APPENDIX D:

Complete list of suspected sources of impairment used in U.S. EPA's Assessment Total Maximum Daily Load (TMDL) Tracking and Implementation System (ATTAINS) database.

Suspected Sources Of Impairment
Above Ground Storage Tank Leaks (Tank Farms)
Accidental Release/Spill
Acid Mine Drainage
Agricultural Return Flows
Agricultural Water Diversion
Agriculture
Airports
Animal Feeding Operations (NPS)
Animal Holding/Management Areas
Animal Shows And Racetracks
Anthropogenic Land Use Changes
Aquaculture (Not Permitted)
Aquaculture (Permitted)
Atmospheric Deposition
Atmospheric Deposition - Acidity
Atmospheric Deposition - Nitrogen
Atmospheric Deposition - Toxics
Auction Barns
Ballast Water Releases
Barge Canal Impacts
Baseflow Depletion From Groundwater Withdrawals
Brownfield (Non-NPL) Sites
Cargo Loading/Unloading
CERCLA NPL(Superfund) Sites
Changes In Ordinary Stratification And Bottom Water Hypoxia/Anoxia
Changes In Tidal Circulation/Flushing
Channel Erosion/Incision From Upstream Hydromodifications
Channelization
Chemical Leak/Spill
Clean Sediments
Coal Mining
Coal Mining (Subsurface)
Coal Mining Discharges (Permitted)
Combined Sewer Overflows
Commercial Districts (Industrial Parks)

Suspected Sources Of Impairment
Commercial Districts (Shopping/Office Complexes)
Commercial Harbor And Port Activities
Confined Animal Feeding Operations - CAFOS (Point Source)
Confined Animal Feeding Operations (NPS)
Construction
Construction Stormwater Discharge (Permitted)
Contaminated Groundwater
Contaminated Sediments
Contribution From Downstream Waters Due To Tidal Action
Cooling Water Intake Structures (Impingement Or Entrainment)
Cranberry Production
Crop Production (Crop Land Or Dry Land)
Crop Production (Irrigated)
Crop Production (Non-Irrigated)
Crop Production With Subsurface Drainage
Dairies
Dam Construction (Other Than Upstream Flood Control Projects)
Dam Or Impoundment
Deicing (Storage/Application)
Discharges From Biosolids (Sludge) Storage, Application Or Disposal
Discharges From Municipal Separate Storm Sewer Systems (MS4)
Discharges From Offshore Oil And Gas Exploration (Permitted)
Dredge Mining
Dredging (E.G., For Navigation Channels)
Drought-Related Impacts
Dry Weather Flows With NPS Pollutants
Erosion And Sedimentation
Erosion From Derelict Land (Barren Land)
Forced Drainage Pumping
Forest Roads (Road Construction And Use)
Freshets Or Major Flooding
Golf Courses
Grazing In Riparian Or Shoreline Zones
Groundwater Loadings
Habitat Modification - Other Than Hydromodification
Hardrock Mining Discharges (Permitted)
Harvesting/Restoration/Residue Management
Heap-Leach Extraction Mining
Highway/Road/Bridge Runoff (Non-Construction Related)
Highways, Roads, Bridges, Infrastructure (New Construction)

Suspected Sources Of Impairment
Historic Bottom Deposits (Not Sediment)
Historical Source, No Longer Present
Hydrostructure Impacts On Fish Passage
Illegal Dumps Or Other Inappropriate Waste Disposal
Illicit Connections/Hook-Ups To Storm Sewers
Impacts From Abandoned Mine Lands (Inactive)
Impacts From Geothermal Development
Impacts From Hydrostructure Flow Regulation/Modification
Impacts From Land Application Of Wastes
Impacts From Resort Areas
Impervious Surface/Parking Lot Runoff
Inadequate Instream Habitat
Industrial Land Treatment
Industrial Point Source Discharge
Industrial Thermal Discharges
Industrial/Commercial Site Stormwater Discharge (Permitted)
Internal Nutrient Recycling
Introduction Of Non-Native Organisms (Accidental Or Intentional)
Lake Fertilization
Landfills
Leaking Underground Storage Tanks
Legacy/Historical Pollutants
Littoral/Shore Area Modifications (Non-Riverine)
Livestock (Grazing Or Feeding Operations)
Loss Of Riparian Habitat
Loss Of Wetlands
Low Head Dams
Low Water Crossing
Managed Pasture Grazing
Manure Lagoons
Manure Runoff
Marina Boat Construction
Marina Boat Maintenance
Marina Dredging Operations
Marina Fueling Operations
Marina Related Shoreline Habitat Degradation
Marina/Boating Pumpout Releases
Marina/Boating Sanitary On-Vessel Discharges
Marinas And Recreational Boating
Mill Tailings

Suspected Sources Of Impairment
Mine Tailings
Mining
Motorized Watercraft
Mountaintop Mining
Municipal (Urbanized High Density Area)
Municipal Point Source Discharges
Municipal Point Source Impacts From Inadequate Industrial/Commercial Pretreatment
Natural Conditions - Water Quality Standards Use Attainability Analyses Needed
Natural Sources
Natural-Beaver Dams/Log Jams
Natural-Drought
Natural-Flood
Naturally Occurring Organic Acids
Natural-Snowmelt
Non-Metals Mining Discharges (Permitted)
Non-Point Source
NPS Pollution From Military Base Facilities (Other Than Port Facilities)
NPS Pollution From Military Port Facilities
Off-Road Vehicles
On-Site Treatment Systems (Septic Systems And Similar Decentralized Systems)
Open Pit Mining
Other Marina/Boating On-Vessel Discharges
Other Recreational Pollution Sources
Other Shipping Releases (Wastes And Detritus)
Other Spill Related Impacts
Other Turf Management
Package Plant Or Other Permitted Small Flows Discharges
Pesticide Application
Petroleum/Natural Gas Activities
Petroleum/Natural Gas Production Activities (Permitted)
Pipeline Breaks
Placer Mining
Point Source(S) - Unspecified
Pollutants From Public Bathing Areas
Post-Development Erosion And Sedimentation
Potash Mining
Rangeland Grazing
RCRA Hazardous Waste Sites
Recreation And Tourism (Non-Boating)
Reduced Freshwater Flows

Suspected Sources Of Impairment
Reduction In Baseflow
Releases From Waste Sites Or Dumps
Removal Of Riparian Vegetation
Residential Districts
Runoff From Forest/Grassland/Parkland
Rural (Residential Areas)
Salt Storage Sites
Saltwater Intrusion
Sand/Gravel/Rock Mining Or Quarries
Sanitary Sewer Overflows (Collection System Failures)
Seafood Processing Operations
Sediment Resuspension (Clean Sediment)
Sediment Resuspension (Contaminated Sediment)
Septage Disposal
Sewage Discharges In Unsewered Areas
Shallow Lake/Reservoir
Shipbuilding, Repairs, Drydocking
Silviculture Activities
Silviculture Harvesting
Silviculture, Fire Suppression
Site Clearance (Land Development Or Redevelopment)
Source Unknown
Sources Outside State Jurisdiction Or Borders
Specialty Crop Production
Spills From Trucks Or Trains
Streambank Erosion
Streambank Modifications/Destabilization
Subsurface (Hardrock) Mining
Surface Mining
Surface Water Diversions
Surface Water Withdrawals
Total Retention Domestic Sewage Lagoons
Transfer Of Water From An Outside Watershed
UIC Wells (Underground Injection Control Wells)
Unknown Point Source
Unpermitted Discharge (Domestic Wastes)
Unpermitted Discharge (Industrial/Commercial Wastes)
Unrestricted Cattle Access
Unspecified Domestic Waste
Unspecified Land Disturbance

Suspected Sources Of Impairment
Unspecified Unpaved Road Or Trail
Unspecified Urban Stormwater
Upstream Impoundments
Upstream Source
Upstream/Downstream Source
Urban Development In Riparian Buffer
Urban Runoff/Storm Sewers
Wastes From Pets
Water Diversions
Waterfowl
Watershed Runoff Following Forest Fire
Wet Weather Discharges (Non-Point Source)
Wet Weather Discharges (Point Source And Combination Of Stormwater, SSO Or CSO)
Wetland Drainage
Wildlife Other Than Waterfowl
Woodlot Site Clearance
Woodlot Site Management
Yard Maintenance

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Suspended solids-associated toxicity of hydraulic fracturing flowback and produced water on early life stages of zebrafish (*Danio rerio*)☆

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ABSTRACT

Hydraulic fracturing flowback and produced water (HF-FPW), which contains polyaromatic hydrocarbons (PAHs) and numerous other potential contaminants, is a complex wastewater produced during the recovery of tight hydrocarbon resources. Previous studies on HF-FPW have demonstrated various toxicological responses of aquatic organisms as consequences of combined exposure to high salinity, dissolved organic compounds and particle/suspended solids-bound pollutants. Noteworthy is the lack of studies illustrating the potentially toxic effects of the FPW suspended solids (FPW-SS). In this study, we investigated the acute and sublethal toxicity of suspended solids filtered from six authentic FPW sample collected from two fracturing wells, using a sediment contact assay based on early-life stages of zebrafish (*Danio rerio*). PAHs profiles and acute toxicity tests provided initial information on the toxic potency of the six samples. Upon exposure to sediment mixture at two selected doses (1.6 and 3.1 mg/mL), results showed adverse effects in larval zebrafish, as revealed by increased Ethoxyresorufin-O-deethylase (EROD) activity. Transcriptional alterations were also observed in xenobiotic biotransformation (*ahr*, *pxr*, *cyp1a*, *cyp1b1*, *cyp1c1*, *cyp1c2*, *cyp3a65*, *udpgt1a1*, *udpgt5g1*), antioxidant response (*sod1*, *sod2*, *gpx1a*, *gpx1b*) and hormone receptor signaling (*esr1*, *esr2a*, *cyp19a1a*, *vtg1*) genes. The results demonstrated that even separated from the complex aqueous FPW mixture, FPW-SS can induce toxicological responses in aquatic organisms' early life stages. Since FPW-SS could sediment to the bottom of natural wetland acting as a continuous source of contaminants, the current findings imply the likelihood of long-term environmental risks of polluted sediments on aquatic ecosystems due to FPW spills.

1. Introduction

Hydraulic fracturing (HF) is a major method of recovering unconventional oil and gas resources in the United States and Canada (Vengosh et al., 2014). Despite the economic and energy security benefits, the practice of HF has raised significant public concerns regarding potential contamination of surface water (Lauer et al., 2016; Vengosh et al., 2014) and shallow groundwater aquifers (Llewellyn et al., 2015; Osborn et al., 2011). One of the significant environmental issues related to HF is the risk of spills associated with the generation and transport of large volumes of HF Flowback and Produced Water (HF-FPW), which is the process-affected water returned to the surface after well stimulation (Alessi et al., 2017; Zhong et al., 2021a). Though initially used for terrestrial extractions, HF has also expanded its scope to offshore hydrocarbon extraction in estuarine, shallow, and deep-sea marine environments (Zhong et al., 2021b), rising concerns on its ecological and toxicological impacts (Folkerts et al., 2020b). HF-FPW is highly heterogeneous due to the variability of natural chemicals contributed by the fractured geologic formation, the fracturing fluid chemicals injected into the wellbore, and subsurface transformations of those chemicals.

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Generally, HF-FPW is high in salts, metals, organic compounds, and suspended particles/suspended solids (He et al., 2017a). These contaminants' concentrations often exceed environmental guidelines for surface water quality (Vengosh et al., 2014). It has been reported that millions of liters of HF-related wastewater have been accidently released into aquatic environments in North America (Maloney et al., 2017; Patterson et al., 2017), which could have long-term impacts on the health of the aquatic ecosystem (Akob et al., 2016; Cozzarelli et al., 2017).

Our group has recently published several studies based on real HF-FPW samples obtained from the Duvernay Formation, Alberta, Canada. Significant toxicity of various HF-FPW samples has been demonstrated using zebrafish (Folkerts et al., 2017a, 2017b; He et al., 2017a, 2018a), rainbow trout (Blewett et al., 2017b; He et al., 2017b), *Daphnia magna* (Blewett et al., 2017a), *Lumbriculus variegatus* (Folkerts et al., 2020a, 2020b), *Coryphaena hippurus* (Folkerts et al., 2020b) and *in vitro* cell lines (He et al., 2018b) as exposure models. In many cases, particle-rich raw samples possessed greater toxicity than filtered samples (particle-free). For example, raw samples resulted in more potent acute toxicity, antioxidant stress, and hormone receptor signaling disruption potentials than filtered or activated charcoal treated samples in exposed zebrafish and rainbow trout embryos (He et al., 2017a, 2017b). *Daphnia magna* exposed to diluted raw FPW samples showed higher mortality and lower fecundity than activated charcoal treated samples or salt-water surrogates (Blewett et al., 2017a). However, these toxicological responses are most likely the consequences of combined exposure to high salinity, dissolved organic toxicants and particle/suspended solids -bound pollutants. A previous study using organic extracts from FPW samples (aqueous and particulate phases) demonstrated the non-salt-related developmental toxicity in exposed zebrafish embryos (He et al., 2018a). Another study using *in vitro* cell lines showed that organic pollutants in FPW displayed receptor-binding activity through signaling pathways, including the aryl hydrocarbon receptor, estrogen receptor and androgen receptor (He et al., 2018b). Most of the polycyclic aromatic hydrocarbons (PAHs) were found bound to particulate phases of HF-FPW samples, which was consistent with the more potent aryl hydrocarbon receptor (AhR) agonistic effects determined in particulate phases compared to those in aqueous phases.

However, our knowledge of the toxic effects of the particle/suspended solids fraction of FPW remains limited. In the scenario that FPW is accidently released into an aquatic ecosystem, both salts and dissolved organic compounds are likely to be diluted and/or transported from the spill site relatively rapidly. Simultaneously, the suspended solids deposited from FPW would likely settle to the bottom of the water body in the region of the spill. Therefore, FPW-associated sediment may perform as a source of contaminants and present a long-term chronic effect on aquatic organisms.

Sediments act as sinks of hydrophobic, persistent and hazardous compounds entering the aquatic ecosystem (Liu et al., 2013; Perelo, 2010). At the sediment: water interface, these compounds can be made available to benthic organisms and organisms in the water column. Investigations combining chemical analyses and biological effects tests are therefore of high priority in the context of sediment toxicity research (Hollert et al., 2009). Organic extractions of sediments can transfer the full spectrum of chemicals adsorbed to the sediments to the dissolved phase, neglecting the bioavailability of sediment contaminants (Rocha et al., 2011). To address this issue, sediment contact assays have been adopted to provide realistic scenarios that consider bioavailability and mimic in situ exposure conditions (Schiwy et al., 2020). Using suspended solids or sediments spiked with pollutants of interest, teratogenic, developmental and other toxicological effects have been investigated in various fish species (Barjhoux et al., 2017; Mu et al., 2017; Müller et al., 2021). Sediment contact assays have been developed as a useful tool and applied widely in sediment quality assessment and toxicity prediction using various model organisms (Brinkmann et al., 2015; Feiler et al., 2013; Koglin et al., 2016; Schertzinger et al., 2019).

The objective of this study was to assess the potential toxicity of FPW suspended solid (FPW-SS) samples collected at three time points from two wells (Well A and Well B). PAHs content in FPW-SS samples were analyzed as surrogates of model contaminants. Sediment contact assays were performed on zebrafish embryos using FPW-SS mixed with clean silica sand to make artificial sediment. Acute toxicity was evaluated by lethal concentrations (LC_{50}) for each FPW-SS sample. Sublethal toxicity was assessed by measuring embryonic ethoxyresorufin-O-deethylase (EROD) activity and fold-changes of mRNA abundance of a battery of genes pertinent to biotransformation, antioxidant response, and hormone receptor signaling by quantitative real-time polymerase chain reaction (Q-RT-PCR).

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma-Aldrich (Oakville, Canada). Parent PAHs standards and sixteen internal standards (deuterium-labeled) were obtained from Wellington Laboratories (Guelph, Canada), and 16 alkyl PAHs standards were obtained from Chiron AS (Trondheim, Norway). Silica sand (grand size $<$ 300 μm) was purchased from Plainsman Ltd. (Edmonton, Canada).

2.2. Sample preparation and characterization

FPW samples were collected from two hydraulically fractured wells located in the Devonian-aged Duvernay Formation (Well A and Well B located near Fox Creek, Alberta, Canada). Time zero was defined as the initial time of pressure release following fracturing, reported here as hours post-stimulation (hps). All samples were temporarily stored in sealed plastic buckets without headspace in a ventilated storage room kept at 20 °C prior to filtration. FPW-SS were collected by vacuum filtration within 1 month after sample arrival, and all filtered samples were stored in amber glass bottles at −20 °C before exposure experiments. Briefly, glass fiber filters (0.4 μm, Glass Fiber Store) were freeze-dried over 24 h in advance, weighed, and used to filter samples (1 L per filter, 10 L for filtering each sample). For each FPW-SS sample, the particle sizes, total organic content (TOC-SS), and inorganic elements/ions were also analyzed. More details are presented in SI. Some key physic-chemical information is summarized in Table 1.

2.3. PAHs analysis

PAHs extraction and profile analysis were conducted following our

Table 1
Summary of key physic-chemistry information of FPW samples collected for the current study. Total concentrations of 10 EPA parent PAHs and 4 alkylated PAHs (i.e., 1-Methylfluorene, 1-Methylphenanthrene, 3,6-Dimethylphenanthrene and 1-Methylpyrene) were determined in FPW-SS samples. Data on total organic carbon (TOC) and total dissolved solids (TDS) are obtained from a companion study (He et al., 2018a).

Parameters	Well A			Well B		
	A1	A2	A3	B1	B2	B3
Collection time (hps)	1	48	144	1	48	136
TOC (mg/L)	11800	271	212	4710	282	240
TDS (mg/L)	5310	164000	192000	145000	162000	175000
SS (g/L)	2.2816	1.0606	0.3785	2.0125	0.7782	1.0031
TOC-SS (w/w %)	2.59	0.86	1.05	2.97	1.63	1.84
Σ_{10} PAH (ng/g)	480	210	4400	5300	5200	6900
Σ_4 alkyl-PAH (ng/g)	470	140	5900	6500	5600	16000
Total PAHs (ng/g)	950	350	10000	12000	11000	23000

previous studies (He et al., 2018b; Zhang et al., 2016). Briefly, PAHs were extracted by accelerated solvent extraction and cleaned-up by solid-phase extraction. The final extract was analyzed by GC-MS following the method established in the previous study. Details are provided in SI.

2.4. Sediment contact exposure

Sediment contact assay was performed following the method described in Schiwy et al. (2014) with minor modifications. Briefly, to make the exposure sediment, each FPW-SS sample was weighted and topped up to a total mass of 3 g with silica sand (grand size < 300 μm) and mixed by a mortar to ensure a homogenous mixture. In total 7 dilution concentrations (0.8, 1.6, 3.1, 6.3, 12.5, 25 and 50 mg/mL), together with silica sand control, were tested for FPW-SS from both Well A and Well B. Each well of the 6-well plate contained 3 g of exposure sediment or silica sand with 5 mL facility water. Parallel experiments using facility water only were conducted to ensure that the silica sand had no effect (Table S4). After 24-h of equilibrium of exposure sediment at 25 ± 1 °C, fertilized zebrafish embryos (wild type Strain AB, 1 h post-fertilization, hpf) were randomly placed (5 embryos per well). Static exposures were conducted (25 ± 1 °C; 16 h/8 h light/dark cycle) with constant shaking at 50 rpm until 96 hpf. Water quality parameters were checked in the overlying water at the beginning and end of exposure to ensure that the observed toxic effects were not caused by hypoxia or other factors (Viganò et al., 2020). All the exposures were repeated for 5 replications (n = 5, in total 25 embryos for each concentration). At the end of the exposure, the mortality rate of embryos as LC_{50} values (concentrations resulting in 50% mortality) was calculated using Toxicity Relationship Analysis Program (TRAP v1.30).

The following sublethal exposures for embryonic EROD assay and gene expression analysis also followed the same design except that 25 fertilized embryos were exposed in a glass Petri dish with 15 g of homogenized exposure sediment mixture and 25 mL facility water. The test concentrations were 1.6 and 3.1 mg/mL (NOEC of A1). At the end of 96 h-exposure, 10 hatched larvae (96 hpf) from the 1.6 and 3.1 mg/mL groups were collected for embryonic EROD assays. The remaining larvae in the 3.1 mg/L group were snap-frozen and stored at −80 °C for analysis of mRNA abundance by Q-RT-PCR. Other details are presented in SI.

2.5. Embryonic EROD assay

EROD assay is a rapid quantitative assay of the cytochrome P450 1A enzyme activity for detecting agonistic contaminants towards AhR (Whyte et al., 2000). A non-destructive EROD assay using zebrafish embryo/larvae was performed following a previous study (He et al., 2017a). Briefly, at the end of the exposure, 10 hatched larvae (96 hpf) from each exposure group were incubated with 7-ethoxyresorufin solution, and excreted resorufin in the solution was measured using a VICTOR3V 1420 Multilabel Counter (PerkinElmer, MS, USA). EROD activity in exposed group was normalized against the silica sand control to generate relative fold changes. More details are provided in SI.

2.6. Quantitative real-time PCR assay

Q-RT-PCR assays were performed following a previous study (He et al., 2018a). Briefly, total RNA was extracted from 10 to 15 hatched larvae and converted to cDNA for transcriptional analysis. Relative fold change of mRNA abundance was measured by ΔΔCt method using elongation factor 1a (*elf1a*) as the reference gene (ABI 7500 Real-Time PCR System). Details are provided in SI. Gene name, abbreviation, sequences of primers, efficiency, and GeneBank reference numbers are listed in Table S1.

2.7. Statistics

Statistical analyses were conducted using the GraphPad Prism statistical software (GraphPad Prism 8). All data are expressed as mean ± standard error of the mean. The statistical differences of LC_{50} of FPW-SS samples from each well were analyzed using the Litchfield-Wilcoxon method (Litchfield and Wilcoxon, 1949). Spearman analyses were performed to determine the correlation coefficient (r) between several physic-chemical properties of FPW-SS samples and toxicity endpoints. Statistical differences were considered significant at $p < 0.05$. Other statistics details are provided in SI.

3. Results

3.1. FPW-SS characterizations

The results of physical analysis and the total metal concentrations of the FPW-SS samples obtained from two wells at different collection time are presented in Table 1 and Table S2. During the first flowback stage (from 1 to 48 hps), the total organic carbon (TOC) in the raw samples decreased sharply (44-fold for well A, 17-fold for well B), then continue to decrease gently in the following time for both wells. Well A had a much lower TDS (5310 mg/L) than well B (145000 mg/L) at flowback initiation, followed by 31-fold increase to over 164000 mg/L after 48 hps. Well B displayed a relatively mild increase in TDS level, holding at over 140000 mg/L throughout the whole process. For both Well A and Well B, TOC-SS (w/w%) was the highest (2.59% and 2.97%) when flowback commenced, which then decreased by 3-fold and 2-fold, respectively, at 48 hps. At the end of flowback, TOC-SS levels showed a slight recovery for both wells (Table 1).

The FPW-SS samples were dominated by sand as solid components (73.1% in A1 and 68.4% in B1) (Table S2). Fig. S1 illustrates the particle distribution (%) in solid composition (clay, slit and sand) in samples over three collection periods of each well. Within the course of flowback, sand proportion showed a gradual decrease in well A, but was characterized by a big decline followed by a slight fluctuation in Well B. The selected elementary and ion concentrations (mg/kg) of each FPW-SS sample are shown in Table S2.

3.2. PAHs profile

Ten out of sixteen parent PAHs (EPA priority pollutants) were detected in the FPW-SS samples. Four alkyl PAHs, 1-Methylfluorene, 1-Methylphenanthrene, 3,6-Dimethylphenanthrene and 1-Methylpyrene were also frequently detected. Total concentrations of parent and alkyl PAHs are shown in Table 1, and detailed concentrations for each detected PAH species are shown in Table S3. Based on the number of aromatic rings, profiles of detected PAHs in FPW-SS samples were categorized into four groups (3-ring, 4-ring, 5-ring and 6-ring) (Fig. S2). At the final collection time point, fluorene was the dominant parent PAH and 1-methylphenanthrene was the dominant alkyl PAH in sample A3; chrysene was the dominant parent PAH and 3,6-dimethylphenanthrene was the dominant alkyl PAH in sample B3. During the whole process, the concentrations of Σ_{10} PAHs and Σ_4 alkyl-PAHs in Well B were much higher than those in Well A (Table 1). Throughout the collection process, 3-ring PAHs dominated total PAHs (>50%) for both Well A and Well B. In Well A, the highest proportion of 3-ring PAHs (70.35%) occurred at 144 hps. For Well B, the proportion of 3-ring PAHs ranged from 72.4% to 79.9%. Over time, a declining trend (from 14% to 1.6%) was observed for 5-ring PAHs, namely benzo[b]fluoranthene in Well A samples, while 4-ring species showed a stable proportion of around 30%. In Well B, 5-ring PAHs displayed a relatively low proportion of no more than 3% at all collection time, with the proportion of 4-ring species fluctuating between 18.8% and 24.4%. In general, the 6-ring PAHs occurred at relatively low proportions (<2%) for both Well A and Well B, with the exception of 10% in sample A2 (Fig. S2).

Fig. 1 shows the total PAHs concentrations coupled with benzo(a) pyrene toxic equivalence quotient (BaP TEQ, ng/g) in FPW-SS from Well A and Well B over time. For both wells, BaP TEQ displayed an increasing trend, peaking at the final collection point. The BaP TEQ concentration of the B3 sample (258.3 ng/g) far exceeded that of the A3 sample (102.3 ng/g).

3.3. Acute toxicity

Zebrafish embryos were applied for sediment contact exposures from 1 hpf to 96 hpf. As illustrated by the dose-response curves (Fig. 2), exposure to FPW-SS from Well A and Well B resulted in concentration-dependent acute toxicity to zebrafish embryos. For Well A, a significantly lower LC_{50}, 7.21 (5.39, 9.65; lower and upper 95% confidence interval, same below) mg/mL, was obtained for the A1 sample than for the A2 and A3 samples, for which the LC_{50} values were 17.14 (12.33, 23.83) and 12.48 (9.25, 16.84) mg/mL, respectively. In contrast, no significant differences in the LC_{50} value were found among samples B1, B2, and B3, where the LC_{50} values were 9.49 (7.10, 12.69), 14.08 (10.32, 19.21) and 12.17 (8.75, 16.93) mg/mL, respectively (Fig. 2, Table S4). Exposure to 50 mg/mL A1 and B1 FPW-SS samples caused 100% death to zebrafish embryos (details provided in Table S4). Significant correlations exist between mortality of exposed embryos and TOC-SS of FPW-SS samples at three exposure concentrations (Spearman $r = 0.9276$ for 12.5 mg/mL, Spearman $r = 0.8407$ for 25 mg/mL and Spearman $r = 0.8827$ for 50 mg/mL). Over different collection points, greater TOC-SS resulted in higher mortality among the above exposure groups (Fig. S3). However, there were no correlations between total PAH content and acute toxicity across all tested concentrations ($p > 0.05$).

3.4. Embryonic EROD activity

In the present study, EROD activity (expressed as fold change compared to the control group) was measured to examine the CYP1A activities of zebrafish larvae after sublethal exposure to Well A or Well B FPW-SS at sublethal doses (1.6 and 3.1 mg/mL) (Fig. 3). In Well A group, significant increases of EROD activity were detected in A1 (1.9 ± 0.1-fold), A2 (1.8 ± 0.1-fold), and A3 (5.0 ± 0.4-fold) at the high dose (3.1 mg/mL), as well as in A3 (2.4 ± 0.2-fold) at the low dose (1.6 mg/mL) compared to the control (Fig. 3a). In contrast, exposure to Well B FPW-SS led to significant increases of EROD activity in all 6 treatments compared to the control, namely B1 (3.7 ± 0.2-fold), B2 (3.6 ± 0.3-fold), and B3 (9.4 ± 0.7-fold) at the high dose, as well as B1 (1.8 ± 0.1-fold), B2 (1.9 ± 0.1-fold), and B3 (2.4 ± 0.1-fold) at the low dose (Fig. 3b).

Statistical analyses between Well A and B were further performed to compare EROD activities induced by FPW-SS collected from similar time points (Fig. S4). In the low dose group (Fig. S4a), the B1 and B2 treatments exhibited significantly stronger EROD activity than the A1 and A2 treatments, respectively, whereas larvae exposed to A3 and B3 samples displayed similar EROD activities. In the high dose group (Fig. S4b), EROD activities were significantly greater in fish exposed to Well B samples at all time points, compared to exposure to Well A samples. In addition, Spearman rank correlation analyses provided signatures of EROD activities of exposed embryos in relation to total PAH content (Fig. S5a) and BaP-TEQ (Fig. S5b) of FPW-SS samples. Embryonic EROD activities had significant positive correlation with total PAH content at both low dose ($r = 0.6641$, $p < 0.0001$) and high dose ($r = 0.7926$, $p < 0.0001$). Strong correlations were found between embryonic EROD activities and BaP-TEQ levels at both low dose ($r = 0.8468$, $p < 0.0001$) and high dose ($r = 0.9144$, $p < 0.0001$) (Table S6).

3.5. Gene expression

Exposure to FPW-SS significantly altered the expression of a battery of genes related to xenobiotic biotransformation in zebrafish larvae. These genes included the aryl hydrocarbon receptor (*ahr*), pregnane X receptor (*pxr*), cytochrome P450 1 family members (*cyp1a, cyp1b1, cyp1c1, cyp1c2*), cytochrome P450 2 family members (*cyp2aa1, cyp2aa2, cyp2aa12*), cytochrome P450 3A65 (*cyp3a65*), uridine diphosphate (UDP) glucuronosyltransferase 1A1 (*udpgt1a1*) and UDP glucuronosyltransferase 5G1 (*udpgt5g1*) (Fig. 4, Table S5). The results showed that the expression of *ahr* increased significantly in all FPW-SS treatments (A1∼A3, B1∼B3) compared to their respective control, with the greatest expression level in B3 (3.7-fold). The expression of *pxr* showed significant increases in A1, A3, B1 and B3 and no changes in A2 and B2. The same pattern occurred in the cytochrome P450 1 family (*cyp1a, cyp1b1, cyp1c1, cyp1c2*), with significant elevation in expression levels occurring in all FPW-SS treatments (A1∼A3, B1∼B3) and the greatest expression level in B3. Significant increases were observed in A1, A3 and B3 for *cyp2aa1*, while the expressions of *cyp2aa2* and *cyp2aa12* both increased significantly in A3 and B3. The expressions of *cyp3a65*, *udpgt1a1* and *udpgt5g1* displayed significant increases in all FPW-SS treatments compared to the control. In addition, the expression of *cyp1a* displayed a rising trend over time for both Well A (A1∼A3) and Well B (B1∼B3), while *cyp1b1*, *cyp1c1* and *cyp1c2* displayed a similar increasing trend from A1 to A3.

The expressions of several antioxidant response -related genes were altered in exposed fish larvae at 96 hpf (Fig. 4, Table S5). The expression of glutathione *S*-transferases family members (*gstp1* and *gstm*), glutathione synthetase (*gss*), glutathione-disulfide reductase (*gsr*) and catalase (*cat*) were unchanged in all FPW-SS treatments despite a slight

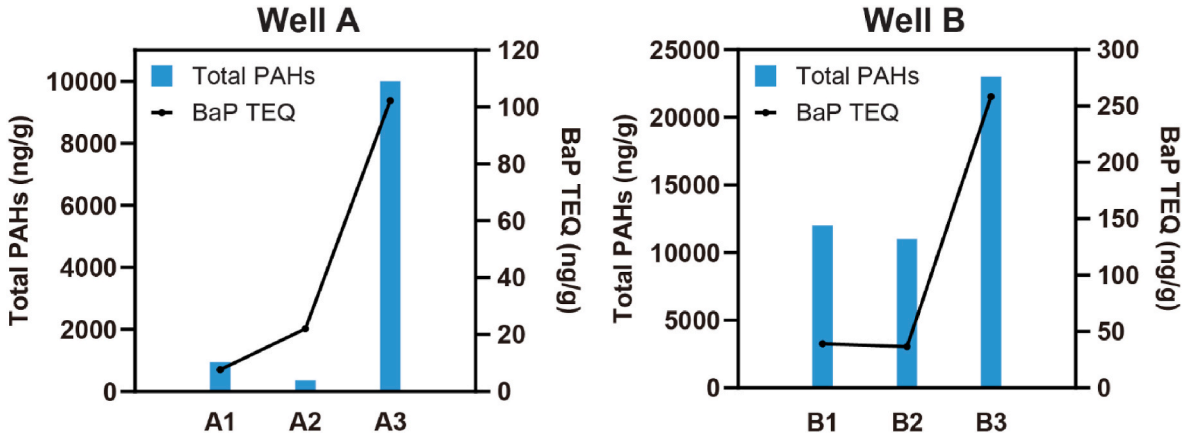

Fig. 1. Total concentrations (ng/g) and the benzo(a)pyrene toxic equivalence quotient (BaP TEQ, ng/g) of 16 EPA priority parent PAHs (10 out of 16 detected) and 4 alkylated PAHs in FPW-SS samples.

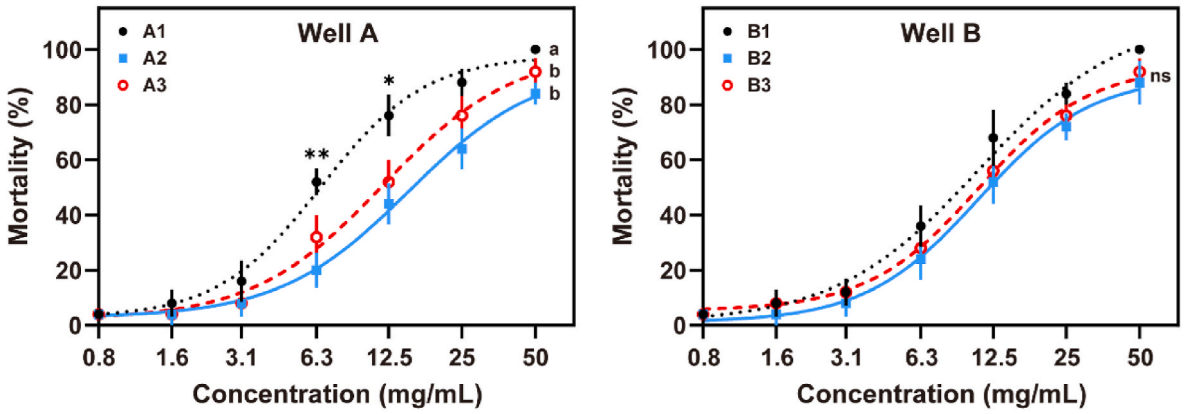

Fig. 2. Mortality of zebrafish (*Danio rerio*) embryos exposed to FPW-SS collected from two wells (Well A and Well B) at three time points (A1, A2, A3 and B1, B2, B3, respectively) at the end of the 96-h sediment contact assay. Asterisks represent any significant difference observed across the samples under any specific concentration (n = 5, Kruskal-Wallis test, $*p < 0.05$, $**p < 0.01$). Lower case letters indicate significant differences among LC_{50} values (Litchfield-Wilcoxon method). All LC_{50} values obtained from Well B displayed no significant difference (ns).

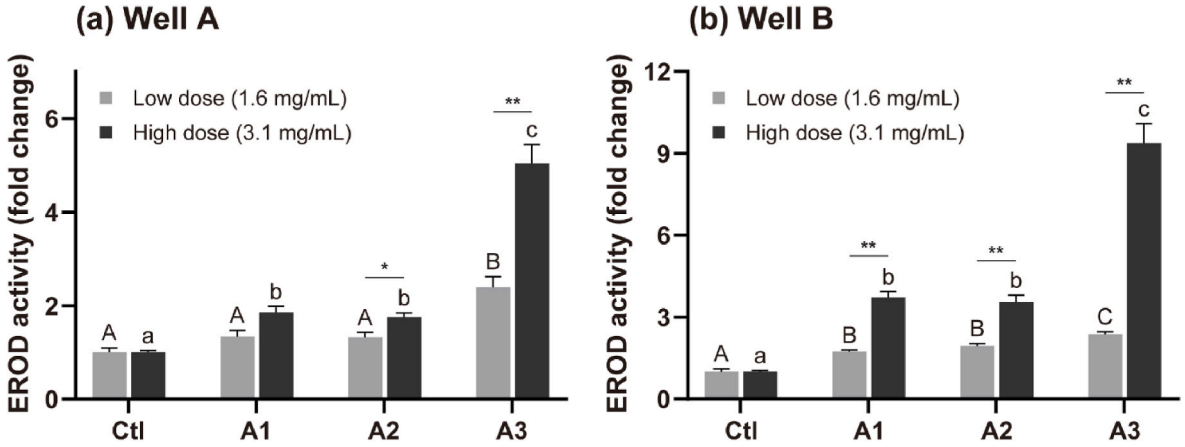

Fig. 3. EROD activity in silica sand control (Ctl) and exposed zebrafish embryos at the end of the 96-h sediment contact assay, expressed by relative fold changes against that of the sand control. Bars represent mean values and standard error of the mean. Upper-case and lower-case letters indicate significant differences among sand control and exposure groups for low and high doses, respectively ($p < 0.05$, ANOVA). Asterisks indicate significant differences from the control (Ctl) (n = 5, one-way ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$).

increase of *gss* expression in B1. The superoxide dismutase family members (*sod1* and *sod2*) exhibited significant increases in expression levels in all FPW-SS treatments compared to the control. For the glutathione peroxidase family members (*gpx1a* and *gpx1b*), *gpx1a* expression significantly increased in A3 and B3, and *gpx1b* expression showed significant increases in A3, B2 and B3. In contrast, significant decreases were observed in A1 and B1 for both *gpx1a* and *gpx1b*.

The mRNA levels of genes relevant to endocrine activity were also determined in exposed fish larvae, including estrogen receptor (ERs) family members (*esr1*, *esr2a*, *esr2b*), androgen receptor (*ar*), cytochrome P450 19A1a (*cyp19a1a*), cytochrome P450 19A1B (*cyp19a1b*) and vitellogenin 1 (*vtg1*) (Fig. 4 and Table S5). No change of expression was observed for *ar* or *cyp19a1b*. However, the expression of *esr1* and *esr2a* increased significantly in A1-A3 and B1, with the greatest level observed in A1 (2.5-fold for *esr1* and 6.6-fold for *esr2a*, respectively). A significant increase in expression occurred in A1 and A2 for *esr2b*. For expression of *cyp19a1a*, the results showed significant increases in A1-A3, with as high as an 8-fold increase in A1. While the expression of *vtg1* was significantly higher than the control in all FPW-SS treatments except in B3, a sharply decreasing trend with time was observed for A1-A3. Similarly, the expressions of *esr2a*, *esr2b* and *cyp19a1a* also tended to decrease with sample collection time for Well A (i.e., from A1 to A3).

Correlation relationships between sample BaP-TEQ and expression of tested genes were revealed by Spearman rank analyses (Table S6). Positive correlations were found between sample BaP-TEQ and several genes including *ahr* ($r = 0.6394$, $p = 0.0001$), *cyp1a* ($r = 0.8625$, $p < 0.0001$), *cyp1b1* ($r = 0.7791$, $p < 0.0001$), *cyp1c1*($r = 0.8310$, $p < 0.0001$), *cyp1c2* ($r = 0.9099$, $p < 0.0001$), *gpx1a* ($r = 0.6821$, $p < 0.0001$), while expressions of *esr2a* ($r = -0.7227$, $p < 0.0001$), *cyp19a1a* ($r = -0.6799$, $p < 0.0001$) and *vtg1* ($r = -0.7520$, $p < 0.0001$) were negatively correlated with BaP-TEQ level of FPW-SS samples.

4. Discussion

4.1. FPW characteristics and carcinogenic potential

For both wells, at the initial stage of flowback, the FPW-SS samples (A1 and B1) were characterized by the relatively high TOC concentrations with more coarse particles; while the TOC-SS decreased a little at the middle stage and increased again at the later stage with more fine particles returning to the surface. In addition, the concentrations of TDS exhibited an increasing trend with the time of flowback for both wells (Table 1). A possible explanation for these changes is that during the flowback process, the earlier FPW volumes return to the surface are likely to contain higher proportion of organic compounds and coarse sands initially injected as additives and proppants in fracturing fluid,

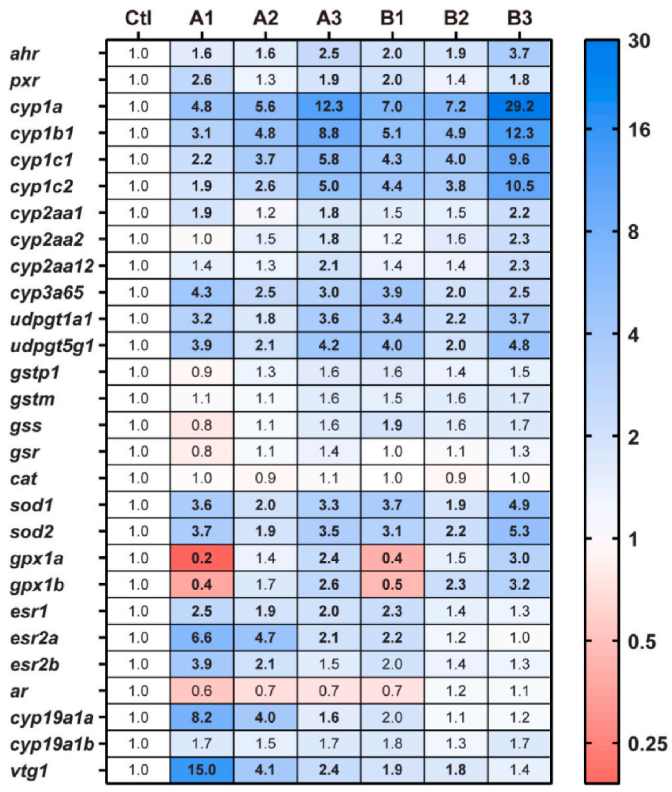

Fig. 4. Heat map showing fold-changes of xenobiotic biotransformation, antioxidant response and hormone receptor signaling-related genes determined by Q-RT-PCR in *Danio rerio* embryos after exposure to 3.1 mg/L of FPW-SS samples. Relative fold changes of mRNA abundance were calculated versus silica sand control (Ctl). Color scale ranges from blue to red which denotes the up- or down-regulated of the genes, respectively. Bold numbers depict significant differences from control group (n = 5, one-way ANOVA, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

including a considerable number and concentration of unknown compounds (He et al., 2017a; Sun et al., 2019). With the increase of flowback time, the proportion of fracturing fluid was gradually replaced by formation water containing a higher salinity, fine particles and natural petroleum associated organics (He et al., 2018a, 2018b). Further characterization is needed to identify the anthropogenic and natural organic compounds associated with FPW-SS.

Due to high hydrophobicity, PAHs tend to adsorb on and accumulate in the particle/suspended solids of FPW samples (He et al., 2017a). The application of the benzo(a)pyrene (BaP)-toxic equivalent factor to PAHs concentrations (e.g., carcinogenic equivalents, BaP-TEQ) can provide an initial toxicity assessment of the FPW-SS sample collected in our study. For Well A, the concentrations of total PAHs experienced a decrease, then an increase, peaking at the end of the flowback phase (A3). Interestingly, the BaP-TEQ value of Well A showed an increasing trend during the same process. This inconsistency was caused by the occurrence of more potent PAH congeners (compounds with higher molecular weight). For Well B, consistently increasing trends were found in the concentrations of total PAHs and the BaP-TEQ value with time, suggesting that the concentrations of natural PAHs increased with the flowback process. Among all samples, the maximum concentrations of total PAHs as well as maximum BaP-TEQ value were found in B3. Furthermore, the BaP-TEQ value of B3 exceeded that of A3 more than 2-fold, indicating higher PAH concentrations in Well B, which is consistent with a companion study (He et al., 2018b). Lacking in correlation between total PAH concentrations and BaP-TEQ ($p > 0.05$) (Table S6) indicated the complicated composition of FPW-SS and the potential carcinogenic effects displayed by other compounds other than detected PAHs.

4.2. FPW-SS associated acute toxicity

Exposure to FPW-SS led to dose-response effects on embryo-larval toxicity in zebrafish. The 96 h-LC_{50} was determined for each well at each collection time (Table S4). The LC_{50} value of the A1 sample was significantly lower than that of A2 and A3 (i.e., toxicity: A1>A3≈A2), suggesting an intense lethal effect at the beginning of the flowback process. The substantial toxicity of the A1 sample could be caused by the highest proportion of 5-ring PAHs (14.1%) (Fig. S2), which have less immediate bioavailability through the aqueous phase but are likely to accumulate within sediments (Wang et al., 2001). The toxic effect of Well B samples remained unchanged temporally with no significant differences between B1, B2 or B3, probably attributed to the similar proportions of low molecular weight PAHs (72%–80%).

The strong positive correlation between embryonic mortality at 12.5 mg/mL ($r = 0.9276$), 25 mg/mL ($r = 0.8407$) and 50 mg/mL ($r = 0.8827$) exposure levels with total organic carbon content (TOC-SS) suggested enhanced acute toxicity of suspended solid samples as a result of higher loads of anthropogenic (e.g., fracturing additives) and naturally occurred organic contaminants (e.g. PAHs). It suggested that the organic contaminants associated with FPW-SS, including but not limited to PAHs, could pose lethal effects to developing zebrafish embryos. The level of total organic contaminant could be reduced by removing suspended solids, thereby partially mitigating the acute lethal effects of FPW samples (He et al., 2017a). The present study further marked FPW-SS as a valid vector of contaminants and a non-negligible pollution source to the aquatic ecosystem, especially samples collected at the initial stage of hydraulic fracturing flowback.

4.3. EROD activity

The aryl hydrocarbon receptor (AhR) signaling pathway plays a vital role in response to environmental contaminants (e.g., PAHs), triggering the downstream gene including *cyp1a*, which can be measured through the enzymatic activity of CYP1A-encoded ethoxyresorufin-O-deethylase (EROD) (Larsson et al., 2014; Santana et al., 2018; Whyte et al., 2000). In the current study, EROD activities in larvae exposed to the high dose were significantly enhanced compared to the control for both wells. Out results show moderate correlations between the total concentrations of PAHs and embryo EROD activity at both doses (Table S6, Figs. S5–a). Strong correlation was found between EROD activity at both doses and BaP-TEQ levels of FPW-SS samples (Table S6, Figs. S5–b). During flowback, EROD activity increased with increasing BaP-TEQ values, suggesting increased potency of AhR agonistic effects from contaminants in collected FPW-SS samples. Similar to EROD activity, expression of *cyp1a* was significant up-regulated in all FPW-SS treatments compared to the control, with variation pattern strongly correlated to that of EROD activity at the same dose. Total PAH concentrations and BaP-TEQ of samples were also found to correlate with the expression of *cyp1a*. Activation of AhR pathway by FPW-SS exposure can be further elucidated by elevated expression of *cyp1a* and other members of the cytochrome P450 family 1 and 2. The elevated expressions of *cyp1b1*, *cyp1c1* and *cyp1c2* in all treatments suggested the occurrence of AhR-dependent regulation upon FPW-SS exposure. Positive correlations were found between these genes and BaP-TEQ level (Table S6, Fig. S6), implying increasing content of AhR inductors in samples with greater carcinogenicity.

The EROD activity has been reported to vary upon interactions of xenoestrogens and oestradiol both *in vivo* (Bianchini and Morrissey, 2018; Kais et al., 2017; Strobel et al., 2015) and *in vitro* (Crump et al., 2016; Petersen et al., 2017; Wang et al., 2020). Previous studies have demonstrated that exposure to PAHs and dioxin-like compounds could induce EROD activity in various teleost species. Xu et al. (2018) reported

substantial elevation of EROD activity in zebrafish larvae exposed to dioxin-like compounds, while responses of medaka larvae to parallel exposure were implicit at lower doses, displaying species-specific action manner. In avian, elevated EROD activity was reported in the high dose group relative to controls after ingestion of PAH-contaminated food, confirming aryl hydrocarbon receptor (AHR) activation in wild-caught sanderling (Bianchini and Morrissey, 2018). Both *in vivo* and *in vitro* EROD assays have proved to be effective and efficient in screening and detection of dioxin-like activity in field samples (Kais et al., 2017; Vieira et al., 2019).

4.4. Gene expression

Xenobiotic biotransformation. The biotransformation process was catalyzed by phase I enzymes including Cytochrome P450 isoforms (CYPs) and phase II enzymes such as glutathione-S-transferase (GST), sulfotransferase (SULT) and UDP-glucuronosyl transferase (UGT) (Santana et al., 2018). The CYP supergene family plays a crucial role in the metabolism of many xenobiotics and endogenous substances (Nekvindova et al., 2020; Zhang et al., 2015). In the 51 CYP gene families that have been identified in zebrafish, there are 2 major groups—CYP genes involved in the synthesis, activation, or inactivation of endogenous regulatory molecules (CYP 5–51) and CYP genes involved in the oxidation of xenobiotics, drugs, and fatty acids (CYP 1–4) (Goldstone et al., 2010; Saad et al., 2016). Many xenobiotic-metabolizing CYP can be induced via transcription factors (aryl hydrocarbon receptor, Ahr; pregnane-X-receptor, Pxr and others) (Goldstone et al., 2010). Several phase I enzymes (e.g., CYP3s) and phase II enzymes (e.g., UDPGT, GSH) have been identified as primary targets of Pxr activation (Kubota et al., 2015). Pxr also interacts with factors binding to the antioxidant response element, thereby up-regulating the expression of phase II conjugating enzymes such as UDPGTs (Higgins and Hayes, 2011).

In the present study, significant up-regulation of genes involved in Phase I of biotransformation, e.g., *cyp1a*, *cyp1b1*, *cyp1c1*, *cyp1c2*, *cyp3a65* and *pxr*, were observed in the exposed zebrafish embryo. As genes involved in the Phase II of biotransformation (i.e., detoxifying phase), the transcripts abundance of *udpgt1a1* and *udpgt5g1* were also significantly greater in embryos exposed to FPW-SS compared to controls. Furthermore, the expression level of *ahr* and *pxr* were also significantly greater in embryos exposed to FPW extracts compared to the control. These results suggested the organic contaminants associated with FPW-SS (including but not limited to PAHs) displayed significant agonistic activity and exerted their toxic effect through the AhR and PXR signaling pathways. AhR activation and developmental toxicity were observed in zebrafish exposed to soil extracts containing parent, unsaturated and oxygenated PAHs (Wincent et al., 2015). In addition, PAHs can also be activated by cytochrome family enzymes to form mutagenic metabolites, including diol epoxides, quinones, and radical species (Xue and Warshawsky, 2005). Further study is needed to investigate the potential genotoxicity of FPW-SS.

Antioxidant response. Organisms, including fishes, have antioxidative systems that use different mechanisms to counteract overproduced ROS. These systems tend to inhibit radical formation and include the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione-dependent peroxidase (GPX), which are critically important in the detoxification of radicals to nonreactive molecules (Van der Oost et al., 2003). PAHs are known to induce oxidative stress and antioxidant responses in both invertebrates and vertebrate species following exposure (Folkerts et al., 2020a; Han et al., 2014). Our result demonstrated a transcriptional regulation by elevated mRNA levels of *sod1* and *sod2* in embryonic zebrafish. The expression of these two genes was significantly up-regulated in all FPW-SS treatments, indicating the antioxidant response as (one of) the toxicity mechanism(s) of FPW-SS. Interestingly, in A1 and B1 samples that initially contained lower total PAHs levels, the gene expression of *gpx1a* and *gpx1b* was down-regulated, which is not consistent with the up-regulation of other antioxidant response genes such as *sod1* and *sod2*. Given the upregulation of *vtg* by A1 and B1 samples, which indicated significant estrogenicity, a possible interpretation can be that the expression of *gpx* in exposed zebrafish were influenced by some unknown endocrine disrupting compounds in A1 and B1 samples. Similar inhibition on *gpx* expression has also been reported in *Micropterus salmoides* with EE2 feed treatment (Colli-Dula et al., 2014) and zebrafish exposed to endocrine active riverbed sediments (Viganò et al., 2020). Nevertheless, our results confirmed zebrafish embryo exposed to FPW-SS could display impairment in antioxidant response. However, further study is needed to characterize the potential interactions between antioxidant and estrogenic activity in response to exposure to complex environmental mixture like FPW.

Hormone receptor signaling. FPW likely contains compounds that cause disruptive effects on hormone receptor signaling (Webb et al., 2014; Folkerts et al., 2020a, 2020b). Many vertebrate and invertebrate studies have shown that PAHs negatively impact reproductive processes (Folkerts et al., 2020a; Head et al., 2015). Previous studies have revealed the consequences of hormone receptor signaling disruption following exposure to petroleum-contaminated waters (Salaberria et al., 2014) and flowback and produced waters (He et al., 2018a), where PAHs are significant fractions. Estrogenic and androgenic activities have been reported for hydraulic fracturing chemicals *in vivo* and *in vitro* (Kassotis et al., 2020, 2016; 2015; Nagel et al., 2020). Following this rationale, the expression of seven hormone receptor signaling-related genes were examined, of which at least five have shown distinct expression alternations in exposed fish larvae.

In zebrafish, three estrogen receptor (ER) isoforms have been identified so far, including *esr1*, *esr2a*, and *esr2b*. It has been demonstrated that these three isoforms can be altered at the transcriptional level and used as an indicator of estrogenic effects (Söffker and Tyler, 2012; Song et al., 2020). In addition, the aromatase (CYP19), which converts androgens to estrogens, is well known to play essential roles in fish steroidogenesis and sex differentiation (Simpson et al., 1994). Fish aromatase is coded by two separate genes, the gonad-specific cyp19a1a and brain-specific cyp19a1b (Lau et al., 2016). The upregulation of the aromatase gene(s) has been linked to various environmental estrogenic compounds, including flame retardants, pesticides, and petroleum-related produced water (Cheshenko et al., 2006; He et al., 2018a). Moreover, the synthesis of vitellogenin can be significantly induced by estrogens in laboratory experiments, and therefore serves as a sensitive biomarker of environmental estrogenic effects (Brion et al., 2004). In the current study, upregulation was observed in the expression of *esr1*, *esr2a* and *esr2b*, suggesting estrogenic compounds are present in FPW-SS. Furthermore, upregulated expression of the gonadal aromatase isoform *cyp19a1a* coupled with *vtg1* also supported the hormone receptor signaling disruptive capacity of FPW-SS samples. In our study, the highest fold increase in expression of *cyp19a1a* and *vtg1* was found in sample A1, indicating FPW-SS obtained from A1 had the most substantial estrogenic efficacy. Generally, for both wells, the initial samples (A1 and B1) displayed stronger estrogenic efficacy, followed by the subsequent samples. This trend does not correspond with the trends of PAHs or BaP-TEQ in the two wells. This implies that total PAHs or BaP-TEQ may not be appropriate markers of estrogenic activity of the suspended solids of FPW sample. Further study is needed to characterize the portfolio of estrogenic compounds in FPW-SS to develop a more comprehensive understanding of the estrogenic activity disruption effects and risks associated with FPW-SS.

5. Conclusion

This study demonstrates that FPW-SS exerted significant toxicity on developing zebrafish embryos using a sediment contact assay. Through analyzing FPW with different chemical compositions, we showed that FPW-SS samples collected from two hydraulically fractured wells displayed distinct temporal patterns of toxicological potentials. The

induction of EROD activity, together with the impaired expression of various genes related to xenobiotic biotransformation and antioxidant response suggested that the activation of AhR (and potentially PXR) might play an important role in FPW-SS toxicity. In addition, the altered gene expressions related to hormone receptor signaling also suggested the presence of endocrine disrupting chemicals in FPW-SS. Future chemical analysis is needed to determine the toxic components other than PAHs in FPW-SS. In future control and containment of FPW contamination in accidental release events, special attention should be focused on the pollutants that come from early stages of flowback.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117614.

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Understanding the effects of hydraulic fracturing flowback and produced water (FPW) to the aquatic invertebrate, *Lumbriculus variegatus* under various exposure regimes

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ABSTRACT

Hydraulic fracturing of horizontal wells is a cost effective means for extracting oil and gas from low permeability formations. Hydraulic fracturing often produces considerable volumes of flowback and produced water (FPW). FPW associated with hydraulic fracturing has been shown to be a complex, often brackish mixture containing a variety of anthropogenic and geogenic compounds. In the present study, the risk of FPW releases to aquatic systems was studied using the model benthic invertebrate, *Lumbriculus variegatus* and field-collected FPW from a fractured well in Alberta. Acute, chronic, and pulse toxicity were evaluated to better understand the implications of accidental FPW releases to aquatic environments. Although *L. variegatus* is thought to have a high tolerance to many stressors, acute toxicity was significant at low concentrations (*i.e.* high dilutions) of FPW (48 h LC50: 4–5%). Chronic toxicity (28 d)of FPW in this species was even more pronounced with LC50s (survival/reproduction) and EC50s (total mass) at dilutions as low as 0.22% FPW. Investigations evaluating pulse toxicity (6 h and 48 h exposure) showed a significant amount of latent mortality occurring when compared to the acute results. Additionally, causality in acute and chronic bioassays differed as acute toxicity appeared to be primarily driven by salinity, which was not the case for chronic toxicity, as other stressors appear to be important as well. The findings of this study show the importance of evaluating multiple exposure regimes, the complexity of FPW, and also shows the potential aquatic risk posed by FPW releases.

1. Introduction

The combination of hydraulic fracturing with horizontal drilling has become a common practice in the extraction of oil and gas around the world over the past decade (Goss et al., 2015). For example, it is estimated that 25,000–30,000 new wells were drilled using these techniques in the United States between 2011 and 2014 alone (Environmental Protection Agency, 2015). Chemicals used in the process can vary from well to well and can include various biocides, well stabilizers, surfactants, and cross-linkers, among others (Environmental Protection Agency, 2015; Stringfellow et al., 2014). A large portion of this fluid system (which would contain the aforementioned chemicals and large volumes of water) returns to the surface in the first few days after well stimulation and is referred to as flowback. The fluids that remain in the well and return to the surface afterward (which is estimated to be 30–80% of injected water volume) are referred to as produced water. Although produced water is characterized as different than flowback, as it typically possesses characteristics more similar to the water stored in the formation; characterizing them separately is difficult as it can be influenced by a variety of factors (Alessi et al., 2017). Additionally, in the field these differences are not all that

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important as flowback and produced water are commonly collected together as a single waste product (moving forward collectively referred to as 'FPW'). The resulting FPW is typically brackish or hyper-saline and can contain a variety of potential constituents from both fracturing process (e.g. biocides, polyacrylamide) and the geologic formation constituents including: salts, metals, organics (e.g. polycyclic aromatic hydrocarbons (PAHs)), and naturally occurring radioactive materials (NORMs) (Stringfellow et al., 2014).

The hydraulic fracturing process is heavily regulated in most countries and industry environmental safety procedures have improved over time. However, due the increased development, the total number of FPW spills or other releases continues to increase [1,2, 4–6]. To date, the majority of the research regarding the risks of FPW spills/releases has focused on public health with little focus on the short and longer-term risks to the environment. Research results evaluating the effects of FPW releases to aquatic organisms have only become available in the last few years. Although limited research has been conducted, it has conclusively demonstrated that even low volume FPW releases into the aquatic environment will have significant sub-lethal and lethal effects on aquatic biota (Blewett et al., 2018, 2017a, 2017b; Delompré et al., 2019b, 2019a; Folkerts et al., 2019, 2017a, 2017b; He et al., 2018, 2017).

To date, one of the largest data gaps in the available research on FPW effects to the aquatic environment is associated with how and in what manner most releases occur (i.e. the type of release, the composition of spilled fluid, and the size of releases (or what is an environmentally-relevant release of FPW)). The US EPA recently reported that of the spills studied by the agency, few spills reached surface water or ground water (18 of 225 studied), but of those that did, most were larger in volume (such as the 11 million L spill from a broken pipeline in North Dakota) (Environmental Protection Agency, 2015). The report also stated that chronic releases (from impoundments or unlined pits) can and do occur and could have long-term impacts to these systems (Environmental Protection Agency, 2015). Currently, a majority of the environmental research evaluating the toxic nature of FPW has focused on the fish species over a 48–96 h time period (Blewett et al., 2017b; Delompré et al., 2019b; Folkerts et al., 2019, 2017b; He et al., 2018), which may not effectively assess the aquatic risk associated with different release scenarios. Outside of this work, the aquatic risk of FPW to invertebrate species has only been evaluated for the pelagic crustacean, *Daphnia* (Blewett et al., 2018, 2017a; Delompré et al., 2019a; Folkerts et al., 2019), only one of which investigated the chronic effects (Blewett et al., 2017a). Additional research understanding acute risk over various time frames, pulse doses, and chronic releases to aquatic life, namely benthic invertebrates, is warranted to complement the currently available literature for understanding the potential aquatic risks of FPW.

The objective of the current study was to better understand the toxicity of acute, pulse, and chronic exposures to FPW to the aquatic benthic invertebrate, *Lumbriculus variegatus*. To accomplish this objective, three experiments were conducted which evaluated short duration and high volume releases (as would be expected at the release point), pulse doses (as would be expected on a larger part of a lotic system as it is flushed downstream), and also chronic releases (as would be expected from impoundment leaks and other unintended slow releases rather than spills). Additionally, as FPW waste varies with time of flowback, FPW was evaluated and characterized at five different times of flowback from the wellhead.

2. Methods

Organisms and FPW collection. *Lumbriculus variegatus* was originally obtained from Environment Canada in Ottawa, Ontario. All organisms were held at 20 ± 1 °C under a 16 h light/8 h dark photoperiod. *L. variegatus* were cultured in dechlorinated city of Edmonton tap water (details of composition can be found in previous work (Blewett et al., 2017b)). Culture organisms were maintained in shredded paper towel and fed three times weekly with solutions prepared from dry algae (AlgaeMax Wafers; New Lite International, Inc, Homestead, FL, USA) and trout chow (Ewos Canada Limited, Surrey, BC).

FPW samples (referred to as 100% FPW) were collected from a hydraulically fractured well (post-stimulation) located outside Fox Creek, Alberta, Canada (Devonian-aged Duvernay Formation) by Encana Corporation (Calgary, AB, CAN). Samples were transported back to the University of Alberta and stored in a dark room at room temperature until use. In this study, FPW from five time points (referred to as time since the initiation of well flowback) were evaluated for both acute and chronic toxicity: 1.33, 24, 48, 72, and 228 h.

Chemical analysis and water quality. Chemical analysis of each of the well sampling times (100% FPW samples) was conducted for a suite of various compounds including trace metals, polycyclic aromatic hydrocarbons, total dissolved solids, total nitrogen, and total organic carbon. The FPW chemistry of all of the five well sampling times is reported in the supplemental information (Tables S1–S2). The chemistry of three of these samples, 1.33 h, 72 h, and 228 h, were previously reported in Folkerts et al. (2019). The analytical chemistry for the 24 h and 48 h well sampling time have not been previously reported and are provided in the supplemental information. Analytical chemistry was not conducted for the dilutions due to time and cost restraints. Dilutions were confirmed via analysis of conductivity. Conductivity was constant in each of the separate bioassay studies as shown in overall low CV values (discussed further in the discussion). Although some slight differences were noted in conductivities, mostly at the higher concentrations, the correlation for conductivity over the range of the dilutions used for all studies was strong for each FPW sampling time (Fig. S1; $R^2 > 0.9$). A description of the methods used in chemical extraction and analysis by GC-MS and HPLC-Orbitrap MS for the 100% FPW can be found in the supplemental information. In all bioassays, water quality was evaluated for conductivity (μS cm^{-1}), pH, and dissolved oxygen (% saturation). Dissolved oxygen was measured using an YSI Model 85 m (YSI Incorporated, Yellow Springs, OH, USA, while conductivity and pH were measured using Omega PHH-7200 (Omega Engineering Canada, St-Eustache, QC, Canada).

Bioassays. Three different bioassays were performed as part of this study including acute (LC50 and LT50), pulse, and chronic bioassays. Bioassays were conducted using unfiltered dilutions of each FPW sample time point. In bioassays, adult *L. variegatus* were collected from the culture and used directly. For each bioassay, ten organisms were used per replicate and five replicates were performed for each dilution treatment. Dilutions of FWP were prepared using the same facility water as the cultures.

Acute bioassays. LC50 bioassays were conducted using five dilutions (2%, 4%, 6%, 8%, and 10%) for each FPW sampling time (1.33, 24, 48, 72, and 228 h) using five different exposure regimes (1, 3, 6, 24, and 48 h). This bioassay was conducted with 30 mL of testing solution in 50 mL vials. Organisms were not fed, no substrate was used, and no water changes conducted during this bioassay. As subsequent studies were going to be conducted over a long period of time (i.e. chronic study), an additional study evaluating the addition of sand as a substrate was conducted. In this bioassay, only two post-stimulation intervals were evaluated (1.33 and 228 h interval) and only for 48 h. Additionally, in this bioassay the addition of aeration was also evaluated in a separate set of replicates with both well sampling times. This study was conducted in larger vessels (100 mL) and used 60 mL of diluted fluid with 15 g of sand

per replicate. Water quality (including dissolved oxygen, conductivity, and pH) was measured in the samples at the beginning and conclusion of both bioassays (*i.e.* at 48 h). Concentrations of 10 and 20% FPW were also assessed for their LT50 for all five well sampling intervals. The same bioassays procedures as the acute study with no sand were used in the LT50 bioassay as well. Mortality was assessed in these bioassays every minute until 100% mortality was observed in each replicate.

Pulse bioassays. Pulse bioassays were conducted for two well sampling times (1.33 h and 228 h). These two times showed the highest toxicity in the chronic bioassays and represented distinct flowback (1.33 h) and produced water (228 h) samples. Two pulses were evaluated including a 6-h and a 48-h pulse. Organisms were first added to individual replicates containing pre-prepared solutions of the FPW (30 mL of FPW dilution in 60 mL beakers) and then after the appropriate time frame was reached (6 or 48 h), organisms were rinsed, removed, and placed into clean vessels. The remaining portion of the bioassay used the same exposure vessels (250 mL jars), volumes (200 mL water and 30 g sand), and regime (28 d) as the chronic bioassay. Water changes, water quality, feeding, and endpoints evaluated were all conducted in the same manner as the chronic bioassay (which is discussed below).

Chronic bioassays. Chronic bioassays were conducted over 28 d in 250 mL jars with 200 mL of diluted FPW per replicate and 30 g of sand per replicate. Five dilutions (0.1%, 0.5%, 1%, 2%, and 5%) were evaluated for each FPW sampling time (1.33 h, 24 h, 48 h, 72 h, 228 h). Concentrations utilized in this bioassay were determined based on the acute exposure bioassays described earlier. Water changes (with a freshly prepared FPW dilution) occurred every other day, with half of the solution (~100 mL) being replaced per replicate. On this day, water quality (before the water change) and feeding (of the abovementioned culture foods) also occurred. Organisms were evaluated for survival/reproduction and growth (via total mass) at the end of the experiment. Organisms were collected at the conclusion of the bioassay and placed into pre-weighed aluminum pans. Pans were dried at 60 °C for three days before being weighed using a Sartorius CP225D analytical balance (Sartourius AG, Goettingen, Germany) to determine dry mass of the organisms.

Bioaccumulation analysis. L. variegatus tissue samples were collected at the end of the acute FPW bioassay that evaluated the addition of air and sand (which evaluated only the 1.33 h and 228 h well sampling times). Tissue was weighed in pre-weighed 1.5 mL Eppendorf tubes following collection (wet weight), air dried for 48 h, and then measured for dry weight. Tissues were then transferred into 15 mL conical tubes and digested using 5 mL trace grade nitric acid and allowed to digest for 48 h in an oven at 55 °C. Following digestion, 5 mL ultrapure water was added to the solution to achieve a total volume of 10 mL. Digested tissue samples were analyzed for sodium (Na) by Atomic absorption spectrometry (ThermoFisher Scientific, Waltham, MA, USA), using US EPA method 7000B (United States Environmental Protection Agency, 2007). Sodium standards (and associated standard curves) were made using reconstituting trace metal basis sodium chloride that was acidified with trace grade nitric acid. To control for possible interferences, all samples were diluted using potassium chloride solution. Using an autosampler, samples were measured for sodium using an ICE 3000 Series AA Spectrometer (ThermoFisher Scientific, Waltham, MA, USA). Sodium concentrations that exceeded the standard curve were diluted using a ThermoFisher auto diluter (ThermoFisher Scientific, Waltham, MA, USA). All samples were run in duplicate and concentrations were calculated as the concentration of total sodium per gram of dry weight of organism.

Statistical analysis. Dose response curves and associated LC50s for acute toxicity testing were calculated using Prism software (GraphPad Software Inc. CA, USA), while LC50s for the chronic bioassay were calculated using R software (R Development Core Team, 2009) and the dose response function (Ritz et al., 2006; Ritz and Streibig, 2005). In both circumstances, the Akaike's information criteria were used to select the most appropriate dose-response functions in each of the bioassays. It should be noted that for total mass EC50 calculations, a total mass of zero was used where 100% mortality occurred (which some have warned could bias (*i.e.* survival bias) the results (Gagliardi et al., 2019)). Prism was also used to conduct two-way ANOVA analysis to compare the different well sampling time treatments in acute and chronic bioassays, and also to compare treatments with and without aeration. Similarly, this software was used for linear regression analysis. If significant differences were noted, a Dunnett's multiple comparison test (using the R software) was employed to compare the individual treatments with the control treatment (with $p < 0.05$ indicating significant differences).

3. Results

Acute toxicity. The LC50 values for *L. variegatus* at all five FPW sampling times (1.33, 24, 48, 72, and 228 h) increased with increasing exposure duration (1, 3, 6, 24, and 48 h). The LC50 values for the first three exposure durations (1, 3, and 6 h) ranged from 4 to 8%; while the LC50 values for the 24 and 48 h exposure period were lower, ranging from 4.1 to 4.8% for the five well sampling times. LC50 values for the five sampling times within a bioassay exposure period were all very similar with only a few exceptions (Fig. 1). It should be noted that although the dilution intervals were narrow in these bioassays (2% between each treatment), the overall lack of acute toxicity differences between the well sampling times was not surprising, as partial mortality was evident in only 11 of 29 (38%) treatments. The lack of partial mortality or what has been referred to as the "all or nothing" response is not a new phenomenon (Kreutzweiser et al., 1994). As expected, the LC50 values decreased with increasing bioassay exposure time, although the differences between the 1 h and 48 h LC50 were generally within a factor of two.

The water quality was evaluated after the conclusion of the 48 h exposure period. The pH ranged from 7.2 to 8.0, with lower values corresponding to lower dilution amounts (i.e. higher FPW concentration). The conductivity ranged from 4.9 to 21.5 mS cm^{-1}, with higher values corresponding to lower dilutions and well sampling events that occurred at later times (*i.e.* 228 h). The dissolved oxygen in these bioassays was generally greater than 60%, with a few exceptions in the higher concentrations. As such, the addition of oxygen via aeration (i.e. 'air' in Table 1) and sand (which was needed in chronic bioassays) was also evaluated (in the 1.33 and 228 h well sampling times) over a 48 h exposure period. The 'no air' treatments in this bioassay had similar pH (ranging from: 7.8–8.2) and conductivities (ranging from: 5.0–24.9 mS cm^{-1}) as those from the previous LC50 bioassays. The pH and conductivity of the aerated samples were all slightly higher than the 'no air' treatments, but still within 10% of one another. Dissolved oxygen in these treatments, however, were considerably different, as aerated samples were all above 90% saturation, while those with 'no air' ranged from 22.8 to 82.2% saturation. Regardless, the addition of neither 'air' nor sand greatly affected the toxicity of FPW to *L. variegatus* (Table 1).

Interestingly, in even the shortest exposure period tested (1 h), 100% mortality occurred in the highest concentration tested (10%) for all well sampling treatments. As a result, we decided to evaluate the acute toxicity of higher amounts of FPW (or less diluted samples) using an LT50 bioassay (10% and 20% dilution of FPW; Table 2). The pH in the 10% and 20% FPW dilution bioassays ranged from 7.8 to 8.0 and 6.7 to 7.7, respectively. The conductivity of the 10% and

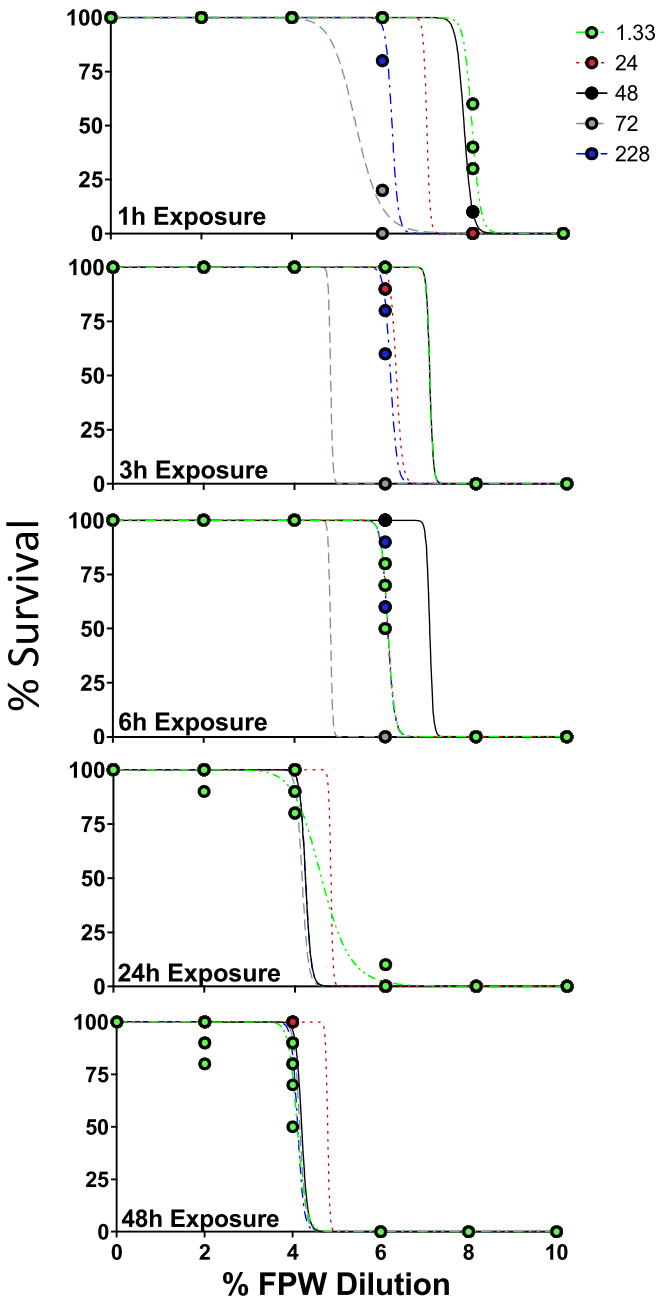

Fig. 1. Dose response curves and associated LC50s (survival) in *Lumbriculus variegatus* for testing with FPW, with each circle depicting a replicate. Five well sampling times (1.33, 24, 48, 72, and 228 h) were tested using five exposure periods (1, 3, 6, 24, and 48 h). Values are presented as percent dilution of 100% unfiltered FPW.

20% FPW dilution ranged from 29.2 to 37.6 and 65.8 to 83.0 mS cm^{-1}, with higher conductivities corresponding to later well sampling events (i.e. 228 h). LT50 values for 10% and 20% dilutions of FPW, regardless of well sampling time, ranged from 12 to 20 min and <3 to 4 min, respectively. In both LT50 bioassays, dissolved oxygen was >70%. In these bioassays, the effect of well sampling times was more apparent as the LT50 differences between the earliest (1.33 h) and the latest (228 h) sampling times had non-overlapping confidence intervals.

Pulse toxicity. Rather than evaluating pulse toxicity for all well sampling times, we chose to evaluate two pulse events (6 and 48 h pulse) for two well sampling times (1.33 and 228 h; Table 3).

Table 1
The 48 h LC50 (and 95% confidence intervals) for *Lumbriculus variegatus* when exposed to FPW in the presence or absence of sand and/or aeration for two well sampling times (1.33 and 228 h). LC50 values are presented as percent dilution of 100% unfiltered FPW.

		Well Sampling Time (h)	
		1.33	228
48h LC50 (% FPW)	**Without Air & Sand**	4.69 (3.77–5.61)	4.81 (3.79–5.83)
	With Air & Sand	5.06 (0.84–9.28)	4.89 (4.86–4.91)
	Without Air & With Sand	5.60 (4.33–6.86)	4.89 (4.86–4.91)

Table 2
The LT50 (and 95% confidence intervals) of 10 and 20% FPW (for well sampling times of 1.33, 24, 48, 72, and 228 h) to *Lumbriculus variegatus*. LT50 values are presented as time in minutes.

Well Sampling Time (h)	LT50 (min)	
	20% Dilution	10% Dilution
1.33	3.57 (3.36–3.78)	19.1 (18.93–19.28)
24	<3 _	18.99 (18.82–19.16)
48	<3 _	14.53 (14.42–14.63)
72	<3 _	14.52 (14.25–14.80)
228	<3 _	12.47 (12.35–12.59)

Survival and growth of *L. variegatus* were then evaluated after a total of 28 d. Growth and reproduction in control individuals was not as productive as in the chronic experiments (10.5 organisms in comparison to 10 initially used; 0.014 g total mass in comparison to the 0.010 g initially used). Regardless of the lack of reproduction and growth in the bioassay, toxicity was similar to that observed in their respective acute toxicity bioassays (Table 1) with the 48 h pulse causing a lower LC50 than the 6 h pulse. In this bioassay, the LC50s dropped 20–34% when compared to the acute bioassay, a product of latent mortality in the study. Interestingly, the EC50 for total mass in this bioassay was not considerably lower than the LC50, which could be a product of the lack of growth in the bioassay. The water quality in this bioassay was similar to the acute study during the pulse event, after which time pH (8.0–8.2), dissolved oxygen (72.5–80.3%), and conductivity (0.44–0.55 mS cm^{-1}) were maintained at control levels (and new clean fresh water was used to replenish the replicates). The presence of latent mortality was not surprising as it is well known that possibly 'effected' organisms in an acute study may not recover even if exposure is discontinued (Zhao and Newman, 2004), which was the case in the current study.

Chronic toxicity. In the control replicates the number of individuals nearly doubled (16.4 organisms in comparison to 10 initially used) and their total mass was more than doubled (0.025 g total mass in comparison to the 0.011 g initially used). The toxicity in response to chronic FPW exposure significantly increased with increasing FPW concentrations (Table 4). A two-way ANOVA showed that significant differences were noted between the concentrations and between the different sampling times. In comparison to the acute study, the 1.33 h well sampling time showed the highest chronic toxicity for both endpoints (LC50: 0.53% and $EC50_{total\ mass}$: 0.22%), while the lowest toxicity was in the 24 (LC50: 3.31% and $EC50_{total\ mass}$: 2.78%) and 48 h (LC50: 2.89% and $EC50_{total}$

Table 3
The 28 d pulse LC50 and EC50 (total mass) of FPW (% dilution) for *Lumbriculus variegatus* for two well sampling times (1.33 and 228 h) as well as the respected LC50 value for the acute study for each pulse event (6 and 48 h). The numbers in parenthesis are the 95% confidence intervals for the LC50 or EC50 values.

Endpoint	Test Duration	Pulse Duration	Well Sampling Time (h)	
			1.33	228
LC50 (%)	6 h	–	6.18 (5.09–7.26)	6.19 (5.41–6.97)
LC50 (%)	48 h		4.69 (3.77–5.61)	4.81 (3.79–5.83)
LC50 (%)	28 d	6 h	4.62 (0.09–9.15)	4.98 (2.06–7.90)
EC50 - Total Mass (%)			4.45 (2.31–6.59)	5.06 (0.49–9.63)
LC50 (%)	28 d	48 h	3.67 (3.02–4.33)	3.18 (2.63–3.73)
EC50 - Total Mass (%)			4.44	3.51

Table 4
Chronic toxicity (# of organisms and total mass) of FPW (% dilution) to *Lumbriculus variegatus*. Five well sampling times (1.33, 24, 48, 72, and 228 h) were tested using five dilution concentrations (0.1, 0.5, 1, 2, and 4% FPW) under a 28 d exposure. Endpoint responses (# of organisms and total mass) that are shaded indicate a significant difference between that FPW dilution and the control. Dissolved oxygen and conductivity cells that are shaded indicate those that exceeded thresholds based on public literature (Dissolved oxygen reached <10% saturation on a given day (Mattson et al., 2008); Conductivity was >5 mS cm^{-1} (Lob and Silver, 2012)).

Well Sampling Time (h)	FPW Dilution (%)	# of Organisms		Total mass (mg)		Dissolved Oxygen (% Saturation)		Conductivity (mS/cm)	
		MEAN	STDEV	MEAN	STDEV	MEAN	STDEV	MEAN	STDEV
1.33	0.1	16.8	1.5	15.5	4.2	66.5	10.3	1.0	0.1
1.33	0.5	9.4	0.9	10.7	4.2	42.4	12.3	2.1	0.1
1.33	1	1.0	1.0	0.6	0.1	23.9	17.7	3.4	0.2
1.33	2	1.4	1.9	1.0	0.6	23.6	15.1	6.1	0.2
1.33	4	0.0	0.0	NA	NA	25.0	13.5	12.1	0.4
24	0.1	15.4	1.1	24.9	5.7	71.7	4.8	1.0	0.1
24	0.5	19.0	1.6	23.9	3.9	65.0	10.5	2.1	0.1
24	1	21.4	2.1	25.3	2.2	63.0	7.9	3.8	0.3
24	2	16.6	2.2	19.5	7.4	46.0	13.6	6.7	0.3
24	4	5.3	1.9	4.8	2.9	29.5	22.6	13.0	0.4
48	0.1	19.4	2.3	24.3	5.0	68.8	8.3	1.0	0.1
48	0.5	18.0	1.4	18.2	1.9	68.5	6.8	2.2	0.1
48	1	19.2	2.2	20.4	3.9	66.3	7.0	3.9	0.2
48	2	17.8	2.9	10.5	1.4	66.5	8.6	7.1	0.2
48	4	0.8	1.8	1.1	--	67.1	7.0	13.4	0.4
72	0.1	17.0	1.9	24.5	2.9	73.9	5.6	0.9	0.1
72	0.5	18.8	2.3	15.0	2.7	66.6	7.8	2.3	0.2
72	1	15.0	2.7	12.2	2.5	60.1	10.5	4.0	0.2
72	2	9.4	0.5	6.4	1.2	59.3	7.2	7.4	0.1
72	4	0.0	0.0	NA	NA	56.4	8.6	14.0	0.6
228	0.1	14.4	1.9	20.7	3.5	73.6	6.4	1.0	0.1
228	0.5	18.0	2.5	16.8	0.7	67.2	8.8	2.4	0.1
228	1	11.8	1.8	8.3	1.3	60.2	8.8	4.4	0.2
228	2	9.8	0.4	6.9	1.5	62.1	7.3	8.0	0.3
228	4	0.0	0.0	NA	NA	55.5	7.1	14.8	0.3

$_{mass}$: 1.65%) sampling times. The two final sampling times had similar toxicities to one another (72 h LC50: 2.07% and $EC50_{total\ mass}$: 0.84%; 228 h LC50: 2.15% and $EC50_{total\ mass}$: 0.76%). Overall, the chronic LC50s were 1.50–8.85 fold lower than the acute LC50s (48 h exposure data) for the five sampling times. In a few circumstances, low concentrations of FPW had significantly higher reproduction than the control (such as the 1% FPW in 24 h well sampling interval which showed over a two fold increase). However, the total mass in these events, showed no differences suggesting that these individuals were smaller overall.

Similar water quality trends to the acute bioassays were observed in the chronic study with the exception of lower dissolved

oxygen levels (Table 4). In short, pH was generally lower in dilutions with higher amounts of FPW (7.6–8.2), while conductivity was higher (0.58–14.8 mS cm^{-1}). These concentrations were consistent throughout the 28d bioassay period (CV for pH and conductivity was <1.6% and <13%, respectively). Dissolved oxygen, on the other hand, significantly decreased throughout the study in all treatments and dropped as low as 4.9% in the dilutions with high amounts of FPW for a subset of well sampling times.

Bioaccumulation. Body residues of sodium (which was one of the trace elements recorded at the highest concentrations in the samples (Flynn et al., 2019; Folkerts et al., 2019)) were measured in *L. variegatus* at the conclusion of the acute bioassay that evaluated the addition of aeration. No differences in toxicity, solution conductivity, or accumulated sodium were noted for a given FPW treatment with or without aeration for both well sampling times (1.33 and 228 h), and thus aerated and non-aerated treatments were pooled together for analysis. The bioaccumulation of sodium in *L. variegatus* for all dilutions was found to significantly correlate to the conductivity of those dilutions (Fig. 2). Sodium accumulation doubled in the 4% dilution of FPW when compared to control (control organisms (mean ± stdev): 550 ± 116 mg g dry wt^{-1}; 1.33 h well sampling time treatment at a 4% dilution: 1142 ± 214 mg g dry wt^{-1}; 228 h well sampling time treatment at a 4% dilution: 1070 ± 242 mg g dry wt^{-1}).

4. Discussion

Toxicity of FPW. The acute toxicological response of *L. variegatus* to FPW was found to be similar to that reported by Folkerts el al. [15] for the three samples (1.33, 72, and 228 h) which were analyzed in both studies (48 h LC50: 3.1–4.2% (Folkerts et al., 2019); 48 h LC50: 4.1–4.8% (present study)). In that study, *L. variegatus* was more sensitive to FPW contamination than rainbow trout (for both embryos and juvenile fish), even though a longer exposure period was utilized in the fish bioassays (96 h). The most sensitive species tested was *Daphnia magna*, which was the only other invertebrate evaluated (48 h LC50: 0.76–1.58%). To date, the sensitivity of only a few species to FPW have been evaluated, with most of those species considered quite tolerant of pollution. Folkerts et al. (2019), only addressed acute toxicity (48 or 96 h exposures), which is a common theme in the majority of the available FPW toxicity literature (Blewett et al., 2018, 2017b; Delompré et al., 2019b, 2019a; Folkerts et al., 2019, 2017a, 2017b; He et al., 2018, 2017). The chances of acute exposure occurring in the field are highly unlikely, especially in a lotic system. The LT50 results (Table 2) and pulse study (Table 3) results suggest that high exposure events and pulse exposures would result in high toxicity and latent mortality issues, respectively. These results combined provide a much clearer picture of the aquatic risk of FPW spills especially at the point of impact, where dilutions as low as 10% would cause dramatic consequences to aquatic life.

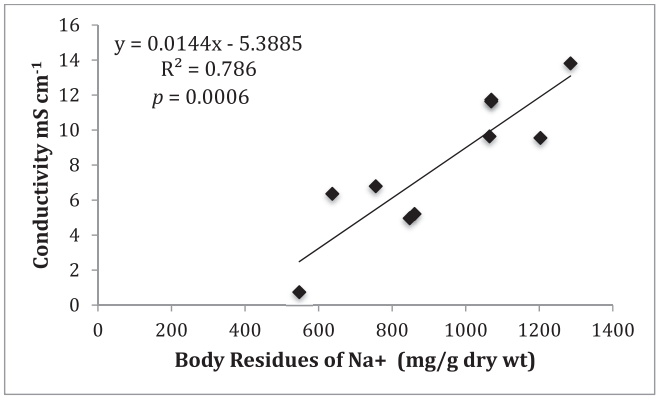

Fig. 2. Linear regression of sodium concentrations in the body of *L. variegatus* in relationship to the conductivity of that treatment. Each data point represents the mean conductivity and body residue for a given treatment for either the 1.33 or 228 h well sampling times. The modeled equation, r^2, and p value are provided.

Outside of acute studies, to our knowledge, there has only been one study that has addressed the chronic risk of FPW releases to aquatic biota (Blewett et al., 2017a). In this study, a dilution of 0.04% FPW resulted in a 3-fold decrease in clutch size of *D. magna* over a 21 d period, which coincided with the observed delay in time to first brood. The dilution required to cause a chronic health effect to *D. magna* was considerably lower than those for *L. variegatus*, as LC and EC50 values ranged from 0.22 to 3.31% in the present study. Regardless, these FPW release amounts illustrate the significant hazard associated with FPW and show the substantial need for further work understanding species sensitivity in relation to FPW toxicity.

Causality of FPW. Although not the primary focus of the study, insights into causality inferred from the present study are in close alignment with previous work. Dilutions of FPW that showed greater than 10% mortality after 48 h exposure all had conductivity values (a common proxy for salinity) above 10 mS cm^{-1} (with one exception, 2% FPW of well sampling time 1.33 h, which showed 22% mortality at a conductivity of 5 mS cm^{-1}). It should also be noted that one sample with a conductivity of greater than 10 mS cm^{-1}did not exhibit toxicity (4% FPW for well sampling time 48 h had a conductivity of 10.1 mS cm^{-1}), otherwise all dilutions that had conductivities below 10 mS cm^{-1} showed less than 10% mortality. These acute values for conductivity are quite similar to previous work examining road salts (NaCl) with other invertebrates, which showed that a large range of insect species were unaffected by conductivity levels up to 10 mS cm^{-1} (Blasius and Merritt, 2017). Furthermore, the bioaccumulation results (Fig. 2) showed high accumulation of sodium, which increased with amounts of FPW. It should be noted, that the bioaccumulation bioassay only analyzed a single ion (sodium) and a variety of other ions associated with salinity, such as calcium and chloride, and other elements of concern (such as barium and strontium) were also elevated in these FPW samples and would also most likely accumulate in the body (Table S1).

The addition of dissolved oxygen had no effect on the survival of these organisms, suggesting that at least in regards to acute toxicity that decreases in dissolved oxygen does not appear to be a driving factor. Low levels of dissolved oxygen were not expected to cause acute toxicity, as past work has shown that dissolved oxygen concentration as low as 0.7 mg O_2 L^{-1} (<10% saturation) caused no mortality to *L. variegatus* over a 10 d exposure period (Mattson et al., 2008). Although salinity appears to be the main driving force-governing toxicity, other stressors cannot be dismissed as metals, trace elements, and PAHs and other organics are also present (Fig. S2, Tables S1–2). The role of these constituents will be discussed in further detail in the chronic risk of FPW discussion below.

In our chronic study, dilutions with conductivities above 5 mS cm^{-1} also showed deleterious effects to *L. variegatus* (8 of 10 dilutions with >5 mS cm^{-1} showed significant decreases in reproduction and 9 of those showed significant decreases to total mass; Table 4). In a few dilutions, conductivities of less than 5 mS cm^{-1}, still exhibited chronic toxicity to *L. variegatus* (3 of 15 dilutions for reproduction and 7 of 15 for total mass). Conductivities in these dilutions ranged from 1.0 to 4.4 mS cm^{-1}. A chronic study evaluating the effects of road salt to *Chironomus riparius* (also considered a tolerant species) showed that conductivity values of

~5 mS cm^{-1} affected survival and/or emergence (Lob and Silver, 2012). These conductivities did not elicit toxic effects in other treatments suggesting other stressors are at play in these dilutions.

In contrast, to the acute study, dissolved oxygen could also be a possible stressor of concern in a subset of dilutions, namely for the FPW collected at 1.33 h. Dissolved oxygen in a few of the dilutions of FPW (1% and 2% as well as in the 24 h 4%) dropped to single digits (<10%) for multiple days with the average below 25% (highlighted in Table 4). Although studies have suggested that dissolved oxygen concentrations as low as ~10% had no effect on survival of this species (Mattson et al., 2008), in combination with other stressors over 28 days it may play a role in toxicity. More concerning is that dissolved oxygen levels could be lower in an environmental scenario as ~50% of the water of each replicate was being changed every other day in the present study. The 1.33 h treatment was collected at the earliest FPW sample collected for this study and most closely resembles a true flowback sample rather than a produced water sample. FPW (especially flowback samples) are typically high in dissolved organic carbon as well as anthropogenic substances, which was the case in the present study (Tables S1–S2, Fig. S2 (Alessi et al., 2017; Sun et al., 2019; Thurman et al., 2014)). In the 1.33 h sample, total organic matter was more than 20 fold higher than in the 228 h sample (Table S1), and contained higher amounts of many PAHs (Table S2). The high organic matter means it is highly likely that this sample has a high chemical oxygen demand resulting in the observed low dissolved oxygen. In addition to salinity and dissolved oxygen issues, other stressors could also contribute to the FPW toxicity. For instance, strontium ranged from 673 to 904 mg L^{-1} in 100% FPW. Even at a 10% dilution the concentration would be 67.3–90.4 mg L^{-1}, which is more than six times higher than the reported chronic effects benchmark (HC5: 10.7 mg L^{-1} (Mcpherson et al., 2014)). Outside of inorganic constituents, organic compounds may also be a product of the observed effects. PAH concentrations in 100% FPW were quite low in all FPW samples (and would be even lower in the diluted samples; Table S2) and are unlikely to be a driver of toxicity to *L. variegatus*, which is quite tolerant of PAH toxicity (Berry et al., 2003). However, other compounds, such as polyethylene glycols (PEGs) and ethoxylates (EOs), are also present and could be affecting toxicity and/or bioavailability of other constituents in the FPW matrix (Fig. S2). Unfortunately, a full hazard assessment of all the constituents, such PEGs and EOs, in a FPW sample to determine causality is difficult as many constituents either do not have available toxicity information, are not able to be characterized analytically, or even are completely transformed into new compounds during the hydraulic fracturing process (Kahrilas et al., 2016). Coupled with this is the fact that numerous chemicals are present simultaneously as a complex mixture in a brackish medium, and so determining causality and risk becomes challenging.

Study species choice. *L. variegatus* is more commonly used as a bioaccumulation test species, rather than for acute and chronic toxicity testing due to its high tolerance to various stressors (Chessman and McEvoy, 1998; United States Environmental Protection Agency, 2000). With that being said, the use of *L. variegatus* as a test species in this study had a couple of advantages. As this species is generally quite tolerant to stressors, it likely provides a conservative scenario of aquatic effects in the event of an accidental FPW release. For instance, a release that would result in 10% FPW in an aquatic system (which caused a 50% reduction of survival in *L. variegatus* in less than 15 min) would eradicate the most sensitive invertebrate species within minutes. For instance, the 10-d conductivity EC20 for mayflies (endpoint: total abundance via a mesocosm study; EC20: 0.28 mS cm^{-1}) would be a more than 100 fold lower than conductivities found in this dilution (conductivity ranging from 29.75 to 37.6 mS cm^{-1}). Similarly, the US EPA derived field based specific conductance benchmark (0.3 mS cm^{-1}) is much lower than the conductivities found to elicit acute and chronic effects to *L. variegatus* (Clements and Kotalik, 2016; Cormier et al., 2013; United States Environmental Protection Agency, 2011). The low benchmark developed by the EPA further strengthen our conclusions that salinity is the likely driver of toxicity in FPW exposures to this species, but also confirms the need for further work with more sensitive invertebrate species.

The second advantage of using this test species is that although *L. variegatus* is considered a quite tolerant species overall, how this organism copes with salinity stress (a major driver for FPW) would be different than those invertebrate species evaluated in past studies. To our knowledge, studies to evaluate the risk (especially chronic risk) of FPW to invertebrates are those that have evaluated it in crustaceans (namely *Daphnia*). Crustaceans, such as *Daphnia magna*, are typically quite tolerant of salinity stress, thus evaluating other invertebrates is critical to better understanding implications of the elevated salinity of FPW. Lastly, this species although not commonly used for toxicity bioassays it is commonly used in bioaccumulation testing. The bioaccumulation bioassay showed the accumulation of salt ions in the body of *L. variegatus*. Although this was a small component of the work, it highlights the amount of salt accumulation that was occurring with subtle increases in the amount of FPW present. This work may serve as a foundation for future research investigating bioaccumulation issues associated with FPW.

Study design implications. The toxicity and determinations of causality for FPW has been evaluated in previous studies in three manners (1) via the direct assessment of produced FPW (Blewett et al., 2018, 2017a, 2017b; Delompré et al., 2019b, 2019a; Folkerts et al., 2019, 2017a, 2017b; He et al., 2018, 2017) as conducted herein; (2) through evaluations of known chemicals in FPW (i.e. 'spiked' studies and literature values (Campa et al., 2018; Gandhi et al., 2018; Sano et al., 2005)); or (3) through assessments of biota in areas of known FPW releases (Grant et al., 2016; Papoulias and Velasco, 2013). All three types have their advantages and disadvantages when evaluating the aquatic risk that FPW poses when entering an aquatic system. For instance, in the present study, the assessment of FPW was conducted over multiple well sampling events but only for a single well in Alberta. This well, located in the Duvernay formation, would not only most likely be quite different from other wells in this formation (as shown in past work (He et al., 2017)), but would be quite different from those found in other formations such as the Marcellus, Fayetteville, Barnett, Bakken, and Denver-Julesburg shales (Flynn et al., 2019). For example, FPW collected from wells in the Duvernay formation had higher levels of total dissolved solids in the present study (~150,000 mg L^{-1}) than those FPW samples collected in the Denver – Julesburg basin (22,500 mg L^{-1} (Lester et al., 2015)). Outside of the geogenic chemical differences in shale formations, the geology and chemistry of the formation would also differ and may result in different chemicals being needed for the hydraulic fracturing process resulting in unique chemical signatures in each FPW. Additionally, given that hydraulic fracturing fluids are provided by a variety of service providers, the exact composition is quite variable and certain constituents are considered proprietary information.

The use of field-collected FPW has several advantages to understand the toxic nature of FPW, the most important being the ability to capture the full complexity of FPW in our toxicity assays. The numerous studies that have assessed risks of FPW by recreating chemical profiles from lists and/or using spiked bioassays are only able to evaluate risk for a subset of chemicals found in fracturing fluid and FPW. Similarly, those studies evaluating sites impacted by FPW cannot conclusively rule out stressors outside of FPW contamination (*i.e.* non-FPW contaminants). However, studies

using individual spiked chemicals and assessment of spill areas also have their strengths as they provide causality information for individual components of FPW and provide environmental realism when spills occur, respectively. Policy and mitigation efforts must evaluate all three types of studies to effectively understand the impacts of releases of FPW, as all three provide useful information in addressing aquatic risk.

5. Conclusions

In the present study, the acute, chronic, and pulse risk of FPW to *L. variegatus* was addressed. Additionally, the study showed that the constituents and toxicity of FPW changed temporally with the time of flowback at the wellhead. Perhaps the most important finding is that low volumes of FPW can cause substantial aquatic chronic effects to invertebrates. This would assume that the release is uniform throughout the aquatic system; the pulse and acute data from the present study (namely the LT50 work) highlight the significant aquatic impacts at the point of release. Work to evaluate the aquatic risk of FPW is still in its infancy, and further work investigating exposure scenarios, species sensitivity, and other shale formations will provide much needed information into monitoring, assessing, and mitigating the impact of FPW releases.

Declaration of competing interest

The authors of this study declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113889.

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Effects of alkylphenols (C_4-C_7) on the reproductive system of Atlantic cod (*Gadus morhua*).

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II. *Development of analytical methods for detection of low levels of alkylphenols in oil produced water and biological tissues*

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Abstract

The work in this thesis is a part of a strategic work at the Institute of Marine Research, which aims to develop a variety of methods to be used in investigating potential endocrine disruption in Atlantic cod (*Gadus morhua*).

Produced water, a by-product of offshore oil production, contains significant amounts of alkylphenols (APs). Many studies have shown that long-chain para-substituated APs cause endocrine disruption in freshwater fish, but relatively little is currently known about their long-term effects on the biology of marine fish. Here we describe the results from two experiments studing in detail the effects of some APs present in produced water on the reproductive potential of first-time spawning Atlantic cod. Groups of cod were fed pastes containing four APs (4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol), at different concentrations for either 4 months (experiment 1) or 5 weeks (experiment 2). AP-exposed fish were compared to unexposed fish and to fish fed paste containing natural estrogen (17 β-estradiol). The results of the present study suggest that multiple mechanisms underlie the responses in the AP treated cod. The exposure to APs influences the plasma concentration of several male and female sex hormones and the egg yolk precursor protein, vitellogenin, in Atlantic cod. This study also shows that AP-exposure down to 20 μg/kg body burden interferes with the maturation of the sex organs, and that this effect is likely caused by disruption of the sex hormone system. There were also found effects of the AP treatment on the hepatic P450 systems (CYP1A and CYP3A) as well as glutathione, glutathione-related enzymes and changes in the lipid composition in liver and brain membranes.

Even though the concentrations used in our experiments are higher than may be reasonably expected as the result of oil production alone, measurements of actual AP levels in the sea indicate that APs may still be a significant risk factor in the marine environment.

List of Abbreviations

11KT	11-ketotestosterone
11β-HSD	11β-hydroxysteroid dehydrogenase
17α,20-βP	17α, 20β-dihydroxy-4-pregnen-3-one
17β-HSD	17β-hydroxysteroid dehydrogenase /oxidoreductase
20β-HSD	20β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
AhR	Aryl hydrocarbon receptor
AP	Alkylphenol
APE	Alkylphenol ethoxylates
APEC	Alkylphenol carboxylates
AR	Androgen receptor
BCF	Bioconcentration Factor
BP	Butylphenol
BPG	brain-pituitary-gonadal axis
BHT	2,6-di-*tert*-butyl-4-methylphenol
cAMP	Adenylate cylase
CAR	Constitutive androstane receptor
CAT	Catalase
CNS	Central-nervous-system
Da	Dopamin
DCM	Dichloromethane
E2	17β-estradiol
EDC	Endocrine disrupting chemicals
ER	Estrogen receptor
EROD	Ethoxyresorufin-O-deethylase
FSH	Follicle-stimulating hormone
GABA	γ-aminobutyric acid
GC-MS	Gas Chromatography-Mass Spectrometry
GC-ECD	Gas Chromatography-Electron capture detector
GC-FID	Gas Chromatography-Flamme ionisation detector
GnRH	Gonadotropins releasing hormones
GR	Glutathione reductase
GSH	Glutathione
GSI	Gonadosomatic index
GST	Glutathione S-transferase
GTH	Gonadotropine
HepP	Heptylphenol
HexP	Hexylphenol
HSI	Hepatosomatic index
ICE 182,780	7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]-17β-estradiol
LC	Lethal concentration
LH	Luteinising hormone
LOEL	Lowest observed effect level
MAPK	Mitogen activated protein kinase
NSC	Neural stem cells
NOAEL	No observable adverse effect level
NP	Nonylphenol
NPE	Nonylphenol ethoxylates
OP	Octylphenol
P45011β	11β-hydroxylase

P450c17	17α-hydroxylase/17,20 lyase
P450C21	21-hydroxylase
P450scc	P450 cholesterol side chain clevage
PGC	Primordial germ cell
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PNEC	Predicted no effect concentrations values
PP	Pentylphenol
PUFA	Poly unsatureted fatty acids
PXR	Pregnane X receptor
QSAR	Quantitative structure – activity relationship
ROS	Reactive oxygen species
SFA	Saturated fatty acids
SOD	Superoxide dismutase
SPE	Solid phase extraction
StAR	Steroidogenic acute regulatory protein
SULT	Sulfotransferases
T	Testosterone
UDPGT	Uridine diphospho-glucuronosyltransferases
VTG	Vitellogenin
Zrp	*Zona radiata* protein

CONTENTS

1. Introduction

1.1 Background

There is need for more knowledge of the effects on the marine environment due to discharges to the sea from the offshore oil and gas industry. Norway is currently in a period where discharges of produced water from the petroleum sector are increasing rapidly as the oil fields ages. Produced water is defined as the water that comes up with oil and gas from sea bed reservoirs, separated on the platform from the oil and discharged into the sea. In 2004 it was estimated that 143 million m^3 of produced water was released (OLF, 2005). The prognoses show that the increasing trend will continue until 2011, and the discharges may reach 180 million m^3 a year before it starts to decline. More knowledge of the long-term effects on the marine environment are essential for the authorities to ensure a healthy development of this sector and to coordinate the exploitation of Norwegian 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 lead to changes in biological diversity or in the marine ecosystem.

In 1997, the Institute of Marine Research started the project "The hormonal effects of alkylphenols on cod (*Gadus morhua*)" that aimed to clarify potential harmful effects of alkylphenols (APs) on cod. Significant quantities of APs are released into the sea by petroleum installations as a result of discharges of produced water. It has been shown that APs may have estrogenic (feminising) effects on fish and animals, resulting in reproductive disturbances. The question was whether cod, Norway's most important commercially fished species, might be similarly affected. Experiments with long-term exposure of cod by environmental relevant doses of selected APs were therefore carried out.

1.2 Brief overview of the endocrine regulation of fish reproduction

The endocrine system plays a essential role for a successful reproduction, and is involved in multiple reproductive functions in vertebrates, like: sex differentiation and development of sexual organs, initiation of puberty, development of secondary sexual characteristics, sexually behavior and controlling the reproductive cycles.

There are more than 24000 different fish species and between these there are large variety both in mechanism of sex determination and reproductive physiology (Nelson, 1994). In thise thesis, the focus will be on gonochoristic teleosts, like cod (gonochoristic: species with separate sexes, the male and female reproductive organs being in different individuals, as opposed to hermaphroditic, gynogenetic, and hybridogenetic).

Sex determination and sex differentiation.

Sex determination and sex differentiation are defined as two different, but closely connected processes (reviewed in Devlin and Nagahama, 2002). The sex determination is the primary control (often predetermined genetically at fertilization) that leads to sex differentiation, the development and expression of the male or female phenotypes (development of testis or ovary). In fish embryos, the germ cells are only present as undifferentiated primordial germ cells (PGCs) and are similar for both sexes, and will later differentiate into oogonia (females) or spermatogoina (Males). Sex differentiation takes first place after hacthing, but there are large differences between species where in the larvae development this takes place. However, it appears to be common that there are a relative short “critical period” where the fish larvae is especially sensitive for the hormonal signals that initiate cell differentiation of PGC and somatic gonadal cells. The endocrine regulation of sex differentiation is not completely understood, but it involves a complex interplay between the brain and gonad and it is clear that sex steroid plays a very important role. Steroid producing enzymes (chapter 1.3) can be detected prior to sex differentiation and especially aromatase, the enzyme responsible

of the last steep in the estrogen synthesis, is found present in the brain (of both sexes) and the gonad (for females) in the time of sex differentiation (Devlin and Nagahama, 2002). Since the appearance of steroid synthesizing cells requires cells differentiation, it is unlikely that the steroids themselves are the primary factors involved in determination of sex ("who came first the hen or the egg"?). Nevertheless, steroid production is very closely correlated with early steps of gonadal differentiation. This is clearly showed by the adverse affect on sex differentiation that can be caused by interfering with the steroid balances. For example, inhibition of estrogen synthesis in early development using aromatase inhibitors can cause masculinization while treatment with exogenous estrogen can cause feminization in many fish species.

The essential role of steroid hormone makes the sex differentiation event vulnerable for endocrine disruption as will bediscussed later in the thesis.

Puberty.

Puberty is the development that brings an immature juvenile to a mature adult reproductive system (Schulz and Goos, 1999). The timing of puberty is in addition to genetic factors also controlled by a variety of external stimuli like photoperiod, water temperature and availability of food. The pubertal maturation is synchronized via the brain-pituitary-gonadal (BPG) axis, and the onset of puberty starts with stimulation of the synthesis of the neuroendocrine decapeptide gonadotropins-releasing hormones (GnRH) in the brain (Welzien et al., 2004; Whitlock et al., 2006). These do in turn control the secretion of gonadotropins (GTH) from the pituitary (follicle-stimulating hormone (FSH) and luteinising hormone (LH)). The GTHs are heterdimeric glycoproteins, consisting of a common glycoprotein α-subunit and a hormone-specific β-subunit. The GTHs are transported by the blood to the gonads and binds to specific membranes reseptors on the gonadal somatic celles, Leydig and Sertoli cells in testis and thecal and granulosa cells in the ovary. The GTHs stimulate the maturation of the gonads and cause these to produce sex steroid hormones, 17 β-estradiol (E2) and

testosterone (T) in female fish; T and 11-ketotestosterone (11KT) in males. Sex steroids have important feedback effects on secretions of hormones from the pituitary and the brain, but are also required in the gonads for germ cell maturation (for details, see (Baroiller et al., 1999; Nagahama, 2000)). Puberty is the first step into oogenesis and spermatogenesis.

Oogenesis

After sex differentiation at larvae stages, the oogonia increase in numbers in the gonads through mitotic proliferation. Oogenesis begins at puberty, when a portion of the oogonia entry into meiosis and becomes primary oocytes. The meiosis is arrested in diploytene stages of prophases I, and the oocyte stays like that through out the growth phases, and until final oocyte maturation where the first meiotic division is completed. The second meiotic division of the oocyte is first completed after fertilization. Together with the onset of previtellogenic growth the folliculogenesis is started and the ovarian follicle is formed (figure 1). In the follicle the oocyte is covered with granulosa cells, which in turn is surrounded by thecal cells. A part of the formation of the follicle is the zonagenesis. Eggshell proteins, *zona radiator* proteins (Zrp) are synthesized in the liver under the influence of E2 and transported to the ovary and incorporated in the corian around the ooctye. Vitellogenisis is the major growth phases of the oocyte and account for as much as 90 % of the final egg weight. Vitellogening (VTG) is a glycophospholipoprotein and the main source of yolk proteins and lipids in the growing oocyte. VTG is, like Zrp, synthesized in the liver in response to E2 and transported by the blood and taken up by the oocyte through receptor-mediated endocytosis (Tyler and Sumter, 1996; Tyler et al., 1999).

The BPG axis plays a central role in regulating the oogenesis by controlling the synthesis of sex steroids (T and E2) that in turn are stimulating the oocyte growth (illustrated in figure 3). In the end of vitellogenisis the BPG axis stimulates a shift in the steroidogenesis of the ovary from synthesizing E2 to produce maturation-inducing

steroids (MIS), which leads to the breakdown of germinative vesicles, maturation of the oocyte and ovulation. At least two different steroid hormones have been identified to induce final maturation in teleost: 17α,20β,-dihydroxy-4-pregnen-3-one (17α,20β-P) and 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P) (Nagahama, 1997).

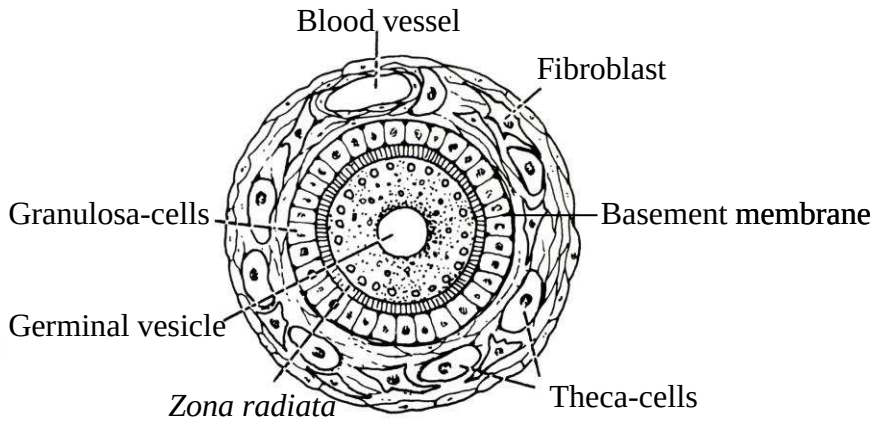

Fig 1. *Diagram of a primær ovarian follicle in fish. The oocyte is covered by zone radiata (also known as chorion and wich lather becomes the egg shell). The oocyte is closely connected with granulosa cells by microvilli located in the chorionic pores. The oocyte and granulosa cells are separated from the surrounding theca cells, blood vessel and fibroblast by a basement membrane. (Illustration: Stein H. Mortensen, IMR).*

Spermatogenesis

During spermatogenesis the male germ cells go through four major phases:
1). Mitotic proliferation, where the Spermatogonial stem cells undergo a specific number of mitotic cycles, leading to both new stem cells and differentiated spermatogonia. 2). Meiosis, where the differentiated spermatogonia undergo meiosis and becomes primary spermatocytes, secondary spermatocytes and finally haploid spermatides. 3). Spermiogenesis, where spermatides are transformed into flagellated spermatozoa. 4). Sperm maturation, where nonfunctional spermatozoa develop into mature spermatozoa (fully capable of motility and fertilization) (Schulz and Miura,

2002; Miura and Miura, 2003; Welzien et al., 2004). The germ cells development is depending on close association with Sertoli cells. Sertoli cells enclosed the germ cells into so called spermatocyst. Each spermatocyst contains clone of germ cells that all are in the same stages of development (figure 2). Cell-Cell communication through gap junctions between Sertoli-Sertoli, Sertoli-germ and germ-germ cells in the spermatocyst is essential for the spermatogenesis. This junctional complexe does together with the basement membrane result in a blood-testis barrier, isolating the germ cells to a Sertoli cell determinated enviroment. The testis lobules are separated by connective tissue containing fibroblast, blood vessels and Leydig cells.

Sex steroids play an important role several places in the spermatogenesis. E2 is part of the regulation of spermatogonia renewal, spermatogonial proliferation toward meiosis is promoted by 11-KT and sperm maturation is regulated by 17α,20β-P (MIS) (Miura and Miura, 2003). Figure 3 gives a simplified schematic diagram of hormone regulation through the BPG axis.

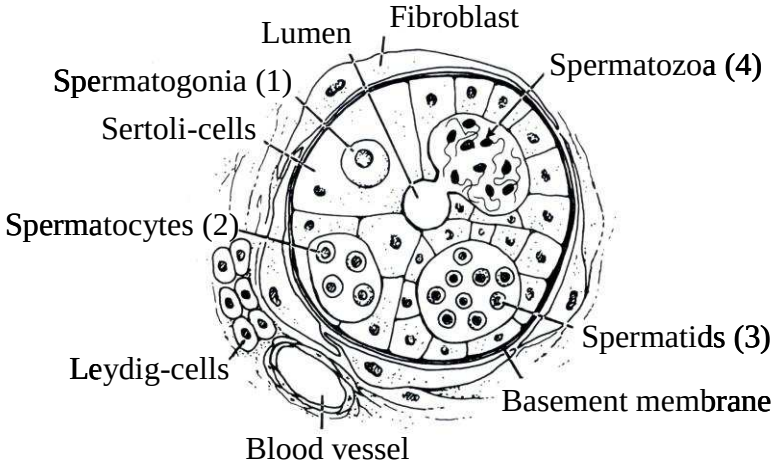

Fig. 2. *Cross-section of testicular lobule with spermatocysts (germ cells surrounded by Sertoli-cells) containg the different stades of sperm development. The number shows the chronological order in development. (Illustration: Stein H. Mortensen, IMR).*

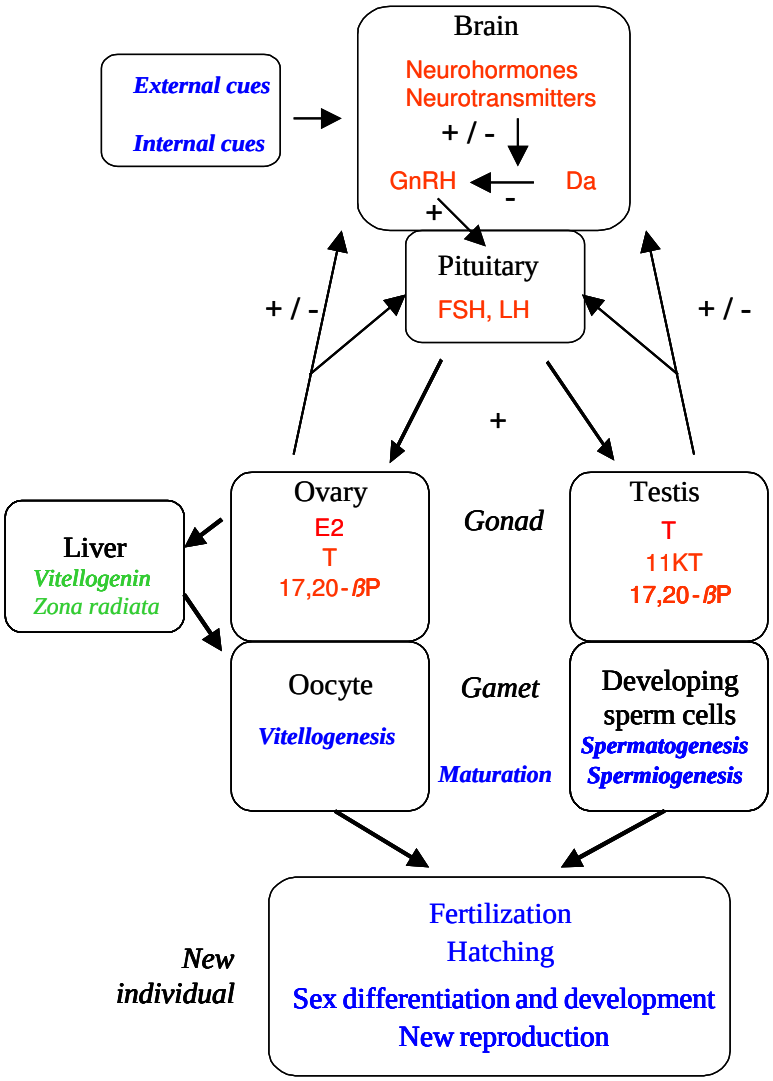

Fig. 3*. The reproductive system of fish and possible sites of action of contaminants. The brain-pituitary-gonadal axis is aktivated by external stimuli (like temperature, photoperiod, pheromones, social behavere, etc) and internal stimuli (biological cloks, nutritional status, etc). The hormonal system is regulated by a series of complex feedback mechanisms between the organs involved. (Da = dopamine; GnRH = gonadotropin-releasing hormone; FSH = follicle-stimulating hormone; LH = luteinising hormone; E2 = 17β-Estradiol; T = testosterone; KT = 11-ketotestosterone; 17,20-βP = 17α, 20β-dihydroxy-4-pregnen-3-one). Black – structures, red – hormones/neurotransmitter, green – protein, blue – processes.*

1.3 Steroid biosynthesis

The steroidogenesis is a complex process converting cholesterol into biological active steroids. The biosynthesis of steroid hormones is mainly happening in the gonads (ovaries and testes), the adrenals and the brain (Kime, 1987; Nagahama, 2000; Schumacher et al., 2003).

Figure 4 shows the biosynthesis pathways, even though it looks very complex it is only involving a small numbers of enzymes:

- Desmolase/lyase that cleave the side chain between carbon 20 – 22 (P450scc = $P450_{c11A}$) and carbon 17 – 20 ($P450_{c17}$).
- Hydroxylases that incorporate hydroxyl groups at different places ($P450_{11\beta}$, $P450_{c17}$, $P450_{C21}$).
- Hydroxysteroid dehydrogenase/oxidoredutase that oxidize hydroxyl-groups into keto-groups or reducing keto-groups to hydroxyl-groups (3β-HSD, 11β-HSD, 17β-HSD, 20β-HSD).
- Aromatase that converts androgens into aromatic estrogens ($P450_{arom} = P450_{C19}$).

The steroidogenic enzymes are located both in the mitochondria and in the endoplasmic reticulum and the synthesis involves transport between the different organelles. The rate-limiting step in the steroidogenesis is the transport of cholesterol between the outer and inner mitochondria membrane where the P450scc is located and the first conversion of cholesterol to pregnenolone takes place. Cholesterol cannot move over the intermembranal space by itself, but is actively transported by the steroidogenic acute regulatory (StAR) protein (Stocco and Clark, 1996).

In the teleost gonads both the StAR and the steroidogenic enzymes are regulated by the GTHs (FSH and LH). The seasonal pattern of FSH and LH differ between different species (Hellqvist et al., 2006), but there are some common mechanisms. In female fish increasing secretion of GTH from the pituitary glands stimulates increased synthesis of sex steroids (E2, T) in the gonads. Plasma levels of E2 and T are rising during vitellogenesis and peaks just before the start of spawning. Changes in the GTH signal then create a shift in the steroidogenesis by down-regulation of $P450_{arom}$ and up-regulating 20β-HSD, leading to a drop in E2 production and a rise in 17α,20β-P (inducing final maturation of the oocyte) (Senthilkumaran et al., 2004). Similar regulation of the steroid synthesis is also seen throughout the spermatogenesis (Schulz and Miura, 2002). The synthesis of E2 in the ovary is mediated by a two-cell system, where thecal cells in the outer follicular layer are converting cholesterol into T. T is secreted from the thecal cells and taken up by the granulosa cells in the inner follicular layer, where T is aromatized into E2 (Nagahama, 1994). In the testis, all steroid synthesis occurs in the Leydig cells.

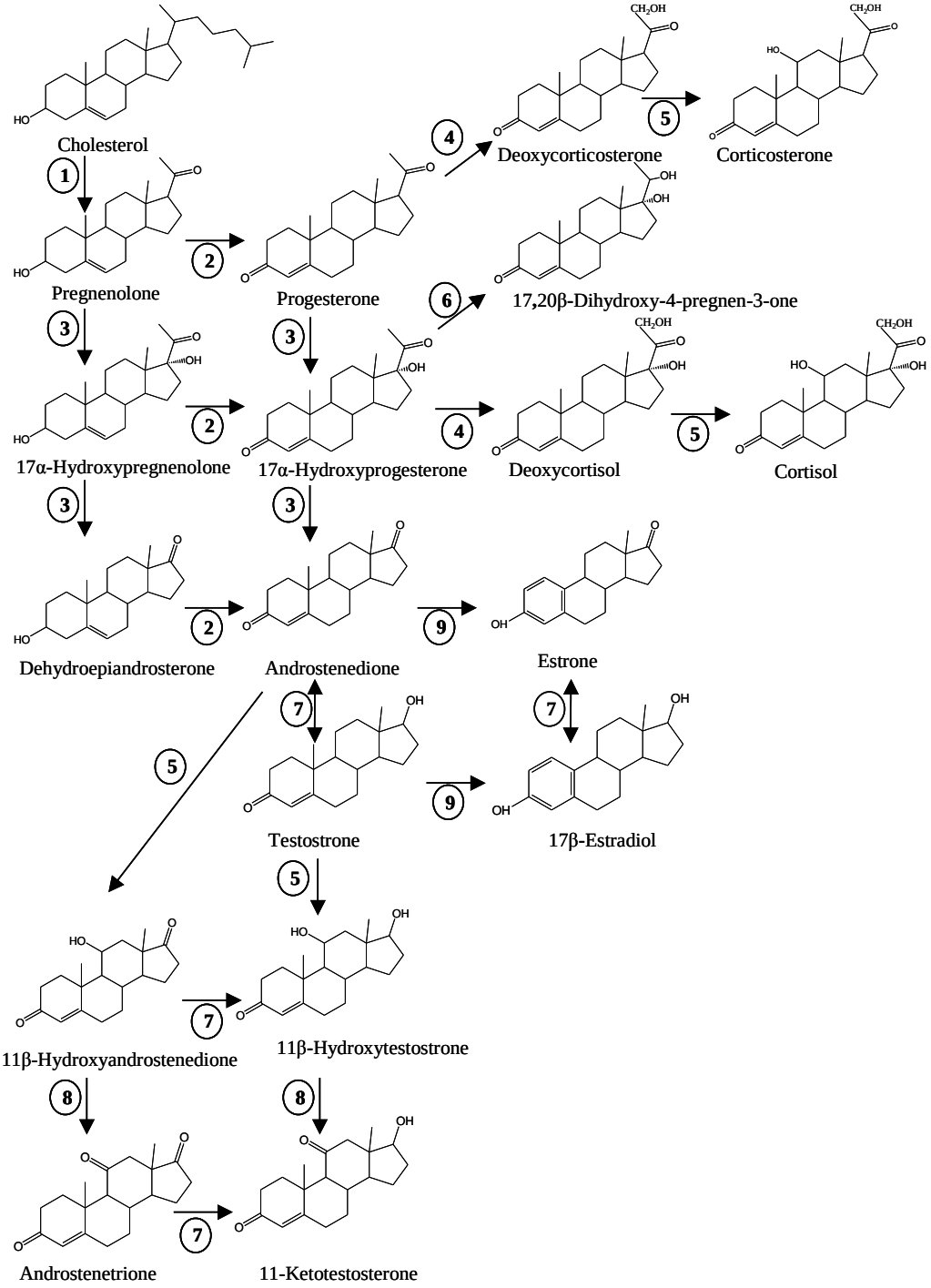

Fig. 4. *Biosynthesis pahtways of steroids in teleosts. Cholesterol is converted to pregnenolone by the enzyme (1) cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc). The other enzymes involved in the steroid synthesis are: (2) 3β-hydroxysteroid dehydrogenase (3β-HSD); (3) 17α-hydroxylase/17,20 lyase ($P450_{c17}$); (4) 21-hydroxylase ($P450_{C21}$); (5) 11β-hydroxylase ($P450_{11\beta}$); (6) 20β-hydroxysteroid dehydrogenase (20β-HSD); (7) 17β-hydroxysteroid dehydrogenase-oxidoredutase (17β-HSD); (8) 11β-hydroxysteroid dehydrogenase (11β-HSD); (9) aromatase ($P450_{arom}$).*

1.4 Distribution and reproduction physiology of Atlantic cod

There are a number of separated stocks of Atlantic cod spread all over the north Atlantic from the coast of Newfoundland Canada/USA in west to the Barents Sea in east. Atlantic cod is a cold-water species and the southern distribution limit is in the English Channel (ICES, 2005). The Arcto-Norwegian cod in the Barents Sea is the largest cod stock in the world and is one of the few stocks that are in reasonably good conditions. Many of the other cod stocks have experienced a dramatic decline since the late 1980s, and several stocks have collapsed and have problems recovering (Myers et al., 1996; Cook et al., 1997; Fu et al., 2001). In the Norwegian part of the Atlantic, the North Sea cod stock is now at a historically low size (Cook et al., 1997; Rice, 2006). The main factor of the collapse in the cod stocks is overfishing. However, there are speculation on whether ecosystem regime shifts, probably driven by climate changes, can be the reason for the lack of recovery that are observed (Gao, 2002; Beaugrand et al., 2003; Alheit et al., 2005). This study has been initiated by the question if pollution and especially endocrine disrupting chemicals (EDC) from the oil industry discharges play a role in the poor recruitment of the North Sea cod.

The Atlantic cod is an asynchronous batch-spawner. The ovary of the cod contains therefore ooctyes at many different stages of development through out the oogenesis and the process of vitellogenesis, final maturation and ovulation are ongoing paralleled in the spawning period. Large cod can spawn 20 batches of eggs over a period of 6-8 weeks from February to April (Kjesbu et al., 1996). The cod have small eggs and a very high fecundity. It is normal that large cod spawn more than 2 million eggs. The cod, as a species (or in local terms, as a stock) has a long spawning season of more than two months and sometimes as long as three months (Brander, 1993). However, in UK waters as many as two thirds of the eggs are spawned during a period of four to six weeks. The spawning season appears to be centred on the period of plankton blooms, with *Calanus finmarchicus* as an important species (Brander, 1994). It 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 (Ellertsen et al., 1986; Gotceitas et al., 1996).

The photoperiod is considered the most important factor for the timing of the sexual maturation of the cod (Norberg et al., 2004). Vitellogenesis starts in October (Kjesbu and Holm, 1994) but the main oocyte growth phase is in the month just prior to spawning. As for other teleost species, the oocyte grow by taking up VTG, which is synthesized in the liver and regulated by E2 (Silversand et al., 1993). The steroid hormone levels in the plasma reflect well the timing of maturation and spawning of cod. In female cod, the E2 levels rise from < 1 ng/ml early in the vitellogenesis up to 40 ng/ml prior to spawning. Testosterone follows a similar seasonal fluctuation as E2, but with lower concentrations (maximum 3-4 ng/ml) (Norberg et al., 2004). Male fish mature earlier that the females and the males often have testis with running sperm many weeks before the spawning. The plasma levels of T and 11-KT are strongly correlated with testis growth (Dahle et al., 2003).

Aquacultured cod mature much earlier than wild fish, and it is normal that farmed cod are first-time spawners at the age of 2 years due to optimal food conditions (Karlsen et al., 1995). Wild cod on the other hand mature between 4 and 8 years old (Norwegian coastal cod and Arcto-Norwegian cod, respectively) (Godo and Moksness, 1987).

1.5 Effects of pollution on reproduction

Aquatic pollution may have severe effects at several different levels in the reproductive cycle of fish (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. Chemicals with “estrogen mimicking” effects have caused most concern (Reviewed by (Arukwe and Goksoyr, 1998)). Growing attention is now also paid to other classes of hormones, such as the androgen system (Kelce and Wilson, 1997; Fang et al., 2003) and the thyroid hormones

(Oberdorster and Cheek, 2001; Brown et al., 2004). Among the xenobiotics that have been shown to have estrogen-disrupting effects (whether agonistic or antagonistic) we find APs, phthalates, bisphenol A, chlorinated hydrocarbons such as polychlorinated biphenyls (PCBs), dioxins and pesticides such as chlordane, dieldrin, DDT and its metabolite DDE (Arukwe and Goksoyr, 1998).

The endocrine apparatus is a complex system with many factors and is therefore liable to suffer disturbances at many levels as described by the general definition 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" (Kavlock et al., 1996).

The high degree of "plasticity" in the sexual development of fish results in the existence of "critical windows" in early life stages. During these periods fish are particularly sensitive to effects from EDCs. 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 estrogens (Piferrer, 2001).

The sensitivity of early life stages to the effects of estrogen is also reflected in results from field works. 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 a number of freshwater fish species (Jobling and Tyler, 2003) and saltwater fish (Matthiessen, 2003).

The yolk protein VTG is a sensitive biomarker, widely used in studies of the effects of estrogen 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. Estrogen

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 estrogen 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 (Jobling and Tyler, 2003). Abnormally high levels of VTG have also been found in saltwater fish: flounder (*Platichthys flesus*) caught off the British coast (Allen et al., 1999a; Allen et al., 1999b; Lye et al., 1997; Kirby et al., 2004; Kleinkauf et al., 2004) and near offshore installations in the UK sector of the North Sea (Matthiessen et al., 1998), flounder (*Pleuronectes yokohamae*) and goby (*Acanthogobius flavimanus*) caught in coastal areas around Japan (Hashimoto et al., 2000, Ohkubo et al., 2003), swordfish (*Xiphias gladius*) and red mullet (*Mullus barbatus*) in the Mediterranean (De Metrio et al., 2003; Fossi et al., 2004; Martin-Skilton et al., 2006b) and cod from the North Sea (Scott et al., 2006).

Unlike the great deal of interest that has been shown in estrogenic effects and feminisation of male fish, there are only a few reports of masculinizing 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 (Beullens et al., 1997). It has also been shown that discharges of wastewater from papermills can contain substances with androgenic or anti-estrogenic effects (Bortone et al., 1989; Bortone and Cody, 1999; Bortone and Davis, 1994; 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 and Thomas, 1999; Thomas, 2000; Tokumoto et al., 2005). Similarly, there is little information in the literature regarding disruptions of the thyroid hormones in fish (Oberdorster and Cheek, 2001; Zhou et al., 2000; Brown et al., 2004).

In spite of the relative comprehensive list of field studies given above a, 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 and Kavlock, 1997; Oberdorster and 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.

1.6 Alkylphenols in the aquatic environment.

Most of the research in this field has dealt with the two long-chain APs nonylphenol (NP) and octylphenol (OP). These are derivatives of degradation products of the non-ionic surfactants known as alkylphenol ethoxylates (APE). APE consist of an alkylphenol group, principally NP (82%) but also OP or dodecylphenol, coupled to long ethylene oxide chains (see (Nimrod and Benson, 1996b)). APE is and has been utilised in a large number of products, including herbicides, paint 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 tons (Renner, 1997). In Norway, the use of APE has been very limited, and has fallen significantly during the 90s, from 615 tons in 1995 to 113 tons in 2000 (www.SFT.no, 2001). The use of NP, OP and their APEs has been forbidden in Norway since January 2002 (www.miljoverndepartementet.no, 2001). The EuropeanUnion is also planning to forbid the use of these substances (Directive 2003-53-EC, 2003). The APE and APs are on the Oslo-Paris Commission's (OSPAR) list of toxic chemicals, which ought to be phased out.

The long-chain APEs have low toxicity and have no hormone-mimicking effects. However, they are broken down gradually and relatively rapidly in waste-treatment plants into the more resistant alkylphenol mono- and di-ethoxylates AP1E and AP2E

and the short-chain carboxylic acid derivatives (the alkylphenol carboxylates AP1EC and AP2EC). These are in turn partly broken down into pure APs (Nimrod and Benson, 1996b). A large proportion of these degradation products finally end up in the aquatic environment. There have been concern about AP in environment for more than two decades (McLeese et al., 1981; Giger et al., 1984; Waldock and Thain, 1986), but the research and monitoring of AP was intensified in middle of the 1990es. NP and OP have now been found in a large number of freshwater systems all over the world, in water concentrations of up to 644 µg/l in particularly highly polluted areas, but with typical values from ng/l to the low µg/l range. In sediment, concentrations are found up to 60 mg/kg (Ying et al., 2002).

The APs are transported by the rivers and eventually ends up in the marine environment. Measurements of seawater from coastal areas near cities and river estuaries have shown concentrations of up to 9 µg/l (table 1), while values from sediment samples can be as high as 15 mg/kg at exposed sites (table 2). The effuents from the great rivers are the main sources of AP into the oceans (Heemken et al., 2001; Stachel et al., 2003; Jonkers et al., 2005a). However, NP, OP and their APE have been found in atmospheric samples (Dachs et al., 1999; VanRy et al., 2000; Cincinelli et al., 2003; Berkner et al., 2004; Xie et al., 2006). It is therefore also possible that air-sea exchanges contribute to distribution of AP into the sea (Xie et al., 2006). The concentrations of APs in the open sea are, as one should expect, much lower than in coastal areas. Kannan *et al.* (1998) found very low levels of NP in the Sea of Japan (0.002 - 0.093 ng/l), while measurement from the North Sea showed significantly higher values. In samples from the German Bight, NP and OP concentrations were found between 0.09 - 4.4 ng/l and 0.013-0.3 ng/l respectively (Bester et al., 2001; Heemken et al., 2001; Xie et al., 2006). In sediment, sampled more than 100 km offshore, concentrations up to 13 µg/kg NP were found (Bester et al., 2001). The concentration of NP in water from the Dutch coastal zone was found to be as high as 1700 ng/l (median concentration 77 ng/l) (Jonkers et al., 2005b).

In addition to the long-chain OP and NP, there are a number of other APs that are used in industrial chemicals and also found in the environment (Remberger et al., 2003). 2,6-Di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene = BHT) is a commonly used antioxidant and stabiliser in large groups of products. BHT is found in river water in concentrations up to 365 ng/l (Kolpin et al., 2002; Fries and Puttmann, 2004) and in marine sediments around the coast of UK in concentrations up to 90 μg/kg (CEFAS, 2006). 4-*tert*-butylphenol (4-*tert*-BP) are widely used in paint, plastics, rubber and glue industry and is found in rivers and coastal areas in both water (up to 2300 ng/l) and sediments (up to 3.2 mg/kg) (Heemken et al., 2001; Kannan et al., 2001; Inoue et al., 2002; Remberger et al., 2003; Uguz et al., 2003; Basheer et al., 2004; Brossa et al., 2004; Kawaguchi et al., 2004; Koh et al., 2006). Other long-chain APs, 4-*n*-pentylphenol (4-*n*-PP); 4-*n*-hexylphenol (4-*n*-HexP) and 4-*n*-heptylphenol (4-*n*-HepP) are reported found in Japanese rivers (30-80 ng/l) (Inoue et al., 2002), coastal waters from Singapore (10-2920 ng/l) (Basheer et al., 2004) and 4-*n*-PP are found in the North Sea in concentration up to 8 ng/l (Heemken et al., 2001).

APs are fully biologically degradable in water, but the degradation rate falls rapidly with increasing chain length. Brendehaug *et al*. (1992) measured the biological degradation of phenols in produced water diluted in seawater, and found that phenol and cresol (methylphenol) degraded very rapidly (only 0.1% remaining after one week), on the other hand, did 33% of the initial concentration of HexP and 60% of HepP still remained after one month (Brendehaug et al., 1992). NP also shows relatively high resistance to biodegradation. In lake water only 9 % was lost by microbial activity in a 57 days experiment (Lalah et al., 2003). Another study of the degradation of NP in seawater indicated a very slow rate at the beginning of the study (0.06% per day), but that the degradation rate increased rapidly after 28 days to 1% per day. This suggests that the microorganisms in the seawater adapt to NP as a substrate after a while. After 58 days, 50% of the original quantity of NP was still in the water (Ekelund et al., 1993).

APs have high capability of sorption to colloidal particles (Johnson et al., 1998) and the primary sink for NP is the sediments. Ahel et al., (1994) found that sediment concentrations were up to 5100 times higher than the concentrations in river water. In addition to high sedimentation rates it is also important that the degradation rate can be very low in surface sediments (Lalah et al., 2003). In anaerobic conditions deeper in the sediment core, the degradation is extremely slow and analysis of sediments cores can give information of the historical discharges of AP (Shang et al., 1999b; Isobe et al., 2001; Hashimoto et al., 2005; Heim et al., 2006; Koh et al., 2006). Profiling the NP distributions in sediment cores from Tokyo Bay showed a maximum of NP in the layer deposited around the mid-1970s (Isobe et al., 2001, Hashimoto et al., 2005). Similarly maximum NP concentrations were found in 1972 sediment cores from Venice Lagoon, Italy (Marcomini et al., 2000). Also in Yeongil Bay, Korea, was the highest sendiment concentrations found in sediment cores from 1971-1980 (Koh et al., 2006). This kind of investigation can track sedimentation of NP all the way back to 1920s in Venice Lagoon, Italy (Marcomini et al., 2000) and 1950s in Tokyo Bay (Isobe et al., 2001).

It is clear that APs (especially NP and OP) are widely distributed in the aquatic enviroment. Analyses of sediment cores from different time periodss show a declining trend in NP concentrations, indicating that the discharges to the enviroment are decreasing. Because APs are biodegradable, the potential environmental problems caused by these substances can disappear in a relatively short time when (if) the production and use of APEs are phased out. There are big differences in the view on AP legislation around the world, the European Union wants to reduce and ban the use of these substances (Directive 2003-53-EC, 2003; OSPAR Commission, 2004), while APEs are still widely used in the USA (Renner, 1997). Increasing use in Asia is also causing concern (Zhou et al., 2003).

Table 1. *Concentration of NP and OP (ng/l) in marine and estuarine waters around the World.*

Location	NP	OP	Reference
Venice lagoon, Italy	200		(Marcomini et al., 1989)
Krka River estuary, Croatia	<20-1200		(Kvestak and Ahel, 1994)
Tyne and Tees estuaries, UK	<80-5200	<100-1300	(Blackburn and Waldock, 1995)
Sea of Japan	0.002 - 0.093		(Kannan et al., 1998)
Tyne and Tees estuaries, UK	30-9050	2-340	(Lye et al., 1999)
Shipyards in Virginia, USA	1.0–6300		(Hale et al., 2000)
North Sea, Germany	1-33		(Bester et al., 2001)
Jamaica Bay, USA	77-416	1.6-8.3	(Ferguson et al., 2001)
North Sea, Germany	0.3-84	0.1-16	(Heemken et al., 2001)
The coast of Spain	150-4100		(Petrovic et al., 2002b)
Tokyo Bay, Japan	10-100		(Hando et al., 2003)
The coast of China	1-10		(Hando et al., 2003)
San Francisco estuary, USA	<0.25-4		(Oros et al., 2003)
Costal water from Singapore	200-2760	10-540	(Basheer et al., 2004)
Coastal area, Okinawa and Ishigaki Islands, Japan	<50-150		(Kawahata et al., 2004)
Tokyo Bay, Japan	0.5-104		(Hashimoto et al., 2005)
Baltic Sea, Germany	2.5-13.8	0.4-0.95	(Beck et al., 2005)
Scheldt and Rhine estuaries, Holland	12-962		(Jonkers et al., 2005a)
North Sea, Holland	31-1700		(Jonkers et al., 2005b)
Ariake sea, Japan	11-49		(Kim et al., 2005)
Saemangeum Bay, Korea	7-298		(Li et al., 2005)
North Sea, Germany	0.09-1.4	0.013-0.3	(Xie et al., 2006)

Table 2. *Concentration of NP and OP (µg/kg dry weight) in marine surface sediments around the world.*

Location	NP	OP	Reference
Barcelona, Spain	6-70		(Chalaux et al., 1994)
Nile estuary, Egypt	19-44		(Chalaux et al., 1994)
10 estruaries, UK	<100-15000		(Blackburn et al., 1999)
Masam Bay, Korea	113-3890		(Khim et al., 1999)
Tyne and Tees estuaries, UK	30-9050	2-340	(Lye et al., 1999)
Strait of Georgia, British Columbia, Canada	280-320		(Shang et al., 1999a)
Jamaica Bay, USA	7-13700	<2-45	(Ferguson et al., 2001)
Shipyards in Virginia, USA	0.5–14100		(Hale et al., 2000)
Tokyo Bay, Japan	30-13000	3-670	(Isobe et al., 2001)
North Sea, Germany	<10-153		(Bester et al., 2001)
Elbe estuary, Germany	370-480		(Heemken et al., 2001)
The coast of Spain	8-1050		(Petrovic et al., 2002b)
Delaware river estuary, USA	0.14-13		(Ashley et al., 2003)
Coastal area, Okinawa and Ishigaki Islands, Japan	<5-44		(Kawahata et al., 2004)
Urdaibai estuary, Spain	140-1100		(Bartolome et al., 2005)
Pearl River estuary and South China Sea, China	59-571	1-18	(Chen et al., 2005)
Scheldt and Rhine estuaries, Holland	3-1026		(Jonkers et al., 2005a)
North Sea, Holland	0.3-86		(Jonkers et al., 2005b)
Tokyo Bay, Japan	2-4560		(Hashimoto et al., 2005)
Bohai Bay, Japan	203		(Hu et al., 2005)
Southern California bight, USA	122-3200	<2-8	(Schlenk et al., 2005)
The coast of UK	<10-5888	<10-530	(CEFAS, 2006)
Yeongil Bay, Korea	2-1430	<1-24	(Koh et al., 2006)
Odense fjord, Denmark	800-3300		(Madsen et al., 2006)

1.7 Alkylphenols and offshore oil and gas production

Historically, large quantities of 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 been as much as 100 tons a year per platform. NP and NPE are found in high concentrations (up to 68 mg/kg) in the sediments around North Sea platforms (CEFAS, 2005, Jonkers et al., 2005b). 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 sectors of the North Sea (Lye, 2000).

In addition to being degradation products of the APEs, APs are natural components of crude oil (Ioppolo-Armanios et al., 1992, Ioppolo-Armanios et al., 1995, Taylor et al., 1997, Rolfes and Andersson, 2001, Bastow et al., 2005). As a result of their solubility in water, a high proportion of APs will be found in the aqueous phase after water/oil separation and discharged into the sea with the produced water. The APs are typically found in concentrations of 0.6 - 10.0 mg/l in produced water. About 80 % of the total amount consists of the most water-soluble APs (phenol and cresol). Of the remaining components, the higher APs from BP - to HepP occur in low concentrations of 0.07 - 237 µg/l (Grahl-Nielsen, 1987; Brendehaug et al., 1992; Røe and Johnsen, 1996; Boitsov et al., 2004).

It is showed that produced water contains estrogen receptor agonists and APs have been identified to be the major contributor to this effect (Thomas et al., 2004a; Thomas et al., 2004b; Tollefsen et al., 2006). *In vitro* screening have found estrogen equivalents form <0.03 – 91 ng E2 /l in produced water from different installations in the UK sector of the North Sea (Thomas et al., 2004a).

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

sea around North Sea offshore installations. One study showed that phenol and lighter APs (C1–C4) occur at the concentrations of 486 and 140 ng/l, respectively (Riksheim and Johnsen, 1994). The discharges of produced water from the Norwegian petroleum sector are continuously increasing with the age of the oil fields, and were in 2004 143 million m^3. In 2004, approximately 13 tons of long-chain ($\geq C_4$) APs were released from installations on the Norwegian continental shelf in connection with discharge of produced water (OLF, 2005).

1.8 Bioconcentration of alkylphenols

NP and OP are both bioconcentrated and have been identified in aquatic organisms in nature. Ahel *et al.* (1993) found concentrations of NP of up to 1600 µg/kg (dry weight) in various freshwater fish in Swiss rivers. NP has been found in carp (*Cyprinus carpio*) caught in Lake Mead, Nevada (up to 184 µg/kg) (Snyder et al., 2001a) and in Cuyahoga River, Ohio (32-920 µg/kg) (Rice et al., 2003). Fish from various lakes in Michigan, USA had tissue concentrations of <3.3 to 29.1 µg/kg NP (Keith et al., 2001). Fish from Japanese rivers have been shown to contain from 1 - 110 µg/kg NP (Tsuda et al., 2000b) and similar concentrations are also found in periphytons, 8-130 µg/kg NP and benthos, 8-140 µg/kg NP (Takahashi et al., 2003). Lower levels NP were found in fish from Chinese rivers (up to 2 µg/kg) (Shao et al., 2005). Breams (*Abramis Brama*) caught in German rivers contain up to 130 µg/kg NP (Klein et al., 2005). Retrospective monitoring of APs in aquatic biota (from the German Environmental Specimen Bank) from 1985 to 2001 shows a decrease of NP concentration in biota from all sampling sites after 1997, the NP content in mussels from the German Bight dropped from 4 µg/kg in 1985 to 1.1 µg/kg in 1995 (Gunther et al., 2001; Wenzel et al., 2004). Wahlberg et al., (1990) found between 200 and 400 µg/kg NP in mussels gathered from the sea near the wastewater outlet of a Swedish plant that produced APE. Molluscs, crustaceans and fish from the Adriatic Sea, Italy, contained 9.5-1431 µg/kg NP and 0.3-4.3 µg/kg OP (Ferrara et al., 2001; Ferrara et al., 2005). NP and OP were found in the bile of red mullet from the Franch coast of the

Mediterranean Sea (Martin-Skilton et al., 2006b). Flounders caught in brackish water outside the rivers Tyne and Tees in England have been shown to contain 5 - 118 µg/kg NP (Lye et al., 1999). NP was not found (above a detection threshold of 100 µg/kg) in fish caught in the British offshore 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 APs of which we are aware of concerned OP and NP. We have found no field studies that have analysed petroleum-related APs.

APs are a highly diverse group of substances in terms of their physico-chemical properties. The water solubility of phenol and the short-chain APs are high, but falls drastically with increasing chain length and therefore increasing hydrophobicity. Table 3 presents an overview of three important physico-chemical properties of importance for the behavior of these substances in the environment, aqueous solubility, the logarithm of the water/octanol partitition coefficient (K_{ow}) and the bioconcentration factor (BCF). A number of studies have shown that OP and NP are readily taken up by fish, both via exposure in the water (Lewis and Lech, 1996; Arukwe et al., 2000b; Ferreira-Leach and Hill, 2001; Pedersen and Hill, 2002; Pickford et al., 2003) and by the food (Thibaut et al., 1998b; Arukwe et al., 2000b; Madsen et al., 2002; Pickford et al., 2003; Madsen et al., 2006). The APs are rapidly metabolised, mainly by phase II enzymes that conjugate intact APs to their corresponding glucuronides. The APs are excreted primarily in the bile and faeces (Ferreira-Leach and Hill, 2001; Thibaut et al., 2002; Smith and Hill, 2004). The APs accumulate particularly in the bile, digestive system and liver, but it has also been shown that AP is taken up by the brain in Atlantic salmon (*salmo salar*) (Arukwe et al., 2000b), rainbow Trout (*Oncorhynchus Mykiss*) (Ferreira-Leach and Hill, 2001; Thibaut et al., 2002), roach (*Rutilus rutilus*) (Smith and Hill, 2004) and cod (Tollefsen et al., 1998). This is of particular interest with respect to hormone-disrupting effects in the central-nervous-system. Studies with PCB have shown that cod (lean fish) are more likely than trout (fat fish) to accumulate lipophilic compounds in the brain (Ingebrigtsen et al., 1990).

Arukwe *et al.* (2000) have compared tissue distributions of NP in salmon following two different exposure regimes, via the water and via food. They found that dosing in the water results in higher uptake and a more regular distribution throughout the body than oral dosing, where NPs are more concentrated around the digestive system. Similarly, Pickford *et al.* (2003) found a 10 fold higher sensitivity for NP in fish exposed via the water compared to oral exposure of corresponding doses.

The bioaccumulation factor (BCF) for long-chain APs (>C4) is in the range of 75 - 1250 (Table 3). In fish, the biological uptake of chemicals with log K_{ow} < 4 (logarithm of the octanol/water partitition coefficient) mainly takes place via the water (theoretically 20 times as fast as uptake via food). For more hydrophobic substances with log K_{ow} > 6, the situation is reversed, with uptake via food being more important (Mackay and Fraser, 2000). As far as the APs are concerned, this means that bioaccumulation in nature properly takes place primarily via uptake through the gills and skin and not by being biomagnified through the food chain. However, little is known about the metabolism of these compounds in organisms that belong to the lower end of the food chain. High BCFs have been reported for NP in estuarine amphipods and this indicates that biomagnification can be an important source of NP in higher trophic levels, such as juvenile fish (Hecht et al., 2004).

***Table 3**. Selection of physicochemical properties of APs that may have relevance for their environmental fate. Aqueous solubility, the logarithm of the water/octanol partitition coefficient (K_{ow}) and the bioconcentration factor (BCF[1]). The overview is from (Shiu et al., 1994, Servos, 1999)*

	Water solubility (mg/l)	Log K_{ow}	BCF in fish	Species	Reference
Phenol	67000-93325	1,46-1,6	17-158	Div. fish	(Servos, 1999)
p-Cresol	1800-53000	1,62-2,06	-		(Servos, 1999)
4-Ethylphenol	5000	2,39-2,58	-		(Servos, 1999)
4-*n*-Propylphenol	1278	3,18-3,20	-		(Servos, 1999)
4-*sec*-BP	-	2,1	37	Salmon	(McLeese et al., 1981)
4-*tert*-BP	580-1848	3,04-3,31	118	Golden Ide	(Freitag et al., 1985)
			125	Cod	(Sundt and Baussant, 2003)
4-*n*-PP	-	-	90	Cod	(Sundt and Baussant, 2003)
4-HexP	-	3,60	346	Salmon	(McLeese et al., 1981)
			592	Cod	(Sundt and Baussant, 2003)
4-*n*-HepP	-	4,00	578	Cod	(Tollefsen et al., 1998)
			520	Cod	(Sundt and Baussant, 2003)
4-*tert*-OP	12,6	4,12	261	Killifish	(Tsuda et al., 2001)
			1134	Roach	(Ferreira-Leach and Hill, 2000)
4-NP	5,4-7	4,20-6,36	75-1250	Div. fish	(Servos, 1999)

[1)] 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 (Mackay and Fraser, 2000).

1.9 Estrogen receptor (ER) and the binding affinities of alkylphenols to ER.

The steroid hormone, E2 is a key regulator of growth, differentiation and physiological functions in a wide number of target tissues, including the male and female reproductive system, neuronal, skeletal and cardiovascular systems. The predominating mechanisms of estrogen action are mediated through binding to the nuclear estrogen receptor (ER), which induces transcription of target genes containing estrogen response element (ERE) (Zhang and Trudeau, 2006). The ER is part of a large nuclear receptor superfamily that shares common structure and function/domains. This receptor family acts as the signal transmitter for most of the known fat-soluble hormones, including steroids (androgen receptor, (AR); progesterone receptor, glucocorticoid receptor, mineralocorticoid receptor), retinoids, thyroid hormones and vitamin D (Mangelsdorf et al., 1995). Other groups of nuclear receptors, so called "Orphan" receptors (the ligands are unknown), pregnane X-receptor (PXR) and

constitutive androstane receptor (CAR) are regulating some of the cytochrome P450 genes and other detoxification genes, (Kretschmer and Baldwin, 2005).

In addition to the classical mechanism of genomic effects there are also increasing evidence for non genomic effects of E2 and other steroids, possible mediated through membrane receptors and secondary messenger cascades (such as release of intracellular Ca^{2+}, mitogen activated protein kinase (MAPK), protein kinase A (PKA) and C (PKC), phospholipase C (PLC), phosphoinositide turnover and adenylate cylase (cAMP)) (Sak and Everaus, 2004, Zhang and Trudeau, 2006). The genomic effect has time delays from hours to days, while the non-genomic mechanism are characterised by very fast signal transmission, from seconds to minutes. Two novel seven-transmembrane spanning steroid membrane receptors, membrane progestin receptor α and membrane estrogen receptor (mER), GRP30, have resently been identified in several vertebrates (Thomas et al., 2006).

In mammals, there have been found two distint forms of nuclear estrogen receptors, ERα and ERβ (Enmark and Gustafsson, 1999). Teleosts have in addition to ERα, two different forms of ERβ (ERβa and Erβb) (Hawkins et al., 2000; Menuet et al., 2002; Hawkins and Thomas, 2004; Sabo-Attwood et al., 2004). The tissue distribution in teleost of the different ERs largely overlaps, and ERs are mainly found in the brain, pituitary gland, liver and gonads (Menuet et al., 2002). There are reported differences in the relative affinity of AP between the ERα and Erβ. In channel catfish, NP showed 100-fold lower affinity for ERα and 10000-fold lower affinity for ERβ than for E2 (Gale et al., 2004). On the other hand, difference in NP affinity to the two ERs in an assay using recombinant human ERα and ERβ, was not observed (Kuiper et al., 1998).

The capability of synthetic non-steroid compounds to bind and activate the ER have been known for more than 70 years (Cook et al., 1933). Dodds and Lawson, (1938) found that among many others compounds, also 4-*tert*-PP and 4-*n*-propylphenol had weak estrogenic effects measured by changes in vaginal cytology in ovariectomized rats. The estrogenicity of APs were further studied by Mueller and Kim, (1978) and many AP isomers were showed to be able to bind and displace E2 from the ER. But it

was first in the beginning of the 1990es, after Soto et al., (1991) had rediscovered the estrogenic effects of NP that the scientific community started the massive focus that have made this group of compounds some of the most intensively studied endocrine disruptors.

Amino acid sequence of the ER and the crystal structures of the E2–ER complex (see figure 5) have together with quantitative structure – activity relationship (QSAR) study of antagonists identified several criteria for high binding affinity to the ER: (1) Phenolic ring with hydrogen-bonding ability; (2) H-bond donor mimicking the 17β-OH and right O-O distance between 3- and 17β-OH; (3) Hydrophobic moiety mimicking the ring structure of E2 (Brzozowski et al., 1997; Sadler et al., 1998; Schmieder et al., 2000; Tanenbaum et al., 1998; Fang et al., 2001; Klopman and Chakravarti, 2003; Tong et al., 2003).

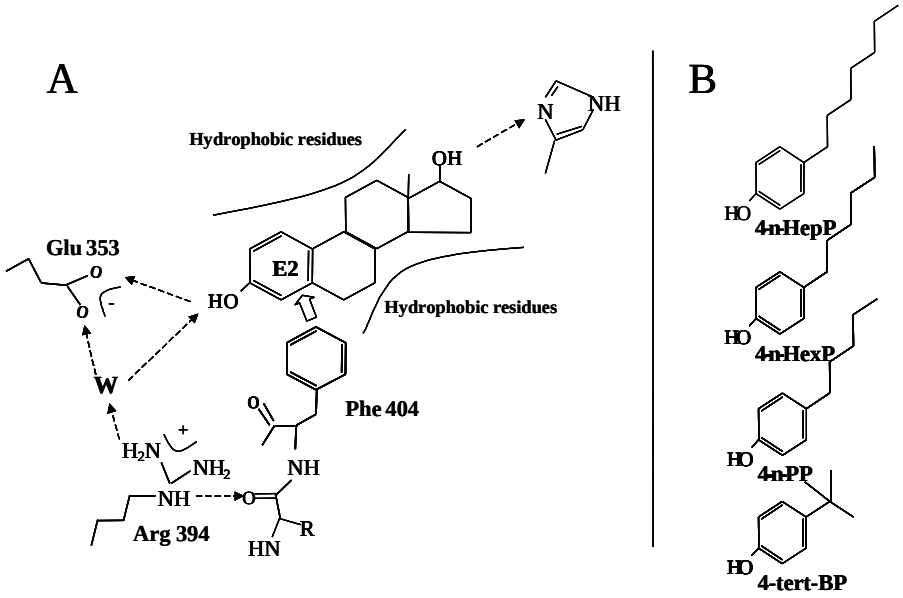

Fig 5. A)*. Model of the ERα-ligand binding domain holding E2. The specific binding domain of the ER is holding the 3-hydroxy-group of E2 in a water (**W**) mediated hydrogen-bonding network involving glutamic acid (**Glu**), arginine (**Arg**) and phenylalanine (**Phe**). The 17-hydroxy-group of E2 is bound by hydrogen bonds to histidine (**His**). In addition, the ligand-binding pocket is covered with hydrophobic amino acids making van der Waals contacts with the carbon skeleton of E2. The figure are taken from the crystal structure of the E2 complex with the human ERα (Tanenbaum et al., 1998).* ***B)****. Chemical structure of the four alkylphenols used in this study: 4-tert-butylphenol (4-tert-BP), 4-n-pentylphenol (4-n-PP), 4-n-hexylphenol (4-n-HexP) and 4-n-heptylphenol (4-n-HepP).*

Beacause of the phenol ring, APs fulfil the must important criteria for binding to the ER. APs do also have the capability of hydrophobic interaction by the alkyl-chain, but as seen on figure 5B the APs are lacking other important structures, like hydrogen-bond donor capability mimicking the 17β-OH of E2. The APs have therefore relatively low affinity to the ER.

In vitro studies (table 4) have found that the size and degree of the branching of the alkyl chain, as well as its position relative to the phenolic hydroxy-group are important for binding affinity to the ER. The most vital factor for high estrogenic activity of APs are that the alkyl chain is in the *para*-position (*para*>*meta*>*ortho*) and that the chain-length is $\geq C_6$. Maximum activity (400 - 6000 times less potent than E2) has been found for $C_6 - C_9$ *para*-substituted tertiary APs, but *para*-substituted C_5, C_4 and C_3 APs are also have weak estrogenic effects (10^5 - 10^7 times less potent than E2) (table 5). Routledge and Sumpter, (1997) found that the tertiary isomers have the highest estrogenic effects (tertiary>secondary=normal), and that 4-*tert*-OP is 60 times more potent than 4-*sec*-OP. Similarly 4-*tert*-HepP is 25 times more potent than 4-*n*-HepP, while 4-*n*-PP on the other hand is three times more potent than 4-*tert*-PP. Other investigations confirm that tertiary isomers are more potent than the normal isomers, but with less difference than found by Routledge and Sumptor (1997). 4-*tert*-OP was 2-10 times more potent than 4-*n*-OP (Tabira et al., 1999; Blair et al., 2000; Schultz et al., 2000). However, the structure of the carbon chain is important. A recent study has examined the estrogenic effects of the alkyl chain of 22 isomers of 4-NP in detail. They found that high "bulkiness" on the β-carbon was the most important factor for the high estrogenic activity and that the activity could differ as much as 3000 times between the most and the least potent 4-NP (Shioji et al., 2006).

In vivo studies also suggest that the estrogenicity of branched APs is higher than that of linear isomers (Pedersen et al., 1999; Chikae et al., 2003).

Table 4. *Estrogen receptor (ER) binding affinities of APs. The most potent isomers are given in bold. IC_{50} value: the concentration of competitor needed to displace half of the bound ligand.*

	1) (Routledge and Sumpter, 1997)	**2)** (Tabira et al., 1999)	**3)** (Blair et al., 2000)	**2)** (Schmieder et al., 2000)	**1)** (Schultz et al., 2000)	**2)** (Hu and Aizawa, 2003)
Compound	Relativ to E2	IC_{50} (M)	IC_{50} (M)	IC_{50} (M)	IC_{50} (M)	IC_{50} (M)
E2	*1*	*2.1 x 10 $^{-9}$*	*9.0 x 10 $^{-10}$*	-	*3.9 x 10 $^{-11}$*	*2.3 x 10 $^{-8}$*
4-dodecylphenol	-	2.0 x 10 $^{-4}$	4.6 x 10 $^{-6}$	9.3 x 10 $^{-4}$	-	-
4-*sec*-decylphenol	1/100000	-	-	7.6 x 10 $^{-6}$	-	-
2-*sec*-decylphenol	Nonactive	-	-	Nonactive	-	-
4-NP	1/30000	**3.7 x 10 $^{-6}$**	**2.4 x 10 $^{-6}$** – 4.7 x 10 $^{-6}$	3.4 x 10 $^{-6}$	-	-
4-*n*-NP	-	4.2 x 10 $^{-6}$	2.8 x 10 $^{-5}$	-	-	**9.5 x 10 $^{-6}$**
4-*tert*-OP	**1/1000**	6.3 x 10 $^{-6}$	6.0 x 10 $^{-6}$	**1.1 x 10 $^{-7}$**	**1.8 x 10 $^{-7}$**	1.4 x 10 $^{-5}$
4-*sec*-OP	1/60000	-	-	-	-	-
4-*n*-OP	-	1.0 x 10 $^{-5}$	1.9 x 10 $^{-5}$	-	1.9 x 10 $^{-6}$	-
2.6-di-butylphenol	Nonactive	-	-	Nonactive	-	-
2.4-di-butylphenol	Nonactive	-	-	-	-	-
4-*tert*-HepP	1/3000	-	-	2.6 x 10 $^{-7}$	-	-
4-*n*-HepP	1/75000	-	-	9.8 x 10 $^{-6}$	-	-
4-*tert*-HexP	1/6000	-	-	5.8 x 10 $^{-7}$	-	-
4-*n*-HexP	-	1.7 x 10 $^{-5}$	-	-	-	-
4-*tert*-PP	1/100000	-	1.7 x 10 $^{-4}$	3.1 x 10 $^{-6}$	4.8 x 10 $^{-6}$	-
4-*n*-PP	1/30000	-	-	1.3 x 10 $^{-5}$	9.5 x 10 $^{-6}$	-
4-*tert*-BP	1/1500000	-	3.7 x 10 $^{-4}$	1.6 x 10 $^{-4}$	-	1.0 x 10 $^{-3}$
3-*tert*-BP	Nonactive	-	-	Nonactive	-	-
2-*tert*-BP	Nonactive	-	-	Nonactive	-	-
4-*sec*-BP	1/3900000	-	2.1 x 10 $^{-4}$	3.9 x 10 $^{-4}$	-	5.8 x 10 $^{-4}$
2-*sec*-BP	-	-	3.2 x 10 $^{-4}$	Nonactive	-	1.4 x 10 $^{-3}$
4-*n*-BP	-	8.5 x 10 $^{-5}$	-	-	-	-
4-*n*-Propylphenol	1/20000000	-	-	2.2 x 10 $^{-3}$	1.5 x 10 $^{-4}$	-
4-Ethylphenol	Nonactive	6.0 x 10 $^{-3}$	1.3 x 10 $^{-3}$	-	Nonactive	1.7 x 10 $^{-3}$
3-Ethylphenol	-	-	6.6 x 10 $^{-4}$	-	-	-
2-Ethylphenol	-	-	> 1.0 x 10 $^{-3}$	-	-	-
Phenol	Nonactive	2.9 x 10 $^{-3}$	-	-	-	9.5 x 10 $^{-2}$

[1] Recombinat Yeast assay (YES screen)
[2] Estrogen receptor competitive-binding assay (Recombinant human oestrogen receptor. hERa)
[3] Estrogen receptor competitive-binding assay (ER from uterine cytosol from Sprague-Dawley rats)

In addition to be an agonist for the ER, APs have also been shown to interfere with several other classes of nuclear receptors. Even though this is not well investigated and the results are not as consistent as for effects on the ER, it tells us that AP can act as a

endocrine disruptor in a much broader way than only being an estrogen mimic (Goksoyr and Male, 2006).

4-*tert*-OP (IC_{50}=5 x 10^{-6} M) and NP (IC_{50}=2.6 x 10^{-6} M) are potent antagonists for AR and can induce anti-androgen effects (Paris et al., 2002; Lee et al., 2003a). On the contrary, Sohoni and Sumpter, (1998) found NP to be a weak agonist to AR. It is also reported that NP and 4-*tert*-OP can be both agonists to PR (Scippo et al., 2004) or PR antagonist (Tran et al., 1996). The thyroid hormone function can be disrupted by APs (Ghisari and Bonefeld-Jorgensen, 2005; Schmutzler et al., 2004). The two orphan nuclear receptors that are involved in regulation of several detoxifications enzymes are also target for AP endocrine disruption, NP is agonist for PXR and CAR (Masuyama et al., 2000, Mikamo et al., 2003, Kretschmer and Baldwin, 2005).

There are also evidence for NP to induce similar effects as E2 via membrane initiated signalling pathways (Loomis and Thomas, 2000; Bulayeva and Watson, 2004; Watson et al., 2005; Wozniak et al., 2005; Thomas and Dong, 2006). The binding affinity of AP to the nuclear ER is about 1000 times weaker than E2, but the effect-concentration of the membrane initiated effect seems to the more equal for E2 and NP. Intracellular Ca^{2+} changes are induced in pituitary tumor cell lines by 10^{-12} M of both E2 and NP within 30 sec of administration, resulting in prolactin (PRL) secretion (Wozniak et al., 2005). Thomas and Dong, (2006) found that NP binds 47 times weaker than E2 to plasma membranes prepared from HEK293 cells transfected with the seven-transmembrane estrogen receptor, GPR30.

1.10 Estrogen-receptor mediated and receptor-independent mechanisms for the biological effects of alkylphenols

Numerous *in vitro* screening systems have been developed to characterise the binding affinity of chemicals to ER (reviewed in (Zacharewski, 1997; Soto et al., 2006)). APs are shown to bind and induce effects through ER, similar to E2. It has also been shown that the effects can be blocked by ER antagonists like tamoxifen or ICI 182,780. The

variety of assays used in studying the estrogenic effects of APs includes: competitive ER binding assay (White et al., 1994); cell proliferation assay, (e.g. E-screen (MCF7-cells) (Soto et al., 1995)); protein expression assay, (e.g. VTG expression in fish hepatocyte culture (Jobling and Sumpter, 1993)); recombinant assays, (e.g. yeast-based screen (YES-screen) (Routledge and Sumpter, 1996) and cell lines (Shelby et al., 1996)).

Recent developments in screening for xenoestrogenic effects are by use of toxicogenomics (Moggs, 2005) where cDNA microarrays containing multiple estrogen-responsive genes can be used both *in vitro* and *in vivo* (Terasaka et al., 2004; Naciff et al., 2005; Terasaka et al., 2006). Such approache clearly show the complexity in estrogen signalling and the disturbers therein. Moggs (2005) reported as many as 3538 genes to be E2-responsive in the mouse uterus, and through gene ontology, the genes are categorised into 35 different biological partways. Toxicogenomics have an enormous potential in providing detailed information regarding the molecular response to xenoestrogens and revealing new biomarkers. Microarray analysis of gene expression profiles in mouse exposed for NP or E2 reveal tissue differences in response to E2 and xenobiotics. The gene expression in the gonade was very similar after E2 and NP exposure, indicating that these effects mainly are induced through the ER or other estrogen receptors. Gene expression in liver, on the other hand was more affected by NP than by E2 and activation of many genes involved in lipid and fatty acids metabolism were only found in the NP groups (Watanabe et al., 2004). Undoubtedly, the "omics" technologies (Genomics, proteomics, metabolomics, lipomics) will also play a very important role in the future for studying endocrine disruption in fish and other aquatic organisms. Today there is still some limitation in that the genome is only sequenced in very few teleosts, but smaller scale DNA microarrays are now available for several model species (Miracle and Ankley, 2005; Moens et al., 2006; Watanabe and Iguchi, 2006). However, the challenge for this approach is the same as for all other use of biomarkers; to create bridges between information of gene expression to physiological and toxicological endpoints that can

be used to extrapolate the effect to fitness of individuals and populations. ("Fitness = the relative contribution of an allele, genotype or phenotype to future generations" Wikipedia, the free encyclopedia).

The following chapters give an overview of different physiological events affected by AP exposure.

1.10.1 Effect of AP on sex differentiation and gonad development.

In non-mammalian vertebrates the genotypic sex can be overridden by exposure to steroid hormones and the sex differentiation are therefore vulnerable to EDC. Exposure to AP in the early life stages of fishes (Gimeno et al., 1996; Gray and Metcalfe, 1997) and amphibians (Kloas et al., 1999; Mosconi et al., 2002) can induce feminization of males and result in intersexuality or higher number of female phenotypes.

AP are stimulating estrogen-dependent uterine growth in rodents (Bicknell et al., 1995). The rat uterotrophic bioassay is validated as "standard *in vivo* method" for screening of xenobiotics by the Organisation for Economic Co-operation and Development (OECD). The lowest observed effect level (LOEL) in the uterotrophic assay is found to be 75 mg/kg/day for NP (Kanno et al., 2003; Owens and Koeter, 2003). Testis development in rats can also be affected by AP, and reduction in testis growth and induction of apoptosis have been reported (Han et al., 2004b; Kim et al., 2004). NP induced apoptosis in rat testis in a similar way as E2 does through the FAS/FASL Pathway (Wang et al., 2003; Han et al., 2004a). Apoptosis is also induced by NP and OP in human embryonic stem cells, and these effects are also related to the FAS/FASL Pathway (Kim et al., 2006b). The FAS-signalling pathway is important in the paracrine-signalling system between Sertoli cells and germ cells (Richburg et al., 2002). In addition to the FAS/FASL pathway, apoptosis can also be induced by a variety of other signal transitions leading to stimulation of calcium flux, cAMP production, PLC activation, inositol phosphate generation and mitochondrial

membrane transition pore permeability. OP and NP also induce apoptosis in Sertoli cell lines by inhibiting endoplasmic reticulum Ca^{+} pumps (Hughes et al., 2000).

Even though the evidences that AP give endocrine disruption in mammalian reproductive tracts are clear, it is important to note that the *in vivo* effects are only seen at relative high doses. Multigenerational studies with rats show a "no observable adverse effect level" (NOAEL) of NP > 100 mg/kg/day for effect on the reproduction development, (Chapin et al., 1999; Nagao et al., 2001; Tyl et al., 2006).

The reproductive system of fish seems more sensitive for AP exposure than that of the mammalians. Concentrations down to 5 μg/l levels of NP or OP are inhibiting the spermatogenesis of male fish, resulting in reduced testis growth, trigging of necrosis and apoptosis and altertion of testis morphology (Jobling et al., 1996; Gimeno et al., 1998; MilesRichardson et al., 1999; Weber et al., 2002). The oogenesis in the female fish is also affected by APs, but at higher dose than what is seen for the male fish. As example, 100 μg/l NP reduces the ovary weight and increases follicle atresia in zebrafish (Weber et al., 2003). End point like fertilization success from life cycle tests with zebrafish comfirm the high teleost sensibility for AP, EC_{50} values = 28 μg/l for 4-*tert*-OP (Segner et al., 2003b).

1.10.2 Effect of AP on brain and the central-nervous-system (CNS).

Estrogens are one of many neuroactive steroids and play a vital role in many neurophysiologic events such as the sexual differentiation and early development of the brain; feedback effect on brain-pituitary-gonad axis; higher cognitive functions like behavior, memory, etc.; and have neuroprotective effects. (McEwen, 2002; Melcangi and Panzica, 2006). The multiple effects are found mediated both by nuclear ER receptors and membrane signalling pathways. However, much are still not known and there are increasing discoveries of new targets and mechanisms of estrogen effects to the CNS (Toran-Allerand, 2004; Ronnekleiv and Kelly, 2005).

A particular attention has been put on APs and neuroendocrine disturbances on the brain-pituitary-gonadal axis in fish (Jones et al., 1998; Piva and Martini, 1998; Harris et al., 2001; van Baal et al., 2000; Zilberstein et al., 2000; Yadetie and Male, 2002; Maeng et al., 2005; Vetillard and Bailhache, 2006). The underlying mechanisms of the effects of AP on the GnRH and GTH are still unknown and the literature demonstrates contradictory effects in different fish species; Harris *et al.* (2001) found that NP reduces the expression of FSH gene in the pituitary gland and FSH secretion to the plasma in water exposed female rainbow trout, even at very low concentrations (they found a significant effect at the lowest exposure dose; 0.7 µg/l). Similarly, the quantity of LH-mRNA is reduced in the pituitary gland on exposure to NP (8.3 µg/l) (Harris et al., 2001). As opposed to this, Yadetie and Male (2002) stated that intraperitoneal injection of NP (50 mg/kg) strongly induces gene expression of LH in female juvenile Atlantic salmon pituitary gland. No effects were seen in male fish. The gene expression of FSH was unaffected in both sexes (Yadetie and Male, 2002). Injection of low dose (10 mg/kg) of NP induced the GTHα and LHβ mRNA levels in the pituitary gland of juvenile masu salmon (*Oncorhynchus masou*). A high dose (50 mg/kg) did, however, not induce this effect on GTHα and LHβ mRNA, but did instead slightly reduce FSHβ mRNA levels (Maeng et al., 2005). Tilapia (*Oreochromis niloticus*) showed a suppressed expression of FSH mRNA, but not LH mRNA in the pituitary gland after 5 weeks water exposure to NP (10 µg/l) (Zilberstein et al., 2000). In African catfish (*Clarias garipinus*) the amount of LH (protein) was increased in the pituitary gland of both sexes, but not in plasma after 7-14 days water exposure of NP (10 µg/l) (van Baal et al., 2000). *In vitro* studies showed an inhibitory effect of NP on the secretion of LH from African catfish cultured pituitary cells (van Baal et al., 2000). Water esposure of NP (2.2 µg/l to 2.2 mg/l) reduced GnRH in the brain of juvenile rainbow trout in a dose dependent manner (Vetillard and Bailhache, 2006).

The effects from AP on the CNS may be mediated through mimicking estrogenic feedback effects. In general, E2 (and other sex steroids) is known to exert positive feedback effects on LH levels, but there is species related variation. The feedback

control of E2 on FSH levels is much less clear, and both positive, negative or no effects are reported from juvenile teleost (Dufour et al., 2000: Kah et al. 2000). It is important to recognize that estrogen often shows reproductive stage-dependent effects on the gonadotropin secretion (Thomas, 2000).

Bevan et al., (2003) found a high increase of apoptotic cells in the nervous systems of developing tadpoles (*Xenopus laevis*) after low NP exposure (100 nM). This observation is also correlated with increased morphological deformations and high mortality. Stimulation of apoptosis by NP exposure are also found in embryonic murine neural stem cells (NSC) (Kudo et al., 2004). NP disturbs the cell cycle of NSC by accumulation of cells in the G_2/M phase by down-regulation the expression of cyclin A and B1, which are the major regulatory proteins for the G_2 to M transition of the cell cycle. The NP exposure can also lead to apoptosis of NSCs by activating the caspase cascade (Kudo et al., 2004). Apoptosis is also suggested as the mechanism for reduction in tyrosine hydroxylase active cells in the brain of neonatal rats exposed for 4-*n*-OP. The effects are thought to explain the hyperactivity behavior in exposed rats (Ishido et al., 2004).

On the contrary, NP can also disturb the neuronal functions by stimulating the synthesis of catecholamine (dopamine, epinephrine and norepinephrine) in bovine adrenal medullary cells after increased tyrosine hydroxylase activity (Yanagihara et al., 2005). The effects were not inhibited by ER antagonist (ICI182,780) or protein synthesis inhibitors (actinomycin D and cycloheximide), suggesting that NP stimulates tyrosine hydroxylase and catecholamine synthesis in a nongenomic manner. This was confirmed by the finding of effects of short-term treatment (10 min), and the authors suggest that activation of MAP kinase system induces the effects. Behavioral studies show that NP exposure has effects on fear response in rats. This is probably induced through alterations of the catecholamine systems (Negishi et al., 2004).

The neurotransmitter acetylcholine can also be affected by AP exposure, both by inhibition of acetylcholinesterase activity (Talorete et al., 2001) and modulation of the

nicotinic acetylcholine receptor (Nakazawa and Ohno, 2001) or the muscarinic acetylcholine receptor (Jones et al., 1998).

The focus on AP as a environmental problem have mainly been for the *para*-substituated APs, but the APs found in crude oil and in produced water contains a large number of isomers (Ioppolo-Armanios et al., 1995). It may therefore be interesting to draw attention to the *ortho*-substituated APs. Propofol® is the name of the widely used intravenous general anaesthetic, 2,6-diisopropylphenol. Propofol® has an inhibitory effect on the neurotransmiter γ-aminobutyric acid (GABA) by binding to the $GABA_A$ receptor, a property in common with many other general anaesthetics (Trapani et al., 2000). Similar effects are found with other *ortho*-substituted AP analogues to 2,6-diisopropylphenol, like 2,6-dimethylphenol, 2,6-diethylphenol etc. Also mono *ortho*-substituted isomers like 2-isopropylphenol show such effects (Krasowski et al., 2001a; Krasowski et al., 2001b). No studies on the effects of *ortho*-substituted APs in wild life were found in the literature. However, when working with complex mixtures of AP, one should keep in mind that other isomers than *para*-substituted can also have specific biological effects at low concentrations.

1.10.3 Effects of AP on biosynthesis and metabolism of steroids.

In addition to affecting the steroid biosynthesis indirectly through the GTH and the brain-pituitary-gonadal axis, APs can also act directly on steroidogenesis enzymes.

In vitro studies with Leydig cells from rats show that 4-*tert*-OP has a biphasic effect on T biosynthesis, with induction of T synthesis at low concentrations (1 and 10 nM) and a reduction of T synthesis at high concentrations (100 - 2000 nM). By using different steroid precursors like 22(R)-hydroxycholesterol, pregnenolone, progesterone and androstenedione as substrate for the T synthesis, inhibitory effects of OP were shown early in the biosynthesis of $P450_{scc}$, 3β-HSD and $P450_{c17,}$ but not 17β-HSD, (the enzyme that converts androstenedione to T). No similar effect was seen with E2 and the effects of OP were not inhibited by ER antagonist (ICE 182,780), demonstrating

that these effects are not modulated through the ER (Murono et al., 1999; Murono et al., 2001). NP are also found to decrease T synthesis by inhibiting $P450_{C17}$ *in vitro* in cells from rat testis, but only minimal effects were seen on T-dependent endpoints *in vivo* (Laurenzana et al., 2002a). 4-*tert*-PP, 4-*tert*-OP and 4-NP are inhibiting $P450_{scc}$ and the hydroxylases ($P450_{11\beta}$, $P450_{c17}$, $P450_{C21}$) in human andrenocortical H295R cells, resulting in a decrease in cortisol secretion (Nakajin et al., 2001). In microsomes from carp testis, NP had no effects on 17β-HSD, but increased 20β-HSD activity dramatically and induced production of MIS (17α,20β-P) (Thibaut and Porte, 2004). Expression of $P450_{11\beta}$ mRNA was completely inhibited in the testis of medaka (*Oryzias latipes*) exposed to ≥ 413 µg/l 4-*tert*-PP (Yokota et al., 2005). Similar inhibition of $P450_{11\beta}$ mRNA is found in the brain of salmon exposed to NP (Arukwe, 2005). The same study also found induction of StAR protein mRNA and $P450_{scc}$ in the brain of NP exposed salmon, showing a possible stimulation of the early steps of the steroid synthesis (**Chapter 1.3**). Several studies have found that NP are inducing aromatase ($P450_{arom}$) mRNA expression in the brain (Kazeto et al., 2003; Kazeto et al., 2004; Meucci and Arukwe, 2006a) and the liver (Min et al., 2003), but not in the gonad (Kazeto et al., 2004) of fish.

Beside effects on the biosynthesis, an increase or a reduction of the metabolic elimination rate can also alter the steroid levels. NP is an agonist of the PXR and the CAR, and may therefore alter several phase I, II and III enzymes that are impotant for the metabolism of natural steroids (Masuyama et al., 2000; Mikamo et al., 2003; Kretschmer and Baldwin, 2005; Meucci and Arukwe, 2006b). PXR and CAR are regulating many important phase I cytochrome P450 enzymes (CYP2A, CYP2B, CYP2C and CYP3A), phase II enzymes, like uridine diphospho-glucuronosyltransferases (UDPGT), glutathione-S-transferases (GST) and sulfotransferases (SULT), and phase III transporters (multidrug resistance proteins that are active in transporting polar metabolites across the membranes for excretion). NP exposure increases the hepatic microsomal progesterone hydroxylase activity and CYP3A proteins in rat liver (Lee et al., 1996). Gender-specific induction of

cytochrome P450s is seen in NP treated mice. NP exposure increase expression of CYP1B subfamily members in both males and females, but CYP3A is exclusive down regulated in the females and CYP2A is induced only in the males (Hernandez et al., 2006). Arukwe et al., (1997a) found that low levels of NP induce steroid hydroxylase activity, but high doses inhibit the activity *in vivo* in juvenile Atlantic salmon. They also found a reduction in CYP1A, CYK2K-like and CYP3A-like proteins in the highest exposed group (125 mg NP/kg) together with reduction in UDPGT activity. Jurgella et al., (2006) demonstrated that NP (100 μM) did not effects E2 metabolism in neither liver nor kidny tissue from lake trout (*Salvelinus namaycuch*). OP (100 μM) inhibit E2 metabolism in the liver tissue but not in kidney tissue. As seen from this discussion is it not clear if AP increase or decrease steroid metabolism, some investigations have found increased metabolism (Baldwin et al., 2005), other have found reduction or no effects (Laurenzana et al., 2002b; Vaccaro et al., 2005; Jurgella et al., 2006).

The effects of APs on the steroidogenic or metabolic enzymes, either by direct inhibition or by altering the gene-expression and protein synthesis (up or down) may affect the seasonal pattern of steroids that is so important for synchronising all the reproductive events. Induction of $P450_{arom}$ can increase the production of E2, and unnatural high levels of E2 have been reported in juvenile male flounders (Mills *et al.* 2001), male and female fathead minnow (Giesy *et al.* 2000) exposed to OP and NP. Offspring of NP exposed rainbow trout had increased levels of E2 in males and T in females, even though the offspring were grown in clean water for 3 years (Schwaiger et al., 2002). However, reduction in E2 and other steroids in plasma have also been reported as results of AP exposure. Arukwe *et al.* (1997) found a reduction in the plasma levels of E2 in juvenile Atlantic salmon at relatively low AP concentrations (1 and 5 mg/kg, injected into the abdomen) but found no effect at higher concentrations (25 and 125 mg/kg). Female rainbow trout exposed to NP has reduced E2 concentration in plasma, but only at the high doses (85.6 μg/l) (Harris *et al.* 2001). NP exposure reduced androgen and estrogen levels in plasma and testis of juvenile male

turbot, while no effect was found in female turbot (Labadie and Budzinski, 2006). In the same study, the amount of glucoronidated steroids in the bile was reduced in male fish, indicating that the drop in steroid concentration was not a result of increased metabolism.

The effects of AP on the steroidogenesis are rather contradictory and several studies find biphasic responses with different doses. However, a lack of a linear dose-response relationship is typical for the steroid system where the nature of the response often is different with low and high doses of steroids. For example, low doses of E2 and testosterone stimulate the secretion of gonadotropin in fish, while is inhibited by high doses (Jalabert *et al.* 2000).

1.10.4 Effects of AP on the immune system

Estrogen plays an important role in the immune system and are involved in differentiation and maturation of T-cells in the thymus and B cells in the bone marrow, and has other immunoregulatory properties as secretion of cytokines and production of antibodies (Sakazaki et al., 2002). NP have been found to mimic estrogenic effects on the immune system like inhibition of lymphocyte mitogenesis (Sakazaki et al., 2002) and to induce thymocyte apoptosis (probably by the FAS/FASL pathway) (Yao and Hou, 2004, Yao et al., 2005, Yao et al., 2006). NP inhibit lipopolysaccharide induced nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production in mouse macrophages (You et al., 2002, Hong et al., 2004). 4-*tert*-OP increases the production of the pro-inflammatory cytokine, interleukin-4 in T-cells. The effect was blocked by a calcineurin inhibitor, FK506, but not by the ER antagonist ICI 182.780, showing that the effect was activated by the Ca^{2+}-calcineurin partway independent of ER (Lee et al., 2003b, Lee et al., 2004). NP is found *in vitro* to have inhibiting effects on one of the key enzymes, cyclooxygenase-1 (COX-1), that converts arachidonic acid (20:4 n-6) to prostaglandines. This can also affect the immune system since prostaglandins play a

central role in regulation of inflammation, together with many other physiological processes (Fujimoto et al., 2005). Multi generation exposure experiments on rats show that NP alters the activity of splenic natural killer cell and increases the numbers of splenocyte subpopulations in second generation (F_1), while no effect was seen in first generation, (F_0) (Karrow et al., 2004). NP has also myelotoxic potency in F_1 male rats (Guo et al., 2005). The expermints found that the effects on the immune system were gender-specific. Although we not are aware of any studies on effects of AP on the immune responses in teleost, AP may probly also interact with the immune systems of fish.

1.10.5 AP induction of oxidative stress and DNA damage.

Ortho-substituted APs have good antioxidant properties because of the ability to stabilise free radicals and thereby reduce autooxidation. BHT is a well known antioxidant. A side effect of the anaesthetic, 2,6-diisopropylphenol (propofol®) is also that it protect cells against oxidative stress (De la Cruz et al., 1999). Many other phenolic compounds also have antioxidant properties, including estrogen. It has been suggested that the neuroprotective effects that are found for estrogens are mediated by antioxidant activity, even though it is not likely to be the most important mechanism (Amantea et al., 2005). As for most antioxidants, phenols can have the opposite pro-oxidant effect leading to production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), and induction of lipid autooxidation. There is several defence systems that can be activated for protection against oxidative stress: glutathione, glutathione peroxidases, superoxide dismutase (SOD) and catalase (CAT). NP exposure of male rats is found to increase H_2O_2 generation and lipid peroxidation in the sperm. This increase in oxidative stress is over-activating the antioxidant defense systems, resulting in reduced activity of CAT, SOD, glutathione peroxidase and glutathione reductase (Chitra et al., 2002). Formation of hydroxy radicals is also found in rat striatum (Obata and Kubota, 2000) and rat Sertoli cells (Gong and Han, 2006) after NP exposure . The NP induced inhibition of

cell growth in bacteria and yeast cultures can be suppressed by adding antioxidants showing that the NP effects possibly are associated with ROS generation (Okai et al., 2000a, Okai et al., 2000b). Similar does DJ-1 (a protein having anti-oxidative function) protect against NP induced cell death in cultured medaka cells (Li et al., 2006).

Both estrogens and APs can be metabolized to catechols, phenoxyl radicals, o-quinones, and semiquinone radicals, all of which could cause damage to cells through alkylation or oxidation of cellular macromolecules including DNA (Krol and Bolton, 1997; Schweigert et al., 2001; Bolton, 2002). DNA damages can in the ultimate consequences lead to cancer. Studies of biotransformation show that the majority of NP are rapidly conjugated at the phenol group by glucuronidase followed by excreted through the bile (Lewis and Lech, 1996). But small amounts of AP are also oxidated to catechols, and covalently bound residues are found in trout (1.7 % of the total labelled NP) and in rudd (*Scardinius erythrophtalmus*) (12-62 % of total 4-tert-OP) (Coldham et al., 1998, Pedersen and Hill, 2000).

The metabolism of AP into reactive metabolites shows a potential for DNA damage. However, NP is not carcinogenic by itself (Sakai, 2001). NP are, on the other hand, shown to promote rat lung carcinogenesis, possibly via mechanisms involving DNA damage caused by ROS (Seike et al., 2003). Absence of promoting effects by NP have been seen in other carcinogenesis models, like thyroid carcinogenesis (Son et al., 2000b, Son et al., 2000a), and prostate carcinogenesis (Inaguma et al., 2004). Even inhibitory effects of NP are reported for rat ovarian carcinogenesis (Tanaka et al., 2002) and 4-*n*-OP and NP are reducing mammary tumor development (Han et al., 2002). DNA damages in human sperm and lymphocytes after NP exposure have been found by the Comet assay (Anderson et al., 2003). DNA damage after 4-*n*-NP exposure is found in larvae of barnacle, an aquatic invertebrate (Atienzar et al., 2002). Exposure of turbot to 30 µg/l NP for 3 weeks did not give chromosomal damage, determined as micronuclei frequency in the fish erythrocytes. On the other hand, a mixture of North Sea oil + APs (oil related isomers) , induced a very high micronuclei

frequency showing genotoxicity (Bolognesi et al., 2006). 24 hours exposure to high doses of NP (890 μg/l) to juvenile sea bass induced erythrocytic nuclear abnormalities (Teles et al., 2004).

1.10.6 Effects of AP on the cell membrane

Evidence of APs as membrane active compounds including membrane swelling, increase in fluidity, lowering of the phase transition temperature and increased ion permeability have been established from two intensively studied APs: the antioxidant BHT (Lanigan and Yamarik, 2002) and the intravenous anaesthetic Propofol® (Singer, 1977; James and Glen, 1980; Tsuchiya, 2001). There are also good support of *para*-substituted long-chain APs can be related to membrane effects that are independent of the estrogenic pathways. NP provokes vesiculation of the Golgi apparatus of epidermis cells from fish at concentration of 20 μM (Lamche and BurkhardtHolm, 2000). Similarly, 4-*tert*-BP and 4-*tert*-OP cause formation of lipid droplets and other changes in Leydig cell membrane structures of rats (Haavisto et al., 2003). Schwaiger et al., (2000) suggest that anaemia found in NP exposed fish is a consequence of an interaction between NP and the erythrocyte membrane. NP increases membrane permeability of mitochondria membranes to protons and act therefore as an uncoupler of the oxidative phosphorylation (Bragadin et al., 1999). Mitochondrial depolarization by NP has also been suggested as one of the mechanisms behind NP induced thymocyte apoptosis (Yao et al., 2006). Several investigations have shown that APs disrupt Ca^{2+} homeostasis by affecting Ca^{2+} membrane channels (Michelangeli et al., 1990; Beeler and Gable, 1993; Ruehlmann et al., 1998; Hughes et al., 2000; Logan-Smith et al., 2002; Kirk et al., 2003; Khan et al., 2003; Lee et al., 2003b; Walsh et al., 2005; Wang et al., 2005). Gap junctional intercellular communication is reduced in murine Sertoli cell line by NP, the effect is partly explained by reduction in phosphorylation of connexin 43 (Aravindakshan and Cyr, 2005), but the gap junction may also be affected by changes in the membrane lipid bilayer (Cascio, 2005).

1.10.7 Alkylphenols and their potential effects on fish reproduction and recruritment.

As discussed in the previous chapters is it quite clear that APs can interfere with a large number of biological pathways. There are particularly good evidence from laboratory studies that AP can induce endocrine disruption and alter the reproduction in fish, and there are also many indications that NP in combinations with other EDCs are involved in reproductive disruption in wild freshwater fish (Jobling and Tyler, 2003) and marine fish (Matthiessen, 2003). Linking the impacts of EDCs with effects on population levels is however still lacking and is one of the largest challenges within this scientific field (Mills and Chichester, 2005).

Table 8 (Appendix 1) gives an overview of 176 *in vivo* laboratory studies that have been investigating effects of APs on teleost fish. The majority of the literature is related to freshwater fish and the toxicology model species dominate, with Cyprinids (zebrafish, fathead minnow, sheephead minnow and goldfish), Salmonides (trout and salmons) and Beloniformes (medaka) constituting for more than 60 % of the total reports. It is therefore clear that our knowledge of the effects of ECDs on teleost only cover a limited number of the more than 24000 different teleost. Especially, there is lacking information on endocrine disruption on marine fish, even though the numbers of reports are increasing rapidly.

Table 8 lists the species, exposure regime and lowest effect concentrations for the different effect parameters, including: toxicity data (lethal dose), growth inhibition, inappropriate production of VTG in male and juvenile fish, inhibited ovarian or testicular development (lower GSI), abnormal blood steroid concentrations, up regulation of ER, alteration in pituitary hormones, alteration in sterodogenisis enzymes, intersexuality and/or feminisation of the gonads, skewed sex ratio, changes in male and/or female maturation, increased ovarian atresia, decreased sexual behaviour in males, reduced spawning success, reduced hatching success and/or larval survival, altered growth and malformations in early development. Some of these measurements can directly be correlated to adverse endpoints like survival, growth,

morphological development and reproduction. These endpoints tell about the fitness of the individual and the effect concentrations can be transferred into "predicted no effect concentrations values" (PNECs) necessary for modelling the risk for damages on population levels. On the other hand, many of the biomarkers are not easily linked to adverse effects.

Many of the studies on the effects of APs on fish are short-time experiments presenting results with different biomarkers. These experiments are very useful in identifying which compounds that are having endocrine disruption effects and it can give mechanistic information, but it is difficult to transfer results from such studies into fitness parameters. One example; APs induce VTG in fish at doses down to 0.1-5 μg/l (Jobling et al., 1996; Fent et al., 2000; Hemmer et al., 2001; Kashiwada et al., 2002). Induction of VTG is the most used estrogen specific biomarker, because of its very high sensitivity and clear link to estrogenic effects. However, the relationship between VTG induction and adverse effects on fish reproduction is unclear. Pathological effects in liver and kidney have been seen in connection with very high VTG induction after exposure of high potent estrogens like E2 or ethynylestradiol (Herman and Kincaid, 1988; Folmar et al., 2001; Palace et al., 2002), but these effects are found after million-fold increase of VTG, resulting in plasma concentrations at the high mg/ml levels. Exposure to weak xenoestrogens like APs is mostly resulting in lower-level induction of VTG and the impact of this is not well defined (Mills and Chichester, 2005).

The optimal experimental design for EDC testing is full life-cycle tests, where multiendpoints are used for investigation of both developmental and reproductive effects. In these tests, the fish are exposed from embryos till the stages of sexual maturation and through the spawning periods. In some cases also multigenerational studies are preformed and the exposure is continued on the second generation. Of practical reasons, full life-cycle tests have only been done on small fish with short generation time (≤ 4 month) like the zebrafish, fathead minnow and medaka. However, even with small laboratory fish that mature rapidly, full-life toxicity tests require very long experiment time and are very costly and work intensive. Therefore most studies

are done by partial life-cycle test, where the experiments focus on special sensitive periods in the fish life. Embryonic and larval development, especially during the critical stages of sexual differentiation and gonadal development, has received much attention. Similarly, many experiments with adult fish are done in the time of vitellogenisis and gonadal maturation.

The lethal concentrations (LC) of NP are found to be between 18-940 µg/l for different species and developmental stages. New hatched fish larvae are most sensitive for the acute toxicity of AP, while the LC for juvenile and adult fish are over 100 µg/l for NP (table 7).

Full-life-cycle test shows that NP and OP exposure reduces the reproduction potential in zebrafish at 28-100 µg/l (Hill and Janz, 2003; Segner et al., 2003b) and in medaka at 2-50 µg/l (Gray and Metcalfe, 1997; Gray et al., 1999b; Yokota et al., 2001; Knorr and Braunbeck, 2002; Seki et al., 2003b), while the AP with shorter chain length, like 4-*tert*-PP are less potent, inducing reproduction disturbance around 200 µg/l both in medaka (Seki et al., 2003b) and fathead minnow (Panter et al., 2006). These effect concentrations are in good agreement with the results from partial life-cycle tests, but there are some differences in sensitivity between different species. The lowest adverse effect concentrations reported in the literature are in rainbow trout. Lahnsteiner *et al*. (2005) found that 60 days of exposure to 750 ng/l NP completely inhibits male semen production and doses down to 130 ng/l NP significant reduced semen production.

As discussed in **Chapter 1.6** NP is found in freshwater systems, mostly in the concentration range ng/l to the low µg/l, but up to 644 µg/l in highly polluted areas. In seawater the concentrations are lower, from low ng/l to 9 µg/l. The environmental water concentrations correspond well with reported levels of NP found in wild freshwater fish, being of the order 1-1600 µg/kg, in view of the fact that the BCF are reported to occur from 75-1250 (table 3). As seen here NP is found at exposed sites in the natural environment at concentrations high enough for adverse effects on fish to occur, but the majority of the measurements are below the known PNEC values. It has

been suggested that APs in the marine environment are partly responsible for testicular abnormalities and VTG induction in male flounder (*Platichthys flesus*) from the UK estuary (Lye et al., 1999), but no clear evidence is available. It is important to note that real environmental exposure will always be a complex mixture of many different compounds, never APs alone. Nevertheless, there have been several attempts to use the available toxicological data to risk assessment. Brown *et al* (2003, 2005) have estimated that long-time exposure (20 years) to 30 μg/l NP could lead to severe decline in population levels of freshwater fish. The U.S. Environmental Protection Agency estimated that NPs PNEC values for freshwater organisms is 28 μg/l (acute toxicity) and 6.6 μg/l (chronic toxicity) and NPs PNEC values for saltwater organisms are 7 μg/l (acute toxicity) and 1.7 μg/l (chronic toxicity) (EPA, 2005). These PNEC values agree with those of Staples *et al.* (2004) who estimated the chronic effect value of NP to be 5.7 μg/l.

1.11 Analytical methods for determination of alkylphenol in biological tissue and produced water.

APs can be analyzed with different chromatographic and electrophoresis methods (reviewed in (Lee, 1999, Petrovic et al., 2002a)). For analysis of the whole profile of individual isomers of Aps, gas chromatography (GC) is preferred because of the high-resolution power. Many are analysing phenols directly without derivatisation both with GC-FID (Ioppolo-Armanios et al., 1992; Chee et al., 1996; Lye et al., 1999) or GC-MS (Giger et al., 1981; Bhatt et al., 1992; Wheeler et al., 1997; Gunther et al., 2001; Espejo et al., 2002).

The APs contain an “active” hydrogen atom and are therefore often converted to thermally stable and less polar compounds to improve their chromatographic performance before GC. Most of the derivatization methods are used together with GC-MS or for the halogenated derivatives with GC-ECD as the detectors. Many different derivatization techniques are used for GC these involve: Akylation to methyl

ethers (Bolz et al., 2000, Fiamegos et al., 2003), 3,5-bis(triflurомethyl)benzyl ethers (Cheung and Wells, 1997), pentaflurobenzyl ethers (Chalaux et al., 1994; Nakamura et al., 2000; Doerge et al., 2002) and 4-tetrafluoropyridyl derivatives (Kojima et al., 2003); silylation to trimethylsilyl ethers (Heberer and Stan, 1997; Mol et al., 2000; Li et al., 2001; Guenther et al., 2002). There are also several arylation based derivatization methods of phenols. Among the most used methods are acetylation (Llompart et al., 1997; Louter et al., 1997; Croley and Lynn, 1998) and pentafluorobenzoyl derivatization (McCallum and Armstrong, 1973; Renberg, 1981; Granmo et al., 1986; Wahlberg et al., 1990; Bao et al., 1996; Kuch and Ballschmiter, 2001; Xiao et al., 2001; Bianchi et al., 2002). Other not so common arylation methods are determination of phenols in crude oil as ferrocenecarboxylic acid esters using GC withatomic emission detection (Rolfes and Andersson, 2001) and extractive derivatization of phenols in oil produced water with methylchloroformat (Grahl-Nielsen and Landgren-Skjellerudsveen, 1982).

Table 9 (Appendix 2) gives an overview of different methods developed for analysing AP in biota samples, and table 10 (Appendix 2) shows methods for AP analysis in produced water.

2. Aims of the Thesis

The work in this thesis is a part of a strategic work at the Institut of Marine Research, which aim to develop a variety of methods to be used in investigating potential endocrine disruption in Atlantic cod. The main concern that initiated the project were whether discharges of APs from the oil industry give endocrine disruption in Atlantic cod and thereby effect the reproduction and recruitment of cod and other species in the North Sea.

The major aims of this thesis are:

- Development of analytical methods for determination of low levels of APs in produced water and fish tissue (**Paper I, Paper II)**
- To study long-term effects of selected *para*-substituted APs (4-*tert*-BP, 4-*n*-PP, 4-*n*-HexP; 4-*n*-HepP) on the reproduction of male and female cod. The objective of this investigation has been to study a wide spectra of biological end points (growth and morphological development), biomarkers (GSI, plasma steroids, VTG and gonad histology) (**Paper III, Paper IV**), effects on the redox status (glutathione and glutathione-related enzymes) (**Paper V),** effects on the hepatic CYP1A and CYP3A protein expressions and enzyme activities **(Paper VI)** and effects on the phospholipids in the liver and brain (**Paper VII**) of AP exposed cod.

3. List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

Paper I

S. Boitsov, S. Meier, J. Klungsøyr, and A. Svardal. Gas chromatography-mass spectrometry analysis of alkylphenols in produced water from offshore oil installations as pentafluorobenzoate derivatives. *Journal of Chromatography A* 1059 (1-2):131-141, 2004.

Paper II

S. Meier, J. Klungsøyr, S. Boitsov, T. Eide, and A. Svardal. Gas chromatography-mass spectrometry analysis of alkylphenols in cod (*Gadus morhua*) tissues as pentafluorobenzoate derivatives. *Journal of Chromatography A* 1062 (2):255-268, 2005.

Paper III

Sonnich Meier, Tom Einar Andersen, Birgitta Norberg, Geir Lasse Taranger, Anders Thorsen Olav Sigurd Kjesbu, Jarle Klungsøyr and Asbjørn Svardal. Effects of long-term exposure of alkylphenols on the reproductive system of Atlantic cod (*Gadus morhua*). Manuscrip.

Paper IV

Sonnich Meier, Tom Einar Andersen, Birgitta Norberg, Anders Thorsen, Geir Lasse Taranger, Olav Sigurd Kjesbu, Roy Dale, H. Craig Morton, Jarle Klungsøyr and Asbjørn Svardal . Effects of alkylphenols on the reproductive system of Atlantic cod (*Gadus morhua*). *Aquatic toxicology* 81 (2): 207-218 (2007).

Paper V

L. Hasselberg, S. Meier, and A. Svardal. Effects of alkylphenols on redox status in first spawning Atlantic cod (*Gadus morhua*). *Aquatic toxicology* 69 (1):95-105, 2004.

Paper VI

L. Hasselberg, S. Meier, A. Svardal, T. Hegelund, and M. C. Celander. Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*). *Aquatic toxicology* 67 (4):303-313, 2004.

Paper VII

Sonnich Meier, Thorny Cesilie Andersen, Kristin Lind-Larsen, Asbjørn Svardal and Holm Holmsen. Effects of alkylphenols on glycerophospholipids and cholesterol in liver and brain from cod (*Gadus morhua*). *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* 145 (3): 420-430, 2007.

4. General discussion

4.1 Analytical methods for determination of alkylphenols in produced water and biological tissues

The aim of the analytical chemistry study was to develop selective and sensitive methods for determination of APs, from phenol to NP, in produced water (**Paper I**) and biota samples (**Paper II**).

Establishment of analytical methods has three main challenges.

1. Extraction of target compounds.

2. Sample Clean-up to remove matrix effects.

3. Sensitive and selective analysis.

Different techniques for all of the above parts of the method development have been tested.

Extraction of target compounds.

For the biota analysis, cyclic steam-distillation was the first extraction technique tested. This method was originally developed by Veith and Kiwus (1977) for pesticide analysis in water, sediments and biota. The method uses a water distillation to concentrate the analytes and the condensated steam is extracted with a small amount of organic solvent in a special apparatus, before it is transferred back to the distillation chamber. This method have also been used to investigate AP levels in biota (Ahel et al., 1993; Lye et al., 1999; Gunther et al., 2001; Keith et al., 2001; Snyder et al., 2001a). However, even extensive attempts to optimize the steam distillation with out-salting, pH adjustment and extraction time did not make this method work satisfactory for AP spiked cod liver samples. At the best, only an AP recovery of 30% was obtained. It was therefore concluded that steam distillation extraction not is a suitable

method for extraction of APs in extremly lipid-rich samples like cod liver. The focus was thereafter subjected to liquid-liquid extraction and dichloromethane (DCM) was found to be a good solvent for the biota samples with recovery 67-90 % (**Paper II**).

The extraction of AP in produced water was done with solid-phases extraction (SPE). Three different SPE cartridges were tested (500 mg superclean envi-chromp columns (supelco), 200 mg Oasis® HBL and 150 mg Oasis® MAX columns (Waters)). All three cartridges contants polymers of styrene-divinyl, this type of sorbents has been shown to possess better extraction abilities than other typically used ons (e.g. C_{18}) (Liu et al., 2004). The MAX column contains quaternary amino groups bound in the styrene-divinyl polymers, this give these cartridges the ability to extract both acidic and hydrophopic compounds. Oasis Max column were used further because they gave the highest recovery and the purest extracts (**Paper I**).

Sample clean-up.

Biological samples have a very complex matrix containing a high amount of lipids, proteins, etc. Therefore, purification of the extracts is usually necessary. The lipids from the biota extracts were effectively removed by gel permeation chromatography (GPC). It was found that a columns switch method using two GPC columns removed more than 98 % of the lipids in the extracts (**Paper II**) and that thise were satisfactory for GC-MS analysis. The produced water extracts from the Oasis® MAX columns was so clean that it did not need any additionally clean-up before derivatisation (**Paper I**).

There were problems with background contamination of APs, especially from 4-NP in the procedure blanks. It seems that some APs are widely spread in most indoor environments (Rudel et al., 2003) and phenol and *para*-substituted APs (*p*-cresol, 4-*tert*-BP and 4-NP) are intensively used in plastics industry (Cascaval et al., 1996). In our work, 4-NP was found in most of the plastic and rubber products used in the laboratory, including vinyl gloves, rubber stoppers for glass funnels and plastic tubes used for the nitrogen evaporator. However, despite a significant effort to avoid these problems, we still detect small amounts of phenol, cresols, 4-*tert*-BP, 4-*tert*-OP and 4-NP in the blank samples. It is therefore important to have a good and intensive control

of procedural blanks. The trace amounts of APs in blank samples increase the risk of false positive results and the levels of contaminants may limit the use of the analytical method (**Paper II**).

Analysis.

Pentafluorobenzoyl derivatisation was selected for the studies, being a sensitive and selective method for the derivatisation of APs for GC-ECD and GC-MS (McCallum and Armstrong, 1973, Renberg, 1981). The derivatisation methods were optimised by the use of factorial experimental designs. Our results show that pentafluorobenzoyl derivatisation is a good and robust method for analysing *meta*- and *para*-substituted APs. The variations of the 7 parameters tested had no significant influence on the recovery of the long chain *para*-substituted APs, but the *ortho*-substituted and most water-soluble APs were significantly affected. The recoveries of the *ortho*-substituted APs were low. For the most sterically hindered APs (like 2,6-dimethylphenol and 2,3,6-trimethylphenol) the recoveries were less than 10 % even at the most optimal conditions. From this result it was concluded that the pentafluorobenzoyl derivatisation is not suitable for analysis of sterically hindered *ortho*-substituted APs (**Paper I**). Capillary GC with ECD detection was found to be a highly sensitive method for analysis of standard solutions. However, GC-MS-NCI (negative ion chemical ionisation) methods were preferred when analysing real complex samples (produced water and biota) due to matrix effects. The complexity of APs in samples of produced water (**Paper I**) makes it difficult to find good internal standards for GC-ECD. In GC-MS on the other hand, it is possible to use an isotope-dilution method where deuterium-labelled APs are used as internal standards.

Both methods are now in use at the laboratory of the Institute of Marine Research.

4.2 Long-term effect study on alkylphenol effects on the reproduction in cod .

4.2.1 Experimental design

Paper III-VII present the results from a project carried out during 1997-2001 where the goal was to study long-term biological effects of very low concentrations of selected C_4-C_7 APs on sex development in Atlantic cod. The study was carried out under controlled laboratory conditions. The compounds tested were 4-*tert*-BP, 4-*n*-PP, 4-*n*-HexP and 4-*n*-HepP.

Very little is known about the fate of these substances in the marine environment. There are no empirical data on concentrations of long-chain APs in the sea around the North Sea offshore installations (**Chapter 1.8**). We were therefore forced to use models when estimating the levels to which fish may be exposed. Rye et al., (1996) simulates the spread of AP discharges from produced water from the Halten Bank, and calculates the likely uptake by pelagic fish using a model. The model simulates the distribution of total AP discharges from two platforms, and includes biological response (Bioconcentration Factor (BCF) and constants for uptake and elimination). The calculations of a "worst case scenario" show that the body burden of AP in the fish modelled will be up to 10 µg/kg (Rye et al., 1996).

Given the lack of field data, we used the model values indicated in Rye *et al.*(1996) as a basis for choosing the exposure regimes in our experiments. Using an equal mixture of the four components with differing chain lengths (C4 to C7), an attempt has been made to take into account the wide range of different APs found in produced water. The intention of the tests was to dose the fish to a body burden within the range of Rye *et al.*'s estimates. Using the available information, it was concluded that 5 µg/kg of each of the four AP correspond to a fairly realistic dose.

Two independent experiments were carried out (table 5 and table 6): In Experiment I (**Paper III**), two groups of cod were exposed through regular food *per os* with a

mixture of the four APs, from October to the end of January (14 weeks): 0.02 mg/kg in the low dose group and 2 mg/kg in the high dose group. In Experiment II (**Paper IV-VII**) five groups of cod were exposed to the same mixture of APs, ranging between 0.02 and 80 mg/kg APs and a positive control of 5 mg/kg E2, for 1 or 5 weeks. In experiment II the APs were administered to the fish by a plastic tube directly to the stomach. The way of exposere assured that each individual got the same defined dose per unit weight.

Table 5. *Exposure and sampling scheme.*

	Experiment I	Experiment II
Start of exposure	1997-09-30	1999-11-16
Sample 1	1997-10-30	1999-11-23
Sample 2	1997-11-27	1999-12-21
Sample 3	1997-12-16	-
Sample 4	1998-01-26	-

Table 6*. Treatment and doses (sum of 4-tert-BP, 4-n-PP, 4-n-HexP and 4-n-HepP) for the two experiments.*

Groups	Experiment I	Experiment II
Control	Untreated	Untreated
Positive control	-	5 mg E2/kg
AP 1	0.020 mg AP/kg	0.020 mg AP/kg
AP 2	2 mg AP/kg	2 mg AP/kg
AP 3	-	20 mg AP/kg
AP 4	-	40 mg AP/kg
AP 5	-	80 mg AP/kg

The results of the present study suggest a multiple mechanism response in the AP treated cod. The exposure to APs can influence the plasma concentration of several male and female sex hormones and the egg yolk precursor protein, vitellogenin, in Atlantic cod. This study also shows that AP-exposure down to 20 µg/kg body burden interferes with the maturation of the sex organs, and that this effect is likely caused by disruption of the sex hormone system. There were also found effects of the AP treatment on the hepatic P450 systems (CYP1A and CYP3A) as well as glutathione, glutathione-related enzymes and changes in the lipid composition in liver and brain membranes.

4.2.2 Effects of APs on the glutathione-dependent antioxidant system in cod.

The effects of AP on the glutathione-dependent antioxidant system were studied in experiment II (**Paper V**). Total glutathione (reduced + oxidized forms)) increased in the livers of female fish after one week of exposure to APs. Males were not sampled after one week, so we do not know the early response of this group to AP exposure. The second sampling (after 5 weeks exposure) 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 temporary 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. glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH), (which generates NADPH) function adequately under the experimental conditions reported.

Neither the activity of glutathione S-transferase (GST) nor of G6PDH appeared to be affected by AP exposure. This may indicate that neither of these two enzymes is particularly important in AP metabolism. It is known that glucuronidation is the major phase 2 metabolism of APs (Lewis and Lech, 1996; Meldahl et al., 1996; Thibaut et al., 1998a; Arukwe et al., 2000b; Ferreira-Leach and Hill, 2001), and these results may indicate that this metabolic pathway is sufficient to metabolise such quantities of APs as the fish were exposed to in our experiments.

4.2.3 Effects of APs on CYP1A and CYP3A in cod.

In vivo and *in vitro* effects of APs exposure in cod have been studied in **Paper VI**. Fish from Experiment II showed a dose-related increase in hepatic CYP1A and CYP3A protein in male cod, but no effect was observed in the females. However, this increase of CYP1A protein levels in the male fish was not linked to an increase in CYP1A-

mediated ethoxyresorufin-*O*-deethylase (EROD) activity, implying that APs inhibit the CYP1A activity *in vivo*. In addition, *in vitro* studies on the cod hepatocytes showed a strong AP dose-dependent reduction in both the CYP1A and CYP3A activity. Similar effects were also see in NP treated juvenile cod (Hasselberg et al., 2005; Sturve et al., 2006). Kinetic study of recombinant medaka CYP3A activity also found that NP are binding CYP3A and blocking the activity (Kullman et al., 2004). AP effects on the P450 systems are further discussed in (**Chapter 1.10.3, Paper VI**).

4.2.4 Effects of APs on membrane lipids in cod liver and brain.

Paper VII demonstrated that APs and E2 alter the fatty acid profile in the polar lipids of the liver to contain more saturated fatty acids (SFA) and less n-3 polyunsaturated fatty acids (n-3 PUFA) compared with the control. In the brain of the exposed groups, a similar effect was found, although with higher saturation of the fatty acids found in the neutral lipids (mainly cholesterol ester). No effects were found in the polar lipids. The AP and E2 exposure also gave a decline in the cholesterol levels in the brain. The *in vitro* studies showed that APs increased the mean molecular areas of the phospholipids in the monolayers at concentrations down to 5 μM, most likely due to intercalation of the APs between phospholipids molecules. The increase in molecular area increased with the length of the alkyl side chain. There are several other investigations that support that APs can affect the lipid environment in the cell membrane (see **Chapter 1.10.6**). Cakmak et al., (2006) found support for that NP exposure induce a decrease the membrane fluidity by increasing the lipid order in the liver of rainbow trout. This agrees with our finding of increased amount of SFA in the polar lipids. The biological consequences of changes in the lipid compositions of the membrane are unknown and need future studies.

4.2.5 Effects of APs on sex steroids in plasma of cod.

A striking observation from both experiments is that AP exposure brings about a considerable drop in the plasma E2 level in the females even at very AP low doses (0.02 mg/kg). The effects depended on the maturation status of the cod. In experiment I the reduction in E2 was first visible after 2 months of exposure in November. In the low-dose group and the high-dose group the E2 level were 68 % and 44 % of control, respectively. This effect became stronger in December (low-dose 71 % and high-dose 35 % of the control), but the difference was not significant before January. This was probably because the number of fish was to low to give statistically significant effects in November and December (see **table 6, Paper III**). In experiment II the exposure first started in November. The down-regulation of E2 levels by AP treatment was confirmed in this experiment and significant effects were found both after 1 and 5 weeks of exposure (**Paper IV**). The plasma level of T in female fish was also affected, but the results were more ambiguous than for E2.

In male cod, AP exposure also affected the plasma levels of 11-KT and T, basically by lowering the levels. However, like T in the female fish, there were large seasonal variations and no dose-related trend.

One hypothesis explaining reductions in the steroid concentrations may be that AP exposure increases the steroid catabolism. NP is shown to be an agonist of the orphan nuclear receptors, PXR and CAR that are involved in regulation of several detoxification enzymes, such as the CYP2B and CYP3A family members, which are responsible for the metabolism of steroids and this may alter their physiological levels (**Chapter 1.10.3**). The fact that we found a dose-related increase in hepatic CYP1A and CYP3A protein in male cod could indicate an induced metabolism, however, such effect was not observed in the females (**Paper VI**). The increase of CYP1A protein levels in the male fish was not linked to an increase in EROD activity, implying that APs inhibit the CYP1A activity *in vivo*. In addition, *in vitro* studies of cod hepatocytes showed a strong AP dose-dependent reduction in both the CYP1A and CYP3A

activities. The increase in the amount of CYP3A protein may indicate an increase in the potential steroid catabolism in the male cod, but not in the females. A general increase in steroid catabolism does not explain the observed decrease in E2 (female) and 11-KT levels (male), because the level of testosterone was maintained or even increased. There is also support for that AP inhibit phase II enzymes and therby decrease steroid catabolism. Kirk et al., (2003) finds that *para*-substituated APs (C1-C9) reduces E2 sulfations in liver cytosol of chub (*Leuciscus cephalus*). Additionally, NP exposure has an inhibiting effect on glucuronidation of T and E2 in juvenile turbot (*Scophthalmus maximus*) and juvenile cod (Martin-Skilton et al., 2006c) and NP is also reducing E2 sulfation and glucuronidation in carp testicular microsomes (Thibaut and Porte, 2004) and E2 sulfation in liver cytosols from two marine fish, *mullus barbatus* and *Lepidorhombus boscii* (Martin-Skilton et al., 2006a).

Therefore, another explanation to the drop seen in E2 and 11-KT levels could be that AP exposure affects the steroidogenic enzymes (**Chapter 1.10.3, Paper IV**). One interesting finding in the present study has been that there is no clear dose-response relationship for E2 to AP exposure. The group that received the lowest dose (0.02 mg/kg) displayed the same decrease in E2 level as those that received higher doses. This may indicate that E2 down-regulation is a result of exceeding a threshold level. Currently we are doing further work to reveal the mechanism of action of APs on the steroid levels in cod. Several studies indicate that APs disrupt the natural endocrinal feedback system of the fish somewhere in the central nervous system and this affects the secretion of GTH from the pituitary and thereby indicretly affect the steroid synthesis (see **Chapter 1.10.2**).

4.2.6 Effects of APs on VTG in plasma of cod.

A massive induction of VTG in female and male cod following exposure to E2 (5mg/kg) confirms the VTG's sensitivity as a biomarker for estrogen (**Paper IV**). It

confirms previous reports on the cod's suitability as a model organism in these studies (Hylland and Haux, 1997; Hylland, 2000).

We demonstrated that the AP exposure gave a weak induction of VTG in the male cod. Experiment I gave statistically inconclusive VTG values. Nevertheless, more male fish were producing VTG in the exposed groups than in the control (**Paper III**). In Experiment II, there was a weak dose-related induction of VTG (**Paper IV**). It should be noted that even though there was an induction of VTG following AP exposure, the induction was several thousand times lower than in the fish exposed to E2.

Some surprising seasonal differences were observed in the control groups. November samples from both experiments showed higher plasma VTG levels in the males than fish from the other samples. After 1 week in Experiment II (November), measurable quantities of VTG were found in as much as 80% of the fish in the control group, while at the end of December (5 weeks) only 30% of the control fish had detectable levels of VTG in their plasma. This may be the effect of normal seasonal fluctuations of endogenous E2.

E2 plays an important role in the early part of spermatogenesis, regulating the renewal of spermatogonia (Miura and Miura, 2003). In the few studies reporting E2 in plasma from male teleost fish, concentrations are generally below 1 ng/ml (eel (*Anguilla japonica*): 0.5 ng/ml (Miura et al., 1999); huchen (*Hucho perryi*): 0.35 ng/ml (Amer et al., 2001); flounder (*Platichthys flesus*): 1.2 ng/ml (Scott, 2000); carp (*Cyprinus carpio*): 0.25 ng/ml (Villeneuve et al., 2002): Atlantic cod: 0.04-0.37 ng/ml (Scott et al., 2006). It is an unanswered question if these naturally occurring levels of E2 can be enough to induce VTG in male fish. Scott et al., (2006) measure induction of VTG in blood plasma of male cod caught in the North Sea and other areas around UK and Norway. A positive relationship between VTG and fish size was reported, but there were not found any correlation between plasma concentrations of E2 and VTG. It is suggested that large cod are exposed for estrogenic compounds through the food chain. Difference in the feeding ecology between large cod (feeding close to the bottom after

large prey) and small cod (feeding on free-swimming organisms in the water column) may explain the observed differents in VTG levels.

In aquaculture, there may be other sources of estrogenic substances. In mature male aquaculture cod VTG concentrations of 6.7 ± 4.5 µg/ml have been found and this is higher than generally found in wild fish (CEFAS, 2005). This points to the presence of elevated estrogen levels in farmed fish. At present, it can only be speculated regarding the reason for this. It is possible that phytoestrogens found in commercial fish diets play a role. In experimental settings, it is also possible that natural estrogen or its metabolites, secreted through urine from female fish, influence the male hormone levels. The recent findings of low, natural E2 levels in male fish, as well as the potency of E2 as a VTG inductor, may indicate that VTG is unsuitable as a biomarker for xenoestrogens at very low concentrations. Our data suggest that the hormone system may be affected by environmental contaminants at very low concentrations, while VTG-induction is only moderately susceptible to such influence. As research moves towards effect-studies of lower and lower concentrations of contaminants, we believe it will be necessary to use additional biomarkers for estrogenic substances than VTG induction.

4.2.7 Do AP exposure have adverse effects on the reproduction of cod

The results from the present thesis (**Paper III and IV**) show a reduction in steroid levels, ovary growth and testis maturation status at the lowest concentration tested, 20 µg/kg nominal body burden (sum of four APs), but it is not clear if these effects are causing adverse effects on the cod reproduction. Table 7 gives corresponding water concentrations and body burden (assumes a BCF factor of 500), useful when comparing the doses of different exposure regimes. Back calculations of the 20 µg/kg are equivalent to a theoretical total concentration of 40 ng/l, which are very low effect concentrations and below levels reported from other studies. Our finding need to be

confirmed in water exposure experiments, but it brings to attention to that there can be large differences in the response to AP between different species and that effects concentrations may be down in the ng/l levels.

Table 7. *Corresponding water concentration and body burdens, using BCF = 500*

Water concentration		Body burden
1 ng/l	↔	0.5 µg/kg
40 ng/l	↔	20 µg/kg
100 ng/l	↔	50 µg/kg
1 µg/l	↔	500 µg/kg
100 µg/l	↔	50 mg/kg

Experiment II showed considerable effects on the pattern of maturation of the testis in males (**Paper IV**). There was an increase in the amount of spermatogonia. There also appeared to be an increase in the amount of spermatocytes and a reduction in spermatozoa. Similar effects after AP exposure have been seen in a number of other fish species, but it still remains to find out if the changes in maturation status are leading to reduced sperm quality and thereby affecting the cability of the male cod to fertilize the eggs.

As discused above, the most significant effects found from these experiments are an anti-estrogenic effect of the APs, possibly by APs causing a down-regulation of natural E_2 synthesis (**Paper III and IV**). Normally, there is a direct relationship between E2, vitellogenin and gonadal growth. It is therefore not surprising that low E2 levels were accompanied by a drop in gonadal weight. The fish from the positive control group aborted the oocyte maturation, resorbing the oocytes through atresia. The groups exposed to APs did not show an increased occurrence of atresia. However, the oocytes had a significant reduction in oocyte diameter. Histologically, the oocytes seem to develop slowly but otherwise normally. The oocytes of the exposed groups were in the beginning of vitellogenesis at a time when the controls were in late vitellogenesis. The significantly smaller oocytes in the exposed groups predicted delayed spawning.

The natural spawning time for the North Sea cod stock are in spring, around the time of the initiation of the seasonal plankton development. The copepod *Calanus*

finmarchicus is a key zooplankton species in Norwegian waters, and early developmental stages (nauplius larvae) of this species are the main prey for fish larvae (Sundby, 2000). Variation in the timing of the plankton development *versus* the spawning and larval development of fish has been considered in the match-mismatch hypothesis to be a major cause for variable recruitment of fish (Cushing, 1990; Beaugrand et al., 2003; Platt et al., 2003). A delay in start of spawning, as estimated for our AP-exposed fish increases the chances of the eggs being spawned too late relative to the optimum. Data from the Baltic Sea show that over the last decade, there has been a shift towards spawning several weeks later in the season than has been the case in the past (Wieland et al., 2000). The temporal overlap between the developing Baltic cod larvae and their prey has decreased since the mid-1980s and this coincides with a massive reduction in the recruitment of this cod stock. (Hinrichsen et al., 2005). The main theories trying to explain the shift in spawning times are changes in water temperature during the period of gonadal maturation, density-dependent processes related to the size of the spawning stock, and food availability. But as shown in the present work, endocrine disrupting chemicals also effect the maturation of the gonads. This indicates that EDCs may be at least partially responsible for the changes seen in fish populations in the highly polluted Baltic Sea.

As support for such a theory, long-term monitoring (1988-2000) in the Baltic Sea of the gonadal size of female perch (*Perca fluviatilis*) revealed a strong trend towards decreased GSI. Pollution are suggested to play an important role as causative for this phenomenon (Hansson et al., 2006). Similarly, Noaksson *et al.* found a delay in the gonadal maturation of wild female perch, roach (*Rutilus rutilus*) and brook trout (*Salvelinus fontinalis*) (Noaksson et al., 2001; Noaksson et al., 2003; Noaksson et al., 2005) living in lakes receiving leakage water from old refuse dumps. The compounds causing endocrine disruption are not identified. The observed reduction in plasma steroid levels (T and E2) in combination with decreased GSI is, however, similar to the findings in cod in the present publication.

In medaka, it has been found that exposure to NP results in reduced realised fecundity (Gronen et al., 1999; Shioda and Wakabayashi, 2000a). These authors also noted a tendency for fewer eggs to hatch when an unexposed female spawned with an exposed male. This aspect has not been examined in our study, but there is every reason to look further into it. If exposure reduces both realised fecundity and the proportion of eggs that actually hatch, this would increase the effects of delayed spawning.

It is not possible from our results to conclude that the AP exposure is resulting in advanced effects on the cod reproduction, but the findings presented clearly calls for more studies.

4.2.8 Bioaccumulation and oral uptake of APs in cod

Our decision to expose fish through their food in our experiments was due to the practical and environmental consequences of exposure through the water. Large fish were used, and they were kept in large tanks. The fish require a continuous supply of large quantities of water (20000 l/h). A large-scale exposure experiment would require large amounts of APs and the building of costly infrastructure to properly handle the discharged water.

The body burden of 5µg AP/kg was expected to be equivalent to the quantity that the fish might absorb if exposed to seawater with 10 ng/l of the individual APs. This simplified calculation assumes a BCF factor of 500 for all four APs. 5 µg/kg is equivalent to a theoretical total concentration (sum of all four APs) of 40 ng/l. This concentration is lower than the levels that have previously been reported to affect the endocrine system in fish.

It may be argued that oral exposure results in APs being distributed in the body to a lesser extent than with exposure through the water. This should, however, result in an underestimation of the effects of the exposure. There seems to be no reason to suggest

that oral exposure leads to increased bioaccumulation. Consequently, our results more likely underestimate than overestimate the effects of AP exposure. Furthermore, the concentrations of APs actually found in the tissues are more likely overestimated than underestimated. On this background, it is clear that the present findings represent a minimum of expected detrimental effects of exposure to the tested concentrations of AP. Sundt and Baussant (Sundt and Baussant, 2003) compared the uptake and tissue distribution of the four APs used in our study in cod, using oral and waterborne exposure. They found that the bioconcentation from seawater was much higher than via absorption through the gut wall. A similar situation is also found in fathead minnows (*Pimephales promelas*) (Pickford et al., 2003). These studies suggest that the actual AP body burden in the current experiment can be only 10% of the nominal body burden and, furthermore, that the exposure level giving significant disruptions in the reproductive system of female cod may be as low as 2 µg/kg body burden, which is equivalent to 4 ng/l in the seawater.

4.3 Alkylphenols from offshore oil production

Limited data have been available on the contents of long-chain APs (C4 - C7) in produced water (Brendehaug et al., 1992; Røe and Johnsen, 1996). The analytical methods generally used (GC-MS of underivatised phenols with cluster analysis of all isomers with specific masses) have low selectivity and overestimations are likely to have occurred. Methods with higher sensitivity and more selective detection, have now been developed (**Paper 1**). A large number of APs are found in an average produced water sample. Theoretically, there can be hundreds of isomers of C_4-C_9 APs. Most of them are not commercially available. Only the long-chain, *para*-substituted APs have significant estrogenic effects. *Ortho*-substituted, *meta*-substituted and short-chain APs have very little or no estrogen effect (Routledge and Sumpter, 1997). At the IMR, an effort is currently made to synthesise as many as possible of the long-chain *para*-substituted AP isomers. We hope to be able to identify and quantify more of the *para*-

substituted AP from C_4 to C_9 and then estimate the total estrogenic potential from APs in produced water. Until such data are available, it will only be possible to obtain long-chain AP concentrations of a few standard compounds or as the sum of all isomers. The method described in **Paper 1**, have been used for determination of APs in produced water from 9 different oil fields on the Norwegian sector and the total concentrations of APs > C_4 are found to be in the range of 5-81 µg/l (in preparation). Thomas *et al* used *in vitro* methods (yeast estrogen screen, YES) to detect estrogen receptor (ER) agonists in produced water from the North Sea oil installation (Thomas et al., 2004b; Thomas et al., 2004a). They found that produced water contains ER agonists in amounts corresponding to E_2 equivalents from the low ng/l and up to 91 ng/l. This corresponds well with the levels of APs, considering that APs are in the order of 1000 times weaker ER agonist than E2 (Routledge and Sumpter, 1997).

The produced water is rapidly diluted after being discharged from the platform. Computer simulations show 30 and 100 times dilution 10 m and 100 m from the outlet, respectively. Further dilution is, however, slower, and the model showed that 1:1000 dilution occurred as far as 1000 m from the outlet (Neff, 2002). The results from the computer model and field data indicate that dispersed oil may be found in concentrations from 1-3 µg/l in an area with a radius of 50 to 100 km around the largest oil fields in the North Sea (Rye et al., 1998). This corresponds to a dilution factor of approx. 1:10.000.

There are no empirical data available on concentrations of long chain APs in the sea around North Sea offshore installations, but as discussed above, the concentrations in the marine environment should be low. On the other hand, the discharges are continuous and prognoses indicate increased discharges as the oil field age. Little is known about the fate of long-chain APs in produced water after it enters the sea. The degradation rate of APs falls rapidly with increasing chain length and APs have high sedimentation rates (**Chapter 1.6**). Measurement of long chain APs in seawater and sediment around oil installations should therefore be a priority in future risks assessment studies.

The results from the presented experiments have been used in a theoretical study titled: “Risk Assessment of reproductive effects of alkyl phenols in produced water on fish stocks in the North Sea” (Myhre et al., 2005). The modeling is done using the DREAM software (developed by Sintef, RF-Akvamiljø and TNO, Delft, the Netherlands). The model includes the combined discharges from three major Norwegian oil fields (Tampen, Ekofisk and Sleipner). The fish stock distributions (cod, saithe and haddock, from the international bottom trawl surveys (IBTS) database) and a Predicted No Effect Concentration (PNEC) for APs of 4 ng/l were used as basis data for the calculations. The total amount of APs>C_4 discharged from all the oil installation was estimated to be 25.6 kg/day, dissolved in 364.300 m^3/day produced water. The conclusion of the risk assessment was: “The overall results of the simulations with DREAM show that there is no significant risk potential. In other words there were no fish particles, which accumulated APs above the critical body burden of 2 µg/kg in any of the simulations. The highest accumulated body burden in any of the fish particles was 0.09 µg/kg” (Myhre et al., 2005). This new modelling work indicates that the article of Rye *et al.* (1996) overestimated the body burden and that the doses used in these experiments may not be expected to arise from produced water discharges alone. However, both models are encumbered with uncertainty, primarily because the fate of the long chain APs in the sea is not known. It is reasonable to believe that these relatively hydrophobic substances will bind to biological particles in the sea. This may affect distribution, degradation and also uptake of APs in the food chain. All these unknown factors urge for proper field studies in order to be able to forecast the impact of these biologically active chemicals on the marine environment.

4.4 Conclusions.

The results presented in this thesis have shown that cod is sensitive to AP contamination of the environment. Even though the concentrations used in our experiments are higher than may reasonably be expected as the result of oil production alone, measurements of real AP levels in the sea indicate that APs may still be a significant factor in the marine environment. Only when the environmental fate of the long-chain APs has been more thoroughly understood, the APs may possibly be ruled out as a significant detrimental factor of proper growth and development of the relevant fish populations. Collecting information about the concentrations of long-chain APs in the open water and in marine sediments is mandatory for sound evaluations of the environmental effects of long-chain APs.

4.5 Future perspectives.

New experiments have been conducted in 2005 to supplement and clarify the results from the present study. These include exposure to lower doses of APs to provide knowledge about the true "no effect" concentration. Furthermore, the fish have also been exposed to real produced water with its natural high complexity of components. Cod have also been reared through spawning after long-time exposure (20 weeks) to APs and produced water, aiming to confirm the estimated delay in spawning shown in this thesis, as well as searching for any effects on realised fecundity and fertilisation.

To begin elucidating the complex mechanisms involved in the response to APs, pitutitary gene expression related to FSH and LH will be analysed, as well as aromatase activity in the gonads and the brain. Steroid profiles will be analysed in blood plasma, gonads and brain, together with the amount of conjugated steroids in the bile.

Appendix 1. *In vivo* studies of the effects of alkylphenols on teleost fish

Table 8*. In vivo studies of the effects of alkylphenols on teleost fish. The table provides an overview of various measurement variables and the lowest effect dose that has response. (where concentrations are quoted per kg, the substance was administered by injection or oral; when quoted per litre, the substance was provided in the water*

Species, dose and exposure time	**Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis**	**Other effects**	**Reference**
Order: Cypriniformes			
Family: Cyprinidae			
Juvenile Carp (*Cyprinus carpio*). 100, 320 and 1000 µg/l 4-*tert*-PP at different times in the early lift stages.	Oviduct formed in testis by exposure during sexual differentiation. Reduction in number of primary sex cells.		(Gimeno et al., 1996; Gimeno et al., 1997; Gimeno et al., 1997)
Mature carp. 32 µg/l 4-*tert*-PP for 3 month.	Reduced number of spermatogenetic cysts. Reduced GSI after three months of exposure.		(Gimeno et al., 1998)
Zebrafish (*Danio rerio*). Fed NP containing food corresponding 40 µg/fish/day for 21 days.	Induction of VTG.		(Allner et al., 1999)
Fathead minnow (*Pimephales promelas*). 0.05–3.4 µg/l NP for 42 days.	1.1-3.4 µg/l NP increase Sertoli cell hypertrophy and necrotic aggregates of germ cells in testes.		(MilesRichardson et al., 1999)
Fathead minnow. 0.05–3.4 µg/l NP for 42 days	0.1 µg/L NP induced a 10-fold raise of E2 in female and induced VTG in the female fish.	BCF for NP from 203-268.	(Giesy et al., 2000)
Fathead minnow. 0.6–63 µg/l NP for 3 weeks.	Induction of VTG at 8 µg/l NP in males and at 58 µg/l NP in females. Doses of NP above 48 µg/l NP inhibited reproduction completely. (measured conc.)	Reduction in the prominence of secondary sexual characteristics in males at 8 µg/l NP.	(Harries et al., 2000)
Juvenile sheepshead minnow (*Cyprinodon variegatus*). Acute toxicity test for NP.		96-hr. LC50 = 142	(Lussier et al., 2000)
Juvenile carp.1–15 µg/l NP for 70 days.		Effects on blood parameters. Enlarged erythrocytes, and possible anaemia from 10 µg/l NP.	(Schwaiger et al., 2000)
Sheepshead minnow. Exp1 0.6 – 43 µg/l NP. Exp 2: 6 and 60 µg/l NP for 16 days. Samples were taken up to 96 days post-exposure.	Induction of VTG above 5 µg/l NP. VTG mRNA rapidly diminishes, but plasma VTG clearance is concentration and time dependent and can be detected months after exposure.		(Hemmer et al., 2001; Hemmer et al., 2002)
Pre-larval zebrafish embryos. 24 hr. exposure for 22 µg/l NP.	Minor but significant change in anterior-posterior distribution of primordial germ cells.		(Willey and Krone, 2001)
Juvenil carp. Injected with 1, 10 and 100 mg/kg NP, repeated after 7 days. Sampling after 7 days.	Induction of VTG at 100 mg/kg NP.		(Casini et al., 2002)
Transgene zebrafish. 220 µg/l NP for 96 hr.	No induction of estrogen receptor (ER)-mediated luciferase gene expression in the transgene fish.	Uptake of NP after 96 hr. 8940 µg/kg NP.	(Legler et al., 2002)
Male carp. 0.05–5.4 µg/l NP for 28-31 days.	No changes in steroid (E2 and T) or VTG levels in plasma. No differents in GSI.	Detection of 0.5-3.5 mg/kg NP in pooled plasma or tissue samples.	(Villeneuve et al., 2002)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Zebrafish. 10-100 µg/l NP. 60 days post hatch (dph), full life cycle tests.	Induction of VTG mRNA above 30 µg/l NP. Shift in sex ratio towards females at 100 µg/l NP (10 % males and 45 % in control). Two fish with ovatestis were found at 100 µg/l NP. 60 days after end of exposure (120 dph) there were no differens in sex ratio from any groups.		(Hill and Janz, 2003)
Embryos of zebrafish. Exposure (1, 10, 1000 µg/l NP) started one hr. after fertilization and til 48 hr.		10 % of the genes on the 230 gene chip were altered even at 1 µg/l NP. 9 gene were selected as potential biomarkers for NP exposure.	(Hoyt et al., 2003)
Male sheepshead minnow. 11.5–61.1 µg/l 4-*tert*-OP for 24 days. Spawning experiment with exposed male and non exposed females.	Induction of VTG at 11.5 µg/L OP. Testis anomalies in male at 33.6 µg/L OP.	Reduction in viable eggs when the male have been exposed for 33.6 µg/L OP. No effects on second generation: embryonic development, egg hatching or fry survival.	(Karels et al., 2003)
Juvenile zebrafish. 2.2, 22 and 220 µg/l NP for 3 days.		Up-regulation of whole fish transcript of aromatase CYP19A2 gene (mainly found in the brain and pituitary) at 2.2 µg/l NP. CYP19A1 (mainly found in the gonads) were unaffected.	(Kazeto et al., 2003; Kazeto et al., 2004)
Fathead minnow. Study of the route of exposure: 2 weeks exposure for 1-50 µg/L NP or 100–1000 µg/kg/day NP oral exposure.	VTG mRNA induced at 50 µg/l NP and 500 µg/kg/day NP. VTG found in plasma also at 10 µg/l NP.	It was concluded that exposure via the water gives a 10–fold higher sensitivity that via exposure by the diet.	(Pickford et al., 2003)
Zebrafish. 1-38 µg/l 4-*tert*-OP for 78 days, full life cycle tests	Fertilization success EC50=28 µg/l OP.		(Segner et al., 2003b; Segner et al., 2003a)
Embryonic zebrafish. 100 and 1000 µg/l NP for 48 or 72 hr.		30 % of the proteins from embryo homogenates were specific to the NP groups (two-dimensional electrophoresis, 2DE).	(Shrader et al., 2003)
Zebrafish and rainbow trout. 12.5-100 µg/l 4-*tert*-OP and 20-500 µg/l NP for 3 weeks.	Rainbow trout were 3 times more sensitive than zebrafish to OP and NP. Induction of VTG at 30 µl/L OP and 100 µl/l NP.		(Van den Belt et al., 2003)
Zebrafish. 2 days post-hatch (dph) – 60 dph. 10, 30 and 100 µg/l NP.	Delayed gametogenesis in both male and female from 100 µg/l NP. Increased ovarian follicle atresia.	Kidney pathology at 10 µg/l NP.	(Weber et al., 2003)
Male goldfish (*Carassius auratus)*. 0.1–100 µg/l NP in 28 days.	Induction of VTG at 100 µg/l NP.	No effect on CYP1A or GST.	(Ishibashi et al., 2004)
Female zebrafish. 30–500 µg/l NP for 3 weeks.	Induction of VTG at 250 µg/l NP. Reduced GSI at 500 µg/l NP.		(Van den Belt et al., 2004)
Fathead minnow. 0.4-7 µg/l NP and 1.5-35 µg/l OP. Mixtures of different xenobiotic. Exposure for 14 days.	EC_{50} for VTG induction 7 µg/l NP and 48 µg/l for OP. Additive effect of mixtures.		(Brian et al., 2005)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Male zebrafish. 500 µg/l 4-*tert*-OP for 7 and 15 days.	Induction of VTG. No difference in testis histology	Increased peroxisomal surface and numerical densities and activities of the peroxisomal β-oxidation enzyme acyl-CoA oxidase (AOX) in the liver. No induction of catalase activity.	(Ortiz-Zarragoitia and Cajaraville, 2005)
Juvenile goldfish. 22 and 220 µg/l NP for 3 weeks.	Induction of VTG at 22 µg/l NP. 22 µg/l NP affects the steroids levels in plasma: decrease in androgens (ELISA with cross reaction of many androgens) and increase of E2.	Induction of gene expression of ERβ-1. Accumulation of NP in liver and muscle.	(Soverchia et al., 2005)
Male carp. 690, 1380 and 2300 µg/l 4-*tert*-BP for 4 weeks. Sampling after 7, 14 , 21 and 28 days.	Reduced Male GSI ≥ 690 µg/l 4-*tert*-BP. Induction of VTG ≥ 690 µg/l 4-*tert*-BP. Totally loss of histo-morphology architecture in testis at all concentrations (disintegration of the lumen wall and necrosis of spermatozoa).	96 hr-LC = 6900 µg/l 4-tert-BP. Increased HIS and reno-somatic index ≥ 690 µg/l 4-tert-BP. Pathological changes was found in liver tissue ≥ 690 µg/l 4-tert-BP (hyperplasia of connective tissue, decrease in the number of hepatocytes, increased vacuolization in extracellular space and fibrous lesions). Decrease in alkaline phosphatase and aspartate aminotransferase activity ≥ 690 µg/l 4-tert-BP. Increrase alanine aminotransferase and acid phosphatase activity ≥ 690 µg/l 4-tert-BP.	(Barse et al., 2006)
Male fathead minnow larvae. 5 µg/l NP for 64 days.	No effects on VTG or GSI.	No effect on larvae survival. The NP exposed showed increased competitive reproductive behavior.	(Bistodeau et al., 2006)
Juvenile carp. 5, 50 and 500 ng/l NP or injection of 50 mg/kg NP. Sampling after 24 hr and 96 hr.	Gene expression analysis using a carp cDNA microarray (consisting of 960 hormone-responsive and gender-associated gene fracments) found that 111 genes were regulated in the liver of NP exposed fish. Examples of significant upregulated genes were: VTG, cytochrome c oxidase and cytochrome P450 monooxygenase (CYP2K1v2). No dose response data are giving.		(Moens et al., 2006)
Fathead minnow embryos. 56, 180 and 560 µg/l 4-*tert*-PP for up to 107days post hatch (dph), some fish were exposed for 30 or 60 dph and followed in clean water on to 107 dph.	No effects on hatching succes or survival, but a delay in time to hatch for the 560 µg/l PP. Induction of VTG at 180 µg/l PP in both female and males. Lower GSI in females at 560 µg/l PP. All female and no testis development at 560 µg/l PP. Intersex and ovarian-like cavity were found in all male fish at 180 µg/l PP.	Lower condition factor in females at all doses. No effect on liver or kidney histologi.	(Panter et al., 2006)
Mature zebrafish. 0.1, 1, 10, 50, 100 and 500 µg/L NP for 3 weeks.	Induction of VTG in male fish at 100 µg/l NP. Reduced GSI for females at 500 µg/l NP. Prenatal exposure of females to 50 µg/L of NP resulted in effect on the offspring by inhibition of CAT D activity, decrease of eggshell thickness and elevation of malformation rate.		(Yang et al., 2006)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Zebrafish. 10, 25 and 75 µg/l for 3 weeks through out the spawning period	Reduction of egg production at all concentrations.		(Zoller, 2006)
Adult rare minnow (*Gobiocypris rarus*). 3, 10 and 30 µg/l NP for 28 days.	Induction of VTG in males at 10 µg/l NP. Ovatestis was found in some males at 30 µg/l NP.	Pathological changes were found in liver and renal tissue of the 30 µg/l NP group (hypertrophy of hepatocytes, damages to cellular structure and accumulation of eosinophilic material).	(Zhai et al., 2007)
Order: Siluriformes.			
Family: Ictaluridae.			
Cannel catfish (*Ictalurus punctatus*). Injection of 79 and 237 mg/kg NP, sampling on day 7.	Induction of VTG in males at 237 mg/kg NP.		(Nimrod and Benson, 1996a)
Cannel catfish. Injected three times (day 1, 4 and 7) with 60 mg/kg NP, the fish were sampled on day 10.	Induction of VTG in males.	Up regulation of ER in the liver.	(Nimrod and Benson, 1997)
Cannel catfish. Seven different diets containing 10 –100 mg/kg NP, sampling after 7 and 21 days		Phagocyte function was enhanced by 10 mg/kg NP on day 7.	(Rice et al., 1998)
Family: Claridae			
African catfish (*Clarias garipinus*). 10 µg/l NP		Increase in LH content in the pituitary of male catfish.	(van Baal et al., 2000)
Order: Salmoniformes			
Family: Salmonids			
Juvenile Atlantic salmon (*salmo salar*). Acute toxicity test for 10 different Aps		96-h LC50 was from 0.13 mg/l for NP to 0.74 mg/l for 4-sec-BP.	(McLeese et al., 1981)
Rainbow trout males. 0.6 – 44 µg/l 4-*tert*-OP; 1.0 – 54.3 µg/l NP, 3 weeks exposure.	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.		(Jobling et al., 1996)
Rainbow trout. 10 – 250 µg/l NP for 72 hr.	Induction of VTG gene expression at 10 µg/l NP.	LC50 was194 µg/l NP.	(Lech et al., 1996,Ren et al., 1996)
Juvenile Atlantic salmon. 1 – 125 mg/kg NP injected. Sampling after 14 days	Induction of Zrp and VTG at 1 mg/kg NP. Reduction in plasma concentrations of E2 at 1 and 5 mg/kg NP. No effects at higher doses.	Dose-related reduction in EROD. Higher activity of steroid-hydrogenase at 1 and 5 mg/kg NP, lower at higher doses.	(Arukwe et al., 1997b; Arukwe et al., 1997a)
Atlantic salmon. 130 mg/kg NP injected once a week in 30 days under smoltification	Increased calcium and protein levels in plasma =>induction of VTG.	Inhibited progress of smoltification.	(Madsen et al., 1997)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Juvenile Atlantic salmon. 25 mg/kg NP injected. Sampling after 14-30 days	Induction of Zrp and VTG.		(Arukwe et al., 1998; Arukwe et al., 2000a; Arukwe et al., 2001b; Arukwe et al., 2001a)
Juvenile female rainbow trout. 1-50 µg/l NP and 4-*tert*-OP for 22-35 days, the fish were followed in 431 days.	Increased GSI at 50 µg/l NP.	Growth inhibition at 10 µg/l of both NP and OP.	(Ashfield et al., 1998)
3 cold water fish (rainbow trout, apache trout, lahanton trout) and 4 warm water fish (fathead minnow, razorback sucker, bonytail chub, colorado squawfish). 50-220 µg/l NP for 96 hr.		220 µg/l NP decrease brain muscarinic cholinergic receptor (MChR) in three cold water species.	(Jones et al., 1998)
Juvenile rainbow trout. Injection of 5 and 50 mg/kg 4-*tert*-OP	Induction of Zrp at both concentration OP.	Up regulation of E2 binding capacity in the liver at both concentrations.	(Knudsen et al., 1998)
Male rainbow trout and adult roach (*Rutilus rutilus*). 1-100 µg/l 4-*tert*-OP	VTG induction at 100 µg/l OP for rainbow trout. Roach was 10 times less sensitive.		(Routledge et al., 1998)
Juvenile rainbow trout. 25-100 µg/l NP for 21 days.	Induction of VTG at 50 µg/l NP. NP exposure did not affect plasma levels of cholesterol, pregnenolone or T levels.		(Tremblay and Van der Kraak, 1998)
Juvenile Atlantic salmon. 125 mg/kg NP injected. Samples after 14 days.	Immunohistochemical identification of VTG in liver.	No histopathological changes.	(Arukwe et al., 1999)
Juvenile Atlantic Salmon feed with 300 mg NP /kg food for 4 weeks or injected with 80 mg/kg NP, sampling after 14 days.	No significant induction of VTG. No difference in sex ratio.		(Norrgren et al., 1999)
Rainbow trout. Comparing branched 4-*tert*-OP, 4-NP and linear 4-*n*-OP, 4-*n*-NP. Injection with 50 mg/kg (sampling after 12 days) and water exposure with 150 µg/l for 9 days.	VTG induction only for the branched Aps and not for the normal Aps.		(Pedersen et al., 1999)
Juvenile Atlantic salmon. 5-125 mg/kg NP injected. Sampling after 2, 4 or 7 days.	Induction of VTG and Zrp mRNA and protein at 25 mg/kg NP in liver.	Up-regulation of ER mRNA in liver at 25 mg/kg NP.	(Yadetie et al., 1999)
Rainbow trout. 1-10 µg/L NP during the embryonic, larval and juvenile life stage for 1 year.	Induction of VTG at 1 µg/l NP and ZRP at 10 µg/l NP. No testisova was observed and the sex-ratios were unchanged.		(Fent et al., 2000; Ackermann et al., 2002)
Juvenile rainbow trout. 0.32-100 µg/l NP for 21 days.	Induction of VTG 16 µg/l NP. No effect on GSI.	Increases in hepatosomatic index at 53 µg/l NP.	(Thorpe et al., 2000)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Female rainbow trout. 0.7, 8.3, and 85.6 µg/L NP, for 18 weeks.	Induction of VTG above 8.3 µg/l. Reduction in E2 in plasma at 85.6 µg/l NP. Decrease in GSI at 85.6 µg/l NP.	Plasma FSH levels and FSH gene expression in the pituitary were reduced at the lowest dose employed (0.7 µg/L NP). Pituitary LH content was significantly lower in fish exposed to 85.6 µg/L NP, and LH gene expression was suppressed in fish exposed to 8.3 and 85.6 µg/L NP.	(Harris et al., 2001)
All-male embryos of Rainbow trout. 10 µg/l and 100 µg/l NP, 1 h exposure per day (in 10 days) from eyed stage to hatching.	*In situ* hybridization showed high levels of estrogen receptors (ER) in embryos receiving 100 µg/l NP. No effect on testis morphology was found (8 months after exposure).		(Madigou et al., 2001)
Juvenile rainbow trout. 2.4 – 24 µg/l NP for 14 days.	Induction of VTG above 6.1 µg/l NP.		(Thorpe et al., 2001)
Chinook salmon (*Oncorhynchus tshawytscha*). 0.1-10 µg/L NP, 29 days post hatching (DPH), fish were allowed to grow until 103 and 179 DPH.	No effect on sex differentions or gonadal morfologi.		(Afonso et al., 2002)
Juvenile rainbow trout. 25-50 mg/kg 4-*tert*-OP injected at day 1, 4 and 7. Samples were taken on day 9.	Induction of VTG in both sexes at both concentrations.	Down-regulation of P450 protein levels in liver (CYP2K1, CYP2M1, CYP3A27). No effect on EROD.	(Katchamart et al., 2002)
All-male amago salmon. 100 µg/l NP for 50 days after hatching	38 % of the fish were completely feminizated. 37 % had intersex gonads.		(Nakamura et al., 2002)
Mature rainbow trout. Intermittently exposed for 1 and 10 µg/l NP (10 days a month in 4 months).	Induction of VTG in males at 1 µg/l NP. The F_1 generation had significant increased E2 in the male fish and T in the females. VTG was induced in F_1 female. One feminized male at 1 µg/l NP and 2 at 10 µg/l NP.	Increased mortality of early egg stage at 1 µg/l NP. Decreased hatching rate at 10 µg/l NP.	(Schwaiger et al., 2002)
Rainbow trout. 0.25-53 µg/l NP for 14 days.	Induction of VTG at 16 µg/l NP.		(Tyler et al., 2002)
Juvenile Atlantic Salmon. Injected with 10, 50 and 125 mg/kg NP. Sampled after 3 days.	Induction of VTG and Zrp in males at 10 mg/kg NP and in females at 50 mg/kg NP.	Induction of LHβ mRNA in the pituitary of the females (not in the males) at 50 and 125 mg/kg NP. Induction of pituitary specific transcription factor (Pit-1) mRNA at 125 mg/kg NP. No effect gene expression of SFHβ, GH or prolactin.	(Yadetie and Male, 2002)
Atlantic salmon smolt. 5-20 µg/L NP for 7 days during the migration period.	No significant induction of VTG.	No effect on gill Na^+K^+ATPase or hypoosmo regulatory.	(Moore et al., 2003)
Juvenile rainbow trout. 0.4-50 mg/kg 4-*tert*-OP administered orally for 11 days.	Induction of VTG at 30 mg/kg OP. Significant correlation between concentration of OP in liver and VTG in plasma.	Only 1-2 ‰ of the total amount of OP was found in the liver and muscle 24 h after last administration.	(Pedersen et al., 2003)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Atlantic salmon smolt. Two 24 h pulses (days 1 and 5) of 20 µg/l NP. Sampling after 1, 2 and 3 month.		Reduced weight after the exposure for 2 and 3 months. Reduction in plasma insulin-like growth factor I (IGF-I) concentrations.	(Arsenault et al., 2004)
Atlantic salmon. 6 serial injection with 120 mg/kg NP over 20 days. The fish were released in a natural stream.	Induction of VTG in both female and males.	Impaired smolt development and survival and delayed downstream migration.	(Madsen et al., 2004)
Juvenile Atlantic salmon. 5, 15 and 50 µg/l NP for 7 days.		Gene expression of brain StAR mRNA was induced at 15 µg/l NP but not at 5 or 50 µg/l NP. P450scc mRNA was induced at 5 and 15 µg/l NP, but not at 50 µg/l NP. CYP1A mRNA was induced at 5 and 15 µg/l NP, but reduced at 50 µg/l NP. CYP3A mRNA was induced at all NP concentrations.	(Arukwe, 2005)
Rainbow trout. 1-250 µg/l NP for 21 days.	Induction of VTG at 13 µg/l NP.		(Dussault et al., 2005)
4 salmonidae and 13 other fish species. Acute toxicity test of NP. 96 hr.		96 hr. LC50 from 50 µg/l – 460 µg/l NP.	(Dwyer et al., 2005)
Juvenile coho salmon (*Oncorhynchus kisutch*). Fed with NP (0.002 – 2000 mg/kg) for 4 weeks in freshwater before transferred to seawater.	No effects on plasma levels of thyroid hormones (T_4 or T_3).	No effect was observed on osmoregulatory performance or subsequent growth performance. Fast elimination via the biliary-fecal pathway.	(Keen et al., 2005)
Rainbow trout. 100 – 750 ng/l NP during the spawning period (60 days).	Semen production was completely inhibited at 750 ng/L NP, and reduced at 280 and 130 ng/l NP (sperm density, sperm motility and sperm fertility were not affected).	There was lower larval survival over 280 ng/L NP. NP did not directly affect sperm motility or fertilizability at any of the concentrations.	(Lahnsteiner et al., 2005)
Sockeye salmon smolt. Injected with 15 and 150 mg/kg NP at day 0 and day 2. The fish were sampled at day 7.		NP had different effects on gene expression of Erα at different times during smolting. In March and May NP did not have any effects, but in April NP did decrease liver and gill Erα mRNA.	(Luo et al., 2005)
Female juvenile Masu salmon (Oncorhynchus masou). Injection of 10 or 50 mg/kg NP and sampling after 3 days.	Induction of VTG mRNA in liver at 50 mg/kg NP.	Low dose of NP (10 mg/kg) induced the GTHα and LHβ mRNA levels. High dose of NP (50 mg/kg) slightly reduced FSHβ mRNA levels.	(Maeng et al., 2005)
Juvenile Atlantic salmon. Injected with 0.5, 2, 10, 40 or 150 mg/kg NP for times over 11 days during parr-smolt transformation. After 14 days the fish were exposed to saltwater.	Induction of VTG and total calcium in plasma at 150 mg/kg NP.	Lower salinity tolerance at 150 mg/kg NP. Decreased plasma insulin-like growth factor I (IGF-I) at 150 mg/kg NP. Decreased plasma thyroxine at 10-150 mg/kg NP after 7 days exposure, but only at 150 mg/kg NP after 14 days. Plasma cortisol levels were not affected by any of the treatments.	(McCormick et al., 2005)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Rainbow trout. 220 µg/l NP for 3 weeks		Fourier transform infrared spectroscopy (FT-IR) analysis showed compositinal and structural changes in the liver of NP exposed fish. Decrease in glycogen and protein levels and increase in hepatic lipids (especially triacylglycerides). The FT-IR spectra also indicated a decrease in membrane fluidity after increased lipid order in the membranes.	(Cakmak et al., 2006)
Juvenile Atlantic salmon. 5, 15 and 50 µg/L NP for 7 days.	Induction of VTG and Zrp mRNA in liver at 15 µg/l NP.	In the brain the gene expression of ERα and ERβ decreased after 3 days, but increased after 7 days exposure to 5-50 µg/l NP. Brain aromatase mRNA was also induced after 7 days exposure to 5-50 µg/l NP. In the liver the gene expression of CYP3A, CYP1A, PXR and AhR were induced after 7 days exposure to 5-50 µg/l NP	(Meucci and Arukwe, 2006c; Meucci and Arukwe, 2006b)
Juvenile female rainbow trout. 2.2 µg/l – 2200 µg/l NP	Induction of VTG gene expression in the lever at 2200 µg/l NP, No effect on ER mRNA	Dose-dependent reduction of sGnRH2 gene expression in the brain, no effect on sGnRH1 or ER mRNA levels	(Vetillard and Bailhache, 2006)
Juvenile female rainbow trout. 40 and 80 µg/l NP for 5 days.		The exposed fish showed changes in behaviour and were were more likely to be attacked by other fish, and were less successful when competing for food resources than control fish. The behavioural effects of the NP exposure do not appear to be related to its estrogenic potential, as there were no effects seen with the estrogen-positive control.	(Ward et al., 2006)
Order: Gadiformes			
Family: Gadidae			
Juvenile Atlantic cod (*Gadus morhua*). Injected with 220 mg/kg NP, sampled after 1-40 days.	Induction of VTG.		(Hylland and Haux, 1997)
Juvenile Atlantic cod. 96 - 806 µg/l 4-*n*-HepP for 168 hr.		168-hr. LC50 = 518 µg/l. Biological half-life ($t_{1/2}$)=13 hr. and BCF = 555	(Tollefsen et al., 1998)
Juvenile Atlantic cod. 29 µg/l NP for 3 weeks.		No induction of micronuclei in peripheral erythrocytes.	(Bolognesi et al., 2006)
Juvenile Atlantic cod. 29 µg/l NP for 3 weeks.	Induction of VTG and Zrp.	SELDI-TOF found 146 peaks (peptides) that were significantly altered (up or down) in the plasma of the exposed cod compared with control.	(Larsen et al., 2006)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Juvenile Atlantic cod. 29 µg/l NP for 3 weeks.	No effect on T and E2 levels in plasma.	No effect on P450 aromatase activity in ovaries. Reduced glucuronidation of E2 (UGT-E2), no effect on UGT-T. No effect on E2-sulfotransferase activity.	(Martin-Skilton et al., 2006c)
Juvenile Atlantic cod. 29 µg/l NP for 3 weeks.		Decrease of hepatic CYP1A and CYP3A levels, decrease in EROD activity. Increase in conjugation enzyme GST activity.	(Sturve et al., 2006)
Mature Atlantic cod. Fed with food containing an AP mixture (4-*tert*-BP, 4-*n*-PP, 4-*n*-HexP, 4-*n*-HepP) corresponding to 0.02 and 2 mg/kg/day for 4 months.	Reduction of E2 and T in the plasma of female at both doses. Induction of VTG in males.		This study (Paper III)
Mature Atlantic cod. Oral exposure for 5 weeks with an AP mixture (4-*tert*-BP, 4-*n*-PP, 4-*n*-HexP, 4-*n*-HepP) 0.02 mg/kg/week - 80 mg/kg/week.	Significant effects at lowest dose. Reduction of E2 and T in the plasma of female, Reduced 11-KT in male plasma. Induction of VTG in males. Delayed ovary growth, lower GSI and smaller oocytes. Increase in the amount of spermatogonia and a reduction in the amount of spermatozoa in testis.	Increased weight loss at 20 mg/kg/week AP for females. Increased hepatic total glutathione in females after 1 week exposure, an effect not seen after 4 weeks. Increased glutathione reductase catalytic activities in both males and females at 0.02 mg/kg/week AP for 4 weeks. The glutathione *S*-transferase activity was only affected in male fish at 0.02 mg/kg/week AP, and glucose-6-phosphate dehydrogenase activity increased in female fish exposed to 0.02 mg/kg/week AP for 1 week. Dose dependent increase of hepatic CYP1A and CYP3A protein levels in males, but not in females. No increase in EROD activity in any of the sexes.	This study (**Paper IV-VII**)
Order: Atheriniformes			
Family: Atherinosidae			
Inland silverside (Menidia beryllina). Acute toxicity test for NP.		96 hr. LC50 = 70 µg/l NP	(Lussier et al., 2000)
Order: Beloniformes			
Family:Adrianichthyidae			
Medaka (*Oryzias latipes*). Full life-cycle exposure, 50 and 100 µg/l NP from hacthing to 3 months.	50% of the male fish from 50-µg/L NP and 86% of the males in the 100-µg/L NP developed testis-ova. The ratios of males to females in the control treatment (2:1, M:F) and the 100 µg/L NP treatment (1:2, M:F) were different.	The medaka embryo/larval LC50 for NP was 460 µg/l NP.	(Gray and Metcalfe, 1997)
Medaka. 0.5, 0.8, 1.9 µg/l NP from hacthing to 1 month. Sampled one month after end of exposure.	No alteration in sex ratios was observed. There was no depreciation in reproductive capability measured by fecundity, viability of eggs, or hatchability of eggs.		(Nimrod and Benson, 1998)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Medaka. Full life-cycle exposure, 10 – 100 µg/l 4-*tert*-OP from hacthing to 3 month.	Ovotestis at 50 and 100 µg/l OP.	Reduced pairing activity (25 µg/l OP). Poor hatching or deformities in offspring of exposed fish (10 – 100 µg/l OP).	(Gray et al., 1999a; Gray et al., 1999b)
Embryo medaka. 0 – 1000 µg/l 4-*tert*-OP from fertilization to day 17 (swim-up)		LC50 = 450, 830 and 940 µg/l OP (3 replicate experiment.	(Gray and Metcalfe, 1999)
Medaka . 10 – 100 µg/l OP for 21 days.	Dose-dependent and reversible induction of VTG at 20 µg/l OP. Ovotestis in two fish (one at 74 µg/l OP and one at 230 µg/l OP). More spermatogonia (A and B) at concentrations above 41 µg/l OP.	Larval survival correlated with VTG serum levels. Unexposed female fish together with exposed males spawned fewer eggs (50%) than controls. Abnormal embryonic development.	(Gronen et al., 1999)
Medaka. 20 µg/l NP (two different sources of NP) for 4 days	Induction of VTG in males. No difference between the different NPs.		(Foran et al., 2000)
Medaka. 150 – 1500 µg/l BP; 6.6 – 66 µg/l NP for 2 weeks.		Reduced hatching fraction with pairs of which the male had been exposed to 66 µg/l NP.	(Shioda and Wakabayashi, 2000a)
Male Medaka. 6.6 – 66 µg/l NP for 2 weeks.		Decrease in the number of hatching from eggs fertilized by male fish dosed 66 µg/l NP	(Shioda and Wakabayashi, 2000b)
Medaka. 0.1 – 100 µg/l NP for 5 weeks.	Female specific proteins (VTG + ZR + ?) induced at 0.1 µg/l NP (immunodetection). Measurable concentration at 100 µg/l NP. Abnormal gonadal development in two (of seven) individuals at 100 µg/l NP.	Reduced survival at more than 50 µg/l NP. LC50 for larvae 130 µg/l NP, and 860 µg/l NP for mature fish.	(Tabata et al., 2001)
Medaka. 4.2-183 µg/l NP. Full life-cycle exposure for 105 days. The offspring (F_1) was followed until 60 d posthatch.	Feminization, altered sex distribution at 51.5 µg/l NP and observation of ovotestis 17.7 µg/l NP. Intersex was found in sekund generation (F_1) from the parents treated with 8.2 µg/l NP.	Embryo survival were lower at 183 µg/l NP. Larval mortality was increased at 17.7 µg/l NP.	(Yokota et al., 2001)
Medaka. 2, 20 or 50 µg/l NP for 7 days.	Induction of VTG at 20 µg/l NP.		(Islinger et al., 2002)
Medaka. 0.1, 10 or 100 µg/l NP for 5 weeks.	Induction of VTG at 0.1 µg/l NP. IC50 values for inhibition to egg hatching were 850 µg/l NP.	72 hr. LC50 = 850 µg/l NP (males); 870 µg/l NP (females); 130 µg/l (recently hatched larvae)	(Kashiwada et al., 2002)
Medaka. 2-50 µg/l OP. Full life-cycle exposure. Exposed for 2-4 hr. post fertilization until maturity (12-13 weeks of exposure)	Ovotestis induced by 50 µg/l OP Shift in sex ratio towards females > 2 µg/l OP.	Cross-mating experiment, mating exposed males with control females resulting in up to 11 % lower fertilization rate. Increased mortality in the progeny derived from exposed females (>20 µg/l OP) and unexposed males. Growth inhibition at > 50 µg/l OP. Increase in mortality both before and after hatch at > 2 µg/l OP.	(Knorr and Braunbeck, 2002)
Medaka. 5-500 µg/l NP for 6 days.	ZRP mRNA was induced at 50 µg/l NP.		(Lee et al., 2002)
Medaka. 100 µg/l NP for 6 weeks.	Increased cellular apoptosis in spermatocytes, Sertoli cells and Leydig cells, but not in spermatids		(Weber et al., 2002)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Male medaka. 0.02 - 40 mg/g diet of 4-*tert*-OP, NP and 4-*n*-NP. The fish were fed for 7 days (≈0.06 g pr 10 fish pr day).	EC_{50} for VTG induction 2600 µg/kg OP, 940µg/kg NP. 4-*n*-NP did induce VTG to a lower extent than the branched isomers.	There was high mortality in the group fed 40 mg/kg diet.	(Chikae et al., 2003)
Medaka. 24.8-184 µg/l NP. Eight breeding pairs were exposed for 3 weeks.	Induction of VTG in both sexes at 50.9 µg/l NP. Ovotestis induced by all concentrations. GSI reduced at 184 µg/L.	Egg production was decreased in the 101 µg/l group and fertility was decreased at 184 µg/L.	(Kang et al., 2003)
Medaka. 20 and 100 µg/l NP for 2 weeks.	Reduced number of motile spermatozoa after 20 µg/l NP.		(Kawana et al., 2003)
Medaka. 75 µg/l NP for 1, 2, 4 and 10 days.		Rapid induction of ER mRNA in the liver after 1 day and maximum expression after 10 days. Increase in aromatase (CYP19) mRNA expression from day 2 and maximum at day 10. p53 mRNA was increased after 1 days but decrease after day 2.	(Min et al., 2003)
Medaka. 51–931 µg/l 4-*tert*-PP. Full life-cycle test. Exposed from fertilized eggs to 101 d posthatch (F_0). Studied effects on second generation (F_1).	Induction of VTG in males at 51 µg/l PP. Feminization (altered sex distribution and observation of ovotestis) at 224 µg/l PP.	Lethal and sublethal toxicity for F_0 at 931 µg/l PP. In F_1 the lethal and sublethal toxicity were 224 µg/l PP.	(Seki et al., 2003b)
Medaka. 3.3-44.7 µg/l NP and 6.9-94 µg/l 4-*tert*-OP. Exposed fertilized eggs to 60 d post hatch.	Induction of VTG at 11.6 µg/l NP and 11.4 µg/l OP. Feminization (altered sex distribution and observation of Ovotestis) at 11.6 µg/l NP and 48.1 µg/l OP.	Growth inhibition at > 23.5 NP.	(Seki et al., 2003a)
Medaka.100 µg/l NP for 5 weeks.	Induction of VTG. The VTG concentration decreased only slowly after exposure and was not return to initial normal levels even after 5 weeks.		(Tabata et al., 2003)
Mature medaka. 24.8, 50.9, 101 and 181 µg/l for 21 days.	Egg production was decreased at ≥101 µg/l NP and fertility was decreased at 181 µg/l NP. Induction of testis-ova in male was found in all NP concentrations, whereas abnormal spermatogenesis and lower GSI was only seen in the males from the 181 µg/l NP group. VTG was induced in the liver of both sexes ≥50.9 µg/l NP		(Kang et al., 2003)
Medaka. 500 µg/l NP for 1-12 days.	Induction of VTG in the liver and in the testis (immunohistochemically).		(Kobayashi et al., 2005)
Transgenic medaka. 0.01 - 403 µg/l NP for 16 days.	No induction of the estrogen-responsive choriogenin H gene (fused to the green fluorescent protein gene). No induction of VTG mRNA at any concentration.	Increased mortality (62 %) at 80 µg/l NP and 100 % at 403 µg/l NP.	(Scholz et al., 2005)
Male medaka. 50 and 500 µg/l NP for 8 hr.	Induction of VTG II mRNA and ERα mRNA at 500 µg/l NP.		(Yamaguchi et al., 2005)
Medaka. 62-783 µg/l 4-*tert*-PP Exposed fertilized eggs for 60 d post hatch.	Feminization (altered sex distribution) at ≥ 238 µg/l PP.	Expression of Cytochrome P450 11β-hydroxylase ($P450_{11\beta}$) mRNA was completely inhibited by ≥ 413 µg/l PP.	(Yokota et al., 2005)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Medaka. 1-100 µg/l NP from day 1 after hacthing to 100 days.	80 % of the exposed males had ovotestis at 29 µg/l NP (measured conc.) and 5 % at 9 µg/l NP. Mixed secondary sex characteristics in 40 % at 29 µg/l NP and 20 % at 9 µg/l NP.		(Balch and Metcalfe, 2006)
Medaka. 10, 50 and 100 µg/l NP for 21 days in the spawning period.	Induction of VTG in males at 10 µg/l NP. Reduction of eggs at 100 µg/l NP. Reduced fertilisation of eggs at 100 µg/l NP. Reduced hatchability and delayed hatching of eggs from the 100 µg/l NP group.	Increased HSI in both gender at 100 µg/l NP. Increased mortality (40 %) of males at 100 µg/l NP. Matenal transfere of NP into the eggs (2-7 µg/g egg) for the 100 µg/l NP group. (BCF = 30-100).	(Ishibashi et al., 2006)
Medaka. 20 and 100 µg/l NP for 96 hr.	Induction of VTG mRNA and Zrp mRNA after 96 hr. (not after 24 hr.)	75 different genes were found altered (using 3.4 K gene microarray) after 96 h. Dose related respons.	(Kim et al., 2006a)
Order: Cyprinodontiformes			
Family: Poecilidae			
Guppy (*Poecilia reticulata*). 150 µg/l 4-*tert*-OP for 4 weeks.		Males displayed reduced aggressiveness to competitors.	(Bayley et al., 1999)
Mosquitofish (*Gambusia holbrooki*). 0.5–50 µg/l NP for 75 days.	100% female secondary sex characteristics at 50 µg/l NP. At 0.5 and 5 µg/l NP, occurrence of individuals with partially developed gonopodia. Only female or undeveloped gonads at 50µg/l NP, none with testis.	Effect on liver at 50 µg/l NP (reduced lipids, perivascular necrosis, and hepatocytes with pyknotic or hypertrophic cores.	(Dreze et al., 2000)
Platyfish (*Xiphophorus maculatus*). 80 – 1280 µg/l NP for 28 days.	Induction of VTG above 80 µg/l NP. Reduced number of cysts in testis; hypertrophy of Sertoli cells (dose-related response found from 80 µg/l NP).	Free sperm in enlarged ductus spermaticus (dose-related)	(Kinnberg et al., 2000a; Kinnberg et al., 2000b)
Swordtail (*Xiphophorus helleri*). 4 – 100 µg/l NP for 60 days.	VTG-mRNA expressions at lowest dose 4 µg/l NP. Increased apoptosis and necrosis. Lesions in testis at 100 µg/l NP. Minor effects also found at 4 µg/l NP.		(Kwak et al., 2001)
Male guppy. 100 – 900 µg/l 4-*tert*-OP for 30 days.	Reduced GSI at 100 µg/l OP Increased quantity of spermatozoa in ejaculate (from 100 µg/l OP). Variable results among groups.	Reduced size and intensity of sexually attractive orange marks (from 100 µg/l OP).	(Toft and Baatrup, 2001)
Platyfish, neonates, juvenile and mature. 14 µg/l NP for 8 months.	Neonates and juvenile showed a significant delay in gonadal development, and did never develop functioning gonads.	High mortality (35 %) in exposed groups (2% in control). Down regulation of GTH in the pituitary.	(Magliulo et al., 2002)
Guppy. 100, 300 and 900 µg/l 4-*tert*-OP for 60 days.	Reduced number of spermatogenetic cysts at 900 µg/l OP.	60 % mortality at 900 µg/l OP (15 % in the control group).	(Kinnberg and Toft, 2003)
Guppy. 26 µg/l 4-*tert*-OP 26-36 days.	Indications of blocked spermatogonial mitosis for the adult males. There were no effects on the weight, length, gonopodiurn index or sex distribution of the offspring.		(Kinnberg et al., 2003)
Guppy. 0-1600 µg/l 4-*tert*-OP for 96 h and 1.7 – 149 µg/l 4-tert-OP for 90 days.	Reduced GSI of female at 100 µg/l Increased quantity of spermatozoa in ejaculate (from 100 µg/l OP). Variable results among groups.	Reduced size and intensity of sexually attractive orange marks (from 100 µg/l OP). 96 h LC_{50} for OP were 495 µg/l.	(Toft and Baatrup, 2003)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
New born guppies. 100 µg/l NP for 90 days.	Induction of VTG mRNA in liver of both males and females. Gender balance towards female (sex ratio = 0.3 males per female).		(Cardinali et al., 2004)
Male guppies, 10, 60, and 150 µg/L NP for 7-21 days.	Induction of VTG at 60 µg/l NP.		(Li and Wang, 2005)
Family: Fundulidae			
Killifish (*Fundulus heteroclitus*). Embryos and larvae. 107-2140 µg/l NP and 100-2000 µg/l 4-*tert*-OP.		Larvae (96-h LC50 = 204 µg/L NP) are more sensitive than embryos (96-h LC50 = 5 mg/l NP). Sub lethal abnormalities at 40 µg/l NP. NP have higher toxicity than OP.	(Kelly and DiGiulio, 2000)
Male killifish. Injection of 10-50 mg/kg NP and 10-50 mg/kg 4-tert-OP. Sampling after 32 days.	Induction of VTG in males at 10 mg/kg NP and 100 mg/kg OP. Decrease in GSI at 50 mg/kg NP.		(Pait and Nelson, 2003)
Male mummichog (*Fundulus heteroclitus*). 65 µg/l NP for 4 days.	Induction of VTG.		(Garcia-Reyero et al., 2004)
Family: Rivulidae			
Rivulus marmoratus (Hermaphroditic fish). 150 or 300 µg/l NP for 60 days.	No fish exposed to 300 µg/l and only 2 of 9 fish exposed to 150 µg/l developed testicular tissue.		(Tanaka and Grizzle, 2002)
Rivulus marmoratus. 300 µg/l NP for 96 hr.		Induction of glutathione S-transferase gene (GSTa mRNA).	(Lee et al., 2005a)
Rivulus marmoratus. 300 µg/l NP for 96 hr.		Induction of P450 1A (CYP1A) gene.	(Lee et al., 2005b)
Rivulus marmoratus. 300 µg/l NP or 300 µg/l 4-tert-OP for 96 hr.		Brain aromatase gene (cyp19b) were up-regulated and ovarian aromatase (cyp19a) were down-regulated by NP. OP up-regulated both brain and ovarian aromatase.	(Lee et al., 2006b)
Rivulus marmoratus. 300 µg/l NP for 96 hr.		Up-regulation of c-KI-*ras* mRNA in liver and Ha-*ras* in the brain. (The *ras* genes are associated with carcinogen exposure in fish).	(Lee et al., 2006a)
Rivulus marmoratus. 300 µg/l NP for 96 hr.		Down-regulation of androgen receptor (rm-AR) and estrogen receptor (ER-α and ER-β) in the gonads and liver.	(Seo et al., 2006)
Order: Gasterosteiformes			
Family: Gasterosteidae			
Three-spined stickleback (Gasterosteus aculeatus). Acute toxicity test of NP		96-hr LC50 of 370 µg/L	(Granmo et al., 1991).
Order: Scorpaeniformes			

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Family: Agonidae			
Pogge (*Agonus cataphractus*). Acute toxicity test of NP for 96 hr and 7 days.		LC50 was 510 and 360 µg/l for 96 hr. and 7 days exposure, respectively.	(Waldock and Thain, 1986)
Family: Sebastidae			
Rockfish (*sebastes schlegeli*). Injection of 10 and 25 mg/kg NP. Sampling after 12 and 48 hr.	Induction of VTG mRNA in the liver of both females and males at 10 mg/kg NP.		(Jung et al., 2003)
Rockfish. Injection of 10 and 25 mg/kg NP. Sampling after 2-14 days.	Induction of VTG mRNA in the liver of both females and males at 10 mg/kg NP. Maximum VTG mRNA levels 48 hr. in females and 72 hr. in males, after injection. Induction of plasma VTG at 10 mg/kg, maximum after 72 hr. in females and 168 hr. in males.		(Jung et al., 2006)
Order: Perciformes			
Family: Centrarchidae			
Bluegill sunfish (*Lepomis macrochirus*). NP was applied to enclosures every 48 h over a 20-d period (11 applications) at rates of 3, 30, 100, and 300 µg/L.		Significant mortality at 300 µg/l NP. Tissue concentrations of NP from enclosures treated with 3 and 30 µg/L NP ranged from 0.01 to 2.94 µg/g wet weight. BCF = 87 ± 124.	(Liber et al., 1999)
Largemouth bass (*Micropterus salmoides).* 50 mg/kg NP injected. Sampled after 48 hr.	Gene array showed 9 genes increased by a factor 2: four VTG genes, Choriogenin 2 and 3, aspartic protease, signal peptidase, and an unidentified gene. 2 genes were down-regulated: transferrin and one unknown.		(Larkin et al., 2002)
Largemouth bass. Injection of 5 mg/kg and 50 mg/kg NP. Sampled after 2, 7 or 14 days.	Induction of VTG at 5 mg/kg NP.	NP decreased glutathione S-transferases (GST) mRNA levels in the liver, but not GST catalytic activity. No effect on glutathione (GSH) levels. 5 mg/kg NP increased hepatic quinone reductase (QR)	(Hughes and Gallagher, 2004)
Family: Cichlidae			
Tilapia (*Oreochromis niloticus*). 2.2-2200 µg/l NP for 3-5 weeks.		FSHβ mRNA is suppressed and LH release from the pituitary is increased at all concentrations, dose response.	(Zilberstein et al., 2000)
Family: Gobiidae			
Japanese common goby (*Acanthogobius flavimanus*). 5, 25 and 50 µg/L NP for 3 weeks.	Induction of VTG in males 13 µg/L NP (measured conc.).		(Ohkubo et al., 2003)
Black goby (*Gobius niger*). Injected with 50 or 500 mg/kg NP. Sampled after 72 hr.	Induction of VTG at both doses. No effects were seen on testis histologi.	Both doses of NP caused reduction of CYP1A mRNA expression and EROD activity in the liver. Increased expression of AhR in liver.	(Maradonna et al., 2004)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Japanese common goby. 0.2, 1, 5, 25 µg/l NP for 3 weeks	Induction of VTG at 25 µg/l NP.	5 and 25 µg/l NP down regulated the expression of ubiquitin C-terminal hydrolase mRNA in testis, but not in the brain.	(Mochida et al., 2004).
Sand goby (*Pomatoschistus minutus*). 3 –100 µg/l 4-*tert*-OP for 101 days.	Induction of VTG at 31 µg/l OP after 28 days exposure. No effect on GSI, but inhibition of the sperm duct glands at 28 µg/l OP.	195 µg/l OP were acutely toxic, 119 µg/l OP gave 100 % mortality over 40 days. LC_{50} was 29 µg/l OP after 8 weeks exposure.	(Robinson et al., 2004)
Family: Moronidae			
Juvenile sea bass (*Dicentrarchus labrax*). 891 µg/l NP for 2-24 hr.	No effect on cortisol.	Induction of erythrocytic nuclear abnormalities frequency. No effect on EROD or GST.	(Teles et al., 2004)
Male sea bass. Injection with 5 or 50 mg/kg NP. Sampling after 3-28 days.	Induction of VTG only for 50 mg/kg NP. Maximum VTG levels 14 days after injection.	Inhibition of CYP1A at 50 mg/kg NP. No effects on 6β-T hydroxylase (CYP3A) or GST.	(Vaccaro et al., 2005)
Family: Mugilidae			
Juvenile and adults grey mullet (*Liza aurata*). Juveiles were exposed for 25, 100 or 1000 µg/l NP for 7 days. Adults were injected with 0.25 or 250 mg/kg. Sampling after 48 hr or 72 hr.	No induction of VTG was seen after exposure of juveniles. In adults were VTG induced at highst dose.NP	NP decreased CYP1A1 mRNA expression and EROD activity in both juveniles and adults at all concentrations	(Cionna et al., 2006)
Family: Sparidae			
Juvenile gilthead seabream (*Sparus Auratus*). Injected with 100 and 200 mg/kg NP. Sampling after 10 days.	Increased plasma levels of E2 at 200 mg/kg NP.	Plasma glucose and protein levels were not affected. Increased levels of triacylglycerol in plasma at both doses.Reduction in kedney Na^+,K^+-ATPase activity and increases in plasma osmolality at 200 mg/kg NP. Decrease in EROD and increase in GST activity at 200 mg/kg NP.	(Carrera et al., 2007)
Family: Zoarcidae			
Male eelpout (*Zoarces viviparus*). Injected with 10 mg/kg/week and 100 mg/kg /week of NP. Sampled after 25 days.	Dose-dependent induction of VTG. Decrease in GSI. Effects on testicular structure and cytology and germ cells and sertoli cells.		(Christiansen et al., 1998)
Male eelpout. 10-1000 µg/l NP for 3 weeks.	Induction of VTG at 100 µg/l NP.		(Korsgaard and Pedersen, 1998)
Eelpout. 10 mg/kg 4-*tert*-OP injected 4 times.	Induction of VTG.	Upregulation of E2 binding capacity in the liver => induction of receptor levels.	(Andreassen and Korsgaard, 2000)
Eelpout. 25 and 100 µg/l 4-*tert*-OP for 3 weeks.	Induction of VTG at 14 µg/l OP (measured conc.).		(Rasmussen et al., 2002)
Eelpout. 100 mg/kg 4-*tert*-OP three times over 10 days.	Massiv induction of VTG. Reduction in GSI and milt volume. Increased spermatocrit.		(Rasmussen and Korsgaard, 2004)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Eelpout . 0.5 and 25 µg/l 4-*tert*-OP for up to 168 hr.	Induction of VTG at 25 µg/l OP after 168 hr.	Induction of gene expression of ERα mRNA in the liver after 48 hr.	(Andreassen et al., 2005)
Male Eelpout. 10, 50 and 100 µg/l 4-*tert*-OP for 3 weeks.	Induction of VTG at 35 µg/l OP (measured conc.).	100 µg/l OP gave effect on the offspring (viviparous fish).	(Rasmussen et al., 2005)
Order: Pleuronectiformes			
Family: Pleuronectidae			
Flounder (*Platichthys flesus*). 10, 30 and 100 µg/l NP for 3 weeks.	No induction of VTG.	Higher HSI at 30 µg/l NP. Lerthal concentration at 100 µg/l NP.	(Matthiessen et al., 1998)
Flounder. 10-200 mg/kg NP injected once a weeks for two weeks. Sampled after 2 weeks after last injection.	Induction of VTG at 10 mg/kg NP after one week. No effect on GSI	High mortality at 200 mg/kg NP (71 %).	(Christensen et al., 1999)
Larvae of winter flounder (*Pleuronectes americanus*). Acute toxicity test.		96 hr. LC50 = 17 µg/l	(Lussier et al., 2000)
Male summer flounder (*Paralichthys dentatus)*. Injected twice with 2, 20 and 200 mg/kg 4-*tert*-OP. Sampled after 4, 6 and 8 weeks.	Increased plasma E2 and decreased T at all concentrations. Reduced GSI at 200 mg/kg OP.		(Mills et al., 2001)
Male summer flounder. 100 mgkg/week 4-*tert*-OP injected 2 times, sampled after 4-6 weeks.	Retadation of testicular development: Reduced GSI, thickened tubule walls and no developing sperm cysts at 100 mg/kg.	No histopathologically effect in liver or kidney	(Zaroogian et al., 2001)
Flounder. 10, 50 and 100 mg/kg OP administered orally every second day during a period of 11 days	Induction of VTG at 10 mg/kg OP	OP accumulated in liver and muscle, the tissue concentration were positive correlated with VTG induction	(Madsen et al., 2002)
Flounder. Exp.1: 10, 50 and 100 mg/kg 4-*tert*-OP and Exp.2: 1, 2,5, 5, 7,5, 10, 25 mg/kg 4-*tert*-OP administered orally every 2 days for 10 days (5 exposure). Samples taken after day 6 and day 11.	Induction of VTG at 5 mg/kg OP. No effect on GSI	OP was found in both liver, muscle and testis tissue. Only 8% of the OP administered to the group receiving 50 mg/kg OP was retained in the liver and the muscles.	(Madsen et al., 2003)
Male winter flounder. Injected with 100 mg/kg/day NP for 2 days. Sampled after 48 hr.		NP induced testosterone metabolism and induction of CYP3A protein.	(Baldwin et al., 2005)
Eggs and larvae of marbled sole (*Pleuronectes yokohamae*). Acute toxicity test, 0-2430 µg/l NP.		Increased mortality at 68 µg/l NP of eggs. Increased mortality at 13 µg/l NP for larvae (96 hr.). No survival of neither eggs nor larvae over 200 µg/l NP.	(Kume et al., 2006)

Species, dose and exposure time	Vitellogenin (VTG), zona radiata protein (Zrp), steroid, sex differentiation and gametogenesis	Other effects	Reference
Flounder. Oral administration of 50 mg/kg 4-*tert*-OP. Sampled after 2-216 hr.	Induction of VTG after 48 hr. The VTG levels increase until the end of the experiment 9 days after the administration of OP.	OP was found in liver, testis, muscle and plasma 3-18 hr. after administration. The maximum concentrations of OP in liver, muscle and testis were 67, 3.2 and 6.8 µg/g, respectively.	(Madsen et al., 2006)
Family: Scophthalmidae			
Juvenile turbot (*Scophthalmus maximus*). 29 µg/l NP for 3 weeks.	Reduction of plasma and testis levels of androgens (T, 11-KT and androstenedione) and estrogens (E1 and E2). Also biliary levels of steroids were highly depressed in male turbot. No effects of NP were found on the steroid profile of the females.		(Labadie and Budzinski, 2006)
Juvenile turbot (*Scophthalmus maximus*). 29 µg/l NP for 3 weeks.	Induction of VTG and Zrp	Surfaced enhanced laser desoption ionisation-time of flight (SELDI-TOF) found 121 peaks (peptides) that were significantly altered (up or down) in the plasma of the exposed turbot compared with control.	(Larsen et al., 2006)
Juvenile turbot (*Scophthalmus maximus*). 29 µg/l NP for 3 weeks.	Lower levels of T and E2 in plasma	Reduced P450 aromatase activity in ovaries. Reduced glucuronidation of T and E2. No effect on E2-sulfotransferase activity	(Martin-Skilton et al., 2006c)

Appendix 2. Analytical methods for determination of alkylphenols.

Table 9. *Analytical methods for determination of alkylphenol in biota.*

Compounds	Matrix	Extraction	Clean-up	Analysis	LOQ	Reference
NP	mussels	Liq/Liq (DCM)	Partitioning (NaOH:AN)	GC-MS (EI and NCI), pentafluorobenzoyl derivatives	1 µg/kg	(Wahlberg et al., 1990)
NP	Algae, fish and duck	Steam distillation	non	HPLC (fluorimetric)	30 µg/kg dry weight	(Ahel et al., 1993)
4-tert-OP	Rats	Liq/Liq(MTBE)	non	GC-MS (EI)	5-10 µg/kg	(Certa et al., 1996)
NP	Fish	Soxhlet (DCM)	Silica and alumina column	GC-MS (EI)	not given	(Blackburn et al., 1999)
NP	Fish	Liq/Liq (Hex)	Florisil column	GC-MS (EI)	10 µg/kg	(Liber et al., 1999)
NP and OP	Fish	Steam distillation	Alumina column	GC-FID and GC-MS (EI)	not given	(Lye et al., 1999)
4-tert-OP	Fish	MASE	SPE (aminopropyl)	LC-MS (APCI)	10 µg/kg – 50 µg/kg	(Pedersen and Lindholst, 1999)
NP and OP	Fish	Liq/Liq (AN)	Partitioning (Hex:AN)	GC-MS (EI)	2 µg/kg (OP), 20 µg/kg (NP)	(Tsuda et al., 1999)
NP	Fish and mussels	MSPD	Direct in the extraction	HPLC (fluorimetric)	10-30 µg/kg	(Zhao et al., 1999)
NP and OP	Mussels	Soxhlet (Hex)	GPC	GC-MS (EI)	1 µg/kg (OP), 13 µg/kg (NP)	(Bennett and Metcalfe, 2000)
NP and OP	Mussels	MSPD	Alumina column	GC-FID and GC-MS (EI)		(De Voogt et al., 2000)
NP and OP	Fish	Liq/Liq (AN)	Partitioning (Hex:AN), Florisil PR	HPLC (fluorimetric)	1 µg/kg (OP), 2 µg/kg (NP)	(Tsuda et al., 2000a)
NP and OP	Mussels	Liq/Liq (DCM)	Silica column	GC-MS (EI)	3 µg/kg	(Cathum and Sabik, 2001)
NP	Food	Steam distillation	HPLC (silica column)	GC-MS (EI), silylated derivatives	27 µg/kg	(Gunther et al., 2001)
NP	Fish	Steam distillation	HPLC (silica column)	GC-MS (EI)	3,3 µg/kg	(Keith et al., 2001)
NP	Fish	Steam distillation	HPLC (silica column)	GC-MS (EI)	4,8 µg/kg	(Snyder et al., 2001a)
NP	Fish	Soxhlet (DCM)	Silica column	HPLC (fluorimetric)	67 µg/kg	(Snyder et al., 2001b)
NP	Fish	Soxhlet (Hex)	SPE (aminopropyl)	HPLC (UV)	10 µg/kg	(Corsi and Focardi, 2002)
NP	Fish	ASE (DCM)	GPC and SPE (aminopropyl)	HPLC (fluorimetric)	5 µg/kg	(Datta et al., 2002)
NP	Rats	Liq/Liq(AN)	SPE (silica column)	LC-MS (APCI) pentafluorobenzyl derivatives	220 µg/kg	(Doerge et al., 2002)
NP and OP	Fish	ASE	Florisil column	LC-MS (ESI)	5 µg/kg (OP), 20 µg/kg (NP)	(Tavazzi et al., 2002)

Table 7. continue on the next page

Table 7 continue

Compounds	Matrix	Extraction	Clean-up	Analysis	LOQ	Reference
NP	Fish	Steam distillation	HPLC (silica column)	GC-MS (EI)	3,3 µg/kg	(Kannan et al., 2003)
NP	Mussels	MASE (Ac:Hex)	Silica column	GC-MS (EI), pentafluorobenzyl derivatives	3 µg/kg	(Sabik et al., 2003)
NP and OP	Fish	ASE (cyclohex/EA 95:5)	GPC and SPE (Aminopropyl)	GC-MS-MS (EI) silylated derivatives	0.2 µg/kg (OP), 2 µg/kg (NP)	(Wenzel et al., 2004)
NP and OP	Oyster	Steam distillation	None	GC-MS (EI)	20 µg/kg	(Cheng et al., 2005)
NP and OP	Fish	Liq./Liq. (AC-Hex 3.5:1)	Alkali extraction (AN/NaOH)	GC-MS (EI)	0.5 µg/kg (OP), 8 µg/kg (NP)	(Ferrara et al., 2005)
HP, OP, NP and DP	Fish	Steam distillation	SPE (aminopropyl)	LC-MS/MS (ESI)	3-4 µg/kg	(Keen et al., 2005)
NP and OP	Fish	Liq./Liq. (AN)	Florisil column	HPLC (fluorimetric)	5 µg/kg (OP), 9 µg/kg (NP)	(Mao et al., 2006)
32 APs from phenol to NP	Fish	Liq./Liq. (DCM)	GPC	GC-MS (NCI) pentafluorobenzoyl derivatives	1-23 µg/kg	**Paper II**

Techniques: ASE = Accelerated solvent extraction; GPC = Gel permeation chromatography; Liq: Liq = Liquid: liquid extraction; MASE = Microwave-assisted solvent extraction, MSPD = Matrix solid-phase dispersion; SPE = Solid phase extraction; **Solvent**: AC = Acetone; AN = Acetonitrile; DCM = Dichloremethane; EA = Ethylacetate; Hex = Hexane; MTBE = Methyl-tert-butylether;

Tabel 10. *Analytical methods for determination of alkylphenol in produced water.*

Compounds	Extraction	Clean-up	Analysis	LOQ	Reference
C_0-C_2 AP	Liq./Liq.(DCM)	None	GC-MS (EI)	Not given	(Grahl-Nielsen, 1987)
Clusters of C_0-C_7	SPE (C_{18})	None	GC-MS (EI)	Not given	(Brendehaug et al., 1992)
C_0-C_3 AP	SPE (C_{18})	None	GC-MS (EI)	Not given	(Bennett et al., 1996)
Clusters of C_0-C_7	SPE (C_{18})	None	GC-MS (EI)	Not given	(Røe and Johnsen, 1996)
32 APs (C_0-C_9)	Liq./Liq.(DCM)	None	GC-MS (EI)	1-1010 ng/l	(Durell et al., 2002)
31 APs (C_0-C_9)	SPE (MAX)	None	GC-MS (NCI) pentafluorobenzoyl derivatives	3-1051 ng/l	**Paper I**
4-APs (C_0-C_9)	Liq./Liq.(DCM)	None	GC-MS (EI)	11-980 ng/l	(Thomas et al., 2004a)

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Rationale for exposure regimens

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

2.2. Cod eggs

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

2.3. Description of the PW exposure experiments

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

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

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

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

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

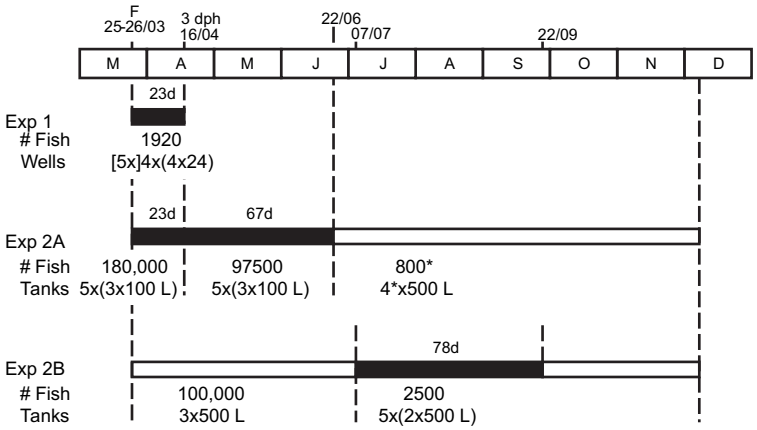

Fig. 1. Schematic representation of Experiments 1, 2A and 2B. Experiment 1 was conducted in 24-well trays and ended on 16/04 (3 dph). Fish in Experiment 2A were maintained in 100 L tanks during the PW exposure phase. At the end of the exposure, 200 randomly selected fish from the M-PW, L-PW, U and E_2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

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

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

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

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

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

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

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

2.5. Collection and treatment of PW

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

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

2.6. Feeding

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

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

2.7. Temperature and light regimen

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

2.8. Chemical analysis of water and fish

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

2.9. Sampling

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

2.10. Sampling for biomarker analysis

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

2.11. ELISA of CYP1A and Vtg

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

2.12. Statistical analyses

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

3. Results

3.1. Chemical analysis of the PW

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

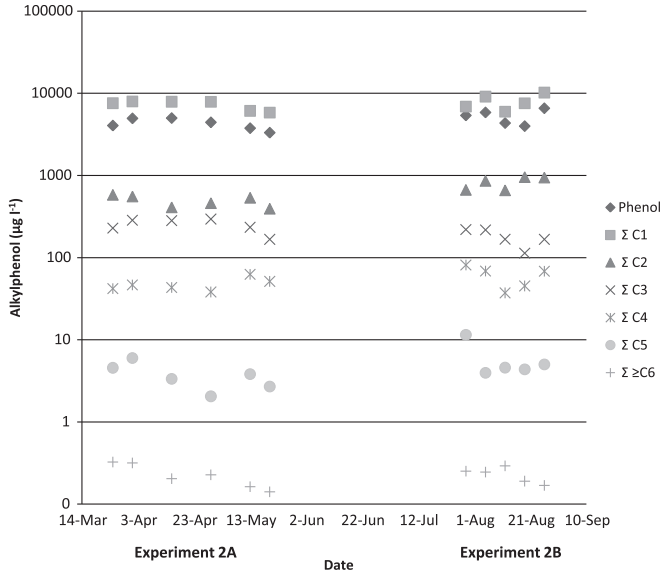

Fig. 2. Stability of the AP concentrations (µg l^{-1}) in the undiluted PW over time. The results are given as the sum of all isomers at each molecular size.

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

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

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

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

3.3. PAH measurements

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

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

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

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

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

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

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

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

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

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

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

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

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

3.6. Effects of PW on larvae and early juveniles

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

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

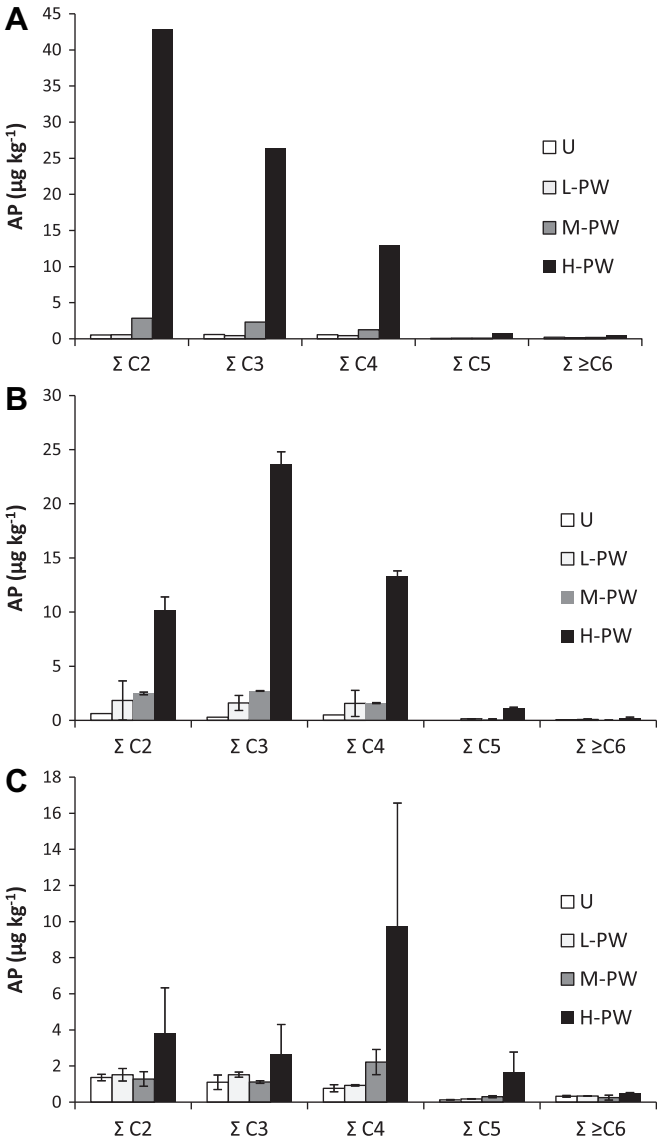

Fig. 3. Tissue concentration of APs (μg kg^{-1} wet weight) in yolk sac larvae (A), early juveniles (B), and juveniles (C). The results are given as the sum of all isomers at each molecular size. For yolk sac larvae measurements were performed on only one sample ($n = 1$) pooled from each of the three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

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

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

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

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

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

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

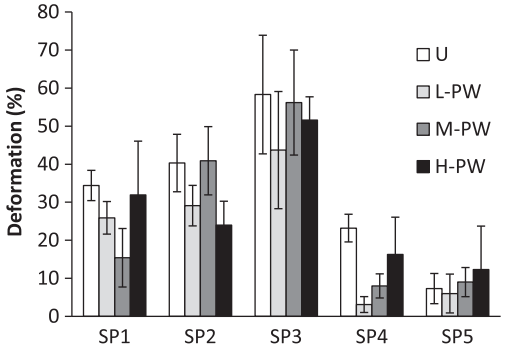

Fig. 4. Percentage of lethal deformities seen throughout the entire embryo stage in Experiment 1. Eggs from each of the 5 spawning pairs (SP1-5) were incubated individually in the wells of 24-well plates (4 parallel plates were analysed for each treatment) filled with the same PW concentrations used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

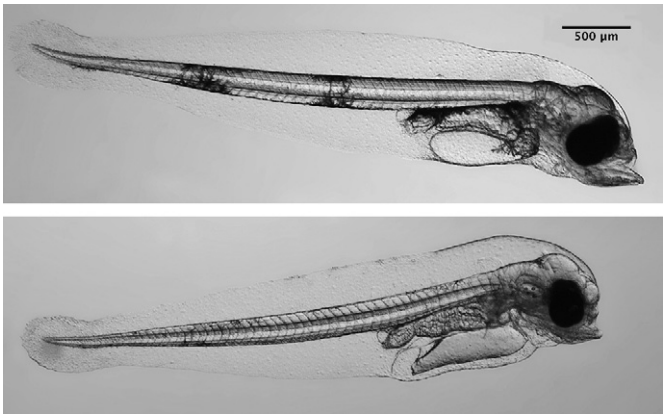

Fig. 5. Representative larvae (4 dph) from the U (A) and H-PW (B) groups following exposure to PW at the embryo stage. The average length of the larvae at 4 dph was 4.5 mm. The lack of pigment and the jaw deformation can clearly be seen on the larvae from the H-PW group. Pictures were retouched in Adobe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

3.8. Expression of Vtg and CYP1A

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

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

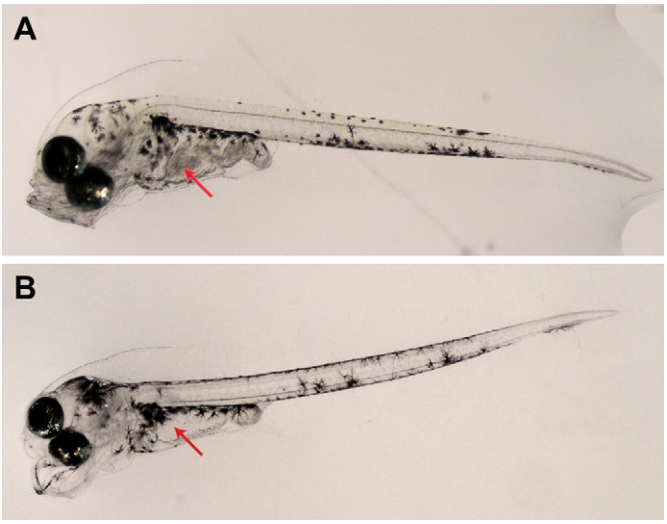

Fig. 6. Representative larvae (14 dph) from the U (A) and H-PW (B) groups. The average length of larvae at 14 dph was 5 mm. An arrow shows the stomach. Zooplankton can clearly be seen in the stomach of the larvae from the U group (A), but not in the stomach of the larvae from the H-PW group (B). The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

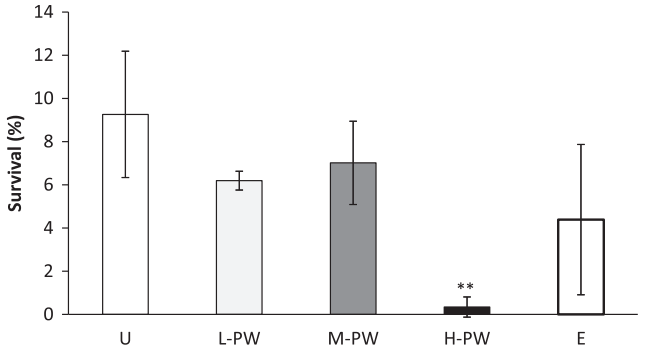

Fig. 7. Survival and growth in Experiment 2A. Survival after 90 days of exposure during the egg and larval stage.

4. Discussion

4.1. PW exposure and rationale behind the experimental design

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

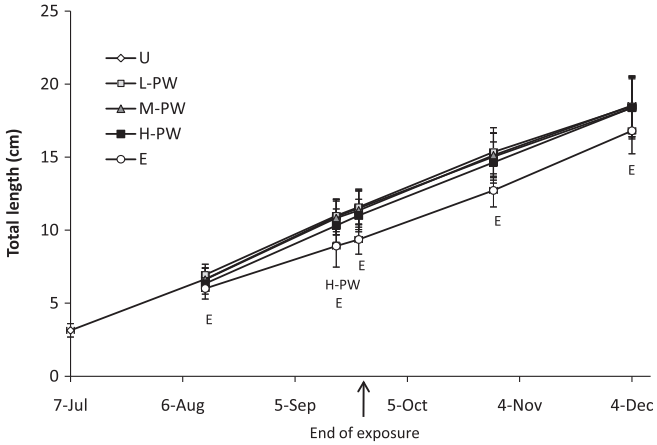

Fig. 8. Growth in Experiment 2B. Growth of juveniles, measured as total length (cm), both during and after exposure (July–December).

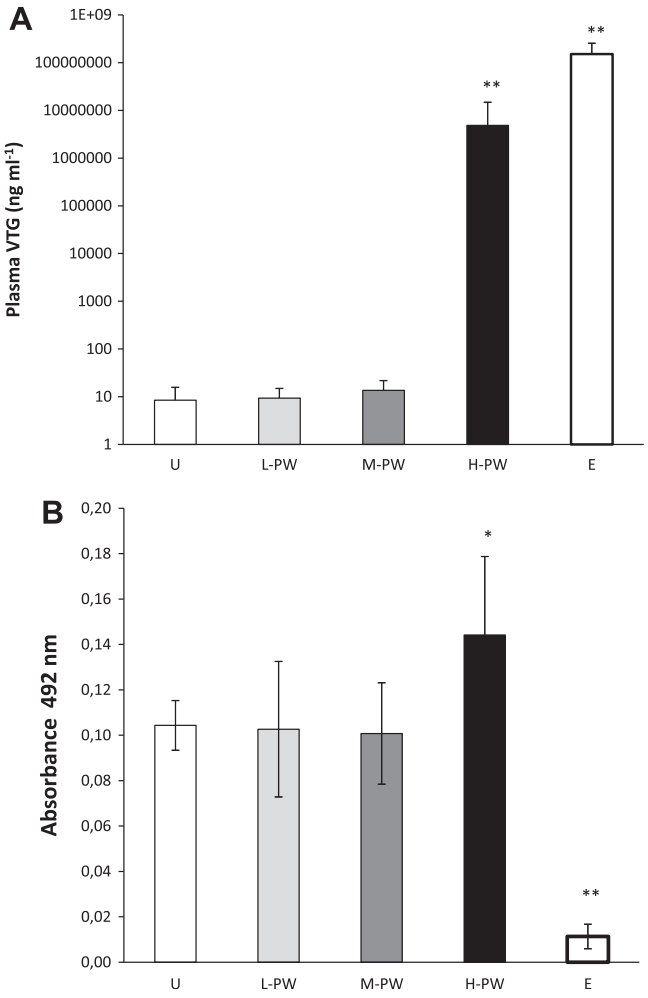

Fig. 9. A. Concentration of Vtg in serum as measured by ELISA. B. Levels of CYP1A in liver as measured by ELISA.

4.2. Uptake of APs into fish tissues

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

4.3. Effects of PW on cod embryos

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

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

4.4. Effects of PW on cod larvae and early juveniles

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

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

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

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

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

4.5. Effects of PW on cod juveniles

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

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

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

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

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

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

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

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

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

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

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

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The Future of Natural Gas

AN INTERDISCIPLINARY MIT STUDY

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MIT Energy Initiative

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While the members of the advisory committee provided invaluable perspective and advice to the study group, individual members may have different views on one or more matters addressed in the report. They are not asked to individually or collectively endorse the report findings and recommendations.

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To view supplementary papers, go to
http://web.mit.edu/mitei/research/studies/natural-gas-2011.shtml

Foreword and Acknowledgements

The Future of Natural Gas is the fourth in a series of MIT multidisciplinary reports examining the role of various energy sources that may be important for meeting future demand under carbon dioxide (CO_2) emissions constraints. In each case, we explore the steps needed to enable competitiveness in a future marketplace conditioned by a CO_2 emissions price or by a set of regulatory initiatives. This report follows an interim report issued in June 2010.

The first three reports dealt with nuclear power (2003), coal (2007) and the nuclear fuel cycle (2010 and 2011). A study of natural gas is more complex than these previous reports because gas is a major fuel for multiple end uses — electricity, industry, heating — and is increasingly discussed as a potential pathway to reduced oil dependence for transportation. In addition, the realization over the last few years that the producible unconventional gas resource in the U.S. is very large has intensified the discussion about natural gas as a "bridge" to a low-carbon future. Recent indications of a similarly large global gas shale resource may also transform the geopolitical landscape for gas. We have carried out the integrated analysis reported here as a contribution to the energy, security and climate debate.

Our primary audience is U.S. government, industry and academic leaders, and decision makers. However, the study is carried out with an international perspective.

This study is better as a result of comments and suggestions from our distinguished external Advisory Committee, each of whom brought important perspective and experience to our discussions. We are grateful for the time they invested in advising us. However, the study is the responsibility of the MIT study group and the advisory committee members do not necessarily endorse all of its findings and recommendations, either individually or collectively.

Finally, we are very appreciative of the support from several sources. First and foremost, we thank the American Clean Skies Foundation. Discussions with the Foundation led to the conclusion that an integrative study on the future of natural gas in a carbon-constrained world could contribute to the energy debate in an important way, and the Foundation stepped forward as the major sponsor. MIT Energy Initiative (MITEI) members Hess Corporation and Agencia Naçional de Hidrocarburos (Colombia), the Gas Technology Institute (GTI), Exelon, and an anonymous donor provided additional support. The Energy Futures Coalition supported dissemination of the study results, and MITEI employed internal funds and fellowship sponsorship to support the study as well. As with the advisory committee, the sponsors are not responsible for and do not necessarily endorse the findings and recommendations. That responsibility lies solely with the MIT study group.

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Abstract

Natural gas is finding its place at the heart of the energy discussion. The recent emergence of substantial new supplies of natural gas in the U.S., primarily as a result of the remarkable speed and scale of shale gas development, has heightened awareness of natural gas as a key component of indigenous energy supply and has lowered prices well below recent expectations. This study seeks to inform discussion about the future of natural gas, particularly in a carbon-constrained economy.

There are abundant supplies of natural gas in the world, and many of these supplies can be developed and produced at relatively low cost. In North America, shale gas development over the past decade has substantially increased assessments of resources producible at modest cost. Consequently, the role of natural gas is likely to continue to expand, and its relative importance is likely to increase even further when greenhouse gas emissions are constrained. In a carbon-constrained world, a level playing field — a carbon dioxide (CO_2) emissions price for all fuels without subsidies or other preferential policy treatment —maximizes the value to society of the large U.S. natural gas resource.

There are also a number of key uncertainties: the extent and nature of greenhouse gas emission mitigation measures that will be adopted; the mix of energy sources as the relative costs of fuels and technologies shift over time; the evolution of international natural gas markets. We explore how these uncertainties lead to different outcomes and also quantify uncertainty for natural gas supply and for the U.S. electricity fuel mix.

The environmental impacts of shale development are challenging but manageable. Research and regulation, both state and Federal, are needed to minimize the environmental consequences.

The U.S. natural gas supply situation has enhanced the substitution possibilities for natural gas in the electricity, industry, buildings, and transportation sectors.

In the U.S. electricity supply sector, the cost benchmark for reducing carbon dioxide emissions lies with substitution of natural gas for coal, especially older, less efficient units. Substitution through increased utilization of existing combined cycle natural gas power plants provides a relatively low-cost, short-term opportunity to reduce U.S. power sector CO_2 emissions by up to 20%, while also reducing emissions of criteria pollutants and mercury.

Furthermore, additional gas-fired capacity will be needed as backup if variable and intermittent renewables, especially wind, are introduced on a large scale. Policy and regulatory steps are needed to facilitate adequate capacity investment for system reliability and efficiency. These increasingly important roles for natural gas in the electricity sector call for a detailed analysis of the interdependencies of the natural gas and power generation infrastructures.

The primary use of natural gas in the U.S. manufacturing sector is as fuel for boilers and process heating, and replacement with new higher efficiency models would cost-effectively reduce natural gas use. Natural gas could also substitute for coal in boilers and process heaters and provide a cost-effective alternative for compliance with Environmental Protection Agency (EPA) Maximum Achievable Control Technology standards.

In the residential and commercial buildings sector, transformation of the current approach to efficiency standards to one based on full fuel cycle analysis will enable better comparison of different energy supply options (especially

natural gas and electricity). Efficiency metrics should be tailored to regional variations in climate and electricity supply mix.

Within the U.S. market, the price of oil (which is set globally) compared to the price of natural gas (which is set regionally) is very important in determining market share when there is the opportunity for substitution. Over the last decade or so, when oil prices have been high, the ratio of the oil price to the natural gas price has been consistently higher than any of the standard rules of thumb. If this trend is robust, use of natural gas in transportation, either through direct use or following conversion to a liquid fuel, could in time increase appreciably.

The evolution of global gas markets is unclear. A global "liquid" natural gas market is beneficial to U.S. and global economic interests and, at the same time, advances security interests through diversity of supply and resilience to disruption. The U.S. should pursue policies that encourage the development of such a market, integrate energy issues fully into the conduct of U.S. foreign policy, and promote sharing of know-how for strategic global expansion of unconventional gas production.

Past research, development, demonstration, and deployment (RDD&D) programs supported with public funding have led to significant advances for natural gas supply and use. Public-private partnerships supporting a broad natural gas research, development, and demonstration (RD&D) portfolio should be pursued.

Chapter 1: Overview and Conclusions

PURPOSE AND OUTLINE OF THE STUDY

Despite its vital importance to the national economy, natural gas has often been overlooked, or at best taken for granted, in the debate about the future of energy in the U.S. Over the past two or three years this has started to change, and natural gas is finding its place at the heart of the energy discussion.

There are a number of reasons for this shift. The recent emergence of substantial new supplies of natural gas in the U.S., primarily as a result of the remarkable speed and scale of shale gas development, has heightened awareness of natural gas as a key component of indigenous energy supply and lowered prices well below recent expectations. Instead of the anticipated growth of natural gas imports, the scale of domestic production has led producers to seek new markets for natural gas, such as an expanded role in transportation. Most importantly for this study, there has been a growing recognition that the low carbon content of natural gas relative to other fossil fuels could allow it to play a significant role in reducing carbon dioxide (CO_2) emissions, acting as a "bridge" to a low-carbon future.

Within this context, the MIT study of *The Future of Natural Gas* seeks to inform the discussion around natural gas by addressing a fundamental question: *what is the role of natural gas in a carbon-constrained economy?*

In exploring this question, we seek to improve general understanding of natural gas, and examine a number of specific issues. How much natural gas is there in the world, how expensive is it to develop, and at what rate can it be produced? We start from a global perspective, and then look in detail at U.S. natural gas resources, paying particular attention to the extent and cost of shale gas resources, and whether these supplies can be developed and produced in an environmentally sound manner.

Having explored supply volumes and costs, we use integrated models to examine the role that natural gas could play in the energy system under different carbon-constraining mechanisms or policies. It is important to recognize that *the study does not set out to make predictions or forecasts of the likelihood or direction of CO_2 policy in the U.S.* Rather, we examine a number of different scenarios and explore their possible impacts on the future of natural gas supply and demand.

Natural gas is important in many sectors of the economy — for electricity generation, as an industrial heat source and chemical feedstock, and for water and space heating in residential and commercial buildings. Natural gas competes directly with other energy inputs in these sectors. But it is in the electric power sector — where natural gas competes with coal, nuclear, hydro, wind, and solar — that inter-fuel competition is most intense. We have, therefore, explored in depth how natural gas performs in the electric power sector under different scenarios. We have also taken a close look at the critical interaction between intermittent forms of renewable energy, such as wind and solar, and gas-fired power as a reliable source of backup capacity.

We look at the drivers of natural gas use in the industrial, commercial, and residential sectors, and examine the important question of whether natural gas, in one form or another, could be a viable and efficient substitute for gasoline or diesel in the transportation sector. We also examine the possible futures of global natural gas markets, and the geopolitical significance of the ever-expanding role of natural gas in the global economy. Finally, we make recommendations for research and development priorities and for the means by which public support should be provided.

HIGH-LEVEL FINDINGS

The findings and recommendations of the study are discussed later in this chapter, and covered in detail in the body of this report. Nevertheless, it is worth summarizing here the highest level conclusions of our study:

1. *There are abundant supplies of natural gas in the world*, and many of these supplies can be developed and produced at relatively low cost. In the U.S., despite their relative maturity, natural gas resources continue to grow, and the development of low-cost and abundant unconventional natural gas resources, particularly shale gas, has a material impact on future availability and price.

2. Unlike other fossil fuels, natural gas plays a major role in most sectors of the modern economy — power generation, industrial, commercial, and residential. It is clean and flexible. *The role of natural gas in the world is likely to continue to expand under almost all circumstances*, as a result of its availability, its utility, and its comparatively low cost.

3. In a carbon-constrained economy, the relative importance of natural gas is likely to increase even further, as it is one of the most cost-effective means by which to maintain energy supplies while reducing CO_2 emissions. This is particularly true in the electric power sector, where, in the U.S., *natural gas sets the cost benchmark against which other clean power sources must compete to remove the marginal ton of CO_2*.

4. In the U.S., a combination of demand reduction and displacement of coal-fired power by gas-fired generation is the lowest-cost way to reduce CO_2 emissions by up to 50%. For more stringent CO_2 emissions reductions, further de-carbonization of the energy sector will be required; but *natural gas provides a cost-effective bridge to such a low-carbon future*.

5. Increased utilization of existing natural gas combined cycle (NGCC) power plants provides a relatively, low-cost short-term opportunity to reduce U.S. CO_2 emissions by up to 20% in the electric power sector, or 8% overall, with minimal additional capital investment in generation and no new technology requirements.

6. Natural gas-fired power *capacity* will play an increasingly important role in providing backup to growing supplies of intermittent renewable energy, in the absence of a breakthrough that provides affordable utility-scale storage. But in most cases, increases in renewable power generation will be at the expense of natural gas-fired power *generation* in the U.S.

7. The current supply outlook for natural gas will contribute to greater competitiveness of U.S. manufacturing, while the use of more efficient technologies could offset increases in demand and provide cost-effective compliance with emerging environmental requirements.

8. Transformation of the current approach to appliance standards to one based on full fuel cycle analysis will enable better comparison of different energy supply options in commercial and residential applications.

9. Natural gas use in the transportation sector is likely to increase, with the primary benefit being reduced oil dependence. Compressed natural gas (CNG) will play a role, particularly for high-mileage fleets, but the advantages of liquid fuel in transportation suggest that *the chemical conversion of gas into some form of liquid fuel may be the best pathway to significant market penetration*.

10. International gas trade continues to grow in scope and scale, but its economic, security and political significance is not yet adequately recognized as an important focus for U.S. energy concerns.

11. Past research, development, demonstration, and deployment (RDD&D) programs supported with public funding have led to significant advances for natural gas supply and use.

BACKGROUND

The Fundamental Characteristics of Natural Gas

Fossil fuels occur in each of the three fundamental states of matter: in solid form as coal; in liquid form as oil; and in gaseous form as natural gas. These differing physical characteristics for each fuel type play a crucial part in shaping each link in their respective supply chains: from initial resource development and production through transportation, conversion to final products, and sale to customers. Their physical form fundamentally shapes the markets for each type of fossil fuel.

Natural gas possesses remarkable qualities. Among the fossil fuels, it has the lowest carbon intensity, emitting less CO_2 per unit of energy generated than other fossil fuels. It burns cleanly and efficiently, with very few non-carbon emissions. Unlike oil, natural gas generally requires limited processing to prepare it for end use. These favorable characteristics have enabled natural gas to penetrate many markets, including domestic and commercial heating, multiple industrial processes, and electrical power.

Natural gas also has favorable characteristics with respect to its development and production. The high compressibility and low viscosity of natural gas allows high recoveries from conventional reservoirs at relatively low cost, and also enables natural gas to be economically recovered from even the most unfavorable subsurface environments, as recent developments in shale formations have demonstrated.

These physical characteristics underpin the current expansion of the unconventional resource base in North America, and the potential for natural gas to displace more carbon-intensive fossil fuels in a carbon-constrained world.

On the other hand, because of its gaseous form and low energy density, natural gas is uniquely disadvantaged in terms of transportation and storage. As a liquid, oil can be readily transported over any distance by a variety of means, and oil transportation costs are generally a small fraction of the overall cost of developing oil fields and delivering oil products to market. This has facilitated the development of a truly global market in oil over the past 40 years or more.

By contrast, the vast majority of natural gas supplies are delivered to market by pipeline, and delivery costs typically represent a relatively large fraction of the total cost in the supply chain. These characteristics have contributed to the evolution of regional markets rather than a truly global market in natural gas. Outside North America, this somewhat inflexible pipeline infrastructure gives strong political and economic power to those countries that control the pipelines. To some degree, the evolution of the spot market in Liquefied Natural Gas (LNG) is beginning to introduce more flexibility into global gas markets and stimulate real global trade. The way this trade may evolve over time is a critical uncertainty that is explored in this report.

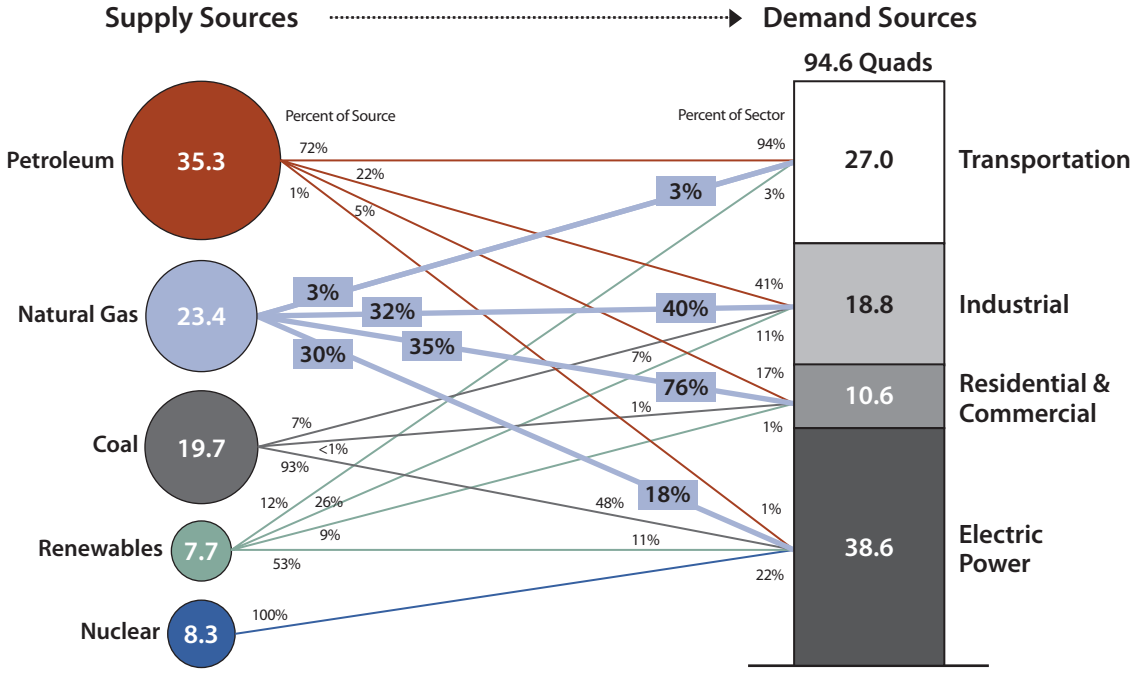
The Importance of Natural Gas in the Energy System

Natural gas represents a very important, and growing, part of the global energy system. Over the past half century, natural gas has gained market share on an almost continuous basis, growing from some 15.6% of global energy consumption in 1965 to around 24% today. In absolute terms, global natural gas consumption over this period has grown from around 23 trillion cubic feet (Tcf) in 1965 to 104 Tcf in 2009, a more than fourfold increase.

Within the U.S. economy, natural gas plays a vital role. Figure 1.1 displays the sources and uses of natural gas in the U.S. in 2009, and it reveals a number of interesting features that are explored in more detail in the body of this report. At 23.4 quadrillion British thermal units (Btu)[1], or approximately 23 Tcf, gas represents a little under a quarter of the total energy supply in the U.S., with almost all of this supply now coming from indigenous resources. Perhaps of more significance, is the very important role that natural gas plays in all sectors of the economy, with the exception of transport. Very approximately, the use of natural gas is divided evenly between three major sectors: Industrial, Residential and Commercial, and Electric Power. The 3% share that goes to transport is almost all associated with natural gas use for powering oil and gas pipeline systems, with only a tiny fraction going into vehicle transport.

In the Residential and Commercial sectors, natural gas provides more than three-quarters of the total primary energy, largely as a result of its efficiency, cleanliness, and convenience for uses such as space and hot water heating. It is also a major primary energy input into the Industrial sector, and thus the price of natural gas has a very significant impact on the competitiveness of some U.S. manufacturing industries. While natural gas provided 18% of the primary fuel for power generation in 2009,

Figure 1.1 Sources and Use of Primary Energy Sources in the U.S. with Natural Gas Highlighted (quadrillion Btu), 2009



Source: EIA, *Annual Energy Outlook, 2009*

it provided 23% of the produced electricity, reflecting the higher efficiency of natural gas plants. As will be seen later in this report, natural gas-fired capacity represents far more than 23% of total power generating capacity, providing a real opportunity for early action in controlling CO_2 emissions.

A Brief History of Natural Gas in the U.S.

The somewhat erratic history of natural gas in the U.S. over the last three decades or so provides eloquent testimony to the difficulties of forecasting energy futures, particularly for natural gas. It also serves as a reminder of the need for caution in the current period of supply exuberance.

The development of the U.S. natural gas market was facilitated by the emergence of an interstate natural gas pipeline system, supplying local distribution systems. This market structure was initially viewed as a natural monopoly and was subjected to cost-of-service regulation by both the Federal government and the states. Natural gas production and use grew considerably under this framework in the 1950s, 1960s, and into the 1970s.

Then came a perception of supply scarcity. After the first oil embargo, energy consumers sought to switch to natural gas. However, the combination of price controls and tightly regulated natural gas markets dampened incentives for domestic gas development, contributing to a perception that U.S. natural gas resources were limited. In 1978, convinced that the U.S. was running out of natural gas, Congress passed the Power Plant and Industrial Fuel Use Act (FUA) that essentially outlawed the building of new gas-fired power plants. Between 1978 and 1987 (the year the FUA was repealed), the U.S. added 172 Gigawatts (GW) of net power generation capacity. Of this, almost 81 GW was new coal capacity, around 26% of today's entire coal fleet. About half of the remainder was nuclear power.

By the mid 1990s, wholesale electricity markets and wellhead natural gas prices had been deregulated; new, highly efficient and relatively inexpensive combined cycle gas turbines had been deployed; and new upstream technologies had enabled the development of offshore natural gas resources. This contributed to the perception that domestic natural gas supplies were sufficient to increase the size of the U.S. natural gas market from around 20 Tcf/year to much higher levels. New gas-fired power capacity was added at a rapid pace.

Between 1989 after the repeal of the FUA and 2009, the U.S. added 306 GW of generation capacity, 88% of which was gas fired and 4% was coal fired.[2] Today, the nameplate capacity of this gas-fired generation is significantly under utilized, and the anticipated large increase in natural gas use has not materialized.

By the turn of the 21st century, a new set of concerns arose about the adequacy of domestic natural gas supplies. Conventional supplies were in decline, unconventional natural gas resources remained expensive and difficult to develop, and overall confidence in gas plummeted. Natural gas prices started to rise, becoming more closely linked to the oil price, which itself was rising. Periods of significant natural gas price volatility were experienced.

This rapid buildup in natural gas price, and perception of long-term shortage, created economic incentives for the accelerated development of an LNG import infrastructure. Since 2000, North America's rated LNG capacity has expanded from approximately 2.3 billion cubic feet (Bcf)/day to 22.7 Bcf/day, around 35% of the nation's average daily requirement.

This expansion of LNG capacity coincided with an overall rise in the natural gas price and the market diffusion of technologies to develop affordable unconventional gas. The game-changing potential of these technologies, combined with the large unconventional

resource base, has become more obvious over the last few years, radically altering the U.S. supply picture. We have once again returned to a period where supply is seen to be abundant. New LNG import capacity goes largely unused at present, although it provides a valuable supply option for the future.

These cycles of perceived "feast and famine" demonstrate the genuine difficulty of forecasting the future and providing appropriate policy support for natural gas production and use. They underpin the efforts of this study to account for this uncertainty in an analytical manner.

Major Uncertainties

Looking forward, we anticipate policy and geopolitics, along with resource economics and technology developments, will continue to play a major role in determining global supply and market structures. Thus, any analysis of the future of natural gas must deal explicitly with multiple uncertainties:

- *The extent and nature of the greenhouse gas (GHG) mitigation measures that will be adopted*: the U.S. legislative response to the climate threat has proved quite challenging. However, the Environmental Protection Agency (EPA) is developing regulations under the Clean Air Act, and a variety of local, state, and regional GHG limitation programs have been put in place. At the international level, reliance on a system of voluntary national pledges of emission reductions by 2020, as set out initially in the Copenhagen Accord, leaves uncertainty concerning the likely structure of any future agreements that may emerge to replace the Kyoto Protocol. The absence of a clear international regime for mitigating GHG emissions in turn raises questions about the likely stringency of national policies in both industrialized countries and major emerging economies over coming decades.

- *The likely technology mix in a carbon-constrained world*, particularly in the power sector: the relative costs of different technologies may shift significantly in response to RD&D, and a CO_2 emissions price will affect the relative costs. Moreover, the technology mix will be affected by regulatory and subsidy measures that will skew economic choices.

- *The ultimate size and production cost of the natural gas resource base*, and the environmental acceptability of production methods: much remains to be learned about the performance of shale gas plays, both in the U.S. and in other parts of the world. Indeed, even higher risk and less well-defined unconventional natural gas resources, such as methane hydrates, could make a contribution to supply in the later decades of the study's time horizon.

- *The evolution of international natural gas markets*: very large natural gas resources are to be found in several areas outside the U.S., and the role of U.S. natural gas will be influenced by the evolution of this market — particularly the growth and efficiency of trade in LNG. Only a few years back, U.S. industry was investing in facilities for substantial LNG imports. The emergence of the domestic shale gas resource has depressed this business in the U.S., but in the future, the nation may again look to international markets.

Of these uncertainties, the last three can be explored by applying technically grounded analysis: lower cost for carbon capture and storage (CCS), renewables, and nuclear power; producible resources of different levels; and regional versus global integrated markets. In contrast, the shape and size of GHG mitigation measures are likely to be resolved only through complex ongoing political discussions at the national level in the major emitting countries and through multilateral negotiations.

The analysis in this study is based on three policy scenarios:

1. A business-as-usual case, with no significant carbon constraints;

2. GHG emissions pricing, through a cap-and-trade system or emissions tax, leading to a 50% reduction in U.S. emissions below the 2005 level, by 2050.

3. GHG reduction via U.S. regulatory measures without emissions pricing: a renewable portfolio standard; and measures forcing the retirement of some coal plants.

Our analysis is long term in nature, with a 2050 time horizon. We do not attempt to make detailed short-term projections of volumes, prices, or price volatility, but rather focus on the long-term consequences of the carbon mitigation scenarios outlined above, taking into account the manifold uncertainties in a highly complex and interdependent energy system.

MAJOR FINDINGS AND RECOMMENDATIONS

In the following section we summarize the major findings and recommendations for each chapter of the report.

Supply

Globally, there are abundant supplies of natural gas, much of which can be developed at relatively low cost. The mean projection of remaining recoverable resource in this report is 16,200 Tcf, 150 times current annual global natural gas consumption, with low and high projections of 12,400 Tcf and 20,800 Tcf, respectively. Of the mean projection, approximately 9,000 Tcf could be developed economically with a natural gas price at or below $4/Million British thermal units (MMBtu) at the export point.

Unconventional natural gas, and particularly shale gas, will make an important contribution to future U.S. energy supply and CO_2 emission-reduction efforts. Assessments of the recoverable volumes of shale gas in the U.S. have increased dramatically over the last five years, and continue to grow. The mean projection of the recoverable shale gas resource in this report is approximately 650 Tcf, with low and high projections of 420 Tcf and 870 Tcf, respectively. Of the mean projection, approximately 400 Tcf could be economically developed with a natural gas price at or below $6/MMBtu at the wellhead. While the pace of shale technology development has been very rapid over the past few years, there are still many scientific and technological challenges to overcome before we can be confident that this very large resource base is being developed in an optimum manner.

Although there are large supplies, global conventional natural gas resources are concentrated geographically, with 70% in three countries: Qatar, Iran, and Russia. There is considerable potential for unconventional natural gas supply outside North America, but these resources are largely unproven with very high resource uncertainty. Nevertheless, unconventional supplies could provide a major opportunity for diversification and improved security of supply in some parts of the world.

The environmental impacts of shale development are challenging but manageable. Shale development requires large-scale fracturing of the shale formation to induce economic production rates. There has been concern that these fractures can also penetrate shallow freshwater zones and contaminate them with fracturing fluid, but there is no evidence that this is occurring. There is, however, evidence of natural gas migration into freshwater zones in some areas, most likely as a result of sub-standard well completion practices by a few operators. There are additional environmental

challenges in the area of water management, particularly the effective disposal of fracture fluids. Concerns with this issue are particularly acute in regions that have not previously experienced large-scale oil and natural gas development, especially those overlying the massive Marcellus shale, and do not have a well-developed subsurface water disposal infrastructure. It is essential that both large and small companies follow industry best practices; that water supply and disposal are coordinated on a regional basis; and that improved methods are developed for recycling of returned fracture fluids.

Natural gas trapped in the ice-like form known as methane hydrate represents a vast potential resource for the long term. Recent research is beginning to provide better definition of the overall resource potential, but many issues remain to be resolved. In particular, while there have been limited production tests, the long-term producibility of methane hydrates remains unproven, and sustained research will be required.

MAJOR RECOMMENDATIONS

Government-supported research on the fundamental challenges of unconventional natural gas development, particularly shale gas, should be greatly increased in scope and scale. In particular, support should be put in place for a comprehensive and integrated research program to build a system-wide understanding of all subsurface aspects of the U.S. shale resource. In addition, research should be pursued to reduce water usage in fracturing and to develop cost-effective water recycling technology.

A concerted coordinated effort by industry and government, both state and Federal, should be organized so as to minimize the environmental impacts of shale gas development through both research and regulation. Transparency is key, both for fracturing operations and for water management. Better communication of oil- and gas-field best practices should be facilitated. Integrated regional water usage and disposal plans and disclosure of hydraulic fracture fluid components should be required.

The U.S. should support unconventional natural gas development outside U.S., particularly in Europe and China, as a means of diversifying the natural gas supply base.

The U.S. government should continue to sponsor methane hydrate research, with a particular emphasis on the demonstration of production feasibility and economics.

U.S. Natural Gas Production, Use, and Trade: Potential Futures

In a carbon-constrained world, a level playing field — a CO_2 emissions price for all fuels without subsidies or other preferential policy treatment — maximizes the value to society of the large U.S. natural gas resource.

Under a scenario with 50% CO_2 reductions to 2050, using an established model of the global economy and natural gas cost curves that include uncertainty, the principal effects of the associated CO_2 emissions price are to lower energy demand and displace coal with natural gas in the electricity sector. *In effect, gas-fired power sets a competitive benchmark against which other technologies must compete in a lower carbon environment*. A major uncertainty that could impact this picture in the longer term is technology development that lowers the costs of alternatives, in particular, renewables, nuclear, and CCS.

A more stringent CO_2 reduction of, for example, 80% would probably require the complete de-carbonization of the power sector. This makes it imperative that the development of competing low-carbon technology continues apace, including CCS for both coal and natural gas. It would be a significant error of policy to crowd out the development of other, currently more costly, technologies because of the new assessment of the natural gas supply. Conversely, it would also be a mistake to encourage, via policy and long-term subsidy, more costly technologies to crowd out natural gas in the short to medium term, as this could significantly increase the cost of CO_2 reduction.

The evolution of global natural gas markets is unclear; but under some scenarios, the U.S. could import 50% or more of its natural gas by 2050, despite the significant new resources created in the last few years. Imports can prevent natural gas-price inflation in future years.

MAJOR RECOMMENDATIONS

To maximize the value to society of the substantial U.S. natural gas resource base, U.S. CO_2 reduction policy should be designed to create a "level playing field," where all energy technologies can compete against each other in an open marketplace conditioned by legislated CO_2 emissions goals. A CO_2 price for all fuels without long-term subsidies or other preferential policy treatment is the most effective way to achieve this result.

In the absence of such policy, interim energy policies should attempt to replicate as closely as possible the major consequences of a "level playing field" approach to carbon-emissions reduction. At least for the near term, that would entail facilitating energy demand reduction and displacement of some coal generation with natural gas.

Natural gas can make an important contribution to GHG reduction in coming decades, but investment in low-emission technologies, such as nuclear, CCS, and renewables, should be actively pursued to ensure that a mitigation regime can be sustained in the longer term.

Natural Gas for Electric Power

In the U.S., around 30% of natural gas is consumed in the electric power sector. Within the power sector, gas-fired power plants play a critical role in the provision of peaking capacity, due to their inherent ability to respond rapidly to changes in demand. In 2009, 23% of the total power generated was from natural gas, while natural gas plants represented over 40% of the total generating capacity.

In a carbon-constrained world, the power sector represents the best opportunity for a significant increase in natural gas demand, in direct competition with other primary energy sources. Displacement of coal-fired power by gas-fired power over the next 25 to 30 years is the most cost-effective way of reducing CO_2 emissions in the power sector.

As a result of the boom in the construction of gas-fired power plants in the 1990s, there is a substantial amount of underutilized NGCC capacity in the U.S. today. In the short term, displacement of coal-fired power by gas-fired power provides an opportunity to reduce CO_2 emissions from the power sector by about 20%, at a cost of less than $20/ton of CO_2 avoided. This displacement would use existing generating capacity, and would, therefore, require little in the way of incremental capital expenditure for new generation capacity. It would also significantly reduce pollutants such as sulfur dioxide (SO_2), nitrous oxide (NO_X), particulates, and mercury (Hg).

Natural gas-fired power generation provides the major source of backup to intermittent renewable supplies in most U.S. markets. If policy support continues to increase the supply of intermittent power, then, in the absence of affordable utility-scale storage options, additional natural gas *capacity* will be needed to provide system reliability. In some markets, existing regulation does not provide the appropriate incentives to build incremental capacity with low load factors, and regulatory changes may be required.

In the short term, where a rapid increase in renewable generation occurs without any adjustment to the rest of the system, increased renewable power displaces gas-fired power generation and thus reduces demand for natural gas in the power sector. In the longer term, where the overall system can adjust through plant retirements and new construction, increased renewables displace baseload generation. This could mean displacement of coal, nuclear, or NGCC generation, depending on the region and policy scenario under consideration. For example, in the 50% CO_2 reduction scenario described earlier, where gas-fired generation drives out coal generation, increased renewable penetration as a result of cost reduction or government policy will reduce natural gas generation on a nearly one-for-one basis.

MAJOR RECOMMENDATIONS

The displacement of coal generation with NGCC generation should be pursued as the most practical near-term option for significantly reducing CO_2 emissions from power generation.

In the event of a significant penetration of intermittent renewable production in the generation technology mix, policy and regulatory measures should be developed to facilitate adequate levels of investment in natural gas generation capacity to ensure system reliability and efficiency.

END-USE GAS DEMAND

In the U.S., around 32% of all natural gas consumption is in the Industrial sector, where its primary uses are for boiler fuel and process heat; and 35% of use is in the Residential and Commercial sectors, where its primary application is space heating. Only 0.15% of natural gas is used as a vehicle transportation fuel.

Industrial, Commercial, and Residential

Within the Industrial sector, there are opportunities for improved efficiency of the Industrial boiler fleet, replacing less-efficient natural gas boilers with high-efficiency, or super-high efficiency boilers with conversion efficiencies up to 94%. There are also opportunities to improve the efficiency of natural gas use in process heating and to reduce process heating requirements through changes in process technologies and material substitutions.

Our analysis suggests that conversion of coal-fired boilers in the Industrial sector to high-efficiency gas boilers could provide a cost-effective option for compliance with new hazardous air pollutant reductions and create significant CO_2 reduction opportunities at modest cost, with a potential to increase natural gas demand by up to 0.9 Tcf/year.

Natural gas and natural gas liquids (NGL) are a principal feedstock in the chemicals industry and a growing source of hydrogen production for petroleum refining. Our analysis of selected cases indicates that a robust domestic market for natural gas and NGLs will improve the competitiveness of manufacturing industries dependent on these inputs.

Natural gas has significant advantages in the Residential and Commercial sectors due in part to its cleanliness and life cycle energy efficiency. However, understanding the comparative cost effectiveness and CO_2 impacts of different energy options is complex. Comparison of

end-use or "site" energy efficiencies can be misleading, since it does not take into account full system energy use and emissions (such as the efficiency and emissions of electricity generation). However, quantitatively accounting for the full system impacts from the "source" can be challenging, requiring a complex end-to-end, full fuel cycle (FFC) analysis that is not generally available to the consumer or to the policy maker.

Consumer and policy maker choices are further complicated by the influence of local climatic conditions and regional energy markets. The primary energy mix of the regional generation mix fundamentally affects "site versus source" energy and emissions comparisons. And the local climate has a major influence on the best choice of heating and cooling systems, particularly the appropriate use of modern space conditioning technologies such as heat pumps. Consumer information currently available to consumers does not facilitate well-informed decision making.

Expanded use of combined heat and power (CHP) has considerable potential in the Industrial and large Commercial sectors. However, cost, complexity, and the inherent difficulty of balancing heat and power loads at a very small scale make residential CHP a much more difficult proposition.

MAJOR RECOMMENDATIONS

Improved energy-efficiency metrics, which allow consumers to accurately compare direct fuel and electricity end uses on a full fuel cycle basis, should be developed.

Over time, these metrics should be tailored to account for geographical variations in the sources of electric power supply and local climate conditions.

Transportation

The ample domestic supply of natural gas has stimulated interest in its use in transportation. There are multiple drivers: the oil-natural gas price spread on an energy basis generally favors natural gas, and today that spread is at historically high levels; an opportunity to lessen oil dependence in favor of a domestically supplied fuel, including natural gas-derived liquid fuels with modest changes in vehicle and/or infrastructure requirements and reduced CO_2 emissions in direct use of natural gas.

CNG offers a significant opportunity in U.S. heavy-duty vehicles used for short-range operation (buses, garbage trucks, delivery trucks), where payback times are around three years or less and infrastructure issues do not impede development. However, for light passenger vehicles, even at 2010 oil–natural gas price differentials, high incremental costs of CNG vehicles lead to long payback times for the average driver, so significant penetration of CNG into the passenger fleet is unlikely in the short term. Payback periods could be reduced significantly if the cost of conversion from gasoline to CNG could be reduced to the levels experienced in other parts of the world such as Europe.

LNG has been considered as a transport fuel, particularly in the long-haul trucking sector. However, as a result of operational and infrastructure considerations as well as high incremental costs and an adverse impact on resale value, LNG does not appear to be an attractive option for general use. There may be an opportunity for LNG in the rapidly expanding segment of hub-to-hub trucking operations, where infrastructure and operational challenges can be overcome.

Energy density, ease of use, and infrastructure considerations make liquid fuels that are stable at room temperature a compelling choice in the Transportation sector. The chemical conversion of natural gas to liquid fuels could provide an attractive alternative to CNG. Several pathways are possible, with different options yielding different outcomes in terms of total system CO_2 emissions and cost. Conversion of natural gas to methanol, as widely practiced in the chemicals industry, could provide a cost-effective route to manufacturing an alternative, or supplement, to gasoline, while keeping CO_2 emissions at roughly the same level. Gasoline engines can be modified to run on methanol at modest cost.

MAJOR RECOMMENDATIONS

The U.S. government should consider revision to its policies related to CNG vehicles, including how aftermarket CNG conversions are certified, with a view to reducing up-front costs and facilitating bi-fuel CNG-gasoline capacity.

The U.S. government should implement an open fuel standard that requires automobile manufacturers to provide tri-flex fuel (gasoline, ethanol, and methanol) operation in light-duty vehicles. Support for methanol fueling infrastructure should also be considered.

Infrastructure

The continental U.S. has a vast, mature, and robust natural gas infrastructure, which includes: over 300,000 miles of transmission lines; numerous natural gas-gathering systems; storage sites; processing plants; distribution pipelines; and LNG import terminals.

Several trends are having an impact on natural gas infrastructure. These include changes in U.S. production profiles, with supplies generally shifting from offshore Gulf of Mexico back to onshore; shifts in U.S. population, generally from the Northeast and Midwest to the South and West; and growth in global LNG markets, driven by price differences between regional markets.

The system generally responds well to market signals. Changing patterns of supply and demand have led to a significant increase in infrastructure development over the past few years with West to East expansions dominating pipeline capacity additions. Infrastructure limitations can temporarily constrain production in emerging production areas such as the Marcellus shale — but infrastructure capacity expansions are planned or underway. Demand increases and shifts in consumption and production are expected to require around $210 billion in infrastructure investment over the next 20 years.

Much of the U.S. pipeline infrastructure is old — around 25% of U.S. natural gas pipelines are 50 years old or older — and recent incidents demonstrate that pipeline safety issues are a cause for concern. The Department of Transportation (DOT) regulates natural gas pipeline safety and has required integrity management programs for transmission and distribution pipelines. The DOT also supports a small pipeline safety research program, which seems inadequate given the size and age of the pipeline infrastructure.

Increased use of natural gas for power generation has important implications for both natural gas and electric infrastructures, including natural gas storage. Historically, injections and withdrawals from natural gas storage have been seasonal. Increased use of natural gas for power generation may require new high-deliverability natural gas storage to meet more variable needs associated with power generation.

MAJOR RECOMMENDATIONS

Analysis of the infrastructure demands associated with potential shift from coal to gas-fired power should be undertaken.

Pipeline safety technologies should be included in natural gas RD&D programs.

END-USE EMISSIONS VERSUS SYSTEM-WIDE EMISSIONS

When comparing GHG emissions for different energy sources, attention should be paid to the entire system. In particular, the potential for leakage of small amounts of methane in the production, treatment, and distribution of coal, oil, and natural gas has an effect on the total GHG impact of each fuel type. The modeling analysis in Chapter 3 addresses the system-wide impact, incorporating methane leakage from coal, oil, and natural gas production, processing, and transmission. In Chapter 5 we do not attempt to present detailed full-system accounting of CO_2 (equivalent) emissions for various end uses, although we do refer to its potential impact in specific instances.

The CO_2 equivalence of methane is conventionally based on a Global Warming Potential (GWP)[3] intended to capture the fact that each GHG has different radiative effects on climate and different lifetimes in the atmosphere. In our considerations, we follow the standard Intergovernmental Panel on Climate Change (IPCC) and EPA definition that has been widely employed for 20 years. Several recently published life cycle emissions analyses do not appear to be comprehensive, use common assumptions, or recognize the progress made by producers to reduce methane emissions, often to economic benefit. We believe that a lot more work is required in this area before a common understanding can be reached. Further discussion can be found in Appendix 1A.

MAJOR RECOMMENDATIONS

The EPA and the U.S. Department of Energy (DOE) should co-lead a new effort to review, and update as appropriate, the methane emission factors associated with natural gas production, transmission, storage, and distribution. The review should have broad-based stakeholder involvement and should seek to reach a consensus on the appropriate methodology for estimating methane emissions rates. The analysis should, to the extent possible: reflect actual emissions measurements; address fugitive emissions for coal and oil as well as natural gas; and reflect the potential for cost-effective actions to prevent fugitive emissions and venting of methane.

MARKETS AND GEOPOLITICS

The physical characteristics of natural gas, which create a strong dependence on pipeline transportation systems, have led to local markets for natural gas, in contrast to the global markets for oil.

There are three distinct regional gas markets: North America, Europe, and Asia, with more localized markets elsewhere. The U.S. gas market is mature and sophisticated, and functions well, with a robust spot market. Within the U.S. market, the price of oil (which is set globally) compared to the price of natural gas (which is set regionally) is very important in determining market share when there is the opportunity for substitution. Over the last decade or so, when oil prices have been high, the ratio of the benchmark West Texas Intermediate oil price to the Henry Hub natural gas price has been consistently higher than any of the standard rules of thumb.

International natural gas markets are in the early stages of integration, with many impediments to further development. While increased LNG trade has started to connect these markets, they remain largely distinct with respect to supply patterns, pricing and contract structures, and market regulation. If a more integrated market evolves, with nations pursuing gas production and trade on an economic basis, there will be rising trade among the current regional markets and the U.S. could become a substantial net importer of LNG in future decades.

Greater international market liquidity would be beneficial to U.S. interests. U.S. prices for natural gas would be lower than under current regional markets, leading to more gas use in the U.S. Greater market liquidity would also contribute to security by enhancing diversity of global supply and resilience to supply disruptions for the U.S. and its allies. These factors ameliorate security concerns about import dependence.

As a result of the significant concentration of conventional gas resources globally, policy and geopolitics play a major role in the development of global supply and market structures. Consequently, since natural gas is likely to play a greater role around the world, natural gas issues will appear more frequently on the U.S. energy and security agenda. Some of the specific security concerns are:

- Natural gas dependence, including that of allies, could constrain U.S. foreign policy options, especially in light of the unique American international security responsibilities.

- New market players could introduce impediments to the development of transparent markets.

- Competition for control of natural gas pipelines and pipeline routes is intense in key regions.

- Longer supply chains increase the vulnerability of the natural gas infrastructure.

MAJOR RECOMMENDATIONS

The U.S. should pursue policies that encourage the development of an efficient and integrated global gas market with transparency and diversity of supply.

Natural gas issues should be fully integrated into the U.S. energy and security agenda, and a number of domestic and foreign policy measures should be taken, including:

- **integrating energy issues fully into the conduct of U.S. foreign policy, which will require multiagency coordination with leadership from the Executive Office of the President;**

- **supporting the efforts of the International Energy Agency (IEA) to place more attention on natural gas and to incorporate the large emerging markets (such as China, India, and Brazil) into the IEA process as integral participants;**

- **sharing know-how for the strategic expansion of unconventional resources; and**

- **advancing infrastructure physical- and cyber-security as the global gas delivery system becomes more extended and interconnected.**

RD&D

There are numerous RD&D opportunities to address key objectives for natural gas supply, delivery, and end use:

- improve the long-term economics of resource development as an important contributor to the public good;
- reduce the environmental footprint of natural gas production, delivery, and use;
- expand current use and create alternative applications for public policy purposes, such as emissions reductions and diminished oil dependence;
- improve safety and operation of natural gas infrastructure;
- improve the efficiency of natural gas conversion and end use so as to use the resource most effectively.

Historically, RD&D funding in the natural gas industry has come from a variety of sources, including private industry, the DOE, and private/public partnerships. In tandem with limited tax credits, this combination of support played a major role in development of unconventional gas. It has also contributed to more efficient end use, for example in the development of high-efficiency gas turbines.

Today government-funded RD&D for natural gas is at very low levels. The elimination of rate-payer funded RD&D has not been compensated by increased DOE appropriations or by a commensurate new revenue stream outside the appropriations process. The total public and public-private funding for natural gas research is down substantially from its peak and is more limited in scope, even as natural gas takes a more prominent role in a carbon-constrained world.

While natural gas can provide a cost-effective bridge to a low carbon future, it is vital that efforts continue to improve the cost and efficiency of low or zero carbon technologies for the longer term. This will require sustained RD&D and subsidies of limited duration to encourage early deployment.

MAJOR RECOMMENDATIONS

The Administration and Congress should support RD&D focused on environmentally responsible domestic natural gas supply. This should entail both a renewed DOE program, weighted towards basic research, and a complementary industry-led program, weighted towards applied research, development, and demonstration, that is funded through an assured funding stream tied to energy production, delivery, and use. The scope of the program should be broad, from supply to end use.

Support should be provided through RD&D, and targeted subsidies of limited duration, for low-emission technologies that have the prospect of competing in the long run. This would include renewables, CCS for both coal and gas generation, and nuclear power.

CONCLUSION

Over the past few years, the U.S. has developed an important new natural gas resource that fundamentally enhances the nation's long-term gas supply outlook. Given an appropriate regulatory environment, which seeks to place all lower carbon energy sources on a level competitive playing field, domestic supplies of natural gas can play a very significant role in reducing U.S. CO_2 emissions, particularly in the electric power sector. This lowest-cost strategy of CO_2 reduction allows time for the continued development of more cost-effective low or zero carbon energy technology for the longer term, when gas itself is no longer sufficiently low carbon to meet more stringent CO_2 reduction targets. The newly realized abundance of low-cost gas provides an enormous potential benefit to the nation, providing a cost-effective bridge to a secure and low carbon future. It is critical that the additional time created by this new resource is spent wisely, in creating lower-cost technology options for the longer term, and thereby ensuring that the natural gas bridge has a safe landing place in a low carbon future.

NOTES

[1]One quadrillion Btu (or "quad") is 1015 or 1,000,000,000,000,000 British thermal units. Since one standard cubic foot of gas is approximately 1,000 Btu, then 1 quad is approximately 1 Tcf of gas.

[2]EIA 2009 Annual Energy Review, Figure 45.

[3]Global-warming potential (GWP) is a relative measure of how much heat a given greenhouse gas traps in the atmosphere.

Chapter 2: Supply

INTRODUCTION AND CONTEXT

In this chapter, we discuss various aspects of natural gas supply: how much natural gas exists in the world; at what rate can it be produced; and what it will cost to develop. Following the introduction and definitions, we look at production history, resource volumes, and supply costs for natural gas — first from a global perspective, and then focusing in more detail on the U.S., paying particular attention to the prospects for shale gas. We then discuss the science and technology of unconventional gas, the environmental impacts of shale gas development, and finally the prospects for methane hydrates.

NATURAL GAS AND THE RECOVERY PROCESS

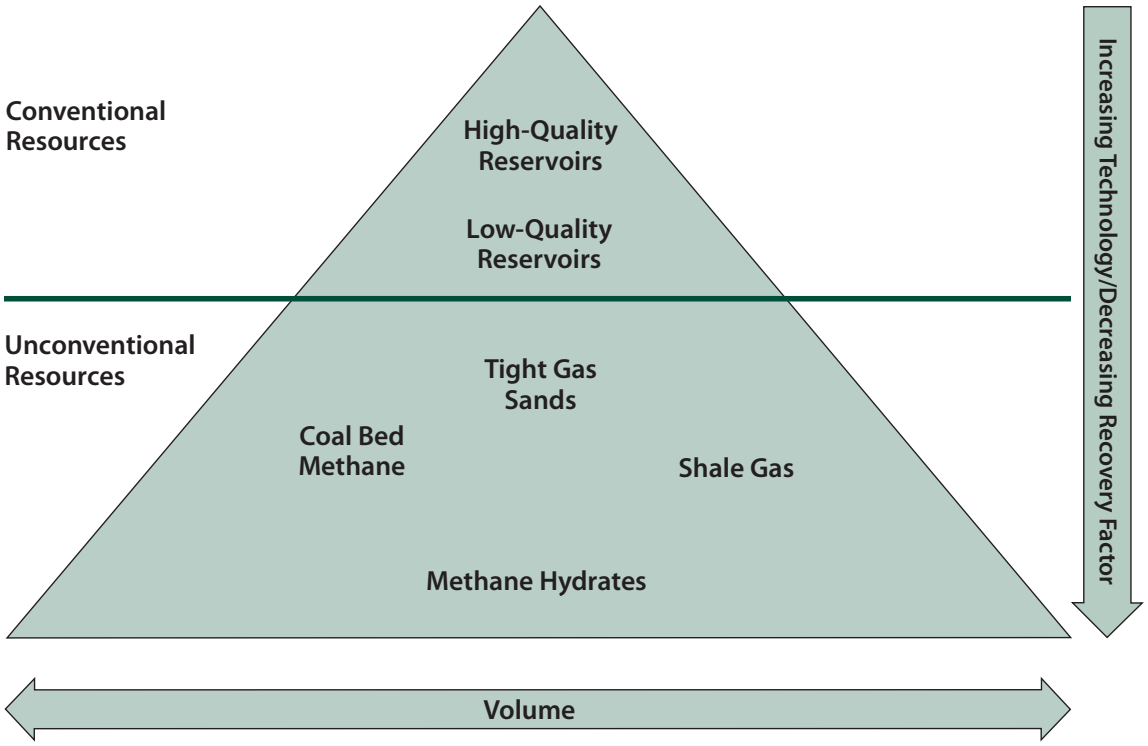
The primary chemical component of natural gas is methane, the simplest and lightest hydrocarbon molecule, comprised of four hydrogen (H) atoms bound to a single carbon (C) atom. In chemical notation, this is expressed as CH_4 (the symbol for methane). Natural gas may also contain small proportions of heavier hydrocarbons: ethane (C_2H_6); propane (C_3H_8), and butane (C_4H_{10}); these heavier components are often extracted from the producing stream and marketed separately as natural gas liquids (NGL). In the gas industry, the term "wet gas" is used to refer to natural gas in its raw unprocessed state, while "dry gas" refers to natural gas from which the heavier components have been extracted.

Thermogenic[1] natural gas, which is formed by the application, over geological time, of enormous heat and pressure to buried organic matter, exists under pressure in porous rock formations thousands of feet below the surface of the earth. It exists in two primary forms: "associated gas" is formed in conjunction with oil, and is generally released from the oil as it is recovered from the reservoir to the surface — as a general rule the gas is treated as a by-product of the oil production process; in contrast, "non-associated gas" is found in reservoirs that do not contain oil, and is developed as the primary product. While associated gas is an important source, the majority of gas production is non-associated; 89% of the gas produced in the U.S. is non-associated.

Non-associated gas is recovered from the formation by an expansion process. Wells drilled into the gas reservoir allow the highly compressed gas to expand through the wells in a controlled manner, to be captured, treated, and transported at the surface. This expansion process generally leads to high recovery factors from conventional, good-quality gas reservoirs. If, for example, the average pressure in a gas reservoir is reduced from an initial 5,000 pounds per square inch (psi) to 1,000 psi over the lifetime of the field, then approximately 80% of the Gas Initially In Place (GIIP) will be recovered. This is in contrast to oil, where recovery factors of 30% to 40% are more typical.

Gas is found in a variety of subsurface locations, with a gradation of quality as illustrated in the resource triangle in Figure 2.1.

Figure 2.1 GIIP as a Pyramid in Volume and Quality. Conventional reservoirs are at the top of the pyramid. They are of higher quality because they have high permeability and require less technology for development and production. The unconventional reservoirs lie below the conventional reservoirs in this pyramid. They are more abundant in terms of GIIP but are currently assessed as recoverable resources — and commercially developed — primarily in North America. They have lower permeability, require advanced technology for production, and typically yield lower recovery factors than conventional reservoirs.

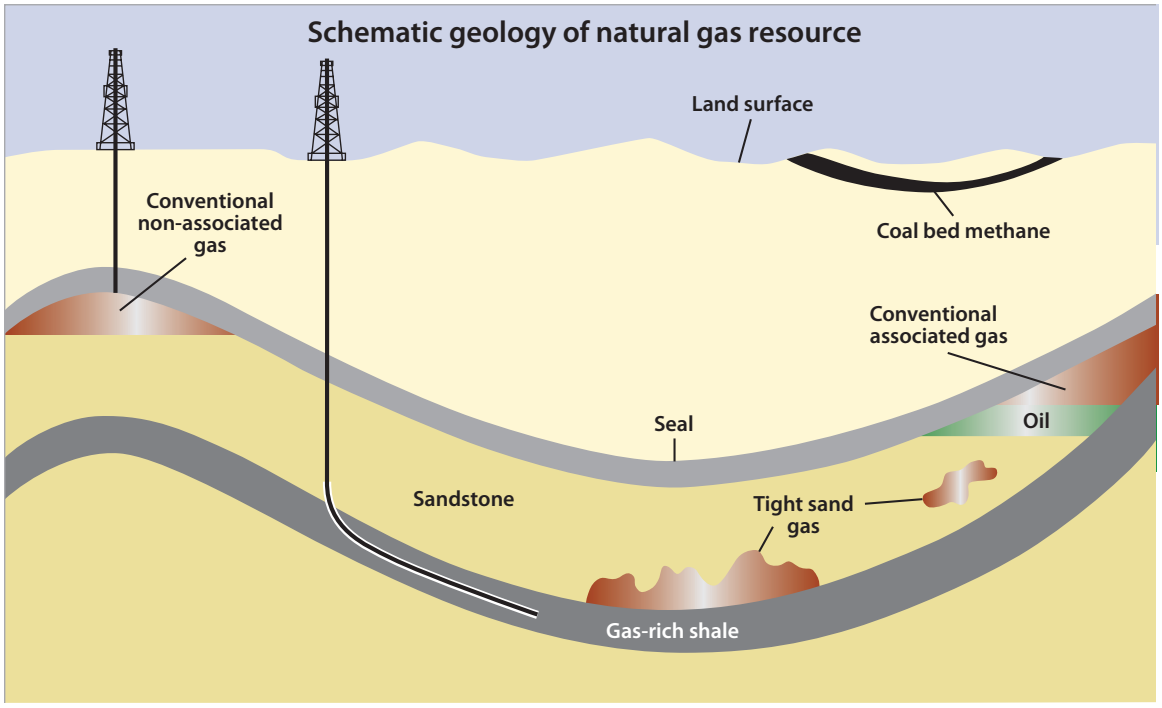


Adapted from Holditch 2006

Conventional resources exist in discrete, well-defined subsurface accumulations (reservoirs), with permeability[2] values greater than a specified lower limit. Such conventional gas resources can usually be developed using vertical wells, and generally yield the high recovery factors described above.

By contrast, unconventional resources are found in accumulations where permeability is low. Such accumulations include "tight" sandstone formations, coal beds (coal bed methane or CBM) and shale formations. Unconventional resource accumulations tend to be distributed over a larger area than conventional accumulations and usually require advanced technology such as horizontal wells or artificial stimulation in order to be economically productive; recovery factors are much lower — typically of the order of 15% to 30% of GIIP. The various resource types are shown schematically in Figure 2.2.

Figure 2.2 Illustration of Various Types of Gas Resource



Source: U.S. Energy Information Administration

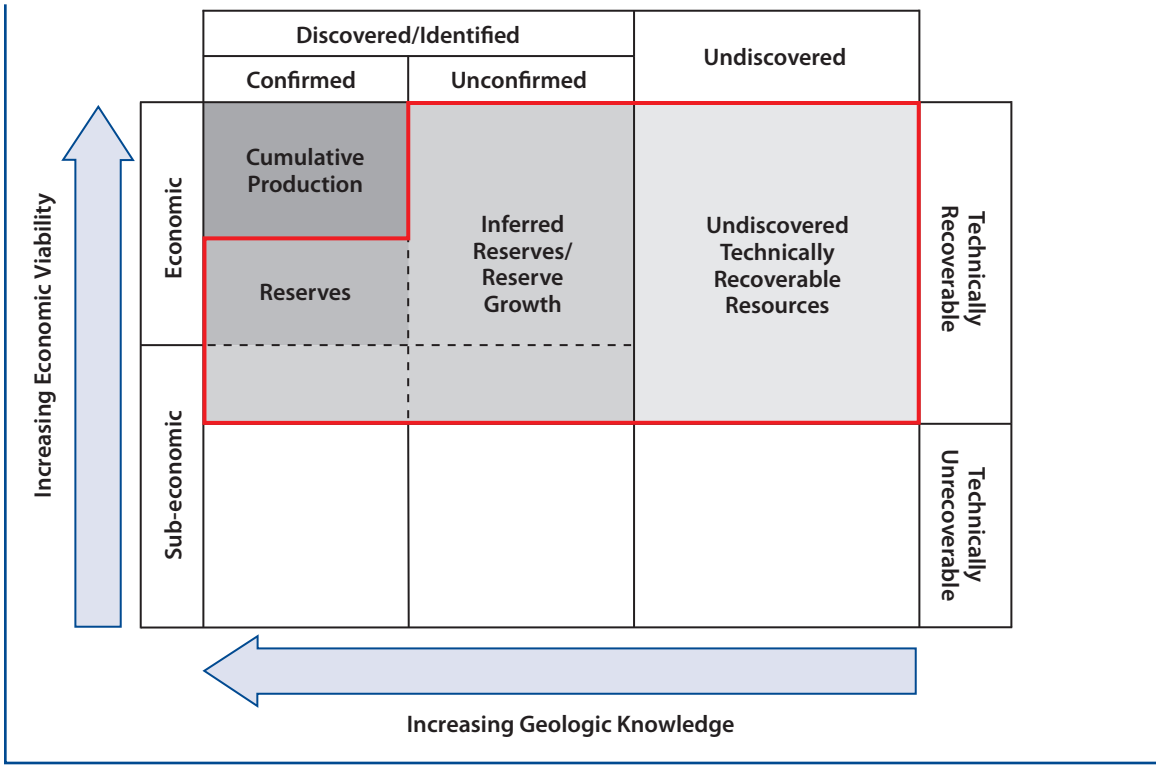
RESOURCE DEFINITIONS

The complex cross-dependencies between geology, technology, and economics mean that the use of unambiguous terminology is critical when discussing natural gas supply. In this study, the term "resource" will refer to the sum of all gas volumes expected to be recoverable in the future, given specific technological and economic conditions. The resource can be disaggregated into a number of sub-categories; specifically, "proved reserves," "reserve growth" (via further development of known fields), and "undiscovered resources," which represent gas volumes that are expected to be discovered in the future via the exploration process.

Gas resources are an economic concept — a function of many variables, in particular the cost of exploration, production, and transportation relative to the price of sale to users.

Figure 2.3 illustrates how proved reserves, reserve growth, and undiscovered resources combine to form the "technically recoverable resource," that is, the total volume of natural gas that could be recovered in the future, using today's technology, ignoring economic constraints.

Figure 2.3 Modified McKelvey Diagram, Showing the Interdependencies between Geology, Technology, and Economics and Their Impacts on Resource Classes; Remaining Technically Recoverable Resources Are Outlined in Red



The methodology used in analyzing natural gas supply for this study places particular emphasis in two areas:

1. Treating gas resources as an economic concept — recoverable resources are a function of many variables, particularly the ultimate price that the market will pay. A set of supply curves has been developed using the ICF[3] Hydrocarbon Supply Model with volumetric and fiscal input data supplied by ICF International (ICF) and MIT. These curves describe the volume of gas that is economically recoverable for a given gas price. These curves form a primary input to the integrated economic modelling in Chapter 3 of this report.

2. Recognizing and embracing uncertainty — uncertainty exists around all resource estimates due to the inherent uncertainty associated with the underlying geological, technological, economic, and political conditions. The analysis of natural gas supply in this study has been carried out in a manner that frames any single point resource estimate within an associated uncertainty envelope, in order to illustrate the potentially large impact this ever-present uncertainty can have.

The volumetric data used as the basis of the analysis for both the supply curve development and the volumetric uncertainty analysis were compiled from a range of sources. In particular, use has been made of data from work at the United States Geological Survey (USGS), the Potential Gas Committee (PGC), the Energy Information Agency (EIA), the National Petroleum Council (NPC), and ICF.

GLOBAL SUPPLY

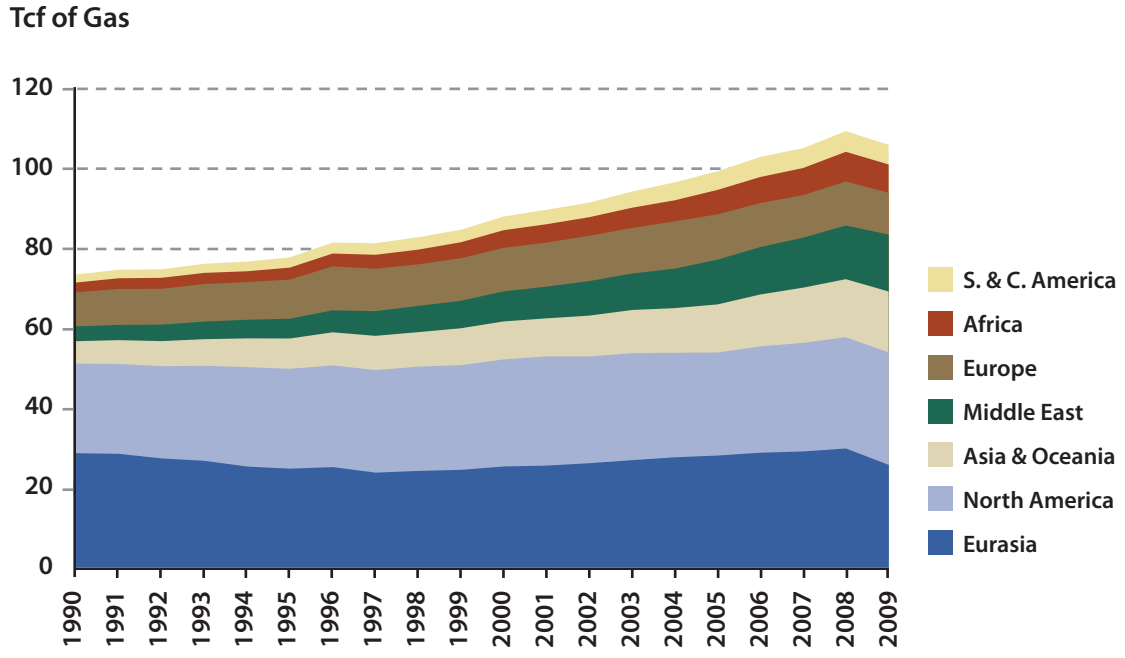
Production Trends

Over the past two decades, global production of natural gas has grown significantly, rising by almost 42% overall from approximately 74 trillion cubic feet (Tcf)[4] in 1990 to 105 Tcf in 2009. This is almost twice the growth rate of global oil production, which increased by around 22% over the same period. Much of the gas production growth has been driven by the rapid expansion of production in areas that were not major gas producers prior to 1990. This trend is illustrated in Figure 2.4, which shows how growth in production from regions such as the Middle East, Africa, and Asia & Oceania has significantly outpaced growth in the traditional large producing regions, including North America and Eurasia (primarily Russia).

Figure 2.5 compares the 1990 and 2009 annual production levels for the 10 largest gas-producing nations (as defined by 2009 output). In addition to demonstrating the overwhelming scale of the U.S. and Russia compared to other producing countries, this figure illustrates the very significant growth rates in other countries. The substantial growth of new gas producing countries over the period reflects the relative immaturity of the gas industry on a global basis outside Russia and North America, the expansion of gas markets, and the rise in global cross-border gas trade.

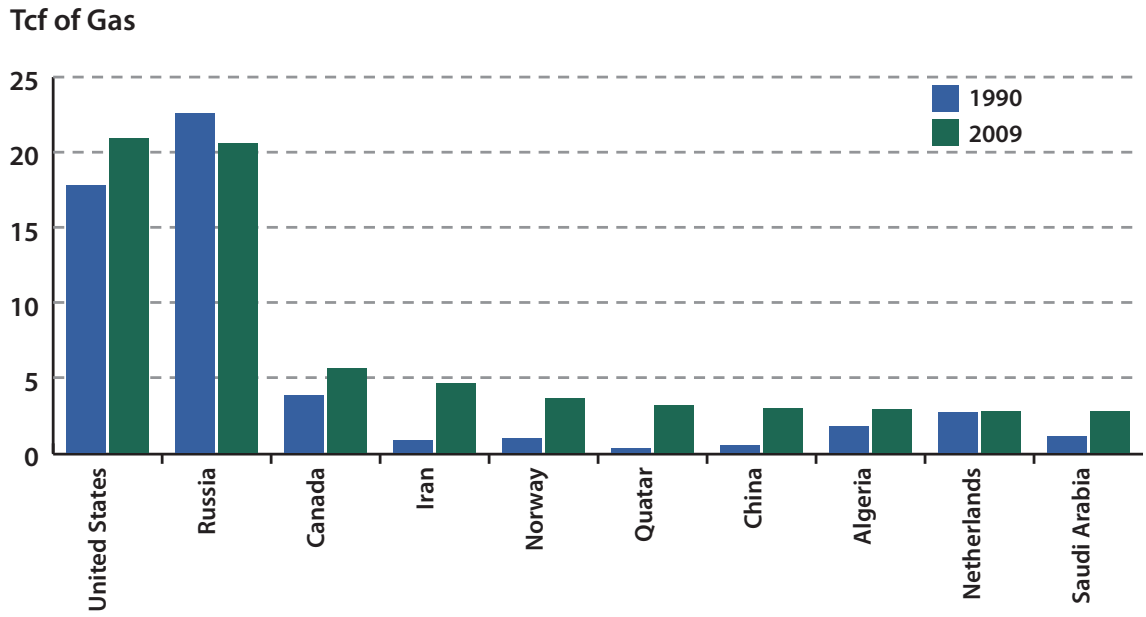
Between 1993 and 2008, global cross-border gas trade almost doubled, growing from around 18 Tcf (25% of global supply) to around 35 Tcf (32% of global supply). Most of the world's gas supply is transported from producing fields to market by pipeline. However, the increase in global gas trade has been accelerated by the growing use of Liquefied Natural Gas (LNG), which is made by cooling natural gas to around -162°C. Under these conditions, natural gas becomes liquid, with an energy density 600 times that of gas at standard temperature and pressure — and it can be readily transported over long distances in specialized ocean-going

Figure 2.4 Trends in Annual Global Dry Gas Production by Region between 1990 and 2009



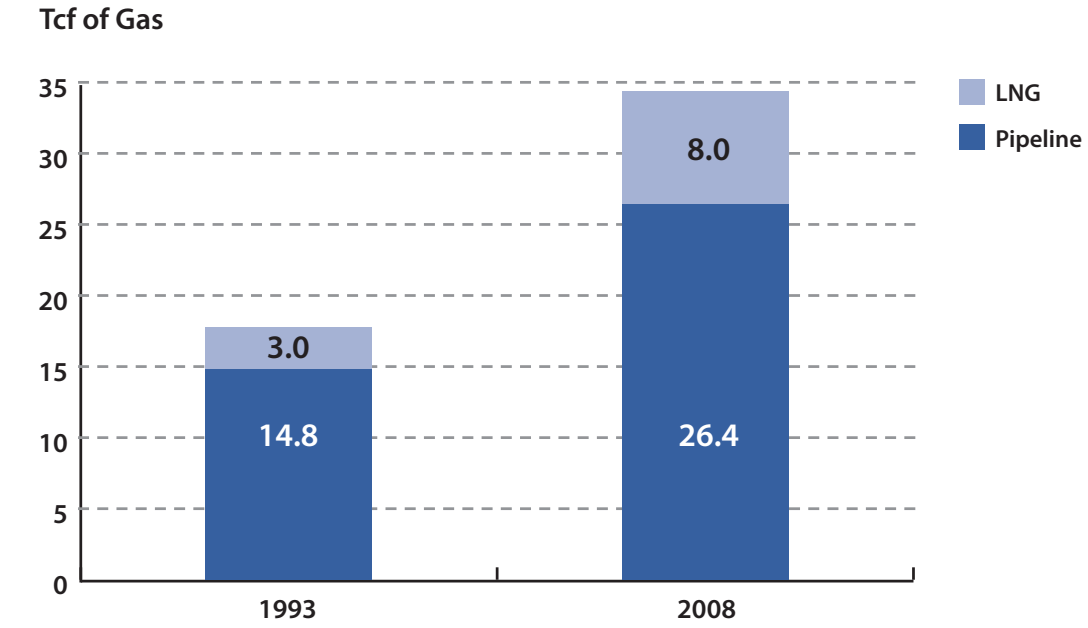
Source: MIT; U.S. Energy Information Administration

Figure 2.5 Comparison of 1990 and 2009 Natural Gas Production Levels for the Top 10 Natural Gas Producing Nations (as defined by 2009 output)



Source: MIT; U.S. Energy Information Administration

Figure 2.6 Global Cross-Border Gas Trade



Source: MIT; U.S. Energy Information Administration