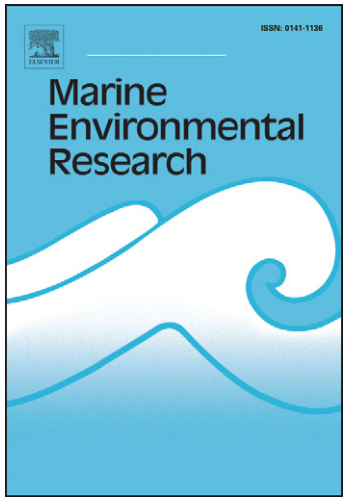
Candidate biomarker discovery in plasma of juvenile cod () exposed to crude North Sea oil, alkyl phenols and polycyclic aromatic hydrocarbons (PAHs)

Anneli Bohne-Kjersem, Arnfinn Skadsheim, Anders Goksøyr, 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)

Anneli Bohne-Kjersem, Arnfinn Skadsheim, Anders Goksøyr, Bjørn Einar Grøsvik

PII: S0141-1136(09)00086-5
DOI: 10.1016/j.marenvres.2009.06.016
Reference: MERE 3355

To appear in: *Marine Environmental Research*

Received Date: 13 February 2009
Revised Date: 19 June 2009
Accepted Date: 24 June 2009

Please cite this article as: Bohne-Kjersem, A., Skadsheim, A., Goksøyr, A., Grøsvik, r.E., Candidate biomarker discovery in plasma of juvenile cod (*Gadus morhua*) exposed to crude North Sea oil, alkyl phenols and polycyclic aromatic hydrocarbons (PAHs), *Marine Environmental Research* (2009), doi: 10.1016/j.marenvres.2009.06.016

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,ξ], Arnfinn Skadsheim[2], Anders Goksøyr[1,3], and Bjørn Einar Grøsvik[1,4]

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

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

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

[4]Institute of Marine Research, PO Box 1870, N-5817 Nordnes, Bergen, Norway

[ξ]Present address: Det Norske Veritas, Johan Berentsensvei 109-111, N-5020 Bergen, Norway

Corresponding author: Anneli Bohne-Kjersem

Email: anneli@.bohnekjersem.net

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

Fax: +47 55 58 96 83

Keywords: Atlantic cod, biomarkers, crude oil, plasma, produced water, proteomics

Abstract

In this study we have investigated protein changes in plasma of juvenile Atlantic cod (*Gadus morhua*) induced by crude North Sea oil and North Sea oil spiked with alkyl phenols and polycyclic aromatic hydrocarbons, a surrogate produced water composition. Using a proteomic approach, we identified 137 differentially expressed proteins at different levels of crude oil exposure. Many of the induced protein changes 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 apoptosis. Although the number of individuals and samples in this study is limited within each treatment group, the protein changes observed in this study represent a first screening for potential biomarker candidates in cod plasma reflecting potential effects of crude oil and produced water exposure on fish.

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, containing a great variety of compounds, including dissolved inorganic salts, minerals and heavy metals, dissolved and dispersed oil compounds, as well as 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 mgL^{-1} (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 accidents and threats of blow-outs or 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).

The development of oil fields may represent a conflict with fisheries interests. In the North Sea, many of the oil and gas installations are situated in areas inhabited by large fish populations. According to a recent risk assessment, the risk area in the vicinity of oil platforms could contain up to twice the average fish density as 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, for example during early development. Adequate biomonitoring tools, such as sensitive biomarkers, that can detect and monitor the effect of low chronic exposure as well as larger acute exposures are therefore of great importance to regulatory authorities, as a basis for better environmental risk assessment.

Here, we have studied the effects of crude North Sea oil singly or spiked with PAHs and alkyl phenols on cod using a proteomic approach. By using two-dimensional electrophoresis (2DE) and image analysis we identified protein changes in cod plasma, that were considered 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

Acetic 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 (ACN), methanol and trifluoroacetic acid (TFA) were all purchased from Merck (Darmstadt, Germany). Ethanol was obtained 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 (by, MO, US). Ampholine™ 3,5-10, DryStrip Cover fluid, and IEF Electrode strips were purchased from GE Healthcare (Uppsala, Sweden).

Ammonium dodecyl sulfate, SDS, Agarose, 30% Acrylamide/Bis solution, 37.5:1, Ammonium persulfate, temed and Precision Plus Protein™ standard were purchased from Bio-Rad (Hercules, CA, US). Trypsin was purchased from Promega (Madison, WI, US), while Poros 20R2, Reverse Phase packing, was purchased from Applied Biosystems (Foster City, CA, US). GelLoader tips were provided by Eppendorf (Hamburg, Germany), and siliconised tubes were provided by Sorenson BioScience,

Inc (Salt Lake City, 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 in 530 litre tanks and fed commercial cod pellets (Skretting). Feeding was stopped two to three days before sampling in order to encourage accumulation 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 from 33-34 ppt and 8-12 ºC during the acclimation 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 experimental tanks one week prior to the start of the oil exposure. Five fish were sampled each time from each tank. The fish were sacrificed with a blow to the head and dissected for sampling of blood and liver tissue. In order to minimize stress during the experiment, length and weight were only measured at the sampling time. The mean total wet weight of the fish was 40.5 ± 12.3 g and the mean length was 16.7 ± 1.5 cm. Blood samples were taken by a heparin-coated syringe which was inserted between the first and second caudal fin and towards the chorda until the main blood vessel was hit. The blood sample was centrifuged at 2000 g for 5 minutes at 4 °C. Thereafter the plasma was removed by a pipette to cryo-vials and frozen at - 78 °C.

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 mg oil kg^{-1} seawater (= mgL^{-1}= ppm)– low levels, 0.25 mgL^{-1}– medium levels, and 1.0 mgL^{-1}– high levels of crude oil, as well as one group exposed to 1.0 mgL^{-1} crude oil spiked with polyaromatic hydrocarbons and alkyl phenols (for details see below). Only samples from day 3 and day 24 were used in the present study.

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 (mgL^{-1}), and the dispersion was distributed via a manifold with overflow to a second set of manifolds with overflows. These secondary manifolds received the 5 mgL^{-1} oil dispersion at various rates through peristaltic pumps. Seawater diluting the oil dispersion was added by gravity and controlled by valves from a header tank with overflow. The overflow ensured stable pressure in the water supplies in the system. Four different solutions involving dispersed crude oil were made in addition to seawater. The levels of hydrocarbons in water and of hydrocarbon uptake in fish following the exposure, are reported in Skadsheim (2005).

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 mgL^{-1} seawater per sum alkyl phenols 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 mgL^{-1} 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 mgL^{-1} oil in seawater emulsion plus the diluted spike composition. The final amount of the acetone carrier in the spike exposure solution was 20 µg acetone L^{-1} water. Hydrocarbon levels in water and the level of hydrocarbon uptake in fish following the exposure are reported in Skadsheim (2005).

Methods

2DE

The protein concentration of the plasma 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 the manufacturer's guidelines. The strips were equilibrated for 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 (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 V, 400 mA per 12 gels, 20°C for 45 min. in a mini-Dodeca unit (Bio-Rad) prior to silver-staining (Heukeshoven & Dernick, 1988). Preparative gels were run in an Ettan Dalt Six/Twelve unit at 1W per 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.

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).

Mass spectrometry

The excised protein spots were in-gel digested according to the method of Shevchenko et al. (1996) prior to mass spectrometry. 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 ngμl^{-1}) 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.

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). SPITC diluted in 20 mM NaHCO3, pH 8.6 to a concentration of 10 $\mu g\mu l^{-1}$ was added to the peptide solution and incubated at 30 min. at 50°C. The reaction was terminated by adding 1 µl 5% TFA. The SPITC-derivated proteins were then applied to nano-columns packed with 20Poros R2 (Gobom et al., 1999). The Column was washed with 0.1% TFA and eluted with matrix, α-cyano-4-hydroxycinnamic acid (CHCA) in 70% ACN/0.1% TFA on to the steel target prior to MALDI-ToF-ToF (4700 Proteomics Analyser, Applied Biosystem, Foster City, 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 $nLmin^{-1}$, 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 cod EST database used for protein identification included 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 Unifob (Bergen, Norway), 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

20 individual plasma samples were screened on 2DE and analysed for differentially expressed proteins utilising the Ludesi 2DE Image Analysis Service. The dataset comprise 9 male individuals and one female individual sampled after 3 days of exposure, and 10 male individuals sampled after 24 days of exposure. The limited number of samples within each group (n=1-3) is due to varying distribution of males and females among the five individuals sampled within each treatment group. Although sex was not documented to influence protein patterns here, we tried to use only male fish for the present study. On average, approximately 840 proteins were detected on the 2DE-gels. Screening analysis gave 78% average correctness, at both day 3 and day 24 of exposure. The estimated total number of correctly identified significant changes ($p<0.05$) were 465.

Biomarker candidates were identified by comparing the global protein expression of silver-stained mini-gels of individuals from 10 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 137 protein changes occur at the lowest level of oil exposure (≤0.06 mgL^{-1} crude oil) (Table 1 and 2).

MS-MS

Of the 137 protein spots on the silver-stained mini-gels evaluated to be differentially expressed upon exposure, 29 proteins were positively identified by MS/MS (Table 3).

After 3 days of exposure (Table 1), fibrinogen (#7013, #7709) was increased in protein level by surrogate produced water exposure. Another protein identified as an alpha-2-antiplasmin (#7550) was down-regulated after 3 days of crude oil exposure.

After 24 days of exposure (Table 2), enolase alpha (#7011) was down-regulated by crude oil and surrogate produced water exposure. Fibrinogen (#7032) was up-regulated at higher levels of crude oil and surrogate produced water exposure. Alpha-2-antiplasmins, A2A, (#7581, #7584, #7587, # 7597) appeared to be up-regulated by 24 days of 1.0 mgL^{-1} crude oil and surrogate produced water exposure. Tropomyosin (#7043), serotransferrin (#7192), hemopexin (#7409), prothrombin (#7285, #7329) and fetuin B (#7098, #7285, #7345) was 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), was up-regulated by 0.25 mgL^{-1} of crude oil. The prepro-apolipoprotein A (#8580) was shown to be up-regulated at crude oil levels $\geq$25 mgL^{-1} and by surrogate produced water exposure, while NTPase (#7810) was found to be down-regulated by 1.0 mgL^{-1} 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 $\geq$1.0 mgL^{-1}.

Apolipoprotein B (#7178) was shown to be up-regulated by 0.25 mgL^{-1} crude oil exposure and by surrogate produced water exposure.

Discussion

This proteomics-based study of protein expression profiles after exposure of juvenile Atlantic cod 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.

Chemical analysis of the water samples by fluorescence demonstrate that mean values of 45-72% of the nominal doses of total hydrocarbons could be recorded over the exposure period, whereas GC/MS analysis detected 80-100% of total PAH (Skadsheim, 2005). Bile PAH metabolites reflected a clear and significant correlation between exposure levels and levels of metabolites in the bile when measured by fixed fluorescence and by GC/MS (ibid.).

Although the number of individuals and samples in this study is limited within each treatment group, all the ANOVA analyses 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 cod plasma that will be further analysed and verified in future exposure studies based on a larger number and broader range of samples.

Image analysis and changes in the protein pattern

Using a 2D-gel based strategy in toxicoproteomics, 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. Good warping- and normalisation-functions prior to spot detection and matching, are therefore crucial to the final result. 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 far better than the average correctness of that of PDQuest, another image analysis software used in our lab. 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.

Biomarker candidates

After 3 days of exposure, fibrinogen was up-regulated after surrogate produced water exposure and there was a down-regulation of A2A following crude oil exposure. After 24 days of exposure, we observed a down-regulation of alpha enolase and prothrombin, and an up-regulation of alpha-2-antiplasmin and fibrinogen, proteins which are involved in the fibrinolytical system. These changes suggest an effect on

the fibrinolytical system following crude oil exposure. Alpha-1-antitrypsin (A1AT) was up-regulated.

The observed changes of expression of A1AT and alpha-enolase also indicate an effect on the immune system. A1AT is known to be linked to immunological 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 involved in a large variety of infectious and autoimmune diseases, microbial tissue invasion, endothelial injury, activation of the complement classical pathway, perturbations 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-(pregnancy zone protein)-resembling protein after crude oil exposure ($\geq$0.25 mgL^{-1}) 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 of oil hydrocarbons or other components of crude oil on the fertility of fish.

Tropomyosin forms complexes with several different 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 in turn lead to an overload of iron (Bradbury, 1997, 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 lipid metabolism. 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 affect 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 mgL^{-1} 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 osteogenesis (Binkert et al.,1999). The apparent down-regulation of these proteins may thus have several adverse effects in the exposed fish.

When evaluating 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 biomarker development with limited amount of samples available for biomarker discovery. Hence, the results from this study may include a number of false positives, although we chose to use a filter of >2 fold-change, in addition to p-values <0.05 (ANOVA) for selecting candidates for further processing. Further analysis, verification, 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, our strategy involves producing 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 mechanisms of action of oil hydrocarbons and alkyl phenols in fish.

Conclusion

We found the Ludesi software to provide robust image analysis for biomarker candidate discovery. 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 observed also at low levels of exposure for many of the proteins, which indicates effects on fish at lower levels of exposure than previously reported. However, their use as future biomarkers has to be tested at a much larger scale in future studies. 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 compared to single biomarkers alone.

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, gave us access to more than 50. 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 was 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. Aquatic Toxicology 51(2):241-258.

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

Binkert C, Demetriou M, Sukhu B, Szweras M, Tenenbaum HC, Dennis JW. 1999. Regulation of osteogenesis by fetuin. Journal of Biological Chemistry 274:28514-20.

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

Bradbury MW. 1997. Transport of iron in the blood-brain-cerebrospinal fluid system. Journal of Neurochemistry 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. Analytical Biochemistry 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. Reproductive Toxicology 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. Journal of Inorganic Biochemistry 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. Comparative Biochemistry and Physiology part C Toxicology and Pharmacology 130(2):249-258.

Gobom J, Nordhoff E, Mirgorodskaya E, Ekman R, Roepstorff P. 1999. Sample Purification and Preparation Technique Based on Nano-scale Reverse Phase Columns for the Sensitive Analysis of Complex Peptide Mixtures by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry. Journal of Mass Spectrometry 34:105-116.

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. Oncology Reports 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.

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. Marine Environmental Research 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. Seminars in Thrombosis and Hemostasis 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. Journal of Mass Spectrometry 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). Aquatic Toxicology 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-dimensional electrophoresis of proteins. Journal of Biological Chemistry 250(10):4007-4021.

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

OSPAR 2006. OSPAR Recommendation 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. Analytical Chemistry 75(3):663-670.

Rifai N, Gerszten RE. 2006. Biomarker discovery and validation. Clinical Chemistry 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. Aquatic Toxicology 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. Biochemical and Biophysical Research Communications 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. Analytical Chemistry 73:1917-1926.

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

Skadsheim A 2005. Cod stocks exposed to crude oils: Uptake, metabolism and biomarker effects. Report AM-2004/005. IRIS-Akvamiljø, Norway (ISBN 82-8192-007-6).

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.*). Comparative Biochemistry and Physiology part C Vol 113 C(2):257-265.

Steen H, Mann M. 2004. The ABC's (and XYZ's) of peptide sequencing. Nature Reviews in Molecular Cell Biology 5(9):699-711.

Tam SP, Archer TK, Deeley RG. 1985. Effects of estrogen on apolipoprotein secretion by the human hepatocarcinoma cell line, HepG2. Journal of Biological Chemistry 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. Autoimmunity Reviews 6(3):176-182.

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

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

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

Table 1: Relative protein expression levels in cod plasma following 3 days of exposure to crude oil and surrogate produced water[a]

Spot # [b]	Relative protein expression levels					Protein identity
	Control (82,83[c])	0.06 mgL^{-1} CO[d] (84,85,86)	0.25 mgL^{-1} CO (87,88,89)	1.0 mgL^{-1} CO (90)	1.0 mgL^{-1} sPW[e] (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**	**2.4**	**5.8**	**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[f])**
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**

[a]Summary of relative protein changes in cod plasma following exposure found by image analysis (Ludesi Interpreter) of 2D gels. **Bold** font type indicates a significant up-regulation (ANOVA: $p < 0.05$, fold change ≥ 2 in protein expression) of a protein as compared to control (or lowest detectable level in any group, if control is below detectable level). *Italics* is used for significant down-regulation (ANOVA: $p < 0.05$, fold change <0.5 in protein expression) of a protein as compared to control. n=1-3 in groups (identified by sample no. in parentheses).

[b]Spot no. are shown with 2D localization in Fig. 1.

[c]sample of female individual

[d]CO - crude oil treated juveniles.

[e]sPW - surrogate produced water (1.0 mgL^{-1} crude oil plus alkylphenol and hydrocarbon spike)-treated juveniles

[f]A2A - Alpha-2-antiplasmin

Table 2: Relative protein expression levels in cod plasma following 24 days of exposure to crude oil and surrogate produced water[a]

Spot # [b]	Relative protein expression levels					Protein identity
	Control (62,63)	0.06 mgL^{-1} CO [c] (68)	0.25 mgL^{-1} CO (69,70)	1.0 mgL^{-1} CO (74,75,76)	1.0 mgL^{-1} sPW [d] (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 [e]**
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**
7051	1	1.9	*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*	**Apolipoprotein 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** **Unnamed**
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*	
7665	1	*0.5*	0.7	*0.3*	*0.3*	**Hypothetical** **APO B** **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**	

[a]Summary of relative protein changes in cod plasma following exposure found by image analysis (Ludesi Interpreter) of 2D gels. **Bold** font type indicates a significant up-regulation (ANOVA: $p< 0.05$, fold change ≥ 2 in protein expression) of a protein as compared to control (or lowest detectable level in any group, if control is below detectable level). *Italics* is used for significant down-regulation (ANOVA: $p< 0.05$, relative fold change <0.5) of a protein as compared to control. n=1-3 in groups (identified by sample no. in parentheses).

[b]Spot no. are shown with 2D localization in Fig. 1.

[c]CO - crude oil treated juveniles.

[d]sPW - surrogate produced water (1.0 mgL^{-1} crude oil plus alkylphenol and hydrocarbon spike)-treated juveniles

[d]Protein abbreviations: 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 in cod plasma 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
7032[2]	CAF96585	Unnamed protein product (fibrinogen) [Tetraodon nigroviridis]	67	6	18	5.0/40	4.96/47,3	Fibrinogen
7043[2]	CAF98296[ns]	Unnamed protein product (tropomyosin) [Tetraodon nigroviridis][ns]	52/29	2	14	6.1/145	5.01/35,2	Tropomyosin
7098[2]	CAF98106	Hypothetical protein LOC563663 (fetuin B) [Danio rerio]	137	9	16	4.4/100	5.05/52,9	Fetuin B
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) [Tetraodon nigroviridis]	298/29	9/426	13	4.9/90	5.99/56.1	Fetuin B
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) [Danio rerio]	326	11	21	4.4/80	5.05/52.9	A2A
7581[2]	NP_001073479.1	Hypothetical protein LOC563663 (A2M) [Danio rerio]	326	11	21	4.5/95	5.05/52.9	A2A
7584[2]	NP_001073479.1	Hypothetical protein LOC563663 (A2M) [Danio rerio]	326	11	21	4.4/95	5.05/52.9	A2A
7597[2]	NP_001073479.1	Hypothetical protein LOC563663 [Danio rerio]	326	11	21	4.3/95	5.05/52.9	A2A
7665[2]	NP_001025233.1	Hypothetical protein LOC321166 [Danio rerio]	645/28	27/	84	4.6/40	5.11/412,6	Lipoprotein, function unknown
	CAA57449.1	Apolipoprotein B [Salmo salar]	675/28	17/	38	4.6/40	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 threshold 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 cod plasma proteins on the 2DE gel.

A 2DE gel of an individual sample of juvenile cod plasma exposed to 1.0 mgL^{-1} 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 protein spots with significant changes (ANOVA: $p<0.05$, fold change ≥ 2) from all the different treatment groups (0.06 mgL^{-1}, 0.25 mgL^{-1} and 1.0 mgL^{-1} crude oil, and crude oil spiked with PAH and alkyl phenols) are shown.