Effects from offshore oil production: chronic exposure of fish to produced water

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Abbreviations

AhR – aryl hydrocarbon receptor

ALA-D – δ-aminolevulininc acid dehydratase

AOX – acyl-CoA oxidase

AP - Alkylphenol

cDNA – complementary deoxyribonucleic acid

CYP1A – cytochrome P450 1A

DNA – deoxyribonucleic acid

EC50 – effective concentration 50

EROD – 7-ethoxyresorufin *O*-deethylase

EST – expressed sequence tag

GC/MS – gas chromatography / mass spectrometry

GSI – gonadosomatic index

GST – glutathione S-transferase

HPLC – high pressure liquid chromatography

K_{ow} – octanol:water partitioning coefficient

mRNA – messenger ribonucleic acid

LMS – lysosomal membrane stability

LOD – limit of detection

MXR – multixenobiotic resistance

NOEC – no observed effect concentration

PAH – polycyclic aromatic hydrocarbon

PBDE – polybrominated diphenyl ether

PCB – polychlorinated biphenyls

PCR – polymerase chain reaction

PEC – predicted environmental concentration

PNEC – predicted no effect concentration

PPAR – peroxisomal proliferator-activated receptor

RNA – ribonucleic acid

SPMD – semipermeable membrane device

UGT – uridine diphosphate-glucuronosyltransferase

UV – ultraviolet

XRE – xenobiotic response element

Contents

1 Introduction

1.1 Oil production and environmental risk

Exploration and production of oil on the Norwegian continental shelf was initiated by the discovery of the oil field Ekofisk over 40 years ago. In the following years, expansions in the number of oil fields led to a steady increase in Norwegian oil production, culminating in the year 2000 with the record production of 187.9 million m^3, equivalent to 3.1 million barrels of oil per day. This production volume put Norway among the leading exporters of oil in the world. Since then the output of oil has declined to 131.8 million m^3 in 2007, a reduction of 30% in 7 years. In the same time, the production of gas has increased by more than 80% (OLF). The Norwegian government has confined the core activities of oil and gas production to the Norwegian and the North Sea, exceeding 50 oil fields, but recent years decline in oil output have led to a drive for expansion of oil exploration into pristine areas, such as the Barents Sea.

In the wake of oil production follows large discharges to the environment. Emissions to air include greenhouse gases such as carbon dioxide and methane, in addition to nitrogen oxides, sulphuric oxides and volatile organic compounds. Discharges to sea originate both from the drilling process (cuttings, drilling fluids and other additives) and from the exploitation process, including displacement and drainage water, accidental spills and produced water. Produced water represents by far the largest discharge to sea (162 million m^3 in 2007 (OLF, 2008)), now exceeding the oil production volume. In addition, produced water discharges from the Danish, Dutch and UK sectors add to the total amount received by North Sea waters (UK discharge in 2005 was 241 million m^3 (Oil & Gas UK)).

While emissions to air generally correspond to production output, the discharge of produced water generally increases as the reservoir relative oil output decrease. As pressure is reduced when oil is extracted from the reservoir, water is injected to maintain pressure and thus oil flow. Both injected and naturally occurring reservoir water follows the oil to the production facility, where it is separated and either reinjected into the reservoir or discharged to sea after cleaning procedures. Approximately 85% of the extracted produced water is discharged to sea. The water is in equilibrium with oil, meaning that it contains compounds derived from, and in a composition similar to, the crude oil. As the ratio of water to oil increases in the well with increasing age of the oil

field, the volume of extracted and discharged produced water will therefore increase correspondingly.

In 1996, Norway introduced a target of zero environmentally harmful discharges of natural compounds from the petroleum industry (White paper no. 58 (1996-1997) and no. 25 (2002-2003), The Norwegian Ministry of the Environment). This meant that there was a need to implement methods for detection of environmental effects, as well as a need for an increased knowledge of whether the observed effects were harmful or not. Effects of discharges to sea from activities related to oil production have been studied for decades, including effects of drill cuttings on benthic communities (e.g. Davies et al., (1984); Grant and Briggs, (2002); Schaanning et al., (2008)), effects of produced water on marine organisms (Gamble et al., 1987; Strømgren et al., 1995; Stephens et al., 2000) and long-term effects of accidental oil spills, of which the best known (but not the largest) being the Exxon Valdez loss in Prince William Sound, Alaska (Payne et al., 2008). However, whether or not effects may be detected is obviously depending on the sensitivity of the applied methods.

Early laboratory experiments often focused on short-term (acute) effects of single compounds determining responses such as EC50s. The EC50 is an estimate of the concentration required to reach a fixed endpoint in 50% of the test organisms. In toxicity testing, the assessed endpoint was often mortality, which is not a very sensitive or environmentally relevant effect. The use of such crude response measurements was generally replaced by estimating the highest concentration of a chemical where no effects were observed (NOEC), which is a more relevant approach for environmental protection purposes. By comparing the predicted concentration of a chemical in the environment (PEC) to the predicted concentration of which no effect is observed (PNEC), a risk quotient may be calculated. A risk quotient (PEC/PNEC) above 1 indicates an increased likelihood for an effect in the environment.

By using models based on risk quotients and hazard classifications (and other known properties of the chemical), environmental impact factors have been calculated for compounds in produced water. Produced water is a saline containing a complex mixture of thousands of compounds such as metals, organic acids, phenols, radionuclides, production chemicals, dissolved hydrocarbons and dispersed oil (Utvik, 1999). The environmental impact factor model identified components in the dissolved hydrocarbons group, namely low molecular weight polycyclic aromatic hydrocarbons (PAHs) and alkylphenols (APs), to be the most significant contributors to environmental risk in produced water (Johnsen et

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al., 2000). Also, PAHs and some APs (nonylphenol, octylphenol) are on the European Union Water Framework Directive list of priority substances (counting 33 entries) to be dealt with at source, in the most economically and environmentally effective manner (EU, 2008).

Although cleaning strategies are applied, significant amounts of PAHs and APs still reach the environment due to the large discharge volumes of produced water. The composition of natural compounds and synthetic chemicals may vary between oil fields due to different geophysical properties between areas. The produced water is normally discharged in the upper part of the water column, and the temperature may be elevated compared to normal North Sea temperatures. Physical factors such as density and temperature therefore tend to section the plume into depth layers close to the surface. In addition, wind and sea currents may cause this plume to be directional. This is of importance when characterising exposure to aquatic organisms. Generally, the most concentrated produced water exposure will be confined to organisms living in the free water masses. In addition, the impact of exposure may also be determined by their mobility. Thus, produced water exposure on native organisms is not easily describable, and is probably best illustrated as highly variable or pulsating.

1.2 PAHs in the North Sea and their effects on fish

1.2.1 Sources and concentrations of PAHs in the North Sea

PAHs are aromatic compounds made from two or more fused benzene rings. Two main categories of PAH contamination of the aquatic environment has been described: pyrogenic and petrogenic. Pyrogenic sources are those including combustion of hydrocarbons or indeed any organic material (engine exhaust, fires and aluminium smelting resulting in atmospheric deposition and wastewater effluents) whereas petrogenic sources include discharges from petroleum related activities as well as natural sources (oil seeps, erosion of coal/peat/oil shale deposits, oil spills, discharges of oil tanker ballast water and produced water, coal-fired power plants and sewage treatment plants). Pyrogenic and petrogenic discharges will to a large extent comprise different PAHs, which make it possible to estimate the main sources in a particular area by comparing their relative contribution. Petrogenic discharges are dominated by low-molecular weight PAHs, similar to the parent crude oil pattern, often with high abundance of alkyl substituents (Neff,

2002). Pyrogenic PAHs are produced at high temperatures through incomplete combustion, and will be dominated by four-, five-, and six-ringed PAHs (Neff, 2002).

PAHs from pyrogenic and petrogenic sources may behave very differently in the environment, as pyrogenic PAHs will commonly be more or less tightly bound to particles (soot etc.), whereas petrogenic are dissolved or loosely bound to particles and therefore comparatively more available to marine organisms (Farrington, 1986). The heavier and more hydrophobic PAHs tend to adsorb to particulate organic matter and may therefore be concentrated in sediments. The main source of PAHs to the North Sea is activities related to petroleum exploitation, producing a petrogenic PAH profile. However, in other parts of the world, sources such as natural oil seeps (Allen et al., 1970) or coal in sediments (Achten and Hofmann, 2009) may be significant contributors producing a similar petrogenic profile.

The concentration of PAHs in the North Sea has been estimated through different sampling techniques and modelling (Utvik et al., 1999; Durell et al., 2006) at different depths and distances relative to the discharge point (Utvik and Johnsen, 1999; Harman et al., submitted). This work has demonstrated a PAH concentration gradient towards oil production platforms. Measured concentrations in seawater indicate that produced water may be diluted several thousand times, even at short distances from the installation. Concentrations of selected compounds as measured in produced water and estimated in the North Sea are presented in table 1.

Despite pre-discharge cleaning processes and evaporation, it is evident that low-molecular-weight PAHs are discharged to sea as dissolved components in produced water. Subsequently, additional processes may further degrade or modify discharged components, such as sunlight (depending on season and latitude) and microbes. Sunlight may reduce the toxicity of the compounds through their breakdown, but it may also increase toxicity to marine organisms (Pelletier et al., 1997; Schirmer et al., 1998), a process known as phototoxicity. Studies on the photodegradation of several oil types have shown that naphthalenes and its methylated derivates are widely altered, whereas phenanthrene, dibenzothiophene and their derivates are more recalcitrant (Jacquot et al., 1996). Microbes also degrade PAHs, but the rate and efficiency of environmental breakdown is not well known (Wammer and Peters, 2005; Doyle et al., 2008). Microbial degradation could also lead to bioactivation and an increase in toxicity (Neff, 2002).

1.2.2 Effects of PAHs on fish

Biological uptake of PAHs is generally considered to be correlated to the PAH lipophilicity (K_{ow}), but may also depend on the extent of alkylation (Jonsson et al., 2004) and the bioavailability of each component (Utvik and Johnsen, 1999; Baussant et al., 2001). In fish, PAHs may be taken up directly from water (bioconcentration) or via the diet (Grung et al., 2009). Some organisms, such as molluscs, may accumulate PAHs in tissue due to a relatively inefficient metabolism. PAHs may therefore be quantified in their tissues to estimate recent exposure. Fish, on the other hand, have an efficient metabolism (see Van Der Oost et al., (2003)) and readily excrete most PAHs. Therefore, PAH metabolite concentrations in bile (rather than tissue levels) are used to indicate exposure (Aas et al., 2000b).

In contrast to estimating PAH exposure through quantification of their metabolites in bile, effect measurements implicate the quantification of biological changes. The liver in fish and digestive gland in shellfish are generally the main sites for xenobiotic metabolism, which is why these organs have received the most attention for this purpose. Nevertheless, depending on the toxicokinetics and dynamics of the chemical, other target organs may be as relevant for biological effect measurements.

In fish, most xenobiotics will be modified by endogenous enzymes and transporters categorized into the phase I, II and III biotransformation systems (Xu et al., 2005). Phase I and II metabolism generally increase water solubility of a xenobiotic and enhance excretion. The phase III system includes membrane protein pumps, in this context commonly referred to as multixenobiotic resistance (MXR), transporting a wide range of substrates out of the cell and thereby decreasing the intra-cellular concentration (Bard, 2000). The phase III system has not commonly been accounted for in environmental monitoring although its effects might be significant (Smital et al., 2004). Components of all three systems have been applied in studies of how PAHs affect fish (e.g. Celander et al., 1993; Beyer et al., 1997; Bard et al., 2002).

The biological effects of PAHs in fish include genotoxicity (French et al., 1996; Ericson et al., 1998; Aas et al., 2000a), impairment of reproduction (Johnson et al., 1997; Monteiro et al., 2000), oxidative stress (Sturve et al., 2006), modification of the immune system (Reynaud and Deschaux, 2006) and developmental toxicity (Incardona et al., 2006). A chronic mesocosm study showed that flounder (*Platichthys flesus*) exposed to

PAHs via sediment developed histopathological lesions (Vethaak et al., 1996), and a tentative causal link between exposure to PAHs and the development of liver cancer has been shown in English sole (*Pleuronectes vetulus*) from Puget Sound (Washington, USA) (Myers et al., 2003). The latter is currently the most comprehensive field study in linking effects in fish to PAH contamination, spanning several levels of biological organisation, although long term effects of contaminants have also been extensively studied in North Sea fish populations (Vethaak and Ap Rheinallt, 1992; Hylland et al., 2006a; Hylland et al., 2006b; Hylland et al., 2008).

The link between PAH exposure and liver cancer includes regulation of the phase I enzyme cytochrome P450 1A (CYP1A), which is now the best characterised and most commonly used enzyme for monitoring effects of PAHs. This enzyme may be quantitatively induced through binding of the aryl hydrocarbon receptor (AhR). Ligand binding activates the AhR which translocates to the nucleus and binds to xenobiotic responsive elements (XRE) in the DNA (Whitlock, 1999). This may activate transcription of a battery of genes containing XREs, including CYP1A. The transcripts may then be translated into e.g. CYP1A proteins enhancing further metabolism of PAHs. In addition to such detoxification processes, CYP1A may also increase PAH toxicity through bioactivation. These mechanisms have been well studied in fish regarding high-molecular-weight PAHs, such as benzo[a]pyrene and other AhR ligands (Beyer et al., 1997), but the effects of petrogenic PAHs on phase I and phase II systems in fish are not well known (Hylland, 2006). At high concentrations, some PAHs have been shown to exert toxicity independently of the well characterised AhR pathway (Incardona et al., 2005).

Some components of the phase II system also contain XREs, such as UDP-glucuronosyl transferase (UGT) and glutathione S-transferase (GST), and may therefore be responsive to AhR ligands. They are generally less compound specific and sensitive than the phase I system (Van Der Oost et al., 2003). These enzymes are conjugating enzymes, attaching endogenous groups to phase I products or directly to xenobiotics to facilitate their excretion. Most PAHs and APs excreted to bile are conjugated by these enzymes.

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1.3 Alkylphenols in the North Sea and their effects on fish

1.3.1 Sources and concentrations of APs in the North Sea

As mentioned above, alkylphenols are another group of compounds in produced water thought to contribute significantly to environmental risk. Alkylphenols may be formed through the degradation of alkylphenol ethoxylates, which have been widely used as detergents, UV stabilizers and plasticizers in a variety of products (Nimrod and Benson, 1996; Ying et al., 2002). In recent years, the use of alkylphenol surfactants has been phased out in the offshore industry, hence alkylphenols in produced water discharges are derived from naturally occurring components of oil or gas.

Alkylphenols are composed of a phenol group with an attached hydrocarbon chain, which may vary in length, structure and position, of which alkylphenol nomenclature is derived (e.g. 4-*tert*-butylphenol, 4-*n*-octylphenol). Their water solubility largely depends on their attached alkylchain. Concentrations of alkylphenols in produced water have been monitored by the oil companies for over a decade, but recent method developments have facilitated alkylphenol analysis with sufficiently low detection limits for levels present in seawater (Boitsov et al., 2004). Recently, the concentrations of several alkylphenols have also been determined in produced water (Boitsov et al., 2007), and in the recipient where elevated concentrations of short-chained APs were observed closer to an oil production platform (Harman et al., submitted). The AP concentration in produced water generally increases with decreasing length of the alkyl-chain, but variability between locations has been found to be greater for short-chained alkylphenols than for long-chained alkylphenols (Boitsov et al., 2007).

1.3.2 Effects of alkylphenols on fish

Studies on alkylphenols have been focussed on a few compounds, such as octylphenol and nonylphenol, due to the early discovery of their endocrine disrupting properties (Sumpter and Jobling, 1993; White et al., 1994; Nimrod and Benson, 1996). Octylphenol and nonylphenol may bind to the estrogen receptor, causing inhibition of testicular growth and feminization of male fish (Sumpter, 1995; Jobling et al., 1996), as well as disruption of

female ovarian development (Harris et al., 2001). Recently, more effort has been put into the identification and effect assessment of estrogenic acting chemicals in produced water (Lye, 2000). The estrogenic activity of APs depends on their structure (Routledge and Sumpter, 1997), and as the bulk of APs in produced water are short-chained they have received increased focus. Several short- and moderate chained alkylphenols in produced water have been shown to bind *in vitro* to the estrogen receptor in fish (Thomas et al., 2004; Tollefsen and Nilsen, 2008) and induce effects associated with estrogenicity (Tollefsen et al., 2008b). The estrogen receptor agonist activity is primarily associated with water soluble compounds in produced water (Tollefsen et al., 2007). As for PAHs, APs can be taken up in fish directly from water or via the diet (Grung et al., 2009; Sundt et al., 2009).

Even at low levels, C_4 to C_7 phenols may have a negative impact on steroid levels and gonad development in Atlantic cod (*Gadus morhua*) (Meier et al., 2007b), as well as effects on membrane lipid composition in liver and brain (Meier et al., 2007a). Endocrine disruption of C_4 and C_5 alkylphenols has been documented in carp (*Cyprinus carpio*) (Gimeno et al., 1996; Barse et al., 2006). Furthermore, moderate chained APs may cause oxidative stress and affect phase I system components (Hasselberg et al., 2004a; Hasselberg et al., 2004b; Sturve et al., 2006) in addition to being cytotoxic to hepatocytes (Tollefsen et al., 2008a).

Table 1. *PAH and AP concentrations measured in produced water (PW) or in the vicinity of North Sea oil production platforms. For comparison, nominal exposure concentrations in the low dose group are presented in the right hand column.*

Compound	PW (µg/L) (median – quartiles)	Troll[4] (ng/L)	Ekofisk[5] (ng/L)	Statfjord B[6] (ng/L)	Ekofisk[7] (ng/L)	Low dose group (ng/L)
Naphthalene	445 (410, 478)[1] 310 (263, 330)[2]	18.8	4.9	46.7	a	155
Phenanthrene	23 (18, 27)[1] 16 (12, 23)[2]	2.47	1.24	nd	2.4	8
Fluorene	12 (7, 16)[1] 12 (10, 16)[2]	1.25	1.26	3.1	0.82	6
Dibenzothiophene	6 (4, 8)[1] 4 (2, 6)[2]	0.29	0.18	nd	0.31	2
Pyrene	5 (4, 6)[1] 0.7 (0.4, 0.9)[2]	0.09	0.11	nd	0.06	0.6
Acenaphthene	2 (1, 3)[1] 2 (1, 2)[2]	0.34	0.16	nd	0.15	0.9
Acenaphthylene	na[1] 0.005 (0.005, 0.005)[2]	0	0	4.74	0.07	0.003
Anthracene	na[1] 0.7 (0.3, 0.9)[2]	0	0.05	Nd	<0.04	0.4
C1-naphthalenes	340 (290,390)[1] 340 (270, 450)[2]	12.3	5.98	109.3	6.8	170
C2-naphthalenes	195 (180, 213)[1] 189 (166, 230)[2]	5.08	3.99	116.8	8.0	95
C3-naphthalenes	110 (95, 125)[1] 113 (103, 160)[2]	5.86	3.47	187.8	16.6	57
C1-phenanthrenes	25 (20, 31)[1] 24 (10, 28)[2]	3.83	3.19	Nd	6.3	21
C2-phenanthrenes	12 (8, 18)[1] 26 (8, 30)[2]	3.14	3.15	Nd	5.0	32
C3-phenanthrenes	9 (8, 11)[1] 4 (2, 5)[2]	1.97	2.23	87.15	b	2
C1-dibenzothiophenes	9 (7, 10)[1] 5 (1, 10)[2]	0.75	0.68	nd	1.33	2
C2-dibenzothiophenes	7 (7, 9)[1] 7 (1, 12)[2]	0.84	0.79	nd	1.1	3

Table 1 continued.

Compound	PW (µg/L) (median – quartiles)	Troll[4] (ng/L)	Ekofisk[5] (ng/L)	Statfjord B[6] (ng/L)	Ekofisk[7] (ng/L)	Low dose group (ng/L)
Phenol	4820 (2663, 6328)[1] 1400 (1167, 1712)[2] 2033 (1153, 2080)[3]	na	na	na	na	700
C1-phenols	2745 (1493, 3593)[1] 1500 (637, 2008)[2] 2311 (1580, 2697)[3]	na	na	na	5.1	750
C2-phenols	630 (485, 800)[1] 600 (330, 804)[2] 250 (241, 420)[3]	na	na	na	16.2	315
C3-phenols	85 (75, 100)[1] 100 (66, 220)[2] 82 (61, 148)[3]	na	na	na	1.7	62
C4-phenols	20 (20, 23)[1] 40 (14, 64)[2] 16 (12, 16)[3]	na	na	na	0.2	18

[1] Oseberg/Troll (Utvik, 1999), n=4

[2] Oseberg/Troll – Ekofisk – Asgård – Statfjord/Snorre/Gullfaks (OLF, 2004) as presented in (Holth et al., 2008), n=16

[3] Statfjord/Snorre/Gullfaks (Boitsov et al., 2007), n=3

[4] Troll B Station 5 (Durell et al., 2006), SPMD based calculations

[5] Ekofisk Station 4 (Durell et al., 2006), SPMD based calculations

[6] Statfjord B Station 1 (Utvik and Gãrtner, 2006), SPMD based calculations

[7] Ekofisk Station 5 (Harman et al., submitted), SPMD based calculations

na = not assessed

nd = below detection limit

1.4 Predicting chronic effects in fish from sublethal markers

When assessing biological effects of contaminants for the conservation of the environment, the aim would be to detect effects before they develop into adverse conditions for organism, population or ecosystem health. Such early warning signals may be biochemical, physiological or histological changes and are often referred to as biomarkers (Peakall, 1994). In the past, observations of adverse biological effects have triggered large retrospective studies to identify causative agents and have included the assessment of increased occurrence of liver cancer in farmed fish (Sinnhuber et al., 1978) or in fish collected in contaminated areas (Dawe et al., 1964; Malins et al., 1985), observations of hermaphroditic fish inhabiting waters receiving sewage treatment effluents (Purdom et al., 1994) or changes in ecosystem composition in marine environments (Gray et al., 1990; Jackson et al., 2001).

However, the use of early warning signals (biomarkers) implies a prospective approach, often spanning several levels of biological organisation. Although it is generally thought that molecular changes precede effects at higher levels and thus are the most sensitive, this concept has yet to be confirmed, as demonstrated by e.g. Brian et al., (2007).

Due to a lack of well defined links between effects at different levels of biological organisation, the ecological relevance will generally increase with increasing biological complexity. On the other hand, the contaminant specificity is often decreased as several distinct lower level effects may lead to similar effects in individuals or populations, such as impairment of reproduction. Thus, the ideal early warning signal would be an easily measurable, contaminant specific effect at a low level of biological organisation with a causal link to adverse effects in individuals, populations or ecosystems.

Several of the currently applied biomarkers have not been directly associated with higher level adverse effects, but represent effects in well known pathways (such as vitellogenin (Hylland et al., 2006a) and ALA-D (Larsson et al., 1985)). For the reasons mentioned, interpretation of early warning signals often involves use of the precautionary principle, potentially triggering false alarms. The usefulness of biomarkers to predict higher level effects have been questioned, pointing out the challenges in interpretation and extrapolation of results (Forbes et al., 2006). However, some studies have shown the usefulness of biomarkers in long term monitoring, such as the modification of steroid

metabolism in several fish species living in the vicinity of municipal refuse dumps (Noaksson, 2003), or the assessment of EROD activity and GSI in predicting PAH induced population level effects in perch (*Perca fluviatilis*) on the Swedish Baltic coast (Hanson et al., 2009a; Hanson et al., 2009b). The use of a set of biomarkers could provide an early warning signal on fish-health through a weight of evidence approach (Hylland et al., 2009; Sanchez and Porcher, 2009). In addition to the biochemical markers already introduced, the assessment of lysosomal membrane stability and peroxisomal proliferation have been suggested as promising early warning signals in monitoring campaigns due to their sensitivity and wide contaminant specificity (Viarengo et al., 2007).

During the last 15 years, Norwegian coastal and offshore areas have been monitored for biological effects of contaminants in several studies and programmes, such as the Joint Assessment Monitoring Programme (JAMP; OSPAR convention) (Ruus et al., 2003), the BECPELAG workshop (Hylland et al., 2006b) and the Water Column Monitoring programme (Hylland et al., 2008). Contaminant related effects have been observed both in fish along the coast and offshore, although effects have been more evident along the coast (Hylland et al., 2006a; Hylland et al., 2008; Hylland et al., 2009). During the offshore monitoring programmes, indications of DNA damage in fish have been observed (Balk et al., 2006; Hylland et al., 2006a) in addition to moderate effects on phase I system components (Förlin and Hylland, 2006). The indications of biological effects from offshore oil production highlighted the need for studying chronic effects of environmentally relevant contaminant concentrations.

1.5 Confounding effects and the challenge of extrapolation

A challenge when assessing effects in organisms is to be able to separate effects induced by contaminants from effects induced or modified by other factors. Some of these factors are well characterised and can thus be avoided or compensated for. However, poorly described interfering factors may be detrimental for the interpretation of biological responses. The robustness and usability of a biological effect parameter for environmental monitoring may be evaluated by its contaminant specificity, dose responsiveness and the stability of its background levels.

For several teleost biomarkers, seasonal effects may be a confounding factor, and for fish with an annual reproduction cycle seasonal fluctuations in background levels and responsiveness to contaminants are often observed. For example, CYP1A metabolic activity in fish may be inhibited by estradiol resulting in large differences in response between males and females (Förlin and Haux, 1990; Lindström-Seppä and Stegeman, 1995; Broeg et al., 1999). In flounder collected in the Norwegian Hvaler archipelago throughout a year, it was shown that season, gender and maturation had strong effects on hepatic CYP1A activity and metallothionein concentration (Hylland et al., 1998). In addition, behaviour related to spawning periods may indirectly confound results in environmental monitoring due to e.g. migration of certain cohorts of a population (Eggens et al., 1995). Other factors well known to influence biochemical or physiological responses in fish are water temperature (Lacorn et al., 2001), fish age (Sleiderink et al., 1995) and nutritional status (Wall and Crivello, 1999). To correct for the factors mentioned, different somatic indices are often calculated and compared, such as condition factor, liver somatic index or gonadosomatic index, all providing a numeric value which potentially can be attributed to fitness and reproductive status. It is also possible to account for such factors by sampling outside the reproductive season or at specific time periods of the year (Kammann et al., 2005; Hylland et al., 2009).

Often, knowledge has to be adapted from controlled model systems in order to better understand responses in novel research areas, a concept referred to as extrapolation. For example, results are often extrapolated between populations, species or life-stages of an organism. Also, exposure history generalizations often have to be made, such as extrapolation of effects from single contaminant exposures to predict the effects of multiple contaminant exposures, or the effects of continuous exposures to predict the effects of pulsed exposures. For example, the calculation of environmental impact factors is often based on toxicity assessments of single compound exposures and the assumption that their effects are additive (Barron et al., 2004). It has been well documented that a mixture of chemicals acting through similar or independent modes of action may cause interactions and unexpected effects in organisms such as antagonism, synergism or potentiation at all levels of biological organisation (Finne et al., 2007; Staal et al., 2007; Billiard et al., 2008). This means that the biological effects of single compounds often cannot be predicted by simple addition; in combination the sum of effects may be more or less than expected. One of the best established interactions is the crosstalk between the AhR and the estrogen receptor. AhR agonists may inhibit estrogenic activity, while

estrogenic substances (such as some APs) may inhibit the AhR response (Klinge et al., 1999; Navas and Segner, 2001; Mortensen and Arukwe, 2007). Furthermore, some PAHs have actually been shown to inhibit CYP1A activity (Willett et al., 2001), and AhR agonists to induce estrogenic responses (Mortensen & Arukwe, 2008).

In addition to the above, organisms may physiologically adjust to an exposure load following long term exposure to contaminants. This phenomenon (adaptation) is observed as a decline in response with time even if stress level remains constant and may be a significant confounding factor in environmental monitoring. Responses have been shown to differ between fish species and even between populations (Larsen et al., 2007). Several types of adaptation have been described, separated by their pattern of temporal development (Wu et al., 2005). Adaptation to heavy creosote (PAH) and PCB contaminations has been shown in several native fish populations (Frederick et al., 2007; Nacci et al., 2009). These populations both displayed immune system adaptations to the highly polluted environment, as well as less inducibility of the CYP1A system (Bello et al., 2001). Adaptation to PAH exposure has also been observed in mussels as a reduction in DNA adduct formation over time (Ching et al., 2001).

In summary, depending on the type of organism and how well the effect parameter is characterised, effects may be over- or underestimated if potentially confounding factors are not properly evaluated. Thus, there is a need for further investigation of the effects in fish of mixture toxicity, adaptation and the effects of pulsed exposure.

2 Aims and objectives

The overall aim of this dissertation was to quantify molecular, biochemical and physiological effects in fish following exposure to environmentally relevant concentrations of components of produced water. In addition, the study aimed to clarify the temporal development of biological responses and to provide comparisons of such effects between two fish species. To accomplish this, chronic exposure studies with an environmentally relevant mixture of PAHs and APs were performed using two model fish species, zebrafish (*Danio rerio*) and Atlantic cod (*Gadus morhua*), lasting for 13 and 44 weeks, respectively. Regular sampling was carried out during both studies, and the following objectives were assessed:

1. Quantify concentrations of PAH and AP metabolites in bile and evaluate their use for exposure monitoring.
2. Quantify effects on gross parameters and reproduction, and evaluate their development and potential impact.
3. Quantify changes and temporal development of biomarkers related to produced water contaminants.
4. Characterise changes in gene expression and evaluate their significance for effects at higher levels of biological organisation.
5. Investigate and compare the effects of pulsed exposure in fish to effects of continuous exposure regimes.
6. Compare responses between species and evaluate their use in environmental monitoring.

3 List of papers

The dissertation is based on the following papers, which will be referred to in the text by their roman numerals (**I-IV**):

Paper I

Grung, M., Holth, T.F., Jacobsen, M.R. and Hylland, K. (2009). Polycyclic aromatic hydrocarbon (PAH) metabolites in Atlantic Cod exposed via water or diet to a synthetic produced water. Journal of Toxicology and Environmental Health Part A – Current Issues, **72**(3-4): 254-265.

Paper II

Holth, T.F., Nourizadeh-Lillabadi, R., Blæsbjerg, M., Grung, M., Holbech, H., Petersen, G.I. and Hylland, K. (2008). Differential gene expression and biomarkers in zebrafish (*Danio rerio*) following exposure to produced water components. Aquatic Toxicology, **90**: 277-291.

Paper III

Holth, T.F., Beylich, B., Skarphéðinsdóttir, H., Liewenborg, B., Grung, M. and Hylland, K. (2009). Genotoxicity of environmentally relevant concentrations of water soluble oil components in cod (*Gadus morhua*). Environmental Science & Technology, **43**(9): 3329-3334.

Paper IV

Holth, T.F., Thorsen, A., Olsvik, P. A. and Hylland, K. Long-term exposure of Atlantic Cod (*Gadus morhua*) to components of produced water: growth, reproduction and gene expression. (Submitted June 2009)

4 Materials and methods

4.1 Choice of species

In recent years, as the genetic information of several fish species has become more available, the application of transcriptomics has expanded in aquatic toxicology to characterise effects of traditional and emerging contaminants. The genomes of several teleost species have been fully sequenced, such as the zebrafish, medaka (*Oryzias latipes*), the stickleback (*Gasterosteus aculeatus*) and two pufferfish species (*Fugu rubripes* and *Tetraodon nigroviridis*). The zebrafish is an increasingly used teleost model in toxicogenomics, in addition to its usefulness in human and aquatic biomedicine (Aleström et al., 2006). Due to the transcriptomic tools available for this species, zebrafish was chosen as a model fish species in the current study. In toxicogenomics, the zebrafish has been used for microarray gene expression analysis of several stressor models such as 17 α-ethinylestradiol (Hoffmann et al., 2006), nonylphenol (Hoyt et al., 2003), hypoxia (Ton et al., 2003) and emerging compounds such as PBDEs (Nourizadeh-Lillabadi et al., 2009). Not until recently have the genome wide effects of produced water relevant exposures been studied in zebrafish (Olsvik et al., 2007; Paper II), in addition to analysis of selected genes using the polymerase chain reaction (PCR) (Arukwe et al., 2008). A disadvantage using zebrafish is its small size, making only small amounts of tissue available limiting the number of analyses applicable to each sample. Also, zebrafish are not environmentally relevant to marine and boreal ecosystems.

Atlantic cod was chosen as the second model species due to its ecological relevance in North Sea ecosystems. Research on the gadoid genome has recently been intensified, both because of the high economic value and farming potential of this species as well as being a relevant monitoring species in the North Atlantic. Several projects have generated Atlantic cod EST sequences and stress-related cDNA libraries (Bowman and Trippel; Olsvik), and small tissue- or stress-specific cDNA microarrays have been produced (Kortner et al., 2008; Lie et al., in press-a). Effects on gene expression after exposing Atlantic cod to C_4-C_7 alkylphenols and produced water via the diet have previously been examined using the CodStress array (Lie et al., in press-b). Also, full-length sequencing of the cod genome is underway at the University of Oslo (Norway) (RCN).

4.2 Exposure systems

Although produced water is discharged in large volumes, the dilution upon mixing with seawater results in low environmental levels. To be able to detect exposure related effects, it is therefore crucial to minimize the number of potential confounding factors. To accomplish this, a laboratory study was carefully designed.

It has been shown that cultivated fish, often used for research purposes, may develop DNA adducts at fish farms due to environmental exposure (Hylland et al., 2008). In addition, unintended contamination may be introduced through the diet. Antioxidants, used as preservatives in commercial fish feed (Holaas et al., 2008) may interfere with sensitive exposure related responses. Also, environmental pollutants accumulated in lipids (such as fish liver oil) used for feed may introduce unwanted effects. On the other hand, the use of physiologically acceptable feed (including lipids) is necessary to ensure the health and maturation of the fish in a reproductive experiment. To minimize the impact of such factors, fish used in this study were acclimated for an extended time and pre-exposure samples were collected for determination of eventual background signals. During the acclimation (4 weeks) and experiment period, zebrafish were fed brine shrimps carefully bred to avoid contamination. Cod were acclimated for 6 months after transportation and for additional 30 days after introduction to the exposure system before treatment was initiated. Cod were fed, in addition to Barents Sea white fish fillets, custom made pellets of extra-purified fish liver oil and meal (Paper I, III and IV).

It should be pointed out that even though the treatments were designated low – high, the nominal concentrations in all groups were in the range of environmentally relevant concentrations. A produced water substitute was made by sorting and selecting the most abundant PAHs and APs in produced water by their median concentrations from several installations in the North Sea and southern Norwegian Sea from 2003 (OLF, 2004). By applying dilution factors of 200 and 2000, nominal exposure concentrations were obtained as presented for the low dosed group (2000x dilution) in table 1. Two positive aspects of using synthetic produced water were: (1) the unique control of exposure conditions; thousands of unknown compounds are present in produced water and composition may vary significantly between installations. Therefore, the use of a mixture of the most ubiquitous petrogenic PAHs and APs would provide valid effect data for most petrogenic sources. (2) Produced water is not easily amenable to transport or experimentation, as processes such as freezing, storage and thawing could lead to modification of the produced

water. As components will be modified soon after discharge in natural systems, it has been speculated that such processes might increase the environmental relevance of exposure studies using frozen produced water (Sundt et al., in press), but it is unlikely that identical physical and chemical processes will take place under these two very different conditions. Finally, a very large volume of produced water would be required for chronic flow-through exposure studies. The use of genuine produced water would have been feasible for the smaller scale zebrafish study (Paper II), as the total volume required would just exceed 150 litres during the 13 weeks of exposure. On the other hand, the corresponding volume of produced water required to complete 44 weeks of cod exposure would be approximately 160 000 L (the high exposure group alone were exposed to an equivalent of 100 000 L produced water). As one objective was to compare responses between the two fish species, similar exposures were required, promoting the use of synthetic produced water.

In both studies, all equipment in direct contact with exposure solutions were made of glass, teflon or coated/lined with teflon to reduce adsorption of the less water soluble components. Also, a main header tank was used for sedimentation of particles in inlet water (relevant to cod experiment) as well as separate header tanks for each exposure group for proper mixing of stock solutions with system water (Figure 1). To reduce carry-over contamination of volatile components, exposure units were separated by plastic curtains. The systems were regularly cleaned by flushing and siphoning.
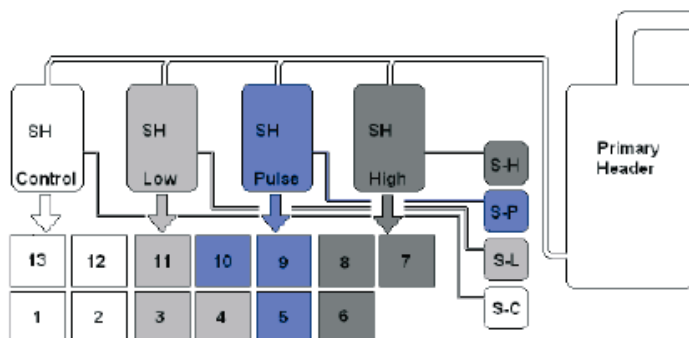

Figure 1. Exposure system setup for both experiments. Control treatment consisted of 4 replicate tanks (1, 2, 12, 13), whereas low (3, 4, 11), pulsed (5, 9, 10) and high (6, 7, 8) treatment consisted of 3 replicates. System water and stock solutions were mixed in secondary header tanks (SH) before distribution to tanks holding the fish.

4.3 Confirmation of water contaminant levels

In the current study, several methods for exposure confirmation were used:

- System water analysis
- Passive sampling devices (cod experiment only)
- *In situ* fluorescence measurements (cod experiment only)

Chemical analysis (GC/MS) of stock solutions from both exposure studies verified nominal concentrations. The compound ratio to nominal stock concentrations was 0.94 ± 0.04 and 0.97 ± 0.01 for zebrafish and cod, respectively (average $\pm$ SE). Analyses of system water in the zebrafish study demonstrated significant differences between the treatments, but the measured concentrations were only approaching 50% of nominal concentrations. Median values were approximately 10% of nominal concentrations (Paper II). A water sampling approach was also performed during the cod study, following a thoroughly prepared sampling scheme, but due to unknown reasons the quantification of compounds was unsuccessful. As the same analytical method was used for both studies, this might indicate matrix effects or unknown differences in post-sampling handling.

Semipermeable membrane devices (SPMDs) were successfully used for monitoring PAH concentrations in water during the first 4 weeks of the cod study (Harman et al., 2009). In general, the SPMDs validated exposure concentrations to within 30% of the nominal exposure concentrations (Figure 2). Naphthalene, alkylated naphthalenes and alkylated phenanthrenes were reduced compared to nominal concentrations. A probable cause might be evaporation as they are the more volatile compounds. In addition, bacteria may selectively utilize certain PAHs as an energy source. No contamination by these compounds was detected in the control group, although concentrations of other compounds (acenaphthene, phenanthrene, anthracene, pyrene, and C2-dibenzothiophenes) were found at >10% of low dose concentrations. The most probable cause was a low background contamination of seawater as the measured concentrations for most of these compounds were above nominal concentrations in the low dose group as well. However, it was clear that the background levels of PAHs were too low to influence determinations in the high dose group.
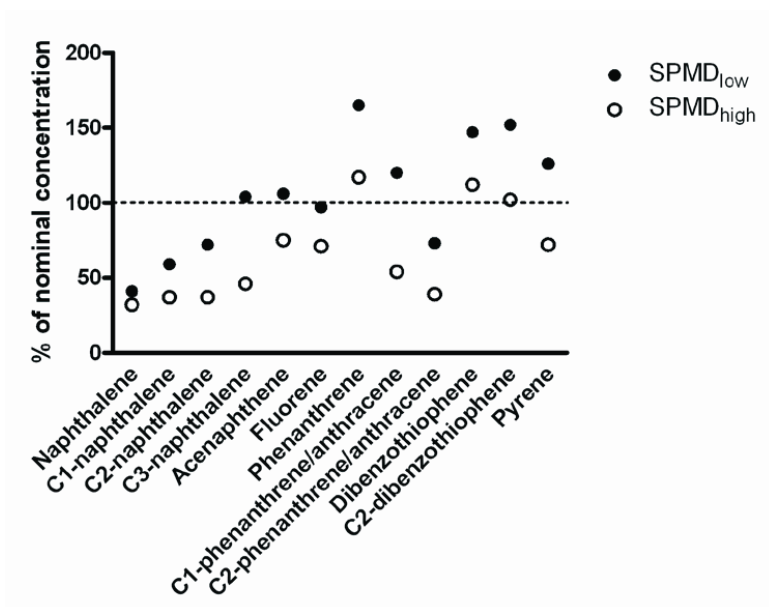
Figure 2. *Effective concentrations presented as percentages of nominal concentrations for several PAHs. Data is based on passive sampling (SPMDs) in the low (closed circles) and high (open circles) exposure groups during the first 4 weeks of the cod experiment.*

Although the water sampling approach in the cod experiment was unsuccessful in determining water concentrations, the SPMD approach demonstrated that nominal exposure concentrations were achieved. It would have been useful to have included passive sampling devices in the zebrafish experiment as well, but due to the lower flow rate and minute compound quantities in this experiment, the risk of altering the exposure concentrations were considered to be high.

In addition to the above methods, PAH concentrations were monitored regularly during the cod experiment using an *in situ* enviroFlu-HC fluorometer (TriOS, Oldenburg, Germany). The fluorometer used excitation/emission wavelengths of 254/360 nm. The fluorometer was calibrated to a dilution series of the stock solution, and a dose related response was observed throughout the study. A protective cage was attached to the fluorometer to reduce fish interference. The pulsed exposure group was not monitored by SPMDs, but differences between pulse on and off were clearly observed using the fluorometer. The use of *in situ* fluorometers appears to be a suitable method for efficient day-to-day monitoring of low levels of PAHs in exposure studies, and added valuable information of exposure conditions in the current study.

Taken together, the ratio between actual and nominal concentrations was lower than expected in the zebrafish study. In the cod study, the ratios appeared to be compound specific and generally within 30% of nominal concentrations, as observed by SPMDs. The low ratios for some compounds could have been caused by evaporation or substrate specific bacterial degradation. Higher ratios may have been caused by elevated background exposure. In total, the exposure levels in both studies were determined to be in an environmentally realistic range.

4.4 Metabolites of PAHs and APs in bile

The measurement of contaminant metabolites in fish bile is a commonly used method for monitoring exposure. It has been successfully applied for characterising PAH exposure in a range of species, such as eel (*Anguilla anguilla*) (Ruddock et al., 2003), flounder (Richardson et al., 2001), and cod (Aas et al., 2006). Feeding status is known to impact fish metabolism and PAH metabolite concentrations, thus procedures for normalising metabolite data to e.g. bile biliverdin or protein content has been suggested (Collier and Varanasi, 1991). In the current study, values were standardised to bile weight, as additional errors may be introduced using protein standardisation methods (Aas et al., 2000b). Also, fish were starved for 2 days prior to sampling in an attempt to balance the fish' feeding status and to ensure accumulation of bile.

The small volume of bile in zebrafish reduced the sensitivity of the analysis compared to the analysis of cod bile. The average bile volume obtained was 0.32 mg corresponding to approximately 0.32 µl of bile. In comparison, the sample size for analysis of cod bile was 25 µl. When sampling the zebrafish, the entire gall bladder was excised and frozen to prevent evaporation of bile fluid. Efforts were focussed on measurements of 1-OH-pyrene using HPLC, due to the high relative accumulation of this metabolite in bile (Paper I) as well as the high method sensitivity for this compound. The lower weight limit for reliable metabolite quantification was approximately 0.02 mg bile and samples below this limit were not included in further calculations.

Phase II metabolism of xenobiotics in fish may produce glucuronide, sulphate and glutathione conjugates. For determination of individual PAH and AP metabolites using GC or HPLC, metabolites are often hydrolyzed due to few available standards of conjugated metabolites (ICES, 2005). In addition, conjugated metabolites are not amenable to GC analysis due to their low volatility. Samples were therefore hydrolyzed and derivatized

prior to GC analysis to improve both compound volatility and method sensitivity. Conjugated metabolites could be assessed using HPLC, although their separation would depend more on conjugated groups than mother compounds, resulting in poor separation. Hence, both GC and HPLC analysis involved a prior hydrolysis of attached endogenous groups by a mixture of glucuronide and sulphate deconjugating enzymes. PAHs and APs conjugated to glutathione were not determined, possibly resulting in an underestimation of metabolite levels. In plaice, glucuronidation has been found to be the major phase II pathway for benzo(a)pyrene conjugation, followed by glutathione and sulphatation (Leaver et al., 1992). In other species, proportion of glutathione conjugates can be significant although they also tend to be retained in liver (Varanasi et al., 1987).

4.5 Growth, reproduction and biochemical markers

Several population level parameters monitored in cod could not be assessed in zebrafish. Gonadosomatic index was not monitored due to the continuous spawning of female zebrafish. Also, as molecular and biochemical parameters were to be measured in zebrafish livers, they were instantly snap-frozen on liquid nitrogen to prevent modification or degradation of the targets. Therefore, zebrafish liver somatic index was not assessed. In addition, not enough blood could be obtained from zebrafish to assess hematocrit levels.

Due to the large difference in life histories, methods applied for monitoring reproduction was different for zebrafish and cod. Zebrafish are asynchronous, indeterminate batch spawners with females scattering clutches of eggs over a substrate. In the current study, a cage designed to collect eggs was used for day-to-day assessment of fecundity. Cod are group-synchronous, determinate batch spawners (Murua and Saborido-Rey, 2003), releasing their eggs in the free water masses. Cod fecundity was estimated by measurement of oocyte diameters (auto-diametric fecundity method) (Thorsen and Kjesbu, 2001). In addition, cod were stripped for egg and sperm (if running) for an additional 4 weeks and fertilization success assessed *in vitro*. Although hormonal injection could be used to trigger maturation and ovulation (Suresh et al., 2000), this was not an option in the current experiment as one of the assessed endpoints was time to start of spawning, in addition to the risk of affecting other endpoints.

Methods for determining CYP1A concentration and DNA adduct formation were successfully applied to both species. However, plasma vitellogenin or hepatic EROD activity assays was modified due to the limited amount of zebrafish tissue. Vitellogenin

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concentration was therefore determined in head and tail homogenates based on the method by (Holbech et al., 2001). An assay for analysis of zebrafish hepatic microsomal EROD activity was tested using HPLC with fluorescence detection (535/585 nm) to increase sensitivity. Although some reliable results were obtained using this method, it was unsuccessful in determining resorufin concentrations in the majority of samples due to interferences of unknown substances on the chromatograms.

It has been questioned whether the ^{32}P-postlabeling method for measuring DNA adduct concentrations would detect adducts based on few aromatic rings (two to three) (Aas et al., 2003). The method has been found to detect adducts from 4-ring PAHs as well as from styrene oxide, a 1-ring compound (Skarphéðinsdóttir, pers. comm.). In addition, elevated levels of DNA adducts using the ^{32}P-postlabeling method have been found in eel concurrent with bioaccumulation of two- and three-ring PAHs (Van Der Oost et al., 1994). Although the specificity of the method does not appear to be well characterised, a dose-dependent detection of adducts in the current study was obvious. In addition, a reduced sensitivity for two- and three-ring adducts could result in an underestimation of adduct concentrations in the current study.

In addition to previously published results, lysosomal membrane stability (LMS) and peroxisomal proliferation markers (AOX) were determined in 6 male and 6 female head kidney samples from Atlantic cod after 0, 2, 16 and 32 weeks of exposure. LMS has been shown to be a sensitive biomarker for produced water discharges in mussels (Bilbao et al., 2006a) and native saithe (*Pollachius virens*) (Bilbao et al., 2006b). LMS has also been used successfully in combination with other biomarkers to assess the size of environmental impact of dredging activities (Sturve et al., 2005). Measurements of AOX have been successfully applied to several marine species and induction is generally regarded as an indication of organic contaminant pollution (Cajaraville et al., 2003). Head kidney was used as the LMS method was unachievable in liver due to the high fat content. LMS was determined as described in Broeg et al., (1999) and AOX according to the method by Small et al., (1985). To my knowledge, this is the first time LMS and AOX have been measured in head kidney from Atlantic cod.

4.6 Transcriptomics

In Paper II and IV, a zebrafish oligo library (Compugen, Rockville, MD) and a cod cDNA library (Lie et al., in press-a) were used, respectively. The zebrafish array contained approximately 16,000 unique 65-mer oligonucleotide sequences of genes of known and unknown functions. For comparison, the cod array contained approximately 750 unique ESTs, included on the array for their properties of response to stress. The large size of the zebrafish array made the potential for discovery driven research large, as was reflected by the number of differentially expressed genes as well as the number of enriched clusters identified by gene set enrichment analyses. The massive down-regulations of gene transcripts as observed in exposed zebrafish were not apparent in exposed cod. This could have been due to the lower number of genes and the design of the cod array.

Large differences in fold-change values were also observed between species. This could reflect true inter-species differences or differences introduced by the applied methods, such as amplification and labelling approaches or platform properties. It has been shown that the two platforms (oligonucleotide and cDNA microarrays) may yield differences in both specificity and sensitivity (Hollingshead et al., 2005). On the other hand, microarray results from both studies were validated by an independent method; quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). This method include reverse transcription of mRNA to complementary DNA, which in combination with the polymerase chain reaction provides sensitive detection and quantification of mRNA levels in a large dynamic range (Mullis, 1990; Bustin et al., 2005). In the current studies, the same RNA samples as in the corresponding microarray experiment were used for first-strand cDNA synthesis. The range of fold-change values from corresponding microarray and RT-qPCR experiments were in agreement, thus the large fold-change differences between species were confirmed.

Potential interfering effects in qPCR experiments have recently been pointed out in combination with a proposal for minimum required documentation (Bustin et al., in press). The most critical factors affecting the correlation between microarray experiments and qPCR have been suggested to be cDNA synthesis conditions (Deprez et al., 2002), microarray data filtering (Morey et al., 2006), and normalization procedures (Vandesompele et al., 2002). In the current qPCR experiments, gene expression was normalized to the expression of selected reference genes. Other possibilities for

normalizing gene expression exist, such as to RNA, DNA or sample quantity. The three reference genes tested (Paper IV) have previously been demonstrated to be stable in several tissues from Atlantic cod (Olsvik et al., 2008). Nevertheless, reference genes should be thoroughly evaluated for each experiment as only two of the three reference genes appeared stable and could be used for normalization. In the zebrafish study, β-actin was selected and evaluated as it has been shown to be stable in this species (Tang et al., 2007; McCurley and Callard, 2008).

In both experiments, oligo d(T) primers were used, although alternatives exists (such as random hexamer or gene specific primers) each with specific advantages and limitations (Deprez et al., 2002). Also, an unspecific fluorescent probe detecting double-stranded DNA (amplicons) was used (SYBR), thus non-target amplification of cDNA template could not be discriminated. To control for mispriming or qPCR primer dimerization, melting curve analysis was performed and reaction efficiencies were evaluated for each primer pair (1.9-2.1). Also, negative controls (non-template and non-enzyme) were included to control for DNA contamination in reagents and RNA samples, respectively.

5 Results and discussion

5.1 Did metabolites in bile confirm exposure?

A dose dependent relationship between water concentrations and pyrene metabolites in zebrafish bile was clearly observed, but after 7 weeks of exposure metabolite levels in the high exposure group appeared to have decreased slightly (Figure 3). Also, a dose related trend was observed after 1 week of exposure, but there was no significance following statistical testing. As previously mentioned, the small amount of zebrafish bile reduced the sensitivity of the metabolite determination thus variability was expected. The results could however also indicate variability in the exposure system. The ratio of 1-OH-pyrene levels between zebrafish and cod bile were 0.2 to 0.5 (depending on exposure group), corresponding to the ratio of measured water concentrations between the two experiments. This indicated further that exposure levels in the zebrafish study were below nominal values. Thus, the measurement of pyrene metabolites in zebrafish bile proved a valuable tool in evaluating exposure conditions, even on an individual basis.
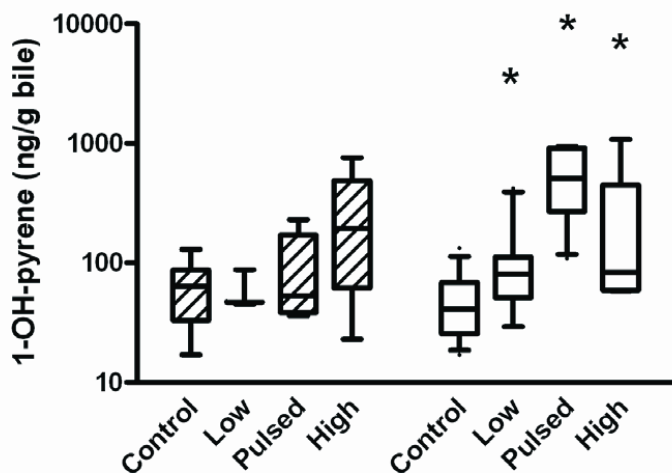

Figure 3. *1-OH-pyrene (determined by HPLC) in zebrafish bile after 1 (hatched) and 7 weeks of exposure. Significant differences (p<0.05; Dunnet's) from control group at each sampling are indicated with an asterisk. (Data representing 7 weeks of exposure adapted from Holth et al., (2008)).*

In cod, a larger quantity of bile was available and a more elaborate analysis could be performed. For most metabolites a relationship to dose was observed (Paper I and IV), and it was shown that the bile metabolite levels in cod from the low dosed group corresponded well with levels observed in Atlantic cod caged near an oil production platform in the North Sea (Aas et al., 2006). The relationship to dose was dependent on the relative lipophilicity (log K_{ow}) of the compounds (Harman et al., (2009); Paper I). Thus, poor relationships between metabolites in bile and water concentrations observed for compounds such as phenol, cresol and naphthalenes could have been due to their volatility. Another possibility could be that their favoured route of elimination was other than excretion to bile (e.g. urine). Overall, the results demonstrated that for most metabolites, concentrations in bile were reliable markers of low level PAH and AP exposure in Atlantic cod.

Table 2. *PAH and AP metabolites identified in cod bile (GC/MS-ToF) from the high exposure group (water exposed fish except for C1-OH-anthracene). Metabolites not present, as determined by co-injection of their standards, are also indicated. Table adapted from Paper I.*

Exposure group	Group of isomers quantified	Number of compounds quantified	Metabolites identified by co-chromatography	Recovery spike samples (%)	Metabolites not present in bile extracts
AP	Phenol	1	Phenol	125	
	p-cresol	1	*p*-cresol	106	
	4-ethylphenol	1	4-ethylphenol	127	
	4-*n*-propylphenol	1	4-*n*-propylphenol	90	
	4-*tert*-butylphenol	1	4-*tert*-butylphenol	100	
PAH	OH-naphthalenes	2	1-OH-naphthalene	66	
			2-OH-naphthalene	65	
	OH-fluorene	1	2-OH-fluorene	62	9-OH-fluorene
	OH-phenanthrenes / anthracenes	4	1-OH-phenanthrene	70	3-OH-phenanthrene 4-OH-phenanthrene 9-OH-phenanthrene
	OH-pyrene	1	1-OH-pyrene	193	
Alkylated PAH	C1-OH-naphthalenes	2		115	2-methyl-1-OH-naphthalene
				68	2-naphthalenemethanol
	C2-OH-naphthalenes	2		115	2-naphthalene-ethanol
	C3-OH-naphthalenes	2			
	C1-OH-phenanthrenes / anthracenes	5 (water exposed)		90	9-(hydroxymethyl)-anthracene
	C1-OH-anthracene	1 (orally exposed)			
	C2-OH-anthracene	1			

In addition to quantification of PAH and AP metabolites in bile, isomers of hydroxylated metabolites were identified for most of the compounds using GC/MS-ToF (Table 2). This qualitative analysis of PAH metabolites was performed on samples from the high exposure group and revealed that monohydroxy metabolites was the dominating form. This was in contrast to results by e.g. Goksøyr et al., (1986) which found dihydrodiols to be the dominating metabolites of phenanthrene in Atlantic cod. The reason for this is unclear, although in the current study, cod were exposed to a mixture of several PAHs and APs via water whereas in the study by Goksøyr et al., (1986), fish were intraperitoneally injected with phenanthrene only. Thus, differences in uptake due to exposure route could be a cause for the observed differences. The exposure to a mixture of PAHs and APs could also have led to a predominant monohydroxylation of the compounds, although the introduction of several PAHs and APs did not interfere with the quantity of metabolites formed (Paper I).

5.2 Impact of exposure on fish health and reproduction

Results indicated that environmental realistic concentrations of PAHs and APs had only minor effects on parameters related to health in zebrafish and Atlantic cod (Paper II and IV). Condition factor (Fulton's K) of male zebrafish was reduced compared to the control group at the end of the exposure period, indicating exposure-induced stress. A similar tendency was also observed in female zebrafish, although apparent differences were not statistically significant. Concurrent with the difference in condition factor, an apparent increase in female cumulative fecundity was observed in zebrafish from exposed groups. Neither the reduction in condition factor nor the increase in female cumulative fecundity appeared to be dependent on exposure dose or regime. Several other reproductive parameters in F0 zebrafish were investigated but were not found to be affected by the treatments.

Developmental effects (dead or malformed larvae) were observed in the F1 generation of exposed zebrafish. The malformations appeared as dorsal curvatures, previously attributed to e.g. 3- and 4-ring PAH toxicity (Incardona et al., 2005; Incardona et al., 2006). Similarly, gender specific effects on growth in addition to F1 generation mortality and deformations (curved spines) have been observed in zebrafish after exposure

to PFOS (Du et al., 2009). It should be noted that the total percentage of affected larvae in the current study was low (1%). In addition, eggs and larvae were reared in PAH and AP concentrations corresponding to the parental generation, thus it was not possible to elucidate whether the effect was due to maternal transfer or direct exposure. Maternal transfer of PAHs has been observed in fish exposed to high concentrations of naphthalene (Pollino et al., 2009) and anthracene (Hall and Oris, 1991).

Assessment of condition factor, liver somatic index, gonadosomatic index and hematocrit in Atlantic cod did not indicate any impact on general health, but an increased time to spawning was observed in the females receiving pulsed exposure (Paper IV). In addition, the onset of spawning (*in vivo*) was assessed for an additional 30 days, whereas concurrent successful stripping of eggs and sperm were more frequent in the control group than in any treatment group. While 17 *in vitro* fertilizations were achieved in the control group, only 5 were achieved in the exposed groups due to a lower number of successfully stripped females. Fluorescent aromatic compounds in bile have been associated to ovarian atresia in English sole (Johnson et al., 1997), whereas exposure of cod to APs via the diet have been shown to delay maturation without apparent oocyte atresia (Meier et al., 2007b). Stressed cod may also withhold eggs in the ovarian lumen (Thorsen et al., 2003). Whether the delayed spawning was caused by ovarian atresia, retainment of eggs in the ovarian lumen or other mechanisms is not known. Another explanation for a delayed maturation could be disruption of the hypothalamic – pituitary – gonadal axis, as has been shown in rainbow trout (*Oncorhynchus mykiss*) exposed to nonylphenol (Harris et al., 2001).

5.3 Were cellular processes affected?

5.3.1 Contaminant metabolism and elevated levels of DNA adducts

Cytochrome P450 1A

Metabolism and excretion of PAHs and APs did not appear to affect hepatic CYP1A protein concentration in zebrafish (Paper II). The metabolism of PAHs in fish has been associated with induction of phase I enzyme activity (Billiard et al., 2002), and it has been shown that the AhR receptor pathway in zebrafish is responsive following exposure to the water soluble fraction of oil (Arukwe et al., 2008). Nevertheless, the water soluble fraction of oil may also contain dispersed oil and therefore high-molecular weight PAHs known to be effective AhR agonists. Thus, the results indicate that low-molecular-weight PAHs were not efficient inducers of the AhR pathway, as has been observed for naphthalene and fluoranthene (McKee et al., 1983; Willett et al., 2001), or that antagonists were present, such as some PAHs and APs (Sturve et al., 2006).

In female cod, the induction of CYP1A activity (EROD) in the low dose group following short-term exposure indicated effects of AhR agonists (Paper III). However, after long term exposure the induction in EROD activity was reduced to background levels. In the high dosed group, an apparent reduction compared to fish in the control group indicated adaptation and/or the presence of antagonists. On both sampling occasions, fish in the pulsed group were receiving control treatment and no effects in CYP1A activity were observed. It is not known whether effects were present at other time points. It should also be pointed out that the measured EROD activity levels (maximum 30 pmol min^{-1} mg^{-1} protein) were low compared to levels measured in wild Norwegian coastal cod, where a background EROD level between 10 and 100 pmol/min/mg protein has been regarded as baseline activity outside spawning periods (Hylland et al., 2009). Compared to these levels, the impact on EROD activity in the current study would be regarded as reflecting normal metabolism.

Formation of DNA adducts

Adduct formation was assessed in zebrafish after 0, 1, 3 (control and high only) and 7 weeks of exposure (unpublished results, Table 3). Overall, levels were low and close to the detection limit of the ^{32}P-postlabeling method. It has been suggested that levels above the detection limit could indicate contaminant related formation of DNA adducts in field-sampled fish (Aas et al., 2003). After 7 weeks of exposure, an apparent dose related trend was observed, but levels in female zebrafish from the high dose treatment were no different than those measured in controls after 1 week of exposure.

Table 3. *Hepatic DNA adducts in female zebrafish as determined by the ^{32}P-postlabeling method. Livers from 4 individuals from each replicate tank were pooled prior to measurements, thus individual n represent values from replicate tanks (except zero time samples which are individual samples). The rightmost column presents the limit of detection (LOD) for the remaining samples where DNA adducts were undetected.*

Type	Weeks of exposure	Total n	n with adducts	DNA adducts	DNA adducts (LOD)
Zero time (mean ± SD)	0	10	4	0.47 ± 0.06	1.22 ± 0.30
Control	1	3	2	1.72, 1.83	0.51
Low	1	3	2	0.68, 0.57	0.54
Pulse	1	3	1	0.85	0.72, 0.47
High	1	3	2	0.64, 0.85	0.66
Control	3	3	2	0.49, 1.02	2.34
High	3	3	1	1.22	1.59, 2.06
Control	7	3	2	0.51, 0.99	0.44
Low	7	3	2	0.67, 0.97	1.13
Pulse	7	3	0	-	0.59, 0.68, 1.10
High	7	3	2	1.70, 1.83	0.98
			Grand average (mean ± SD)	0.98 ± 0.46	1.00 ± 0.60

High levels of DNA adducts were apparent in female cod from the high exposure group in the current study (Paper III). The maximum observed level (72.5 nmol adducts mol^{-1} normal nucleotides; hereafter nmol/mol) were comparable to levels observed in fish after acute environmental oil exposure e.g. the "Erika" oil spill resulting in 92-290 nmol mol^{-1} in native common sole (*Solea solea*) (Amat et al., 2006) or in cod exposed for 30 days to 1 ppm dispersed crude oil (approx 100 nmol mol^{-1}) (Aas et al., 2000a)). In the latter study, although DNA adducts formed in the 1 ppm group in a matter of days, adducts were not evident in the low dose group (0.06 ppm) until 30 days of exposure. In the current study, DNA adducts were formed in the low and high dosed group between 16 and 44

weeks of exposure. This suggested that the formation of DNA adducts, although being a sensitive parameter, might be underestimated when applied in short term monitoring campaigns using caged fish.

DNA adducts were also detected in two female cod in the control group after 44 weeks of exposure, but the levels in these fish (0.65 and 2.07 nmol/mol) were both within the range of normal background values previously observed in native Atlantic cod (Aas et al., 2003). Nevertheless, the autoradiograms showed that these spots was situated in the PAH radioactive zone (Amat et al., 2006), suggesting them to be PAH originated adducts. The reason for this could be that the fish received a low background level of certain water-borne PAHs, as previously discussed.

Cod in the pulsed group developed DNA adducts (<1.6 nmol/mol) at a similar level as the control fish. This result was surprising as the PAH metabolites in bile demonstrated levels similar to the high exposure scenario throughout the study. This suggested that a continuous exposure load may be an important factor for formation of DNA adducts, at least at concentrations relevant to offshore oil production areas.

No apparent relationship between hepatic CYP1A activity (EROD) in female cod and formation of DNA adducts was observed in the current study, which could have implications for the use of these biomarkers in monitoring programmes. For example, in a recent study, DNA adducts were only analysed in samples with induced EROD activity (Schnell et al., 2008). Results from the same study showed that the site with highest observed DNA adduct levels was the site with lowest EROD activity and highest 1-OH-pyrene in bile. Although fish from groups with high levels of DNA adducts had the lowest EROD activities (Paper III), these two parameters were determined on different sampling points and thus not in the same individuals. The possibility that there is a link between the two responses can therefore not be excluded.

5.3.2 Effect on lysosomal stability

Lysosomes are organelles containing enzymes for digestion of endogenous and exogenous substances. The permeability of the lysosome membrane may be affected by chemicals leading to the release of hydrolytic enzymes. In flounder, it has been proposed that measurement of hepatic lysosomal membrane stability (LMS) has predictive capabilities of ecologically relevant endpoints via the support of carcinogenic processes (Kohler and Pluta, 1995; Kohler et al., 2002), and that fish intermittently exposed to chronic anthropogenic stress may have lower potential or need a longer period to recover from the effects of pollution (Broeg et al., 2002).

Cod from the pulsed and high treatment groups had significantly reduced LMS compared to fish from zero time, control or low dosed group (Figure 4; Beckius et al., in prep). No differences were observed between gender or exposure time. The responses developed already after 2 weeks of exposure and remained stable until week 32 (last sampling point). The maximum labilization time observed in this study was 20 minutes in fish from the zero time sampling, control group and low exposure group. It has been suggested that in fish, values less than 10 minutes indicate the onset of liver histopathologies, 20-30 minutes indicate compensating stress and values over 40 minutes reflect an unaffected state (Viarengo et al., 2007). Labilization times determined in head kidney from unaffected sea bass (*Dicentrachus labrax*) were in the range of 30-35 minutes (Romeo et al., 2000). Thus, given the previous classification, a stress response was observed in cod head kidney even in control individuals. On the other hand, labilization time may vary significantly between tissues and species (Broeg et al., 2002). No adaptation to the exposure was apparent even after 32 weeks of exposure, promoting the use of LMS as a robust biomarker in cod head kidney. This is also in accordance with the results of chronic studies on other fish species (Broeg et al., 2002). Hence, LMS appears promising for monitoring of produced water impacts on cod, although it should be noted that the method did not discriminate between low and control exposure. More studies on the use of LMS in head kidney of cod are recommended as there is a need for comparative results.
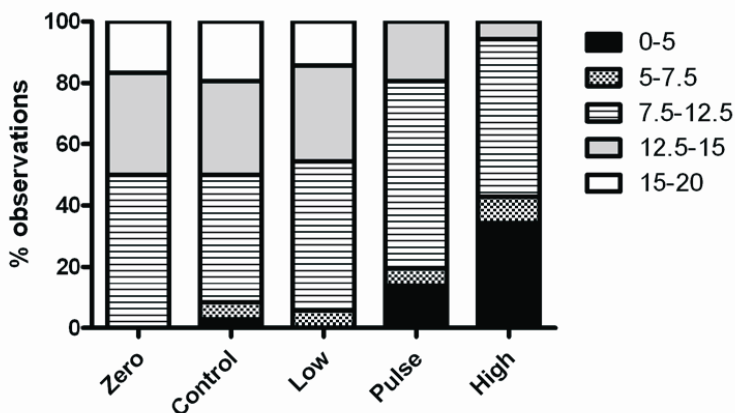
Figure 4. Lysosomal membrane stability (LMS) determined in cod. As no differences were observed between genders or over time, data represent values in both males and females integrated over the exposure period of 32 weeks (last sampling point). Pulse and dosed group were different from zero, control and low (Dunnet's, p<0.005). Labilization time was categorized in minutes as indicated in legend.

5.3.3 Enzymatic activity in peroxisomes

Peroxisomes are ubiquitous organelles involved in cellular metabolic processes such as lipid metabolism. They may expand quantitatively as a response to pollutants binding to peroxisome proliferator-activated receptors (PPARs) with a concomitant induction of certain enzyme activities, such as acyl-CoA oxidase (AOX) (reviewed in Cajaraville et al., (2003)). Chemicals with such properties are often called peroxisome proliferators, and include both PAHs and APs. Effects of peroxisomal proliferation in fish are not well characterised, although a connection to endocrine disruption might be based on receptor cross-talk (Ibabe et al., 2005; Batista-Pinto et al., 2009). In mammals, PPARs have been shown to be implicated in cell differentiation and carcinogenesis (Sertznig et al., 2007).

In the current study, both sex and time were identified as factors modifying AOX activity in cod head kidney. Males had lower enzymatic activity than females, and a temporal reduction in AOX activity was apparent. However, after 2 weeks of exposure, a dose related pattern was observed in males showing significant induction in the high dosed group (Dunnet's, $p < 0.05$). No obvious exposure related differences were observed in males or females at other sampling times. The maximum AOX activity levels in the current study were similar to, or lower than, hepatic levels in saithe (Bilbao et al., 2006b) and cod (Bilbao et al., 2006a) from a reference area in the North Sea. Thus, although it appeared

that AOX activity in cod head kidney to some extent was affected by the treatments, the overall sensitivity was poor and the variability was large.

5.4 Were higher level effects related to changes in gene expression?

As previously discussed only weak effects on reproduction were observed in female zebrafish. The apparent increase in female cumulative fecundity was not related to exposure dose. On the other hand, expression of genes related to reproduction was clearly affected (Paper II). Gene transcript levels coding for several egg-specific proteins (zona pellucida) in addition to an androgen synthesizing enzyme, 17β-hydroxysteroid dehydrogenase, were down-regulated. In a study by Meier et al., (2007b), C_4-C_7 alkylphenols were shown to affect steroid levels in fish in a dose independent manner. In addition, recent studies have indicated that androgens may influence fecundity in fish (Kortner et al., 2009). Therefore, a tentative explanation to the observed response in reproduction could involve effects on steroid metabolism.

Vitellogenin mRNA expression and levels of circulating vitellogenin protein appeared not to be affected. Although some short-chained alkylphenols have been shown to bind to the rainbow trout estrogen receptor, their affinity compared to the endogenous ligand 17β-estradiol is low (Tollefsen and Nilsen, 2008). The levels of APs in produced water may not be sufficient to cause vitellogenin induction in fish (also see Sundt et al., (2008)). It has also been shown in several fish species that some reproductive parameters may be even more sensitive than the induction of vitellogenin production (Gimeno et al., 1998; Hill and Janz, 2003).

In zebrafish, effects were observed in both condition factor and lipid metabolism genes (Paper II). In a study by Smolders et al. (2003), lipid energy budgets were identified as the most sensitive endpoint from a range of gross indices in zebrafish exposed to effluents, and were correlated to other endpoints such as condition factor, respiration and reproductive output. This relationship has also been demonstrated in carp exposed to effluents (Smolders et al., 2004) and was further confirmed by the observation of differential expression of lipid metabolism genes (Moens et al., 2007). It appears likely that the differential regulation of lipid metabolism genes might be linked to the reduction in

condition factor. Two genes related to lipid metabolism were suggested as potential biomarkers in zebrafish (fatty acid synthase and CCAAT enhancer-binding protein-alpha).

In cod, the only affected parameter with relevance at a population level was a delayed time to spawning in female cod receiving pulsed exposure. On the level of gene expression, equivocal effects were observed on male zona pellucida gene regulation and vitellogenin mRNA expression was generally below the detection limit (Paper IV). In cod, zona pellucida genes appear to be under estrogenic control (Oppenberntsen et al., 1992), and concurrent regulation with vitellogenin genes in response to environmental pollution has been observed (Olsvik et al., 2009). The equivocal expression of estrogen responsive genes in cod suggested that population level reproductive effects were (a) unrelated to estrogen regulated pathways, or (b) more sensitive endpoints than vitellogenin induction, or a combination of both.

No exposure related effects were observed in gross health parameters in Atlantic cod, nor in the expression of related genes. It has been shown that APs may affect growth and change the fatty acid profile of polar lipids in Atlantic cod liver (Meier et al., 2007a), demonstrating the responsiveness of these pathways in Atlantic cod, which potentially could affect the expression of genes involved in lipid and fatty acid metabolism. This may indicate antagonistic chemical interactions or that environmentally relevant levels of produced water were too low to induce effects in these pathways in Atlantic cod.

Effects were observed in AhR-related genes in cod, but levels and activity of CYP1A protein were not related to this gene expression or to the formation of DNA adducts. Although the formation of PAH originated DNA adducts in fish has previously been linked to induction of CYP1A protein (Myers et al., 2003), to my knowledge this link has not been extended to the level of gene expression. The reason for this might be that for long-lived proteins, such as CYP1A, gene expression and protein levels may not always correspond (Kloepper-Sams and Stegeman, 1994; Kammann et al., 2008). These studies in combination with results from the current study indicate that gene expression of CYP1A does not appear as a useful indicator to predict DNA adduct formation. However, as also observed by George et al., (2004), the measurements of CYP1A mRNA or protein levels might still be useful as exposure markers.

5.5 Did zebrafish and Atlantic cod respond similarly?

In the following section, response similarities and differences between species will be discussed. A summary of endpoints and observed effects are given in Table 4.

Table 4. *Overview of parameters assessed in zebrafish and cod after exposure to components of produced water. Effects are indicated by "+".*

Endpoint	Zebrafish		Cod	
	Dose response	Temporal trend	Dose response	Temporal trend
Metabolites in bile	+[1]	-	+	-
Somatic indices	-	+	-	-
Reproduction	+	-	+	na
F1 generation effects	+	na	na	na
Vitellogenin protein	-	na	na	na
CYP1A	-[2]	-[2]	+[3]	+[3]
DNA adducts	-	-	+	+
LMS	na	na	+	-
AOX	na	na	-	-

[1]1-OH-pyrene
[2]CYP1A quantity
[3]CYP1A activity (EROD)
na = not available

In both zebrafish and cod, clear dose related responses were observed in pyrene metabolites. Although interspecies teleost metabolism of PAHs is thought to be similar, differences have been observed between closely related species such as dab (*Limanda limanda*) and flounder (Kammann, 2007). In that same study, normalisation of 1-hydroxypyrene to biliverdin appeared to eliminate the difference. Other studies have indicated that differences between species might be small, and that normalisation of metabolite data has little effect on results (Vuorinen et al., 2006). For the purpose of monitoring exposure conditions, the assessment of pyrene metabolites in bile normalised to bile weight provided valuable information for both species in the current study.

The exposures did not appear to have a large impact on gross parameters or reproduction in neither species. Zebrafish appeared to be more sensitive than cod for changes in condition factor and lipid metabolism, and genes involved in zebrafish lipid metabolism pathways appeared to contain relevant endpoints for characterising effects of

components of produced water. As previously discussed, zebrafish might be a sensitive species for effects on lipid metabolism and health parameters. In addition, simultaneous exposure of cod and turbot (*Scophthalmus maximus*) to dispersed crude oil and APs suggested that cod might be more resilient for effects in gross parameters than other species (Martin-Skilton et al., 2006). A reason for this species difference might be due to differences in lipid storage strategies, of which cod have large lipid reserves in liver and thus might be less sensitive to changes in such systems.

Although the difference in DNA adduct formation between species was unexpected, the low exposure levels implied a long latency period for DNA adduct formation in cod (Paper III). It might be that the duration of the zebrafish experiment was not sufficient for formation of elevated amounts of adducts. Also, cod has been shown to be a sensitive species for DNA adduct formation (Aas et al., 2000a; Paper III). In addition, other tissues than liver might be more sensitive to DNA adduct formation in zebrafish (Hsu and Deng, 1996). Species differences in PAH disposition or rate of biotransformation (phase I or II systems) are well known and has been associated with differences in DNA adduct formation and prevalence of liver neoplasms in fish (Collier et al., 1992; Willett et al., 1997). Differences in DNA adduct patterns after oil exposure have been observed between species even within the same taxonomic family such as cod and polar cod (*Boreogadus saida*) (Aas et al., 2003), suggesting differences in toxicodynamics and/or kinetics to be significant factors. Similarly, differences in toxicokinetics have also been observed in several flatfish species exposed to the same pollutant load (Eggens et al., 1996; Anulacion et al., 1998).

In summary, differences in responses were observed between zebrafish and cod at all levels of biological organisation, suggesting that effects of produced water exposure might be difficult to extrapolate between these two species, and that choice of model species for assessing ecological impact of produced water exposure must be carefully evaluated.

6 Conclusions and perspectives

The results of this study demonstrated that environmentally relevant levels of components of produced water may affect condition factor, maturation, biochemical processes and gene expression in fish. The usefulness of bile PAH and AP metabolite measurements to evaluate exposure was demonstrated in two fish species.

The development of biomarker responses was shown to depend on exposure regime as well as exposure period. Lysosomal stability (LMS) in cod kidney was related to dose, the effects were observed early (within two weeks) and remained at a stable level throughout the exposure period in fish receiving both continuous and pulsed exposure. Thus, LMS appeared to be a useful marker for effects in Atlantic cod.

Formation of DNA adducts in female cod liver was also related to dose, but in contrast to LMS, more than 16 weeks was required for formation of significant levels. This parameter may therefore be underestimated following short-term exposures, such as most offshore fish caging studies (often 4-6 weeks). Although a time-dependent increase was observed, it also required a continuous exposure regime, which is not often observed in the environment.

Other biomarkers were demonstrated either to adapt or appeared to be insensitive to the exposures. CYP1A activity (EROD) in female cod was responsive on occasion, but a reduction of activity over time was observed. Protein levels of vitellogenin and hepatic CYP1A in zebrafish, as well as AOX in cod kidneys, were not affected in the current study.

Gene transcription of several distinct cellular mechanisms was clearly affected in both species, and a predominance of differentially expressed genes in zebrafish was down-regulated. This indicated that down-regulation of responsive pathways may be as important or more important than up-regulation.

As both presence and absence of effects following pulsed exposure were apparent (DNA adduct formation; oocyte maturation; condition factor), the effects of exposure regime on biomarkers used in monitoring of offshore produced water discharges should be further investigated.

Response differences between the two species suggested that extrapolation between species could lead to under- or overestimation of effects depending on the parameter assessed. Effects in zebrafish following exposure to components of produced water could not be directly extrapolated to effects in Atlantic cod.

In conclusion, the results of this study showed that exposure to environmentally relevant levels of PAHs and APs had clear effects on fish (LMS, DNA adducts, maturation, condition factor) , and that some markers currently used in environmental monitoring appeared not to be sufficiently sensitive for detecting effects of low contaminant levels (CYP1A, vitellogenin, AOX), or were adapted over time (EROD). In addition, effects on gene expression suggested that yet other pathways could be affected. There is thus a need for sensitive as well as alternative methods to better characterize the effects of produced water discharges.

Sensitive methods exist for early detection of DNA damage in single cells (comet) as well as methods for determining fatty acid profiles and metabolism of steroids. Also, genetic tools should be further developed for species or taxonomic groups ecologically relevant to the North Sea as well as the Barents Sea. In addition, transcriptomic approaches indicated effects on several processes crucial to fish health, such as disease susceptibility, carcinogenesis, steroid metabolism and neurological alterations, which should be further investigated.

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