Proteome changes in Atlantic cod (*Gadus morhua*) exposed to oil and produced water:

Discovery of biomarker candidates for environmental monitoring

Anneli Bohne Kjersem

Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

July 2007

Scientific environment

This Ph.D project is funded by Total E&P Norway, and was in 2005 integrated into a larger research project within the PROOF program by the Norwegian Research Council.

My supervisors and those contributing the most to this project are Prof. Anders Goksøyr (Department of Molecular Biology, University of Bergen) and Dr. Bjørn Einar Grøsvik (present address Institute of Marine Research, Bergen, Norway).

Laboratory exposures of juvenile cod exposed to crude North Sea oil, alkyl phenols and poly-aromatic hydrocarbons were conducted at IRIS Akvamiljø (Melkjarvik, Norway), set up and carried out by Arnfinn Skadsheim.

Sonnich Meier planned, set up and carried out the laboratory exposures of cod larvae and fry exposed to produced water and oestradiol, and these exposures were performed at the Industry Lab at the High-technology Centre in Bergen. Gunnar Nyhammer, Department of Biology, the University of Bergen, was also deeply involved in running these laboratory exposures.

MS-MS analysis were performed both at the Proteomic Platform at the University of Bergen, PROBE, and at the University of Southern Danmark (SDU) in Odense, Denmark. Magnus Artnsen, Olav Mjaavatn, Hilde Garberg, and Clive De Santos at PROBE were responsible for training, trouble-shooting and running samples on the Q-ToF. Andrea Maria Lorentsen and Nicolai Bache at the Protein Chemistry Group at SDU have provided training as well as run samples on the MALDI-ToF-ToF. LIMS and training using LIMS was provided by Kristian Flikka at the Computational Biological Unit (CBU) at the University of Bergen.

The newly developed cod EST database used in this study is formed from and based on the work of Pål Olsvik´s group at NIFES (the Norwegian Institute for Research on Seafood and Nutrition, Bergen), Frank Nilsen´s group at the Institute of Marine

Research (Bergen), as well as the work of Anders Lanzén and Pål Puntervoll at CBU, the University of Bergen.

Karen Anita Aa did her Master degree as an integrated part of the project working on the study of cod fry liver exposed to produced water and oestradiol.

Acknowledgements

This PhD would never have been completed without the efforts of several people. First of all I would like to thank my two supervisors Prof. Anders Goksøyr and Dr. Bjørn Einar Grøsvik for this wonderful opportunity, for all the time and effort they have contributed to this work, and for all their encouragement and guidance. My thanks also go to Total E&P for funding and support.

Thanks also to the "Lab 2 family" and all my friends and colleges at MBI for creating such a nice working atmosphere and for all the good laughs, to my fellow Ph.D. students Christina Tolfsen and Karin Berg for the good companionship and the fruitful discussions over the years, and to Karen Anita Aa for choosing Lab 2 and this project for her Master and all her efforts during this period. Anne Elisabeth Madsen, our lab manager, Magnus Blø and Tina Søfteland also deserves thanks for technical assistance.

I also wish to thank my co-authors Arnfinn Skadsheim, Nicolai Bache, Sonnich Meier, Gunnar Nyhammer, and Peter Roepstorff for all the hard work and enthusiasm they have put into this. I enjoyed so much working with you all and I am impressed by your work!

I would like to acknowledge Magnus Arntsen, Clive de Santos, Hilde Garberg and Olav Mjaavatn at PROBE, and Kristian Flikka, Pål Puntervoll and Anders Lanzén at CBU for providing technical assistance and training.

To all of my friends: Thank you for being there!

Finally I would like to thank my family:

Mamma, the completion of this work is first and foremost thanks to you! Thanks for looking after me and my little family. My thanks also go to my big brother Rolf André for always being so positive and enthusiastic; making me believe everything is possible. The support and interest in my work of my family in law is also highly appreciated. Thanks also to Jorunn Kristine, Tirill and Edvard, and Guro, Geir and Janne Beathe for all good hugs and moments during these years. To Eyvind and Ane: I am so grateful to have you in my life! Thanks for being there, - you are everything and I love you so much!

Abstract

Produced water is the most dominating source of continuous oil discharges to the sea offshore. It contains a wide range of compounds such as alkyl phenols, polyaromatic hydrocarbons (PAHs), heavy metals, carboxylic acids and other organic compounds, which may cause harm by themselves or in synergy with other compounds present in produced water. Worldwide there is a growing concern that contamination of the marine environment may constitute an important contribution to the reported decline in harvested populations of marine fish species, and early life stages have been reported to be more sensitive to xenobiotic exposure than adult fish.

Proteomics were applied to identify changes in the proteome of Atlantic cod (Gadus morhua) exposed to crude North Sea oil and North Sea produced water at different life stages and during early development. Apparent protein changes were identified and linked to possible signalling pathways and mechanisms involved in the biological response of fish following exposure. Exposure to North Sea crude oil and produced water appeared to induce a large number of changes, also at low levels of exposure. More than 40 of the 137 protein changes detected in plasma of juvenile cod following exposed to crude oil and surrogate produced water appeared at the lowest level of exposure, 0.06 ppm crude oil. Almost all of the protein changes detected in whole fry and fry liver following produced water exposure occurred at the lowest levels of produced water, 0.01% and 0.1% produced water.

In total 137 proteins appeared to change in plasma of juvenile cod exposed to crude oil and oil spiked with PAHs and alkyl phenols. 84 proteins appeared to change in whole cod fry exposed to produced and oestradiol from egg to fry stage whereas 105 proteins appeared to change in liver of cod fry exposed to produced and oestradiol. Comparing the results from produced water exposure with oestradiol exposure,

suggest that some of the responses may be related to endocrine disrupting effects. However, about 25% - 50% of the produced water affected proteins did not change following oestradiol exposure.

The biomarker candidates found in this study includes a vide range of different protein previously been reported to be involved in a variety cellular and molecular functions, indicating effects on the immune system, the fibrinolytical system, iron hemostasis, fertility, metabolism, perception, morphology and development.

The levels of exposure used in this study are supposed to reflect both acute discharges and long-term, chronic exposures, levels of exposure close to back-ground levels and distances far from the platforms, as well as higher levels of exposure comparable to the expected concentrations in close proximity of the oil installations offshore. The biomarker candidates discovered in this study will have to be verified and validated for use as biomarkers in environmental monitoring in future studies.

The PhD project “Proteome changes in Atlantic cod (Gadus morhua) exposed to oil and produced water: Discovery of biomarker candidates for environmental monitoring” is funded by Total E&P Norway and was carried out at the Department of Molecular Biology, The University of Bergen, Norway.

Abbreviations

A1AT	Alpha-1-antitrypsin
A2A	Alpha-2-antiplasmin
ANOVA	Analysis of variance
AP	Alkyl phenols
APE	Alkylphenolethoxylate
APO B	Apolipoprotein B
BA	Benzene alcohol
blast	Basic local alignment search tool
BSA	Bovine serum albumin
BXT	Benzene, xylene, toluene
cCBB	collodial Coomassie Brilliant Blue
CYP1A	Cytochrome P450
1DE	One-dimensional gel electrophoresis
2DE	Two-dimensional gel electrophoresis
dpf	Days post fertilization
E2	17β oestradiol
ELISA	Enzyme-linked immuno-sorbent assay
ERA	Environmental risk analysis
ESI	Electrospray ionisation
EST	Expressed sequence tag
EU	European Union
FT-MS	Fourier transformation mass spectrometry
GNP	Gross National Product
HC	Hydrocarbons
HELCOM	The Helsinki Commission: Baltic marine environment protection commission
Hsc	Heat shock cognate
Hsp	Heat shock protein
ICAT	Isotope-coded affinity tags
IPG	Immobilized pH-gradient
LC	Liquid chromatography

MALDI	Matrix-assisted laser desorption/ionisation
MS	Mass spectrometry
MudPIT	Multidimensional protein identification technology
OLF	Oljeindustriens landsforeningen (The Norwegian Oil Industry Association)
OSPAR	The Oslo-Paris Commission: Convention for the protection of the marine environment of the North-East Atlantic
PAH	Poly-aromatic hydrocarbons
p-blast	Protein blast
Prepro-APO A	Prepro-apolipoprotein A
PSI	Position-specific iterated
PW	Produced water
PZP	Pregnancy zone protein
QA	Quality assurance
SELDI	Surface-enhanced laser desorption/ionisation
SILAC	Stable-isotope labelling in cell culture
sPW	Surrogate produced water
ToF	Time-of-flight
Vtg	Vitellogenin
WHO	World Health Organization

Contents

1. Introduction

1.1 The Petroleum Industry

1.1.1 Production and the History of the Petroleum Industry

The Norwegian oil adventure started in 1969, when a Phillips Petroleum mobile drilling rig struck oil and gas in the Ekofisk field, ca 250 kilometres out of Stavanger (Hansen et al. 1984). The production on the Ekofisk began in 1971.

Since the beginning of the Norwegian oil adventure in 1969, oil and gas for more than 5000 billion NOK has been produced. Norway is today the world's third largest exporter of oil and the world's eighth largest exporter of gas. Of the total Norwegian export, the oil and gas export accounts for approximately 50 %, contributing to approximately 25 % of the Norwegian GNP. Today 50 oil fields are operative in the Norwegian zone, producing approximately three million barrels of oil every day, and 85 billion Sm^3 of gas on an annual basis. Never before have the investments in the Norwegian petroleum industry been greater: In 2006 more than 100 billion NOK are estimated to have been invested on the Norwegian shelf, and 2100 billion NOK have so far been invested in developing the Norwegian oil and gas industry (OLF 2006a).

1.1.2 Production and Distribution

Offshore oil and gas installations are densely distributed in the North Sea along the Norwegian coast and at the Norwegian Continental Shelf (Figure 1, OLF 2006b).

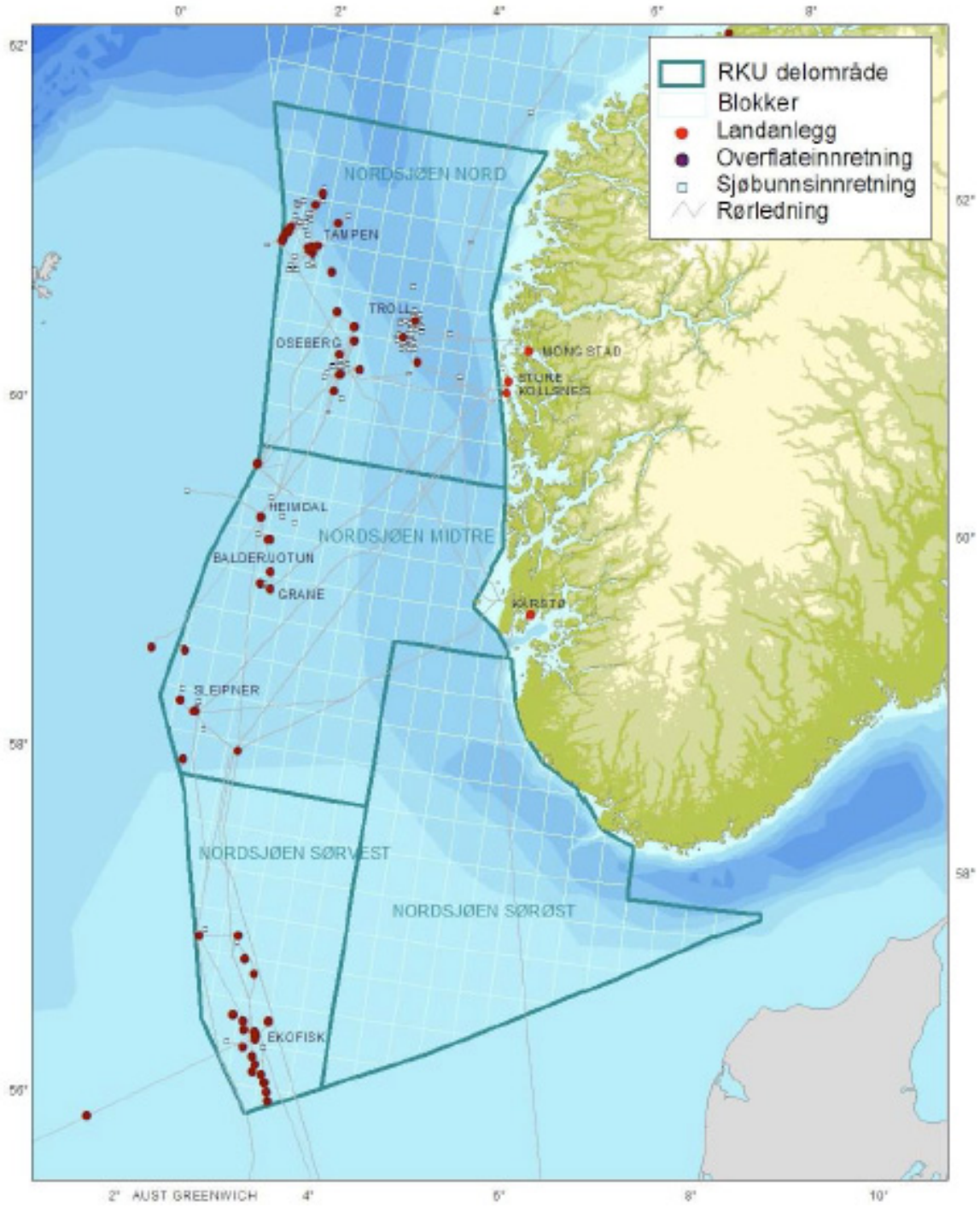


Figure 1: Oil installations at the Norwegian Continental Shelf, the North Sea. Oil installations are marked as red dots, whereas connecting pipelines are lined in grey. Reproduced with permission from OLF (OLF 2006b).

The oil production has increased over the years, and large amounts of oil produced place Norway among the world's largest producers of oil (Figure 2).

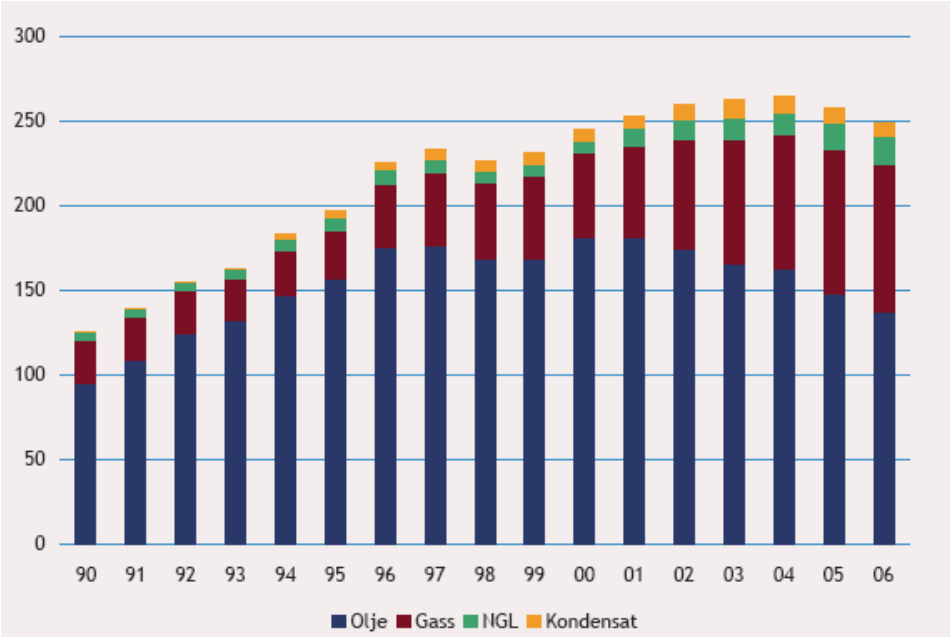


Figure 2: **The annual production of oil and gas on the Norwegian Continental Shelf, the North Sea.** Production of oil (million Sm^3) is marked in blue and production of gas (million Sm^3) is marked in red. Reproduced with permission from OLF (OLF 2007).

Prognoses estimate a continued increase in total oil and gas production in the North Sea to 2008. Long-term estimates calculate a decrease in oil production, whereas the gas production is estimated to be continuously high in the future.

1.1.3 Risk Assessment and Management

Ecological/ environmental risk assessment (ERA) is defined as the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi 1994; van der Oost et al. 2003) The risk assessment process identify and quantify the risk resulting from a special use or occurrence of a chemical compound, of which risk analysis determines the risk of a certain situation, whereas risk assessment examines a solution to the problem (van der Oost et al. 2003).

In the past decades, formal risk analysis has played an increasing influential role in public policy, from local and national to international level. In the early 1970s, risk analysis evolved into a major policy decision tool. It has become a dominating tool for energy, environment, health, and safety decisions, both public and private (Kammen and Hassenzahl 1999).

Long-term effects of discharges to the sea from the petroleum activities has for many years been a hot topic both in research and in the Norwegian society as a whole. There is a key concern of an overall impact on the marine environment that may disturb a sustainable exploration of our natural resources (Waldichuk 1979; Worm et al. 2006). Hence, research programmes and risk analysis has been initiated to aid risk management: PROOF, a 2001-2009 research programme by the Norwegian Research Council has as a main objective to increase knowledge concerning the long-term effects of discharges from the petroleum activities. The purpose is to provide a better basis for the authorities to make decisions when coordinating the petroleum activities, such as in Lofoten and the Barents Sea, with other uses (e.g. fishing) and protection of the marine environment.

Previously, studies monitoring biological effects of the North Sea have reported effects of contamination on fish embryos and the early development of pelagic fish (Cameron et al. 1996). Malformation rates higher than average was found under the influence of estuaries and in the coastal areas of the southern part of the North Sea (ibid.). The regional pattern of malformation rates of pelagic fish embryos observed in the North Sea was also found to be in good agreement with the distribution of several chlorinated hydrocarbons and with the distribution of heavy metals.

Other reviews have been studying the ecological effects of oil spills and subsurface blowouts specifically (Hamdoun et al. 2002; Rye et al. 2000; Teal and Howarth 1984). One of these studies conclude that under unfavourable conditions, the overlap between the hydrocarbon concentration fields and the geographical distribution of larvae in the North Sea along the west coast of Norway, may cause a significant impact on a population level (Rye et al. 2000).

There is also in these risk analyses an on-going discussion to what extent oil discharges are distributed and diluted in the environment. (Moore and Dwyer 1974) concluded that 1-2 days of weathering significantly reduces the toxic effects of oil spills. This was contradicted in the review by (Teal and Howarth 1984). Calculations from the Bravo blow-out in the Ekofisk field in the North Sea in 1977 estimated about 20 x 10^3 tons of oil to be blown in the air, resulting in an oil slick covering about 40 000 km^2 as 12 x 10^3 tons of oil fell back onto the sea surface. Up to 300 µg/l of oil were detected within 18 km of the blow-out.

In 1997 the parliament white paper No- 58 (1996-1997) “Environmental Policy for a Sustainable Development” introduced the zero-discharge concept. Based on this report a goal of zero environmentally harmful discharges to sea by year 2005 was established. As a general rule no environmentally harmful substances should be discharged, be it added chemical substances or natural occurring chemical substances. The goal applies to all offshore operations encompassing both drilling and well operations and production as well as discharges from pipelines.

1.1.4 Future Petroleum industry and Exploration in Polar areas

After more than 40 years of oil and gas activities in Norway, approximately 30% of the estimated petroleum resources have been explored. Existing and new developments with an estimated production of a total volume equivalent to the volume of petroleum resources already produced have been approved. It is assumed that an additional 6 percent can be recovered by new discoveries in existing fields, and yet, that another 6 percent can be produced if methods and technology is developed to increase the recovery rate in existing fields. The Norwegian Petroleum Directorate projects that 26 percent of the remaining resources on the Norwegian Shelf have not yet been discovered (OLF 2006a).

More than 60 exploration wells have been drilled in the Barents Sea area since 1980. The parliament white paper No- 38 “Oil and Gas activities” (2001-2002) stipulates further requirements regarding oil and gas activities in Lofoten and the Barents Sea, such as zero produced water discharges during normal operations for all new, not yet approved, field developments in Nordland VI, all areas north of the 68º N, and in areas where the environment is particularly vulnerable. Nor must there be any discharges to the sea during drilling of wells, with the exception of the top-hole section. Drilling waste has to be re-injected or brought to land unless other solutions are better for the environment or for safety.

Studies investigating the effects of oil discharges on artic marine species have found the artic species to be more sensitive to these pollutants than more temperate species (Olsen et al. 2007), emphasising the increased vulnerability of the Artic environment.

1.2 Produced Water and Discharges Offshore

1.2.1 Discharges offshore

There are three main sources of continuous discharges of oil into the sea offshore: Produced water, displacement water and drainage water (Figure 3). Oil discharges may also occur from jet water used for cleaning process equipment, in connection with accidents, or as fallouts from oil droplets following the burning of oil in connection with well testing and well maintenance.

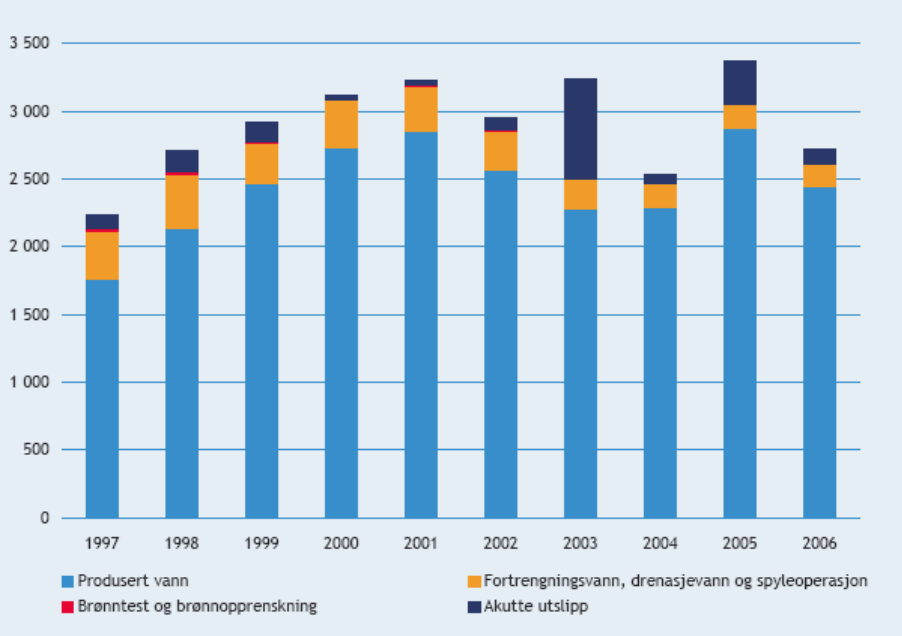


Figure 3: Discharges of oil to the North Sea on the Norwegian Continental Shelf. Discharges of oil to sea are produced water (light blue), testing of wells (red), displacement and drainage water (orange) and acute discharges (dark blue), in tonnes. Reproduced with permission from (OLF 2007).

In 2006 the total amount of oil discharges at sea was 2717 tonnes. Produced water (approximately 90%), displacement water, drainage water and sand jetting counted together for 96% of these discharges, while 4% was related to acute discharges and fall-outs during burning of oil in connection with well-testing and well clean-up (OLF 2007).

The EU legislation is based on the recommendations and work of the Barcelona Convention (Mediterranean Sea), the Bucharest Convention (the Black Sea), the Baltic Marine Environment Protection Commission (HELCOM), and the OSPAR convention (North-East Atlantic). Norway is obliged as a member of the OSPAR convention to follow these international standards. Since 1986, the standard of 40 mg/L for the average concentration of hydrocarbons in effluents discharged from platforms have been applied (PARCOM 8/12/1, paragraph 5.37, (OSPAR 1986)). As from 1 January 2007, a standard of 30 mg/L of dispersed oil in produced water were to be applied (OSPAR Recommendation 2006/4, (OSPAR 2006)). At the Norwegian Continental Shelf in the North Sea the average hydrocarbon content in produced water was 16.9 mg/L in 2006 (OLF 2007). The goal of zero effect discharges is also implemented by OSPAR, and the OSPAR convention also have stated a goal of cessation of discharges, emissions and losses of hazardous substances by the year of 2020 (OSPAR; Sintra statement 1998).

1.2.2 Produced water

The production of produced water is increasing and is normally very high during the last phase of the oil production. Fields that produce gas and condensate products produce relatively small amount of produced water. When the wells start to produce larger amounts of water they are normally shut down.

Produced water contains a great variety of compounds, such as dissolved inorganic salts, minerals and heavy metals, dissolved and dispersed oil compounds, organic compounds such as carboxylic acids and phenols from the formation water (Neff 2002). The five most common organic discharges are poly-aromatic hydrocarbons (PAH), BXT (Benzene, Xylene, Toulene), phenols, alkyl phenols, and carboxylic acids (Figure 4).

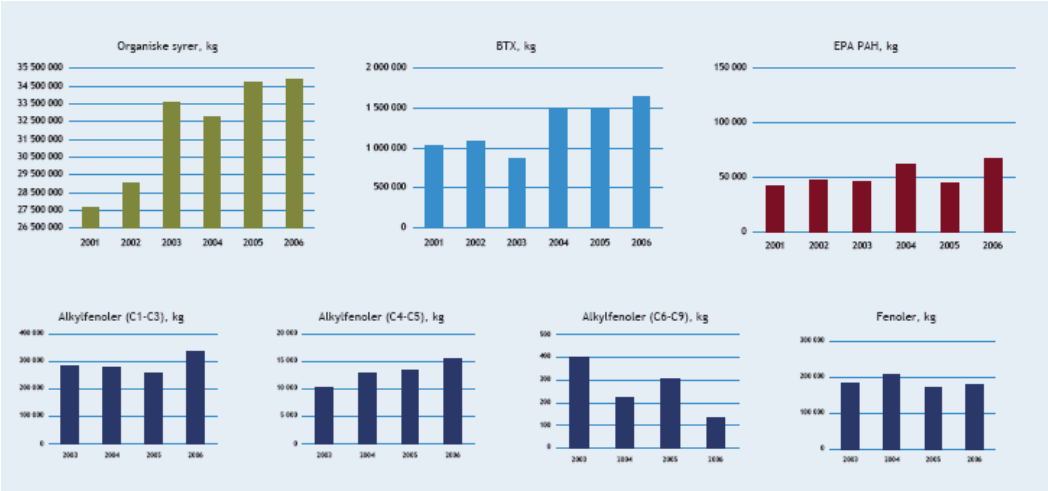


Figure 4: The major contributors to hydrocarbon discharges through produced water. Green: organic acids, light blue: BTX, red: EPA. Bottom left: Alkyl phenols of varying chain length; C1-C3: number one from the left, C4-C5: number two from the left, and C6-C9: number three from the left. Bottom right: Phenols. The measures are in kilo (kg). Reproduced with permission from OLF, (OLF 2007).

The most common heavy metals released during drilling (Figure 5) are impurities from barite: Arsenic (As), lead (Pb), cadmium (Cd), copper (Cu), chromium (Cr), mercury (Hg), nickel (Ni) and zinc (Zn) (ibid). The composition of produced water will vary from one platform to another and during the field's lifetime. In 2004, produced water accounted for 90 per cent of the total oil discharges to the sea from the petroleum industry (OLF 2005).

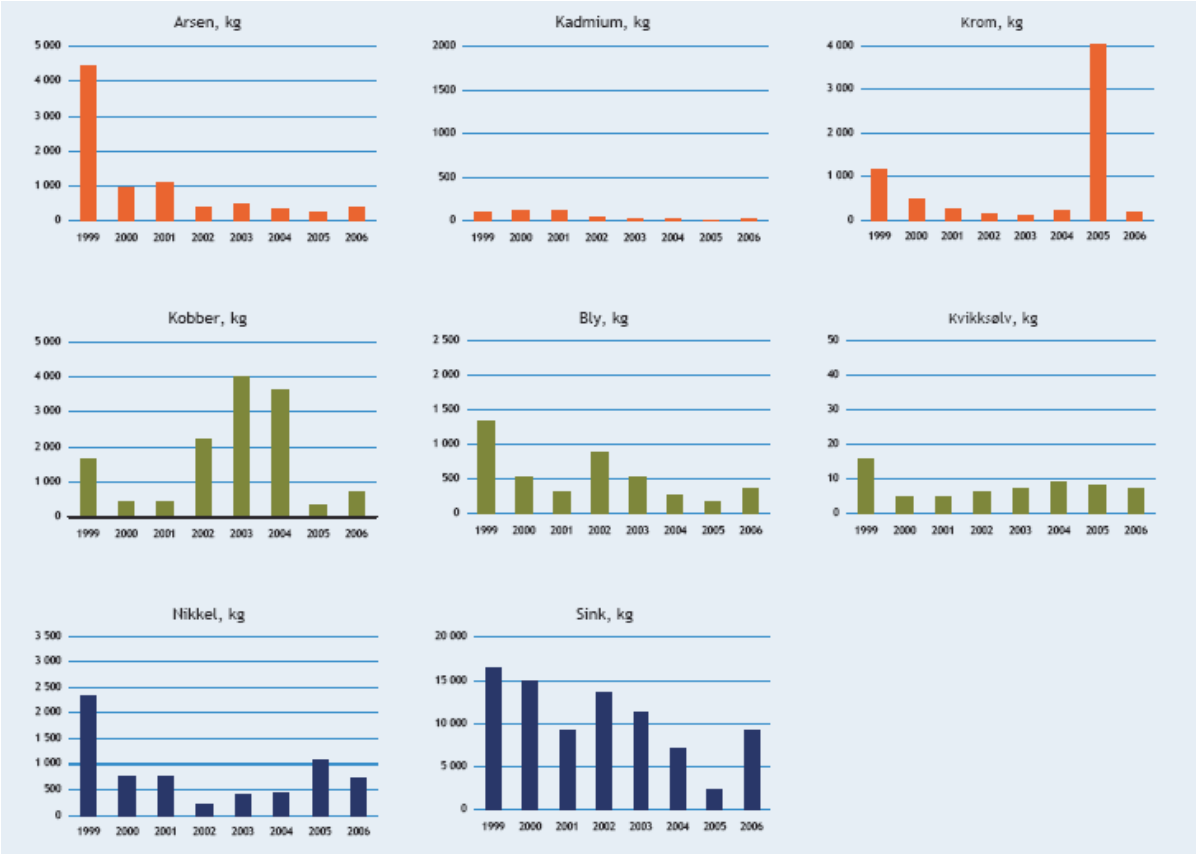


Figure 5: Discharges of heavy metals through produced water to the North Sea, the Norwegian Continental Shelf. From top to bottom, left to right: Arsenic, cadmium, chromium, copper, lead, mercury, nickel and sink. The measures are in kilo (kg). Reproduced with permission from OLF (OLF 2007).

The average oil concentration in the discharges of produced water is reduced. However, the discharges of produced water are also expected to increase in the future as several large oil fields on the Norwegian shelf have reached a mature production phase with continually rising water cuts. Hence, the total amount of hydrocarbons discharged into the sea increases.

Alkyl phenols

Alkyl phenols (APs) are biodegraded products of alkylphenolethoxylates (APEs), which are widely used as detergents, emulsifiers, solubilisers, wetting agents, dispersant in paints, formulated pesticides and herbicides, personal care products and

food packaging (Lamche and Burkhardt-Holm 2000; Naylor 1992). APs are natural components of crude oil (Ioppolo-Armanios et al. 1992) are released into the marine environment from offshore oil production and from municipal wastewater (Naylor 1992). The most water-soluble APs constitute 80% of the alkyl phenols in produced water, whereas higher APs (C_2-C_7) make up the rest (Boitsov et al. 2004). The biodegradation rate of APs is found to decrease markedly with increasing length of the alkyl chain. Alkyl phenols are reported to affect the reproductive system (Meier et al. 2007) and the redox status (Hasselberg et al. 2004; Hasselberg et al. 2004) in Atlantic cod. APs were shown to induce a number of effects in exposed female fish, such as impaired oocyte development, reduced oestrogen levels, and delayed spawning. In the same study, AP-exposure of male fish lead to effects such as reduced levels of 11-keto-testosterone, induction of vitellogenin (Vtg), impaired testicular development, increased amounts of spermatogonia and reduced amount of spermatozoa present (Meier et al., 2006). Some of these effects have previously also been reported from other studies with APs (Arukwe et al. 1997; Cardinali et al. 2004; Christiansen et al. 1998; Jobling et al. 1996; Schwaiger et al. 2002).

Polynuclear/polycyclic Aromatic hydrocarbons, PAHs

PAHs are hydrocarbon compounds consisting of multiple, fused aromatic rings (Harvey 1997). They are released into the environment as point source pollutants, e.g. oil spills (Neff 1985), or as non-point source of pollutant, primarily by atmospheric deposition (Lunde and Bjorseth 1977) and are considered one of the most widespread organic pollutants. PAHs are formed by incomplete combustion of fuels and organic material, such as wood, coal, diesel, crude oil, fat, tobacco or tar (Dabestani et al. 1995; Neff 1979; Neff 1985). It may be worth mentioning that the different types of combustion yield different distributions of PAHs.

Benzene and naphthalene are often included as PAHs, even though they are not formally PAHs. PAHs of three rings or more have low water solubility in water and a low vapour pressure. The water solubility decreases approximately an order of

magnitude for each additional ring (Neff 1979; Neff 1985). PAH toxicity is very structure dependent (Cerneglia and Heitkamp 1989; Neff 1979; Neff 1985). PAHs are lipophilic (Jonsson 2003), and some are shown to carcinogenic (Santodonato 1997), mutagenic (Machala et al. 2001), and teratogenic (Guillette and Guillette 1996; Guillette et al. 1996). One PAH compound, benzo[a]pyrene was actually the first chemical carcinogen to be discovered (Shimkin et al. 1951). PAH is reported to affect steroid hormone levels in exposed fish (Monteiro et al. 2000; Nicolas 1999).

Heavy metals

Large amount of heavy metals is discharged through produced water (Figure 5). Metals are in general toxic by altering enzyme activities through binding to the functional groups (sulfhydryl, carboxyl, imidazol, etc) of the enzymes or by displacing the metal associated with the enzyme (Viarengo 1985). The redox cycling of heavy metals and their interactions with organic pollutant are major contributions to oxidative stress resulting from aquatic pollution (Ahmad et al. 2005).

Arsenic (As), is very similar to phosphorus and will partly substitute for phosphorus in biochemical reactions, making it poisonous (Patlolla and Tchounwou 2005). It is known as a carcinogen (Eisler 2004; Jarup 2003). The metabolic interferences by arsenic may lead to death by multi-system organ failure (Hall et al. 1991; Tsuji et al. 2004).

Lead (Pb) and **mercury** (Hg) are both potent neurotoxins (Baatrup 1991; Castoldi et al. 2001; Castoldi et al. 2001; Jarup 2003), which accumulate in soft tissue and brain over time. In this context it should be mentioned that one of the most important sources of mercury exposure to humans is through fish consumption. Hence, pregnant and breast-feeding women as well as young children are in many areas given the advice to restrict their intake of fish and seafood (Castoldi et al. 2001; Ginsberg and Toal 2000; Inskip and Piotrowski 1985; Koos and Longo 1976). Mercury is transformed by bacteria to organic mercury, which cause toxic effects.

Embryotoxicity and teratogenicity of organic mercury compounds have previously been observed in numerous systems such as fish, birds and mammals (Leonard et al. 1983). Lead is also known to cause blood disorders as well as brain disorders (Goldstein 1992).

Cadmium (Cd) exposure is found to cause spectra of adverse effects in humans (for reviews: (Bertin and Averbeck 2006; Jarup 2003): Acute intoxication have been reported to affect testes, liver and lungs. Chronic exposure has been reported to cause obstructive airway diseases, emphysema, end-stage renal failure, diabetic and renal complications, altered blood pressure, bone disorders and immune-suppression.

Copper (Cu) is poisonous (Eife et al. 1999) and may cause oxidative stress (Livingstone 2001; Pandey et al. 2001; Parvez et al. 2003).

Zinc (Zn) accumulation in the brain is also reported as a prominent feature of advanced Alzheimer disease (AD) (Religa et al. 2006).

Chromium (Cr) and **nickel** (Ni) are reported to enhance UV radiation-induced carcinogenesis in mice (Uddin et al. 2007). Nickel compounds are well established carcinogens (Chen and Costa 2006; Chen et al. 2005).

BXT (Benzene, Xylene, Toluene)

Benzene, C_6H_6, is carcinogenic and is an important industrial solvent and precursor in the production of drugs, plastics, synthetic rubber, and dyes. It is also a natural constituent of crude oil, but is generally synthesised from other compounds present in petroleum. Benzene has also been used as a petrol additive. It is a known human carcinogen and major health effects of benzene rise from chronic exposure through the blood, it damages the bone marrow and can cause a decrease in red blood cells, leading to anaemia (Snyder 2000; Snyder 2000). Benzene can also cause excessive bleeding and depress the immune system (Hsieh et al. 1991).

Xylenes are a group of benzene derivatives. They occur naturally in petroleum and coal tar, and are produced from petroleum for use as solvent, in the production of polymers, as a cleaning agent, in pesticide, in paint and varnishes. Xylene affects the brain, and is also known to cause irritations and breathing difficulties (Riihimaki and Savolainen 1980; Rosengren et al. 1986).

Toluene is a common solvent (Kim et al. 2005). However, chronic inhalation of toluene has been linked to brain damage (Benignus 1981; Benignus 1981; Foo et al. 1990). Toluene is metabolised by cytochrome P450-mediated mixed-function oxidase system (Nelson et al. 2004; Nelson et al. 1987) to benzyl alcohols (BA) (Tassaneeyakul et al. 1996).

1.3 Atlantic Cod (*Gadus morhua*)

The Atlantic cod, *Gadus morhua*, belongs to the family *Gadidae*, of the order *Gadiformes*, the class *Actinopterygii*, and the phylum *Chordata* (Moyle and Cech Jr. 1988). It is an important food fish and a pelagic fish of historically great economic importance (Kurlansky 1998). The North Sea stock is primarily fished by European Union members and Norway, while the fishing in the Barents Sea is dominated by Norway and Russia. Atlantic cod feed mostly on krill, small crustaceans and fish, while the adult fish feed mostly on fish species such as capelin and herring

1.3.1 Distribution

In the western Atlantic Ocean cod has a distribution from North Carolina, USA, and round both of the coasts of Greenland; in the eastern Atlantic it is found from the Bay of Biscay north to the Artic Ocean, including the North Sea (Figure 6), areas around Iceland and the Barents Sea (Hall et al. 2004).

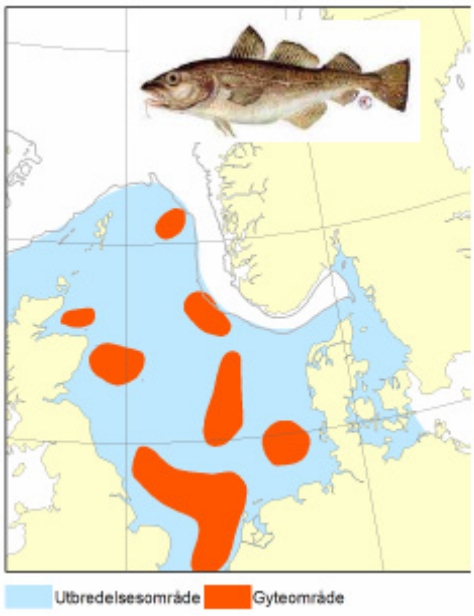


Figure 6: Distribution area and spawning area of Atlantic cod within the North Sea. Distribution area (marked blue) show that cod are well distributed within the North Sea and that the cod spawn (marked orange) in large areas within the North Sea. Reproduced with permission from OLF, (OLF 2006b).

1.3.2 Spawning

Cod has a long spawning season that last for more than two months (April-June), and sometimes as long as three (Brander 1994; Hall et al. 2004). The spawning season appears to be centred on the period of plankton blooms (Brander 1994), which is important to ensure good larvae survival by ensuring that as many eggs as possible hatch when food is available and predation is minimal (Platt et al. 2003). Cod have small, pelagic eggs (0.8-3 mm) and spawn in several batches throughout the spawning season (Kjesbu 1989). Cod larvae drift from spawning areas offshore with sea currents to more coastal areas.

1.3.3 Developmental stages and metamorphosis

The basic developmental mechanisms of teleosts are controlled by genetic and environmental factors (for review, see (Falk-Petersen 2005). In cod, the main organs and organ systems become functional by first feeding and differentiate during the

larval stage and metamorphosis. The differentiation of cells, tissues, organs and organ systems proceeds continuously from the early embryonic phase throughout metamorphosis in accordance with a gradual improvement of functionality (Falk-Petersen 2005).

At the egg stage, the blastula forms after hours or a couple of days depending on the incubation temperature (Timmermans 1987). Organogenesis (4-10 days post-fertilisation, dpf) starts when the three germinal layers ectoderm, mesoderm and endoderm have been established and gastrulation (2-3 dpf) has finished. In cod, hatching gland cells develop in the head and eye region until hatching (10-12 dpf) (Adoff 1986).

Cod larvae hatch at 70-80 dº from 1.2 to 1.4 mm large eggs, at length 4-4.5 mm and dry weights around 0.05 mg. Exogenous feeding starts long before the yolk is exhausted (Harboe et al. 1990). At hatching, liver, pancreas and gallbladder are present (Morrison 1993). The kidney, excretory organs and the primary lymphoid organ are also present at this stage (Padrós and Crespo 1996). Also, the germinal cells forming the gonad are present at the yolk sac stage (Morrison 1993). However, the spleen and thymus appear later (Padrós and Crespo 1996).

After about 2 days the mouth of the cod larvae opens and the cod jaw becomes functional after 5 days, when the cod larvae can then take in food particles (Kjørsvik et al. 1991). During the yolk sac period, a range of digestive enzymes appears in the digestive system (Kjørsvik et al. 2004). The swim bladder works as a hydrostatic pump and is normally filled within 8-10 days after hatching in cod. Failure to do so may lead to reduced growth and skeletal deformities (Blaxter 1986). The thyroid follicle is generally apparent, although not necessarily functioning, within few days after hatching.

The larval phase is then initiated by the first exogenous feeding. Marine teleosts are considered one of the fastest growing vertebrates. During the larvae period growth

and development is rapid when the food is abundant, and most of the growth is in the form of protein deposition in the muscle tissue. Tissues and organs increase in size as differentiation gradually proceeds. The skin grows thicker and the sense organs develop and grow more advanced as the movement reaction become faster and organs increase in mass and area becoming more efficient. Respiration and locomotor activity increase as activity increases, depending on muscular and gill development. Hence, swimming performance of the larvae may be related to alterations in body form, musculature and fins (see review by (Falk-Petersen 2005). Close to the first feeding, the interrenals, thyroid follicles and pituitary are present and operative. Hence, prolactin, thyroid and growth hormones and cortisol important to further development and growth are produced (Tanaka et al. 1995). Also the endocrine system is considered to be functioning early in larvae development, and is thought to respond directly to organic nutrients through hormone synthesis, conversion and reception (Funkenstein and Cohen 1996; MacKenzie et al. 1998).

The maturation of larvae morphology and anatomy into the juvenile structure and function is called metamorphosis (Blaxter 1988). During metamorphosis larvae characteristics gradually disappear and the behaviour changes. Metamorphosis is considered to be under neuroendocrine and endocrine control, in which thyroid and growth hormones, prolactin and cortisol are central (Atkinson 1994; Inui et al. 1994). During metamorphosis the thyroid gland is also active and regulated via the pituitary-thyroid axis (Inui et al. 1994). At 11-13 mm in length, corresponding to approximately 50 days post hatching, cod larvae usually enters metamorphosis (Pedersen and Falk-Petersen 1992). The median finfold then disappears. Most larvae are found in mid-water at metamorphosis (Morrison 1993).

The juvenile stage is reached at 20-30 mm length (Falk-Petersen 2005). The lymphoid organs are fully developed in 25 mm cod. When the lymphoid organs become lymphoid, the cod juveniles become immunocompetent (Schrøder et al. 1998).

Endogenous estrogens have previously been shown in several studies to affect testicular development and function during early life stages, both in fish and other species (Aravindakshan et al. 2004; Delbes et al. 2006; Guillette et al. 1995; Guillette et al. 1996; Jobling et al. 1996; Rolland 2000). Several studies have also reported effects of pollution on reproduction and survival of early life stages in teleosts (for review; (Jobling et al. 1996). Cameron et al. (1996) reported significant impacts on early life stages of cod development following exposure to environmental pollutants in the North Sea. They calculated a 50% decrease in newly hatched larvae due to additional mortality. The increase in mortality was most likely caused by observed embryonic malformations following exposure.

1.3.4 The fish physiology – In Brief

The organs within an organism have specialised function and the expression of many specialised proteins are constricted to certain tissues in which they exert these functions. Linking biomarker proteins to their biochemical function in the organisms may be used to assess the health status of the organisms and ultimately the overall environmental risk. Hence, common body tissues and body fluids to use in fish biomarker discovery include blood, mucus, liver, gonads, and gills.

Blood

The fish blood volume is less (2-5% of body volume) compared to other species (approximately 8% of body volume in mammals). The protein level in blood is also somewhat lower compared to e.g. mammals (Satchell and Helle 1992). Similar to other species, blood cells in the circulation include erythrocytes, lymphocytes and thrombocytes. However, in contrast to e.g. human thrombocytes, the fish thrombocytes possess a nucleus. Their function in haemostasis is however the same as in mammals. Haematopoiesis are in teleosts located primarily to kidney, spleen and thymus (an organ specific to fish), although some blood cells are produced in

liver, in parts of the digestive system (submucosa) and in embryonic yolk sac. The fish thymus is characterised by a large content of small lymphocytes, called thymocytes (ibid.).

Special features of blood and the circulation beneficial to biomarker discovery are the transport of sex hormones from the liver to the gonads, the excretion of proteins into the blood stream upon injury and during disease and the easy, non-destructive sampling of blood. In relation to reproductive effects and function of the gonads, plasma steroid concentrations reflect not only the rate of synthesis, but also the rate of deactivation by the liver (Kime 1999). The gonads can signal back their status to the pituitary and hypothalamus by steroids as well as other messengers (Kime 1999). Blood also possess the more obvious features of circulating throughout the organism being in contact with different organs, transporting signalling molecules and hormones, as well as housing cells like the lymphocytes important to the immune system and in mediating an immunological defence. However, the immune response of cod is unusual in that specific antibody response is limited or absent (Magnadottir et al. 2001).

Liver

The liver is relatively large in cod compared to other species (Kryvi 1992). As liver is the most important metabolising organ, a large number of metabolic processes take place here. In respect to biomarker discovery, changes in the level and activities of the biotransformation enzymes are considered the most sensitive effect biomarkers (van der Oost et al. 2003). Major types of xenobiotic biotransformation enzymes, like the CYP1A-system, have been found to be predominantly located in the liver (Bucheli and Fent 1995; Husøy et al. 1996; Stegemann et al. 1992). Important constituents of oogenesis, such as vitellogenin, and in many species including cod, the eggshell zona radiata proteins (or the vitelline envelope proteins), are also produced in the liver and released into the blood for transportation and incorporation in the oocytes (Arukwe and Goksoyr 2003; Tyler et al. 1991). The synthesis of these

proteins is regulated by oestrogen, 17β-oestradiol, E2 (Kime 1999). The intracellular actions of E2 are mediated by estrogen receptors, ERs (see review by (Goksøyr 2006). E2 is also found to have a suppressive effect on CYP1A (Navas and Segner 2001), an important enzyme in the defence system of the cells. However, the E2 inhibitory action on CYP1A does not seem to override xenobiotic induction of CYP1A (ibid.).

Gonads

Fish are considered useful models for the effects of environmental pollution on the vertebrate reproduction for a number of reasons: They are the species most at risk from aquatic pollution, they are readily exposed in the laboratory to environmentally realistic doses, both sexes produce large numbers of gametes which can be readily counted and examined for malformations, fertilisation and hatch rates are readily measured, the offspring and progeny can be easily monitored for developmental abnormalities and fertility function, and the endocrine control of their reproduction is quite well understood (Kime 1999). Exposed wild fish are also easily compared to the laboratory exposed fish, and fish are increasingly used as models for toxicological effects in mammals (for reviews: (Alestrom et al. 2006; Delbes et al. 2006; Rolland 2000). The endocrine system of fish is controlled by a complex of external stimuli, hypothalamic hormones, pituitary hormones, gonadal hormones, and deactivating hormones from the liver (Kime 1999).

Sex steroid hormones play important roles at all stages of the reproductive cycle. In addition to their role in fish reproduction, sex steroids are known to be involved in fish growth, digestion and food utilisation, gut transport, shifts in body composition, intermediary metabolism, osmotic regulation and immunity (Cuesta et al. 2007). Fish differs from mammals in that the major testicular testosterone is 11-ketotestosterone rather than testosterone (Kime 1995) and that oestradiol acts very differently to mammals: It is an ovarian oestrogen, but is predominantly distributed to the liver

where it stimulates the production of vitellogenin, which is incorporated into the oocytes (Kime 1999; Tyler et al. 1991).

1.3.5 The Cod Genome and Proteome

The cod (*Gadus morhua*) is not a model organism and hence, the cod genome is not well sequenced. However, increasing interest and research on this species have resulted in more of the genome being sequenced and more genes being annotated. To date, 1712 core nucleotides and 46005 expressed sequence tags (ESTs) are available through the NCBI database, yielding a total of 1609 peptide/protein sequences in the NCBI database (NCBI, 2007). The haploid cod genome has been estimated to a size of $3.4 \cdot 10^8$ kb (Grøsvik and Raae 1992). Currently, the genomes of the elephant fish (*Callorhinchus milli*), zebrafish (*Danio rerio*), skate (*Leucoraja erinacea*), Japanese medaka (*Oryzias latipes*), Japanese pufferfish (*Takifugu rubripes*) and freshwater pufferfish (*Tetraodon nigroviridis*) are being sequenced or have been recently sequenced (NCBI, 2007). However, they may differ to the Atlantic cod in habitat, genome size and evolutionary history (Moyle and Cech Jr. 1988).

In relation to this, it might be worth mentioning that the Fugu fish have been found to possess a higher number of genes, just above 35,000 genes, than humans and rodents (Southan 2004). However, teleost fish contain larger numbers of duplicated genes compared to lobe-finned fish and tetrapods such as mammals (Taylor et al. 2003)

The restricted access to teleost, non-model organism sequence data challenges protein identification in proteomic studies to a large extent (Snape et al. 2004).

1.4 Biomarkers

1.4.1 Definition of Biomarkers

The term biomarker has received several definitions: It is used to include any measurement reflecting an interaction between a biological system and a potential hazard, being chemical, physical or biological (WHO 1993). A biomarker has also been defined as a change in a biological response (ranging from molecular to cellular to physiological responses and to behavioural responses), which can be related to exposure to or toxic effects of environmental chemicals (Peakall 1994). Others have defined biomarkers as any biological response to an environmental chemical at the sub-individual level, measured inside an organism or in its products, indicating a deviation from the normal status that cannot be detected in the intact organism (van Gestel and van Brummelen 1996).

1.4.2 Fish biomarkers and Environmental Risk Analysis

(Stegemann et al. 1992) and van der Oost et al. (2003) have proposed six criteria a (fish) biomarker has to meet:

- The assay to quantify the biomarker should be reliable, relatively cheap and easy to perform
- The biomarker response should be sensitive to pollutant exposure and/or effects in order to serve as an early warning signal
- Baseline data of the biomarker should be well defined in order to distinguish between natural variability (noise) and contaminant-induced stress (signal)
- The impacts of confounding factors to the biomarker response should be well established
- The underlying mechanism of the relationships between biomarker response and pollutant exposure (dosage and time) should be established

- The toxicological effects of the biomarker, e.g. the relationships between it response and the (long-term) impact to the organism, should be established

Biomarkers should also preferably be non-invasive or non-destructive, to allow or facilitate environmental monitoring of pollution effects in protected or endangered species (Fossi & Marsili, 1997). Benninghoff (2006) have set the following criteria for high-quality biomarkers:

- The biomarker should be inducible or repressible
- The measured response should be specific to chemicals within that class
- The response should have sufficient sensitivity for routine detection
- The biomarker should be highly accurate and reproducible among experiments within a laboratory and among different laboratories and animal models
- The biomarkers should be quantifiable so that the degree of risk can be identified

In order to assess the overall quality of the aquatic environment, biological responses are also examined to estimate the impact of pollutants on the physiological processes. The biological response triggered by exposure may be used to assess the health status of organisms and to obtain early warning signals of environmental risk (Payne et al., 1987). Hence, by linking biomarker proteins to their biochemical function in the organisms the environmental risk may be assessed. Biochemical parameters that are useful in this sense include biotransformation enzymes, oxidative stress parameters, biotransformation products, haematological parameters, immunological parameters, reproductive and endocrine parameters, genotoxic parameters, neurological parameters, histological and morphological parameters (van der Oost et al. 2003).

1.4.3 The Use of Biomarkers in Ecotoxicology

Ecotoxicology deals with the interactions between environmental chemicals and biota, and the adverse effects at different levels of biological organisation. One way to assess the potential effects of contamination in the environment is the use of biomarkers (Bucheli and Fent 1995). Much focus has been on establishing biomarkers that may function as early-warning signals and reflect and/or predict biological responses and effects at a higher level of organisation. This is because effects at a higher hierarchical level are always preceded by earlier changes in the biological process (Bayne et al. 1985; van der Oost et al. 2003). Today the use of biomarkers is incorporated into a multibiomarker approach (Galloway et al. 2004; Larsen et al. 2006) and a system biology (the study of the interactions between the components of biological systems) approach. Historically, ecological risk assessment and the analysis of adverse effects have been based on measured physical and chemical parameters, with limited ability to determine the biological effects of exposure (Kirby 1998; Peakall and Walker 1994). The use of multiple biomarkers, devised to measure a suite of biological effects ranging from the molecular level to e.g. population and ecosystem level, in a systems biology approach is hence vital to the risk assessment (Galloway et al. 2004). An important mechanism to disease development and toxic response and hence, in relation to the multibiomarker approach, is that a single protein can interact with diverse partners under different conditions which results in different biological outcomes, depending on what partner interacts with the protein (Cho et al. 2004; Drewes and Bouwmeester 2003).

In the review of van der Oost et al. (2003), 12 groups of fish biomarkers were evaluated. According to this evaluation, the biotransformation enzymes (phase I and phase II) and the reproductive and genotoxic parameters scored best. In relation to biomarker discovery studies, the typical stress proteins and the anti-oxidant enzymes were evaluated as the worst fish biomarkers, giving the lowest score in the evaluation.

Despite the promise for biomarker discovery by proteomics (see below), their contribution to diagnosis and monitoring has up to date been scarce. This has been attributed to the lack of coherent pipeline of validation using well-established methods of biomarker candidates (Rifai et al. 2006). Hence, prior to use in bio-monitoring and diagnosis, the biomarker candidate have to go through the phases of qualification, verification, assay optimisation, validation and commercialisation, improving the experimental design and increase the efficiency of biomarker development (ibid.).

1.5 Proteomics

1.5.1 Proteomics and biomarker discovery

Proteomics is defined as the large scale study of proteins (Wilkins et al. 1996), or the study of total proteins from a particular cell line, tissue, or organism (Lee and Lee 2004). There are three groups of proteomic studies: Expression proteomics, cell-map proteomics and structural proteomics (Blackstock and Weir 1999; Figeys 2002; Figeys 2002; Lee and Lee 2004; Pandey and Mann 2000). Expression proteomics study the global changes in the protein expression, cell-map proteomics study protein-interactions and structural proteomics is concerned with determining three-dimensional protein structure. However, proteomic studies are challenging, in particular in expression proteomics and in biomarker discovery, both with respect to separation and mass spectrometry analysis: The dynamic range of plasma protein concentrations covers more than ten-fold of magnitude, thus the low abundant proteins are difficult to detect as they are often masked by the high-abundance proteins, particularly albumin (Shen et al. 2006). Also, the 12 most abundant plasma proteins comprise more than 90% of total plasma protein content in plasma. Hence, when analysing the proteome of body fluids like plasma and serum in a state where the low-abundant proteins are also detectable is somewhat challenging. A common

approach to solve this problem is to deplete plasma or serum of the higher abundant proteins like albumin prior to proteome analysis. However, methods like immunoaffinity-based depletion give rise to other problems affecting the proteome analysis:

1. As much as up to 90% of the potential protein biomarkers are associated with the highly abundant carrier proteins in blood, hence these may be lost from the analysis upon depletion.
2. Extensive sampling handling during the depletion process may lead to sample loss, protein degradation and modification artefacts resulting in substantial sample-to-sample variation.
3. Samples are diluted in the process, making them less suitable for downstream analysis.

1.5.2 Toxicoproteomics and Toxicogenomics

Genomics is the study of the genome, which is all the genetic material in the cell. Hence, toxicogenomics is the study of genes or genetic material affected by toxic exposure. DNA micro-arrays may be used to evaluate changes in gene expressions in response to xenobiotic exposure (Benninghoff 2007). Responsive gene sets are identified, and the biological processes associated with these transcriptional profiles are attempted identified and evaluated to gain insight into the network of biological processes specifically involved in the induced response. However, predicting genes accurately from genomic data is still difficult (Eisenberg et al. 2000). This is particularly true for small genes, which can be missed entirely, or genes with little or no homology to other known genes (Pandey and Mann 2000). This necessitates the use of proteomics, as gene products have to be verified as an important step in annotating genomes (Pandey & Mann 2000). Secondly, the changes in gene transcription rates do not necessarily correlate with protein expression or protein activity (Benninghoff 2006). As mRNA levels do not to always correlate directly to

protein level, it may be necessary to determine the protein directly, again necessitating a proteomic approach (Gygi et al. 1999). Proteins are primarily responsible for cellular responses to physical stimuli, as well as the phenotype of the organism (Barrett et al. 2005; Benninghoff 2007). Also, genes do not code for post-translational protein modifications, the proteolysis, recycling or the sequestration of the proteins, which are also vital to the protein function (Barrett et al. 2005; Pandey and Mann 2000). The proteome represents all proteins produced from the genetic material of the cell (Barrett et al. 2005).

1.5.3 Methods of Proteomics

Two-Dimensional Gel Electrophoresis (2DE) and Image Analysis

Two-dimensional gel electrophoresis is perhaps the most widely used method in proteomics due to its high resolution and ability to separate complex protein mixtures (Görg et al. 2000; Klose 1999). One of the strengths of 2DE is the superior separation of modified proteins (Figeys 2002; Mann and Jensen 2003). Since its introduction in the mid-70s (Klose 1975; MacGillivray and Rickwood 1974; O'Farrell 1975), the method and equipment of 2DE have developed substantially (Figeys 2002; Görg et al. 2000; Görg et al. 1988; Rabilloud 2002). In two-dimensional gel electrophoreses the solubilised proteins are isolated according to their isoelectric point, usually in an immobilised pH gradient strip, prior to separation of the proteins according to their molecular mass in a SDS PAGE gel in the second dimension. Estimates of protein separation capacity by 2DE are varying from up to 1,000 proteins (Pandey and Mann 2000) to several thousand (see review by (Cho et al. 2004). The introduction of narrow range IPG strips lead to a revolutionary increase in 2DE resolution: From a previous average of 2000 proteins being separated in 2DE gels using standard IPG strips, up to 10 000 proteins were now possible to separate in a single 2DE gel (Wita Proteomics, Berlin, Germany; (Mitchell 2003). The 2DE method suffers from the incapability of separating very acidic or basic proteins, as well as very large hydrophopic proteins, such as membrane proteins, and either very small or very large

proteins in general (ibid.). However, the difficulties of separating and analysing e.g. large hydrophobic proteins also count for the gel-free based techniques to a large extent as well (ibid.).

In general, three basic staining methods, Colloidial Coomassie Brilliant Blue (cCBB), Sypro Ruby and silver staining are used for staining 2DE gels. These staining methods differ in sensitivity, linear range, and compatibility with mass spectrometry analysis (Gade et al. 2003; Patton 2002). cCBB is most compatible with MS analysis, but regarded as less sensitive. Sypro Ruby has high sensitivity and broad linear range (Nishihara and Champion 2002). Silver staining is the most sensitive, but has an apparently smaller range that hinders reliable quantification (Gade et al. 2003). Unmodified silver staining is also not MS-compatible. However, both Colloidial Coomassie and Sypro Ruby are found to stain some proteins less efficiently (data not shown). In a modified 2DE technique, the samples are dyed with cyanine dyes (Unlu et al. 1997) prior to 2DE, enabling control, treated, and a standard sample to be run on the same gel to minimise technical variation (Patton 2002).

The stained 2DE images are scanned and the global protein expression pattern is analysed and differentially expressed proteins are identified using image analysis software (Aittokallio et al. 2005; Raman et al. 2002). The choice of image analysis software has impact on the final results of the image analysis as the features of different software differ substantially with regard to alignment/ warping, spot segmentation and detection, and normalisation (Aittokallio et al. 2005; Raman et al. 2002).

Liquid chromatography

Due to the limited dynamic range of 2DE-based proteomics, LC-MS-MS is applied in proteomic studies of complex protein/peptide mixture. However, one-dimensional

peptide chromatography does not provide sufficient peak capacity, the mass spectrometers are poor quantitative devices, and the amount of data obtained is enormous and the analysis inherently complicated. Hence, two-dimensional and three-dimensional chromatographic separations of trypsin-digested samples (MudPIT- multidimensional protein identification technology), sometimes pre-fractionated by 1DE, are currently a widely used separation method in proteomics (Aebersold and Mann 2003; Lin et al. 2003). Different tagging techniques, like ICAT (isotope-coded affinity tag) and SILAC (stable-isotope labelling in cell culture), are used in combination with liquid chromatography methods for (relative) quantitation of proteins (Steen and Mann 2004).

Protein micro-arrays and SELDI

Protein micro-arrays are based on miniaturized and parallelized ligand binding assays, which enable the protein identification, quantitation and to study protein-protein-interactions, and are therefore considered a promising tool for proteomic research (Templin et al. 2003). Using protein micro-arrays, proteins are "captured" to a surface, depending on their chemical or biological properties. In SELDI, surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry, the peptides or the proteins captured on a chip, similar in principle to the micro-array, are detected by mass spectrometry, yielding their m/z values (ibid.). SELDI is another proteomic method used in ecotoxicology (Bjornstad et al. 2006; Hogstrand et al. 2002; Knigge et al. 2004; Larsen et al. 2006; Provan et al. 2006). In SELDI, protein chips with various chemical (e.g. hydrophilic, hydrophobic, ion-exchange, metal-binding) or biological (e.g. antibody, DNA, receptor) capture surfaces are used. A fraction of proteins solubilised from tissues or body fluids are then added and bound to the selected capture surface. The appropriate matrix is then added and the proteins are laser desorbed for MS-analysis. The resulting mass spectra reflect protein expression profiles to identify differentially expressed proteins.

However, the bottleneck of protein micro-arrays, and hence SELDI is the limitation of the capture molecules: E.g. in the study of Larsen et al. (2006) Vtg appeared not to bind to the chip under the buffer conditions used. Using SELDI and protein micro-arrays there is also a great risk of loosing large bulky proteins as they are only attached to the surface with a small part of the protein during washing steps. Hence, the spectra were dominated by low molecular weight proteins, in accordance with other studies using the SELDI-method (Bjornstad et al. 2006; Petricoin et al. 2002).

Peptide sequencing by MS/MS

Mass spectrometry is carried out on ionised samples in the gas phase. By definition, a mass spectrometer consists of an ion source, a mass analyser that measures the mass-to-charge ratio (m/z) of the ionised analytes, and a detector that detects the number of ions and their m/z values (Aebersold and Mann 2003). The most common techniques of ionisation is electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) (Karas and Hillenkamp 1988). ESI is often used and coupled with liquid chromatography as it ionises the analytes out of a solution. MALDI sublimates and ionise the sample out of a dry, crystalline matrix via laser pulses (Aebersold and Mann 2003). Characteristically, ESI produce multiple charged ions, which offers many advantages, and less sample is required (Lin et al. 2003). In proteomic research, four basic types of mass analysers are predominantly in use. They differ in design and performance, and each with their own strengths and limitation; the ion trap, the time-of-flight (ToF), the quadrupole and the Fourier transform ion cyclotron (FT-MS) analysers. The analysers can either be used individually or in tandem combination to take advantage of their individual strengths (Aebersold & Mann, 2003). MALDI is often coupled to ToF and ion trap analyzers (Lin et al. 2003).

Mass spectrometry is considered a breakthrough tool for proteomics as it can be used to deduce peptide sequences from small molecular quantities of sample (Lubeck et al.

2002). This breakthrough is mainly due to improvements in ionisation, separation and the coupling to other experimental techniques (ibid.). In general two methods are applied in peptide sequencing: One method correlates the obtained MS spectrum data (m/z-values) to known protein or peptide sequences in a database, while the other *de novo* sequencing method is based on deducing the amino acid sequence from the MS spectrum/data. The amino acid sequence is then used in homology searches in a database for protein identification. The latter one is commonly used for sequencing novel, unknown proteins and their modifications. In an ideal fragmentation process with an ideal instrument, each fragment would be cleaved exactly at the bond of the two amino acids, so that the peptide sequence could be determined by matching the mass difference of the two consecutive ions with the mass of an amino acid. However, the fragmentation process is far from ideal, making the *de novo* sequencing by MS rather time consuming (ibid.). As new high sensitive separation techniques are used to obtain low-abundant protein, new proteins are likely to be captured and detected as promising biomarker candidates. Hence, the identification of these proteins by MS requires other algorithms than the (original) standard database matching algorithms (Altschul et al. 1997; Schaffer et al. 2001; Shevchenko et al. 2001).

Biological Databases and Search Algorithms

Mass spectrometry yields m/z values of the peptides used in protein database searches using dedicated algorithms that would produce peptides of the same molecular mass when cleaved with the same protease. Hence, the quality of the result depends on sample purity, mass accuracy/ instrument accuracy and the number of peptide masses obtained (Lin et al. 2002).

The matching result will also be dependent on the database and the algorithm used for searching. The different database identification approaches are reviewed by Steen and Mann (2004).

Because protein identification relies on matches with sequence databases, high-throughput proteomics is currently confined to species whose genome is well sequenced and where a comprehensive sequence database is available (Aebersold and Mann 2003).

2. Aims of Study

The objective of this study has been to discover new biomarkers that may function as a diagnostic tool for detecting and monitoring the environmental impact of oil and produced water discharges on exposed marine fish.

The three main aims of this project and thesis are:

- Biomarker discovery by using a proteomic-based strategy (Two-dimensional gel electrophoresis (2DE) and image analysis software were applied to identify changes in the proteomic pattern induced by exposure, whereas mass spectrometry analysis was used to identify the biomarker candidates).
- Identifying mechanisms involved in the biological response to exposure.
- Detecting biomarkers and biological effects of oil and produced water during critical windows of cod development, and to study the effects of long-term chronic exposure to oil and produced water.

3. Summary of Results

The thesis is based on the following three manuscripts, which in the following will be referred to by Roman numerals:

Manuscript I

Anneli Bohne Kjersem, Arnfinn Skadsheim, Anders Goksøyr and Bjørn Einar Grøsvik. Candidate biomarker discovery in plasma of juvenile cod (*Gadus morhua*) exposed to crude North Sea oil, alkyl phenols and polycyclic aromatic hydrocarbons (PAHs). Manuscript to be submitted to Aquatic Toxicology.

Manuscript II

Anneli Bohne Kjersem, Nicolai Bache, Sonnich Meier, Gunnar Nyhammer, Peter Roepstorff, Anders Goksøyr and Bjørn Einar Grøsvik. Biomarker candidate discovery in cod fry (*Gadus morhua*) continuously exposed to North Sea produced water from egg to fry. Manuscript to be submitted to Aquatic Toxicology.

Manuscript III

Anneli Bohne Kjersem, Karen Anita AA, Nicolai Bache, Sonnich Meier, Gunnar Nyhammer, Peter Roepstorff, Bjørn Einar Grøsvik and Anders Goksøyr. Biomarker candidate discovery and possible toxicological effects in liver of cod fry (*Gadus morhua*) continuously exposed to produced water. Manuscript to be submitted to Comparative Hepatology

4. General Discussion

The main objectives of this project have been to identify new biomarker candidates that can act as early warning signs and that are sensitive to oil (**Manuscript I**) and produced water (**Manuscript II and III**) exposure for use in future environmental risk assessment, to evaluate the mechanisms underlying possible responses (**Manuscript I-III**), and to possibly identify critical windows in the cod development (**Manuscript II-III**) as well as possible long-term effects of chronic exposure (**Manuscript II-III**). Hence, chapter 4.1.1 will focus on comparing changes in the proteomic patterns detected in the plasma of juvenile cod (**Manuscript I**), whole cod fry ((**Manuscript II**) and cod fry liver (**Manuscript III**), to identify biomarker candidates common to these three body tissues and fluids, as well as to different stages of development. Chapter 4.1.2 will try to draw the overall picture of biological responses that may possibly be induced by exposure to oil and produced water, as indicated by the biomarker candidates, and in relation to this, in chapter 4.1.3 there will be an attempt to link the apparent biological responses from chapter 4.1.2 to early life stages and teleost development. In chapter 4.1.4 the relevance of the findings in this study as well as the link between biomarker candidates from laboratory exposure studies to field studies and real life exposures will be discussed.

4.1 General Discussion

4.1.1 Biomarker Candidate Discovery

Crude oil exposure of juvenile Atlantic cod (**Manuscript I**) appeared to induce 137 changes in the proteomic pattern in blood plasma, resulting in an apparent down-regulation alpha-2-antiplasmin (A2A) after 3 days of crude oil exposure. Fibrinogen appeared to be up-regulated by surrogate produced water (sPW). 24 days of exposure resulted in an apparent up-regulation of alpha-2-antiplasmin (A2A), fibrinogen, alpha-1-antitrypsin (A1AT), prepro-apolipoprotein A (prepro-APO A), and

apolipoprotein B (APO B), while leading to an apparent down-regulation of alpha-enolase, Pzp-resembling proteins (PZP), tropomyosin, serotransferrin, hemopexin, pro-thrombin, and NTPase. Pentraxin appeared only to be induced by crude oil spiked with alkyl phenols and PAHs, and not by crude oil alone. However, about half of these identified biomarker candidates changed significantly (ANOVA: $p<0.05$, fold change≥ 2) in protein expression following low levels of exposure; 0.06 ppm-0.25 ppm crude North Sea (Statfjord B) oil. It should also be mentioned that most of the protein changes observed in this study (**Manuscript I**) by exposure to crude oil appeared also to be affected by crude oil spiked with alkyl phenols and PAHs, which resembles produced water in composition. Hence, these findings support the common belief that hydrocarbons from oil in produced water result in many of the changes observed following produced water exposure and emphasise the importance of the allowed threshold of hydrocarbon content in produced water.

Most of the protein changes detected following oil exposure and reported in **Manuscript I** were not detected in studies on cod fry (**Manuscript II and III**): In whole cod fry exposure to produced water appeared to modify myosin heavy chains and up-regulate fast skeletal muscle alpha-actin, while inducing an apparent down-regulation Hsc71, keratins, as well as alpha-actinin. In comparison, oestradiol treatment appeared to induce an up-regulation of myosin heavy chains and an apparent down-regulation of fast skeletal muscle alpha-actin and an ATPase not affected by produced water. Of the 60 proteins found to be differentially expressed following exposure to produced water, only about half of them appeared to alter in their protein expression following oestradiol treatment (**Manuscript II**). Hence, these finding suggests that there are, as mentioned previously, a variety of compounds present in produced water and that although many of them seem to have a potential endocrine disrupting effect, many of those that cause effects in the organism cause other types of effects than endocrine disruption. It is also worth mentioning that of the 60 proteins found to be differentially expressed following exposure to produced

water, 51 one of them were induced by low levels of produced water (≤0.1%), which is comparable levels to real life exposure in a radius of 0.1-1 km from a platform (refer chapter 4.1.4).

In the last study we investigated the effects of produced water and oestradiol exposure on 6 months old cod fry continuously exposed at larvae stage (**Manuscript III**). In contrast to the study of whole cod fry at an earlier developmental stage (**Manuscript II**), a wider range of protein groups appeared to be differentially expressed in cod fry liver following the same treatment: Similar to the previous study, Hsc 71 and keratins, appeared to be down-regulated by exposure to produced water. Non-muscle tropomyosin and hemopexin as identified in juvenile cod exposed to crude North Sea oil (**Manuscript I**) were also identified and appeared to be down-regulated in this study (**Manuscript III**). Other apparent protein changes following produced water exposure of cod fry at the larvae stage are ß-actin, ribosomal protein large PO (0.01% PW), elongation factors, 14-3-3B1 and E1, 60 S acidic ribosomal protein P0, HSP90, mitofilin, down-syndrome critical region gene 2 product as well as fructose-1,6-bisphosphate aldolase, aldehyde dehydrogenase (0.1% PW), aconitase 1, pyroline-5-carboxylate dehydrogenases, and antiquitin, in addition to those already mentioned. Oestradiol treatment appeared to induce differential expression of a protein predicted similar to selenium-binding protein 1, and alanine-aminotransferase, and in addition to keratin, ß-actin, Hsc71, HSP90, hemopexin, fructose-1,6-bisphosphate aldolase, pyroline-5-carboxylase dehydrogenase, aconitase 1. The protein expression, reported in **Manuscript III**, follows a similar pattern to that reported in **Manuscript II**: Of the 62 proteins detected to significantly change upon produced water exposure, 30 were also significantly altered following oestradiol exposure.

4.1.2 Biological Responses to Oil and Produced Water

In juvenile cod exposed to crude North Sea oil (**Manuscript I**) there appeared to be an effect on the immune system and increased risk of autoimmune diseases, effects on the fibrinolytical system, imbalance in iron homeostasis possibly causing effects from iron overload, effects on spermatogenesis and fertility, altered triglyceride and cholesterol metabolism and induced apoptosis. These are all functions important to sustain health, fitness and reproduction.

However, the effects possibly induced by produced water observed and reported from cod fry exposed to produced water (**Manuscripts II and III**) seem to be more linked to development and perhaps not that surprisingly, to proteins more expressed during early life stages: Foremost there seems to be an effect on skeletal muscle development, myogenesis, from significant changes in protein expression of myosin heavy chain proteins and fast skeletal muscle alpha actin (**manuscript II**). Some of the biomarker candidates detected in whole cod fry also suggest a possible impact on perception and morphology. This may in turn lead to an impairment of general growth and development.

In more developed cod fry (**Manuscript III**) a larger range of proteins and effects seemed to be affected by exposure to produced water: The detected and identified protein changes indicate an effect on morphogenesis and growth (metabolism). Similar to the findings from biomarker discovery in total fry exposed to produced water (**Manuscript II**), several of the findings from this study (**Manuscript III**) also indicate biological responses such as morphology changes, and disturbances of cod development, processes typical or more expressed in the developing fish compared to the juvenile (**Manuscript I**) and adult fish. As already mentioned, the liver performs many important body functions including regulation of metabolism, synthesis of proteins that are transported via the circulation to their target organs, energy storage,

vitamins and trace metals storage, and transformation and excretion of lipids, steroids and xenobiotics. Hence, the differences in possible biological effects suggested by the biomarker candidates discovered in cod fry liver (**Manuscript III**) compared to those suggested from a similar study on plasma of juvenile fish exposed to crude North Sea oil (**Manuscript I**), may as well reflect the difference in function between the liver and blood as the difference in developmental stages, since many of the proteins identified as differentially expressed in the cod liver study (**Manuscript II**) are predominantly distributed in the liver and typical to the liver metabolising function. Hence, some of these biomarker candidates may also be representative biomarker candidates of juvenile and adult cod liver.

4.1.3 Critical Windows and Developmental Stages

The differentiation of cells, tissues, organs and organ systems proceeds continuously from the early embryonic phase throughout metamorphosis in accordance with a gradual improvement of functionality (Blaxter 1988). The heart starts to contract early and have started contraction at the embryonic stage (Falk-Petersen 2005). The eyes of cod larvae become functional during the first week after hatching when first feeding is initiated, and rods seem to be involved in movement perception and may be particularly important in predator avoidance (Falk-Petersen 2005; O'Connell 1981). Germinal cells forming the gonads can be seen at the yolk sac period (Falk-Petersen 2005; Morrison 1993). The simple V-shaped embryonic musculature originates from myotomes laid down during early somitogenesis. The myotomes are simple, segmental and V-shaped at hatch and composed of proteins of the larvae type. Myotomes are added posteriorly during early growth (Falk-Petersen 2005). In medaka (*Oryzias latipes*) the genes encoding myosin heavy chains have previously been found predominantly expressed in embryo and larvae, all belonging to fast skeletal muscles showing a spatiotemporally expression pattern during development

(Ono et al. 2006). In fugu fish (*Fugu rubripes*) alpha-skeletal actin is also localised in skin and gills in addition to fast skeletal muscle and the heart (Venkatesh et al. 1996).

During larval development, tissues and organs undergo gradual differentiation and increase in size. The myotomes become progressively more complex in shape as they interdigit with growth. Post-embryonic growth occurs through different phases such as hypertrophy (increase in muscle fibre diameters) and hyperplasia (recruitment of new muscle fibres) from undifferentiated myoblasts or myosatellite cells (Falk-Petersen 2005). These cells then proliferate and differentiate to form new fibres or are incorporated to pre-existing fibres by processes regulated by myogenic regulatory factors. The muscle development is important to maintain fast cruising and for fast escape responses. Muscles also line, support and have a function to other tissues and organs, which appears to be another reason why muscle development is so important to larvae and cod development. This can be linked to the findings in this study on whole cod fry exposed to produced water at the larvae stage, in which the biomarker candidates myosin heavy chain, fast skeletal myosin heavy chain, fast skeletal muscle actin, and the Hsc71 were found to be differentially expressed following PW treatment (**Manuscript II**), indicating a possible effect on the ontogenesis, and in particular muscle development and function.

During the larval stage the liver also develops, growing in size increasing with mitochondria (Morrison 1993). The larvae also begin to produce their own prolactin, thyroid hormones, growth hormone and cortisol, which are known to be important to further growth and development (Falk-Petersen 2005). The larvae endocrine system is also believed to be functional early in development, whereas the thyroid gland is believed to become active later (ibid.). The fish then enters the metamorphosis, which is under neuro-endocrine and endocrine control and many hormones interact to initiate and complete the metamorphosis. For instance, thyroid hormones stimulate

muscle growth, in which the larvae-type musculature transfer into the adult type and muscle cells move and rearrange (ibid.). Whether any of the observed proteome changes and the possible effects of the changes observed in the study on the exposed cod fry liver (**Manuscript III**) can be linked to hormonal control is not known. Actin, keratin, Hsc 71, Hsp90 domains, the aldolase and the aldehyde dehydrogenase as well as aconitase 1 detected as biomarker candidates in exposed cod fry liver (**Manuscript III**) appeared all to be affected by oestradiol treatment as well as by produced water exposure. Hence, these candidate responses are likely to be under endocrine and thyroid hormone control. For instance Hsp71 is found to be up-regulated in a tissue specific manner during early development (Park and Jameson 2005). Hsp90 and associated proteins are known to play an important part in conforming the ligand binding domain of the nuclear steroid receptors and the AhR, as reviewed by (Pratt 1997). Fructose-1,6-bisphosphate aldolase and aldehyde dehydrogenase are both metabolic enzymes involved in the glycolytic pathway, and aconitases are involved in the metabolism in Krebs cycle, catalyzing conversion of citrate and isocitrate, which are critical to normal physiology and survival(Tong et al. 2007). These findings also have to be viewed in light of the function of the liver. In the liver hormones (or endocrine disruptors) activate or inactivate receptors or molecules important to the receptor function (You 2004). Once activated the ligand-bound receptor may initiate the transcription of genes which are then translated into proteins of important function successively released into the blood stream and transported to another target organs causing an effect or response in that organ.

4.1.4 Laboratory Exposure versus Field Studies

The set-up and levels of exposure used in crude oil and crude oil spiked with alkylphenol and PAHs (**Manuscript I**) relates to the findings of Aas et al. (2000) in which analysis of PAH parent compounds in liver samples and metabolite compounds in bile of oil-exposed Atlantic cod revealed a peak in PAH parent

concentration in liver following 3 days of exposure which afterwards decreased towards the end of exposure, whereas PAH metabiltes and CYP1A enzyme activity were increasing throughout the period of exposure. In this study the range in biomarker responses observed corresponded to a chronic exposure of 0.06 ppm, 0.25 ppm and 1.0 ppm dispersed crude oil, as used in our study (**Manuscript I**).

As already mentioned the total hydrocarbon (HC) level allowed discharged through produced water is 30 mg/L (30 ppm), whereas the actual average discharge of HC content in produced water is approximately 16.9 mg/L (OLF 2005). Processes such as weathering and photo-oxidation, as well as salinity, temperature and ocean currents all influence the actual amount of hydrocarbons and other compounds that the fish are exposed to through produced water released into the marine environment. Hence, the level of 1-0.01% produced water used in these exposure studies (**Manuscripts II and III**) is meant to reflect discharge scenarios of produced water similar to a distance of approximately 0.1-10 km from the point of discharge, i.e. the oil platform. However, this distance and hence the dilution of produced water is dependent on a number of factors as already mentioned. It is also worth mentioning that simulation data suggest an environmental risk from discharges of produced water only in close vicinity to the point of discharges. However, studies also show that fish, including cod, tend to aggregate close to the oil installations (Myhre et al. 2005).

4.1.5 Methodology

Pilot-studies were performed prior to all the three studies, testing sample preparation protocols, protein load, IPG strip pH range, SDS-PAGE pore size (gel percentage) and staining (sensitivity, linear range). Those parameters gaining the best 2DE resolution as well as the most informative 2DE pattern was chosen for further analysis. The power of analysis is dependent on the number of individuals as well as

the number of replicates included in the analysis, to ensure that the findings are not a result by chance, but actual responses. Any missing values will weaken the strength and power of the test. In 2DE analysis, the number of samples is the main contributing factor to the power of the analysis, as a larger number of samples most likely cover more of the range of the response, decreasing the number of missing values. The number of individuals included in the 2DE analysis is varying, dependent on number of samples available. In the first study (**Manuscript I**) only 2-3 individuals were included in each treatment group, which is borderline to what can be analysed using statistical methods and to determine differentially expressed proteins. However, the result is strengthened by the number of treatment groups and hence, the total number of treated individuals and gels included in each statistical analysis. In the first study (**Manuscript I**) 8 treated individuals/gels were compared to 2 control samples. In the next study (**Manuscript II**) 15 treated individuals were compared to 4 control samples as 4 individuals/gels within each treatment group were used for the statistical analysis. 7 individuals/gels per treatment group were used for statistical studies in the last study (**Manuscript III**) so that in total 27 treated animals/gels were compared to 7 control samples. Hence, the number of individuals analysed and included in the statistical analysis in each study should be sufficient for detecting real differentially expressed proteins. It should also be emphasised that this is the first phase of discovery in biomarker development, in which the differentially expressed proteins will be quantified and verified using a larger number and a broader range of samples in future analysis (Rifai et al. 2006).

The statistical analysis used in this study includes one-way ANOVA (**Manuscript I-III**) and hierarchical clustering analysis (**Manuscript II-III**). ANOVA is based on the three assumptions of independence, normality and homogeneity of variance so that the error residuals are independently, identically, and normally distributed. In the case of differentially expressed proteins in 2DE gels the spot volumes are in many cases not normally distributed, but it suffice that the mean values are normally

distributed. Following spot detection the data are normalised to minimize the errors rising from technical variation. The assumption of a protein being differentially expressed is based on the two criteria of $p<0.05$ in ANOVA analysis and a fold change ≥ 2. The criteria of fold change ≥ 2 arise from the biological variation having to exceed the technical variation (Barrett et al. 2005). The average technical variation in 2DE analysis is found to be 16-39%, where as the biological variation range from to 22-55% (Molloy et al. 2003). Cluster analysis can be used either as a quality control measure to determine that the identified protein changes really are differentially expressed or to link protein changes that are differentially regulated in the same way. By clustering analysis the spots are clustered according to how closely they are related and how similar their protein expression profiles are. In our study hierarchical clustering analysis was used to verify the differential expression of proteins (**Manuscript II-III)**. Our findings from the ANOVA-analysis were confirmed by the hierarchical clustering analysis.

The fragmentation and hence the MS-MS data may also be influenced by random fluctuations during analysis. However, the measurement and the protein identity results are not that vulnerable to these fluctuations as the whole pattern of peptide fragments obtained is used for database searches and protein identification, in addition to using dedicated algorithms that combine biological sense and statistics, taking into the consideration the likelihood of the result to occur by chance. It should also be mentioned that the instruments are well calibrated prior to use as well as during analysis. However, protein identities obtained using these methods depend on the database as well as the search algorithm used. Dedicated search algorithms reflecting the biology and nature of the data (e.g. PSI-blast versus p-blast, choice of scoring matrices etc.) and well annotated, non-redundant databases, containing the most sequence data related to species of interest should be used.

There are many possible artefacts in 2DE-analysis possibly having an impact on the outcome: The protein concentration as measured by Bradford may not be accurate: Protein concentrations not measured in the same run or on the same plate are related to BSA standards of possible different batches and varying concentrations. The BSA is also viscous possibly resulting in different protein amounts being transferred by the pipette and loaded to the IPG strips. The isoelectric focusing, the transfer of proteins from the first to the second dimension and the SDS-PAGE used in the second dimension are all very sensitive to slight variations in concentration, to contaminations as well as alterations in pH, temperature and concentration of the buffers used during the run of the 2DE analysis. The differential protein expression analysis is related to the total amount of protein on the gel. Hence, if a group of proteins are heavily up-regulated this will result in other proteins being wrongly detected as differentially expressed as the ratio between the amount of these proteins and the amount of total protein is significantly altered. The result of 2DE analysis is also very dependent on the linearity and sensitivity of the staining used and to some extent the scanner/densitometer used for scanning. The detection of differentially expressed proteins is more prone to error at the threshold of sensitivity and linearity of the staining used. This also counts at the borderline of the detection range of the instruments.

4.2 Conclusion

- More than 40 of the 137 protein changes detected in plasma of juvenile cod following exposed to crude oil and surrogate produced water (**Manuscript I**) appeared at the lowest level of exposure, 0.06 ppm crude oil.

- 51 of the 60 protein changes detected in whole larvae following produced water exposure (**Manuscript II**) occurred at the lowest levels of produced water, 0.01% and 0.1% produced water. Similarly, 53 of the 62 protein changes detected in cod fry liver of fry exposed to produced water at fry stage

(**Manuscript III**) occurred at the lowest levels of exposure, 0.01% and 0.1% produced water.

- In total 137 proteins appeared to change in plasma of juvenile cod exposed to crude oil and oil spiked with PAHs and alkyl phenols (**Manuscript I**). 84 proteins appeared to change in whole cod fry exposed to produced water and oestradiol from egg to fry stage (**Manuscript II**), whereas 105 proteins appeared to change in liver of cod fry exposed to produced water and oestradiol (**Manuscript III**).

- Biomarker candidates of crude oil exposure of cod in plasma are alpha-2-antiplasmin, fibrinogen, alpha-1-antitrypsin, preapolipoprotein A, NTP-ase, Pzp-resembling proteins, tropomyosin, serotransferrin, hemopexin, pro-thrombin, and apolipoprotein B, as suggested from proteomic analysis of juvenile cod exposed to crude oil (**Manuscript I**).

- Biomarker candidates of produced water exposure of developing cod are myosin heavy chains and fast skeletal myosin, fast skeletal muscle alpha-actin, keratins, Hsc71, and alpha-actinin, as suggested from proteomic studies on whole cod fry exposed to produced water at the larvae stage (**Manuscript II**), as well as Hsc71, Hsp 90, keratin, ß-actin, 14-3-3B1 and E1, ribosomal proteins, elongation factors, mitofilin as well as a fructose-1,6-bisphosphate aldolase, an aldehyde dehydrogenase, aconitase 1, pyroline-5-carboxylate dehydrogenase and antiquitin, as suggested from proteomic studies on liver of cod fry exposed to produced water at fry stage (**Manuscript III**).

- Comparing the results from produced water exposure with oestradiol exposure, suggest that some of the responses may be related to endocrine disrupting effects. However, about 25 % (**Manuscript II**) and 50% (**Manuscript III**) of the produced water affected proteins did not change following oestradiol exposure.

- These results suggest that one subset of biomarkers could be applied during the early developmental stages such as the embryo, larvae, and fry stage (**Manuscripts II and III**), whereas another, independent subset of multi-biomarkers could be applied on juvenile and adult cod (**Manuscript I**) to detect and monitor the effects of produced water on cod or marine fish, taking into account the organ of study available.

- A multi-biomarker approach is preferable as the results seem to indicate that the vast number of chemicals within oil and produced water may cause harm by themselves, inducing individual effects as well as interacting effects with other compounds present in crude oil and produced water. A multi-biomarker approach may be suggested also as the produced water composition varies from field to field, and hence the induced changes in protein expression will probably differ, mirroring the dose-effect relationship, as well as the set of biological responses detected.

- These results also suggest that a systems biology approach should be applied to understand and integrate possible effects, since effects, as suggested by the discovery of these biomarker candidates, may be effects of a common origin, detectable as an initial response in the liver which can lead to more extensive effects in other organs at a later stage. This includes endocrine disrupting effects by impaired hormone balance and/or by signalling between cells within a tissue and between organs, or metabolites and altered enzyme activities causing secondary effects elsewhere or within the same organ at a later stage.

4.3 Future Perspectives

These biomarker candidates will have to be validated for use of as biomarkers in ERA through a multi-step pipeline, as suggested by Rifai et al. (2006) for biomarkers in clinical use. Hence, the next step will be to develop better methods for quantifying these biomarker candidates to verify their differential expression upon oil and/or

produced water exposure. One way to achieve this is to develop antibodies specific to these differentially expressed proteins that can be used in quantitative assays, such as ELISA, that are rapid, cheap and easy in use, enabling the analysis of a large number of proteins within a week. The expression of these biomarker candidates should also be verified and their use as biomarkers validated using a broader range of samples and controls, which should also include a large number and range of field samples.

Advances within this field of research are also strongly dependent on the sequencing of the cod genome and other fish genomes as well as annotation of sequences. The availability of annotated sequences will aid mechanistic studies as well as the discovery of new biomarkers as other valuable tools such as genonthology analysis can be used for identifying signalling pathways and protein cascades affected upon exposure.

Trans-generational proteomic studies should also be initiated to investigate effects in the progeny and to verify effects and the extent of effects on the reproductive system and reproductive organs. The findings in this study indicate effects during ontogenesis and possible effects on fertility and reproductive organs, but linking such effects directly to oil and produced water exposure is impossible from these data. However, we have gonad tissue from juveniles exposed to produced water exposed at larvae stage that we plan to study using a proteomic approach to answer some of these questions.

These findings suggest a multi-biomarker approach, e.g. applied on SELDI using chip technology or other protein micro-array techniques (Benninghoff 2007). However, many of the biomarker candidates discovered in this study may be too large and have a conformation not suitable for micro-array techniques (Larsen et al.

2006). Hence, assay optimising and commercialisation at the end of the biomarker pipeline may prove difficult, stressing the need of improving the capacity of protein micro-array or chip technology.

References

Adoff GR. 1986. Anatomical studies of developing eggs and larvae of the cod (Gadus morhua L.). Bergen, Norway:University of Bergen.

Aebersold R, Mann M. 2003. Mass spectrometry-based proteomics. Nature 422(6928):198-207.

Ahmad I, Oliveira M, Pacheco M, Santos MA. 2005. Anguilla anguilla L. oxidative stress biomarkers responses to copper exposure with or without beta-naphthoflavone pre-exposure. Chemosphere 61(2):267-275.

Aittokallio T, Salmi J, Nyman TA, Nevalainen OS. 2005. Geometrical distortions in two-dimensional gels: applicable correction methods. J Chromatogr B Analyt Technol Biomed Life Sci 815(1-2):25-37.

Alestrom P, Holter JL, Nourizadeh-Lillabadi R. 2006. Zebrafish in functional genomics and aquatic biomedicine. Trends Biotechnol 24(1):15-21.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25(17):3389-3402.

Aravindakshan J, Paquet V, Gregory M, Dufresne J, Fournier M, Marcogliese DJ, et al. 2004. Consequences of xenoestrogen exposure on male reproductive function in spottail shiners (Notropis hudsonius). Toxicol Sci 78(1):156-165.

Arukwe A, Goksoyr A. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp Hepatol 2(1):4.

Arukwe A, Knudsen FR, Goksoyr A. 1997. Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. Environ Health Perspect 105(4):418-422.

Atkinson BG. 1994. Metamorphosis: model systems for studying gene expression in postembryonic development. Developmental genetics 15:313-319.

Baatrup E. 1991. Structural and functional effects of heavy metals on the nervous system, including sense organs, of fish. Comp Biochem Physiol C 100(1-2):253-257.

Barrett J, Brophy PM, Hamilton JV. 2005. Analysing proteomic data. Int J Parasitol 35(5):543-553.

Bayne BL, Brown DA, Burns K, Dixon DR, Ivanovici A, Livingstone DA, et al. 1985. The effects of stress and pollution on marine animals:Praeger, New York, USA.

Benignus VA. 1981. Health effects of toluene: a review. Neurotoxicology 2(3):567-588.

Benignus VA. 1981. Neurobehavioral effects of toluene: a review. Neurobehav Toxicol Teratol 3(4):407-415.

Benninghoff AD. 2007. Toxicoproteomics - The next step in the evolution of environmental biomarkers? Toxicological Sciences 95(1):1-4.

Bertin G, Averbeck D. 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie 88(11):1549-1559.

Bjornstad A, Larsen BK, Skadsheim A, Jones MB, Andersen OK. 2006. The potential of ecotoxicoproteomics in environmental monitoring: biomarker profiling in mussel plasma using ProteinChip array technology. J Toxicol Environ Health A 69(1-2):77-96.

Blackstock WP, Weir MP. 1999. Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol 17(3):121-127.

Blaxter JHS. 1986. Development of sense organs and behaviour in teleost larvae with special reference to feeding and predator avoidance. Transaction of the American Fisheries Society 115:98-114.

Blaxter JSH. 1988. Pattern and variety in development. New York, USA:Academic Press.

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

Brander KM. 1994. The location and timing of cod spawning around the British islet. . ICES Journal of Marine Science 51:51-89.

Bucheli TD, Fent K. 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic biosystems. Crit Rev Environ Sci Technol 25:201-268.

Cameron P, Berg J, von Westerhagen H. 1996. Biological effects monitoring of the North Sea employing fish embryological data. Environmental Monitoring and Assessment 40(107-124).

Cardinali M, Maradonna F, Olivotto I, Bortoluzzi G, Mosconi G, Polzonetti-Magni AM, et al. 2004. Temporary impairment of reproduction in freshwater teleost exposed to nonylphenol. Reprod Toxicol 18(4):597-604.

Castoldi AF, Coccini T, Ceccatelli S, Manzo L. 2001. Neurotoxicity and molecular effects of methylmercury. Brain Res Bull 55(2):197-203.

Castoldi AF, Coccini T, Manzo L. 2001. Biological markers of neurotoxic diseases. Funct Neurol 16(4 Suppl):39-44.

Cerneglia CE, Heitkamp MA. 1989. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment. Boca Raton, FL, USA:CRC Press, Inc.

Chen H, Costa M. 2006. Effect of soluble nickel on cellular energy metabolism in A549 cells. Exp Biol Med (Maywood) 231(9):1474-1480.

Chen H, Davidson T, Singleton S, Garrick MD, Costa M. 2005. Nickel decreases cellular iron level and converts cytosolic aconitase to iron-regulatory protein 1 in A549 cells. Toxicol Appl Pharmacol 206(3):275-287.

Cho S, Park SG, Lee DH, Park BC. 2004. Protein-protein interaction networks: from interactions to networks. J Biochem Mol Biol 37(1):45-52.

Christiansen T, Korsgaard B, Jespersen A. 1998. Effects of nonylphenol and 17 beta-oestradiol on vitellogenin synthesis, testicular structure and cytology in male eelpout Zoarces viviparus. J Exp Biol 201(Pt 2):179-192.

Cuesta A, Vargas-Chacoff L, Garcia-Lopez A, Arjona FJ, Martinez-Rodriguez G, Meseguer J, et al. 2007. Effect of sex-steroid hormones, testosterone and estradiol, on humoral immune parameters of gilthead seabream. Fish Shellfish Immunol.

Dabestani R, Ellis KJ, Sigman ME. 1995. Photodecomposition of anthracene on dry surfaces: products and mechanism. Journal of Photochemistry and Photobiology A: Chemistry 86:231-239.

Delbes G, Levacher C, Habert R. 2006. Estrogen effects on fetal and neonatal testicular development. Reproduction 132(4):527-538.

Depledge MH, Fossi MC. 1994. The role of biomarkers in environmental assessment (2). Ecotoxicology 3:161-172.

Drewes G, Bouwmeester T. 2003. Global approaches to protein-protein interactions. Current Opinion in Cell Biology 15:199-205.

Eife R, Weiss M, Barros V, Sigmund B, Goriup U, Komb D, et al. 1999. Chronic poisoning by copper in tap water: I. Copper intoxications with predominantly gastointestinal symptoms. Eur J Med Res 4(6):219-223.

Eisenberg D, Marcotte EM, Xenarios I, Yeates TO. 2000. Protein function in the post-genomic era. Nature 405(6788):823-826.

Eisler R. 2004. Arsenic hazards to humans, plants, and animals from gold mining. Rev Environ Contam Toxicol 180:133-165.

Falk-Petersen IB. 2005. Comparative organ differentiation during early life stages of marine fish. Fish Shellfish Immunol 19(5):397-412.

Figeys D. 2002. Functional proteomics: mapping protein-protein interactions and pathways. Curr Opin Mol Ther 4(3):210-215.

Figeys D. 2002. Proteomics approaches in drug discovery. Anal Chem 74(15):412A-419A.

Foo SC, Jeyaratnam J, Koh D. 1990. Chronic neurobehavioural effects of toluene. Br J Ind Med 47(7):480-484.

Funkenstein B, Cohen I. 1996. Ontogeny of growth hormone protein and mRNA in the gilthead sea bream Sparus aurata. Growth regulation 6:16-21.

Gade D, Thiermann J, Markowsky D, Rabus R. 2003. Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium Pirellula sp. strain 1. J Mol Microbiol Biotechnol 5(4):240-251.

Galloway TS, Brown RJ, Browne MA, Dissanayake A, Lowe D, Jones MB, et al. 2004. A multibiomarker approach to environmental assessment. Environ Sci Technol 38(6):1723-1731.

Ginsberg GL, Toal BF. 2000. Development of a single-meal fish consumption advisory for methyl mercury. Risk Anal 20(1):41-47.

Goksøyr A. 2006. Endocrine disruptors in the aquatic environment: Mechanisms of toxicity and their influence on reproductive processes in fish. Journal of Toxicology and Environmental Health Part A 69(1-2):175-184.

Goldstein GW. 1992. Neurologic concepts of lead poisoning in children. Pediatr Ann 21(6):384-388.

Grøsvik G, Raae AJ. 1992. The genome size and the structure and the content of ribosomal RNA genes in Atlantic cod 8Gadus morhua L.). Comp Biochem Physiol 101(3):407-411.

Guillette LJ, Crain DA, Rooney AA, Pickford DB. 1995. Organization Versus Activation - the Role of Endocrine-Disrupting Contaminants (Edcs) during Embryonic-Development in Wildlife. Environmental Health Perspectives 103:157-164.

Guillette LJ, Jr., Guillette EA. 1996. Environmental contaminants and reproductive abnormalities in wildlife: implications for public health? Toxicol Ind Health 12(3-4):537-550.

Guillette LJ, Jr., Pickford DB, Crain DA, Rooney AA, Percival HF. 1996. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. Gen Comp Endocrinol 101(1):32-42.

Gygi SP, Rochon Y, Franza BR, Aebersold R. 1999. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19(3):1720-1730.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, et al. 2000. The current state of two-dimensional electrophoresis with immobilized pH-gradients. Electrophoresis 21:1037-1053.

Görg A, Postel W, Günther S. 1988. Two-dimensional electrophoresis. Electrophoresis 9:531-546.

Hall D, Beattie D, Grossman S, Campbell C. 1991. Multisystem failure of the arsenic-poisoned patient. Dimens Crit Care Nurs 10(3):151-159.

Hall TE, Smith P, Johnston IA. 2004. Stages of embryonic development in the Atlantic cod Gadus morhua. J Morphol 259(3):255-270.

Hamdoun AM, Griffin FJ, Cherr GN. 2002. Tolerance to biodegraded crude oil in marine invertebrate embryos and larvae is associated with expression of a multixenobiotic resistance transporter. Aquat Toxicol 61(1-2):127-140.

Hansen TB, Lange OJ, Lavik H, Olsen WH. 1984. Offshore adventure: A pictorial history of the Norwegian Petroleum Industry:Oslo Universitetsforlaget.

Harboe T, Næss T, Naas KE, Rabben H, Skjoldal L. 1990. Age of Atlantic halibut larvae (Hippoglossous hippoglossous L.) at first feeding. In: ICES CM19990/F53 (mimeo)1990.

Harvey RG. 1997. Polycyclic aromatic hydrocarbons. New York:Wiley-VHC, Inc.

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

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

Hogstrand C, Balesaria S, Glover CN. 2002. Application of genomics and proteomics for study of the integrated response to zinc exposure in a non-model fish species, the rainbow trout. Comp Biochem Physiol B Biochem Mol Biol 133(4):523-535.

Hsieh GC, Sharma RP, Parker RD. 1991. Hypothalamic-pituitary-adrenocortical axis activity and immune function after oral exposure to benzene and toluene. Immunopharmacology 21(1):23-31.

Husøy A-M, Myers MS, Goksøyr A. 1996. Cellular localization of cytochrome P450 (CYP1A) induction and histology in Atlantic cod (Gadus morhua L.) and European flounder (Platichthys flesus) after environmental exposure to contaminants by caging in Sørfjorden, Norway. Aquatic Toxicolgy 36:53-74.

Inskip MJ, Piotrowski JK. 1985. Review of the health effects of methylmercury. J Appl Toxicol 5(3):113-133.

Inui Y, Miwa S, Yamano K, Hirano T. 1994. Hormonal control of flounder metamorphosis.

Ioppolo-Armanios MI, Alexander R, Kagi RI. 1992. Identification and analysis of C_0-C_3 phenols in Australian crude oils. Org Geochem 18:603-609.

Jarup L. 2003. Hazards of heavy metal contamination. Br Med Bull 68:167-182.

Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbout trout (*Onchorhynchus mykiss)* exposed to oestrogenic alkylphenolic chemicals. . Environ Toxicol Chem 15:194-202.

Jonsson G. 2003. Determination of PAH metabolites in fish bile; Evaluation, quality assurance and utilisation of analytical methods Bergen, Norway:University of Bergen.

Kammen DM, Hassenzahl DM. 1999. Should We Risk It? Exploring Environmental, Health and Technological Problem Solving
Princeton, NJ:Princeton University Press.

Karas M, Hillenkamp F. 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 60(20):2299-2301.

Kim D, Chae JC, Zylstra GJ, Sohn HY, Kwon GS, Kim E. 2005. Identification of two-component regulatory genes involved in o-xylene degradation by Rhodococcus sp. strain DK17. J Microbiol 43(1):49-53.

Kime DE. 1995. The effects of pollution on reproduction in fish. Rev Fish Biol Fish 5:52-96.

Kime DE. 1999. A strategy for assessing the effects of xenobiotics on fish reproduction. Sci Total Environ 225(1-2):3-11.

Kirby M. 1998. Looking forward, looking back. Med J Aust 168(8):393-395.

Kjesbu OS. 1989. The spawning activity of of cod, Gadus morhua L. . J Fish Biol 34:195-206.

Kjørsvik E, Pittman K, Pavlov D. 2004. From fertilization to the end of methamorphosis-functional development. In: Culture of cold-water marine fish (Moksness KaO, ed). Oxford, UK:Blackwell Publishing Ltd., 204-239.

Kjørsvik E, van der Meeren T, Kryvi H, Arnfinnsson J, Kvenseth P. 1991. Early development of the digestive tract of cod larvae (Gadus morhuaL.) during start feeding and starvation. Journal of Fish Biology 38:1-15.

Klose J. 1975. Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 26(3):231-243.

Klose J 1999. Large-gel 2-D electrophoresis. Methods Mol Biol 112:147-172.

Knigge T, Monsinjon T, Andersen OK. 2004. Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery in blue mussels (Mytilus edulis) exposed to polyaromatic

hydrocarbons and heavy metals under field conditions. Proteomics 4(9):2722-2727.

Koos BJ, Longo LD. 1976. Mercury toxicity in the pregnant woman, fetus, and newborn infant. A review. Am J Obstet Gynecol 126(3):390-409.

Kryvi H. 1992. Generell anatomi. In: Fiskens fysiologi (Døving K, Reimers E, eds):John Griegs forlag, 8-32.

Kurlansky M. 1998. Cod: A biography of the fish that changed the world. London, Uk:Jonathan Cape.

Lamche G, Burkhardt-Holm P. 2000. Changes in apoptopic rate and cell viability in three fish epidermis cultures after exposure to nonylphenol and to a wastewater sample containing low concentrations of nonylphenol. Biomarkers 5(3):205-218.

Larsen BK, Bjornstad A, Sundt RC, Taban IC, Pampanin DM, Andersen OK. 2006. Comparison of protein expression in plasma from nonylphenol and bisphenol A-exposed Atlantic cod (Gadus morhua) and turbot (Scophthalmus maximus) by use of SELDI-TOF. Aquat Toxicol 78 Suppl 1:S25-33.

Lee WC, Lee KH. 2004. Applications of affinity chromatography in proteomics. Anal Biochem 324(1):1-10.

Leonard A, Jacquet P, Lauwerys RR. 1983. Mutagenicity and teratogenicity of mercury compounds. Mutat Res 114(1):1-18.

Lin D, Tabb DL, Yates JR, 3rd. 2003. Large-scale protein identification using mass spectrometry. Biochim Biophys Acta 1646(1-2):1-10.

Livingstone DR. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 42(8):656-666.

Lubeck O, Sewell C, Gu S, Chen X, Cai DM. 2002. New computational approaches for de novo sequencing from MS/MS experiments. Proceedings of the IEE 90(12).

Lunde G, Bjorseth A. 1977. Polycyclic aromatic hydrocarbons in long range transported aerosols. Nature 268:518-519.

MacGillivray AJ, Rickwood D. 1974. The heterogeneity of mouse-chromatin nonhistone proteins as evidenced by two-dimensional polyacrylamide-gel electrophoresis and ion-exchange chromatography. Eur J Biochem 41(1):181-190.

Machala M, Ciganek M, Blaha L, Minksova K, Vondrack J. 2001. Aryl hydrocarbon receptor-mediated and estrogenic activities of oxygenated polycyclic aromatic hydrocarbons and azaarenes originally identified in extracts of river sediments. Environ Toxicol Chem 20(12):2736-2743.

MacKenzie DS, van Putte CM, Leiner KA. 1998. Nutrient regulation of endocrine function in fish. Aquaculture 161:3-25.

Magnadottir B, Jonsdottir H, Helgason S, Bjornsson B, Solem ST, Pilstrom L. 2001. Immune parameters of immunised cod (Gadus morhua L.). Fish Shellfish Immunol 11(1):75-89.

Mann M, Jensen ON. 2003. Proteomic analysis of post-translational modifications. Nature Biotechnology 21(3):255-261.

Meier S, Andersen TC, Lind-Larsen K, Svardal A, Holmsen H. 2007. Effects of alkylphenols on glycerophospholipids and cholesterol in liver and brain from

female Atlantic cod (Gadus morhua). Comp Biochem Physiol C Toxicol Pharmacol 145(3):420-430.

Mitchell P. 2003. In the pursuit of industrial proteomics. Nature Biotechnology 21:233-237.

Molloy MP, Brzezinski EE, Hang J, McDowell MT, VanBogelen RA. 2003. Overcoming technical variation and biological variation in quantitative proteomics. Proteomics 3(10):1912-1919.

Monteiro PR, Reis-Henriques MA, Coimbra J. 2000. Plasma steroid levels in female flounder (Platichthys flesus) after chronic dietary exposure to single polycyclic aromatic hydrocarbons. Mar Environ Res 49(5):453-467.

Moore SF, Dwyer RL. 1974. Effects of oil on marine organisms: a critical assessment of publisheddata. Water Res 8(10):819-827.

Morrison M. 1993. Histology of the Atlantic cod, Gadus morhua: An atlas. Canadian Special Publication of Fisheries and Aquatic Sciences Part 4 Eleutheroembryo and larva 119C:596.

Moyle PB, Cech Jr. JJ. 1988. Fishes: An introduction to ichthylogy. Englewood Cliffs, New Yersey, USA:Prentice-Hall, Inc.

Myhre LP, Bausant T, Sundt R, Sanni S, Vabø R, Skjoldal HR, et al. 2005. Risk Assessment of reproductive effects of alkyl phenols in produced water on fish stocks in the North Sea AM-2004/018.

Navas JM, Segner H. 2001. Estrogen-mediated suppression of cytochrome P4501A (CYP1A) expression in rainbow trout hepatocytes: role of estrogen receptor. Chem Biol Interact 138(3):285-298.

Naylor CG, Mieure, J.P., Adams, W.J., Weeks, J.A., Castaldi, F.J., Ogle, L.D., Romano, R.R. 1992. Alkylphenols ethoxylate in the environment. Journal of the American Oil Chemists' Society 69(7):695-703.

Neff JM. 1979. Polycyclic hydrocarbons in the aquatic environment. Sources, fates and biological effects. London:Applied Science Publishers.

Neff JM. 1985. Polycyclic aromatic hydrocarbons Washington:Hemisphere Pup corp.

Neff JM. 2002. Bioaccumulation in marine organisms. Effect of contaminants from oil well produced water.: Elsevier Science Publishers, Amsterdam.

Nelson JHL, Krogh ET, Gill CG, Friesen DA. 2004. Monitoring the TiO2-photocatalyzed destruction of aqueous environmental contaminants at parts-per-trillion levels using membrane introduction mass spectrometry (MIMS). Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering 39(9):2307-2317.

Nelson MJ, Montgomery SO, Mahaffey WR, Pritchard PH. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl Environ Microbiol 53(5):949-954.

Nicolas JM. 1999. Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. Aquatic Toxicology 45(2-3):77-90.

Nishihara JC, Champion KM. 2002. Quantitative evaluation of proteins in one- and two-dimensional polyacrylamide gels using a fluorescent stain. Electrophoresis 23:2203-2215.

O'Connell CP. 1981. Development of organ systems in the northern anchovy Engraulis mordax and other teleosts. American Zoologist 21:429-446.

O'Farrell PH. 1975. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250(10):4007-4021.

OLF. 2005. Miljørapport 2004. Stavanger, Norway: Oljeindustriens landsforening (OLF).

OLF 2006a. Faktahefte. Stavanger, Norway: Oljeindustriens landsforening (OLF).

OLF 2006b. RKU-rapport. Stavanger, Norway: Oljeindustriens landsforening (OLF).

OLF 2007. Miljørapport 2006. Stavanger, Norway: Oljeindustriens landsforening (OLF).

Olsen GH, Sva E, Carroll J, Camus L, De Coen W, Smolders R, et al. 2007. Alterations in the energy budget of Arctic benthic species exposed to oil-related compounds. Aquat Toxicol 83(2):85-92.

Ono Y, Liang C, Ikeda D, Watabe S. 2006. cDNA cloning of myosin heavy chain genes from medaka Oryzias latipes embryos and larvae and their expression patterns during development. Dev Dyn 235(11):3092-3101.

OSPAR. 1986. PARCOM Recommendation 86/1 of a 40 mg/l Emission Standard for Platforms.

OSPAR. 2006. OSPAR Recommendation 2006/4 amending OSPAR Recommendation 2001/1 for the management of produced water from offshore installations.

Padrós F, Crespo S. 1996. Ontogeny of the lymphoid organs in the turbot Scophthalmus maximus; a light and electron microscope study. Aquaculture 144:1-16.

Pandey A, Mann M. 2000. Proteomics to study genes and genomes. Nature 405:837-846.

Pandey S, Ahmad I, Parvez S, Bin-Hafeez B, Haque R, Raisuddin S. 2001. Effect of endosulfan on antioxidants of freshwater fish Channa punctatus Bloch: 1. Protection against lipid peroxidation in liver by copper preexposure. Arch Environ Contam Toxicol 41(3):345-352.

Park SY, Jameson JL. 2005. Minireview: transcriptional regulation of gonadal development and differentiation. Endocrinology 146(3):1035-1042.

Parvez S, Sayeed I, Pandey S, Ahmad A, Bin-Hafeez B, Haque R, et al. 2003. Modulatory effect of copper on nonenzymatic antioxidants in freshwater fish Channa punctatus (Bloch.). Biol Trace Elem Res 93(1-3):237-248.

Patlolla AK, Tchounwou PB. 2005. Cytogenetic evaluation of arsenic trioxide toxicity in Sprague-Dawley rats. Mutat Res 587(1-2):126-133.

Patton WF. 2002. Detection technologies in proteome analysis. J Chromatogr B Analyt Technol Biomed Life Sci 771(1-2):3-31.

Peakall DW. 1994. Biomarkers:the way forwardin environmental assessment. Toxicol. Ecotoxicol. News. 1:55-60.

Peakall DW, Walker CH. 1994. The role of biomarkers in environmental assessment (3). Ecotoxicology 3:173-179.

Pedersen T, Falk-Petersen IB. 1992. Morphological changes in cod with special reference to the development of the stomach and pyloric caeca. Journal of Fish Biology 41:449-461.

Petricoin EE, Paweletz CP, Liotta LA. 2002. Clinical applications of proteomics: proteomic pattern diagnostics. J Mammary Gland Biol Neoplasia 7(4):433-440.

Platt T, Fuentes-Yaco C, Frank KT. 2003. Marine ecology: Spring algal bloom and larval fish survival. Nature 423(6938):398-399.

Pratt WB. 1997. The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. Annu Rev Pharmacol Toxicol 37:297-326.

Provan F, Bjornstad A, Pampanin DM, Lyng E, Fontanillas R, Andersen OK, et al. 2006. Mass spectrometric profiling - a diagnostic tool in fish? Mar Environ Res 62 Suppl:S105-108.

Rabilloud T. 2002. Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. Proteomics 2(1):3-10.

Raman B, Cheung A, Marten MR. 2002. Quantitative comparison and evaluation of two commercially available, two-dimensional electrophoresis image analysis software packages, Z3 and Melanie. Electrophoresis 23(14):2194-2202.

Religa D, Strozyk D, Cherny RA, Volitakis I, Haroutunian V, Winblad B, et al. 2006. Elevated cortical zinc in Alzheimer disease. Neurology 67(1):69-75.

Rifai N, Gillette MA, Carr SA. 2006. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 24(8):971-983.

Riihimaki V, Savolainen K. 1980. Human exposure to m-xylene. Kinetics and acute effects on the central nervous system. Ann Occup Hyg 23(4):411-422.

Rolland RM. 2000. Ecoepidemiology of the effects of pollution on reproduction and survival of early life stages in teleosts. Fish and fisheries 1:41-72.

Rosengren LE, Kjellstrand P, Aurell A, Haglid KG. 1986. Irreversible effects of xylene on the brain after long term exposure: a quantitative study of DNA and the glial cell marker proteins S-100 and GFA. Neurotoxicology 7(3):121-135.

Rye H, Johansen Ø, Reed M, Ekrol N, Deqi X. 2000. Exposure of fish larvae to hydrocarbon concentration fields generated by subsurface blowouts. Spill Science and Technology Bulletin 6(2):113-123.

Santodonato J. 1997. Review of the estrogenic and antiestrogenic activity of plocyclic aromatic hydrocarbons: Relationship to carcinogenity. Chemosphere 34(4):835-848.

Satchell G, Helle KB. 1992. Sirkulasjonssystemet. In: Fiskens fysiologi (Døving K, Reimers E, eds):John Griegs forlag, 146-166.

Schaffer AA, Aravind L, Madden TL, Shavirin S, Spouge JL, Wolf YI, et al. 2001. Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res 29(14):2994-3005.

Schrøder MA, Villena AJ, Jørgensen TØ. 1998. Ontogeny of lymphoid organs and immunoglobulin producing cells in Atlantic cod (Gadus morhua L.). Developmental and comperative Immunology 22(5/6):507-517.

Schwaiger J, Mallow U, Ferling H, Knoerr S, Braunbeck T, Kalbfus W, et al. 2002. How estrogenic is nonylphenol? A transgenerational study using rainbow trout (Oncorhynchus mykiss) as a test organism. Aquat Toxicol 59(3-4):177-189.

Shen C, Li L, Chen JY. 2006. A statistical framework to discover true associations from multiprotein complex pull-down proteomics data sets. Proteins 64(2):436-443.

Shevchenko A, Sunyaev S, Loboda A, Shevchenko A, Bork P, Ens W, et al. 2001. Charting the proteomes of organisms with unsequenced genomes by Maldi-Quadrupole Time-of-Flight Mass Spectrometry and BLAST homology SEARCHING. Anal Chem 73:1917-1926.

Shimkin MB, Koe BK, Zechmeister L. 1951. An instance of the occurrence of carcinogenic substances in certain barnacles. Science 113(2945):650-651.

Snape JR, Maund SJ, Pickford DB, Hutchinson TH. 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquat Toxicol 67(2):143-154.

Snyder R. 2000. Overview of the toxicology of benzene. J Toxicol Environ Health A 61(5-6):339-346.

Snyder R. 2000. Recent developments in the understanding of benzene toxicity and leukemogenesis. Drug Chem Toxicol 23(1):13-25.

Southan C. 2004. Has the yo-yo stopped? An assessment of human protein-coding gene number. Proteomics 4(6):1712-1726.

Steen H, Mann M. 2004. The ABC's (and XYZ's) of peptide sequencing. Nat Rev Mol Cell Biol 5(9):699-711.

Stegemann JJ, Brouwer M, Richard TDG, Förlin L, Fowler BA, Sanders BM, et al. 1992. Molecular responses to environmental contaminations: enzyme and protein systems as indicators of chemical exposure and effects. Chelsea, MI, USA:Lewis publishers.

Tanaka M, Tanangonan JB, Tagawa M, de Jesus EG, Nishida H, Isaka M. 1995. Development of the pituitary, thyroid and interrenal glands and application of endocrinology to the rearing of marine fish larvae. Aquaculture 135:111-126.

Tassaneeyakul W, Birkett DJ, Edwards JW, Veronese ME, Tassaneeyakul W, Tukey RH, et al. 1996. Human cytochrome P450 isoform specificity in the regioselective metabolism of toluene and o-, m- and p-xylene. J Pharmacol Exp Ther 276(1):101-108.

Taylor JS, Braasch I, Frickey T, Meyer A, Van de Peer Y. 2003. Genome duplication, a trait shared by 22000 species of ray-finned fish. Genome Res 13(3):382-390.

Teal JM, Howarth RW. 1984. Oil spill studies: A review of ecological effects. Environmental Management 8(1):27-44.

Templin MF, Stoll D, Schwenk JM, Potz O, Kramer S, Joos TO. 2003. Protein microarrays: promising tools for proteomic research. Proteomics 3(11):2155-2166.

Timmermans LPM. 1987. Early development and differentiation in fish. Sarsia 72:331-339.

Tong WH, Li K, Uhrigshardt H, Rouault TA. 2007. The role of mitochondrial and cytosolic iron-sulfur cluster biogenesis in maintainence of mammalian iron homeostasis. American Journal of Hematology 82(6):521-521.

Tsuji JS, Benson R, Schoof RA, Hook GC. 2004. Health effect levels for risk assessment of childhood exposure to arsenic. Regul Toxicol Pharmacol 39(2):99-110.

Manuscript I

Candidate biomarker discovery in plasma of juvenile cod (*Gadus morhua*) exposed to crude North Sea oil, alkyl phenols and polycyclic aromatic hydrocarbons (PAHs)

Anneli Bohne Kjersem[1], Arnfinn Skadsheim[2], Anders Goksøyr[1,3], Bjørn Einar Grøsvik[1,4]

[1]Dept. of Molecular Biology, University of Bergen, PB 7800, N-5020 Bergen, Norway

[2]International Research Institute of Stavanger AS, Mekjarvik 12, 4070 Randaberg, Norway

[3]Biosense Laboratories AS, Thormöhlensgt. 55, N-5020 Bergen, Norway

[4]Institute of Marine Research, N-5000 Bergen, Norway

Corresponding author: Anneli Bohne Kjersem

Email: anneli.bohne.kjersem@mbi.uib.no

Phone: +47 55 58 45 00/ +47 90 09 44 57

Fax: +47 55 58 96 83

Keywords: Atlantic cod, biomarkers, crude oil, produced water, image analysis

Abstract

The three main sources of continuous oil discharges offshore are produced water, displacement water and drainage water, which in addition to acute discharges and fall-outs (3%) resulted in a total of 2530 tonnes of oil being discharged to the sea at the Norwegian Continental Shelf in 2004 (OLF; 2005). In the North Sea, many of the oil and gas installations are situated in areas inhabited by large fish populations. Oil discharges and spills in these areas may therefore have the potential to affect the fish stocks, possibly also at more sensitive early life stages. However, the effects of oil and produced water are not well studied, and there is a growing concern that the increasing oil activity offshore may negatively affect fish health and reproduction. In this study we have investigated protein changes in juvenile cod (*Gadus morhua*) induced by crude North Sea oil and North Sea oil spiked with alkyl phenols and PAHs, a surrogate produced water composition, using a proteomic approach. Two different image analysis softwares, Ludesi Analysis Center/Ludesi Interpreter and PDQuest Advanced, were used and compared for detection of changes in the protein expression. This comparison revealed that the choice of image analysis software is crucial to detection of differentially regulated proteins. Using Ludesi, we were able to identify 137 differentially expressed proteins at different levels of crude oil exposure. Many of the protein changes induced occurred at low levels of exposure. The results obtained with protein expression profiles after exposure to oil and surrogate produced water indicate effects on fibrinolysis and the complement cascade, the immune system, fertility-linked proteins, bone resorption, fatty acid metabolism as well as increased oxidative stress, impaired cell mobility and increased levels of proteins associated with apopotosis.

Introduction

The three main sources of continuous oil discharges offshore are produced water, displacement water and drainage water (OLF, 2005). Of these, produced water is by far the most dominant, and contains a great variety of compounds, such as dissolved inorganic salts, minerals and heavy metals, dissolved and dispersed oil compounds, organic compounds such as carboxylic acids and phenols from the formation water (Neff, 2002). The maximum permitted concentration of oil in discharged produced water is 30 mg/L (30ppm) (OSPAR, 2006). The most well studied compounds of produced water, polycyclic aromatic hydrocarbons (PAHs) and alkyl phenols, have been reported to cause severe effects on marine wildlife (Nicholas, 1999; Aas et al., 2000; Evanson & van der Kraak, 2001; Schwaiger et al., 2002; Cardinali et al., 2004; Meier et al., 2007).

In addition to the release of compounds through routine discharges of produced water, the threat of accidents of oil tankers spilling large amounts of oil offshore are incidents of great concern. Previously, negative effects from oil spills have been reported (Solé et al., 1996, Carls et al., 2001, Jewett et al., 2002, Lee and Anderson, 2005).

Norway is today one of the world's ten largest producers of oil. It is Norway's largest export industry, followed by seafood export from fisheries and aquaculture. The development o oil fields in Norwegian waters may represent a potential conflict with fisheries interests. In the North Sea, many of the oil and gas installations are situated in areas inhabited by large fish populations. A recent risk assessment found that parts

of the risk area in the vicinity of oil platforms could contain approximately twice the average fish density of the whole North Sea area (Myhre et al., 2005). Oil discharges and spills in these areas may therefore have the potential to affect a large number of individual fish for prolonged periods, possibly also at more sensitive life stages of development. Better biomonitoring tools, such as sensitive biomarkers, that can detect and monitor the effect of low chronic exposure as well as larger acute exposures are for these reasons of great importance to regulatory authorities.

Cod (*Gadus morhua*) is an important species in North-Atlantic fisheries, as well as a new species for aquaculture developments in Northern waters. Declining cod stocks in the North Sea has raised concerns that this may be due to a combination of anthropogenic factors, including overexploitation, climate change and pollution (Jackson et al., 2002; Worm et al., 2006). Potential conflicts with both coastal industry and a petroleum industry expanding into Northern waters makes it important to understand how effluents from offshore petroleum activities, from industrial and urban (sewage) effluents may affect growth, reproduction, and health of this species. This study is part of a larger project aiming to provide new biomarkers for pollution exposure in cod. One aim of this project has been to provide new tools for environmental monitoring, giving both a deeper mechanistic understanding of exposure effects, and an improved basis for risk assessment.

Here, we have studied the effects of crude North Sea oil and crude North Sea oil spiked with PAHs and alkyl phenols to cod using a proteomic approach. By using two-dimensional electrophoresis (2DE) and image analysis we identified protein changes in cod, that we consider as promising biomarker candidates for the biological

effects of oil and produced water to fish. The proteins were isolated from 2DE-gels and identified by mass spectrometry (MS), searching a new cod genome database based on expressed sequence tags (ESTs).

Materials and methods

Materials

Acidic acid, Coomassie brilliant blue CBB G250, ortho-phosphoric acid, urea, thiourea, tris (hydroxymethyl)-ammoniummethane, sodium carbonate, formic acid, formaldehyde, sodium acetate trihydrate, glutardialdehyde, 25% v/v, sodium thiosulphate pentahydrate, silver nitrate, $EDTAxNa_2x2H_2O$, ammonium sulphate, acetonitril, methanol and thiofluoro acetic were all purchased from Merck (Damstadt, Germany).

Ethanol was purchased from Arcus Kjemi (Oslo, Norway).

Bovine Serum Albumin, BSA, 3-(3-colamidopropyl)-dimetylammonio-1-propansulfoanat, CHAPS, Triton X-100, DL-dithiothreitol, DDT, iodoacetamide, alpha-cyano-4-hydroxycinnamic acid, CHCA and 4-sulfophenyl isothiocyanate, sodium salt monohydrate, SPITC, were purchased from Sigma Aldrich (MO, US).
Ampholine^{TM} 3,5-10, DryStrip Cover fluid, and IEF Electrode strips were purchased from GE Healthcare.

Ammonium dodecyl sulfate, SDS, Agarose, 30% Acrylamide/Bis solution, 37.5:1, Ammonium persulfate, temed and Precision Plus Protein^{TM} standard were purchased from Bio-rad (CA, US).
Trypsin was purchased from Promega (WI, US), while Poros 20R2, Reverse Phase packing, was purchased from Applied Biosystems (CA, US). GelLoader tips were

provided from Eppendorf (Hamburg, Germany), and siliconised tubes were provided from Sorenson, BioScience, Inc (UT, US). Peptide Calibration Standard and MTP384 Target plate polished steel TF were purchased from Bruker Daltonics (Leipzig, Germany).

Experimental design

Fish

Juvenile cod (0-group) were purchased from an aquaculture facility, Sagafjord in Leirvik at Stord, Norway (North Sea Norwegian coastal origin), four weeks prior to the start of the exposure (October, 2002). The fish were maintained until use in 530 litre tanks and fed commercial cod feed pellets (Skretting) twice daily during normal working days, and once over the weekend. Feeding was stopped two to three days before sampling in order to encourage storage of bile in the gall bladder. The sea water was taken from the 78 m depth water intake at Akvamiljø (Mekjarvik, Stavanger, Norway). The water salinity and temperature ranged respectively from 33-34 ppt and 8-12 ºC during the storage period. During the exposure period the temperature ranged from 10 – 11.5 ºC, whilst the salinity was as for the storage period. The fish were carefully transferred to avoid skin lesions and to minimize behavioural stress to the five experiment tanks one week prior to the start of the oil exposure. Five fish were sampled each time from each tank. Past final sampling length and weight was measured for the first time in order to minimize stress during the experiment. At sampling, the mean total wet weight was 40.5 ± 12.3 g and length 16.7 ± 1.5 cm. The fish were killed with a blow to the head and dissected for sampling.

Exposure

The cod were exposed for 24 days, and sampled at day 3, 14 and 24. Five exposures were set up including control. The cod were exposed to three levels of crude oil; 0.06 ppm – low levels, 0.25 ppm – medium levels, and 1.0 ppm – high levels of crude oil, as well as one group exposed to 1.0 ppm crude oil spiked with polyaromatic hydrocarbons and alkyl phenols (for details see below).

Oil

The oil dispersions were made in the continuous flow system (CFS, using Statfjord B field crude oil. The oil was in each experiment dispersed in seawater as droplets with mean and median diameters in the 11-13 μm range. Initially the oil was dispersed at 5 mg oil/kg seawater (ppm), and the dispersion was distributed via a manifold with overflow to a second set of manifolds with overflows. These secondary manifolds received the 5 ppm oil dispersion at various rates through peristaltic pumps. Seawater diluting the oil dispersion was added by gravity and regulated by valves from a header tank with overflow. The overflows ensured stable pressure in the water supplies in the system. Four different solutions involving dispersed crude oil were made in addition to seawater.

Surrogate produced water

The spike was initially made as a concentrated stock solution comprised of the hydrocarbons dissolved in acetone. The spike hydrocarbon mixture in acetone was first pumped into a mixing flask that also received seawater. This first step of dilution generated a concentration of 0.8 mg/litre seawater per sum APs or PAHs. This solution was then fed by gravity and valve control to a second mixing flask and a

manifold, which provided a final concentration at 0.2 ppm hydrocarbon solution of each of the two spike substance groups. This flask also received an oil emulsion fed via a peristaltic pump to provide the final 1 ppm oil in seawater emulsion plus to the diluted spike composition. The final amount of the acetone carrier in the spike exposure solution was 20µg acetone/litre water.

Methods

2DE

The protein concentration of the samples was determined according to Bradford (1976). For analytical gels, 20 μg of sample was diluted in re-hydration buffer (7M urea, 2M thiourea, 4% CHAPS, 20 mM DTT, 0.5% v/v Triton X-100, 0.5% v/v Ampholine 3-10, bromphenol blue) and applied to IPG strips pH 4-7, 7 cm (GE Healthcare) (O'Farrell, 1975, Görg et al., 1988 & 2000). For preparative gels, 1000 μg of sample was diluted in re-hydration buffer and added to IPG strips pH 4-7, 18 cm. The strips were then re-hydrated for a minimum of 12 hours and focused on a Multiphor II unit (GE Healthcare) according to producer` s guidelines. The strips were equilibrated 15 min. at room temperature in 0.25% DDT-containing SDS equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, bromphenol blue) and 15 min. in a 4.5% iodoacetamide-containing SDS equilibration buffer (O'Farrell, 1975, Görg et al., 1988 & 2000) prior to separation in the second dimension on 9% SDS-PAGE gels (Laemmli, 1970). Analytical gels were run at 200 Volt, 400 mA/12 gels, 20°C for 45 min. in a mini Dodeca unit (Biorad) prior to silver-staining (Heukeshoven & Dernick, 1985). Preparative gels were run in an Ettan DaltSix/ Twelve unit at 1W/ gel, 20°C, for approximately 17 h. The preparative gels were then stained with Collodial Coomassie (Neuhoff et al, 1988).

Gel image analysis

Silver-stained analytical gels and Coomassie-stained preparative gels were scanned on a GS-800 Calibrated Densitometer flatbed scanner (Biorad, CA, US) using PDQuest 7.2.0 software (Biorad, CA, US). The gels were scanned with medium resolution: 63,5 (X) x 63,5 (Y) microns.

PDQuest

The silver-stained gels were processed and analysed in a trial PDQuest version, PDQuest Advanced, version 8.0 (Biorad), downloaded from the Internet (www.biorad.com). The images were cropped prior to analysis. For each series; males - 3 days, males – 14 days, males – 24 days and females- 24 days, automatic spot-detection was performed based on manually selection of a faint spot, a small spot and the largest spot cluster in one representative image from each series. Streaking, background, speckle-removal, and smoothing were adjusted automatically by the programme based on the initial spot selection. The sensitivity was adjusted to give a satisfactory signal-to-noise ratio. We used automated matching, in which all unmatched spots from all the gels were set to be added to a master gel, and all the images were warped prior to matching. A gel from the group of individuals exposed to 1.0 ppm crude oil was selected as master gel within each experiment, as it included the most spots. Each experiment included gels of individuals of the same gender exposed for the same duration of time. As we are looking for changes in the protein pattern that may represent toxicological changes, normalization was disabled in order not to influence the gel analysis, as described by the PDQuest Help function. The matchset and the gel images used for PDQuest analysis were uploaded to the Ludesi

Interpreter (www.ludesi.com) for ANOVA analysis and for comparison with the Ludesi analysis.

Ludesi

The scanned 2DE images were sent to Ludesi Analysis Centre (Sweden) for professional image analysis. At Ludesi Analysis Centre the spots were automatically detected and the results were manually verified and edited where needed. The gels were automatically matched using all-to-all matching; every gel was matched to all other gels, avoiding introduction of bias caused by use of a reference gel. The matching was iteratively improved by optimization of matching parameters and manual editing where needed. Integrated intensities were measured for each spot, background corrected, and then normalized. Normalization removes systematic gel differences originating from variations in staining, scanning time, protein loading, etc, by mathematically minimizing the median expression difference between matched spots.

One-way ANOVA-analysis ($p \leq 0.05$ and fold change ≥ 2) was performed comparing the level of protein expression to level of exposure using Ludesi 2DE Interpreter (www.ludesi.com). However, it should be emphasised that the results of the image analysis were carefully validated and any changes detected in gel areas of poor separation were excluded to limit the number of false positives.

Mass spectrometry

The excised protein spots were in-gel digested according to the method of Shevchenko et al. (1996) prior to mass spectrometry.

De novo-sequencing

4-sulphophenyl isothiocyanate, SPITC, was used to sulfonate trypsin-digested peptides to yield good y-ion sequences at low peptide concentrations for improved protein identification (Marekov and Steinert, 2003). The SPITC-derivated proteins were then applied to nano-coloums packed with 20Poros R2 and eluted with matrix, α-cyano-4-hydroxycinnamic acid (CHCA) on to the steel target prior to MALDI-ToF-ToF (4700 Proteomics Analyser, Applied Biosystem, CA, US). Signal-to-noise ratio was set to 20, mass tolerance was set to ± 0.5 m/z and mass outlet factor was set to 10.

MALDI-ToF-ToF spectra of trypsin-digested peptides and SPITC-treated trypsin-digested peptides were compared, and fragments that by this comparison were found to be sulfonated were manually selected for further fragmentation and de novo-sequencing.

LC-ESI-Q-ToF

Prior to application of sample on the LC-ESI-Q-ToF, 20 µL trypsin-digested protein samples of isolated 2DE-spots were run on home-made C_{18} Stage tip columns (Rappsilber et al., 2003), and the samples were eluted with 1/10 of sample volume of 100% acetonitril (ACN) and dissolved in approximately 2x sample volume of 2% formic acid (FA). 40 µL of the dissolved sample was applied on the ESI-LC-QToF instrument.

The samples were applied to an Ultimate 3000 nano-LC (Dionex Corporation, USA). The nano-LC consist of a C_{18} Pepmap 100 pre-column (Dionex Corporation, USA) with bead size 5µm, pore size 100Å, and a nC_{18} analytical column, C_{18} Pepmap 100 (Dionex Corporation, USA), with bead size 3 µm and pore size 100Å in the first set-up, and with bead size 5µm and pore size 100Å in the second set-up. The flow rate on the analytical column was 300 nL/min, mobile phase A was 2% ACN and 0.1% FA, and mobile phase B was 90 % ACN and 0.1% FA. The following gradient was used: 0-3 min: 5% B; 3-40 min.: 5-50% B; 40-45 min.: 50-95% B; 45-53 min.: 95% B; 53-55 min.: 95-5% B; 55-70 min.: 5% B.

ESI-voltage was 2942 V, cone voltage 100 V and the ion source temperature 110° C. The collision energy followed a step-wise increment profile according to ion mass and charge, the collision energy increasing to 53 kV for 2+ ions and to 42 kV for 3+ ions. Data were acquired sequentially in MS-mode, with scan range (m/z) 450-1100 Da. MS/MS spectra of 3 ions from the MS-scan were recorded, within the mass range of 50-1500 Da. Only parent ions with two or three charges were chosen for MS/MS and the selection width of precursor ion was 2 Da.

The resulting MS/MS data were managed by Mass Lynx 4.0 software (Micromass) and MS_Lims (http://genesis.ugent.be/ms_lims/).

Database searches

The newly developed cod EST database used for protein identification includes more than 50 000 EST sequences from cod from a project at the Institute of Marine Research, Norway, NIFES (Norwegian Institute of Research on Nutrition and Seafood) and groups affiliated to the National Research Council, Canada. The annotation of the sequences was performed by the Computational Biology Unit at UoB, producing approximately 6000 contigs and more than 30 000 singlets. The EST sequences are derived from cDNA libraries of cod exposed to an array of environmental pollutants, such as PCBs and heavy metals, and purified mRNA sequences from several different tissues. RNA was also purified from cod captured in the marinas of Bergen and Trondheim, and therefore assumed to be exposed to a battery of pollutants, as well as cod exposed to hypoxia and hyperoxia (http://www.codstress.olsvik.info/Results.html).

The obtained de-novo sequence data from SPITC-MALDI-ToF-ToF were initially used in BLAST-searches in the in-house developed cod EST-database, and the sequence of the contig(s) and/or singlets which matched the query sequences (minimum two fragments with score>identity threshold score and E-values>0.05) were retrieved and used in a second blast-search in the NCBInr database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) for protein identity (Altschul et al., 1997).

The sequence data from the LC-MS-MS runs were used in MASCOT-searches in the cod EST-database (Perkins et al., 1999, Steen & Mann, 2004) using the following parameters: Missed cleavages: 1; Significance treshold $p<0.05$; MS^{1} mass accuracy: 0.5 Da, MS^{2} mass accuracy: 0.3 Da; Partial modifications: Carbamidomethyl (C) and Oxidation (M). The quality of the matches was manually inspected. The sequence of the contig(s) and/or singlets which matched the query sequences (minimum two fragments with score>identity threshold score and E-values>0.05) were retrieved and used in blast-search in the NCBInr database for protein identity.

Results

2DE and Image Analysis

30 individual plasma-samples were screened on 2DE and analysed for differentially expressed proteins utilising the Ludesi 2DE Image Analysis Service as well as performing the analysis ourselves using the PDQuest Advanced software. The dataset comprise 9 male individuals and 1 female individual (control) sampled after 3 days of exposure, 10 male individuals and 10 female individuals sampled after 24 days of exposure. The limited number of samples within each group is due to varying distribution of males and females among the 5 individuals sampled within each treatment group. In average, approximately 840 proteins were detected on the 2DE-gels by Ludesi, compared to an average of approximately 260 proteins detected by PDQuest. When comparing the results of image analysis of the 30 gels provided by Ludesi Analysis Centre with PDQuest analysis, we found that the number of differentially expressed proteins at a significance level of $p<0.05$ (ANOVA) detected by the two different image analysis softwares differed substantially. The results of the image analyses were manually verified by screening 40 randomly selected proteins from each time series, 10 proteins from each group of exposed individuals. The screening found the average correctness of PDQuest image analysis to be 18% (3 days of exposure) and 30% (24 days of exposure) compared to 78% average correctness, at both day 3 and day 24 of exposure, using Ludesi. Estimated total number of correct identified significant changes ($p<0.05$) were 73 by PDQuest compared to 465 by Ludesi. For these reasons, Ludesi analysis results were preferred and used for selecting biomarker candidates.

Biomarker candidates were identified by comparing the global protein expression of silver-stained mini-gels of individuals from 15 different treatment-groups, the majority of groups consisting of two to three individuals. Protein changes only observed in groups consisting only of one gel/individual sample were excluded. 137 protein changes with a fold change ≥2 and $p<0.05$ using one-way ANOVA were identified as protein changes by crude oil and surrogate produced water exposure (Table 1 and 2). Their localisation on a 2DE-gel is shown in Figure 1. Interestingly, more than 40 of 135 protein changes occur at the lowest level of oil exposure (≤0.06 ppm crude oil) (Table 1 and 2).

MS-MS

Of the 137 protein spots identified on the silver-stained mini-gels to be differentially expressed upon exposure, 29 proteins were positively identified by MS/MS (Table 3). By using a newly developed cod EST-database we increased our success rate of protein identification by mass spectrometry from approximately 10% to >90%. After 3 days of exposure (Table 1), fibrinogen (#7013, #7709) appeared increase in intensity at surrogate produced water exposure. Another protein identified as an alpha-2-antiplasmin (#7550) appeared to be down-regulated after 3 days of crude oil exposure.

After 24 days of exposure (Table 2), enolase alpha (#7011) appeared to be down-regulated at crude oil exposure and after surrogate produced water. Fibrinogen (#7032) appeared to be up-regulated at higher levels of crude oil exposure and surrogate produced water exposure. Alpha-2-antiplasmins, A2A, (#7581, #7584,

#7587, # 7597) appeared to be up-regulated by 24 days of 1.0 ppm crude oil and surrogate produced water exposure. Tropomyosin (#7043), serotransferrin (#7192), hemopexin (#7409), prothrombin (#7285, #7329) and fetuin B (#7098, #7285, #7345) seemed to be down-regulated by crude oil exposure and in the cases of serotransferrin and pro-thrombin also by surrogate produced water exposure. The latter exposure was also found to induce a down-regulation of pentraxin (#7027). Alpha-1-antitrypsin, A1AT (#7811), appeared to be up-regulated by 0.25 ppm of crude oil. The prepro-apolipoprotein A (#8580) was shown to be up-regulated at crude oil levels ≥25 ppm and by surrogate produced water exposure, while NTPase (#7810) was found to be down-regulated by 1.0 ppm crude oil and oil spiked with PAHs and alkyl phenols. A protein similar to pregnancy zone protein, Pzp (#7826), was found to be down-regulated by crude oil exposures ≥1.0 ppm. Apolipoprotein B (#7178) was shown to be up-regulated by 0.25 ppm crude oil exposure and by surrogate produced water exposure.

Discussion

Image analysis and changes in the protein pattern

We used and compared the Ludesi 2DE Image Analysis Service with our own analysis using the PDQuest software, analysing 30 2DE-gels of individual plasma samples of juvenile cod exposed to crude oil. The Ludesi service provided in our view robust image analysis in which the spots of different spot patterns on different 2DE gels appeared to correspond well to the corresponding spots in the other gels. For these, an appropriate normalisation function was applied providing accurate spot detection and protein expression measurement. The average correctness of the Ludesi image analysis was calculated to be by far better than the average correctness of the PDQuest image analysis. Using PDQuest, we had trouble obtaining satisfactory warping (correspondence of 2D gel spot patterns, removal of gel distortions), and satisfactory matching (correspondence of individual spots between different 2DE gels) resulting in poor and inaccurate spot detection.

In general there will always be a gel to-gel variation and a technical variation in addition to the biological variation and changes in the protein pattern caused by xenobiotic exposure (Molloy et al., 2003). The gel to-gel variation includes differences in gel size, in the protein amount loaded to the strips and absorbed by the strips, as well as staining efficiency. A good warping- and normalisation-function prior to spot detection and matching, are therefore crucial to the final result. According to the help-function in PDQuest, normalisation is not to be used where an overall change in protein expression is expected, *´for example when testing for*

toxicity. Normalisation assumes that any overall change is unwanted and removes it`. In the case of PDQuest, we followed this instruction and did not normalise our data and hence, technical variation may have affected the measured spot volumes and intensities by PDQuest.

Biomarker candidates

The results obtained with protein expression profiles after exposure to oil and surrogate produced water indicate effects on fibrinolysis and the complement cascade, the immune system, fertility-linked proteins, bone resorption, fatty acid metabolism as well as increased oxidative stress, impaired cell mobility and increased levels of proteins associated with apopotosis.

Although the number of individuals and samples in this study is limited within each treatment group, all the ANOVA analysis included a minimum of ten gels, and protein changes only apparent in groups of one individual were excluded from the analysis. The protein changes observed in this study may therefore represent a first screening for potential biomarker candidates in plasma that will be further analysed and verified in future exposure studies based on a larger number and broader range of samples, as described by Rifai et al. (2006).

After 3 days of exposure fibrinogen appeared to be up-regulated after sPW exposure and there appeared to be a down-regulation of A2A following crude oil exposure. After 24 days of exposure, we observed an apparent down-regulation of alpha enolase and prothrombin, and an apparent up-regulation of alpha-2-antiplasmin and

fibrinogen, proteins which are all involved in the fibrinolytical system. These biomarker candidates suggest an effect on the fibrinolytical system following crude oil exposure. Alpha-1-antitrypsin (A1AT) appeared to be up-regulated.

Both the apparent changes of expression of A1AT and alpha-enolase also indicate an effect on the immune system. A1AT is known to be linked to immunologic and inflammatory disorders and is also found to be regulated by sex-hormones (Machovich & Horvath, 1981, Yamamoto & Sinohara, 1984, Schwarzenberg et al., 1987). Impairment of alpha-enolase activity is reported to be implemented in a large variety of infectious and autoimmune diseases, microbial tissue invasion, endothelial injury, activation of the complement classical pathway, pertubations of the fibrinolytical system, as well as induction of apoptotic cell death (Terrier et al., 2007). Pentraxin may also indicate an effect on the immune system, as short pentraxins are acute phase proteins, produced in liver in response to inflammation, as reviewed by Bottazi et al., (2006).

We also identified a down-regulation of Pzp-resembling (pregnancy zone protein) after crude oil exposure (≥0.25 ppm) and an up-regulation of A2A. Alpha-2-antiplasmin constitutes a key recognition sequence for cell adhesions (Thomas et al., 2007), and has been reported to enhance cell aggregation and suppress cell mobility (Hayashido et al., 2007). It has also been found to be involved in mammalian spermatogenesis, sperm capacitation, and fertilization, as reviewed by Liu (2007). These changes may indicate an effect from exposure to crude oil on the fertility of fish.

Tropomyosin forms complexes with several dfferent proteins, including actin, involved in cell adhesion to surfaces. The apparent change in tropomyosin expression after crude oil exposure, together with the apparent change in A2M expression may imply effects of crude oil exposure on cell adhesion.

Serotransferrin and hemopexin are important constituents of the iron homeostasis system, regulating cellular iron levels. Both serotransferrin and hemopexin appeared in our study to be down-regulated by crude oil exposure, which might suggest in turn lead to an overload of iron (Bradbury, 1997, Beaumont, 2004, Anderson & Frazer, 2005). In cells, like macrophages, only a slight increase in iron (2-3 fold) is found to affect cell signalling, leading to NO production and activation of the nuclear transcription factor NF kappa B, and cellular function, resulting in a stimulation of the production of reactive oxygen species (ROS) and oxidative stress (Chrichton et al., 2002).

Our results may also indicate an effect of crude oil exposure on the metabolism. In our study, both prepro-apolipoprotein and apolipoproteins B (Apo B) appared to be differentially expressed after 24 days of exposure. Apolipoproteins are important regulators of triglyceride and cholesterol metabolism. Previously, oestrogen has been shown to effect the density distribution of apolipoproteins (Tam et al., 1985).

We also found a NTPase and fetuin B to be down-regulated in the groups of individuals exposed to 1.0 ppm crude oil and surrogate produced water. This NTPase is found in apoptosis as well as being involved in MHC transcription activation. Fetuin B is a protease inhibitor of many functions, such as regulation of insulin and

hepatocyte growth factor receptors, in response to systemic inflammation and in osteogenese. The apparent down-regulation of these proteins may thus have several effects in the exposed fish.

When considering these differentially expressed proteins as biomarker candidates, it is important to be aware of the limited number of individuals within each group. We are in the first phase of discovery with limited amount of samples available for biomarker discovery and hence, the results from this study most likely include a number of false positives. Further analysis and validation of these results will determine whether they are suited for use as biomarkers in the future (Rifai et al., 2006).

As well as trying to identify more of the proteins observed to be differentially expressed in this study, we now plan to produce antibodies directed towards these biomarker candidates. The candidates will be validated for use as biomarkers using antibodies in ELISA assays. The antibodies may also be used in mechanistic studies to gain more knowledge of the underlying response mechanisms.

Conclusion

We have compared the Ludesi 2DE Image Analysis Service and image analysis by ourselves using the PDQuest software, for identifying differentially expressed proteins, induced by crude oil and surrogate produced water exposure. We found the Ludesi software to provide robust image analysis for biomarker candidate discovery. Using PDQuest we did not manage to warp, match or normalise our data satisfactorily.

By using a newly developed cod EST-database we increased our success rate of protein identification by mass spectrometry from approximately 10% to >90%. By this approach we observed 137 proteins to be differentially expressed due to exposure, and 29 of these proteins were successfully identified by mass spectrometry. Taken together, our findings suggest alpha enolase, plasminogen, alpha-1-antitrypsin, alpha-2-macroglobulin, alpha-2-antiplasmin, prothrombin, pentraxin, tropomyosin, serotransferrin, hemopexin, fetuin B, apolipoprotein B, the NTPase and the Pzp proteins as potential biomarker candidates in plasma for the effects of oil and produced water to fish. Changes in protein expression were in this study shown to be induced also at low levels of exposure for many of the proteins, which is a desirable feature of biomarker candidates. However, their use as future biomarkers has to be tested at a much larger scale in future studies. The identified proteins have previously been linked to spectra of severe adverse effects such as infectious and autoimmune diseases, microbial tissue invasion, endothelial injury, activation of the complement classical pathway, perturbations of the fibrinolytical system, bone resorption as well as induction of apoptotic cell death, oxidative stress and impaired fertility. Also many

of the responses seen in this study seem to be somewhat linked to each other, indicating that an array of these biomarker candidates used together may give a better indication of adverse effects in the fish induced by oil and/or produced water.

Acknowledgements

We thank Prof. Peter Roepstorff, Nicolai Bache and Andrea Marie Lorentzen in the Protein Research group at The Southern Danish University, Odense, Denmark, for valuable input, training and access to their MALDI-Tof-Tof instrument, which has been a valuable contribution to our work with cod protein identification by mass spectrometry. LC-MS-MS runs were performed by PROBE, the Proteomic platform at the University of Bergen. We also thank Magnus Artnzen at PROBE for excellent service and valuable input. Pål Olsvik, NIFES, Bergen, Norway, and Frank Nilsen, the Institute of Marine Research, Bergen, Norway, gained us access to more than 45. 000 cod ESTs in the database, and Anders Lanzen and Pål Puntervoll, the Computational Biology Unit made the database searchable, which has been essential to retrieving these results, and this co-operation is highly appreciated. Our work is financially supported by Total Norway AS and the Norwegian research Council (grant 1644243/ S40, 159197/I20 and 165233/S40, and The National Functional Genomics Programme - FUGE).

References

Aas E, Baussant T, Balk L, Liewenborg B, Andersen OK. 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquat Toxicol 51(2):241-258.

Anderson GJ, Frazer DM. 2005. Hepatic iron metabolism. Semin Liver Dis 25(4):420-432. 20(1):68-72.

Bottazzi B, Garlanda C, Salvatori G, Jeannin P, Manfredi A, Mantovani A. 2006. Pentraxins as a key component of innate immunity. Curr Opin Immunol 18(1):10-15.

Bradbury MW. 1997. Transport of iron in the blood-brain-cerebrospinal fluid system. J Neurochem 69(2):443-454.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Cardinali M, Maradonna F, Olivotto I, Bortoluzzi G, Mosconi G, Polzonetti-Magni AM, et al. 2004. Temporary impairment of reproduction in freshwater teleost exposed to nonylphenol. Reprod Toxicol 18(4):597-604.

Carls MG, Babcock, M.M., Harris, P.M., Irvine, G.V., Cusick, J.A., Rice,S.D. 2001. Persistence of oiling in mussel beds after the Exxon Valdez oil spill. Marine Environmental Research 51(2):167-190

Crichton RR, Wilmet S, Legssyer R, Ward RJ. 2002. Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells. J Inorg Biochem 91(1): 9-18.

Evanson M, Van Der Kraak GJ. 2001. Stimulatory effects of selected PAHs on testosterone production in goldfish and rainbow trout and possible mechanisms of action. Comp Biochem Physiol C Toxicol Pharmacol 130(2):249-258.

Görg A, Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Wiess, W. 2000. The current state of two-dimensional electrophoresis with immobilized pH-gradients. Electrophoresis 21:1037-1053.

Görg A, Postel, W., Günther,S. 1988. Two-dimensional electrophoresis. Electrophoresis 9:531-546.

Hayashido Y, Hamana T, Ishida Y, Shintani T, Koizumi K, Okamoto T. 2007. Induction of alpha2-antiplasmin inhibits E-cadherin processing mediated by the plasminogen activator/plasmin system, leading to suppression of progression of oral squamous cell carcinoma via upregulation of cell-cell adhesion. Oncol Rep 17(2):417-423.

Heukeshoven J, Dernick R. 1988. Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. Electrophoresis 9(1):28-32.

Jackson JB, Kirby MX, Berger WH, Bjorndal KA, Botsfjord LW, Bourque BJ, et al. 2002. Historical overfishing and the recent collapse of coastal ecosystems. Science 293:629-637.

Jewett SC, Dean TA, Woodin BR, Hoberg MK, Stegeman JJ. 2002. Exposure to hydrocarbons 10 years after the Exxon Valdez oil spill: evidence from

cytochrome P4501A expression and biliary FACs in nearshore demersal fishes. Mar Environ Res 54(1):21-48.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680-685.
Lee RF, Andersson, J.W. 2005. Significance of cytochrome P450 system responses and levels of bile fluorescent aromatic compounds in marine wildlife following oil spills. Marine pollution bulletin 50(705-723).
Liu Y. 2007. Involvement of plasminogen activatorinhibitor type 1 in spermatogenesis, sperm capacitation, and fertilization. Semin thromb Hemost 33(1):29-40.
Machovich R, Horvath I. 1981. Thrombin and haemostasis: regulation of the biological functions of thrombin. (Facts and perspectives). Haematologia (Budap) 14(4):339-359.
Marekov LN, Steinert PM. 2003. Charge derivatization by 4-sulfophenyl isothiocyanate enhances peptide sequencing by post-source decay matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Mass Spectrom 38(4):373-377.
Meier S, Andersen TE, Norberg B, Thorsen A, Taranger GL, Kjesbu OS. 2007. Effects of alkylphenols on the reproductive system of Atlantic cod (Gadus morhua). Aquat Toxicol 81(2):207-218.
Molloy MP, Brzezinski EE, Hang J, McDowell MT, VanBogelen RA. 2003. Overcoming technical variation and biological variation in quantitative proteomics. Proteomics 3(10):1912-1919.
Myhre LP, Bausant, T., Sundt, R., Sanni, S., Vabø, R., Skjoldal, H.R., Klungsøyr, J. 2005. Risk Assessment of reproductive effects of alkyl phenols in produced water on fish stocks in the North Sea AM-2004/018.
Neff JM. 2002. Bioaccumulation in marine organisms. Effect of contaminants from oil well produced water.: Elsevier Science Publishers, Amsterdam.
Neuhoff V, Arold N, Taube D, Ehrhardt W. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9(6):255-262.
Nicholas JM. 1999. Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. Aquatic Toxicology 45(2-3):77-90.
O`Farrell PH. 1975. High resolution two-dimesional electrophoresis of proteins. J Biol Chem 250(10):4007-4021.
OSPAR. 2006. OSPAR Recommendatio 2006/4 Amending OSPAR Recommendation 2001/1 for the management of Produced water from Offshore Installations. OSPAR 06/23/1-E, Annex 15.
Perkins DN, Pappin, D.J., Creasy, D.M., Cottrell, J.S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data Electrophoresis 20:3551-3567.
Rappsilber J, Ishihama Y, Mann M. 2003. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem 75(3):663-670.
Rifai N, Gerszten RE. 2006. Biomarker discovery and validation. Clin Chem 52(9):1635-1637.
Schwaiger J, Mallow U, Ferling H, Knoerr S, Braunbeck T, Kalbfus W, et al. 2002. How estrogenic is nonylphenol? A transgenerational study using rainbow trout (Oncorhynchus mykiss) as a test organism. Aquat Toxicol 59(3-4):177-189.

Schwarzenberg SJ, Sharp HL, Berry SA, Manthei RD, Seelig S. 1987. Hormonal regulation of serum alpha 1-antitrypsin and hepatic alpha 1-antitrypsin mRNA in rats. Biochem Biophys Res Commun 147(3):936-941.

Shevchenko A, Sunyaev, S., Loboda, A., Shevchenko, A., Bork, P., Ens, W., and Standing, K.G. 2001. Charting the proteomes of organisms with unsequenced genomes by Maldi-Quadrupole Time-of-Flight Mass Spectrometry and BLAST homology SEARCHING. Anal Chem 73:1917-1926.

Shevchenko A, Wilm M, Vorm O, Mann M. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68(5):850-858.

Solé M, Porte, C., Biosca, X., Mitchelmore, C.L., Chipman, J.K., Livingstone, D.R., Albaigés, J. 1996. Effects of the "Aegean Sea" oil spill on biotransformation enzymes, oxidative stress and DNA adducts in digestive gland of the Mussel (*Mytilus edulus L.*). Comp Biochem Physiol Vol 113 C(2):257-265.

Steen H, Mann M. 2004. The ABC's (and XYZ's) of peptide sequencing. Nat Rev Mol Cell Biol 5(9):699-711.

Tam SP, Archer TK, Deeley RG. 1985. Effects of estrogen on apolipoprotein secretion by the human hepatocarcinoma cell line, HepG2. J Biol Chem 260(3):1670-1675.

Terrier B, Degand N, Guilpain P, Servettaz A, Guillevin L, Mouthon L. 2007. Alpha-enolase: A target of antibodies in infectious and autoimmune diseases. Autoimmun Rev 6(3):176-182.

Thomas L, Moore NR, Miller S, Booth NA. 2007. The C-terminus of alpha2-antiplasmin interacts with endothelial cells. Br J Haematol 136(3):472-479.

Tolosano E, Altruda F. 2002. Hemopexin: structure, function, and regulation. DNA Cell Biol 21(4):297-306.

Worm B, Barbier EB, Beaumont N, Duffy JE, Folke C, Halpern BS, et al. 2006. Impacts of biodiversity loss on ocean ecosystem services. Science 314(5800):787-790.

Yamamoto K, Sinohara H. 1984. Regulation by sex hormones of serum levels of contrapsin and alpha 1-antiprotease in the mouse. Biochim Biophys Acta 798(2):231-234.

Table 1: Changes in protein expression induced by 3 days of exposure of crude oil (CO) and surrogate produced water (sPW)

Spot #	Protein expression levels					Protein identity
	Control (82,83[a])	0.06 ppm CO (84,85,86)	0.25 ppm CO (87,88,89)	1.0 ppm CO (90)	1.0 ppm* sPW (91)	
6986	1	5.2	5.9	7.5	4.1	
7013	1	0.5	**0**	**0**	2.3	**Fibrinogen**
7029	1	1.2	1.4	1.9	**0**	
7057	1	0.7	4.2	9.0	6.3	
7087	0	1	0	3.0	0	
7105	1	5.4	7.6	8.1	**0**	
7188	1	0.9	0.7	3.3	6.6	
7264	0	1	2.428	5.878	3.2	
7454	1	0.1	0.4	0.7	0.4	
7526	1	0.8	1.2	6.2	3.1	
7550	1	**0**	0.1	**0**	1.1	**Hypothetical (A2A)**
7561	1	0.5	0.1	6.4	**0**	
7602	1	2.4	2.1	9.8	2.9	
7611	1	2.8	2.4	0.7	8.7	
7632	1	0.2	1.6	0.5	**0**	
7644	1	0.9	4.7	9.7	7.8	
7647	1	0.4	1.4	2.1	0.8	
7667	0	1	2.2	8.0	0	
7709	1	**0**	2.3	**0**	7.7	**Fibrinogen**
7715	1	0.1	**0**	0.5	1.8	
7739	0	1	10.9	0	7.9	
7814	1	0.1	0.3	0.1	0.1	
7842	1	2.7	1.7	1.3	4.1	
7848	1	1.9	9.3	6.8	19.1	
8005	0	0	1	6.1	0	
8039	1	0.8	**0**	4.1	1.9	
8115	1	**0**	1.3	1.8	4.9	
8153	1	0.1	**0**	**0**	2.2	
8411	1	**0**	**0**	**0**	**0**	
8418	0	0	1	4.1	0	
8593	0	1	9.2	37.9	0	
8756	1	5.1	3.3	37.6	**0**	
8769	1	**0**	**0**	10.5	**0**	
8786	1	**0**	**0**	22.0	**0**	
9439	1	**0**	1.3	**0**	**0**	**Fibrinogen**

Overview of significantly altered proteins following exposure found by image analysis. CO- Samples of crude oil treated juveniles. sPW- samples of surrogate produced water-treated juveniles. n=1-3/group.

Red colour is used for a significant up-regulation (ANOVA: $p< 0.05$, fold change ≥ 2 in protein expression) of a protein as compared to control.

Green colour is used for significant down-regulation (ANOVA: $p< 0.05$, fold change ≥ 2 in protein expression) of a protein as compared to control.

Grey colour is used for protein spots not significantly changed in protein expression following treatment.

O – the protein is not expressed in this group.

[a]sample of female individual

A2A; Alpha-2-antiplasmin

Table 2: Changes in protein expression after 24 days of continuous crude oil (CO) and surrogate produced water (sPW) exposure

Spot #	Protein expression levels					Protein identity
	Control (62,63)	0.06 µg/L CO (68)	0.25 µg/L CO (69,70)	1.0 µg/L CO (74,75,76)	1.0* µg/L sPW (79,81)	
6965	1	0	0	0	0	
6971	1	0.4	0.7	0.3	0.6	
6983	1	1.3	0.2	0.9	0.7	
6984	1	0	0	0	0	
6989	1	0	0	0	0	
7001	1	0.3	0.5	0.2	0.6	
7011	1	0	0	0	0	Alpha enolase
7018	1	0.7	0	0	1.3	
7020	1	0.5	1.2	0.1	0.3	A2M
7021	1	0.6	1.1	0.3	0.4	
7027	1	1.1	1.1	1.4	0	PTX
7032	1	2.9	0.4	2.5	2.2	Fibrinogen
7043	1	0.6	0.6	0.2	0.3	Tropomyosin^{ns}
7051	1	19	0.3	1.2	1.9	
7053	1	0.6	0.8	0.3	0.4	
7055	1	1.7	0	2.1	1.9	
7057	1	0	1.5	0.4	0	
7069	1	1.1	2.0	1.6	0.5	
7071	1	0	0	0	0.2	
7072	1	3.2	0	2.0	2.4	
7073	1	0	0	0	0.3	
7077	1	1.9	1.6	1.0	0.4	
7081	1	0.7	1.1	0.4	0.1	
7082	1	0.6	1.4	0.2	0.2	
7084	1	0	0	0	0	
7098	1	1.3	0.6	1.7	2.1	Fetuin B
7125	1	1.7	0	0	1.1	
7140	1	1.6	2.7	1.3	1.2	
7143	1	1.2	0.7	1.2	0.2	
7144	1	6.8	1.2	2.0	0	
7160	1	0.7	1.0	0.4	0.3	
7178	1	0	2.4	0	0.4	Apolipoprotien B
7180	1	0	0	0	0	
7186	1	0	1.2	0	0	
7192	1	0.3	0.9	0	0.1	Serotransferrin
7197	1	0.8	0	0.1	0	A2M
7215	1	1.3	2.5	0.5	0.9	
7228	1	2.7	5.1	1.7	1.7	
7240	1	0	0.9	0.4	0.5	
7241	1	2.0	0.8	2.9	2.1	
7259	1	5.8	0	4.4	0.5	
7285	1	0	0.4	0	1.2	Prothrombin* Fetuin B
7288	1	1.6	0.2	0.2	0	
7294	1	0.2	1.6	0.2	0.6	
7300	1	1.1	0.7	1.8	0.3	
7315	1	2.0	10.0	3.0	0.9	
7329	1	0	0.3	0	0	Prothrombin* Fetuin B
7336	1	2.8	3.4	0.5	1.5	
7341	1	3.5	1.4	2.6	1.9	
7342	1	0	0.2	0	0.4	
7345	1	0.5	0	0.5	0	Fetuin B
7409	1	0	0.1	0.1	0.5	HX

7429	1	14.5	4.9	**0**	4.0	
7433	1	0.1	1.0	0.1	0.3	
7437	1	0.1	**0**	**0**	**0**	
7457	1	**0**	1.1	19.3	**0**	
7477	1	2.3	9.5	**0**	0.3	**A2M**
7506	1	8.3	**0**	8.3	7.6	
7525	1	**0**	0.7	4.3	5.4	
7581	1	1.7	0.6	2.2	2.4	**A2A**
7584	1	1.5	**0**	2.1	2.3	**A2A**
7587	1	1.5	1.0	2.2	2.8	
7597	1	1.0	**0**	1.6	2.1	**A2A**
7633	1	1.2	2.0	**0**	**0**	
	1	0.5	0.7	0.3	0.3	**Hypothetical APO B**
7665						**Uncharacterized**
7678	1	0.9	0.5	0.8	**0**	
7705	1	0.9	2.2	0.5	0.8	
7715	1	4.1	3.9	2.0	2.3	
7725	1	11.9	**0**	7.0	1.0	
7726	1	4.2	3.7	2.2	2.3	
7746	1	**0**	**0**	**0**	**0**	
7748	1	2.1	0.6	**0**	**0**	
7806	1	1.4	1.1	0.9	**0**	
7807	1	**0**	0.2	0.3	0.8	
7810	1	0.7	1.4	0.5	0.3	**NTPase**
7811	1	1.7	3.4	**0**	**0**	**A1AT**
7814	1	**0**	1.4	1.5	**0**	
7826	1	0.6	0.5	0.3	0.6	**PZP**
7843	1	0.6	0.5	1.7	2.1	
7854	1	**0**	**0**	0.2	0.4	
7864	1	0.6	1.1	**0**	0.5	
7878	1	**0**	0.3	1.4	8.5	
7922	1	13.5	**0**	16.1	4.2	
8007	1	1.9	3.4	0.3	0.5	
8033	1	7.0	3.7	1.6	**0**	
8039	1	1.9	7.6	1.5	0.9	
8102	1	**0**	**0**	3.3	10.1	
8162	1	6.6	6.0	1.2	**0**	
8189	0	0	0	1	0	
8381	0	0	1	0	0	
8397	0	0	1	66.8	0	
8409	0	0	0	0.9	1	
8448	0	0	0	0	1	
8580	0	0	1	2.0	2.2	**(Prepro)-APO A-1**
8581	0	0	1	1.0	0	
8636	0	0	0	0.6	1	
8841	0	0	0	2.1	1	
8864	0	1	0	0.1	1.1	
8985	0	0	1	0	0	
9128	0	0	0	0	1	
9244	0	0	0	0	1	
9257	0	0	0	0	1	

Overview of significantly altered proteins following exposure found by image analysis: CO-Samples of crude oil treated juveniles. sPW- samples of surrogate produced water-treated juveniles.. n=1-3/group.
Red colour is used for a significant up-regulation (ANOVA: $p < 0.05$, fold change ≥ 2 in protein expression) of a protein as compared to control.
Green colour is used for significant down-regulation (ANOVA: $p < 0.05$, fold change ≥ 2 in protein expression) of a protein as compared to control.
Grey colour is used for protein spots not significantly changed in protein expression following treatment.

O – the protein is not expressed in this group.
A2A; Alpha-2-antiplasmin, HX; Hemopexin, PTX; Pentraxin, A2M: alpha-2-macroglobulin, APO; apolipoprotein, A1AT; Alpha-1-antitrypsin, PZP; Pregnancy zone protein.

Table 3: Identification of differentially expressed proteins after continuous exposure to crude North Sea oil and surrogate produced water

Spot #	Acc. no	Protein identity	Score[a]	Queries matched[b]	Seq. cov. %	Observed pI/Mr	Theoretical pI/Mr	Putative conserved domains
7011[2]	AAH59511.16[ns]	Enolase 1, (alpha) [Danio rerio][ns]	72/29	2/312	9	5.9/50	6.19/47,4	Enolase
7013[2]	NP_998219	Fibrinogen gamma polypeptide [Danio rerio]	93	12	39	5.0/43,5	5.07/48,8	Fibrinogen
7020[2]	BAA85038	Alpha-2-macroglobulin-1 [Cyprinus carpio]	107	5	17	5.3/94	5.40/160, 8	A2M
7027[1]	BAB69039.1	Pentraxin [Cyprinus carpio]	108	2	7	4.6/25	5.07/25,4	PTX
	CAF96585	Unnamed protein product (fibrinogen)	67	6	18	5.0/40	4.96/47,3	Fibrinogen
7032[2]		[Tetraodon nigroviridis]						
	CAF98296[ns]	Unnamed protein product (tropomyosin)	52/29	2	14	6.1/145	5.01/35,2	Tropomyosin
7043[2]		[Tetraodon nigroviridis][ns]						
	CAF98106	Hypothetical protein LOC563663 (fetuin B)	137	9	16	4.4/100	5.05/52,9	Fetuin B
7098[2]		[Danio rerio]						
7178 [ns,2]	CAA57449.1[ns]	Apolipoprotein B [Salmo salar][ns]	58	2	5	4.6/46	5.24/115,9	APO B
7192[2]	Q92079	Serotransferrin [Gadus norhua]	852	8	62	6.5/75	6.12/67.9	Transferrins
7197[2]	BAA92285	Orla C3-1 [Oryzias latipes]	59	5	10	5.3/84	5.9/184,5	A2M
7285[2]	CAD59688	Prothrombin [Oncorhynchus mykiss]	463/29	12/478	15	4.9/90	6.32/70,5	Prothrombin
7329[2]	CAD59688	Prothrombin [Oncorhynchus mykiss]	660/29	21/426	29	4.9/90	6.32/70,5	Prothrombin
	CAF98106	Unnamed protein product (fetuin B)	298/29	9/426	13	4.9/90	5.99/56.1	Fetuin B
		[Tetraodon nigroviridis]						
7345[2]	CAF98106	Unnamed protein product [Tetraodon nigroviridis]	225/29	6/594	11	4.5/90	5.99/56.1	Fetuin B
7409[2]	XP_691686	Predicted: Hemopexin [Danio rerio]	302/28	9/428	26	4.4/75	6.02/45,4	Hemopexin, HX
7550[2]	NP_001073479.1	Hypothetical protein LOC563663 (A2M)	326	11	21	4.4/80	5.05/52.9	A2A
		[Danio rerio]						
7581[2]	NP_001073479.1	Hypothetical protein LOC563663 (A2M)	326	11	21	4.5/95	5.05/52.9	A2A
		[Danio rerio]						
7584[2]	NP_001073479.1	Hypothetical protein LOC563663 (A2M)	326	11	21	4.4/95	5.05/52.9	A2A
		[Danio rerio]						
7597[2]	NP_001073479.1	Hypothetical protein LOC563663 [Danio	326	11	21	4.3/95	5.05/52.9	A2A
		rerio]						
7665[2]	NP_001025233.1	Hypothetical protein LOC321166 [Danio	645/28	27/	84	4.6/40	5.11/412,6	Lipoprotein, function
		rerio]						unknown
	CAA57449.1	Apolipoprotein B [Salmo salar]	675/28	17/	38		5.24/115,9	APO B

7709[1]	NP_997939	Fibrinogen, B beta polypeptide[h] [Danio rerio]	34.9			5.2/64	8.07/54,4	Fibrinogen
7810[1,ns]	XP_692778.2 [ns]	PREDICTED: hypothetical protein [Danio rerio] [ns]	29.4				6.67/129,5	NTPase
7811[1]	CAD90255.1	alpha-1-antiproteinase-like protein [Oncorhynchus mykiss]	39	(1e-96)		5.2/57	5.86/47,5	A1AT
7826[2]	CAG06475	Unnamed protein product [Tetraodon nigroviridis]	115	7	22	5.2/200	8.25/154,1	A2M
	XP_001341390	Predicted: Similar to Pzp Protein [Danio rerio]	106	7	12		7.9/165,1	PZP
	XP_001343136.1	Predicted: Similar to Pzp Protein [Danio rerio]	87	4	14		8.45/169,7	PZP
8580[1,ns]	AAU87042.1	Preproapolipoprotein A-1 [Gadus morhua][ns]	24.3			4.9/27	5.92/14,9	APO A1*
9439[1,h]	NP_997939	Fibrinogen, B beta polypeptide [Danio rerio][h]	34.1			5.8/63	8.07/54,4	Fibrinogen

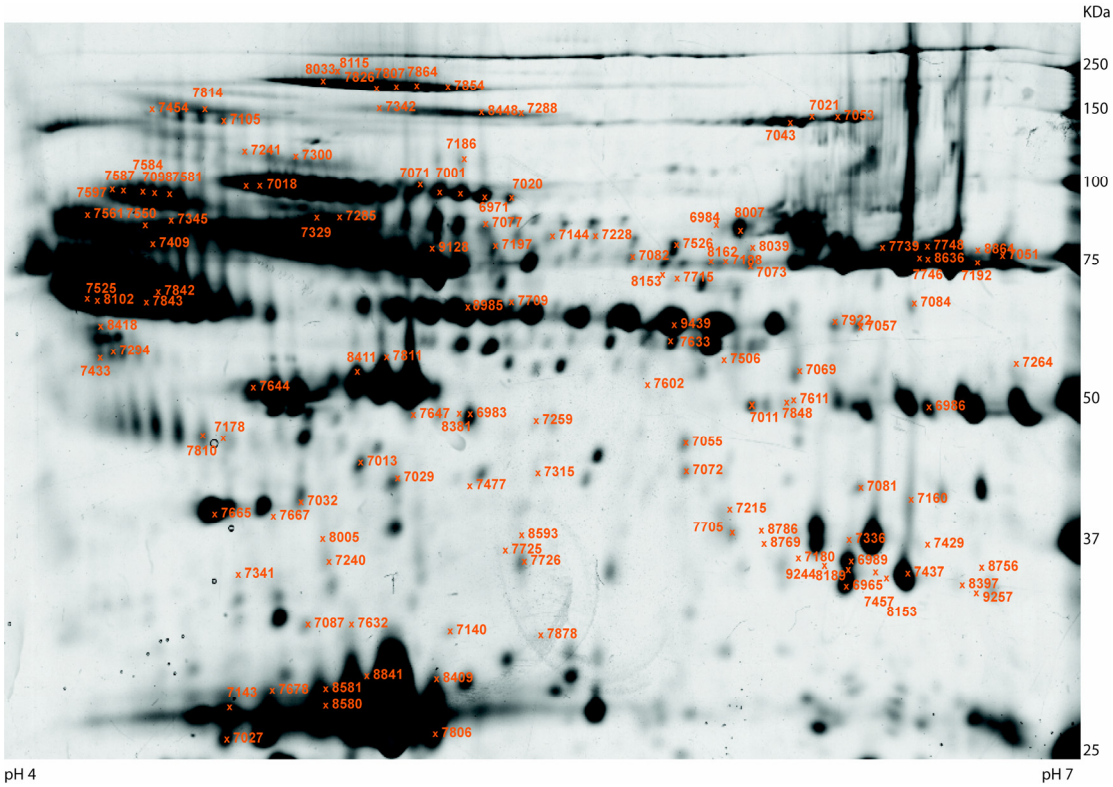
[1]MS method: SPITC-MALDI-ToF-ToF
[2]MS method: ESI-LC-QToF
[ns]non-significant hit: Only one fragemt hit (significant score, $p<0.05$)
[h]The protein was identified as an unnamed protein and was further characterised by homology in blast
[a]The score of query's match in the database/the treshold score of homology (identity)
[b]The number of query fragments that match/the total number of query fragments
Abbreviations: A2M: Alpha-2-macroglobulin, PTX; Pentraxin, APO; Apolipoprotein, A2A; Alpha-2-antiplasmin, HX; Hemopexin, A1AT; Alpha-1- antitrypsin, PZP; Pregnancy zone protein.
The following search criteria were used: Modification: Carbamidomethyl (C), Oxidation (M); Peptide tolerance: 0.5 Da; MS/MS tolerance: 0.3 Da; Enzyme: Trypsin; Monoisotopic. In NCBI-searches taxonomy was specified: Actinopterygii (ray-finned fish). $p<0.05$

Figure legend

Figure 1: Localisation of differentially expressed plasma proteins on the 2DE gel.

A gel of an individual sample of a juvenile cod exposed to 1.0 ppm crude North Sea oil. 20 μg protein were loaded to a 7 cm pH4-7 IPG strip and separated in the second dimension on a 9% SDS PAGE gel. All the 137 significant (ANOVA: $p<0.05$, fold change ≥ 2) from all the different treatment groups (0.06 ppm, 0.25 ppm and 1.0 ppm crude oil, and crude oil spiked with PAH and alkyl phenols) are shown.

Figure 1



Manuscript II

Biomarker candidate discovery in Atlantic cod (*Gadus morhua*) continuously exposed to North Sea produced water from egg to fry

Anneli Bohne Kjersem[1], Nicolai Bache[2], Sonnich Meier[3], Gunnar Nyhammer[4],

[2]Peter Roepstorff, Anders Goksøyr[1,5], Bjørn Einar Grøsvik[1,3]

[1]Department of Molecular Biology, University of Bergen, PB 7800, N-5020 Bergen

[2]Department of Biochemistry and Molecular Biology, the University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

[3]Institute of Marine Research, PB 1870, N-5817 Bergen, Norway

[4]Department of Biology, University of Bergen, PB 7800, N-5020 Bergen

[5]Biosense Laboratories AS, Thormöhlensgt. 55, N-5020 Bergen, Norway

Corresponding author: Anneli Bohne Kjersem

E-mail: anneli.bohne.kjersem@mbi.uib.no

Phone: +47 55 58 45 00

Fax: +47 55 58 96 83

Keywords: Biomarker candidates, egg, fry, larvae, produced water

Abstract

Produced water (PW) is by far the most dominant source of continuous oil discharges to sea. Some of the main pollutants present in produced water are alkyl phenols, poly aromatic hydrocarbons, and heavy metals such as arsenic, mercury, lead, cadmium, and chromium. These have previously been reported to cause adverse effects both in wild life and in humans. Some of these compounds have also been found to exert endocrine disrupting effects, leading to impaired development and reproduction. However, how they act together and how they may affect development and reproduction is not well established for all of these chemicals. Reports from previous studies indicate that early stages of development are more sensitive to xenobiotic exposure. In this study cod were exposed to different levels of North Sea produced water (PW) and 17-ß-oestradiol (E2), a natural oestrogen, from egg to fry stage (90 days). By comparing changes in protein expression following E2 exposure to changes induced by PW treatment, we were able to compare the induced changes by PW to the mode of action of oestrogens. Changes in the proteome in response to exposure in whole cod fry (90 dph) were detected by two-dimensional gel electrophoresis and image analysis and identified by MALDI-ToF-ToF mass spectrometry, using a newly developed cod EST database and the NCBI database. We identified 15 protein changes and 5 new biomarker candidates of produced water (PW) and oestradiol exposure in Atlantic cod fry (*Gadus morhua*). Many of the protein changes occur at low levels (0.01 % and 0.1% PW) of exposure, indicating putative biological responses at lower levels than previously detected. The biomarker candidates discovered in this study may, following biomarker validation, prove useful as diagnostic tools in monitoring exposure and effects of discharges from the petroleum industry offshore, aiding future environmental risk analysis and risk management.

Introduction

Produced water is considered to be by far the most dominant discharge from offshore oil production, containing a variety of compounds, including oil products such as mono-, di- and poly-aromatic hydrocarbons (PAHs), alkyl phenols, and several heavy metals, which have previously been shown to cause adverse effects in fish. Some of these compounds are reported to be endocrine disrupting chemicals (Evanson and Van Der Kraak 2001; Santodonato 1997), affecting steroidogenesis (Evanson and Van Der Kraak 2001; MacLatchy and Van Der Kraak 1995), affecting reproductive organs and reproduction (Meier et al. 2007), and fish egg, larvae, and fry (Hahn 2001; Rolland 2000). PAHs and metals present in produced water are also reported to cause oxidative stress (Hasselberg et al. 2004; Livingstone 2001), possibly resulting in mutagenesis (Machala et al. 2001) and carcinogenesis (Santodonato 1997).

Biological effects of exposure occurring at the early stages of development are of special interest as these stages are found to be more sensitive to xenobiotic exposure (Bern 1992; Colborn et al. 1993). In many teleost species, this period of enhanced vulnerability is from fertilization through the yolk sac embryo stage (Rolland, 2000), which is prior to the larvae stage. However, studies have shown that food and water-borne exposure contributes significantly to fry mortality (Rolland, 2000).

At present, being able to detect and monitor effects of exposure before they threat health and reproduction, causing an effect at population level, is difficult: Most field studies report late occurring adverse effects on cod and other teleost species, often not visible in the exposed fish, but rather in its progeny (see reviews by (Rolland, 2000; Schwaiger, 2002). Also the majority of biomarkers of exposure developed in laboratory studies are not sensitive enough

to detect effects and act as early warning signals of later, broader-reaching effects of chronic exposure. Several field data suggest that adverse effects are induced at lower levels of exposure than known biomarkers can detect (Denslow et al. 2001; Reid and MacFarlane 2003). Hence, there is a need for more sensitive biomarkers that can act as early warning signals to detect and monitor the effects of oil and produced water to marine fish.

In this study, we have exposed cod at early life stages (*Gadus morhua*) to several treatments of produced water (Oseberg C, the North Sea) and 17-ß-oestradiol (E2), a natural oestrogen, for 90 days from egg to fry stage. Our aim has been to investigate produced water-induced changes in cod fry, and, by comparing them to the responses induced by E2, to see if any of these responses represent the same mode of action (MOA).

There has been an unprecedented decline in commercial marine fish species world-wide, resulting in a growing concern for the future viability of fishery resources and prompting a search for causes explaining this decline (Jackson et al. 2002). In many coastal ecosystem cod fish and other marine vertebrates are functionally or entirely extinct. Overfishing is believed to be the main reason for this (Worm et al. 2006), although pollution is considered an important contributing factor to the reported decline (Waldichuk 1979). Although commercially important, little is known about the effects and the mechanisms involved in the biological responses of cod following xenobiotic exposure. Hence, the development of new and more sensitive biomarkers may also contribute with new knowledge important to risk management as drilling and exploration of wells in new areas is progressing.

Materials

Acidic acid, Coomassie brilliant blue CBB G250, ortho-phosphoric acid, urea, thiourea, tris (hydroxymethyl)-ammoniummethane, acetonitril, methanol and thiofluoro acetic were all purchased from Merck (Damstadt, Germany).

Ethanol was purchased from Arcus Kjemi (Oslo, Norway).

Bovine Serum Albumin, BSA, 3-(3-colamidopropyl)-dimetylammonio-1-propansulfoanat, CHAPS, Triton X-100, DL-dithiothreitol, DDT, iodoacetamide, and alpha-cyano-4-hydroxycinnamic acid, CHCA, were purchased from Sigma Aldrich (MO, US).

Ampholine^{TM} 3,5-10, DryStrip Cover fluid, and IEF Electrode strips were purchased from GE Healthcare.

Ammonium dodecyl sulfate, SDS, Agarose, 30% Acrylamide/Bis solution, 37.5:1, Ammonium persulfate, temed and Precision Plus Protein^{TM} standard were purchased from Bio-rad (CA, US).

Trypsin was purchased from Promega (WI, US), while Poros 20R2, Reverse Phase packing, was purchased from Applied Biosystems (CA, US). Siliconised tubes were provided from Sorenson, BioScience, Inc (UT, US). Peptide Calibration Standard and MTP384 Target plate polished steel TF were purchased from Bruker Daltonics (Leipzig, Germany).

Methods

Experimental design

The exposure was carried out for 90 days, starting 25 March 2004 on new spawned eggs and ended at early fry stages the 22 June 2004. The exposure occurred in green, circular tanks with a height of 60 cm, and a diameter of 48 cm. Effective water height in the tanks was about 55 cm, and water volume was 100 l. There were three groups treated with produced water, one group treated with 17β-oestradiol and an untreated control group. There were three parallels for each treatment (totally 15 tanks).

Sixty thousands newly spawned eggs (5 x 12000) from 5 brood stock families (originating from Tysfjord, Northern Norway) were used in each of three parallel exposure groups. The eggs were exposed in the entire embryo development and through hatching. Day-3 after hatching the number of living larvae was measured and the exposure continued on 6000 larvae from each parallel group.

The larvae and early fry were fed with natural zooplankton. The density was 2000 prey pr. liter, and was counted daily. Collection of zooplankton was done by filtering seawater through a Hydrotech filter, and the filtrate was then collected in tank with a diameter on 3 m. The plankton was dominated of nauplii and copepodites of copepods. Two different types of cultivated plankton algae, *Isochrysis galbana* and *Rhodomonas* sp. was supplied to the feeding tanks daily. The temperature in the tanks was hold at 4.8 °C for 30 days and then increased slowly with liner gradient of 0.5 °C a week until 8.6 °C at the end of exposure, (totally 89 day degrees (d°) in embryo stages and 473 d° post hatching). The tanks got a

simulated, natural light regime for Bergen, Norway (60° 25' N, 5° 20' E) using a light control program called LYSSTYR 4.0.

Produced water (PW)

4000 l PW was transported from a North Sea oil platform, Oseberg C (Hydro) to the Institute of Marine Research, Bergen. The PW was bubbled with air for 10 minutes to remove toxic H_2S (g) and then frozen in 25 l containers at -30 °C until use.

The frozen PW (25 l) was thawed at 4 °C and used for 3 days in the exposure system before a new batch of PW was thawed.

Exposure

The xenobiotics were dosed using a fixed ration between two water flows: a sea water flow of 100 ml/min and a "PW flow" of 1 ml/min. The concentration gradient was made by diluting the PW in a header tank before exposure: 1 % PW = pure PW; 0.1 % PW = 1:10 PW; 0.01 % = 1:100 PW. For the positive control, 17β-oestradiol was dissolved in ethanol (9 mg/ml) and 100 µl of this solution was added each l of sea water in the header tank (=> 1000 µg/l).

The "PW flow" was administrated with a 16 channel peristaltic pump (Ismatec MCP) using Tygon tubing.

Sample preparation

Whole individual 90 day cod fry were homogenised in 6x v/w re-hydration buffer (7M urea, 2M thiourea, 4% CHAPS, 20 mM DTT, 0.5% v/v Triton X-100, 0.5% v/v Ampholine 3-10, bromphenol blue) and homogenised using a Potter Elvehjem homogeniser with a teflon®

pestle. The pestle was run up and down 6 times during homogenisation. The homogenised sample was subsequently centrifuged at room temperature at 13 000 g for 20 min. The protein concentration of the samples were determined using a plate reader-modified Bradford` s assay (Bradford 1976).

Two-dimensional gel electrophoresis (2DE)

500 µg of sample was diluted in re-hydration buffer (7M urea, 2M thiourea, 4% CHAPS, 20 mM DTT, 0.5% v/v Triton X-100, 0.5% v/v Ampholine 3-10, bromphenol blue) and added to IPG strips pH 4,5-5,5 (covering the majority of proteins in the sample, results not shown), 18 cm (Görg 1988; Görg 2000; O'Farrell 1975). Four individuals from each treatment group were analysed by 2DE. Due to limited amount of sample, less protein (135-250 µg), of samples exposed to 0.1% produced water were diluted in buffer and added to the IPG strips. Only three individuals from this group were analysed by 2DE. The strips were re-hydrated for a minimum of 12 hours and focused on a Multiphor II unit (GE Healthcare) according to the manufacturer's guidelines. The strips were equilibrated 15 min. at room temperature in 0.25% DTT-containing SDS equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, bromphenol blue) and 15 min. in a 4.5% iodoacetamide-containing SDS equilibration buffer (Görg 1988; Görg 2000; O'Farrell 1975) prior to separation in the second dimension on 9% SDS-PAGE gels (Laemmli, 1970). Gels were run in an Ettan Dalt Twelve unit (GE Healthcare) at 1W/ gel, 20°C, for approximately 17 h. The gels were then stained with Collodial Coomassie (Neuhoff et al. 1988). Coomassie-stained gels were scanned on GS-800 Calibrated Densitometer flatbed scanner (Biorad, CA, US) using PDQuest 7.2.0 software (Biorad, CA, US). The gels were scanned with high resolution: 127.0 x 127.0 microns.

The global protein expression was analysed using Delta 2D image analysis software from Decodon (Greifswald, Germany): TIFF images of the 2DE gel images were up-loaded in Delta 2D and grouped according to treatment. The images were warped using a group-warping strategy and 'exact' warping. A union fusion-gel was produced from all the images within the control, high level exposure (1.0% PW) group and the oestradiol-treated group. No filters were used. Spots were detected on the fusion gel and validated spots were transferred to all the other gel images. Normalisation was performed, based on total intensity including all spots on the gel. The spot data from the all images quantitation table was exported to a cvs-file in Excel, which was successively up-loaded in the Ludesi Interpreter (www.ludesi.com, Lund, Sweden), together with the gel TIFF images. Gel comparison, ANOVA ($p \leq 0.05$ and fold change ≥ 2) and hierarchical clustering analysis (distance function: Pearson correlation; linking method: average; $p > 0.05$) were performed using Ludesi Interpreter. The results of the image analysis were carefully controlled, and any changes detected in difficult gel areas or any changes resulting from bad spot detection, were excluded to limit the number of false positives.

Mass spectrometry

In-gel digestion of protein spots was performed as described by (Shevchenko et al. 1996). Briefly, the excised gel plugs were washed in digestion buffer (50 mM NH_4HCO_3, pH 7.8)/acetonitrile (60/40) and dried by vacuum centrifugation. Modified trypsin (10 ng/µl) dissolved in 50 mM NH_4HCO_3, pH 7.8, was added to the dry gel pieces and incubated on ice for 1 h. After removing the supernatant, additional digestion buffer was added and the digestion was continued at 37 °C over night. The supernatant from the digestion was analysed directly by off-line LC-MALDI without peptide extraction.

Peptide separation was performed using an inert nanoflow-HPLC system from LC-Packings (Ultimate; Switchos2; Famos; LC packings, Amsterdam, The Netherlands) equipped with a Probot MALDI spotting device (LC Packings, Amsterdam, The Netherlands). 10 µl of the in-gel digested protein(s) was acidified with 0.5 µl 10% TFA and loaded onto a home-made 1-cm fused-silica precolumn (100µm i.d., ReproSil-Pur C18 AQ, 3-mm; Dr. Masch GmbH) and then eluted onto a home-made 10-cm fused-silica analytical column (75µm i.d., ReproSil-Pur C18 AQ, 3-mm; Dr. Masch GmbH) using a flow of 200 nL/min and a gradient from 100% solvent A (H_2O/TFA, 100:0.1, v/v) to 50% solvent B (H_2O/acetonitrile/TFA, 20:80:0.1, v/v/v) over 40 min, then 50–100% solvent B in 5 min. Column effluent was mixed in a 1:9 ratio with MALDI matrix (2g/L a-cyano-4-hydroxycinnamic acid in H_2O/acetonitrile/TFA, 30:70:0.1, v/v/v) through a 25 nL mixing tee (fused-silica capillary TSP050375; Composite Metal Services Ltd., UK) and spotted onto a MALDI plate in 30 second fractions. The LC-MALDI preparation was analyzed on an ABI 4700 (Applied Biosystems, Framingham, MA, USA) proteomics analyzer using an automated workflow. A maximum of 10 peptides per spot with a s/n > 80 was automatically selected for MS/MS. CID was performed at collision energy of 1 kV with an indicated collision gas pressure of ~1 $\times 10^{-6}$ Torr.

The spectra were annotated and analyzed using Data Explore™ v. 4.5 (Applied Biosystems) The MS^2 data from the entire off-line LC MALDI experiment were combined into a single mass list using an in-house developed script (Jakob Bunkenborg, SDU, DK). The sequence data from the MS-MS runs (mascot-generic format, mgf files) were used in database searches in the cod expressed sequence tag (EST) database and/or the NCBInr database for protein identity using the MASCOT search engine (Perkins et al. 1999; Steen and Mann 2004). The following parameters were used for the database search: Missed cleavage 1: Significance

threshold $p<0.05$; MS^1 mass accuracy 100 ppm, MS^2 mass accuracy; Partial modifications: Carbamidomethyl (C) and Oxidation (M)

Database searches

The cod EST database used for protein identification includes more than 50,000 EST sequences from cod from a project at the Institute of Marine Research, Norway, NIFES (Norwegian Institute of Research on Nutrition and Seafood) and groups affiliated to the National Research Council, Canada. The annotation of the sequences was performed by the Computational Biology Unit at the University of Bergen, producing approximately 6000 contigs and more than 30,000 singlets. The EST sequences are derived from cDNA libraries of cod exposed to an array of environmental pollutants, such as PCBs and heavy metals, and purified mRNA sequences from several different tissues. RNA was also purified from cod captured in the marinas of Bergen (Norway) and Trondheim (Norway), and therefore assumed to be exposed to a battery of pollutants, as well as cod exposed to hypoxia and hyperoxia, http://www.codstress.olsvik.info/Results.html (Olsvik et al. 2005).

Results

Exposure of cod to 1,0% PW during egg and larvae stage resulted in significant increase in mortality compared to control. At sampling, only 7 individuals (ca. 0.05%) were left in the group of fry exposed to 1.0% PW (ca. 5% survival in the control group). Morphological changes were observed among several of the 1.0% PW-treated fishes, although this number were not found to be significant. There was also a significant difference in the cod fry weight in the 1.0%PW-treated group compared to the E2-treated group. Individuals within the E2-treated group were of significantly lower weight.

The proteomic analysis includes 4 individuals within each treatment-group and 3 individuals within the group treated with 0.1% (medium levels of) PW. Exposure of cod to produced water and E2 from egg to fry stage (90 days) resulted in a number of protein changes (Figure 1). Treatment with 10 μg/l E_2 induced more changes in the 2DE pattern compared to the changes induced by exposure to produced water (Figure 1). The 2DE and image analysis of the global protein expression of whole cod fry exposed to produced water and E2 showed a significant change in protein expression (ANOVA: $p<0.05$; fold change ≥ 2) of 84 proteins (Table 1). Of the 84 protein changes 27 have significantly changed expression after exposure to 0.01% PW, 24 have changed levels after exposure to 0.1% PW, and 9 have changed levels after exposure to 1.0% PW, whereas 24 proteins were shown to have changed levels after exposure to 10 μg/l E_2. Of the 60 protein changes found to be induced by produced water, 24 were not affected by E2 treatment. Hierarchical clustering analysis as shown in Figure 2 demonstrate this pattern. About 25% of the observed protein changes appeared to be unique to oestradiol treatment, whereas 25% appeared to be unique to the produced water treatment, and

one third of the apparent changes induced by produced water appeared to be induced by after treatment with 10 μg/l E_2 (Figure 2).

The differentially expressed proteins were isolated from a control gel and from a gel of an E2 treated sample (Figure 3), and trypsin-digested prior to MS/MS analysis. The MS/MS data were used for database searches for protein identification, in which 13 of totally 24 protein spots were successfully identified (Table 2). In general a minimum of two significant fragments is used as criteria for protein identification. However, some of the biomarker candidates in this study are only identified by one significant fragment, as the fragment sequence covers a long stretch of the sequence of the identified protein, and as the protein identity is strengthened by the identification of similar proteins in that gel area. Several of the proteins in the trains of spots in the upper part of the gel were identified as skeletal muscle myosin heavy chains (MHC), as were several of the differentially expressed protein spots. However, the identified MHCs show some conflicting results regarding protein expression: Those in the upper trains of spots appeared to be up-regulated after exposure to produced water and down-regulated following E2 exposure, whereas those proteins identified as myosin with a lower molecular weight appeared to be down-regulated by PW treatment and up-regulated after exposure 10 μg/l E_2. Hence, these protein changes appear to be protein modifications of myosin. Several others of the differentially expressed proteins were identified as fast skeletal muscle alpha-actin and appeared to be up-regulated by PW exposure and down-regulated by E2 treatment. Hsc71, an Hsp70, appeared to be down-regulated by 0.1% PW. Alpha-actinin was also found to be down-regulated by produced water treatment (both 0.1 % and 1.0 %). Exposure to 0.01 % and 0.1% PW caused a down-regulation of keratin. E2 treatment appeared to down-regulate ATP synthase, which is known to be involved in energy production.

The success rate of the protein identification was approx. 50 %, which is lower than expected based on previous work on juvenile cod plasma and liver samples. The success of protein identification seemed to be linked to protein amount, as the very small spots in the spot trains in the upper parts of the gel were more difficult to identify than the larger spots in the middle- and lower part of the gel. Characteristic to these gel images of whole cod fry is also the large groups of the same protein, such as myosin in the upper part of the gel image. The gel images of whole cod fry samples seem to be dominated by few types of proteins forming large groups of spot trains on the gel.

Discussion

We have detected changes in protein expression of cod fry exposed to produced water and 10 μg/l E_2 by 2DE and image analysis. Changes in the protein expression were also confirmed by the hierarchical clustering analysis. Of the proteins shown to be differentially expressed (ANOVA: $p>0.05$ and fold change≥ 2), most of the changes (27 of 84) occurred following exposure to low level, 0.01%, and medium level (24 of 84), 0.1%, of produced water, corresponding to less than 40 to 4 μg hydrocarbons/L produced water or a distance >100-1000 meters from the point of discharge (oil platform) (Calculations based on (Neff 2002) and (Rye et al. 2000). These results indicate an alteration in part of the cod fry proteome at low levels of produced water and appreciable distances from the point of discharge. As mentioned, we observed a significant increase in mortality and an insignificant increase in morphological changes among individuals exposed to high levels of produced water (1% PW). Whether these observations regarding mortality and malformations can be linked to the observed changes in protein expression is uncertain. However, the alterations in the proteome detected at lower levels of exposure may be construed as early warning signs of the observed effects, such as morphology abnormalities, following high level exposure, as changes and effects at a lower level of organisation always proceed the changes and effects observed at higher levels of organisation (Bayne 1985). This will then strengthen the role of the differentially regulated proteins detected in this study as biomarker candidates in future bio-monitoring. The high number of protein changes apparently induced by low levels of produced water may be another indication of the early life stages being more sensitive to xenobiotics.

More than half of the identified protein changes induced by low levels of exposure (0.01% PW and 0.1% PW), were also shown to be affected by E_2 treatment, indicating an overlap in

these responses. Approximately half of the changes induced by E_2 treatment were also found to be affected by exposure to produced water. Some of the proteins in which expression was altered following exposure to produced water and oestradiol were identified as myosin and alpha actin. Produced water treatment induced a change in protein expression of myosin different to the protein expression induced by oestradiol treatment. In contrast, alpha actin showed a similar response in protein expression after both produced water (0.01 % and 0.1%) and oestradiol treatment, although high levels of PW resulted in a down-regulation of alpha actin.

The myosin isoforms detected are heavy chain myosins and skeletal muscle heavy chain myosin. Previous studies have found the development of skeletal muscles, i.e. myogenesis, in teleosts to go through different phases, in which the spatial differential expression of myosin heavy chain and transcription of myosin heavy chain genes are important to the development and differentiation of myotomes, in particular hyperplasia (muscle fibre recruitment) (Steinbacher et al. 2006). This process is controlled by the transcription factors MEF2 (myocyte-specific enhancement factor), *Myo D* and Myogenin. In medaka (*Oryzias latipes*) the genes coding for myosin heavy chains were found predominantly expressed in embryo and larvae, all belonging to fast skeletal muscles showing a spatiotemporally expression pattern during development (Ono et al. 2006). These genes are also linked to temperature adaptation (Liang et al. 2007). The fast skeletal alpha actin is important in the development of skeletal muscle in fish (Thiebaud et al. 2001; Venkatesh et al. 1996). In fugu fish (*Fugu rubripes*) alpha-skeletal actin is also localised in skin and gills in addition to fast skeletal muscle and the heart (Venkatesh et al. 1996). At the larvae stage increased activity increases the requirements for respiration, and locomotor activity is closely linked to both muscular and gill development (Falk-Petersen 2005). Alpha smooth muscle actin is found in the ovaries of

zebrafish (Van Nassauw et al. 1991), which later in life is involved in the contraction of the ovaries. Alpha actin and myosin are also present in retina rod structures (Chaitin and Burnside 1989; Poplinskaia 1995; Williams et al. 1992). During larvae stage the rods seem to be involved in movement perception and may be particularly important in predator avoidance (Blaxter 1986; O'Connell 1981). Hence, the changes in protein expression (ANOVA: $p<0.05$; fold change ≥ 2) of myosin and alpha actin suggest an impact of produced water on the fast skeletal muscle development, essential for somatic growth, perception, potentially impairing general growth and development of cod fry. These results may also explain the large differences in larvae size observed between cod fry exposed to 1.0% PW and 10 ppb oestradiol. However, the large size of 1.0% PW-treated larvae may be explained by the good access of food on the seven individuals left in the tank as the amount of food was kept constant while the high mortality in this group significantly lowered the number of animals consuming the food. Impairment of growth and development may have economic consequences for fisheries as the biomass of the fish stocks can be reduced.

Produced water resulted in a down-regulation of alpha-actinin. This protein have previously been suggested to provide structural support for the motile photoreceptors and a semipermeable barrier in vertebrate retina (Williams et al. 1990). In teleosts these junctions in the retina help them maintain their orientation with respect to incoming light (ibid.).

Hsc71, a Hsp70, was found down-regulated by produced water. The Hsp70 proteins are known to be able to bind hydrophobic protein domains and function as chaperones that assemble and stabilize multiprotein complexes, translocation of polypeptides across cell membranes, and aiding protein folding (Feder and Hofmann 1999; Young and Hartl 2002). Hsp70 is reported to associate with Hsp90, which is of importance to the conformation of the

ligand binding domain of the nuclear steroid receptors (and the Ah receptor) as reviewed by (Pratt 1997). Thus, the down-regulation of Hsc71 may possibly lead to numerous responses.

Filament proteins, keratin K8b (S2) and cytokeratin 4 (Krt4), was both found to be down-regulated by produced water in this study. The keratins are known to be involved in maintenance of cellular architecture and to provide mechanical resistance against stress (Kirfel et al. 2002). The down-regulation of keratin suggests an effect on tissue integrity. Some studies also report keratins to be involved in apoptosis and signal transduction (Schaffeld and Schultess 2006).

Hence, the most evident responses from produced water exposure seen in this study seem to involve structural and cytoskeletal proteins, and signalling proteins, important to muscle development, rod/retina function, cellular signalling and morphology (tissue integrity). This is also important for swim performance and predator escape (Falk-Petersen 2005).

ATP synthase generates ATP and energy from fatty acid fuels (Crockett et al. 1999). Treatment with 10 μg/l E_2 lowered the protein expression of ATP synthase, which may lead to a slower metabolism. This may in turn lead to an increase in exposure to xenobiotics, as maternal transfer of hydrophobic xenobiotic contaminants sequestered in lipid reserves may accumulate as the conversion of fatty acids slows down (van der Kraak et al. 2001).

Before these changes in protein expression can be applied as biomarkers in future bio-monitoring, they will have to be validated going through a validation process consisting of quantitation, qualification, verification, validation and ultimately assay optimisation (Crockett et al. 1999). The differential protein expressions will need to be confirmed by more targeted

assays and techniques, and in the verification process the analysis is extended to a larger number of samples including a broader range of controls and cases to confirm sensitivity and assess specificity (ibid.).

The MS/MS technique used for protein identification is well developed. However, we believe the reason why we did not manage to identify more of the observed protein changes is lack of sequence data in the database and the limited protein amount in many of the isolated spots, as only 500 μg sample was applied due to scarce amount of sample available.

Conclusion

Most of the detected alterations in the proteome of whole cod fry induced by exposure to produced water were detected following low levels of exposure. Hence the protein changes observed after low level treatment may function as early warning signs that precede possible biological effects observed among individuals exposed to higher levels of produced water. More than half of the observed protein changes induced by produced water appeared to overlap responses found in oestrogen-exposed fry. Myosin heavy chain, fast skeletal muscle alpha actin, Hsc71, alpha-actinin, and keratin are suggested biomarker candidates of the effects of produced water on larvae, and ATP synthase is a biomarker candidate of the effects of E_2 on cod larvae. These biomarker candidates will have to be validated before use as biomarkers in future environmental monitoring. These responses observed indicate an effect of produced water on muscle development, as well as on cod morphology and perception, and an effect of E_2 on the lipid metabolism and growth. Oestrogen exposure also elevated MHC and alpha actin expression in fry, but the biological significance of this response is unclear.

Acknowledgement

This study was supported by the Norwegian Research Council grants 164423/S40 and 141213/720 and by Total E&P Norway. The instruments for off line LC MS and MS/MS were founded by the Danish Research agency through the Danish Biotechnology Instrument Center.

References

Bayne BL, Brown, D.A., Burns, K., Dixon, D.R., Ivanovici, A.,Livingstone, D.A., Lowe, D.M., Moore, M.N., Stebbing, A.R.D., Widdings, J. 1985. The effects of stress and pollution on marine animals:Praeger, New York, USA.

Bern HA. 1992. The development of the role of hormones in development--a double remembrance. Endocrinology 131(5):2037-2038.

Blaxter JHS. 1986. Development of sense organs and behaviour in teleost larvae with special reference to feeding and predator avoidance. Transaction of the American Fisheries Society 115:98-114.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Chaitin MH, Burnside B. 1989. Actin filament polarity at the site of rod outer segment disk morphogenesis. Invest Ophthalmol Vis Sci 30(12):2461-2469.

Colborn T, vom Saal FS, Soto AM. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101(5):378-384.

Crockett EL, Londraville RL, Wilkes EE, Popesco MC. 1999. Enzymatic capacities for beta-oxidation of fatty fuels are low in the gill of teleost fishes despite presence of fatty acid-binding protein. J Exp Zool 284(3):276-285.

Denslow ND, Lee HS, Bowman CJ, Hemmer MJ, Folmar LC. 2001. Multiple responses in gene expression in fish treated with estrogen. Comp Biochem and Physiol Part B 129.

Evanson M, Van Der Kraak GJ. 2001. Stimulatory effects of selected PAHs on testosterone production in goldfish and rainbow trout and possible mechanisms of action. Comp Biochem Physiol C Toxicol Pharmacol 130(2):249-258.

Falk-Petersen IB. 2005. Comparative organ differentiation during early life stages of marine fish. Fish Shellfish Immunol 19(5):397-412.

Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243-282.

Görg A, Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Wiess, W. 2000. The current state of two-dimensional electrophoresis with immobilized pH-gradients. Electrophoresis 21:1037-1053.

Görg A, Postel, W., Günther,S. 1988. Two-dimensional electrophoresis. Electrophoresis 9:531-546.

Hahn ME. 2001. Dioxin toxicology and the aryl hydrocarbon receptor: insights from fish and other non-traditional models. Mar Biotechnol (NY) 3(Supplement 1):S224-238.

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

Jackson JB, Kirby MX, Berger WH, Bjorndal KA, Botsfjord LW, Bourque BJ, et al. 2002. Historical overfishing and the recent collapse of coastal ecosystems. Science 293:629-637.

Kirfel J, Peters B, Grund C, Reifenberg K, Magin TM. 2002. Ectopic expression of desmin in the epidermis of transgenic mice permits development of a normal epidermis. Differentiation 70(1):56-68.

Liang CS, Kobiyama A, Shimizu A, Sasaki T, Asakawa S, Shimizu N, et al. 2007. Fast skeletal muscle myosin heavy chain gene cluster of medaka Oryzias latipes enrolled in temperature adaptation. Physiol Genomics 29(2):201-214.

Livingstone DR. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 42(8):656-666.

Machala M, Vondracek J, Blaha L, Ciganek M, Neca JV. 2001. Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay. Mutat Res 497(1-2):49-62.

MacLatchy DL, Van Der Kraak GJ. 1995. The phytoestrogen beta-sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl Pharmacol 134(2):305-312.

Meier S, Andersen TC, Lind-Larsen K, Svardal A, Holmsen H. 2007. Effects of alkylphenols on glycerophospholipids and cholesterol in liver and brain from female Atlantic cod (Gadus morhua). Comp Biochem Physiol C Toxicol Pharmacol 145(3):420-430.

Neff JM. 2002. Bioaccumulation in marine organisms. Effect of contaminants from oil well produced water.: Elsevier Science Publishers, Amsterdam.

Neuhoff V, Arold N, Taube D, Ehrhardt W. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9(6):255-262.

O'Connell CP. 1981. Development of organ systems in the northern anchovy Engraulis mordax and other teleosts. American Zoologist 21:429-446.

O'Farrell PH. 1975. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250(10):4007-4021.

Olsvik PA, Lie KK, Jordal AE, Nilsen TO, Hordvik I. 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol 6:21.

Ono Y, Liang C, Ikeda D, Watabe S. 2006. cDNA cloning of myosin heavy chain genes from medaka Oryzias latipes embryos and larvae and their expression patterns during development. Dev Dyn 235(11):3092-3101.

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20(18):3551-3567.

Poplinskaia VA. 1995. [The cytostructure and morphogenesis of the rod outer segments]. Ontogenez 26(1):5-21.

Pratt WB. 1997. The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. Annu Rev Pharmacol Toxicol 37:297-326.

Reid DJ, MacFarlane GR. 2003. Potential biomarkers of crude oil exposure in the gastropod mollusc, Austrocochlea porcata: laboratory and manipulative field studies. Environmental Pollution 126(2):147-155.

Rolland RM. 2000. A review of chemically-induced alterations in thyroid and vitamin A status from field studies of wildlife and fish. J Wildl Dis 36(4):615-635.

Rye H, Johansen Ø, Reed M, Ekrol N, Deqi X. 2000. Exposure of fish larvae to hydrocarbon concentration fields generated by subsurface blowouts. Spill Science and Technology Bulletin 6(2):113-123.

Santodonato J. 1997. Review of the estrogenic and antiestrogenic activity of plocyclic aromatic hydrocarbons: Relationship to carcinogenity. Chemosphere 34(4):835-848.

Schaffeld M, Schultess J. 2006. Genes coding for intermediate filament proteins closely related to the hagfish "thread keratins (TK)" alpha and gamma also exist in lamprey, teleosts and amphibians. Exp Cell Res 312(9):1447-1462.

Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, et al. 1996. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc Natl Acad Sci U S A 93(25):14440-14445.

Steen H, Mann M. 2004. The ABC's (and XYZ's) of peptide sequencing. Nat Rev Mol Cell Biol 5(9):699-711.

Steinbacher P, Haslett JR, Six M, Gollmann HP, Sanger AM, Stoiber W. 2006. Phases of myogenic cell activation and possible role of dermomyotome cells in teleost muscle formation. Dev Dyn 235(11):3132-3143.

Thiebaud P, Rescan PY, Barillot W, Ralliere C, Theze N. 2001. Developmental program expression of myosin alkali light chain and skeletal actin genes in the rainbow trout Oncorhynchus mykiss. Biochim Biophys Acta 1519(1-2):139-142.

van der Kraak G, Hewitt M, Lister A, McMaster ME, Munkittrick KR. 2001. Endocrine toxicants and reproductive success in fish. Human Ecological Risk assessment 7(5):1017-1025.

Van Nassauw L, Harrisson F, Callebaut M. 1991. Localization of smooth-muscle markers in the ovaries of some ectothermic vertebrates. Anat Rec 229(4):439-446.

Venkatesh B, Tay BH, Elgar G, Brenner S. 1996. Isolation, characterization and evolution of nine pufferfish (Fugu rubripes) actin genes. J Mol Biol 259(4):655-665.

Waldichuk M. 1979. The assessment of sublethal effects of pollutants in the sea. Review of the problems. Philos Trans R Soc Lond B Biol Sci 286(1015):399-424.

Williams DS, Arikawa K, Paallysaho T. 1990. Cytoskeletal components of the adherens junctions between the photoreceptors and the supportive Muller cells. J Comp Neurol 295(1):155-164.

Williams DS, Hallett MA, Arikawa K. 1992. Association of myosin with the connecting cilium of rod photoreceptors. J Cell Sci 103 (Pt 1):183-190.

Worm B, Barbier EB, Beaumont N, Duffy JE, Folke C, Halpern BS, et al. 2006. Impacts of biodiversity loss on ocean ecosystem services. Science 314(5800):787-790.

Young JC, Hartl FU. 2002. Chaperones and transcriptional regulation by nuclear receptors. Nat Struct Biol 9(9):640-642.

Table 1: Overview of differentially expressed proteins in exposed cod fry

Spot	Control	0.01% PW	0.1% PW	1.0% PW	E2 (10 ppb)
9	1	2.1	1	2.7	0.08
11	1	1.2	0.5	1.5	0.2
17	1	0.02	0.2	3.5	1
61	1	0.2	0.4	1.6	0.4
75	1	0.4	0.8	3.2	0.8
90	1	0.6	0.2	0.6	0.2
88	1	0.6	0.4	1.3	0.4
92	1	0.2	0.3	0.4	0.2
94	1	0.8	0.5	0.7	0.4
107	1	1.4	1.8	0.9	3.6
109	1	1.1	0.2	0.7	0.5
113	1	0.5	0.2	0.6	0.4
118	1	0.2	0.2	1.1	0.07
123	1	2.3	1	2.1	0.8
139	1	0.5	0.5	0.8	0.4
145	1	0.4	0.4	0.4	0.08
234	1	0.7	2.1	0.6	2.0
238	1	0.1	0.8	1.3	2.4
239	1	0.05	0.5	1.0	2.9
241	1	0.4	0.2	0.3	1.7
243	1	0.5	0.4	0.4	2.0
244	1	0.9	0.8	0.4	3.3
247	1	0.2	0.4	0.6	1.6
254	1	0.4	0.3	0.4	1.8
256	1	0.9	0.9	0.5	3.0
257	1	0.8	0.9	0.5	2.2
261	1	0.7	0.4	0.4	1.8
262	1	0.5	0.7	0.4	1.8
265	1	0.9	0.7	1	3.0
308	1	0.5	1.0	1.5	3.7
276	1	0.3	0.8	2.2	0.8
282	1	0.7	2.4	0.6	1.6
283	1	0.6	2.3	0.9	1.5
306	1	0.9	0.9	0.9	3.6
309	1	0.4	0.4	0.4	2.0
325	1	1.9	2.7	0.5	4.7
326	1	1.2	3.8	0.8	4.3
327	1	1.3	1.7	0.4	3.2
329	1	0.5	0.6	0.9	2.6
331	1	0.9	4.7	0.5	4.1
331	1	2.1	2.5	0.4	3.9
334	1	2.3	1.8	0.4	2.7
357	1	3.0	1.4	0.5	2.1
335	1	0.7	1.5	0.8	3.7
337	1	0.7	3.3	0.4	4.6
339	1	1	2.5	0.5	2.5
344	1	0.7	0.1	0.3	1
348	1	0.5	0.4	0.7	0.9
349	1	1.4	0.8	0.6	2.8
352	1	1	2.2	0.5	3.9
357	1	1.5	0.7	0.6	2.3
356	1	1	1.7	0.4	2.1
355	1	0.7	0.7	0.3	1.5
366	1	0.9	1.3	1.2	6.6
371	1	1.6	1.9	0.7	4.6
361	1	1	1	0.4	1.8
372	1	1	1	0.5	2
374	1	2.3	0.7	0.8	1.3

376	1	0.9	1.1	0.9	2.4
384	1	0.4	0.3	0.8	0.6
386	1	1.1	0.4	0.5	0.9
401	1	0.6	1.2	0.7	2.3
402	1	0.8	2.3	0.4	2.4
404	1	1.2	1.9	0.9	2.7
408	1	1.3	2.3	0.7	4.7
409	1	2.3	0.7	0.5	2.9
410	1	0.9	0.5	1	0.3
415	1	1.1	1.1	0.9	4.3
417	1	0.5	0.4	0.6	1.9
418	1	6.3	2.2	0.4	4.4
418	1	1	0.8	0.4	1.9
419	1	0.7	1.3	2.6	6.5
423	1	1.4	1.2	0.9	2.7
423	1	2.0	1.7	0.3	3.7
422	1	1.6	2.2	0.4	2.9
440	1	0.4	0.7	1	3,0
441	1	1.2	1	0.8	4.9
452	1	1.6	6.1	0.9	3.7
455	1	4.3	16.2	1.2	6.4
456	1	0.6	0.3	0.8	0.9
459	1	0.9	1.3	0.7	2.0
442	1	1	1.3	0.7	3.6
460	1	2.2	1.4	0.8	5.4
462	1	2.0	0.9	0.4	1

Overview of significantly altered proteins following exposure found by image analysis: PW-Samples of produced water treated fry. E2- samples of oestradiol-treated fry. n=4/group, except for group 0.1% PW; n=3.
Red colour is used for a significant up-regulation (ANOVA: $p < 0.05$, fold change ≥ 2 in protein expression) of a protein as compared to control.
Green colour is used for significant down-regulation (ANOVA: $p < 0.05$, fold change ≥ 2 in protein expression) of a protein as compared to control.
Grey colour is used for protein spots not significantly changed in protein expression following treatment.

Table 2: Biomarker candidates of produced water and oestradiol- Differentially expressed proteins identified by MS/MS

Spot n°	Accession number	Protein Identity	Queries matched[a]	Database	Score[b]	Seq. cov. %	Theoretical pI/Mw (kDa)
9	XP_708916.1	Myosin, heavy polypeptide 1, skeletal muscle (MHC) [Danio rerio]	29 (177)	NCBI	1586/31	13	5.66/287609.6
94	Q9PTY0	ATP synthase subunit beta [Cyprinus carpio]	11 (67)	Cod EST	421/27	38	5.05/55247.3
188	AAH66728	Krt4 protein [Danio rerio]	4 (150)	Cod EST	58/27	10	5.34/54027.2
243	XP_708916.1	Myosin, heavy polypeptide 1, skeletal muscle (MHC) [Danio rerio]	2 (79)	NCBI	94/31	2	5.66/287609.6
244*	XP_708916.1	Myosin, heavy polypeptide 1, skeletal muscle (MHC)[Danio rerio]	2 (45)	NCBI	63/32	1	5.66/287609.6
247	CAA63300.1	Simple type II keratin k8b (S2) [Oncorhynchus mykiss]	3 (373)	Cod EST	90/27	4	5.14/58976.5
254*	XP_708916.1	Myosin, heavy polypeptide 1, skeletal muscle (MHC) [Danio rerio]	3 (45)	NCBI	51/31	2	5.66/287609.6
344*	AAN77132	Alpha-actinin [Danio rerio][h]	8 (53)	NCBI	179/30	11	5.17/103860.2
366*	BAA09069.1	Myosin heavy chain (MHC) [Cyprinus carpio]	1 (44)	NCBI	39/31	1	5.14/110155.2
386*	P47773	Heat shock cognate 71kDa protein (Hsc71) [Ictalarus punctatus]	6 (49)	NCBI	364/31	12	5.19/71340.4
402*	P49055	skeletal alpha-actin [Carassius auratus]	3 (46)	NCBI	92/31	11	5.23/42141.1
418*	AAM21702	Fast skeletal muscle alpha-actin [Gadus morhua]	4 (47)	Cod EST	74/28	16	5.23/41974.9
441*	AAM21702	Fast skeletal muscle alpha-actin [Gadus morhua]	4 (47)	Cod EST	135/28	17	5.23/41974.9

The following search criteria were used: Modification: Carbamidomethyl (C), Oxidation (M); Peptide tolerance: 100 ppm; MS/MS tolerance: 0.25 Da; Enzyme: Trypsin; Monoisotopic. In NCBI-searches taxonomy was specified: Actinopterygii (ray-finned fish). $p<0.05$

[h]The protein was identified as an unnamed protein and was further characterised by homology in blast

*No preanalaytical LC-run

[a]The score of query's match in the database/the treshold score of homology (identity)

[b]The number of query fragments that match/the total number of query fragments

Figure legends

Figure 1: Comparison of protein changes induced by produced water treatment and oestradiol exposure

Left: Protein changes induced by 1.0% PW (orange) compared to control (blue) detected by 2DE and image analysis in whole fry individuals.

Right: Protein changes induced by 10 µg/L E_2 (orange) compared to control (blue) detected by 2DE and image analysis in whole fry individuals.

Figure 2: Dendogram from hierarchical clustering analysis of differentially expressed proteins

The gel names are given in the left margin, in which the following abbreviations for the different treatments are used; L-low levels (0.01%) PW, M- medium levels (0.1%) PW, H- high levels (1.0%) PW, and E2-oestradiol. n=4/group, 5 groups (except for group 0.1% PW; n=3). The coloured squares represent spots, in which the spots identified to be the same spot on the different gels are aligned vertically. The three-structure in the top of the figure indicate the spot relationships between the gels found from hierarchical clustering. Red colour indicates a strong expression of the spot. Green colour indicates a weak expression of the spot. A darker shade indicates a spot volume closer to the average.

Figure 3: Localisation of the differentially expressed proteins on the 2DE gels

The significant protein changes found by image analysis of PW- and oestradiol-treated whole cod fry (ANOVA: $p>0.05$, fold change ≥ 2) are indicated on representative 2DE gels by their spot numbers. Abbreviations used: PW; produced water-treated sample, E_2; Oestradiol-treated sample

Figure 1

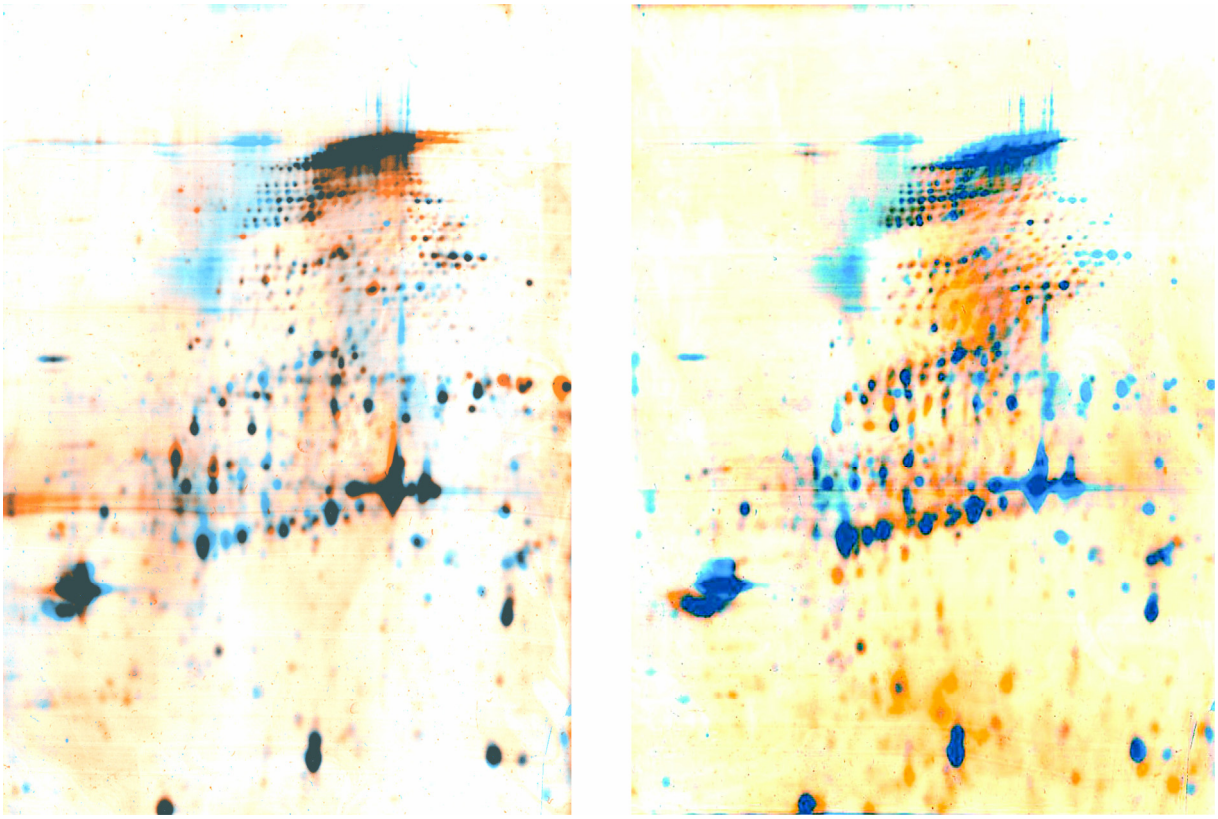


Figure 2

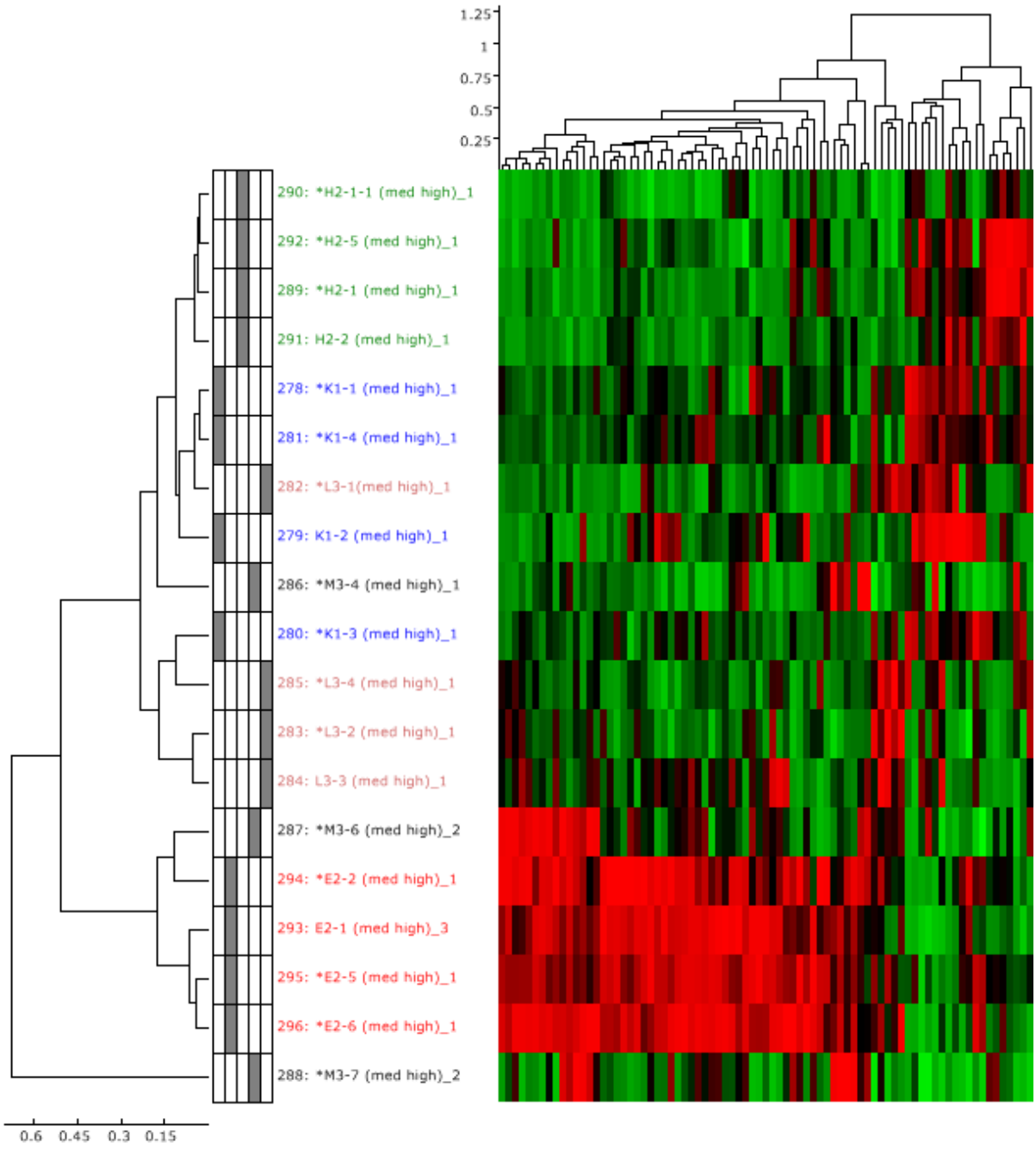
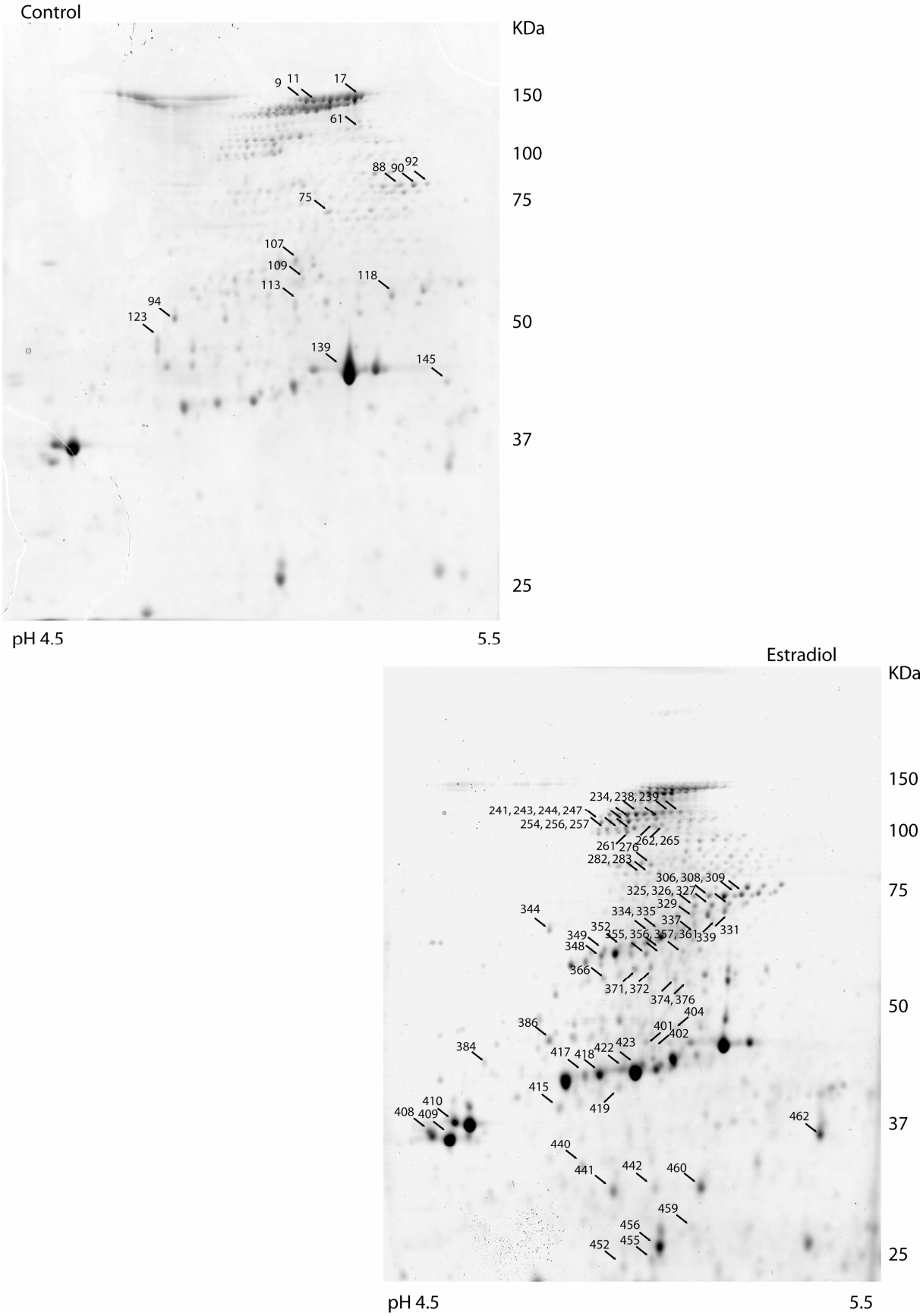

1.25
1
0.75
0.5
0.25
290: *H2-1-1 (med high)_1
292: *H2-5 (med high)_1
289: *H2-1 (med high)_1
291: H2-2 (med high)_1
278: *K1-1 (med high)_1
281: *K1-4 (med high)_1
282: *L3-1(med high)_1
279: K1-2 (med high)_1
286: *M3-4 (med high)_1
280: *K1-3 (med high)_1
285: *L3-4 (med high)_1
283: *L3-2 (med high)_1
284: L3-3 (med high)_1
287: *M3-6 (med high)_2
294: *E2-2 (med high)_1
293: E2-1 (med high)_3
295: *E2-5 (med high)_1
296: *E2-6 (med high)_1
288: *M3-7 (med high)_2
0.6 0.45 0.3 0.15

Figure 3

Fig 2: Isolation and identification of differentially expressed larvae proteins



Manuscript III

Biomarker candidate discovery and possible toxicological effects in liver of cod fry (*Gadus morhua*) continuously exposed to produced water

[1ξ]Anneli Bohne Kjersem, [1]Karen Anita Aa, [2]Nicolai Bache, [3]Sonnich Meier,

[4]Gunnar Nyhammer, [2]Peter Roepstorff, [1,3]Bjørn Einar Grøsvik, and [1,5]Anders Goksøyr

[1]Department of Molecular Biology, University of Bergen, PB 7800, N-5020 Bergen, Norway

[2]Department of Biochemstry and Molecular Biology, the University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

[3]Institute of Marine Research, PB1870, N-5817 Bergen, Norway

[4]Department of Biology, University of Bergen, PB 7800, N-5020 Bergen. Norway

[5]Biosense Laboratories AS, Thormöhlensgt. 55, N-5020 Bergen, Norway

[ξ]Corresponding author: anneli.bohne.kjersem@mbi.uib.no

Keywords: Atlantic cod, biomarker candidates, fry, liver, produced water, proteomics, oestradiol,

Abstract

Produced water is considered to be the most dominant source of discharges offshore. It contains a great variety of compounds, which may cause harm by themselves or in synergy with other compounds present in produced water. Worldwide there is growing concern that xenobiotic exposure of the marine environment may constitute an important contributing factor to the reported decline in harvested populations of marine fish species, and early life stages have been reported to be more sensitive to xenobiotic exposure than adult fish. In this study, cod fry (3,5 months old) were continuously exposed to different levels of produced water and 17-β-oestradiol, a natural oestrogen, for 78 days (until 6 months of age) . The protein pattern of cod fry liver was analysed by two-dimensional gel electrophoresis and image analysis to detect differentially expressed proteins. 2DE spots of differentially regulated proteins were isolated and identified by MALDI-ToF-ToF mass spectrometry analysis, searching NCBI and a newly developed cod EST-database of more than 50,000 ESTs. We detected 105 significant protein changes following exposure to produced water and oestradiol. Approx. 50% of the protein changes occurred at ≥0.1% produced water. Identified protein changes indicate that produced water may affect different molecular and cellular functions such as cellular integrity, signal transduction, and metabolism, as stress, structural, and cytoskeletal proteins, as well as metabolising enzymes appear to change in their protein expression following exposure.

Introduction

The petroleum industry, fisheries and seafood industry represent large economical and social interests. However, there is growing concern worldwide that xenobiotic exposure of the marine environment may constitute an important contributing factor to the reported decline in several populations of marine teleost species [1]. Produced water is by far the most dominant discharge from regular oil and gas production offshore. Although it varies from field to field in amount and composition, it is generally a mixture of formation water contained naturally in the reservoir, injected water used for secondary oil recovery, and treatment chemicals added during production, in the oil-water separation process, and in preparation for re-injection of produced water [2]. The amount of produced water can vary between 2-98% of the gross fluid production. Since 1986, the standard of 40 mg/L for the average concentration of hydrocarbons in effluents discharged from platforms have been applied (PARCOM 8/12/1, paragraph 5.37, [3]). As from 1 January 2007, a standard of 30 mg/L of dispersed oil in produced water were to be applied (OSPAR Recommendation 2006/4, [4]). At the Norwegian Continental Shelf in the North Sea the average hydrocarbon content in produced water was 16.9 mg/L in 2006 [5]As the oil fields grow older, the need of using drilling fluids increases. An attempt to replace the most harmful substances by less harmful compounds, and the reinjection of fluids, is reducing the discharges of harmful substances at sea. Still, produced water contains a great variety of compounds, such as dissolved inorganic salts, minerals and heavy metals, dissolved and dispersed oil compounds, organic compounds such as carboxylic acids and phenols from the formation water [6]. The five most common organic discharges are polyaromatic hydrocarbons (PAH), BXT (benzene, xylene, toluene), phenols, alkyl phenols, and carboxylic acids. The most common heavy metals released during drilling are

impurities from barite: Arsenic (As), lead (Pb), cadmium (Cd), copper (Cu), chromium (Cr), mercury (Hg), nickel (Ni), and zinc (Zn) (ibid).

We have in this study investigated the effects of produced water in liver of cod fry (*Gadus morhua*) continuously exposed to different levels of produced water at the fry stage, and compared these effects to endocrine disrupting (ED) effects by simultaneously exposing a group of larvae to the natural oestrogen 17β-oestradiol (E2). The effects of exposure to produced water and E2 was studied using a proteomic approach to identify effects at a molecular level. Changes in tissue induced by an exposure represent biomarker candidates that may act as early-warning signals, as effects observed at a higher level of organisation in the organisms, and in a population as a whole, is proceeded by changes at a lower level of organisation, e.g. at the protein level.

Alkyl phenols have previously been reported to significantly reduce hatching rates and affect steroidogenesis, altering hormone levels in fish [5-7] and affect reproduction [8]. PAH exposure has also been reported to disrupt endocrine functioning and alter the levels of steroids in fish [9-11]. β-naphtoflavone (BNF), a PAH-like compound, has been shown to alter the normal function of the hypothalamus-pituitary-thyroid (HPT) axis in eel [12] and hence, may impair the systemic hormonal control of reproductive processes [13].

North Sea oil has previously been shown to cause oxidative stress and altering CYP1A and CYP3A protein expression in juvenile Atlantic cod. In the same study, exposure to alkyl phenol resulted in decreased CYP1A and CYP3A protein expression. This result is consistent with findings reported by Hasselberg et al. [14]. Metabolised PAHs and heavy metals may also produce reactive oxygen species (ROS), leading to oxidative stress. However, most of the

PAHs (dominantly 2- and 3-rings PAHs) found in crude oil are not considered strong inducers of the CYP1A [15], although CYP1A induction in cod larvae has been reported after oil hydrocarbon exposure [16], [17]. Oestradiol has previously been shown to be able to modulate the hepatic CYP1A expression in fish [18]. Previous studies investigating the expression of known biomarker illustrate the need of new and more sensitive biomarkers for measuring effects after exposure to oil and produced water on marine species, as many of the biomarkers currently available are unable to detect effects of low, chronic exposure ([19], [20],[21].

Marine teleost species, like the Atlantic cod (*Gadus morhua*), have received increased attention in environmental risk analysis: In addition to being commercially important, fish are sensitive organisms found in the environment where they are directly exposed to toxic substances that may also negatively affect human health [22] In this context, early life stages have been reported to be more sensitive to xenobiotic exposure than adult fish ([23, 24]. It has also been reported that exposure and responses induced at early life stages may not be detected or observed in the exposed animal, but rather in its progeny [5].

Materials

Acidic acid, Coomassie brilliant blue (CBB) G250, ortho-phosphoric acid, urea , thiourea, tris (hydroxymethyl)-ammoniummethane, sodium carbonate, formic acid, formaldehyde, sodium acetate trihydrate, glutardialdehyde, sodium thiosulphate pentahydrate, silver nitrate, EDTAxNa_2x2H_2O, ammonium sulphate , acetonitril, methanol and thiofluoro acetic were all purchased from Merck (Damstadt, Germany). Ethanol was purchased from Arcus Kjemi (Oslo, Norway).

Bovine Serum Albumin, 3-(3-colamidopropyl)-dimetylammonio-1-propansulfoanat, CHAPS, Triton X-100, DL-dithiothreitol, (DTT), iodoacetamide, alpha-cyano-4-hydroxycinnamic acid (CHCA), 4-sulfophenyl isothiocyanate, sodium salt monohydrate, SPITC, were purchased from Sigma Aldrich (MO, US). Ampholine^{TM} 3,5-10, DryStrip Cover fluid, 18 cm IPG strips and IEF Electrode strips were purchased from GE Healthcare. 7 cm IPG strips were purchased from Invitrogen. Ammonium dodecyl sulfate (SDS), Agarose, 30% Acrylamide/Bis solution, 37.5:1, Ammonium persulfate, TEMED and Precision Plus Protein^{TM} standard were purchased from Bio-Rad (CA, US). Trypsin was purchased from Promega (WI, US), while Poros 20R2 and Reverse Phase packing, were purchased from Applied Biosystems (CA, US). GelLoader tips were provided from Eppendorf (Hamburg, Germany), and siliconised tubes were provided from Sorenson, BioScience, Inc (UT, US). Peptide Calibration Standard and MTP384 Target plate polished steel TF were purchased from Bruker Daltonics (Leipzig, Germany).

Methods

Experimental design

The exposure was carried out for 78 days, starting 7th June 2004 on early fry stages (total length 3.1±0.5 cm and weight 0.22±0.09 g) and lasting till 22th September 2004 (total length 11.4±1.3 and weight 13.4±5.4 g). The exposure was done in green**,** quadratic (1m x 1m) 500 l tank. There were three groups treated with produced water, one group treated with 17β-oestradiol (E2) and one untreated control group. There have been two parallels for each treatments (totally 10 tanks).

Fry from 5 broodstock families (originating from Tysfjord, Northern Norway) were used in each of two parallel exposure groups (250 fry in each). The fry were fed with marine dry pellets with increasing size (0.6 mm to 1.5 mm) (Marin 030 and 050, Ewos A/S, Bergen, Norway). The temperature in the tanks was held at 12.0 °C and the tanks got a simulated, natural light regime for Bergen, Norway (60° 25' N, 5° 20' E) using a light control program called LYSSTYR 4.0.

Exposure

Produced water (PW)

4000 l PW was transported from a North Sea oil platform, Oseberg C (Hydro) to the Institute of Marine Research, Bergen. The PW was bubbled with air for 10 minutes to remove toxic H_2S (g) and then frozen down in 25 l containers at -30 °C until use.

Every day, 28 l of PW or diluted PW were transferred to a header tank and used for 24 hours. The chemical exposure was dosed using a fixed ratio between two water flows: a sea water flow of 1 l/min and a "PW flow" of 10 ml/min. The concentration gradient was made by diluting the PW in header tank before exposure: 1 % PW = pure PW; 0.1 % PW 1:10 PW; 0.01 % 1:100 PW.

Oestradiol

For the positive control, 17β-oestradiol was dissolved in ethanol (9 mg/ml) and 100 µl of this solution was added each l of seawater in the header tank (=> 1000 µg/l).

The "PW flow" was administrated with a 16 channel peristaltic pump (Ismatec MCP) using Tygon tubing.

Sample preparation

Fry liver (pooled and individual samples) was homogenised in 4x v/w ice-cold 0.1 M sodium phosphate buffer (pH 7.4) with 0.1 M KCl and 10% glycerol using a Potter Elvehjem homogeniser with a Teflon® pestle [25]. The pestle was run up and down 6 times during homogenisation. The homogenised fry samples were subsequently centrifuged at room temperature at 13,000 g for 20 min. The supernatants containing the resulting S12 fraction were used for analysis. The protein concentration of the samples was determined using a plate-reader modified Bradford`s assay [26] .

Analytical gels of individual samples

20 μg of sample of individual cod fry was dissolved in rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 20 mM DTT, 0.5% v/v Triton X-100, 0.5% v/v Ampholine 3-10, bromphenol blue) and applied to IPG strips pH 4-7, 7 cm (Invitrogen) ([27], [28], [29]. The strips were then rehydrated for a minimum of 12 hours and focused on a Multhiphor II unit (GE Healthcare): Step gradient: Step 1: 0:01h, 200V, 2mA, 5W, 20ºC. Step 2: 1:30 h, 3500V, 2mA, 5W, 20ºC. Step 3: 1:30 h, 3500V, 2mA, 5W, 20ºC. The strips were equilibrated 15 min. at room temperature in 0.25% DTT-containing SDS equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, bromphenol blue) and 15 min. in a 4.5% iodoacetamide-containing SDS equilibration buffer [28], [29] prior to separation in the second dimension on 9% SDS-PAGE gels [30]. Analytical gels were run at 200 Volt, 400 mA/12 gels, 20°C for 45 min. in a mini Dodeca unit (Bio-Rad) prior to silver-staining [31].

Preparative gels of pooled samples

1000 μg of pooled sample was diluted in re-hydration buffer and added to IPG strips pH 4-7, 18 cm [27], [28], [29]. Two replicates of one pooled sample from each treatment group were analysed by 2DE. The strips were re-hydrated for a minimum of 12 hours and focused on the Multhiphor II unit according to the manufaturer's guidelines. The strips were equilibrated 15 min. at room temperature in the 0.25% DTT-containing SDS equilibration buffer and 15 min. in the 4.5% iodoacetamide-containing SDS equilibration buffer prior to separation in the second dimension on 9% SDS-PAGE gels. Gels were run in an Ettan Dalt Twelve unit at 1W/ gel, 20°C, for approximately 17 h. The gels were then stained with Collodial Coomassie [32].

All gels were scanned on GS-800 Calibrated Densitometer flatbed scanner (Biorad, CA, US) using PDQuest 7.2.0 software (Biorad, CA, US). The gels were scanned with medium resolution: 63.5 x 63.5 microns.

The global protein expression was analysed using Delta 2D image analysis software from Decodon (Greifswald, Germany): TIFF images of the 2DE gel images were up-loaded in Delta 2D and grouped according to treatment. The images were warped using a group-warping strategy and exact warping. A union fusion-gel was produced from all the images within the control, high level exposure (1.0% PW), and the E2-treated group. No filters were used. Spots were detected on the fusion gel and validated spots were transferred to all the other gel images. Normalisation was performed, based on total intensity and including all spots on the gel. The spot data from the All images quantitation table was exported to a cvs-file in Excel, which was successively up-loaded in the Ludesi Interpreter (www.ludesi.com, Lund, Sweden), together with the gel TIFF images. Gel comparison, ANOVA ($p \leq 0.05$ and fold change ≥ 2) and hierarchical clustering analysis (distance function: Pearson correlation; linking method: average) were performed using Ludesi Interpreter. The results of the image analysis were carefully controlled, and any changes detected in difficult gel areas or any changes resulting from bad spot detection, were excluded to limit the number of false positives.

Mass spectrometry

In-gel digestions were performed as described by Shevchenko et al. [33]. Briefly, the excised gel plugs were washed in digestion buffer (50 mM NH_4HCO_3, pH 7.8)/acetonitrile (60/40) and dried by vacuum centrifugation. Modified trypsin (10 ng/µl) dissolved in 50 mM NH_4HCO_3,

pH 7.8, was added to the dry gel pieces and incubated on ice for 1 h. After removing the supernatant, additional digestion buffer was added and the digestion was continued at 37°C over night. The supernatant from the digestion was analysed directly by off-line LC-MALDI without peptide extraction.

Peptide separation was performed using an inert nanoflow-HPLC system from LC-Packings (Ultimate; Switchos2; Famos; LC packings, Amsterdam, The Netherlands) equipped with a Probot MALDI spotting device (LC Packings, Amsterdam, The Netherlands). 10 µl of the in-gel digested protein(s) was acidified with 0.5 µl 10% TFA and loaded onto a home-made 1-cm fused-silica precolumn (100µm i.d., ReproSil-Pur C18 AQ, 3-mm; Dr. Masch GmbH) and then eluted onto a home-made 10-cm fused-silica analytical column (75µm i.d., ReproSil-Pur C18 AQ, 3-mm; Dr. Masch GmbH) using a flow of 200 nL/min and a gradient from 100% solvent A (H_2O/TFA, 100:0.1, v/v) to 50% solvent B (H_2O/acetonitrile/TFA, 20:80:0.1, v/v/v) over 40 min, then 50–100% solvent B in 5 min. Column effluent was mixed in a 1:9 ratio with MALDI matrix (2g/L a-cyano-4-hydroxycinnamic acid in H_2O/acetonitrile/TFA, 30:70:0.1, v/v/v) through a 25 nL mixing tee (fused-silica capillary TSP050375; Composite Metal Services Ltd., UK) and spotted onto a MALDI plate in 30 second fractions. The LC-MALDI preparation was analyzed on an ABI 4700 (Applied Biosystems, Framingham, MA, USA) proteomics analyzer using an automated workflow. A maximum of 10 peptides per spot with a s/n > 80 was automatically selected for MS/MS. CID was performed at collision energy of 1 kV with an indicated collision gas pressure of ~1 $\times 10^{-6}$ Torr.

The spectra were annotated and analyzed using Data Explore™ v. 4.5 (Applied Biosystems) The MS^2 data from the entire off-line LC MALDI experiment was combined into a single mass list using an in-house developed script (Jakob Bunkenborg, SDU, DK). The sequence

data from the MS-MS runs (mascot-generic format, mgf files) were used in database searches in the cod EST-database and/or the NCBInr database for protein identity using the MASCOT search engine [34], [35]. The following parameters were used for the database search: Missed cleavage 1: Significance threshold $p<0.05$; MS^1 mass accuracy 100 ppm, MS^2 mass accuracy; Partial modifications: Carbamidomethyl (C) and Oxidation (M).

Database searches

The in-house developed cod expressed sequence tags (EST) database used for protein identification includes more than 50,000 EST sequences from cod obtained from the Institute of Marine Research, Norway, NIFES (Norwegian Institute of Research on Nutrition and Seafood) and groups affiliated to the National Research Council, Canada. The annotation of the sequences was performed by the Computational Biology Unit at the University of Bergen, producing approximately 6000 contigs and more than 30,000 singlets. The EST sequences are derived from cDNA libraries of cod exposed to an array of environmental pollutants, such as PCBs and heavy metals, and purified mRNA sequences from several different tissues. RNA was also purified from cod captured in the harbours of Bergen and Trondheim (Norway), and therefore assumed to be exposed to a battery of pollutants, as well as cod exposed to hypoxia and hyperoxia (http://www.codstress.olsvik.info/Results.html).

Results

By comparing global protein expression obtained by 2DE and image analysis in liver samples of cod fry exposed to produced water or 10 µg/l E2, 105 proteins were found to be significantly differentially expressed following exposure (ANOVA: $p<0.05$, fold change≥ 2) (Table 1 and Figure 1). The analysis includes 7 individuals within each treatment group and 6 individuals in the group treated with 0.1% (medium levels of) PW.

18 proteins were differentially expressed following low level produced water (0.01%, PW) treatment, 35 proteins were differentially expressed following medium level PW (0.1%) treatment, and 9 proteins were found to be differentially expressed following high level (1.0%) PW treatment. Of these 62 proteins affected by produced water, 30 proteins were also differentially expressed upon exposure to 10 µg/l E2. Another 43 proteins were only differentially expressed after E2 treatment and showed no response following PW exposure. These findings are also reflected by the hierarchical clustering analysis (distance function: Pearson correlation; linking method: average). In the dendrogram from the clustering analysis (Figure 2) almost one third of the spots show a strong protein expression (marked red) following E2 exposure, and no apparent change in protein expression following PW-treatment as compared to control (marked more or less the same colour of green). Almost one third of the proteins strongly expressed proteins (marked red) after PW-exposure, is not that well expressed (marked green) after exposure to E2.

Of the 105 significant protein changes (ANOVA: $p<0.05$, FC≥ 2), 42 protein spots were isolated for protein identification by MS/MS. We succeeded in identifying 21 of these, resulting in a success rate of 50 % (Table 2).

Low levels of produced water resulted in an apparent down-regulation of keratin, actin and 14-3-3B1. These proteins were all affected by E2 treatment as well. Hsc71, 14-3-3BI, 14-3-3E1, elongation factor 1, tropomyosin, 60 S acidic ribosomal protein P0, Hsp90, hemopexin, mitofilin, non-muscle tropomyosin and down-syndrome critical region gene 2 product as well as fructose-1,6-bisphosphate aldolase and pyroline-5-carboxylate dehydrogenase, were all down-regulated by medium levels of produced water. Hsc71, HSP90, hemopexin, the aldolase and the dehydrogenase were also affected by E2 treatment. The heat shock proteins and hemopexin appeared to be up-regulated by E2 treatment while the metabolic enzymes appeared to be down-regulated.

High levels of produced water (1.0%) lead to a down-regulation of aconitase 1 and aldehyde dehydrogenase. These proteins appeared to be down-regulated by E2-treatment as well. PW also appeared to induce both an up-regulation and a down-regulation of antiquitin and pyroline-5-carboxylate dehydrogenase, while this protein seemed to be unaffected by E2 exposure.

E2 treatment apparently caused a down-regulation of pyrophosphatases (inorganic) 1, eukaryotic elongation initiation factor, tubulins, glycerol-3-phosphate dehydrogenase, cytochrome P450 2x1, phosphotirsterase, thioredoxin, glucocolate oxidase, S-adenosylhomocysteine hydrolase, alanine-glycosylate aminotransferase and a selenium binding protein1.

As some of these proteins were identified from the same spot as other proteins, their individual expression is not known. Some of these proteins were also identified from more than one spot on the gel showing the same change in protein expression following exposure.

Thus, this strengthens also the likelihood of the apparent change in protein expression of proteins identified from the same spot as other proteins.

Discussion

In this study, using a proteomic strategy, we have detected responses in hepatic protein expression pattern of cod fry exposed to low and environmentally relevant concentrations of produced water during the larvae stage. Of 105 significant protein changes, 53 occurred at low and medium dose exposures. 24 of these were also observed after E2 exposure. Identified protein changes indicate that produced water may affect different molecular and cellular functions such as cellular integrity, signal transduction, and metabolism, as stress proteins, structural proteins, cytoskeletal proteins, and metabolising enzymes appeared to change in their protein expression following the exposures.

Low levels of PW resulted in a down-regulation of keratin S2 and actin, and an up-regulation of a 14-3-3B1. 14-3-3 proteins appear to effect intracellular signaling and mainly bind proteins containing phosphothreonine or phosphoserine motifs (Ferl et al., 2002)(?). Keratins are intermediate filament proteins. Actin microfilaments, tubulin, microtubules, and the intermediate filaments is found important for the maintenance of cellular structure and integrity [36]. Some studies also report keratins to be involved in apoptosis and signal transduction [37]. Actin is functioning in cell-cell adherence junction and cell-cell communication in different cells and tissues, which is considered vital for normal functioning as well as important in development [37]. Actin, and other cytoskeleton proteins have previously been identified as targets of oxidative stress [21], [38] . In the study by [21], actin was found significantly elevated in mussels exposed to flame retardants and lowered, but not significantly, in mussel exposed to crude oil, alone and spiked with alkyl phenols and PAHs, a composition similar to that of produced water. Hence, the down-regulation of keratin S2 and

actin in the liver by low levels of produced water may indicate an effect on liver function and possibly organogenesis.

Hsc71 belongs to the Hsp70 family. The Hsp70 proteins are known to be able to bind hydrophobic protein domains and function as chaperones that assemble and stabilize multiprotein complexes, translocation of polypeptides across cell membranes, and aiding protein folding [39], [40]. Hsp90 and associated proteins (e.g.Hsp70) are known to play an important part in conforming the ligand binding domain of the nuclear steroid receptors (and the Ah receptor (as reviewed by [41]. Hence, a down-regulation of Hsp90 and Hsc71 (Hsp70) may lead to a number of effects in the organism.

Fructose-1,6-bisphosphate aldolase and NAD-dependent aldehyde dehydrogenase are both metabolic enzymes involved in the glycolytic pathway, and the apparent down-regulation of these enzymes in this study indicate an effect of exposure to PW or E2 on the metabolism, which secondarily may affect growth, size and survival of the fish.

Protein P0 is found phosphorylated in the ribosomes, and affect the expression of specific proteins involved in other important functions. Down-regulation of 60 S acidic ribosomal protein P0 and the elongation factors may cause an abruption of protein translation, which may result in many secondary effects. Mitofilin have previously been linked to abnormal mitochondrial function, resulting in decreased cellular proliferation and increased apoptosis [42]. Tropomyosins, together with actin is known to be important in morphogenesis, and tropomyosin isoforms promote microfilament stability [43]. The role of the Down syndrome gene product in liver is not clear.

High levels of produced water (1.0%) lead to a down-regulation of aconitase 1, and an aldehyde dehydrogenase. These proteins appeared also down-regulated by E2-treatment. PW also appeared to induce both an up-regulation and a down-regulation of antiquitin and pyroline-5-carboxylate dehydrogenase (P5C dehydrogenase), while the expression of these protein seemed to be unaffected by E2 exposure. Aconitases are involved in the metabolism in Krebs cycle, catalyzing the conversion of citrate and isocitrate, which is critical to normal physiology and survival, and their activity is affected by iron levels, oxidative stress and Fe-S cluster biogenesis apparatus [44]. As already mentioned, PW-treatment induced a down-regulation of aldehyde dehydrogenase involved in glycolysis. Hence, these results together suggest an impact on metabolism and energy production. Antiquitin is an aldehyde dehydrogenase which has been linked to osmotic regulation and fluidity control in animals [45]. In plants it is also linked to detoxification, but such responses and responses in antiquitin-level following hormone-treatment have not yet been detected in previous animal studies (reviewed by [45]. P5C dehydrogenase is hypothesised to be involved in amino acid metabolism, but the effect of a potential down-regulation of this enzyme is not clear.

E2 treatment appeared to cause altered protein expression of a wide range of proteins. E2 appeared to cause a down-regulation of a selenium-binding protein 1 and an up-regulation of alanine-glyoxylate aminotransferase. The biological effects of these latter responses are not clear. However, alanine amino-transferase levels are used as a measure of liver-function and to diagnose chronic liver diseases in humans [46]. These proteins appeared to be unaffected by exposure to produced water. The apparent down-regulation of metabolic enzymes explain the reduced growth in oestradiol-treated fry observed in this study [6].

Taken together, these results indicate that produced water exposure may induce some of the same responses as E2 exposure, in addition to other biological effects through different modes of action. As already mentioned, produced water contains a large range of different compounds that may cause harm by themselves or in synergy with other compounds present. Hence, these results also indicate that compounds in produced water other than those known to cause endocrine disrupting effects, like PAHs, alkyl phenols, phtalates and PCBs, may also cause harmful effects in marine fish.

E2 treatment also appeared to induce changes in protein expression unparalleled in groups of PW-treated fish. Hence, these protein changes may indicate that the mixture of compounds found in produced water do not have a strong estrogenic effect. These results may also suggest a higher sensitivity of the early life stages compared to juvenile and adult fish, as many of the responses detected in this study have not been identified in any of the few, similar studies done on juvenile and adult teleosts [47, 48]. Hence, more research is needed investigating long-term effects of produced water on early life stages and development in teleosts.

The protein changes detected in this study represent a broad range of proteins and biological responses, and thus, represent potential biomarker candidates. Following this biomarker discovery phase, the candidates will have to be qualified, verified, the assays optimised and customized prior to their eventual use in bio-monitoring and environmental risk analysis [49]. Hence, the next step will be to measure the protein expressions in the different treatment groups and on field samples using a broader range of samples, and using other techniques such as immunoassays in order to verify a differential expression. The effect of produced water and E2 should also be monitored in trans-generational studies to better determine the

final outcome of these effects, as initially mentioned, other studies imply that some effects may be visible rather in the progeny than in the exposed animal itself.

Conclusion

In this study, using a proteomic strategy, we have detected responses in hepatic protein expression pattern of cod fry exposed to low and environmentally relevant concentrations of produced water during the fry stage. Of 105 significant protein changes, 53 occurred at low and medium dose exposures. 24 of these were also observed after oestrogen exposure. Identified protein changes indicate that produced water may affect different molecular and cellular functions such as cellular integrity, signal transduction, and metabolism, as stress proteins, structural proteins, cytoskeletal proteins, and metabolising enzymes appeared to change in their protein expression following exposure.

The protein changes detected in this study represent a broad range of proteins and biological responses, and thus, represent potential biomarker candidates. Following this biomarker discovery phase, the candidates will have to be qualified, verified, the assays optimised and customized prior to their eventual use in bio-monitoring and environmental risk analysis.

Acknowledgements

This study was supported by the Norwegian Research Council grants 164423/S40 and 141213/720 and by TOTAL E&P NORGE AS. The instruments for off line LC MS and MS/MS were founded by the Danish Research agency through the Danish Biotechnology Instrument Center.

References

1. Waldichuk, M., *The assessment of sublethal effects of pollutants in the sea. Review of the problems.* Philos Trans R Soc Lond B Biol Sci, 1979. **286**(1015): p. 399-424.
2. Carroll, M., Pearson, T., Dragsund, E., Gabrielsen, KL. *Environmental status of the Norwegian offshore sector based om the petroleum regional monitoring programme, 1996-1998.* 2000.
3. OSPAR, *PARCOM Recommendation 86/1 of a 40 mg/l Emission Standard for Platforms*, in *Decisions, Recommendations and Other Agreements relating to the Offshore Oil and Gas Industry Strategy*, C.f.t.P.o.t.M.E.o.t.N.-E.A. OSPAR, Editor. 1986.
4. OSPAR, *OSPAR Recommendation 2006/4 amending OSPAR Recommendation 2001/1 for the management of produced water from offshore installations*, in *Decisions, Recommendations and Other Agreements relating to the Offshore Oil and Gas Industry Strategy*, C.f.t.P.o.t.M.E.o.t.N.-E.A. OSPAR, Editor. 2006.
5. Schwaiger, J.,Mallow, U.,Ferling, H., Knoerr, S., Braunbeck, T., Kalbfus, W., Negele, RD., *How estrogenic is nonylphenol? A transgenerational study using rainbow trout (Oncorhynchus mykiss) as a test organism.* Aquat Toxicol, 2002. **59**(3-4): p. 177-89.
6. Meier, S., Andersen, TC., Lind-Larsen, K., Svardal,A., Holsen, H.., *Effects of alkylphenols on glycerophospholipids and cholesterol in liver and brain from female Atlantic cod (Gadus morhua).* Comp Biochem Physiol C Toxicol Pharmacol, 2007. **145**(3): p. 420-30.
7. Arukwe, A., F.R. Knudsen, and A. Goksoyr, *Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens.* Environ Health Perspect, 1997. **105**(4): p. 418-22.
8. Cardinali, M., Maradonna, F., Olivototto, I., Bortoluzzi, G., Palzonetti-Magni, AM, Carnevali, O., *Temporary impairment of reproduction in freshwater teleost exposed to nonylphenol.* Reprod Toxicol, 2004. **18**(4): p. 597-604.
9. Evanson, M. and G.J. Van Der Kraak, *Stimulatory effects of selected PAHs on testosterone production in goldfish and rainbow trout and possible mechanisms of action.* Comp Biochem Physiol C Toxicol Pharmacol, 2001. **130**(2): p. 249-58.
10. Monteiro, P.R., M.A. Reis-Henriques, and J. Coimbra, *Plasma steroid levels in female flounder (Platichthys flesus) after chronic dietary exposure to single polycyclic aromatic hydrocarbons.* Mar Environ Res, 2000. **49**(5): p. 453-67.
11. Nicolas, J.M., *Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants.* Aquatic Toxicology, 1999. **45**(2-3): p. 77-90.
12. Teles, M., Oliveira, M., Pacheco, M., Santos, MA.., *Endocrine and metabolic changes in Anguilla anguilla L. following exposure to beta-naphthoflavone--a microsomal enzyme inducer.* Environ Int, 2005. **31**(1): p. 99-104.
13. Navas, J.M., Zanui, S., Segner, H., Carrillo, M., *Beta-naphthoflavone alters normal plasma levels of vitellogenin, 17 beta-estradiol and luteinizing hormone in sea bass broodstock.* Aquat Toxicol, 2004. **67**(4): p. 337-45.
14. Hasselberg, L.,Meier, S., Svardal, A., Hegelund, T., Celander, MC., *Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (Gadus morhua).* Aquat Toxicol, 2004. **67**(4): p. 303-13.
15. Aas, E., Baussant, T, Balk, L., Liewenborg, B, Andersen, OK., *PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for*

chronic oil exposure: a laboratory experiment with Atlantic cod. Aquat Toxicol, 2000. **51**(2): p. 241-58.
16. Goksoyr, A., Andersson, T., hansson, T, Klungsøyr, J., Zhang, Y., Forlin, L.., *Species characteristics of the hepatic xenobiotic and steroid biotransformation systems of two teleost fish, Atlantic cod (Gadus morhua) and rainbow trout (Salmo gairdneri).* Toxicol Appl Pharmacol, 1987. **89**(3): p. 347-60.
17. Goksoyr, A., Serigstad, B., Solberg, TS., Stegeman, JJ., *Response of Cod (Gadus morhua) Larvae and Juveniles to Oil Exposure Detected with Anti-cod Cytochrome P-450c IgG and Anti-scup Cytochrome P-450E MAb 1-12-3.* Marine Environmental Research MERSDW, 1988. **24**(1-4): p. 31-35.
18. Navas, J.M., Zanui, S., Segner, H., Carrillo, M., *beta-Naphthoflavone alters normal plasma levels of vitellogenin, 17 beta-estradiol and luteinizing hormone in sea bass broodstock.* Aquatic Toxicology, 2004. **67**(4): p. 337-345.
19. Reid, D.J. and G.R. MacFarlane, *Potential biomarkers of crude oil exposure in the gastropod mollusc, Austrocochlea porcata: laboratory and manipulative field studies.* Environmental Pollution, 2003. **126**(2): p. 147-155.
20. Almroth, B.C., Sturve, J., Berglund, A, Forlin, L, *Oxidative damage in eelpout (Zoarces viviparus), measured as protein carbonyls and TBARS, as biomarkers.* Aquatic Toxicology, 2005. **73**(2): p. 171-180.
21. Jonsson, H., Schiedek,D., Goksoyr, A., Grosvik, BE., *Expression of cytoskeletal proteins, cross-reacting with anti-CYP1A, in Mytilus sp exposed to organic contaminants.* Aquatic Toxicology, 2006. **78**: p. S42-S48.
22. Warner, K.E. and J.J. Jenkins, *Effects of 17 alpha-ethinylestradiol and bisphenol a on vertebral development in the fathead minnow (Pimephales promelas).* Environmental Toxicology and Chemistry, 2007. **26**(4): p. 732-737.
23. Bern, H.A., *The Development of the Role of Hormones in Development - a Double Remembrance.* Endocrinology, 1992. **131**(5): p. 2037-2038.
24. Guillette, L.J., Crain, DA., Rooney, AA., Pickford, DB., *Organization Versus Activation - the Role of Endocrine-Disrupting Contaminants (Edcs) during Embryonic-Development in Wildlife.* Environmental Health Perspectives, 1995. **103**: p. 157-164.
25. Nilsen, B., K. Berg, and A. Goksøyr, *Induction of Cytochrome P450 1A (CYPl A) in Fish: A Biomarker for Environmental Pollution*. Methods in Molecular Biology. Vol. 7. 1998: Humana Press. 423-438.
26. Bradford, M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal. Biochem., 1976. **72**: p. 248-254.
27. O'Farrell, P.H., *High resolution two-dimensional electrophoresis of proteins.* J Biol Chem, 1975. **250**(10): p. 4007-21.
28. Görg, A., W. Postel, and S. Günther, *Two-dimensional electrophoresis.* Electrophoresis, 1988. **9**: p. 531-546.
29. Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Wiess, W., *The current state of two-dimensional electrophoresis with immobilized pH-gradients.* Electrophoresis, 2000. **21**: p. 1037-1053.
30. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
31. Heukeshoven, J. and R. Dernick, *Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels.* Electrophoresis, 1988. **9**(1): p. 28-32.

32. Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W. *Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250.* Electrophoresis, 1988. **9**(6): p. 255-62.
33. Shevchenko, A., Wilm, M., Worm, O., Mann, M., *Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels.* Anal Chem, 1996. **68**(5): p. 850-8.
34. Perkins, D.N., Pappin, D.J., Creasey, DM., Cottrell, JS., *Probability-based protein identification by searching sequence databases using mass spectrometry data* Electrophoresis, 1999. **20**: p. 3551-3567.
35. Steen, H. and M. Mann, *The ABC's (and XYZ's) of peptide sequencing.* Nat Rev Mol Cell Biol, 2004. **5**(9): p. 699-711.
36. Ku, N.O., Zhou, X., Toivola, DM., Omary, MB, *The cytoskeleton of digestive epithelia in health and disease.* Am J Physiol, 1999. **277**(6 Pt 1): p. G1108-37.
37. Schaffeld, M. and J. Schultess, *Genes coding for intermediate filament proteins closely related to the hagfish "thread keratins (TK)" alpha and gamma also exist in lamprey, teleosts and amphibians.* Exp Cell Res, 2006. **312**(9): p. 1447-62.
38. Perez, L.M., Milkiewicz, P., Ahmed-Choudhury,J., Elias, E., Ochoa, JE, Pozzi, EJS., Coleman, R., Roma, MG., *Oxidative stress induces actin-cyto skeletal and tight-junctional alterations in hepatocytes by a Ca2+-dependent, PKC-mediated mechanism: Protective effect of PKA.* Free Radical Biology and Medicine, 2006. **40**(11): p. 2005-2017.
39. Feder, M.E. and G.E. Hofmann, *Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology.* Annu Rev Physiol, 1999. **61**: p. 243-82.
40. Young, J.C. and F.U. Hartl, *Chaperones and transcriptional regulation by nuclear receptors.* Nat Struct Biol, 2002. **9**(9): p. 640-2.
41. Pratt, W.B., *The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase.* Annu Rev Pharmacol Toxicol, 1997. **37**: p. 297-326.
42. John, G.B., Shang, Y., Li, L., Renken, C., Mannella, CA:, Selker, JML., Rangell, L., Bennett, J., Zha, J., *The mitochondrial inner membrane protein mitofilin controls cristae morphology.* Molecular Biology of the Cell, 2005. **16**: p. 1543-1554.
43. Gunning, P., R. Weinberger, and P. Jeffrey, *Actin and tropomyosin isoforms in morphogenesis.* Anatomy and Embryology, 1997. **195**(4): p. 311-315.
44. Tong, W.H., et al., *The role of mitochondrial and cytosolic iron-sulfur cluster biogenesis in maintainence of mammalian iron homeostasis.* American Journal of Hematology, 2007. **82**(6): p. 521-521.
45. Fong, W.P., C.H. Cheng, and W.K. Tang, *Antiquitin, a relatively unexplored member in the superfamily of aldehyde dehydrogenases with diversified physiological functions.* Cell Mol Life Sci, 2006. **63**(24): p. 2881-5.
46. Pendino, G.M., Mariano, A., Surace, P., Caserta, CA., Fiorillo, MT., Amante, A., Bruno, S., Mangano, C., Polito, I., Amato, F., *Prevalence and etiology of altered liver tests: a population-based survey in a Mediterranean town.* Hepatology, 2005. **41**(5): p. 1151-9.
47. Aluru, N., Jorgensen, E.H., Maule, AG., Vijayan, MM., *PCB disruption of the hypothalamus-pituitary-interrenal axis involves brain glucocorticoid receptor downregulation in anadromous Arctic charr.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(4): p. R787-93.
48. Olsvik, P.A., Kristensen, T., Waagbo, R., Tollefsen, KE. Rosseland, BO., Toften, H., *Effects of hypo- and hyperoxia on transcription levels of five stress genes and the*

glutathione system in liver of Atlantic cod Gadus morhua. J Exp Biol, 2006. **209**(Pt 15): p. 2893-901.
49. Rifai, N., M.A. Gillette, and S.A. Carr, *Protein biomarker discovery and validation: the long and uncertain path to clinical utility.* Nat Biotechnol, 2006. **24**(8): p. 971-83.

Table 1: Protein expression of differential expressed proteins analysing individual samples exposed to produced water (PW) and 17β-oestradiol(E_2)

Spot	Control	0.01% PW	0.1% PW	1.0% PW	10 ppb E_2
1	1	0.680	0.710	0.948	3.599
2	1	1.060	0.998	1.354	0.464
3	1	0.973	1.364	1.023	2.350
4	1	0.876	0.547	0.556	2.173
5	1	1.601	0.834	0.851	0.470
6	1	1.142	0.863	1.024	4.269
7	1	1.067	0.643	0.325	0.265
8	1	0.620	0.783	0.743	2.198
9	1	0.374	1.469	1.017	0.861
10	1	1.430	0.935	1.018	2.364
11	1	1.228	0.66	1.012	0.133
12	1	1.613	0.232	0.242	2.371
13	1	0.941	0.121	0.612	1.978
14	1	0.759	0.712	0.714	2.041
15	1	0.539	0.895	2.512	1.927
16	1	1.309	0.111	0.114	2.379
17	1	0.745	0.257	0.368	1.123
19	1	1.758	2.0648	1.519	0.69
20	1	0.82	0.204	0.486	3.156
21	1	0.975	1.053	1.026	0.482
22	1	0.943	1.134	2.072	0.443
23	1	1.471	0.286	0.779	1.983
24	1	1.225	0.446	0.718	1.778
25	1	0.991	0.173	0.675	0.894
26	1	1.412	3.500	0.936	2.928
27	1	0.969	0.802	1.298	2.583
28	1	0.422	3.724	1.368	0.385
29	1	1.099	0.481	0.606	1.816
30	1	0.811	0.942	1.091	0.488
31	1	0.481	2.106	2.415	2.088
32	1	0.917	0.486	0.515	1.496
33	1	1.372	0.888	1.002	0.297
34	1	0.857	0.449	0.473	2.482
35	1	2.330	1.421	1.222	5.949
36	1	2.020	0.23	1.065	4.426
37	1	3.938	0.144	1.212	1.151
38	1	0.958	0.821	1.095	0.479
39	1	2.005	0.537	0.668	0.411
40	1	0.769	1.186	0.799	3.220
41	1	1.133	0.89	1.164	2.044
42	1	0.858	0.52	0.985	2.301
44	1	1.129	0.589	0.276	0.759
45	1	1.494	0.516	0.521	2.993
46	1	1.050	0.969	0.927	0.290
47	1	0.514	0.221	1.573	0.469
48	1	2.570	1.226	2.371	7.063
49	1	1.059	1.348	1.224	0.445
50	1	1.125	2.822	0.729	3.038
51	1	2.170	1.391	0.978	0.667
52	1	1.540	0.0978	0.718	6.921
54	1	0.697	1.451	1.172	2.404

55	1	0.275	0.798	1.440	1.087
56	1	2.564	0.213	0.986	1.197
57	1	0.442	0.739	0.952	1.088
58	1	1.466	0.183	0.936	2.114
59	1	0.529	0.550	0.487	0.468
60	1	1.685	1.651	0.837	2.865
61	1	0.889	0.259	0.376	1.139
62	1	1.005	1.169	1.488	4.302
63	1	0.957	1.329	1.038	2.679
64	1	0.684	0.600	0.186	1.716
65	1	0.809	0.429	0.93	1.278
66	1	0.679	0.307	0.183	0.753
67	1	0.889	0.991	0.754	0.383
68	1	1.181	1.158	0.711	2.340
69	1	1.204	0.501	1.050	10.400
70	1	1.110	0.100	0.678	1.110
71	1	1.219	0.315	0.691	2.398
72	1	1.086	0.696	0.763	2.229
73	1	1.183	1.273	1.139	0.481
74	1	0.840	0.887	1.232	0.411
75	1	1.051	1.370	1.098	4.301
76	1	1.204	0.418	0.759	0.533
77	1	1.309	0.193	1.079	5.857
78	1	0.976	0.971	1.120	0.491
79	1	1.234	0.375	1.078	1.372
80	1	1.004	0.368	0.616	1.272
81	1	0.913	0.0328	0.997	0.796
82	1	0.654	0.635	1.004	0.461
83	1	2.799	0.957	4.973	12.344
84	1	0.296	0.455	0.536	0.484
85	1	1.344	0.468	1.832	4.616
86	1	1.143	0.629	0.985	0.474
90	1	0.869	0.312	0.442	0.894
91	1	1.677	1.370	0.180	0.801
92	1	0.867	0.537	3.158	0.557
93	1	1.167	0.669	1.071	2.185
94	1	1.222	1.436	1.151	2.186
95	1	0.921	0.497	0.351	0.491
96	1	1.341	0.241	0.921	2.249
97	1	0.863	1.113	0.948	0.450
98	1	1.421	1.252	0.952	3.342
99	1	1.594	0.367	0.998	3.437
100	1	2.070	2.320	2.447	3.590
101	1	0.731	1.610	1.140	2.519
102	1	0.862	0.445	0.111	0.698
103	1	0.948	0.643	0.614	2.039
104	1	0.954	0.367	0.460	1.309
105	1	1.327	0.775	1.081	3.392
106	1	0.615	0.611	0.981	2.241
107	1	0.464	1.804	1.004	2.417
108	1	1.152	0.505	1.131	2.252
109	1	0.402	1.095	0.823	1.414
110	1	0.957	1.751	0.0923	0.809
111	1	1.129	2.495	2.009	6.870

Red colour indicates a significant increase in relative spot volume (ANOVA>0.05, fold change ≥2).
Green colour indicates a significant decrease in relative spot volume (ANOVA<0.05, fold change ≥2).
Grey colour indicates no significant change in protein expression.
n=4 per group, except for the group 0.1% PW: n=3
Abbreviations: Spot no indicates the spot number from the 2DE gel.

Table 2: Biomarker candidates of produced water and 17-β-oestradiol - Differentially expressed proteins identified by MS/MS

Spot n°	Accession number	Protein Identity	Queries matched[a]	Score[b]	Seq. Cov. %	Theoretical pI/Mw[c]
7	CAJ83333	Aldehyde dehydrogenase 1 family [Xenopus tropicalis][h]	1 (135)	42/29	5	5.80/79.7
	NP_031412.2	Aconitase 1 [Mus musculus][h]	1 (135)	34/29	11	7.23/98.1
12	BAD05136.1	hsc71 [Paralichthys olivaceus]	3 (159)	96/26	2	5.23/71.2
	Q6UFZ9	14-3-3B1 protein [Oncorhynchus mykiss]	9 (64)	280/28	39	4.64/27.5
13	AAZ39049	Antiquitin [Acanthopagrus schlegelli]	2 (158)	51/25	8	5.66/55.4
15	XP_001521350	Similar to pyroline-5-carboxylate dehydrogenase [Ornithirynchus anatinus][h]	1 (158)	30/25	23	8.66/30.3
17	AAQ72491	14-3-3E1 protein [Oncorhynchus mykiss]	9 (51)	214/28	47	4.67/29.3
	Q6UFZ9	14-3-3B1 protein [Oncorhynchus mykiss]	1 (51)	42/28	13	4.64/27.6
	NP_956243.1	Eukaryotic elongation factor 1 beta 2 [Danio rerio]	1 (51)	35/28	5	4.49/24.5
25	NP_958900.1	Tropomyosin 3 [Danio rerio]	4 (32)	113/29	11	4.76/28.8
	Q90YX1	60S acidic ribosomal protein P0	1 (32)	33/29	5	9.94/34.9
31	CAA63300.1	Simple type II keratin K8b (S2) [Oncorhynchus mykiss]	70 (896)	1609/28	57	5.14/59.0
	AAH66728.1	Krt4 protein [Danio rerio][h]	3 (896)	212/28	18	5.34/54.0
34	CAG08708	glucose-regulated protein 94 (HSP90) [Paralichtys olivaceus][h]	4 (51)	49/27	10	4.88/89.3
	XP_691686.2	Hemopexin [Danio rerio]	2 (51)	37/27	10	6.02/45.4
49*	NP_001086976	Hydroxyprostaglandin dehydrogenase 15-(NAD) [Xenopus laevis][h]	3 (56)	127/27	18	6.45/28.7
70	Q6UFZ9	14-3-3B1 protein [Oncorhynchus mykiss]	9 (64)	280/28	39	4.64/27.6
73	NP_001017833.1	Pyrophosphatase (inorganic) 1	18 (136)	691/28	63	5.25/32.6
	NP_998155.1	Eukaryotic translation initiation factor 3, subunit 2 beta (WD40) [Danio rerio]	6 (136)	131/28	20	5.22/36.3
	AAI35837	Pyp protein [Xenopus tropicalis][h]	2 (136)	54/28	4	6.35/37.3
	Q9YHC3	Tubulin beta-1 chain [Gadus morhua][h]	19 (301)	657/28	41	4.79/49.7
74	AAD56401.1	Beta-2 tubulin [Gadus morhua]	8 (301)	571/28	38	4.71/49.6
	NP_942104.1	Tubulin, beta 2C [Danio rerio][h]	1 (301)	533/28	44	4.85/49.8
	NP_001072450.1	Tubulin, beta 4 [Xenopus tropicalis][h]	1 (301)	457/28	36	4.79/49.8
	NP_942104.1	Tubulin, beta 2C [Danio rerio][h]	5 (301)	272/28	42	4.85/49.8
	AAT47549.1	Glycerol-3-phosphate dehydrogenase [Gadus morhua]	6 (301)	172/28	30	5.47/38.0
	NP_001017833.1	Pyrophosphatase (inorganic) 1 [Danio rerio]	7 (301)	128/28	35	5.25/32.6
	AAG30296.1	Cytochrome P450 [Ictalurus punctatus][h]	3 (301)	86/28	10	8.71/56.1
	CAG07103	Unnamed protein product (Phosphotriesterase, PTE) [Tetraodon nigroviridis]	3 (301)	83/28	38	5.84/38.9
	AAH44524	Sb:cd825 protein (thioredoxin) [Danio rerio]	2 (301)	79/28	13	6.32/54.7
	NP_001077011	Hydroxyacid oxidase (glycolate	2 (301)	54/28	13	7.56/40.5

		oxidase) [Danio rerio][h]				
	AAI35837	Pyp protein [Xenopus tropicalis][h]	1 (301)	50/28	4	6.35/37.3
	NP_954688	S-adenosylhomocysteine hydrolase [Danio rerio]	2 (301)	28/28	7	6.33/48.0
80	NP_001001401	Inner membrane protein mitochondrial (mitofilin) [Danio rerio]	4 (286)	83/28	35	5.31/83.2
	NP_001001401	Inner membrane protein mitochondrial (mitofilin) [Danio rerio]	4 (286)	67/28	20	5.31/83.2
	BAD90024.1	Heat shock 90kDa protein 1 beta isoform b (Hsp90) [Oncorhynchus mykiss]	3 (286)	46/28	5	4.92/83.4
84 [dn]	AAH16045	Actin, beta [Homo sapiens][h]	3			5.29/41.8
91	AAZ39049	Antiquitin [Acanthopagrus schlegelli]	2 (158)	51/25	8	5.66/55.4
	XP_001521350	Similar to pyroline-5-carboxylate dehydrogenase [Ornithirynchus anatinus][h]	1 (158)	30/25	23	8.66/30.3
90	NP_001079937	tropomyosin 4 isoform 2 [Danio rerio]	4 (182)	74/27	10	4.63/28.5
	NP_001002714.1	Down syndrome critical region gene 2 [Danio rerio]	1 (182)	49/27	3	4.54/30.7
93*	CAH59400.1	Alanine-glycosylate aminotransferase [Plasticthys flesus]	1 (48)	31	5	8.93/30.2
95[dn]	P53447	Fructose-bisphosphate aldolase B [Sparus aurata][h]	4			8.43/39.6
97 [dn]	XP_001339392	PREDICTED: Similar to Selenium-binding protein 1 [Danio rerio]	4			5.88/51.0
99 [dn]	XP_001521350.1	Similar to pyroline-5-carboxylate dehydrogenase [Ornithorhynchus anatinus][h]	6			8.68/30.3
100	Q6UFZ9	14-3-3B1 protein [Oncorhynchus mykiss]	9 (64)	280/28	39	4.64/27.6

The following search criteria were used: Modification: Carbamidomethyl (C), Oxidation (M); Peptide tolerance: 100 ppm; MS/MS tolerance: 0.25 Da; Enzyme: Trypsin; Monoisotopic. Database: Cod EST. $p<0.05$

[h]The protein was identified as an unnamed protein or hypotetical protein and was further characterised by homology in blast (PAM 30: Existence:9, extension:1).

*No preanalaytical LC-run of sample.

[dn]De novo sequencing: De novo sequences obtained from MS/MS were used in blast-searches in the Cod EST database: $p<0.05$ and identified by homology in the NCBI database (PAM 30: Existence: 9, extension:1).

[a]Score of MS fragments/ treshold score for homology/identity

[b]Fragments of query matched to the database hit/total number of query fragments used in the database search

Abbreviations used: Spot n[o]: spot number; Seq. cov.: sequence coverage; E-value: expect value; pI: isoelectric point; Mw[c]: molecular weight (kDa)

Figure legends

Figure 1: Differentially expressed proteins following exposure to produced water and 17β-oestradiol

The gel represents a pooled sample of liver of cod fry exposed to 1.0% produced water and separated in the first dimension in an 18 cm IPG-strip pH 4-7 and in the second dimension in a 9% SDS-PAGE. The numbers on the gel are the spot numbers, which correspond to the spot numbers in the tables (Table 1 and Table 2). Grey-coloured boxes indicate that these spots are not visible on this gel, as they are expressed on other gels and in samples from other treatment groups.

Figure 2: Dendrogram from hierarchical clustering analysis of differentially expressed proteins in cod fry liver following exposure

The dendrogram shows the grouping of differentially expressed proteins from liver of cod fry exposed to produced water and oestradiol for 78 days. Distance function: Pearson correlation. Linking method: Average. The names in the left margin are the name of the gels: L stands for low dose (0.01%) PW-exposed fry, M stands for medium dose (0.1%) PW-exposed fry, H stands for high dose (1.0 %) PW-exposed fry. 7 individual samples were analysed per group, with the exception of the M group in which only 6 individual samples were analysed. The three-structure on the top of the dendrogram shows the relationship between the differentially expressed proteins. Red colour indicates a strong spot and green colour indicate a weaker spot. A darker shade means that the spot is closer to the average spot volume than a brighter shade of the same colour. n=4 per group, except for the group of 0.1% PW; n=3.

Figure 1

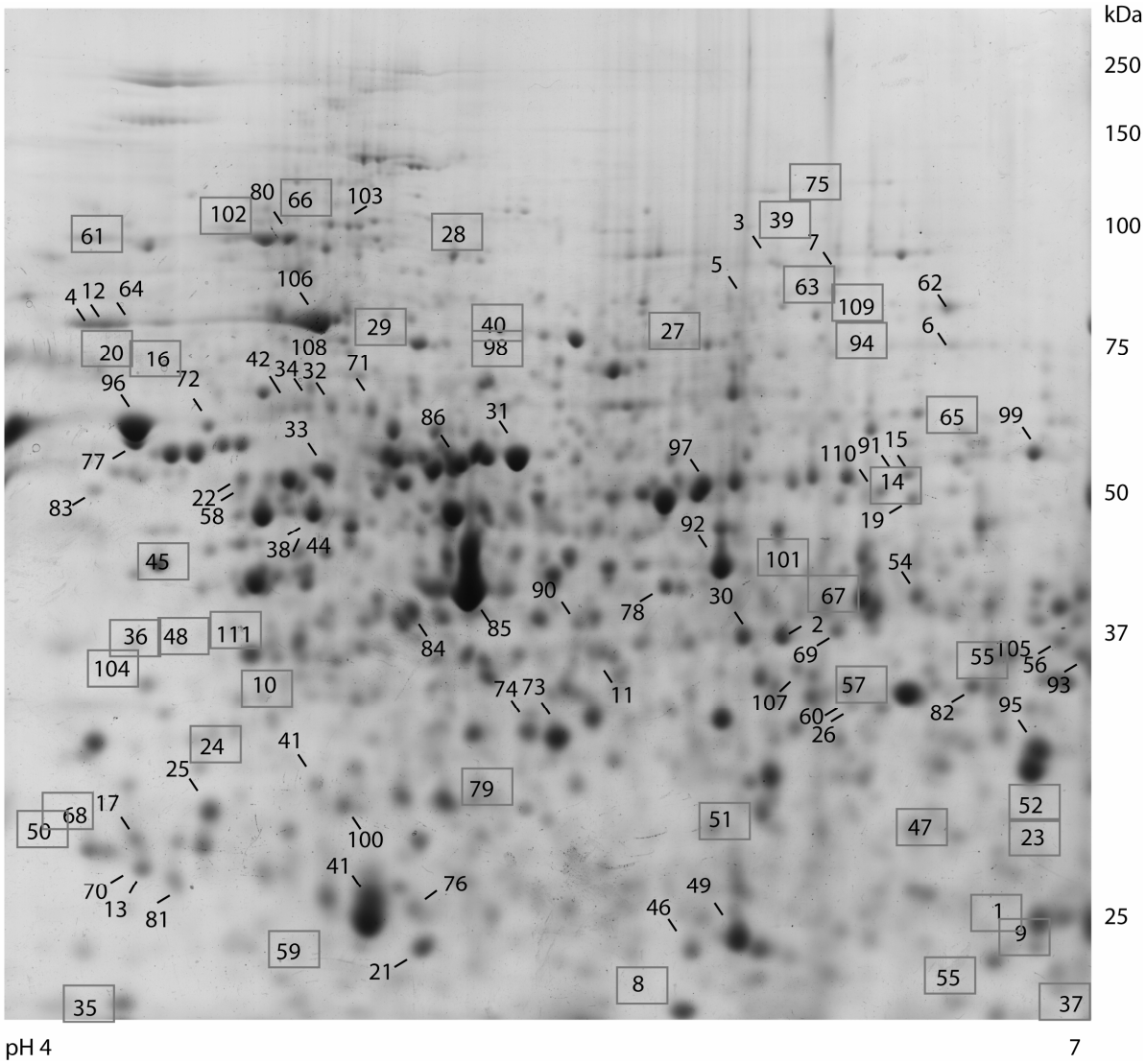
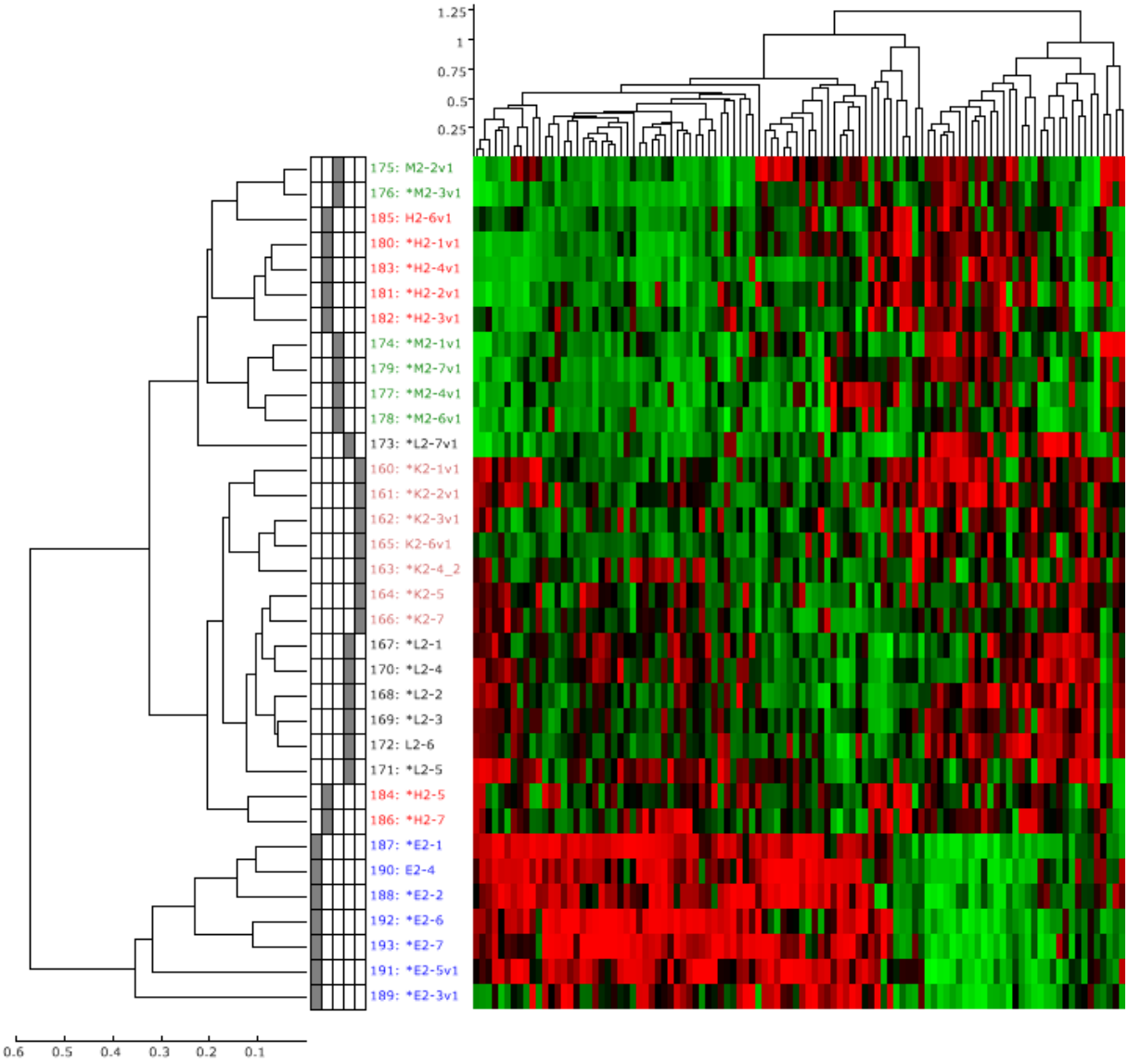

kDa
250
150
100
75
50
37
25
pH 4
7
80
66
103
102
61
28
75
3
39
7
5
63
62
106
4
12
64
109
6
29
40
27
94
20
16
98
42
34
32
108
71
96
72
65
99
86
31
33
97
110
91
15
77
14
22
58
83
19
92
38
44
45
101
54
90
78
30
67
36
48
111
85
84
2
69
55
105
56
93
104
10
74
73
11
107
57
82
95
60
26
24
41
25
79
52
68
17
51
47
23
50
100
41
76
70
13
81
49
46
1
9
59
21
8
55
37
35

Figure 2


1.25
1
0.75
0.5
0.25
175: M2-2v1
176: *M2-3v1
185: H2-6v1
180: *H2-1v1
183: *H2-4v1
181: *H2-2v1
182: *H2-3v1
174: *M2-1v1
179: *M2-7v1
177: *M2-4v1
178: *M2-6v1
173: *L2-7v1
160: *K2-1v1
161: *K2-2v1
162: *K2-3v1
165: K2-6v1
163: *K2-4_2
164: *K2-5
166: *K2-7
167: *L2-1
170: *L2-4
168: *L2-2
169: *L2-3
172: L2-6
171: *L2-5
184: *H2-5
186: *H2-7
187: *E2-1
190: E2-4
188: *E2-2
192: *E2-6
193: *E2-7
191: *E2-5v1
189: *E2-3v1
0.6
0.5
0.4
0.3
0.2
0.1

Errata for
Proteome changes in Atlantic cod (*Gadus morhua*) exposed to oil and produced water:

Discovery of biomarker candidates for environmental monitoring

Anneli Bohne Kjersem

Thesis for the degree philosophiae doctor (PhD)

at the University of Bergen

_____ _____

(signature of candidate) (signature of faculty)

01.08.07

Errata

Manuscript I:

Page 19, line 1: “#7587” should be deleted

Page 19, line 3: “#7098” should read “#7329”

Page 19, line 3: “down-regulated” should be inserted into the sentence: “Apolipoprotein B (#7178) was shown to be up-regulated by 0.25 ppm crude oil and down-regulated by surrogate produced water exposure.”

Manuscript II:

Page 11, line 1: “0.25 Da” should be inserted at the end of the sentence: “MS^2 mass accuracy: 0.25 Da”

Page 12, line 18: “24” should be corrected to “61”

Page 15, line 5 and page 28, line 17: “$p>0.05$” should be corrected to “$p<0.05$”

Page 27, Table 2: “188” should be corrected to “118”

Manuscript III:

Page 12, line 4: “0.25 Da” should be inserted at the end of the sentence: “MS^2 mass accuracy: 0.25 Da”

Page 14, line 1: Sentence should read “Low levels of produced water resulted in an apparent down-regulation of keratin and actin, and an up-regulation of 14-3-3B1.”

Page 14, line 8: Sentence should read “The heat shock proteins, hemopexin and the aldolase appeared to be up-regulated by E2 treatment while the dehydrogenase appeared to be down-regulated.”

Page 14, line 15: Sentence should read “E2 treatment apparently caused a down-regulation of pyrophosphatases (inorganic) 1, eukaryotic elongation initiation factor, tubulins, glycerol-3-phosphate dehydrogenase, cytochrome P450 2x1, phosphotriesterase, thioredoxin, glucolate oxidase, S-adenosylhomocysteine hydrolase and a selenium-binding protein 1, and an up-regulation of alanine-glycosylate aminotransferase.”