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

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1 **Abstract**

2
3 In this study we have investigated protein changes in plasma of juvenile Atlantic cod
4 (*Gadus morhua*) induced by crude North Sea oil and North Sea oil spiked with alkyl
5 phenols and polycyclic aromatic hydrocarbons, a surrogate produced water
6 composition. Using a proteomic approach, we identified 137 differentially expressed
7 proteins at different levels of crude oil exposure. Many of the induced protein changes
8 occurred at low levels of exposure. The results obtained with protein expression
9 profiles after exposure to oil and surrogate produced water indicate effects on
10 fibrinolysis and the complement cascade, the immune system, fertility-linked proteins,
11 bone resorption, fatty acid metabolism as well as increased oxidative stress, impaired
12 cell mobility and increased levels of proteins associated with apoptosis. Although the
13 number of individuals and samples in this study is limited within each treatment
14 group, the protein changes observed in this study represent a first screening for
15 potential biomarker candidates in cod plasma reflecting potential effects of crude oil
16 and produced water exposure on fish.

1 **Introduction**

2
3 The three main sources of continuous oil discharges offshore are produced water,
4 displacement water and drainage water (OLF, 2005). Of these, produced water is by
5 far the most dominant, containing a great variety of compounds, including dissolved
6 inorganic salts, minerals and heavy metals, dissolved and dispersed oil compounds, as
7 well as organic compounds such as carboxylic acids and phenols from the formation
8 water (Neff, 2002). The maximum permitted concentration of oil in discharged
9 produced water is 30 mgL⁻¹ (30ppm) (OSPAR, 2006). The most well studied
10 compounds of produced water, polycyclic aromatic hydrocarbons (PAHs) and alkyl
11 phenols, have been reported to cause severe effects on marine wildlife (Nicholas,
12 1999; Aas et al., 2000; Evanson & van der Kraak, 2001; Schwaiger et al., 2002;
13 Cardinali et al., 2004; Meier et al., 2007).

14
15 In addition to the release of compounds through routine discharges of produced water,
16 the accidents and threats of blow-outs or oil tankers spilling large amounts of oil
17 offshore are incidents of great concern. Previously, negative effects from oil spills
18 have been reported (Solé et al., 1996, Carls et al., 2001, Jewett et al., 2002, Lee and
19 Anderson, 2005).

20
21 The development of oil fields may represent a conflict with fisheries interests. In the
22 North Sea, many of the oil and gas installations are situated in areas inhabited by large
23 fish populations. According to a recent risk assessment, the risk area in the vicinity of
24 oil platforms could contain up to twice the average fish density as the whole North
25 Sea area (Myhre et al., 2005). Oil discharges and spills in these areas may therefore

1 have the potential to affect a large number of individual fish for prolonged periods,
2 possibly also at more sensitive life stages, for example during early development.
3 Adequate biomonitoring tools, such as sensitive biomarkers, that can detect and
4 monitor the effect of low chronic exposure as well as larger acute exposures are
5 therefore of great importance to regulatory authorities, as a basis for better
6 environmental risk assessment.

7
8 Here, we have studied the effects of crude North Sea oil singly or spiked with PAHs
9 and alkyl phenols on cod using a proteomic approach. By using two-dimensional
10 electrophoresis (2DE) and image analysis we identified protein changes in cod
11 plasma, that were considered as promising biomarker candidates for the biological
12 effects of oil and produced water to fish. The proteins were isolated from 2DE-gels
13 and identified by mass spectrometry (MS), searching a new cod genome database
14 based on expressed sequence tags (ESTs).

15

1 **Materials and methods**

2

3 **Materials**

4

5 Acetic acid, Coomassie brilliant blue CBB G250, ortho-phosphoric acid, urea,
6 thiourea, tris (hydroxymethyl)-ammoniummethane, sodium carbonate, formic acid,
7 formaldehyde, sodium acetate trihydrate, glutardialdehyde, 25% v/v, sodium
8 thiosulphate pentahydrate, silver nitrate, EDTA $\times$ Na $_2$ $\times$ 2H $_2$ O, ammonium sulphate,
9 acetonitril (ACN), methanol and trifluoroacetic acid (TFA) were all purchased from
10 Merck (Darmstadt, Germany). Ethanol was obtained from Arcus Kjemi (Oslo,
11 Norway).

12

13 Bovine Serum Albumin, BSA, 3-(3-colamidopropyl)-dimethylammonio-1-
14 propansulfoanat, CHAPS, Triton X-100, DL-dithiothreitol, DDT, iodoacetamide,
15 alpha-cyano-4-hydroxycinnamic acid, CHCA and 4-sulfophenyl isothiocyanate,
16 sodium salt monohydrate, SPITC, were purchased from Sigma Aldrich (by, MO, US).
17 AmpholineTM 3,5-10, DryStrip Cover fluid, and IEF Electrode strips were purchased
18 from GE Healthcare (Uppsala, Sweden).

19

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

1 Inc (Salt Lake City, UT, US). Peptide Calibration Standard and MTP384 Target plate
2 polished steel TF were purchased from Bruker Daltonics (Leipzig, Germany).

3

4 **Experimental design**

5

6 **Fish**

7 Juvenile cod (0-group) were purchased from an aquaculture facility, Sagafjord in
8 Leirvik at Stord, Norway (North Sea Norwegian coastal origin), four weeks prior to
9 the start of the exposure (October, 2002). The fish were maintained in 530 litre tanks
10 and fed commercial cod pellets (Skretting). Feeding was stopped two to three days
11 before sampling in order to encourage accumulation of bile in the gall bladder. The
12 sea water was taken from the 78 m depth water intake at Akvamiljø (Mekjarvik,
13 Stavanger, Norway). The water salinity and temperature ranged from 33-34 ppt and
14 8-12 °C during the acclimation period. During the exposure period the temperature
15 ranged from 10 – 11.5 °C, whilst the salinity was as for the storage period. The fish
16 were carefully transferred to avoid skin lesions and to minimize behavioural stress to
17 the five experimental tanks one week prior to the start of the oil exposure. Five fish
18 were sampled each time from each tank. The fish were sacrificed with a blow to the
19 head and dissected for sampling of blood and liver tissue. In order to minimize stress
20 during the experiment, length and weight were only measured at the sampling time.
21 The mean total wet weight of the fish was 40.5 ± 12.3 g and the mean length was 16.7
22 ± 1.5 cm. Blood samples were taken by a heparin-coated syringe which was inserted
23 between the first and second caudal fin and towards the chorda until the main blood
24 vessel was hit. The blood sample was centrifuged at 2000 g for 5 minutes at 4 °C.
25 Thereafter the plasma was removed by a pipette to cryo-vials and frozen at - 78 °C.

1

2 **Exposure**

3 The cod were exposed for 24 days, and sampled at day 3, 14 and 24. Five exposures
4 were set up including control. The cod were exposed to three levels of crude oil: 0.06
5 mg oil kg⁻¹ seawater (= mgL⁻¹ = ppm)– low levels, 0.25 mgL⁻¹– medium levels, and
6 1.0 mgL⁻¹– high levels of crude oil, as well as one group exposed to 1.0 mgL⁻¹ crude
7 oil spiked with polycyclic aromatic hydrocarbons and alkyl phenols (for details see below).
8 Only samples from day 3 and day 24 were used in the present study.

9

10 **Oil**

11 The oil dispersions were made in the continuous flow system (CFS), using Statfjord B
12 field crude oil. The oil was in each experiment dispersed in seawater as droplets with
13 mean and median diameters in the 11-13 µm range. Initially the oil was dispersed at 5
14 mg oil/kg seawater (mgL⁻¹), and the dispersion was distributed via a manifold with
15 overflow to a second set of manifolds with overflows. These secondary manifolds
16 received the 5 mgL⁻¹ oil dispersion at various rates through peristaltic pumps.
17 Seawater diluting the oil dispersion was added by gravity and controlled by valves
18 from a header tank with overflow. The overflow ensured stable pressure in the water
19 supplies in the system. Four different solutions involving dispersed crude oil were
20 made in addition to seawater. The levels of hydrocarbons in water and of hydrocarbon
21 uptake in fish following the exposure, are reported in Skadsheim (2005).

22

23

24 **Surrogate produced water**

1 The spike was initially made as a concentrated stock solution comprised of the
2 hydrocarbons dissolved in acetone. The spike hydrocarbon mixture in acetone was
3 first pumped into a mixing flask that also received seawater. This first step of dilution
4 generated a concentration of 0.8 mgL^{-1} seawater per sum alkyl phenols or PAHs. This
5 solution was then fed by gravity and valve control to a second mixing flask and a
6 manifold, which provided a final concentration at 0.2 mgL^{-1} hydrocarbon solution of
7 each of the two spike substance groups. This flask also received an oil emulsion fed
8 via a peristaltic pump to provide the final 1 mgL^{-1} oil in seawater emulsion plus the
9 diluted spike composition. The final amount of the acetone carrier in the spike
10 exposure solution was $20 \text{ } \mu\text{g acetone L}^{-1}$ water. Hydrocarbon levels in water and the
11 level of hydrocarbon uptake in fish following the exposure are reported in Skadsheim
12 (2005).

13

14

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 ($10 \text{ ng}\mu\text{l}^{-1}$) 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 NaHCO_3 , pH 8.6 to a concentration of $10 \mu\text{g}\mu\text{l}^{-1}$ 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-derivatized 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 $\pm 0.5 \text{ m/z}$ 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

1 to be sulfonated were manually selected for further fragmentation and de novo-
2 sequencing.

3

4 **LC-ESI-Q-ToF**

5 Prior to application of sample on the LC-ESI-Q-ToF, 20 μ L trypsin-digested protein
6 samples of isolated 2DE-spots were run on home-made C_{18} Stage tip columns
7 (Rappsilber et al., 2003), and the samples were eluted with 1/10 of sample volume of
8 100% acetonitril (ACN) and dissolved in approximately 2x sample volume of 2%
9 formic acid (FA). 40 μ L of the dissolved sample was applied on the ESI-LC-QToF
10 instrument.

11

12 The samples were applied to an Ultimate 3000 nano-LC (Dionex Corporation, USA).
13 The nano-LC consist of a C_{18} Pepmap 100 pre-column (Dionex Corporation, USA)
14 with bead size 5 μ m, pore size 100Å, and a n C_{18} analytical column, C_{18} Pepmap 100
15 (Dionex Corporation, USA), with bead size 3 μ m and pore size 100Å in the first set-
16 up, and with bead size 5 μ m and pore size 100Å in the second set-up. The flow rate on
17 the analytical column was 300 nLmin⁻¹, mobile phase A was 2% ACN and 0.1% FA,
18 and mobile phase B was 90 % ACN and 0.1% FA. The following gradient was used:
19 0-3 min.: 5% B; 3-40 min.: 5-50% B; 40-45 min.: 50-95% B; 45-53 min.: 95% B; 53-
20 55 min.: 95-5% B; 55-70 min.: 5% B.

21

22 ESI-voltage was 2942 V, cone voltage 100 V and the ion source temperature 110° C.

23 The collision energy followed a step-wise increment profile according to ion mass and
24 charge, the collision energy increasing to 53 kV for 2+ ions and to 42 kV for 3+ ions.

25 Data were acquired sequentially in MS-mode, with scan range (m/z) 450-1100 Da.

1 MS/MS spectra of 3 ions from the MS-scan were recorded, within the mass range of
2 50-1500 Da. Only parent ions with two or three charges were chosen for MS/MS and
3 the selection width of precursor ion was 2 Da.

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

6

7 **Database searches**

8

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

21

22 The obtained de-novo sequence data from SPITC-MALDI-ToF-ToF were initially
23 used in BLAST-searches in the in-house developed cod EST-database, and the
24 sequence of the contig(s) and/or singlets which matched the query sequences
25 (minimum two fragments with score>identity threshold score and E-values>0.05)

1 were retrieved and used in a second blast-search in the NCBI database
2 (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) for protein identity (Altschul et al.,
3 1997).

4 The sequence data from the LC-MS-MS runs were used in MASCOT-searches in the
5 EST-database (Perkins et al., 1999, Steen & Mann, 2004) using the following
6 parameters: Missed cleavages: 1; Significance threshold $p < 0.05$; MS¹ mass accuracy:
7 0.5 Da, MS² mass accuracy: 0.3 Da; Partial modifications: Carbamidomethyl (C) and
8 Oxidation (M). The quality of the matches was manually inspected. The sequence of
9 the contig(s) and/or singlets which matched the query sequences (minimum two
10 fragments with score > identity threshold score and E-values > 0.05) were retrieved and
11 used in blast-search in the NCBI database for protein identity.

12

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).

1

2 **MS-MS**

3

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

6

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

10

11 After 24 days of exposure (Table 2), enolase alpha (#7011) was down-regulated by
12 crude oil and surrogate produced water exposure. Fibrinogen (#7032) was up-
13 regulated at higher levels of crude oil and surrogate produced water exposure. Alpha-
14 2-antiplasmins, A2A, (#7581, #7584, #7587, # 7597) appeared to be up-regulated by
15 24 days of 1.0 mgL⁻¹ crude oil and surrogate produced water exposure. Tropomyosin
16 (#7043), serotransferrin (#7192), hemopexin (#7409), prothrombin (#7285, #7329)
17 and fetuin B (#7098, #7285, #7345) was down-regulated by crude oil exposure and in
18 the cases of serotransferrin and pro-thrombin also by surrogate produced water
19 exposure. The latter exposure was also found to induce a down-regulation of
20 pentraxin (#7027). Alpha-1-antitrypsin, A1AT (#7811), was up-regulated by 0.25
21 mgL⁻¹ of crude oil. The prepro-apolipoprotein A (#8580) was shown to be up-
22 regulated at crude oil levels ≥ 25 mgL⁻¹ and by surrogate produced water exposure,
23 while NTPase (#7810) was found to be down-regulated by 1.0 mgL⁻¹ crude oil and oil
24 spiked with PAHs and alkyl phenols. A protein similar to pregnancy zone protein, Pzp
25 (#7826), was found to be down-regulated by crude oil exposures ≥ 1.0 mgL⁻¹.

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

3

4 ***Discussion***

5 This proteomics-based study of protein expression profiles after exposure of juvenile
6 Atlantic cod to oil and surrogate produced water indicate effects on fibrinolysis and
7 the complement cascade, the immune system, fertility-linked proteins, bone
8 resorption, fatty acid metabolism as well as increased oxidative stress, impaired cell
9 mobility and increased levels of proteins associated with apoptosis.

10

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

17

18 Although the number of individuals and samples in this study is limited within each
19 treatment group, all the ANOVA analyses included a minimum of ten gels, and
20 protein changes only apparent in groups of one individual were excluded from the
21 analysis. The protein changes observed in this study may therefore represent a first
22 screening for potential biomarker candidates in cod plasma that will be further
23 analysed and verified in future exposure studies based on a larger number and broader
24 range of samples.

25

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 ($\geq 0.25 \text{ mgL}^{-1}$) 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.

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

5
6 Serotransferrin and hemopexin are important constituents of the iron homeostasis
7 system, regulating cellular iron levels. Both serotransferrin and hemopexin appeared
8 in our study to be down-regulated by crude oil exposure, which might in turn lead to
9 an overload of iron (Bradbury, 1997, Anderson & Frazer, 2005). In cells, like
10 macrophages, only a slight increase in iron (2-3 fold) is found to affect cell signalling,
11 leading to NO production and activation of the nuclear transcription factor NF kappa
12 B, and cellular function, resulting in a stimulation of the production of reactive
13 oxygen species (ROS) and oxidative stress (Chrichton et al., 2002).

14
15 Our results may also indicate an effect of crude oil exposure on lipid metabolism.
16 Both prepro-apolipoprotein and apolipoproteins B (Apo B) appeared to be
17 differentially expressed after 24 days of exposure. Apolipoproteins are important
18 regulators of triglyceride and cholesterol metabolism. Previously, oestrogen has been
19 shown to affect the density distribution of apolipoproteins (Tam et al., 1985).

20
21 We also found a NTPase and fetuin B to be down-regulated in the groups of
22 individuals exposed to 1.0 mgL⁻¹ crude oil and surrogate produced water. This
23 NTPase is found in apoptosis as well as being involved in MHC transcription
24 activation. Fetuin B is a protease inhibitor of many functions, such as regulation of
25 insulin and hepatocyte growth factor receptors, in response to systemic inflammation

1 and in osteogenesis (Binkert et al.,1999). The apparent down-regulation of these
2 proteins may thus have several adverse effects in the exposed fish.

3

4 When evaluating these differentially expressed proteins as biomarker candidates, it is
5 important to be aware of the limited number of individuals within each group. We are
6 in the first phase of biomarker development with limited amount of samples available
7 for biomarker discovery. Hence, the results from this study may include a number of
8 false positives, although we chose to use a filter of >2 fold-change, in addition to p-
9 values <0.05 (ANOVA) for selecting candidates for further processing. Further
10 analysis, verification, and validation of these results will determine whether they are
11 suited for use as biomarkers in the future (Rifai et al., 2006).

12

13 As well as trying to identify more of the proteins observed to be differentially
14 expressed in this study, our strategy involves producing antibodies directed towards
15 these biomarker candidates. The candidates will be validated for use as biomarkers
16 using antibodies in ELISA assays. The antibodies may also be used in mechanistic
17 studies to gain more knowledge of the underlying mechanisms of action of oil
18 hydrocarbons and alkyl phenols in fish.

19

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.

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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

^aSummary 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).

^bSpot no. are shown with 2D localization in Fig. 1.

^csample of female individual

^dCO - crude oil treated juveniles.

^esPW - surrogate produced water (1.0 mgL⁻¹ crude oil plus alkylphenol and hydrocarbon spike)-treated juveniles

^fA2A - Alpha-2-antiplasmin

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

Spot # ^b	Relative protein expression levels					Protein identity
	Control	0.06 mgL ⁻¹ CO ^c	0.25 mgL ⁻¹ CO	1.0 mgL ⁻¹ CO	1.0 mgL ⁻¹ sPW ^d	
	(62,63)	(68)	(69,70)	(74,75,76)	(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	(Prepro)-APO A-1
8580	0	0	1	2.0	2.2	
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	

^aSummary 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).

^bSpot no. are shown with 2D localization in Fig. 1.

^cCO - crude oil treated juveniles.

^dsPW - surrogate produced water (1.0 mgL⁻¹ crude oil plus alkylphenol and hydrocarbon spike)-treated juveniles

^dProtein 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

¹MS method: SPITC-MALDI-ToF-ToF

²MS method: ESI-LC-QToF

^{ns}non-significant hit: Only one fragment hit (significant score, p<0.05)

^hThe protein was identified as an unnamed protein and was further characterised by homology in blast

^aThe score of query's match in the database/the threshold score of homology (identity)

^bThe 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.