of VTG-levels in male fish, sampling 1. Control, positive control (5 ppm E2), 0,02 ppm, 2 ppm, 20 ppm, 40 ppm og 80 ppm alkylphenol exposed groups.

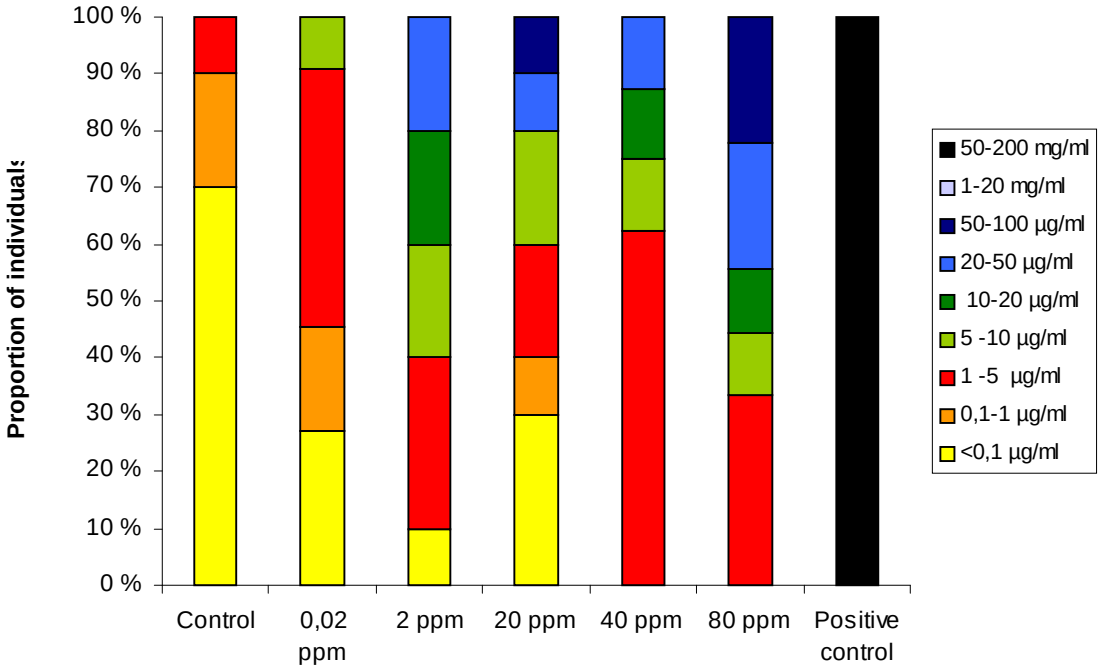


Fig. 30. Interval division of VTG-levels in male fish, sampling 2. Control, positive control (5 ppm E2), 0,02 ppm, 2 ppm, 20 ppm, 40 ppm og 80 ppm alkylphenol exposed groups.

4.4.5 Gonadosomatic index

Long-term exposure to oestradiol (positive controls, sample 2) resulted in a statistically significant decrease in the GSI (Fig. 31). There were no differences in GSI in the other groups.

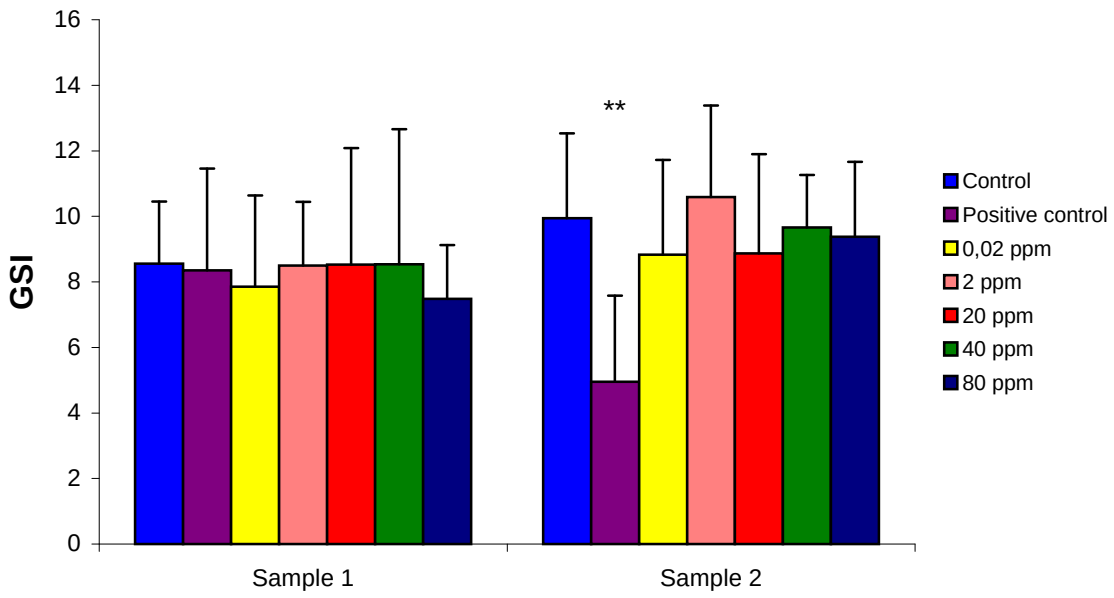


Fig. 31. Gonadosomatic index (GSI) for male fish: control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), ** p≤0,01

4.4.6 Maturation status

Changes in the pattern of testis maturation were found in the exposed groups (0.02 and 80 ppm), as well as in the positive control (Fig. 32 and 33; Table 8). Note the reduction of spermatozoa levels in the exposed groups, and the appearant increase for spermatogonia and spermatocytes. The variance was greatest in the group which had received 0.02 ppm alkylphenols.

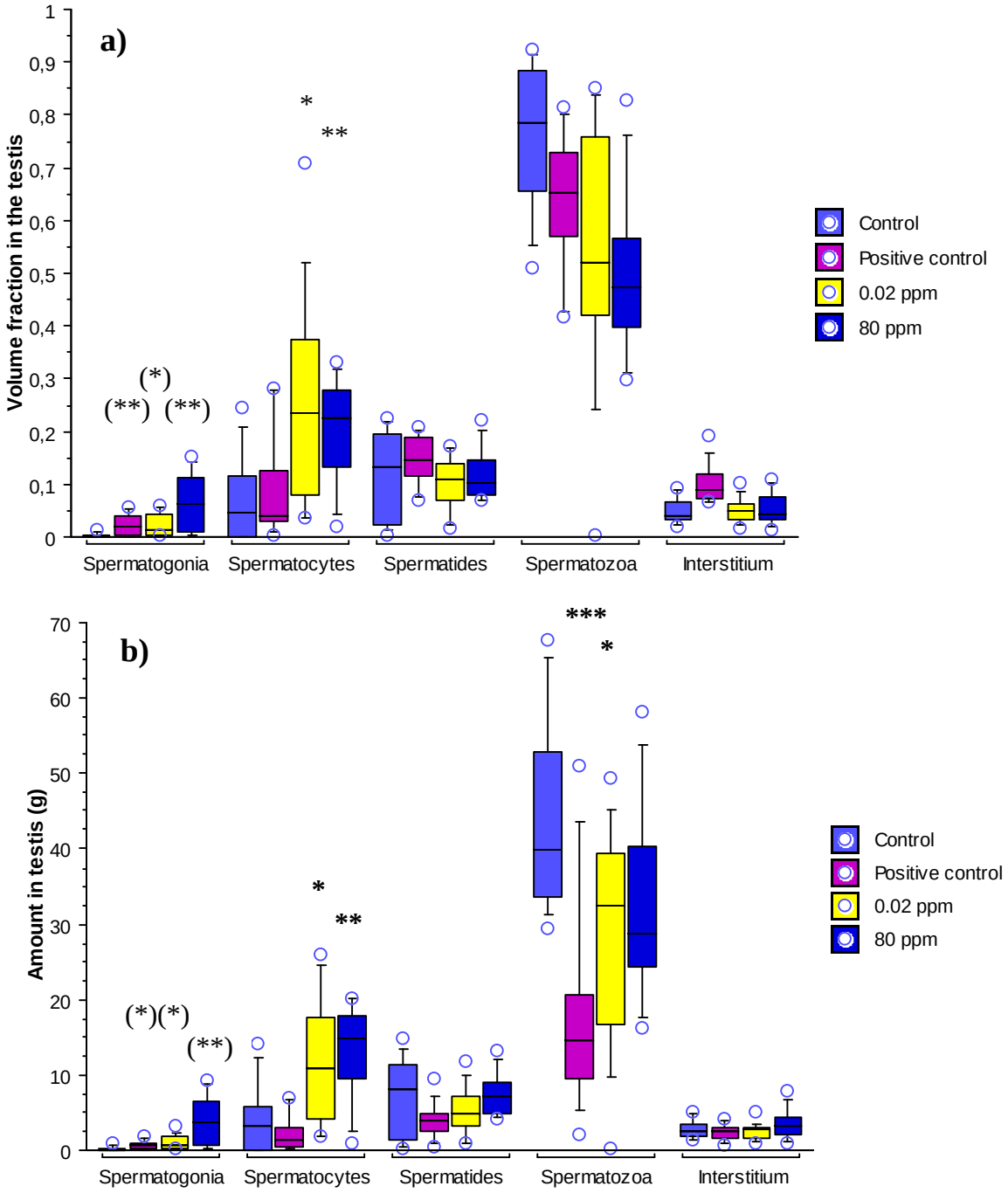


Fig. 32. The impact of alkylphenols on the abundance of cell types in testis. a) Fraction of each cell type in testis. b) Total amount of each cell type in testis (g). (Boxplot: median (line), 25-75 percentile (box), 10-90 percentile (vertical line) and all observations beyond 10 and 90 percentile as separate points). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; (*) og (**): correspondingly, but accompanied by uncertainty in the estimates.

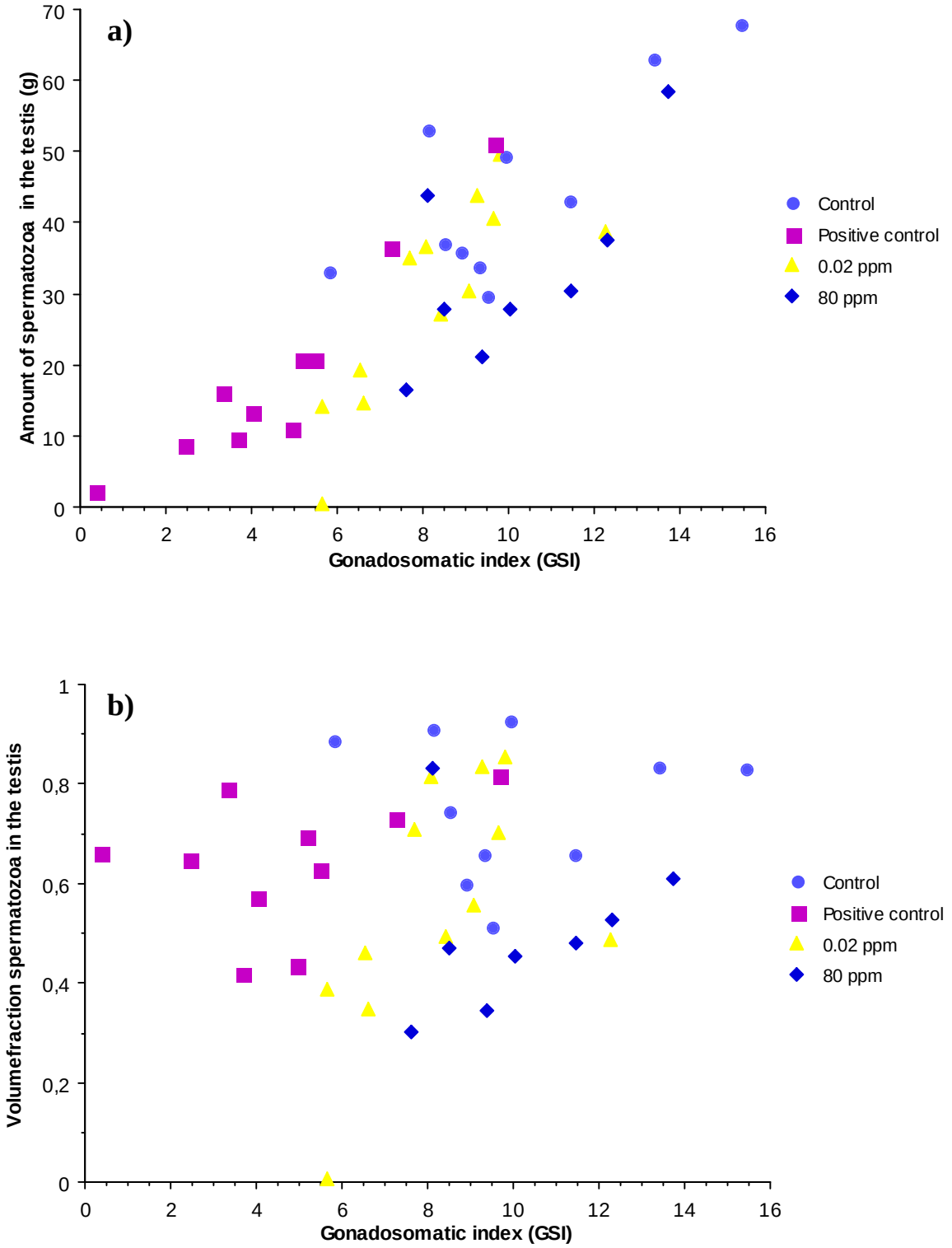


Fig. 33. Alkylphenol exposure affected the amount (a) and fraction (b) of spermatozoa in testis. Oestradiol also caused a reduction of spermatozoa, but in this case there was a concurrent reduction in GSI.

Table 8. Effects on male fish.

Variable		**Control**	**0.02 ppm**	**80 ppm**	**Positive control**
No. of individuals	n	10	12	8	10
Fraction spermatogonia	mean ± SD	0.002±0.004	0.02±0.02	0.07±0.06	0.02±0.02
	p-value *	-	*0.008*	*0.004*	*0.006*
Total amount of spermatogonia (g)	mean ± SD	0.16±0.33	1.07±1.02	3.90±3.49	0.67±0.61
	p-value *	-	*0.014*	*0.004*	*0.032*
Fraction spermatocytes	mean ± SD	0.07±0.09	0.25±0.20	0.20±0.10	0.09±0.10
	p-value *	-	*0.017*	*0.010*	0.641
Total amount of spermatocytes (g)	mean ± SD	4.24±4.84	11.78±8.37	13.21±6.60	2.37±2.49
	p-value *	-	*0.021*	*0.004*	0.293
Fraction spermatides	mean ± SD	0.12±0.09	0.10±0.05	0.12±0.05	0.15±0.05
	p-value *	-	0.497	0.917	0.459
Total amount of spermatides (g)	mean ± SD	7.44±5.29	5.26±3.24	7.48±3.01	3.96±2.49
	p-value *	-	0.249	0.984	0.076
Fraction spermatozoa	mean ± SD	0.75±0.14	0.55±0.25	0.50±0.16	0.64±0.13
	p-value *	-	*0.034*	*0.003*	0.077
Total amount of spermatozoa (g)	mean ± SD	44.41±13.29	28.94±14.49	32.65±13.39	18.88±14.61
	p-value *	-	*0.018*	0.082	*0.0007*
Fraction interstitium	mean ± SD	0.05±0.03	0.05±0.02	0.05±0.03	0.10±0.04
	p-value *	-	0.924	0.759	*0.002*
Total amount interstitium (g)	mean ± SD	2.81±1.29	2.48±1.12	3.47±2.12	2.44±1.08
	p-value *	-	0.531	0.423	0.498

* comparisons between the exposed groups and the control group. Significant values in italic.

4.5 Effects of alkylphenols on the two detoxification systems cytochrome P450 (phase 1 metabolism) and glutathione (phase 2 metabolism)

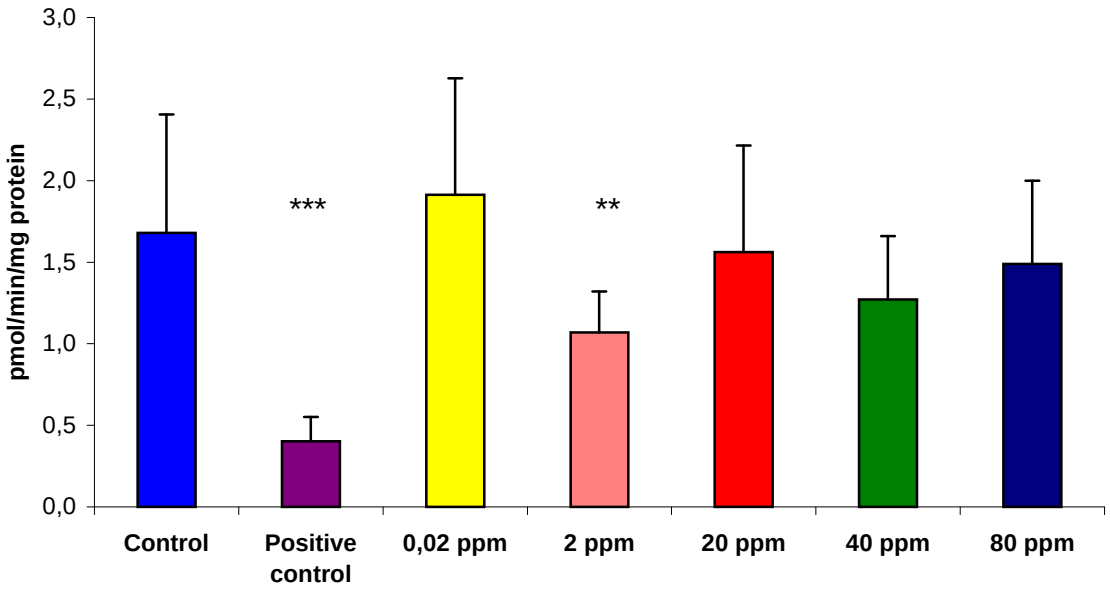


Fig.34. Catalytic activity of CYP1A in liver from male fish after four weeks of exposure (sampling 2) from control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), **p≤0,01, *** p≤0,001.

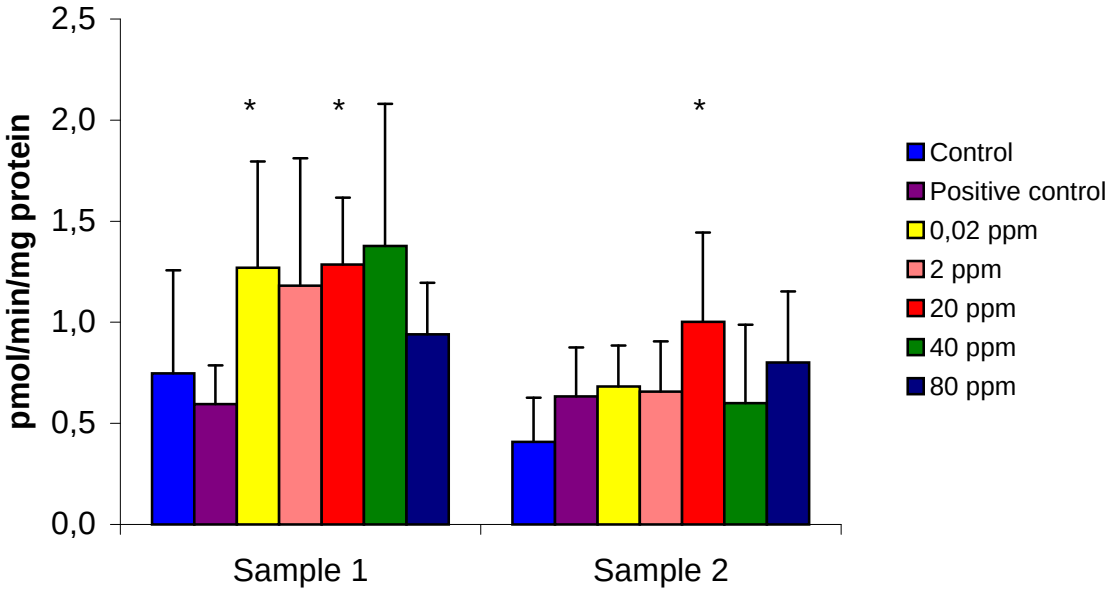


Fig. 35. Catalytic activity of CYP1A in liver from female fish after 1 exposure (sampling 1) and after 4 exposures (sampling 2) from control and exposed groups. Mean values ± standard deviation. Asterisks indicate significant difference from the control group (unpaired t-test), *p≤0,05.

CYP1A

1 2 3 4 5 6 7

CYP3A

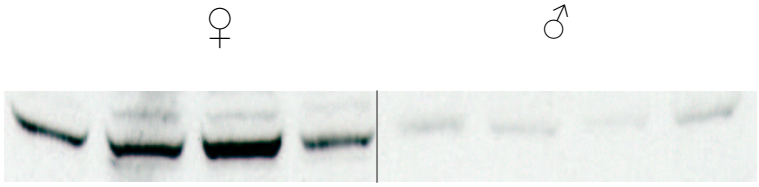
1 2 3 4 5 6 7

Fig. 36. Induction of CYP1A and CYP3A protein expression in liver from male fish exposed for different doses of alkylphenols for four weeks. The amount of protein is contrasted with control and positive control (5 ppm E2). Well 1: Positive control, well 2: Control, well 3: 0,02 ppm, well 4: 2 ppm, well 5: 20 ppm, well 6: 40 ppm, and well 7: 80 ppm.

CYP1A

1 2 3 4 5 6 7 8

CYP3A



1 2 3 4 5 6 7 8

Fig. 37. Protein level of CYP1A and CYP3A in liver from four different non-exposed female fish (well 1-4) compared with ditto number of male fish (well 5-8).

The catalytic activity of CYP1A measured as EROD fell strongly in males exposed to oestradiol for four weeks (positive controls, Fig. 34). In the group of males exposed to alkylphenols, on the other hand, enzyme activity was not affected to any great extent. This is very interesting in view of the fact that both CYP1A and CYP3A protein expression is induced in a dose-dependent manner (Figure 36).

In other words, an increase in the amount of the protein CYP1A does not bring about an increase in enzyme activity in males following exposure to alkylphenols.

There was significantly higher CYP1A activity (EROD) in female fish exposed to 0.02 and 20 ppm in the first sampling, and to 20 ppm in the second (Fig. 35). EROD activity also tended to be higher in the other groups, but the differences were not statistically significant. The response was not dose-dependent. A protein induction response similar that observed in males was not found in female fish (data not shown).

Fig. 37 shows that, under normal conditions, there are major differences in levels of CYP1A and CYP3A between males and females, with the males having the lowest amounts of P450. This does not fit well with the picture of CYP1A catalytic activity, where males tend to have a higher level of activity than females.

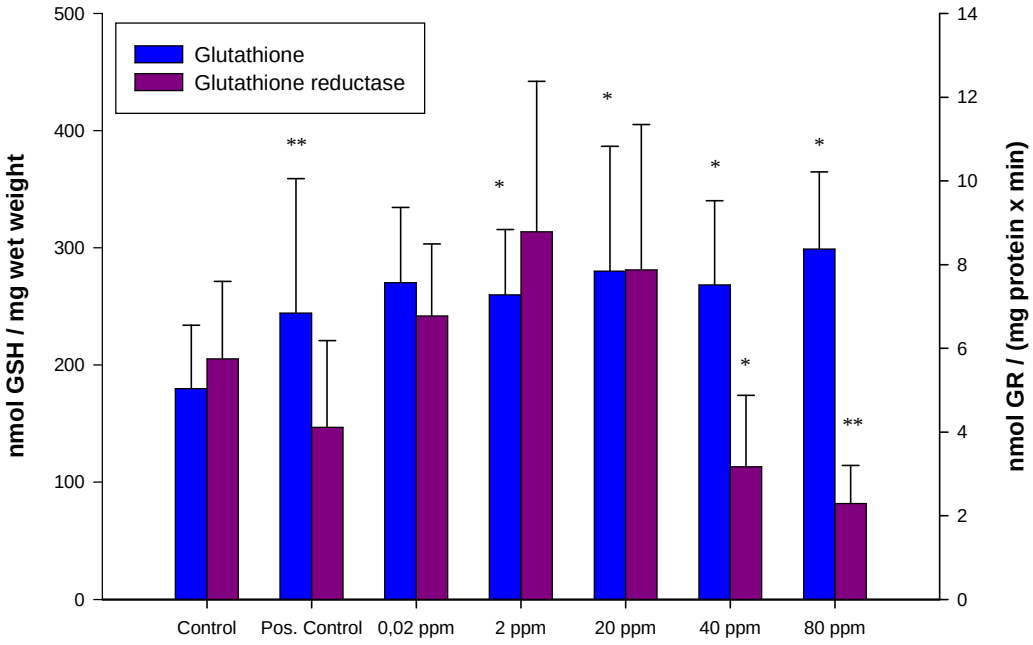


Fig. 38. Glutathione concentration (GSH) and glutathione reductase activity (GR) in liver from female fish sampling 1. Control and exposed groups. Asterisks indicate significant difference from the control group (unpaired t-test), * p≤0,05, ** p≤0,01.

The total amount of glutathione in the liver of female fish rose following a week of exposure to alkylphenols. Glutathione reductase was induced in a biphasic mode, in which activity rose at lower concentrations of alkylphenols and sank at higher concentrations (Fig. 38). The reduced form of glutathione was also measured (results not shown). The ratio of reduced glutathione to total glutathione was relatively constant (Close to 1) at all levels of exposure, which suggests that even if GR activity decreases, its capacity is sufficient to maintain glutathione in its reduced form. The results also indicate that the “pentose phosphate pathway” produces sufficient NADPH to ensure that GR functions adequately.

A comparison of Fig. 38, 39 and 40 shows that the mean level of glutathione was higher in males than females (second sampling), and higher in females from the second than the first sampling.

The activity of two other enzymes that are directly or indirectly involved in glutathione metabolism was also measured. Neither the activity of glutathione S-transferase (GST) nor of glucose-6-phosphate dehydrogenase (G6PDH) seems to have been affected by exposure to alkylphenols (results not shown).

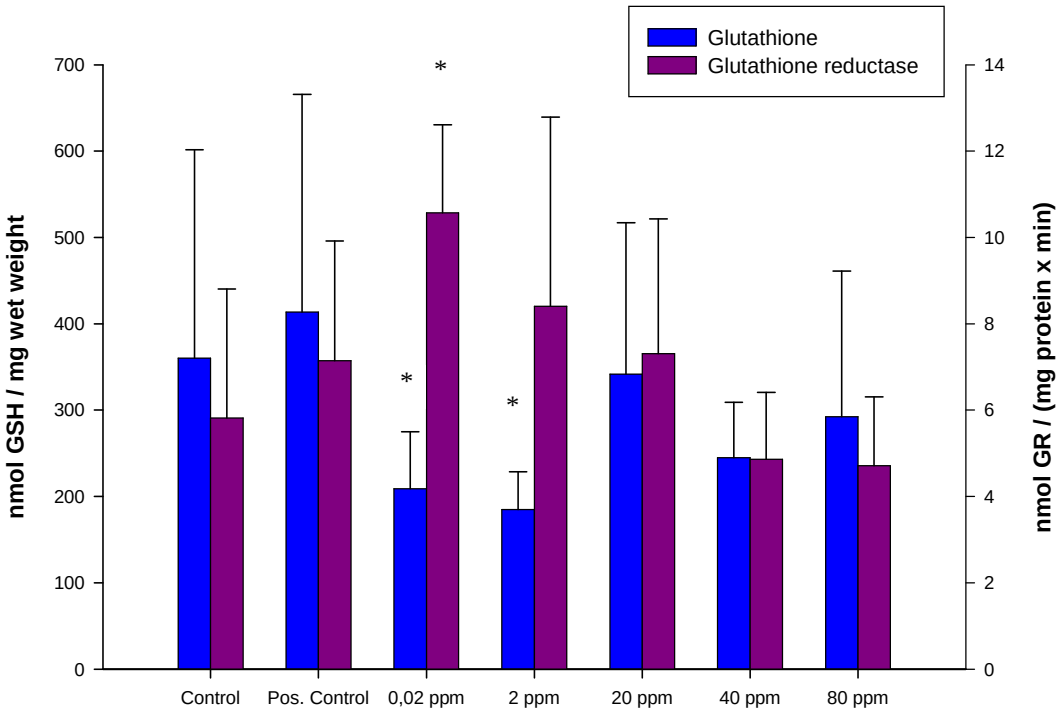


Fig.39. Glutathione concentration (GSH) and glutathione reductase activity (GR) in liver from female fish sampling 2. Control and exposed groups. Asterisks indicate significant difference from the control group (unpaired t-test), * p≤0,05.

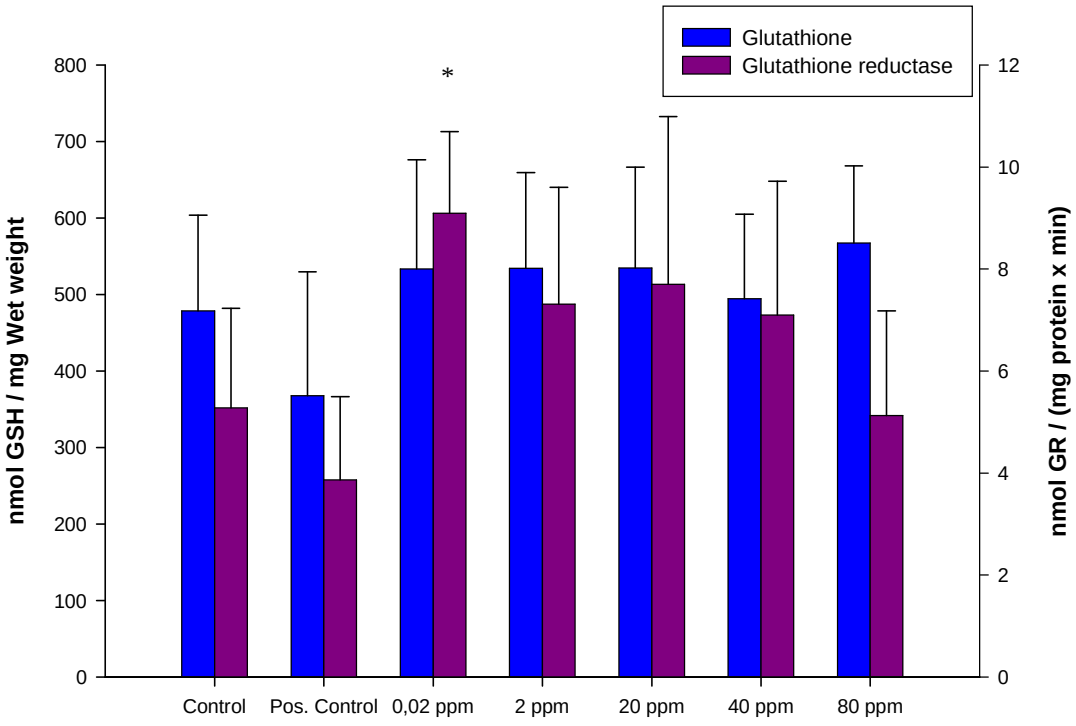


Fig. 40. Glutathione concentration (GSH) and glutathione reductase activity (GR) in liver from male fish sampling 2. Control and exposed groups. Asterisks indicate significant difference from the control group (unpaired t-test), * p≤0,05.

5 Discussion

5.1 Steroids

We found that exposure to alkylphenols had major effects on natural levels of steroids in both male and female fish. We found a very powerful and significant down-regulation of 17ß-oestradiol levels in the exposed groups. In the long-term exposure experiment (experiment 1) this effect was evident as early as November, and it continued, with a rising level of significance, in the next two samplings (December and January) (Fig. 7). In the January sample, the 17ß-oestradiol level of the low-dose group (0.02 ppm) was also significantly lower than the control group (Fig. 7). Similar findings were also made in experiment 2. All groups exposed to alkylphenols in experiment 2 (except the 2 ppm group) displayed low levels of oestrogen. Even though the greatest down-regulation of 17ß-oestradiol level was found at the highest dose (80 ppm), the results did not evidence a clear dose-response relationship (Fig. 18). A fall in plasma 17ß-oestradiol after exposure to nonylphenol has also been observed in salmon (Arukwe *et al.*, 1997) and rainbow trout (Harris *et al.*, 2001). Two other studies of flounder (*Paralichthys dentatus*) (Milles *et al.*, 2001) and fathead minnow (*Pimephales promelas*) (Giesy *et al.*, 2000) found an increase in plasma 17ß-oestradiol on exposure to alkylphenols (octyl- and nonylphenol).

Effects on testosterone were also found in female fish as well as on 11-ketotestosterone and testosterone in males, although the picture is not as clear as it was for the effects of oestrogen. There were large seasonal differences in experiment 1. In October, (following a month of exposure), there were greatly increased levels of testosterone in female (both at 0.02 and 2 ppm) and male fish (0.02 ppm), with an opposite trend in subsequent samplings, with lower testosterone levels in the exposed fish (significantly lower in males in November (0.02 and 2 ppm) and females in December (0.02 ppm). The effects on 11-ketotestosterone were similar to those with testosterone. Experiment 2 showed a decrease in levels of testosterone and 11-ketotestosterone in several of the exposed groups, but there was no clear dose-related trend.

The positive controls (experiment 2) displayed a large decrease in testosterone in both males and females, while the 11-ketotestosterone level was similarly very low in males exposed to large doses of oestradiol. The powerful effects that we found in the positive controls can be explained by the fact that high doses of oestradiol either induce a major increase in steroid metabolism, thereby depressing the concentrations in plasma, or produce a down-regulation of steroid synthesis itself.

As we explained in the Introduction, steroid synthesis is controlled by what is known as the brain-pituitary gland-gonadal axis. This is an extremely complex system that regulates seasonal variations in hormone levels and thereby all the physiological processes that take place during the process of gonadal development. A very large number of mechanisms are involved in this regulation process and there are thus several places at which it can be disrupted. On the basis of our data we cannot say anything about which mechanisms have been disrupted. Many different levels may well be involved.

The brain and the pituitary gland control the synthesis of gonadotrophins; i.e. follicle-stimulating hormone (FSH) and luteinising hormone (LH), which in turn regulate steroid synthesis in the gonads. This gonadotrophin secretion is controlled by a complex regulatory system that consists of several steps at which disruptions can take place (Jalabert *et al.*, 2000). Attention is currently being focused on alkylphenol and neuroendocrine disturbances (Jones *et al.*, 1998; Piva & Martini, 1998; Yadetie *et al.*, 1999b; Harris *et al.*, 2001). These last authors found that nonylphenol reduces the genetic expression of FSH in the hypophysis and FSH secretion to the plasma in the rainbow trout, even at very low concentrations (they found a significant effect even at the lowest dose; 0.7 µg/l). Similarly, the quantity of LH-mRNA is reduced in the hypophysis on exposure to nonylphenol (8.3 µg/l). At higher doses (85.6 µg/l), these workers found a large fall in GSI and plasma 17ß-oestradiol, similar to what we report here. The underlying mechanisms of the effects of alkylphenols on the gonadotrophins are still unknown. At the gonadal level, steroid synthesis is dependent on the receptor system of the gonadotrophins and regulation of the steroid-synthesising enzymes. These steps may also be vulnerable to endocrine disturbances (Arukwe *et al.*, 1997). Alkylphenols and other xenobiotics may well also affect natural steroid metabolism by inducing enzyme systems that participate in common metabolic processes.

One interesting finding of these studies has been that there is no clear dose-response relationship. The group that was administered the lowest dose (0.02 ppm) displayed the same fall in oestradiol level as some of the groups that received higher doses (Fig. 18). This may indicate that down-regulation is a result of exceeding a certain threshold level. Such a lack of dose-response relationship is a typical phenomenon in the steroid system. There are often different responses to low and high doses of steroids. Low doses of oestrogen and testosterone stimulate the secretion of gonadotrophin in fish, while high doses inhibit it (Jalabert *et al.*, 2000). Similarly, differences in the effects of low and high doses of alkylphenols on steroid-synthesising enzymes have been observed (Arukwe *et al.*, 1997). We will in the future work on the basis of the hypothesis that the effects described are a result of disruptions at CNS level, and that this is related to the natural feedback system of the fish. None of the data from these experiments are capable of answering such questions, although the data of Harris *et al.* (2001) provide strong indications in this direction.

5.2 Vitellogenin

Figures 19 and 27 show that exposure to oestradiol leads to a powerful induction of vitellogenin (VTG) in both males and females (positive controls, 5 ppm E2). This confirms that VTG is a sensitive biomarker for the influence of oestrogen, and that cod is a suitable model organism in these studies, as has already been shown by similar studies (Hylland & Haux, 1997; Hylland, 2000).

The results of the experiments on males exposed to alkylphenols showed a clear, dose-related induction of VTG (Fig. 28), but its levels were several thousand times lower than in the fish exposed to E2. Generally speaking, there was a certain amount of variation in VTG concentrations within the groups. Among the consequences of this was that the only significant difference we found in experiment 1 was between the control group and the 2 ppm group of males from the October sampling. Dividing the VTG levels into concentration intervals, however, gives us a fairly clear picture. The experiment 1, which looked at the effects of long-term exposure, 20% of the control group had measurable amounts of VTG (males), while the proportions in the 0.02 ppm and 2 ppm groups were 50 and 70% respectively (Figure 16).

Some surprising seasonal differences were observed in both experiments. In November, plasma VTG levels were generally higher in males from both the control and exposed groups. In the first sampling in experiment 2 (November), measurable quantities of VTG were found in as much as 80% of the control group (Fig. 29), while at the end of December (second sampling) only 30% of the control fish had VTG in their plasma. In spite of higher VTG values in the control group, fish from sample 1 in experiment 2 and from the November sampling in experiment 1 still showed a clear increase in VTG as a result of exposure to alkylphenols.

Vitellogenin and zona radiata protein (ZRP) have been used for many years as biomarkers for oestrogen induction (for review, see Jones *et al.* 2000). Various alkylphenols have been shown in earlier experiments to induce VTG in males, even at very low concentrations: nonylphenol: 5.4 ppb (sheepshead minnow) (Hemmer *et al.*, 2001), octylphenol: 4.8 ppb and nonylphenol: 20.3 (rainbow trout) (Jobling *et al.*, 1996). A major British study of vitellogenin concentrations in male fish (flounder, *Platichthys flesus*) showed that fish caught in the vicinity of oil offshore installations in the UK sector of the North Sea had significantly higher values of VTG than three reference groups taken in “unpolluted” areas (Mathiessen *et al.*, 1998).

5.3 Gonadosomatic index

In female cod, gonadal weight rose from about 2% of body weight at the end of November to 11% just before spawning at the end of January. The large increase in weight is mainly due to the uptake and transport of lipids and proteins from the liver to the gonads, in the form of vitellogenin, as described in the Introduction. Normally, there is a direct relationship between oestrogen, vitellogenin and gonadal growth. It is thus not surprising that low oestrogen levels are followed by a drop in gonadal weight. In experiment 1 there was a lower mean value of GSI (females) in the two exposed groups than in the controls, but the difference was not statistically significant (Fig. 10). In experiment 2, on the other

hand, there was a large and significant fall in GSI in female fish (Fig. 21). In the positive controls (5 ppm E2), we could observe a significant amount of resorption of the gonad, and it is unlikely that this group would have been able to complete spawning. The other groups showed no "external" effects of exposure, although their gonadal weights were considerably lower. In experiment 1 we also looked at spawning success, egg quality and larval survival rates. All groups spawned normally and no abnormalities in terms of survival or growth were observed (Folkvord *et al.*, 1999; Thorsen *et al.*, 1998).

5.4 Maturation

5.4.1 Females

Significant effects were found on oocyte diameter, and thereby also on the time to spawning. This delay may represent an important loss of reproductive potential. A large cod can spawn as many as 20 batches of eggs over a period of around six weeks, with an average of 34 days (SD = 10 days) (Kjesbu *et al.*, 1996). The quality of the eggs spawned tends to fall in the course of the spawning period (Solemdal *et al.*, 1992). The number of eggs in each batch is also smaller at the beginning and the end of spawning (Kjesbu *et al.*, 1996). This means that the period that includes good spawning batches is probably not much longer than three weeks, and for first time spawners it is probably shorter. In such a perspective, a delay of three weeks is considerable. The cod, as a species (or in local terms, as a stock) has a long spawning season of more than two months (sometimes as long as three months) (Brander, 1993, 1994). However, as many as two thirds of the eggs are spawned during a period of four to six weeks in UK waters (Brander, 1994). The spawning season appears to be centred on the period of the plankton bloom, with *Calanus finnmarchicus* as an important species (Brander, 1993, 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. A delay in the mean time of start of spawning increases the chances of the eggs being spawned too late relative to this optimum. In medaka, it has been found that exposure to nonylphenol results in reduced realised fecundity (Groene *et al.*, 1999; Shioda & Wakabayashi, 2000). These authors also noted a tendency for fewer eggs to hatch when an unexposed female spawned together with an exposed male. This aspect has not been examined in our study, but there is every reason to look inti it in future experiments. If exposure reduces both realised fecundity and the proportion of eggs that actually hatch, this would reinforce the effects of delayed spawning.

5.4.2 Males

Experiment 2 showed considerable effects on the pattern of maturation of the testis in males. There was an increase in the amount of spermatocytes and a reduction of spermatozoa. There also appeared to be an increase in the amount of spermatogonia, though the method used did not produce completely reliable estimates for these cells, which make up a relatively small proportion of the testis. However, previous studies have also demonstrated an increase in the occurrence of spermatogonia (in addition to increased mitotic activity in the spermatogonium population) on exposure to octylphenol, nonylphenol and 17ß-oestradiol (Folmar *et al.*, 2001; Giesy *et al.*, 2000; Jobling *et al.*, 1996; Zaroogian *et al.*, 2001). There are also reports of disruptions of the later stages of spermatogenesis. Platy (*Xiphophorus maculatus*) form what are known as spermatozeugmata ("packets" of spermatozoa) which are transferred to the female. Formation of these spermatozeugmata has been shown to be disrupted by exposure to nonylphenol at relatively high doses (Kinnberg *et al.*, 2000a and b). However, their exposure level was considerably higher than in our experiments.

Major morphological changes in the Sertoli cells of the testis in the platy have been observed following exposure to nonylphenol (Kinnberg *et al.*, 2000a and b; Miles-Richardson *et al.*, 1999). Kinnberg *et al.* exposed their fish to high doses of nonylphenol (80 - 1280 µg/l), while Miles-Richardson *et al.* used doses of 0.05 - 3.4 µg/l. They identified effects on Sertoli cells from 1.1 µg/l. The cells displayed considerable hypertrophy, and changed their shape from plate-like to cuboid or cylindrical. There was also a change in the cytoplasm, with the endoplasmic reticulum (ER) developed dilated cisternae, the nucleus became enlarged and spherical, and vacuoles appeared in the cytoplasm.

Hughes *et al.* (2000) report that low concentration in the µM region inhibit the Ca^{2+} pump in the ER membrane in rats. They come to the conclusion that alkylphenols disrupt the Ca^{2+} homeostasis in the testis and thus bring about cell death. In their study, nonylphenol caused apoptosis in isolated TM4 cells, probably as a result of the disruption of Ca^{2+} balance. The findings of Kinnberg *et al*. (2000 a and b) fit in well with this explanatory model, particularly the electron microscopy observations. Combining all these observations, it seems likely that the changes that we observed in the testis may be due to disruption of the calcium balance in the Sertoli cells. This may explain what we observed in our studies, in which the amount of spermatozoa fell, while the amount of spermatocytes increased, and the amount of spermatogonia also appeared to rise. The spermatocytes depend on their association with a Sertoli cell for their further differentiation. Sperm formation is dependent on hormonal signals, to which the Sertoli cells react (Nagahama, 2000). The effects on the Sertoli cells thus also impact the spermatides, and it is thus likely that they will suffer cell death (probably by apoptosis) before they manage to mature into spermatozoa. Previous studies have also reported necrotic cells and phagocytosis in the testicular tubuli (Miles-Richardson *et al.*, 1999), which also suggests 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 exposure doses, is a direct cytotoxic effect of alkylphenols (Kinnberg *et al.*, 2000b; Kime, 1999). An effect of this sort fits well with what we observed in our positive control group, in which there appeared to be a reduction in all types of testicular cells.

Vitellogenin and lesions
A number of studies have reported the appearance of eosinophile (and PAS-positive) areas or lesions in the testis, liver and kidney as a result of exposure to oestradiol and oestradiol mimics (Zaroogian *et al.*, 2001; Herman & Kincaid, 1998; Folmar *et al.*, 2001). This appears to be related to raised plasma vitellogenin levels. We did not observe testicular lesions in our studies. However, it is possible that such lesions would have developed even at the low concentrations we used, if the exposures had continued longer. Nor did we perform PAS staining of our tissue samples, so that it is possible that small lesions occurred without being identified.

Sexual differentiation
In several species, it has been found that long-term exposure to alkylphenols may lead to the formation of oocytes in the testis (Hashimoto *et al.*, 2000; Gray and Metcalfe, 1997; Gimeno *et al.*, 1998). We did not observe this in our study, but this is something that ought to be looked at in more detail. It is likely that cod, which are incapable of changing sex as adults, are only likely to suffer this type of effect if exposure takes place before or during sexual differentiation. We cannot therefore expect to shed any light on this aspect by testing fish that are already sexually differentiated. This would also elucidate whether a change would take place in the male: female ratio following long-term exposure. Such an alteration has been found in medaka (Gray & Metcalfe, 1997), in which the ratio of males to females fell from 2:1 in controls to 1:2 in a group exposed to 100 µg / *p*-nonylphenol. It is not clear whether this is due to disturbances in the sexual differentiation or selective male mortality. Whatever the cause, such a change would have significant implications for the reproductive capacity of the species.

5.5 Effects of alkylphenols on the two detoxification systems: cytochrome P450 (phase 1 metabolism) and glutathione (phase 2 metabolism)

It has been demonstrated in several species that cytochrome P450 CYP1A levels fall significantly in female fish parallel with rising oestrogen levels during the sexual maturation process, and that female fish tend to have a lower level of CYP1A than males (Gray *et al.*, 1999; Elskus *et al.*, 1992; Larsen *et al.*, 1992; Lindstrom-Seppa & Stegman, 1995; Arukwe & Goksøyr, 1997; Williams *et al.*, 1999). The ability of oestrogen to down-regulate the fish CYP1A system has also been well documented by exposure experiments with 17ß-oestradiol (Pajor *et al.*, 1990; Gray *et al.*, 1991; Arukwe *et al.*, 1997; Donohoe *et al.*, 1997; Navas & Segner, 2000). The mechanism that induces the effects of oestrogen on the CYP1A system is not known. It has been suggested that there may exist mutual interactions ("cross-talk") between the aryl hydrocarbon receptor (AhR) and the oestrogen receptor (Safe, 1995), but a great deal remains to be cleared up in this area.

Our measurements of the enzymatic activity of CYP1A (EROD) (Fig. 34 and 35) in these studies agree with the above findings. We found a large drop in EROD activity in the oestrogen-exposed males (positive control group), while the male control group displayed higher EROD values than the female controls. We did not find any clear effects of exposure to alkylphenols on EROD activity in either males or females. No "oestrogen-regulated" fall in enzymatic activity was found in the exposed fish, but on the other hand there was a trend in the direction of raised EROD activity in exposed females. This may be explained by the fact that exposure to alkylphenols results in lower levels of natural oestrogen (17ß-oestradiol), and that the natural inhibition of CYP1A is thus less marked. This reflects a complexity in the effects of P450 CYP1A, whereby alkylphenols may well influence the system both directly via a specific "oestrogen effect" and indirectly by influencing the natural hormone balance.

Measurements of the CYP1A and CYP3A proteins (Fig. 36 and 37) produced a different picture than the one we saw in the measurements of enzyme activity (EROD). The Western blots showed a dose-related increase in CYP1A and CYP3A in males exposed to alkylphenols, while there was no effect on females. Surprisingly enough, we found higher levels of protein in females than males. At this point in time we can offer no explanation of the lack of relationship between protein level and enzyme activity.

Arukwe *et al.* (1997) found that large doses (125 mg/kg) of nonylphenol both reduced EROD activity and the quantity of CYP1A protein in salmon. On the question of the metabolism of progesterone by liver microsomes, they found a bimodal response to alkylphenols. At low doses (1 mg/kg) they found an increase in metabolic rate, which turned into a decrease at high doses (25 and 125 mg/kg). An *in vitro* study on a primary culture of rainbow trout hepatic cells (Navas & Segner, 2000) found no effects of octylphenol exposure on EROD activity, while a major fall in EROD was observed following exposure to oestrogen. Similarly, Jeong *et al.* (2001) found that exposure to nonylphenol did not affect EROD activity in mice (Hepa-1c1c7 cells). Lee *et al.* (1996 a and b) reported that nonylphenol competitively inhibited the catalytic activity of CYP1A and CYP3A in rat liver microsomes and downregulated the amount of CYP1A protein. On the other hand, the amount of CYP3A protein was found to be upregulated in rats exposed to nonylphenol. CYP3A is also known to be important in steroid metabolism (Buhler and Wang-Buhler, 1998).

Neither the activity of GST nor of G6PDH appeared to be affected by alkylphenol exposure (results not shown). This may indicate that neither of these two enzymes is particularly important in alkylphenol metabolism. It is known that glucuronidation is of particular importance in the phase 2 metabolism of alkylphenols (Lewis & Leach, 1996; Meldahl *et al.*, 1996; Thibaut *et al.*, 1998; Arukwe *et al.*, 2000; Ferreira-Leach & Hill, 2001), and these results may indicate that this metabolic pathway is sufficient to metabolise such quantities of alkylphenols as the fish were exposed to in our experiments.

Total glutathione rose in the livers of female fish after one week of exposure to alkylphenols (Figure 38). Males were not sampled after one week, so we do not know the early response of this group to alkylphenol exposure. The second sampling showed a smaller difference in GSH levels between the control and exposed groups. The level of reduced glutathione was also measured and the ratio of reduced to total glutathione was calculated. This relationship was relatively constant, and was similar in controls, positive controls and the exposed groups. Overall, the results show that there may be a passing effect on glutathione level, but that the redox ratio remains unchanged. High, relatively stable redox ratios also indicate that the system that keeps glutathione in its reduced form, i.e. as GR and G6PDH, (which generates NADPH) functions adequately under the experimental conditions reported here. GR is affected to a certain extent (Figures 38 and 39), which suggests that the exposed fish were being exposed to increased oxidative stress (Stephensen *et al.*, 2001).

The mean level of glutathione was higher in males than females, and higher in female fish from the second sampling than the first (Fig. 38 - 40). This indicates that glutathione status may be dependent on the reproductive cycle, and that there are sex differences in GSH concentration in the cod liver. Similar phenomena are well known from mammalian studies (Sies & Ketterer, 1988; Meister & Anderson, 1983).

5.6 Bioaccumulation, toxicity and uptake

Alkylphenols are acutely toxic to fish. Data for nonylphenol show LC50 values of 17 - 3000 ppb for fish. For invertebrates the LC50 is 20 - 3000 ppb, and for algae, 27 - 2500 ppb. Alkylphenols are rapidly absorbed and accumulate in fish tissues. The bioaccumulation factor (BCF) for long-chain alkylphenols (>C4) is 75 - 1250 (Servos, 1999). A study using cod found a BCF for heptylphenol just below 600 (Tollefsen *et al.*, 1998).

The alkylphenols are metabolised rapidly, first and foremost by phase 2 enzymes that conjugate intact alkylphenols to their corresponding glucuronides. There is also a certain amount of phase 1 metabolism. The alkylphenols are excreted primarily in the bile and faeces. Uptake studies have shown that alkylphenols accumulate particularly in the bile, digestive system and liver, but it has also been shown that alkylphenol is taken up by the brain in salmon (Arukwe *et al.*, 2000), trout (Ferreira-Leach & Hill, 2001) and cod (Tollefsen *et al.*, 1998). This is of particular interest with respect to hormone-disrupting effects. Studies of PCB have shown that cod (lean fish) are more likely than trout (fatty fish) to accumulate lipophilic material in the brain (Ingebrigtsen *et al.*, 1990).

5.7 Alkylphenols in the marine environment

The produced water content of long-chain alkylphenols (C4 - C7) is low, at the lower end of the ppb range (2 - 237 ppb) (Brendehaug *et al.*, 1992). Added to this is the extremely high dilution factor associated with offshore discharges. Concentrations in the marine environment are therefore extremely low. Rye *et al.* (1996) combined a 3D hydrodynamic model (which estimated ocean currents), a 3D "multi-source" numerical model (which estimated the dilution factor for petroleum components) and a biological model to simulate egg and larval drift and swimming fish. Bioaccumulation and bioconcentration (BCF) factors were incorporated in the model. They used this combined model to estimate total concentrations of alkylphenols in fish and larvae occupying an area of the Halten Bank that is affected by discharges of produced water. The results showed that the fish could accumulate a body burden of 1 - 10 ppb.

No empirical data are available to confirm these model-based estimates. All in all, we know little of the fate of long-chain alkylphenols in produced water. The rate of breakdown of alkylphenols falls rapidly with increasing chain length. Brendehaug *et al.* (1992) measured the biological breakdown of phenols in produced water diluted with seawater, and found that phenol and cresol (C1) were broken down very rapidly (only 0.1% remaining after one week), while 33% of the initial concentration of hexylphenol (C6) and 60% of that of heptylphenol (C7) remained after one month. Another study of the breakdown of nonylphenol (C9) in seawater indicated a very slow breakdown at the beginning of the study (0.06% breakdown per day), but that the rate of breakdown increased rapidly after 28 days to 1% per day, suggesting that the microorganisms in the seawater adapt to nonylphenol as a substrate after a while. After 58 days, 50% of the original quantity of nonylphenol was still in the water (Ekelund *et al.*, 1993).

The dose of 5 ppb body burden per day should be equivalent to the quantity that the fish might be expected to take up if they were exposed to seawater with a content of 0.008 ppb of the individual component. This calculation includes a BCF factor of 600. Our dose regime would thus be equivalent to a total theoretical concentration (of all four phenols) of 0.032 ppb. This is an extremely low concentration, much lower that those that have already been reported as having an effect. The true range of concentrations of alkylphenols in areas in which produced water is discharged will have to be obtained and evaluated against the level of 0.032 ppb, if we are to be able to draw any further conclusions about the risk of effects on reproduction in cod stocks. There is also a need for studies of critical time windows for exposure.

6 Summary of important findings

The aims of this study were to find out whether alkylphenols in produced water from the offshore industry are capable of disrupting hormonal processes in cod, and whether these substances affect central enzymatic systems that are generally involved in the metabolism of foreign compounds. Other possible biological effects of alkylphenols were not examined in this study.

Effects on females:
The study demonstrated that alkylphenols reduce oestrogen levels in female fish, even at very low doses. Recalculating these doses into theoretical concentrations in seawater, we found effects at doses as low as 0.032 ppb (exposure at 20 ppb body burden), which was the lowest level studied. The possibility of effects at even lower concentrations cannot be excluded. The oocytes of exposed fish developed more slowly than normal and this effect was evident in their gonadosomatic index, which was lower than in the control group. Cod that had been exposed to the lowest concentration of alkylphenols for five weeks in November - December had an estimated start of spawning that was 21 days later than controls. Such an effect could have a strong influence on the reproductive capacity of the population, and it would be a serious matter if similar delays were demonstrated in natural stocks.

Effects on males:
The level of the male sex hormone testosterone was lower in cod exposed to alkylphenols. These fish also began to produce the egg-yolk protein vitellogenin, which is not normally produced by males. There were also significant differences in the pattern of maturation of the testis. Exposure reduced the proportion of spermatozoa (sperm) and at the lowest dose, the total amount of spermatozoa was also reduced. At the same time, there was a rise in the amount of spermatogonia and spermatocytes (earlier stages of the sperm development). A potential consequence of this is reduced fertilisation capacity, which would reinforce the consequences of a change in the time of spawning in females.

Of the central enzyme systems that participate in the metabolism of foreign substances, both cytochrome P450, glutathione and glutathione reductase were affected, though only to a moderate extent. Glutathione S-transferase and glucose-6-phosphate dehydrogenase were not affected to a demonstrable degree.

7 Future projects on alkylphenols and produced water

1. Carry out an experiment similar to experiment 2 (individual oral dosing), in which groups would be exposed from the beginning of November until after spawning. This would reveal possible changes in the time of spawning and in the level of atresia. Such an experiment could confirm existing indications that alkylphenols postpone the onset of spawning in females, even at low doses, and would also indicate whether the level of atresia increased, with the consequence of fewer eggs being spawned. Male fish also need to be studied more thoroughly. Do they spawn at the same time as the females; how much sperm can they produce (both in terms of volume and number of sperm); are the sperm less motile, and do they have a poor fertilisation capacity? The lowest dose should probably be lowered further. Should there be more individuals in each group?

2. Determine the time frame in the gonadal maturation process in cod that is most sensitive to exposure. If possible, such a study should also include the *previous* spawning season, since it has been shown that in a number of species, the female establishes the foundations of the *following year's* fecundity during or immediately after the spawning season.

3. Study more specifically what happens in the cod (brain, gonads, liver, etc.) during exposure to alkylphenols, that leads to the effects demonstrated in the present studies.

4. Look in more detail at uptake, distribution in the tissue, metabolism and excretion of alkylphenols in a range of fish species (lean vs. fatty fish)

5. Determine the importance of different dose regimes.

6. Perform uptake and membrane studies of alkylphenols in fish cell cultures. Such studies should include follicle cells and oocytes as a minimum. In males, detailed studies of Sertoli cells would be of major importance.

7. Study primary production in areas with a high level of petroleum activities and compare this with control areas. Can the growth of fish larvae and fry be affected by changes in the availability of food or in its composition? Are fish attracted to oil fields by greater availability of food? Is the sex ratio of the larval populations that grow up near petroleum installations affected?

8. Carry out field studies in which alkylphenols are measured in biota and water in defined areas around petroleum installations, as well as general background levels.

9. Compare the time of spawning of populations in the vicinity of platforms with populations inhabiting a similar physical environment (temperature, light, availability of food, and any other variables that might be supposed to affect spawning time).

10. Set out fish in sea-cages in selected offshore fields in order to survey uptake and effects under field conditions.

11. Estimate what proportion of a population, e.g. in the North Sea, that is likely to be exposed to alkylphenols.

12. Study the effects of complete produced water on eggs, larvae, fry and fish, as well as on other organisms of major importance in the ecosystem.

13. Determine the combined effects of the individual components of produced water.

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