Akvamiljø

Biomarker measurements in long term exposures of a model fish to produced water components (PAHs and alkylphenols)

Report AM-01/007

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Scope:

Temporal and dose-related biomarker effects were studied in the small tooth carp sheepshead minnow (*Cyprinodon variegatus*) exposed for five weeks to polyaromatic hydrocarbon (PAH) and C1-C5 alkylphenol (AP) mixtures. The two mixtures were made to reflect the composition of produced water from North Sea oil fields.

Key-words:

Fish, sheepshead minnow, produced water, PAH, alkylphenols, biomarker effects

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Summary

Temporal and dose-related biomarker effects were studied in the small tooth carp sheepshead minnow (*Cyprinodon variegatus*) exposed for five weeks to polyaromatic hydrocarbon (PAH) and C1-C5 alkylphenol (AP) mixtures. The two mixtures were made to reflect the composition of produced water from North Sea oil fields. The present study supplement the fecundity and gamete quality related effect studies reported earlier by Bechmann et al. (2000) and Taban et al. (2000) for the PAH and AP exposures, respectively. In the PAH study, groups of males and females were exposed to five concentrations of the PAH mixture (2, 6, 14, 51 and 214 µg/L ∑PAH), and to a dispersed North Sea crude oil (6 mg/L) as positive control (Bechmann et al. 2000). In the AP study, fish were exposed to five concentrations of the C1-C5 AP mixture (1, 5, 21, 52 and 505 µg/L ∑AP) and to 4-nonylphenol (4-NP, 11 µg/L) as positive control (Taban et al. 2000). All contaminants were administered in a continuous flow exposure system.

In fish exposed to PAH mixture, a dose dependent increase of biliary PAH metabolite fluorescence and hepatic DNA adducts was found. After five weeks of exposure, both these responses were apparent even at the lowest dose of 2 µg/L ∑PAH. A similar effect was detected in oil exposed fish, and the DNA adduct level in these fish was significant also after two weeks recovery in clean water. No induction of hepatic cytochrome P4501A (CYP1A) dependent EROD activity was found in the PAH exposed fish.

In male fish exposed to 4-NP significant induction of plasma vitellogenin (VTG) was measured at all sampling days, including after two weeks recovery. Zona radiata proteins (ZRP) were also induced in NP exposed fish, but the ZRP levels showed a downward trend during the exposure period. In fish exposed to the C1-C5 alkylphenol mixture, male vitellogenesis and zonagenesis was found in a few individuals, but a consistent group-related or dose-dependent trend was not found. Immunohistopathology examinations of VTG induced male fish revealed a strong VTG staining in the interstitial tissue surrounding the tubuli testes, and in the spermatogenic cysts at the testis periphery, possibly representing an early stage in the development of an ovo-testes condition.

Contents

Preface

This study is funded by the Norwegian Oil Industry Association (OLF). The samples of exposed fish analysed originate from two experiments under the DREAM project that were conducted in the facilities of Akvamiljø a/s (Stavanger) by Renee Bechmann and Ingrid Taban. The industrial participants in the DREAM consortium (TotalFinaElf, ENI-AGIP, Norsk Hydro, and Statoil) are acknowledged for allowing spare samples from the two exposure studies to be used for biomarker analyses. RF-Rogaland Research and Akvamiljø a/s contributed with internal funding of some of the sampling activity.

List of symbols

AP	Alkylphenol
APE	Alkylphenol polyethoxylate
Biomarker:	Pollutant responsive parameter in organisms
C1-C5 AP	Alkylphenols with 1-5 carbon sidechains
CHARM	Chemical Hazard Assessment and Risk Management model
DREAM	Dose related Risk and Effect Assessment Model
EIF	Environmental Impact Factor
FF	Fixed wavelength fluorescence
SFS	Synchronous fluorescence spectrometry
NP	Nonylphenol
NOEC	No Observable Effect Concentration
OP	Octylphenol
Oocyte:	Egg cell
Oil E&P	Oil exploration and production
PAH:	Polyaromatic hydrocarbon
VTG:	Vitellogenin (precursor for eggyolk protein)
ZRP:	Zona radiata protein (proteins that form the eggshell)
Xenoestrogen	Estrogenic pollutant compound

Introduction

In the North Sea, the discharge of produced water from offshore oil rigs and production platforms lead to a chronic low-dose contamination of the receiving water mass with polyaromatic hydrocarbons (PAHs), short-chained alkylphenols (APs) and a number of other compounds. The potential of long-term environmental effects of these discharges has gained concern in recent years. The total input of PAH and alkylphenol contaminants in the North Sea environment originates from numerous sources related to domestic activities, land-based industries, shipping, as well as the activities related to oil exploration and production (E&P). The volume of produced water released from oil platforms and installations in the Norwegian sector of the North Sea has increased from 20 to 100 million tons in the period 1993-1999 (SFT). The increase will continue during the next decade. The E&P industry must therefore evaluate whether these discharges pose a threat to the biological resources in the central North Sea environment. An important part of this work is the development of better analytical tools for assessment of long-term/low-dose impacts of various pollutant chemicals originating from the produced water discharges. The PAHs and the short-chained APs are examples of pollutant classes for which this information is needed.

PAHs are organic compounds composed of two or more benzene rings fused together. This is the class of organic pollutants released in largest quantities to the environment. PAHs are generated mostly from the incomplete combustion all kinds of carbon-containing materials and from discharges of crude oils and other liquids and materials derived from fossil fuel (Lyons 1997; Utvik 1999). Some PAHs are strong carcinogens in humans and wildlife, but due to a number of factors that determines the physical, chemical and toxicological characteristics, the PAHs appear as a highly diverse group of chemicals. With respect to environmental issues, the environmentally most significant PAHs are molecules containing two to seven benzene rings. Most studies of environmental PAH have been directed to the characterisation of the chemical levels in the environmental compartments and in biota. But later studies have focussed increasingly more on the biological effects of PAH exposures in different organisms. Increased levels of PAH-DNA adducts as well as increased incidences of various cellular disorders and even liver cancer have been found in fish populations inhabiting PAH polluted marine recipients, e.g. (Myers et al. 1998). Environmental authorities at national and international levels have prioritised actions to lower the environmental input of PAH. Crude oils contain considerable amounts of PAH, in particular the naphthalene-rich oils. In the North Sea oil industry, the discharges of oil-based drilling-mud and in later years the increased volume of produced water has therefore led to environmental concern. Presently, oil-based drilling mud are normally not discharged in the Norwegian sector, but the question of how to deal with the increasing volume of produced water is still unsolved. In 1999, the reported produced water discharges represented a release of 2467 tons of oil in the Norwegian sector (SFT). The content of dispersed crude oil (and PAHs) in produced water vary considerably between different oilfields, and also with time. The reported median concentration of PAHs in produced water in the Norwegian sector is ca 800 µg/L (Røe and Johnsen 1996). The regulative adopted in the OSPAR conventions demands the produced water to contain dispersed oil at levels below a certain limit (presently 40 mg/L) before it can be released to the environment. A revision of this regulative is ongoing in OSPAR.

Alkylphenols with short side-chains of one to five carbons (C1-C5 APs) are prevalent in crude oils and thus also in produced water discharges. The levels of C1-C5 APs in the produced water plume from North Sea production platforms have been reported sometimes to exceed 300 µg/L (Røe and Johnsen 1996). For 1999, it is estimated that approximately 15 tons of alkylphenols were released in produced water discharges in the Norwegian sector of the North Sea (SFT). The Environmental occurrence of APs may also be attributed to the breakdown of less stable alkylphenol ethoxylates (APEs). This is in particular the case of APs with longer side-chains, since long-chained APEs are among the most widely used groups of surfactants. The nonylphenol ethoxylates are, by far, the most prevalent the APEs used in industry (Renner 1997). It is estimated that the total amount of APEs produced world-wide is about 500,000 tons for being used in detergents, paints, pesticides, textile and petroleum recovery chemicals, metal working fluids, and personal care products. As with the PAHs, APs is a large class of chemical structures with diverse physical, chemical and toxicological characteristics. Much of the APs exploited by industry end up in the aquatic environment. With respect to environmental issues, alkylphenols have been almost synonymous with nonylphenols (NPs) or octylphenols (OPs), or compounds derived from these, i.e. the polyethoxylates (APEs) and polyethoxycarboxylates (APECs). However, in recent studies an increased emphasis has been put on effect studies of APs with sidechains shorter than five carbons, e.g. (Gimeno et al. 1998). NPs and OPs are acutely toxic to fish, invertebrates and algae, but most of the environmental concern recently is due to their potential as endocrine disrupters (Servos 1999). NP and OP molecules bind to the estrogen receptor, and induce responses that normally are induced by the presence of estrogen steroid hormones. In male fish, this estrogenic response can be measured as induction of the female pre-yolk protein vitellogenin (VTG). On a more long-term basis the effect may influence the growth and development of the male gonads. Ova-testes, an inter-sex condition in genetic male fish, have been described in rivers and estuaries chronically contaminated with estrogenic contaminants. The findings of endocrine effects of OP and NP chemicals have contributed to more strict environmental regulations of these substances both onshore and offshore, e.g. in the North Sea oil industry. The discharge of NP and OP derived chemicals from the offshore installations is now prohibited in the Norwegian sector. However, the question that is relevant for the produced water issue is whether the short-chained APs also may cause low-dose/long-term effects in biota.

Several model tools have been developed for environmental risk ranking and toxicity evaluation of chemicals and effluent discharges. In particular the oil industry operating in the North Sea has given model approaches high priority. The internationally harmonised CHARM model (chemical hazard assessment and risk management) is used much for minimising the use of environmentally unfriendly E&P chemicals (Karman and Reerink 1998; Vik et al. 1998). Several other models related to oil industry are presently being developed, such as the Environmental Impact Factor (EIF) and the Dose related Risk and Effect Assessment Model (DREAM). The latter model is in the first version directed towards the prediction of chronic effects related to the discharges of PAHs and short-chained alkylphenols from offshore platforms and installations. Described very briefly, DREAM is a body-burden based risk and effect assessment model with emphasis on chronic effect parameters. The model includes also complex calculations of fate and distribution of the released components in the receiving environment. A series of pollutant exposure experiments are needed to generate input data to the DREAM model, i.e. specific uptake related to exposure concentrations and specific biological effects related to doses of pollutants. Two of the DREAM

experiments also form the basis of the present study, since the samples analysed originates from the same fish specimens as were used in the reproduction-effect studies within the DREAM project. From these experiments, effects in exposed fish related to egg production, hatching time, hatching percent and frequency of larval deformities are to be used as inputs in the DREAM model for estimations of dose-responsive effects (Bechmann et al. 2000; Taban et al. 2000). However, also other effect parameters in the fish, other than the DREAM parameters, may contribute with relevant information about eventual pollutant induced stress conditions in the exposed fish. The so-called environmental biomarker approach allows the use of a wide range of pollutant responsive elements in animals as signals or tools for assessing biological effects of exposures. Broadly defined, a biomarker can be any parameter in an organism that varies due to pollutant, or pollutant mixture, exposure. The biomarker response may be on biomolecular, biochemical, cellular, or physiological levels. By using appropriate biomarkers, a pollutant impact may be identified at an early stage and at a low level of biological organisation, which often is advantageous in pollutant effect investigations. Internationally, a set of early warning biomarker parameters is already being recommended (e.g. by OSPAR) for the environmental risk evaluation of chemicals, mixtures, and effluents. Several of these are used also in the present study.

In the present study, selected biomarker effects were analysed in additional samples from sheepshead minnows being exposed continuously to various concentrations of waterborne mixtures of PAHs and C1-C5 alkylphenols. The objective was to provide a supplement to the reproduction effect endpoints that will be used in the DREAM model. These supplementary effect parameters will also be measured in field studies. In fish exposed to PAH mix and crude oil, PAH metabolites in bile, hepatic EROD activity and hepatic DNA adducts were analysed. In fish exposed to alkylphenol mix and nonylphenol, vitellogenin and zona radiata protein in male plasma, gonadal histology and immunohistochemical localisation of VTG in testes were analysed. The biomarker results are discussed in connection to the reproduction effect data that will be used in the DREAM model. Additionally, a bioassay on hatching success of eggs in the AP mix exposure solutions is presented, and these results are compared to the DREAM effect data where the eggs were hatched in clean water.

Materials and methods

Fish, exposure systems and treatment chemicals

The sheepshead minnow (*Cyprinodon variegatus*, figure 1) was used as study organism in the DREAM studies. This small, estuarine fish species belong to the cyprinodontidae (common family names: tooth carps, killifishes or pulpfishes) and occur normally at the Atlantic and Gulf coasts of the USA. It is suitable for chronic (egg-to-egg) bioassays since it is easily held in the laboratory and produces numerous eggs (in warm water, 25-30°C). The generation time is short (3-4 months) and its small adult size (typical male length 48mm) provides for downscaled fish bioassays. The fish was purchased from Sea Plantations, Salem, Massachusetts, US, and from Aquatic Research Organisms, Hampton, New Hampshire, US. The fish were brought to the Akvamiljø research laboratory (Stavanger, Norway) and acclimated prior to the exposure studies.

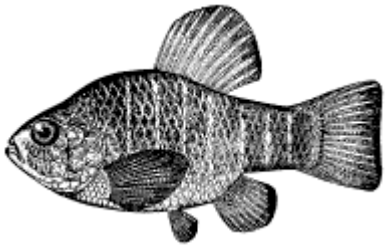
Male   Female

Figure 1. Sheepshead minnow (*Cyprinodon variegatus*) is recommended by the OSPARCOM for reproduction/life cycle tests. The fish is relatively easy to maintain in the laboratory, and has a short life cycle. Fertile eggs may be cultured to mature adults within 3 months in the laboratory. Photo courtesy of Baylink (http://www.baylink.org/).

In the two experiments the fish were exposed to the pollutant chemicals by means of a continuous flow system. The PAH and AP mixtures used for exposure of fish were designed to mimic a typical composition of PAH and AP chemicals in produced water. As positive control treatments, dispersed North Sea crude oil was used in the PAH mix experiment, whereas 4-nonylphenol (Fluka Chemika) was used in the AP mix experiment. A detailed overview of the exposure systems, treatment conditions, exposure concentrations and the experiment sampling groups are provided by Bechmann et al. (2000) for the PAH mix study and by Taban et al. (2000) for the C1-C5 alkylphenol mix study. Only a brief summary is given in the present study. The pollutant concentrations measured in the exposure seawater were as follows: PAH mix: 2, 6, 14, 51 and 214 µg ΣPAH/L. Dispersed crude oil treatment: 6 mg/L (representing 53 µg ΣPAH/L). AP mix: 1, 5, 21, 52, 505 µg ΣAP /L. 4-nonylphenol: 11 µg/L. In both experiments an acetone control (acetone concentration 25µl/L seawater) and two blank controls (seawater only) were included.

Sampling of fish for biomarker analyses

The various groups of sheepshead minnows were first sampled for the DREAM related parameters (egg count, etc.) and were then available for the additional sampling for biomarker parameters. From each group, a maximum of three males and seven females were sampled, the histopathology sampling was performed only on a subset of specimens. After stunning the fish and subsequently recording the fish size, a small volume (10-100 µl) of blood was obtained from the caudal vein by means of a heparinised syringe (insulin type needle). The blood sample was then ejected in a pre-cooled eppendorf sample vial and diluted 10x in ice-cold PBS buffer (pH 7.3). The diluted blood sample was then kept on ice until centrifugation (2000*g* x 4 min at 4°C) for the preparation of (10x diluted) plasma. The diluted plasma fraction was transferred to a new vial and stored at –80°C until analysis of VTG and ZRP. The liver and gallbladder were excised. The gallbladder bile was stored at –80°C until analysis of PAH metabolites. The liver was weighed and then split in sub-samples. One liver sub-sample was homogenised in four volumes of cold homogenisation buffer and centrifuged (12000*g* x 10 min at 4°C) for preparation of the post-mitochondrial supernatant (PMS) and the mitochondrial pellet (which contain nuclei and

mitochondria). The PMS was stored at –80°C until analysis of EROD activity and the mitochondrial pellet was stored at –80°C until analysis of PAH-DNA adducts. Histological samples were obtained by immersing internal organs in ice-cold neutral buffered formalin (formaldehyde 4%, obtained from Norsk Medisinaldepot). Additionally, a whole body fixation was performed in a few specimens by perfusion of fixative in blood circulation system before immersing the whole fish in fix.

Analyses of biomarker parameters

PAH metabolite fluorescence signals in bile were measured with fixed wavelength fluorescence (FF) and synchronous fluorescence spectrometry (SFS) according to the methods described by Aas et al. (2000). The bile samples were diluted 1:1600 in 50 % methanol and then measured in a quartz cuvette on a Perkin Elmer LS50B luminescence spectrometer with slit widths set at 5 nm for both excitation and emission wavelengths. FF fluorescence was detected at the excitation/emission wavelength pairs 290/335 nm, 341/383 nm and at 380/430 nm, denoted $FF_{290/335,}$ $FF_{341/383}$, $FF_{380/430}$, respectively. The signal level in solvent blanks was subtracted. SF was measured in the range 200-500 nm with $\Delta\lambda$ 42 nm.

The hepatic CYP1A level was quantified in post-mitochondrial supernatant (PMS) by means of ethoxyresorufin-*O*-deethylase (EROD) activity according to the protocol described by Nilsen et al. (1998). Measured EROD activities were standardised towards PMS protein in the sample. The protein level was measured according to Bradford (1976) with BSA (Sigma, 50 mg/ml) as standard.

The pellet from preparation of hepatic PMS was used for detection of bulky hydrophobic DNA adducts by means of the ^{32}P-postlabelling assay. DNA was isolated from the pellet sample as described by Reddy and Randerath (1987). The measurement of adducts was conducted according to the protocol of Reichert and French (1994) with salmon sperm DNA as blank sample. Briefly, DNA was enzymatically hydrolysed to deoxyribonucleoside 3'-monophosphates using micrococcal endonuclease and spleen phosphodiesterase. Aliquots of hydrolysed DNA were treated with nuclease P1 (Reddy and Randerath 1986) which selectively hydrolyses normal 3'-mononucleotides to nucleosides, thereby enriching the mixture in adducted 3'-monophosphates. Samples enriched in DNA adducts were then postlabelled using [γ-^{32}P] ATP. The ^{32}P-labelled adducts were then chromatographed on polyethyleneimine-cellulose thin-layer chromatography (TLC) sheets prepared in the laboratory as described by Reichert and French (1994). Total nucleotides were determined by postlabelling an aliquot of DNA hydrolysate followed by one-dimensional TLC of 5'-labeled nucleotides using 0.24 M ammonium sulphate in 8 mM sodium phosphate, pH 7.4, as a solvent and quantitation of the deoxythymidine-3',5'-bisphosphate spot. The ^{32}P-labelled DNA-adducts and DNA bases on the chromatograms were located and quantitated using storage phosphor imaging technology as described by Reichert et al. (1992).

Vitellogenin (VTG) and *zona radiata* proteins (ZRP) were analysed in diluted plasma by means of a semiquantitative indirect Enzyme Linked ImmunoSorbent Assay (ELISA). Primary antibodies for both ELISA assays were obtained from Biosense Laboratories, Bergen, Norway. VTG was detected with monoclonal antibodies against purified Killifish VTG (Biosense product MAb ND-5F8). ZRP was detected with polyclonal antibodies against purified ZRP from salmon (Biosense product PAb 0-173).

The ELISA assays were performed according to protocols described by Oppen-Berntsen et al. (1994) and Arukwe et al. (1997).

For histopathology and immunohistochemistry (VTG and ZRP immunolabelling) the tissues samples were fixed in neutral-buffered formalin (4% formaldehyde final concentration) for several weeks before paraffin-embedding. Tissue sections (3-5 µm thick) were collected onto 3-aminoalkyltriethoxysilane-coated glass slides (Sigma), dried for 48 hours at 37^0C, and deparaffinized. The sections were autoclaved (immersed in citrate buffer, pH 6.0) for 15 minutes at 121^0C before being cooled down for 40 minutes. Endogenous peroxidase was blocked with 0.03% hydrogen peroxide in methanol (Merck) for 30 minutes. After blocking with 20% normal goat serum (Sigma) in phosphate-buffered saline (pH 7.2) to block non-specific binding sites, the sections were immunostained with monoclonal anti Killifish VTG (Biosense MAb ND-5F8) or with polyclonal anti salmon ZRP (Biosense PAb 0-173) (both diluted 1/200) for 1 hour at room temperature. Dilution was performed in PBS containing 1% BSA (Fluka). After incubation with these primary antisera, sections were first incubated with biotinylated universal secondary antibody for 30 minutes (Vector laboratories, Vectastain Elite ABC kit), followed by treatment with Vector Elite ABC. Colour development was performed for 5 minutes using diaminobenzidine (DAB, Sigma). Between the various steps, sections were thoroughly rinsed in PBS containing 0.05% Tween-20 (Merck). Sections were counterstained with Mayer's hematoxylin for 60 seconds, dehydrated and mounted. Each IHC run included a negative serum control in which the primary antibody was omitted.

Hatching success in AP exposed eggs

In the DREAM project, eggs from exposed fish were transferred to clean seawater for evaluation of hatching success (Bechmann et al. 2000; Taban et al. 2000) . However, during the AP experiment an additional study was conducted, in which the hatching success was studied in spawned eggs collected from the AP exposure aquariums. These were not transferred to clean seawater but instead kept in hatching aquaria in the AP exposure solution. Eggs spawned in two dose-groups (21 and 505 µg/L AP mix) as well as from a control group were used for this additional study. The procedure used for scoring hatching success is described by Bechmann et al. (2000) & Taban et al. (2000).

Results

Bile fluorescence in PAH exposed fish

A dose-responsive increase of the biliary fluorescence was observed in the groups of sheepshead minnows exposed the PAH mixture (figures 2, 3 and 4). In particular the detection at the 'pyrene WLP' was sensitive (figure 3). After five week of exposure the elevation of this signal was statistically significant already in the 2 µg/L group. Unfortunately, only one individual bile sample was available from the sheepshead minnows exposed to dispersed crude oil. In this sample, however, all three fluorescence

signals were high, the 380nm/430nm signal even higher than in the fish exposed to the highest dose of PAH mix (figure 4).

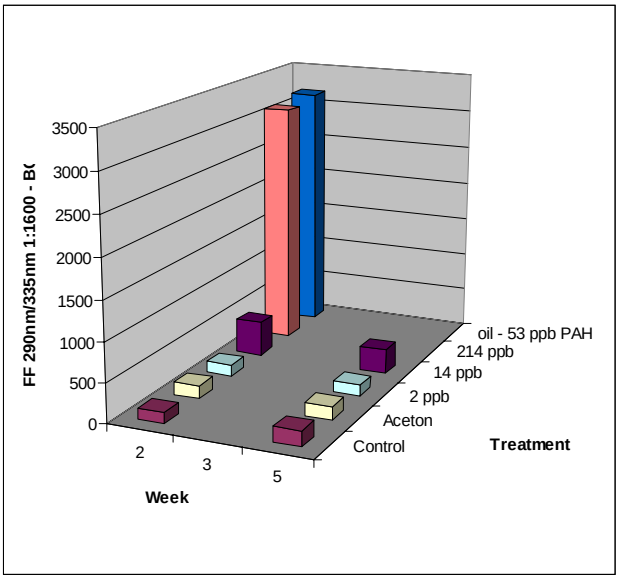


Figure 2. FF fluorescence of diluted bile at the wavelength pair (WLP) 290nm/335nm. Fluorescence at this WLP reflects the presence of metabolites of two-three ring PAHs, in particular the conjugated and hydroxylated naphthalenes.

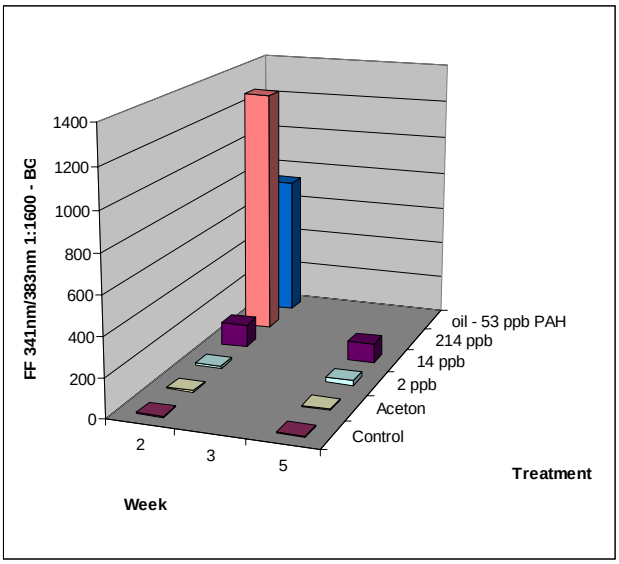


Figure 3. FF fluorescence of diluted bile at the wavelength pair (WLP) 341nm/383nm. Fluorescence at this WLP reflects the presence of metabolites of medium sized (four-ring) PAHs, in particular represented by conjugated and hydroxylated pyrene.

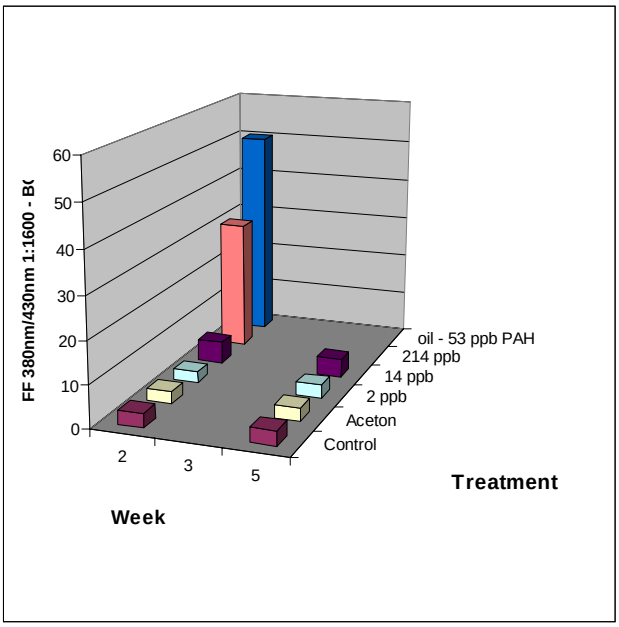


Figure 4. FF fluorescence of diluted bile at the wavelength pair (WLP) 380nm/430nm. Fluorescence at this WLP reflects the presence of metabolites of some larger PAHs, such as conjugated and hydroxylated Benzo[a]pyrene.

Hepatic EROD activity in PAH exposed fish

No induction of hepatic EROD activity was found the fish exposed to PAHs or dispersed crude oil (figure 5). The mean levels detected in the PAH groups was even lower than in the two control groups. The EROD level in the control group was not stable during the five week study period, but showed a downward trend.

DNA adduct analyses in PAH exposed fish

The DNA-adduct levels detected in the hepatic PMS pellet showed a dose-responsive relation to the PAH concentration in the exposure water (figure 6 and figure 7). The level of DNA adducts detected in the 214 µg/L exposure group (figure 6) hints that a plateau in response was approached. The adduct levels measured in the dispersed oil exposed group was high also after two weeks recovery (figure 6 and figure 7J and 7K). The control DNA samples were all very clean with no evidence of background DNA damage (see figure 7A-7C). The actual values of all controls were less than the detection limit of the adduct-analysis (1 nmol/mol).

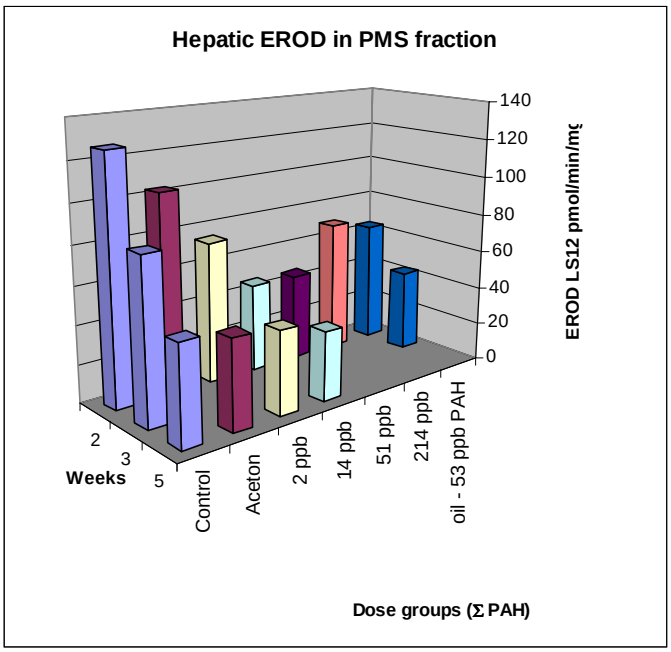


Figure 5. CYP1A dependent EROD activity in liver PMS fraction from fish exposed to various doses of PAH mixture and dispersed crude oil.

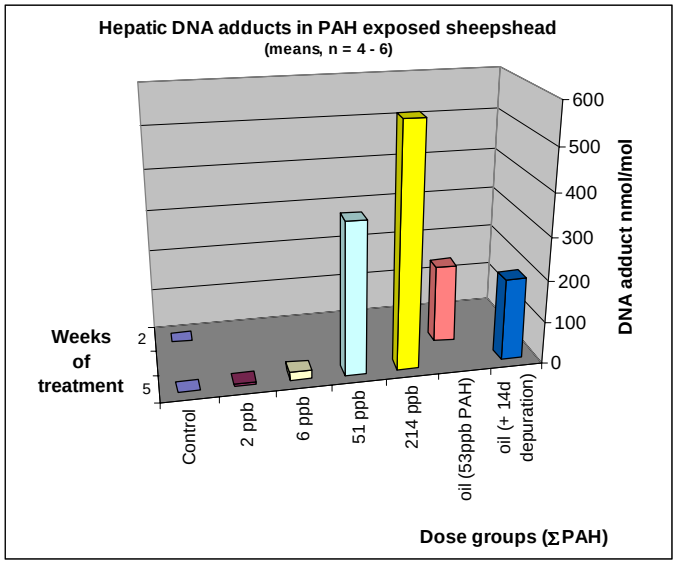


Figure 6. DNA adduct levels measured by ^{32}P-postlabelling in hepatic PMS pellets from sheepshead minnows exposed to various doses of PAH mixture and dispersed crude oil.

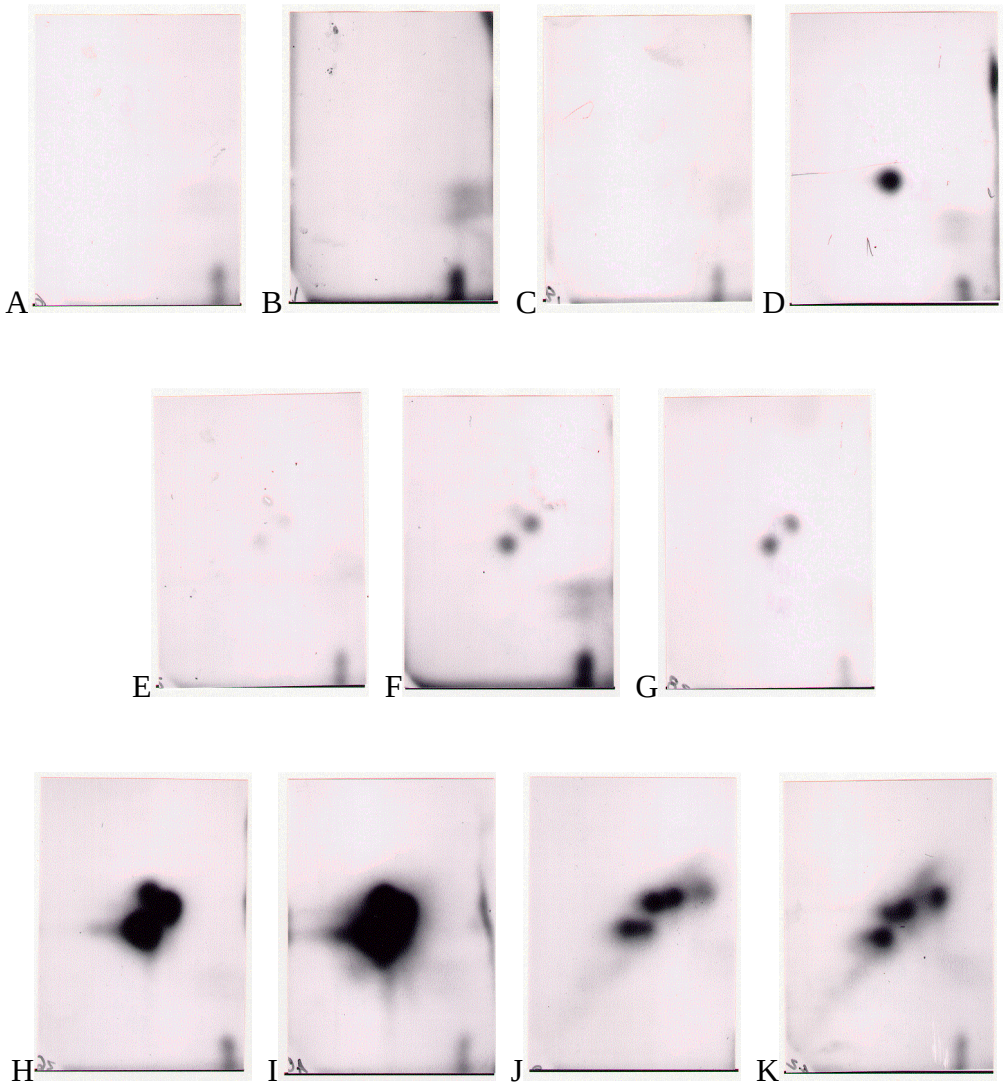


Figure 7. Representative autoradiograms from ^{32}P-postlabelling DNA adduct analyses of hepatic PMS pellets from sheepshead minnows exposed to various doses of PAH mixture and dispersed crude oil. A: control (2wk (exposure of fish)/21hr (developing of autoradiogram)), B: control (2wk/90hr), C: control (5wk/21hr), D: BaP std (positive ref sample), E: 2 ppb (2wk/21hr), F: 2 ppb (2wk/90hr), G: 6 ppb (5wk/21hr), H: 51 ppb (5wk/21hr), I: 214 ppb (5wk/21hr), J: oil (3wk/21hr), K: oil (3wk+2wk depuration/21hr).

VTG and ZRP in AP mix and NP exposed fish

Strong induction of plasma vitellogenin levels were found in male sheepshead exposed to 4-nonylphenol (positive control), whereas there was no consistent effect-trend in males from the groups exposed to the short-chained alkylphenol mixture (figure 8). However, some AP exposed individuals exhibited induced VTG levels, but it seemed not to be any consistency in relation to dose and time. No VTG induction was observed in the two control groups throughout the 5 weeks study period.

The levels of plasma *zona radiata* protein in males from groups exposed to the AP mixture were generally corresponding with the observed VTG levels, but the background signal (noise) was substantially higher (figure 9). This indicates the polyclonal ZRP antiserum to be considerably less specific than the monoclonal antibodies used for detection of VTG proteins. The plasma ZRP levels in the 4-nonylphenol group (positive control) showed a clear downward trend during the exposure period. This was unlike the VTG responses, which were stable throughout the exposure period (Figure 8).

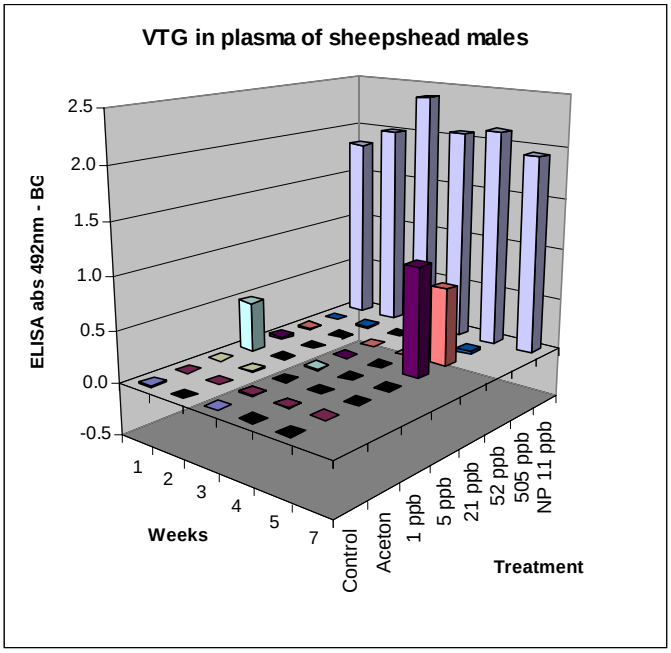


Figure 8. ELISA measurements of plasma vitellogenin levels in male sheepshead minnows exposed to various doses of alkylphenol mix and 4-nonylphenol group (positive control).

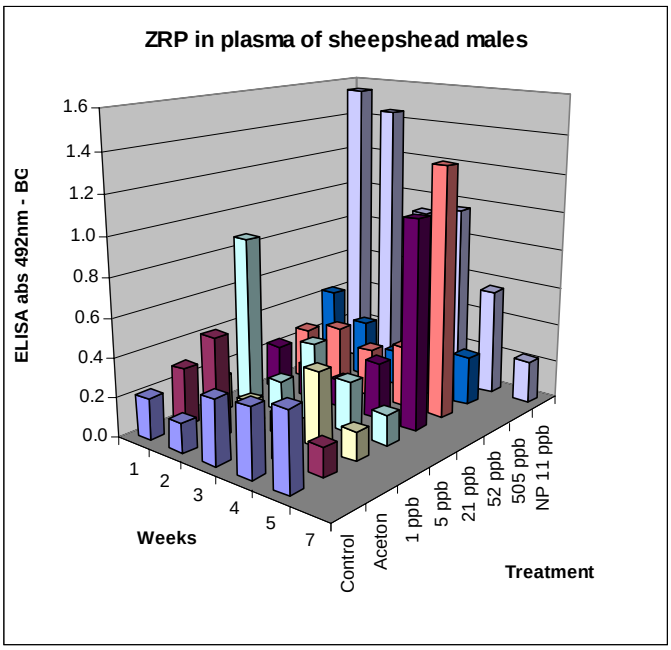


Figure 9. ELISA measurements of plasma *zona radiata* protein levels in male sheepshead minnows exposed to various doses of alkylphenol mix and 4-nonylphenol group (positive control).

Histopathology in AP exposed fish

The histological localisation of VTG proteins by means of immunohistochemical analyses revealed a strong and characteristic staining in male gonads (testes) from fish exposed to 4-nonylphenol. Strongest staining in the testis tissue was observed in Leydig-like cells surrounding the tubuli testes and in the spermatogenic cysts at the testis periphery (figure 10A). Analyses of ovaries from untreated females demonstrated the normal histological localisation of VTG proteins in oocytes of different maturation (figure 10B). The latter was in accordance with the normal histology of female sheepshead minnow earlier reported by Selman and Wallace (1982). The ELISA analyses revealed that a few AP mix exposed males exhibited high VTG levels in plasma. In testes of these males a similar VTG staining as observed in NP exposed males was detected, though distinctly less in the interstitial cells (not shown).

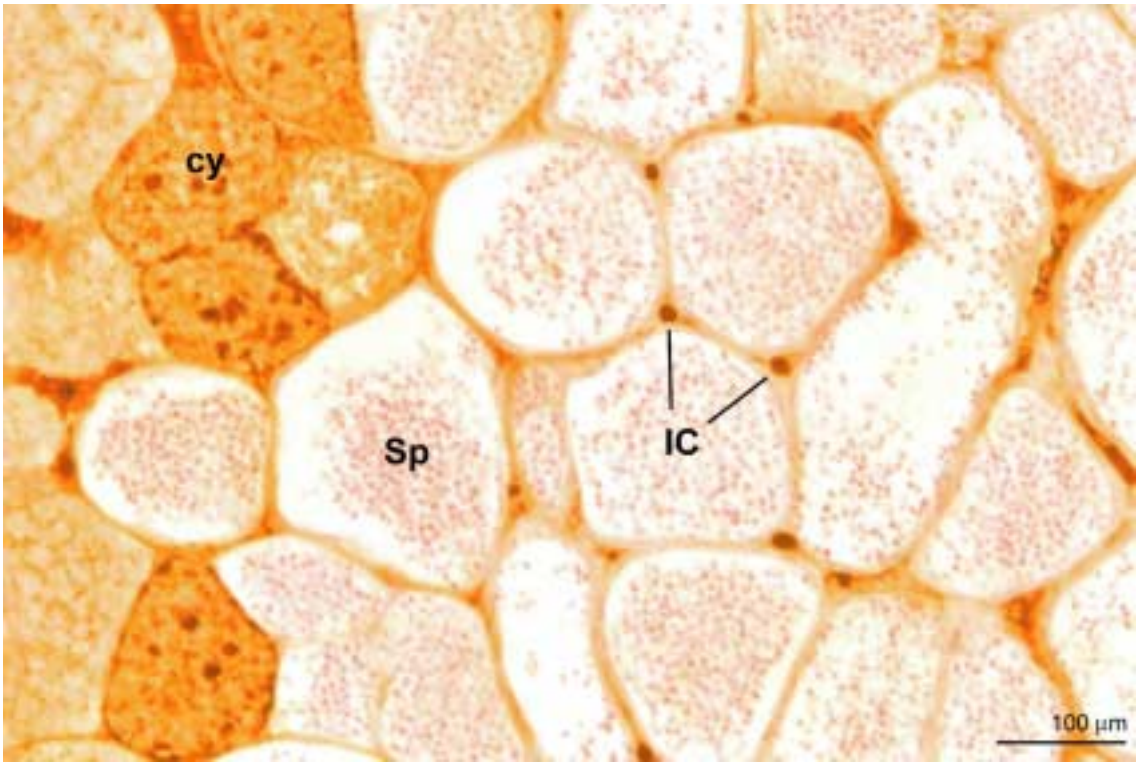


Figure 10A. Light micrograph of a longitudinal section through the outer and middle region of testes from a male sheepshead minnow *Cyprinodon variegatus*, exposed to 4-nonylphenol (11 µg/L) for 5 weeks. Spermatogenic cysts (cy) with Sertoli-cells and various stages of germ cells, and efferent tubuli filled with spermatozoa (Sp) are shown. Leydig-like yellow-brown stained interstitial cells (IC) can be seen between the tubuli.

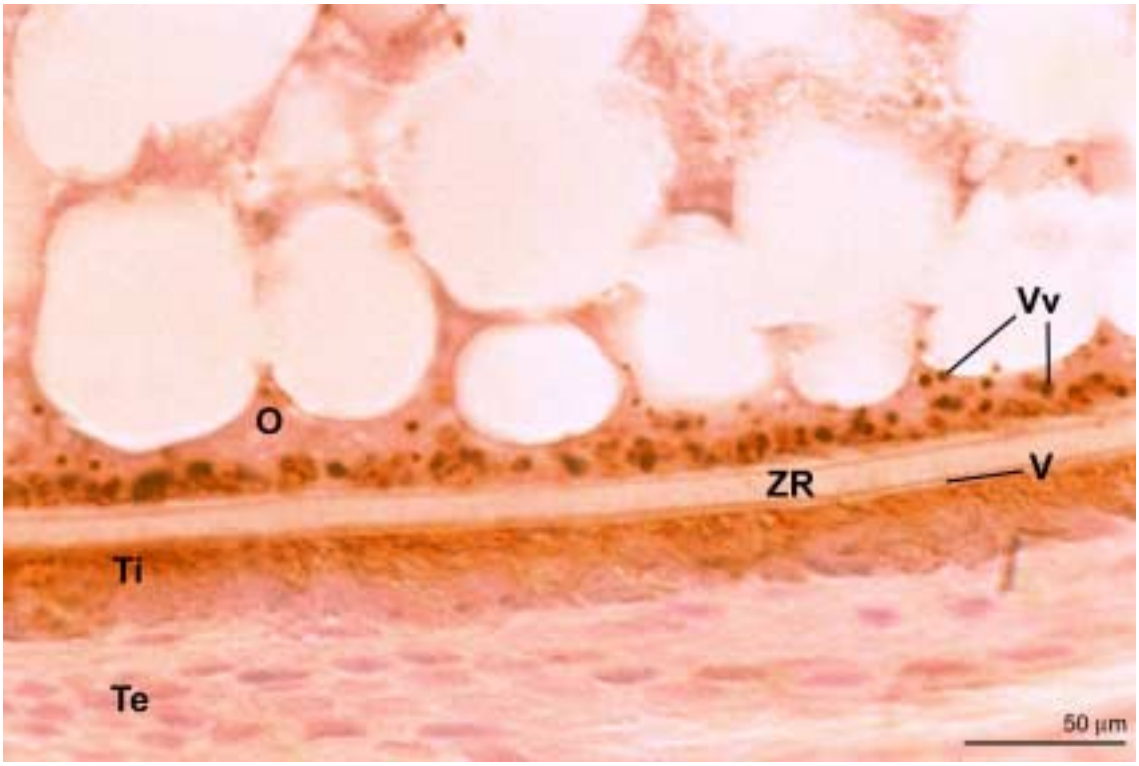


Figure 10B. Light micrograph of a cross-section through the outer region a vitellogenic follicle from female *Cyprinodon variegatus*, showing the oocyte (O) and its surrounding acellular and cellular investments. Te, theca externa; Ti, theca interna; ZR, zona radiata; V, vitelline envelope; Vv, vitellogenin-containing vesicle. The yellow-brown stained vitellogenin can be seen accumulating in the theca interna and in vesicles at the oocyte side of the zona radiata.

Hatching success in AP exposure solutions

In eggs kept for hatching in AP mix exposure solutions a lowered hatching success was found as compared to the corresponding DREAM study treatments, where the exposed eggs were allowed to hatch in clean seawater. In the highest AP exposure concentration (505 µg/L) the effect was apparent at all sampling times, and none of the eggs hatched at the sampling after 3 and 4 weeks of parental exposure (figure 11). Also eggs exposed to 21 µg/L seemed to exhibit a reduced hatching success when compared to the mean of the control groups. In this study, the mean hatching success of three seawater controls (two seawater control groups from the DREAM experiment, and the seawater control from the additional study) was used as the control reference value.

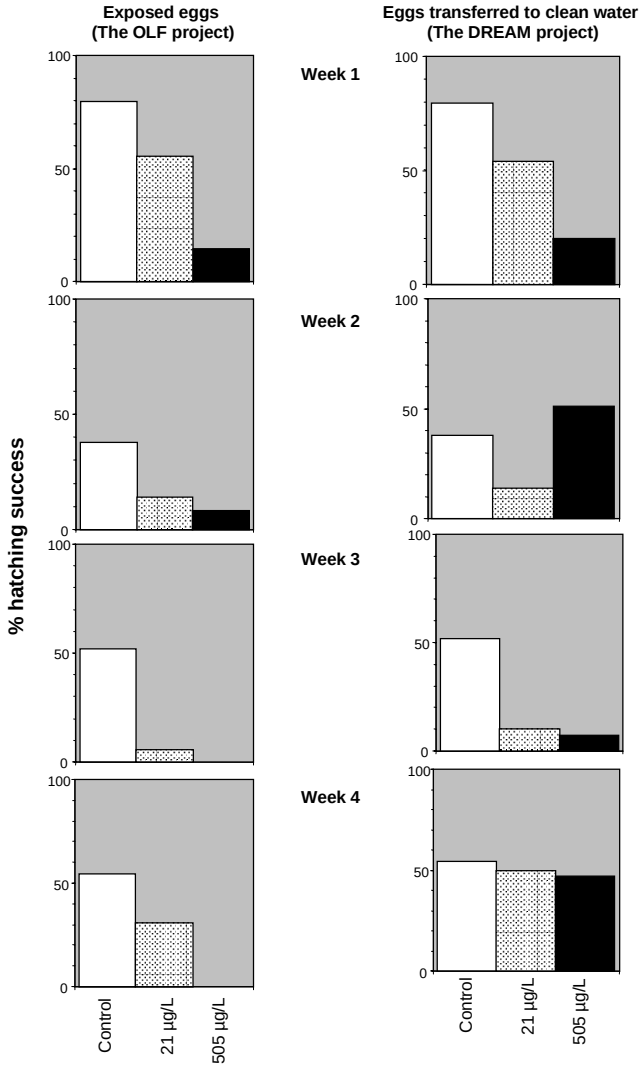


Figure 11. Hatching success for eggs from *Cyprinodon variegatus* exposed to 21 µg/L and 505 µg/L of a mixture of alkylated phenols for 1, 2, 3, and 4 weeks. *Left:* Eggs exposed to the same concentrations as the parent fish. *Right:* Eggs from exposed fish transferred to clean water. The control columns are the mean of the two seawater controls in the DREAM experiment and the seawater control in the OLF part of the experiment.

Discussion

The presently reported study was performed with additional samples from sheepshead minnows exposed during two experiments in the DREAM project (Bechmann et al. 2000; Taban et al. 2000). In these DREAM studies, the effects related to egg production, egg quality, and larval deformities were studied in sheepshead minnows exposed to PAH and AP mixtures whose composition profiles were relatively similar to produced water. The present biomarker study aimed at expanding the available information for evaluating which responses and effects that occurred in the fish. In particular, any relevant biomarker signals that showed a dose-responsive relation to the exposure doses were of interest.

Biomarker responses to PAHs and dispersed crude oil

As shown by the ^{32}P-postlabelling analyses, the exposure of sheepshead minnows to the PAH mixture and to dispersed crude oil caused a significant formation of DNA adducts in the liver tissue. These findings are consistent with other studies showing hepatic DNA adduct formation in fish when exposed chronically to PAHs or crude oils either in the laboratory or in the field, e.g. (Kurelec et al. 1992; Reichert et al. 1998; Harvey et al. 1999; Aas et al. 2000; Aas et al. 2001). Other DNA adduct studies with sheepshead minnow have not been found, but with the related species killifish (*Fundulus heteroclitus*) Willett et al. (1995) reported a dose-dependent increase in formation of DNA adducts in fish treated with 1-50 mg/kg of benzo[a]pyrene. In a field study at sites heavily polluted from industrial activities McCain et al. (1996) found significantly elevated hepatic DNA adduct levels in Gulf killifish (*Fundulus grandis*) and longnose killifish (*F. majalis*) from the most polluted sites. Based on the present results, DNA adduct formation in sheepshead minnows appears to be highly sensitive as biomarker of PAH effect. Not unexpectedly, highest adduct levels were detected in the PAH mix dose-group which received the strongest PAH dose. Interestingly, significant effects of the PAH mix exposure, in particular at the highest exposure concentrations, were also found on egg production and other reproduction related effect parameters in the DREAM study (Bechmann et al. 2000). The sensitivity of the DNA adduct biomarker was illustrated in the present study since in fish from the lowest treatment dose (2 µg/L PAH mix) an elevated level of DNA adducts was apparent. Thus, as compared to the NOEC value (No Observable Effect Concentration) for the most sensitive effect parameter (reduction of the egg production) reported by Bechmann et al. (2000), a significant increase of DNA adducts occurred at least at a 20 times lower PAH concentration. The present study is also interesting with regard to the procedure used for processing samples for the DNA adduct analysis. Due to the small livers of the sheepshead, the DNA adduct analysis was carried out with the hepatic 12,000*g* pellet fraction originating from the procession of 12,000*g* supernatants for the EROD assay. Others have not reported this procedure previously, but the clear results found in the present study clearly indicate this procedure to be feasible.

Interestingly, also the pyrene-related bile fluorescence signal was significantly increased at the lowest PAH-mix dose-level in the present study, illustrating the applicability of the bile fluorescence as a sensitive and cost-effective screening parameter of PAH exposure. The link between PAH-related bile-fluorescence and hepatic DNA adduct

formation in fish have been studied rather extensively by others, e.g. (Stein et al. 1992; van der Oost et al. 1994; French et al. 1996; Myers et al. 1998; Lyons et al. 1999; Aas et al. 2000; Aas et al. 2001). PAHs are readily metabolised in fish, and both the bile fluorescence increase and the adduct formation are mainly attributed to the *in vivo* presence of PAH metabolites. Thus, both these biomarkers reflect a flux of PAH rather than the body burden of unmetabolised parent PAH compounds. The latter has until now most commonly been used in PAH monitoring studies. Due to the metabolic capabilities of the fish, however, detection of parent PAHs often fail to reflect a dose-responsive pattern in exposure studies, or in field investigations at PAH polluted locations. Several international authorities, such as OSPAR and ICES, now recommend the use of bile fluorescence and DNA adducts as relevant biomarkers for evaluating PAH pollutant stress in fish. These two biomarker parameters can apparently be integrated in risk assessment models like DREAM that are based on dose-response relationships

In PAH exposed fish in the present study, hepatic EROD activity was not induced. The EROD even appeared to be decreased as compared to the levels measured in the control fish. This may seem odd since the bile fluorescence and DNA adduct biomarkers very clearly showed that the PAH stress-level was considerable, in particular in the highest dose groups. Induction of cytochrome P4501A (CYP1A) measured as hepatic EROD activity is previously reported in many PAH effect studies in fish, e.g. (Vanderweiden et al. 1994; Willett et al. 1995; Beyer et al. 1997; Sandvik et al. 1997). However, the apparent lack of positive EROD response in the present study is most likely attributed to the PAH mixture used, since this may not have included sufficient amounts of the CYP1A inducing PAHs. About 90% of the PAH mixture consisted of naphthalene and C1-C3 naphthalenes which are not known to be potent CYPIA inducers. Other studies on CYP1A/EROD induction in sheepshead minnow are not available, but a few studies with other species of the killifish family have been reported. Willett et al. (1995) found a strong and dose-responsive induction of hepatic EROD activity (max induction 1529 pmol/mg/min) in killifish (*Fundulus heteroclitus*) exposed directly to 1-50 mg/kg of benzo[a]pyrene, one of the most potent CYP1A inducing compounds. Based on these data, Willett et al. concludes that killifish are highly responsive to CYP1A inducers. It may be expected that also sheepshead is normally CYP1A responsive, but since significant species differences in CYP1A responsiveness have been found previously for rather closely related fish species, e.g. (Eggens et al. 1996), an exposure-effect study with B[a]P or an alternative compound such as β-naphthoflavone (BNF) should be performed before conclusions can be drawn.

Biomarker responses to C1-C5 APs and NP

Unlike the DNA adduct and PAH metabolite responses in the PAH study, no simple first-order dose-responsive biomarker responses were recorded in the fish exposed to the AP mixture. Some of the AP exposed male fish, however, exhibited strong vitellogenesis, observed as high plasma VTG ELISA signals. None of the fish in the seawater or acetone control groups showed any VTG induction, thus ruling out the possibility of analytical and methodological errors. Additionally, the VTG induced AP exposed individuals were also high in ZRP ELISA signals and this confirms the VTG induction observed. The reason why only a few random scattered individuals in the AP exposed groups exhibited an estrogenic effect is not understood, but the results may

indicate that these individuals were more sensitive than the individuals not responding. In the DREAM study reported by Taban et al., certain effects on the reproduction parameters were observed in sheepshead minnows exposed to the C1-C5 alkylphenol mixture, but there was no positive dose-dependency related to the exposure concentration. In fact, the lowest egg production was recorded in the group exposed to the lowest concentration of AP mix (Taban et al. 2000). Possibly, the unclear effects of the AP mix seen both in Taban et al. (2000) as well as in the present study indicate that there was an effect, but it was not detected with the biomarker parameters measured. Thus, the lack of dose-responsiveness should not be regarded as a negative result. With regard to the VTG and ZRP measurements, it is also clear that a larger number of specimens per sampling day (presently n=3) may have given results more in consistence with the exposure concentrations. In the nonylphenol exposed male fish, on the other hand, there was a clear and consistent estrogenic effect of the exposure throughout the exposure period.

The clear induction of VTG and ZRP in the 11µg/L NP exposed group illustrates that sheepshead minnow is clearly responsive towards xenoestrogens. The results also demonstrate that the chronic exposure system and the two ELISA assays performed well. Interestingly, also Taban et al. (2000) found significant effects (on egg production, hatching and larval deformity) in the NP exposed group.

Also a few other recent studies have described the vitellogenic response in sheepshead minnows (Bowman et al. 2000; Folmar et al. 2000; Denslow et al. 2001; Hemmer et al. 2001). These studies have mostly used various estrogens as treatment agents in VTG induction studies, but in Hemmer et al. (2001) also *p*-nonylphenol is used. Hemmer et al. studied dose-responsiveness of VTG mRNA and plasma VTG in sheepshead minnow exposed to *p*-nonylphenol (0.64, 5.38, 11.81, 23.27 and 42.67 µg/L) for 42 days. They found a clear dose-dependent expression of VTG mRNA levels whereas the plasma VTG levels were less dose-dependent, e.g. in the three highest dose groups the plasma VTG levels were equal in the second half of the exposure (Hemmer et al. 2001). They also observed that fish exposed to the lowest NP dose, 0.64 µg/L, exhibited variable plasma VTG levels, which possibly may indicate sensitivity differences among the fish in the same treatment group. This may be a similar phenomenon as observed in the AP mix exposed groups in the present study.

The immunohistochemical examination of male gonads in the NP exposed fish demonstrated that VTG was translocated to specific cellular structures in the tissue surrounding the tubuli testes and granulated in the spermatogenic cysts at the terminal end of the lobules at the testis periphery. Examination of female gonads demonstrated the location for normal incorporation of VTG in the maturing oocytes, as strong staining was observed in the cells of theca interna and in vesicles in the oocyte at the inside of the zona radiata. In the VTG induced individuals from the AP exposed groups a very weak, diffuse staining of the intratubular cells and of the spermatogenic cysts was seen. One exception here was the 5-week group, which had received 148 ppb, where stronger staining could be seen, mainly in the peripheral cysts.

In the testes, the interstitial spaces between the seminiferous tubules are filled with connective tissues, nerves, as well as blood vessels and lymphatic vessels. The interstitial tissue is also where secretory *Leydig cells* are found. These cells produce androgen steroid hormones (e.g. testosterone), responsible for the development of the secondary male sex characteristics. As judged from the present immunoslides, the

strongest VTG staining in the interstitial tissue was located to the blood vessels but, interestingly, also to Leydig like cells. Analogue cellular structures to the male Leydig cells in the female gonad are the hormone producing cells in theca interna, i.e. the inner part of the follicle that surrounds the oocyte. These cells are suggested to produce steroids that subsequently are converted into estradiol. Possibly, the present observation of strong VTG staining of Leydig-like cells in the interstitial tissue between tubuli testes, and in the spermatogenic cysts at the testis periphery, represent an early stage of oocyte formation. Thus, signalling ovo-testes condition development in the vitellogenic males. Clearly, the immunohistochemistry results in the present study illustrate the value of histopathological biomarkers for describing the cellular localisation of effects induced by pollutant compounds. They also indicate the potential of VTG immunohistochemistry of male gonads as an early warning biomarker for development of ovo-testis in xenoestrogen affected male fish.

Hatching in AP mix exposure solutions

In the DREAM study of AP effects on hatching, the parent fish were exposed to AP mix and the eggs were hatched in clean water (Taban et al. 2000). In the present additional hatching study, fertilised eggs were hatched in two concentrations of AP mix contaminated water. Apparently, the extra AP mix treatment resulted in an enhanced inhibitory effect on the hatching, in particular in the highest AP mix concentration. However, it was observed an increased growth of bacteria (or algae) in at the higher concentrations of AP mix. It should not be disregarded that this may have influenced on the hatching success of the AP exposed eggs.

Conclusions

The model fish sheepshead minnow exhibited dose-responsive increase of biliary PAH metabolite fluorescence and formation of hepatic DNA adducts when exposed to a PAH mixture which resembles a typical profile from produced water. The NOEC value for these biomarker signals was 2 µg ΣPAH /L seawater. The responses were of first-order type and it seems that these biomarker parameters can be integrated in risk assessment models like DREAM that are based on dose-response relationships. Hepatic EROD activity, on the other hand, was not increased in the PAH exposed fish.

A mixture of C1-C5 alkylphenols (APs), with a composition typical for produced water, did not produce consistent estrogenic effects (male VTG and ZRP induction) in exposed fish. However, strong induction signals were recorded in some of the exposed individuals, whereas none of the negative control fish were induced. Due to this variation in the AP exposed fish the study is not conclusive with respect to feasibility of estrogenicity biomarkers in risk assessment models with respect to produced water discharges. At the same time there were undoubtedly responses signalling feminisation in some of the AP exposed individuals. A follow up study to clarify this effect phenomenon is therefore recommended.

In fish exposed to nonylphenol a strong and consistent VTG induction was measured throughout the five weeks exposure period. The latter illustrate the feasibility of

sheepshead minnows in effect studies with estrogenic chemicals. The nonylphenol exposure also caused immunohistochemical VTG staining in Leydig-like cells in the interstitial tissue between the tubuli testes, and in the spermatogenic cysts, in the male gonad. Possibly, this effect which reflect an early stage in the development of an ovo-testes condition in the NP exposed fish, and which may be a potential early warning biomarker of this condition in xenoestrogenicity studies.

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