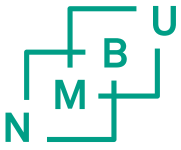
NMBU

Abstract

Expansion of petroleum-related activities in the Arctic has raised concerns about the environment and the adverse effects of potential oil spill on exposed Arctic marine organisms. Additionally, the Arctic is undergoing climatic changes that may compromise the ability of Arctic organisms to manage toxicants after a potential oil spill. Oil effects on aquatic organisms have been well study, but our knowledge about the combined effect of oil exposure and heat stress is limited. The present study examined the effects of oil exposure on 56 polar cod (*Boreogadus saida*) with weight of 28. 9 $\pm$ 18.9 g at elevated temperatures by exposing fish to mechanically dispersed oil at 4 °C (THC_{T0}: 13.5 mg L^{-1}) or 11 °C (THC_{T0}: 9.6 mg L^{-1}) for 2 days. Liver samples were collected after end exposure and after 11 days of recovery and transcriptiome changes were analyzed by Nofimas's genome-wide Atlantic cod oligonucleotide microarray. The rapid hepatic response to oil exposure comprised mainly genes involved in xenobiotic metabolisms and related biochemical processes, but also genes involved in stress responses, reparation, and immunity. The results were validated by measuring the gene expression for eleven of these genes by SYBR Green qPCR. The overall gene expression was slightly higher induced at the elevated temperature. Following 11 days of depuration most of the gene expressions changes had returned to basal level and suggests that polar cod is able to cope with relative brief periods of oil exposure. The study identified sensitive and reliable biomarkers including aryl-hydrocarbon receptor repressor (AHRR) and fibroblast growth factor 7 (FGF7) to assess the impact of oil pollution on polar organisms.

Sammendrag

Utvidelse av petroleumsrelatert virksomhet i Arktis har reist bekymringer om de negative effektene et potensielt oljeutslipp vil ha på miljøet og utsatte arktiske marine organismer. I tillegg til utvidelse av den petroleumsrelaterte virksomhet undergår Arktis klimaforandringer som kan komprimere Arktiske organismers egenskaper til å håndtere giftstoffer etter et eventuelt oljeutslipp. Flere studier har undersøkt oljens effekt på marine organismer, men det er lite kunnskap om den kombinert effekten av oljesøl og temperaturstress. Denne studien undersøkte polartorskens (*Boreogadus saida*) respons til oljesøl ved økt temperatur ved å eksponere 56 fisk med vekst av 28,9 ± 18,9 g til mekanisk dispergert olje ved 4 °C (THC_{T0}: 13.5 mg L^{-1}) eller 11 °C (THC_{T0}: 9.6 mg L^{-1}) i 2 dager. Leverprøver ble innsamlet etter endt oljebehandling og etter 11 dager restitusjon og transkripsjonsenderinger ble undersøkt ved bruk av Nofimas helgenom Atlantisk torsk oligonuklotide mikromatrise. Den raske lever responsen på oljeeksponering bestod hovedsakelig av gener involvert i den xenobiotiske metabolismen og andre beslektede prosesser, men også til en mindre grad gener involvert i stress respons, reparasjon og immunitet. Resultatene ble validert ved å måle genuttrykket til elleve av disse genene ved bruk av SYBR Green qPCR. Generelt var genene litt sterkere uttrykk ved høyest temperatur. Etter 11 dager restitusjon var flesteparten av de forandrete genekspresjonene returnert til normalt nivå som indikerer at polartorsken kan håndtere relative korte perioder av oljeeksponering. Studiet identifiserte sensitive og stabile biomarkører som inkluderer genene aryl-hydrokarbone reseptor repressor (AHRR) og fibroblast vekstfaktor 7 (FGF7) som kan bli brukt til å vurdere påvirkningen oljegiftstoffer har på polare organismer.

Acknowledgment

This study was conducted at Nofima Ås, Norway. I want to express my gratitude and appreciation to Nofima for having me as a master student, for providing me with materials, supports and with instruments.

I would like to express my special thanks to my supervisor Dr. Øivind Andersen for introducing me to the tropic as well as encouragement, useful comments, quick responses and support through the learning process of this master thesis. I would also thank my co-supervisor at Nofima Dr. Aleksei Krasnov for been very helpful and inspiring.

I would also express my deep gratitude to the helpful persons at Nofima's department marine biotechnology lab. Especially thanks to Dr. Gerrit Timmerhaus, Dr. Adrijana Skugor, Hege Munck and Katrine Hånes Kirste for supporting and helping me true my novice at the lab and for their great patience. You have all been to great help.

In addition, thanks to all the useful persons at Akvaplan-niva, Polar Environmental Centre, Tromsø, Norway who provided for the polar cod and let out the experiment. Especially to Dr. Marianne Frantzen and Dr. Lionel Camus. The study would not been possible without you. It would neither been possible without Framsenteret's Miljøgiftflaggskipet for financially support and the 56 polar cod that sacrificed their life for this experiment. Thank you all.

Last but not least a great thanks and appreciation to Katrine Stange Overå for always been there, for supporting all the way and for spending numerous hour reading through and encourage my writing. I couldn't have done it without you.

Department of Animal and Aquacultural Sciences, NMBU

Ås, May 2014

Marte Rosland

Abbreviations

B(a)P	Benzo(a)pyrene
cDNA	Complementary DNA
C_T	Cycle threshold
DNA	Deoxyribonucleic acid
MDO	Mechanically dispersed oil
mRNA	Messenger RNA
NOAA	Northwest Fisheries Science Center
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
qPCR	Quantitative PCR / real-time PCR
RNA	Ribonucleic acid
THC	Total hydrocarbon content

Enzymes

ABC	ATP-binding cassette
AHRR	Aryl-hydrocarbon receptor repressor
CYP	Cytochrome P450
FGF	Fibroblast growth factor
GST	Glutathione S-transferase
HSP	Heat shock protein
MID1IP	Mid1-interacting protein
MRP	Multidrug resistant protein
PAPSS	3-phosphoadenosine 5-phosphosulfate synthase
PSAT	Phosphoserine aminotransferase
PTS	6-pyruvoyl tetrahydrobiopterin synthase
SOD	Superoxide dismutase
Tiparp	TCDD-inducible poly [ADP-ribose] polymerase
UCP	Mitochondrial uncoupling protein
UGT	UDP glucuronosyltransferase
XYLT	Xylosyltransferase

Table of contents

Abstract i

Sammendrag ii

Acknowledgment iii

Abbreviations iv

1. Introduction 1

1.1 Petroleum activity in Arctic 1

1.2 Cruide oil 1

1.3 Heat stress 3

1.4 Biomarkers 4

1.5 Polar cod as model organism 4

1.6 Aim and hypothesis of the thesis 6

2. Materials and Methods 6

2.1 Fish sampling and experimental set-up 6

2.2 RNA extraction 7

2.3 Microarray analysis 8

2.4 qPCR 9

2.5 Data treatment 10

3. Results 11

3.1 Multiple differentially expressed genes 11

3.2 Cellular response; biochemical pathways and physiological processes 13

3.2.1 Xenobiotic metabolism 13

3.2.2 Metabolism of cofactors, aromatic and lipophylic compounds 14

3.2.3 Stress response and reparation 15

3.2.4 Immune genes 16

3.3 qPCR as microarray verification 17

4. Discussion 18

4.1 Combined effect of heat stress and dispersed oil exposure 18
4.2 Depuration 19
4.3 Biochemical pathways and physiological the processes affected 19
4.3.1. PAH metabolism 19
4.3.2. Stress and immune response 20
4.4 Methodological considerations 21
4.5 Conclusion and outlook 22
5. References 23
Supplementary tables 30
Supplementary table 1 30
Supplementary table 2 30
Supplementary table 3 31
Supplementary table 4 32
Supplementary table 5 33
Supplementary table 6 34

1. Introduction

1.1 Petroleum activity in Arctic

Human activity in the Arctic has been modest, but climatic changes and petroleum discovery have initiated an interest to expand this activity (Serreze et al. 2000; USGS 2000). Elevated temperatures have, among other things, created ice free areas for longer periods of times, thereby increasing the passability and decreasing the expenses of constructing an infrastructure (ACIA 2005; Smith & Stephenson 2013). Additionally, US Geological Survey estimated in 2008 that as much as 30 % of the world's undiscovered oil resources and 13 % of the undiscovered natural gas resources are supposed to be located in the Arctic.

Petroleum-related activities in the Arctic have raised environmental issues of concern. Arctic marine organisms can potentially be exposed to oil spills from operational or accidental discharges. The unique environment with subzero temperatures, extended darkness in winter, isolation and sea ice will increase the threat of oil spills. Furthermore, oil particles usually persist longer in colder than in warmer climate. This is due to the fact that oil particles can become trapped in ice pockets, it evaporates slower in cold climate and the harsh environment may delay the oil spill response. Additionally, effective technologies for oil spill cleanup in the Arctic are still under development (Jonsson et al. 2010; AMAP 2008). Hence, oil particles can persist for many years in aquatic habitats.

1.2 Cruide oil

One of the main group of compounds found in crude oil is the ubiquitous polycyclic aromatic hydrocarbons (PAHs) (Incardona et al. 2006). PAH compounds are fused aromatic rings, such as benzo(a)pyrene (B(a)P) (Fig. 1). PAHs effects on organisms have been well studied for more than 40 years by NOAAs Ecotoxicology Program amonge others. In aquatic organisms several PAHs have shown to be linked to cytotoxic, mutagenic, and immunotoxic effects (Sturve et al. 2006). Generally, PAHs is accepted to act through one of two pathways for aquatic organisms; either through *i)* "dioxin-like" toxicity mediated by activation of the aryl hydrocarbon receptor (AHR) or *ii)* "nonpolar narcosis" were toxicity is mediated through non-specific partitioning into lipid bilayers (Incardona et al. 2006).

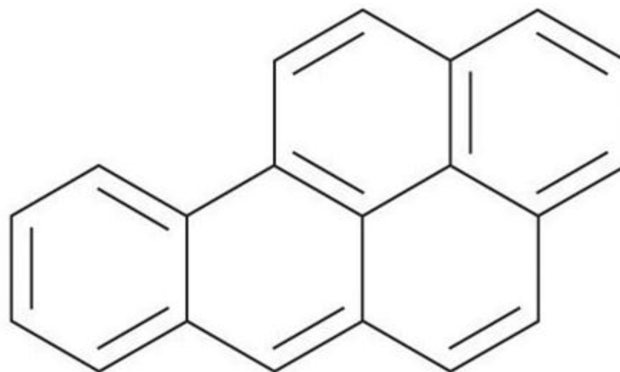

Figure 1: Benzo-a-pyrene ($C_{20}H_{12}$) chemical structure. Benzo-a-pyrene is a PAH compound consisting of a benzene ring fused to a pyrene. (The database and ontology of chemical Entities of Biological Interest (ChEBI) 2014)

Dissolved PAH exposure to aquatic organisms has been found to cause malformations of embryo and larvae. The most well-known responses are increased mortality and delayed development. Furthermore, features such as edema, hemorrhaging, and cardiac abnormalities are found in embryos and larvae exposed to oil (Carls et al. 2008; Hicken et al. 2011; Ingvarsdottir et al. 2012). Dysfunction in the heart is observed as defect in heart rate and rhythm, cardiac contractility, and failed cardiac looping soon after the heart becomes functional. PAHs disrupts the specialized ion channel pores which control the heart rate by allowing molecules to flow in and out of the heart (Brette et al. 2014). Disruption in the development process of the heart causes irreversible dysfunction in the heart, leading to reduced swimming performance and aerobic capacity in the adult fish (Hicken et al. 2011; Ingvarsdottir et al. 2012).

In adult aquatic organisms, PAHs are found to cause DNA damage, growth reduction and endocrine alteration (Caliani et al. 2009; Meador et al. 2006). Furthermore, studies have reported negative effects of PAHs on reproduction, respiration, and the nervous and immune system (Holth et al. 2008; Khan 2013). For instance, reproduction in Atlantic cod (*Gadus morhus*) was reported to be disrupted after exposure to a low concentrations of water-accommodated fractions of a crude oil for periods of 38-92 days as documented by delay in the spawning, spermination, and gonadal development (Khan 2013). Moreover, exposure of Atlantic cod to weathered crude oil and a mixture of persistent organic pollutants for 4 weeks induced CYP1A and a number of genes involved in xenobiotic metabolisms, phospholipid biosynthesis, and antioxidant response (Bratberg et al. 2013).

PAHs are xenobiotics compounds, foreign substrates for an organism, to all aquatic organisms. Detoxification of PAH and other xenobiotics occurs through xenobiotic metabolism. Xenobiotic metabolism can be divided in three phases driven by a specialized enzymatic system (Murphy 2001). Firstly, phase I modification, introduction of polar and reactive group into the xenobiotic substrate by for example oxidation, reduction, and hydrolysis. Secondly, phase II conjugation, addition of charged compound such as glutathione, sulfate, glycine, or glucuronic acid. Thirdly, phase III further modification and excretion, substrates with an anionic group are target for membrane transport and extraction (Fig. 2). Examples of enzymes involved are cytochrome P450 1A (CYP1A) in phase I, glutathione S-transferase (GST) in phase II, and ATP-binding cassette transporters (ABC transporters) in phase III (Murphy 2001).

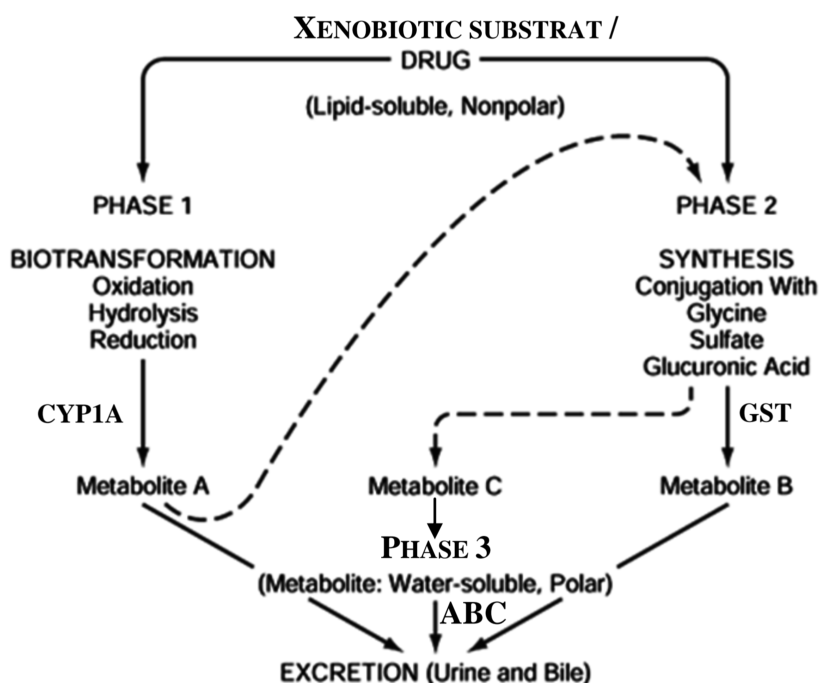

Figure 2: Xenobiotic metabolism pathways. Xenobiotic substrates are detoxified by the three phases of xenobiotic metabolism phase I modification, phase II conjugation and phase III further modification and excretion, driven by a specialized enzymatic system. Edited version of Baden and Rice (1990)

1.3 Heat stress

The sensitivity of Arctic aquatic organisms to toxicants might increase due to climatic changes (Lapointe et al. 2011). Elevated temperature is known to create “biochemical restructuring” in the organisms, involving alteration of the quantity and presence of molecular compounds (Somero & Hochachk 1971). New vital molecules at elevated temperature may be synthesized on expense of adaptive responsive molecules resulting in reduced defense against

pollution. Heat stress has been shown to induces a suite of genes involved in regulation of transcription, nucleosome assembly, chromatin organization, and protein folding in larval zebrafish (*Danio rerio*) (Long et al. 2012). Furthermore, heat stress has been reported to increase mortality and induce phenotypic changes in Atlantic cod and polar cod embryos (Graham & Hop 1995; Skjaerven et al. 2013). At the present, few genome-wide studies on the sensitivity of aquatic organisms' to toxicants at elevated temperature have been conducted. In fathead minnow (*Pimephales promelas*) the combined effect of heat stress an copper exposure were shown to be synergistic with significant altered transcription level (Lapointe et al. 2011).

1.4 Biomarkers

With the expansion of petroleum-related activity in the Arctic and the threat of global warming, reliable biomarkers are needed to assess the impact of anthropogenic threats from pollution on polar organisms. Biomarkers are biological measures which links and evaluates environmental exposure to a health outcome, often identified as strongly up- and down-regulated genes and molecules. Several biomarkers for PAH have been identified in aquatic organisms from sub-tropical to boreal regions. The most well-known PAH biomarkers are involved in the xenobiotic metabolisms, including CYP1A and GST, and in the antioxidant defense, such as superoxide dismutase (SOD) (van der Oost et al. 2003). Heat shock protein (e.g. HSP70) is the most common biomarker for heat stress (Logan & Somero 2011).

Studies identifying PAH biomarkers in Arctic water organisms have not been performed until the last decades (Jonsson et al. 2010; Nahrgang et al. 2008; 2009a; 2009b; 2010a). Studies on polar cod (*Boregadus saida*) showd that the strongest link between PAH exposure and health outcome was found at mRNA level, whereas protein and enzymatic responses were less clear (Nahrgang et al. 2009a). At mRNA level, the strongest and earliest biomarker responses are shown to be hepatic CYP1A, catalase, GST, and glutathione peroxidase (Nahrgang et al. 2009a; Nahrgang et al. 2010b).

1.5 Polar cod as model organism

Polar cod plays a key role in the Arctic marine ecosystem as a crucial food web-anchoring species (Welch et al. 1992). The distribution is spanning the Arctic seas off northern Greenland, Alaska, Canada and Russia (Fig. 3) and is overlapping with the of petroleum-related activity in Arctic. Further, its location in the ice edge, a natural oil spill sink, makes

the polar cod a relevant model species for oil pollution in the Arctic (Jonsson et al. 2010; Stange & Klungsøyr 1997). As a model organism polar cod is a robust fish under laboratory condition (Jonsson et al. 2010) and its high similarity to the Atlantic cod has made it possible to use monitoring tools designed for Atlantic cod on polar cod (Bakke & Johansen 2005; Nahrgang et al. 2009a). Furthermore, polar cod has an optimal temperature between 0 – 4 °C, but it is a robust species well-adapted to seasonal fluctuations in the temperature.

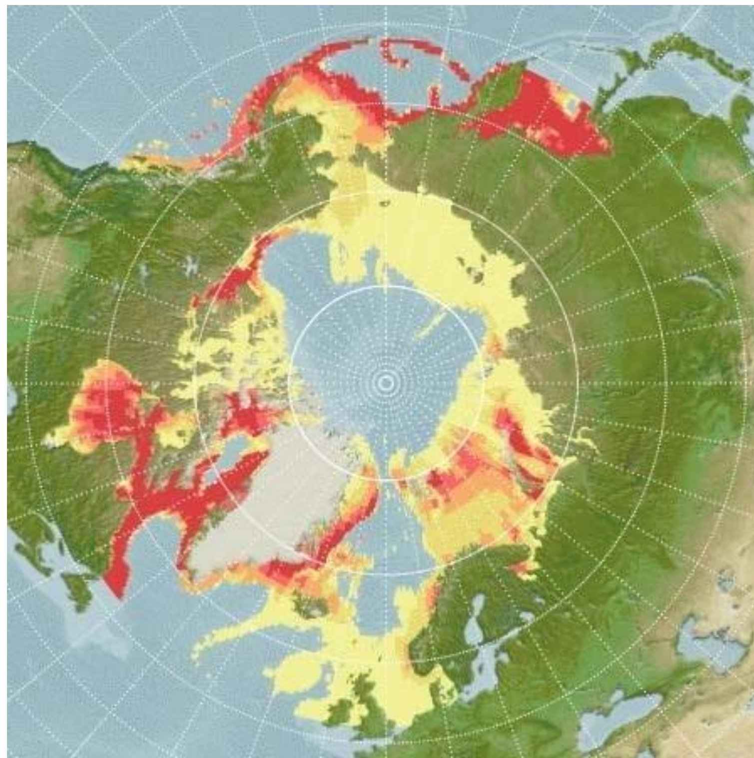

Figure 3: Polar cod's distribution spanning the Arctic seas off northern Greenland, Alaska, Canada and Russia. Red: high density of polar cod, yellow: lower density of polar cod, blue: ocean, and green: continent (Makivik Corporation).

In this study polar cod was used as a model organism to examine the individual and combined effects of oil exposure and thermal stress using hepatic transcriptional analyses. The liver was chosen as study organ due to its role as the main organ for metabolism of toxic oil components. Hepatic cDNA transcriptional expression was analyzed using microarray and the results were validated by quantify the mRNA expression of eleven biomarker candidates by real-time polymerase chain reaction (qPCR).

1.6 Aim and hypothesis of the thesis

The aim of the study was to examine the transcriptional response in polar cod exposed to mechanically dispersed oil and heat stress by using microarray to identify genes with altered expression. Differentially expressed genes would aid interpret biochemical pathways and physiological processes affected. Another objective of this study was to identify sensitive and reliable biomarkers by quantifying selected genes using qPCR that would also validate microarray results.

Based solely on the referred study on fathead minnow (Lapointe et al. 2011) we study we hypothesize that the combined treatment of oil exposure and elevated temperature would trigger the most significant changes in gene expression levels.

2. Materials and Methods

2.1 Fish sampling and experimental set-up

Adult polar cod were caught with a benthic trawl in the Barents Sea in January 2013. The fish were stored onboard in large tanks with running seawater during transportation to Tromsø. At the arrival at Barents Ecotox laboratory, Akvaplan-niva, they were tagged and acclimated to the laboratory conditions in 5,000 L holders with running seawater at 4 °C one month. The fish were treated against diseases caused by e.g. fin erosion and no mortality was observed. A total of 56 fish with weight of 28.9 $\pm$ 18.9 g and length of 16.4 $\pm$ 4.5 cm were randomly distributed to four 120 L experimental tanks (n = 14) prior to the start of the experiment.

The temperature in two tanks was then gradually increased to 11 °C during four days, while the temperature in the two other tanks was kept at 4 °C. After one week all four groups were exposed to either mechanically dispersed oil (MDO, Supplementary table 1,2) or clean seawater (control) for 48 hrs (table 1). The oil exposure treatment were generated according to the protocol developed by CEDRE, France for the DISCOBIOL project (e.g. .Milinkovitch et al. (2013)) and total hydrocarbon concentration (THC) was measured in the exposure tanks at time zero and after end exposure. Seven fish per treatment were sampled at the end of exposure and after 11 days of recovery by keeping the fish in new tanks with clean seawater at either 4 °C or 11 °C. The fish were anaesthetized in metacain and killed with a sharp blow to the head. The liver was dissected, snap frozen in liquid nitrogen and kept at -80 °C prior to analysis at NOFIMA autumn 2013.

Table 1: Experimental setup for the exposure tanks. Polar cod were exposed either seawater (control) or mechanically dispersed oil (MDO) for 2 days at either 4 °C or 11 °C, followed by 11 days of depuration. Samples were collected after end exposure and after recovery. Total hydrocarbon concentration (THC) was measured on time zero (T_0).

Group nr.	Tank	Temperature	THC_{T0} (mg L^{-1})	Treatment	Days of exposure	Days of recovery
1	1	4 °C	0.0	Control	2	-
2						11
3	2	4 °C	0.0	MDO	2	-
4						11
5	3	11 °C	9.6	Control	2	-
6						11
7	4	11 °C	13.5	MDO	2	-
8						11

2.2 RNA extraction

RNA was extracted from liver by resuspending chopped samples (~100 µg) in 1 mL Isol-RNA before homogenization with ceramic beads (1.4 mm zirconium oxide beads) and Precellys®24 (Bertin Technologies, France) for 2x 20 s at 5500 rpm. Ceramic beads function is to mechanically disrupt the cell where Isol-RNA is a lysis reagent designed to facilitate lysis of fatty tissues and inhibit RNases (5 Prime 2007) . To separate nucleic acid form proteins, chloroform was added to the homogenates, mixed and centrifuged (1,200 x g, 4 °C) for 15 min. The upper layer of the supernatant, containing nucleic acid, was transferred to new tubes and equal volume of lysis buffer and of 96 % ethanol was added, vortex and applied on a 96 Well Total RNA filter plate. The plate was centrifuged (1,200 x g) for 2 min to form pellets of aggregated DNA since DNA is insoluble in 96 % alcohols. Total RNA was purified by PureLink® Pro 96 RNA Purification Kit (Life techologies) according to manufacturer's instructions, including the optional one-column DNase digestion to remove genomic DNA.

Hepatic RNA quantity and purity were determined by electrophoresis (2100 Bioanalyzer, Agilent Technologies, CA, US) and spectrophotometry (NanoDrop 1000, Thermo Scientific, MA, US). Bioanalyzer were performed according to the Agilent High Sensitivity DNA kit

Guide on 1 µL RNA for each sample. Bioanalyzer creates an integrated electrical circuit to separating RNA strands by mass-to-charge ratio. RNA concentration and the ribosomal peaks are measured by comparing the migration time for the RNA samples to a ladder standard with known size and concentration in the electrical circuit. NanoDrop measured the intensity of light transmitted through 1 µL droplet samples. Quantity was measured at a wavelength of 260 nm, corresponding to the nucleic acid maximally absorption wavelength. Quality is determined by measuring the A260/A280 ratio where protein absorbs maximally at 280 nm, i.e. ration between absorption of nucleic acid and proteins. The isolated RNA was stored at -80 °C prior to further analysis.

2.3 Microarray analysis

To obtain gene expression profiles of polar cod exposed to mechanically dispersed oil and temperature stress, microarray analyses were performed. Microarray is a monitoring tool measuring the gene expression of a large number of genes simultaneously. Microarray is a DNA chip with tens of thousands of microscopic spots containing DNA probes attached to a solid surface. Probes are short specific DNA sequences. Gene expression rate, measures by fluorescent, depends on the number of target genes hybridized to each spot. Microarray is used in many research areas, such as in diagnostics, environmental toxicology, and ecotoxicology, and is often based on the comparison of healthy and affected samples. This study compared the hepatic gene expression of control groups to oil exposed groups at two different temperatures (4 °C and 11 °C) and time-points (day 2 after end exposure and day 13 after 11 days of recovery) by using Nofima's genome-wide Atlantic cod oligonucleotide microarray ACIQ2 (Agilent Technologies UK Ltd, described by Krasnov et al. (2013)). The 4x44 k format array includes probes to the genomic sequence of Atlantic cod retrieved from Ensembl and Unigene. To demonstrate the applicability of this microarray on polar cod, sequence comparison of relevant genes in Atlantic cod and polar cod were shown to shared 95-99 % identity. Additionally, primers and antibody designed for Atlantic cod have earlier been successfully applied on polar cod due to their similarity in their gene and protein structures (Bakke & Johansen 2005; Nahrgang et al. 2009a; Teletchea et al. 2006).

A total of 32 microarrays were used to analyze 5-6 livers from each experimental group sampled after 2 and 13 days, except for the controls sampled only after 2 days. Obtaning of cDNA and the microarray experiment were performed using Agilent Technologies's Low

Input Quick Amp Labeling Kit, two-color and Gene Expression Hybridization Kit and QUIAGEN's RNeasy Mini Kit, all according to Agilent`s "gene Expression oligo microarrays" –protocol (Agilent Technologies, CA, US). cDNA quantity and quality were determined for each sample by spectrophotometry of 1µL (NanoDrop 1000, Thermo Scientific, MA, US). The experimental groups were hybridized against a common reference RNA pool containing equal RNA concentration from all the 32 experimental groups. Common reference was used to compare expression level across different microarrays (Sterrenburg et al. 2002). For all arrays treated cDNA and common reference were labeled with Cy5 and Cy3, respectively.

2.4 qPCR

To validate the results from the microarray analysis, a SYBR Green qPCR was performed. SYBR Green qPCR measures the present of target genes at the end of each cycle by the non-specific fluorescent dye SYBR Green emitting only light when bound to any double stranded DNA present. qPCR are widely used in medicine and biology, for example in diagnostic uses.

A number of eleven genes were selected for qPCR. The genes were selected based on their transcriptional expression, functional role and novelty. Specific forwards and reverse primer were designed for each gene (table 2) by using the web-based Primer3 (v. 0.4.0) software, with the exception of CYP1A, which had already been successfully designed (Nahrgang et al. 2009a; 2009b). The designed primers were examined for sequence similarity with other sequences at http://ncbi.nig.gov by tBLASTx.

Transcriptional verification by qPCR was performed on four pools each of 5 livers sampled from the treatment groups at day 2. For each pool a total of 4.5 µg RNA was used to synthesis cDNA using Agilent Technologies "AffinityScript qPCR cDNA Synthesis Kit". cDNA synthesis was preformed according to manufacturer's instructions, including the proposed increase in incubation time to 45 min at 42 °C to increase the cDNA yield.

qPCR were performed in 12 µL reactions in the presence of 1µL forward primer, 1 µL reverse primer, 6 µL SYBR Green (Life Technologies Corporation), and 4 µL cDNA. Prior to the qPCR reaction, dried primers were moistened with water equal to 10 x nmoles primer. qPCR reactions were performed in duplicates and a negative control without cDNA were amplified

for each primer set. Ubiquitin was used as a reference primer to normalize the data. qPCR was performed by using LightCycler® 480 Instrument (Roche Applied Science, IN, US) with the following cycles: 95 °C 5 min, then 45 cycles of 95 °C 10 sek, 60 °C 20 sek and 72 °C 30 sek, before final extension of 40 °C 10 sek.

2.5 Data treatment

The microarray slides were scanned by Dr. Gerrit Timmerhaus (Nofima, Ås) using Axon GenePix 4200AL Scanner (MDS Analytical Technologies). Additionally, Gene Pix Pro was used for grid alignment, feature extraction and quantification. The results were further analyzed by Dr. Aleksei Krasnov (Nofima, Ås), using Nofimas's bioinformatic package STARS (Salmon and Trout Annotated Reference Sequences, Krasnov et al. (2011)) and FE (Feature Extraction Software, Agilent Technologies) for respectively data processing and mining and for filtration for low quality spots. Subsequent, the data were lowness normalized of log_2-expression reaction rations. The most significant differentially expressed genes were identified in a two-step selection process. First, the results were analyzed performing t-test ($p < 0.05$) with the parameter: log_2-expression reaction rations > 0.8 (1.75- fold). Secondly, the results were analyzed performing t-test ($p < 0.01$) with the parameter: log_2-expression reaction rations > 1 (2- fold). The first selected genes were used for Ward's method 1-Pearson r cluster analysis, while the second-step-result genes were used for closer inspection of candidate markers and functional interpretation. Numbers of differentially expressed genes were counted and the magnitude of expression changes calculated by $\Sigma(log_2ER)^2$. Based on the results, 11 differentially expressed genes were chosen for qPCR verification.

The mRNA expressions of the selected genes were determined according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) and compared to the microarray values. The method uses the C_T-value, number of PCR cycles at which the fluprescence generated crosses the threshold, to calculate the normalized difference between the oil exposed and control fish genome in a fold change expression. To further compare the correlation between the microarray and qPCR values a Spearmans's rank rho test were conducted by Tinn-R (v 2.4.1.5, GNU General Public License). Lastly, a t-test was performed on the qPCR values to identify significant ($p < 0.03$) altered gene expression.

Table 2: Selected genes and their corresponding PCR primers from *B. saida* for qPCR verification. All sequences are written in 5' → 3' direction. Ubiquition was used as common reference.

Abbreviation	Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
AHRR	Aryl-hydrocarbon receptor repressor b	CAAGCGAATCCAGAGAAACC	GCGTAGAACACCATCCCATC
Tiparp	TCDD-inducible poly [ADP-ribose] polymerase	TCAACATCAAGGAGGGCTTC	AAGAGGAGGGGTGAGGAGAA
ABCG2	ATP-binding cassette subfamily G (WHITE), member 2	TGGCGTACCAGGGAGTAGAT	TTTGGTGTAAGCGATGGACA
Ud2a2	UDP glucuronosyltransferase 2a2	AATGGTTGCCTCAGAACGAC	GCACTTCCAGCCTCAAGATG
CYP1B1	Cytochrome P450 family 1 subfamily B polypeptide 1	TGTCTGGAAGCCTGTCTGTG	CGTTGCCGTATTTCTTAGCC
CYP1A	Cytochrome P450 family 1 subfamily A	CCACCCCGAGATGCAGG	CGAAGGTGTCTTTGGGGA
PSAT1	Phosphoserine aminotransferase 1	TGAGTGTCCTGTGGTCTTCG	ACTTCTGCTTGTTGAGCGTCT
PAPSS2	3-phosphoadenosine 5-phosphosulfate synthase 2	GTGATGGAGGGAGGTGATTG	CAGTGGAGTGAGGCGGTATT
FGF7	Fibroblast growth factor 7	CGGCAAGGAGATGTTTATCG	GAAGTGGGACGCTATGTGCT
CRYGB	Gamma-crystallin B	CATGTCCAACTGCATGTCCT	CATCATCCTGCCCCTGTACT
UCP3	Mitochondrial uncoupling protein 3	GCCATCCTCAAACACAACCT	ATGTAGCGGGTCTTCACCAC
Ub	Ubiquitin	GGCCGCAAAGATGCAGAT	CTGGGCTCGACCTAAGAGT

3. Results

3.1 Multiple differentially expressed genes

The hepatic transcriptional response in polar cod to individual and combined treatment of mechanically dispersed oil and thermal stress was investigated by cDNA microarray. All treatments showed alteration in the genetic expression compared to the control samples (Fig. 4). A total number of 3448 genes with significant ($p < 0.05$) change in gene expression compared to the controls were identified and used for hierarchical clustering (Fig. 4a). The clustered samples almost coincided with the treatments and suggested treatment-related expressional response to oil exposure and temperature. Only one exception was observed; an oil exposed fish at 11 °C on day 13 was assigned to the cluster of control fish at 11 °C on day 2.

A total number of 1517 genes with highly significant ($p < 0.01$) change in gene expression were identified for all experiments and for each treatment the number of significantly expressed genes were counted. The number of differentially expressed genes were calculated by substract number of significant expressed genes of oil exposed to control groups at 4 °C and at 11 °C (Fig 4) and the number was found to be slightly greater at higher temperature (Fig. 4b). The hepatic response was substantially reduced after 11 days recovery, and a slightly greater reduction was observed at higher temperature (Fig. 4b,c). The magnitude of expression (Fig. 4c) showed the same pattern as number of differentially expressed genes (Fig. 4b).

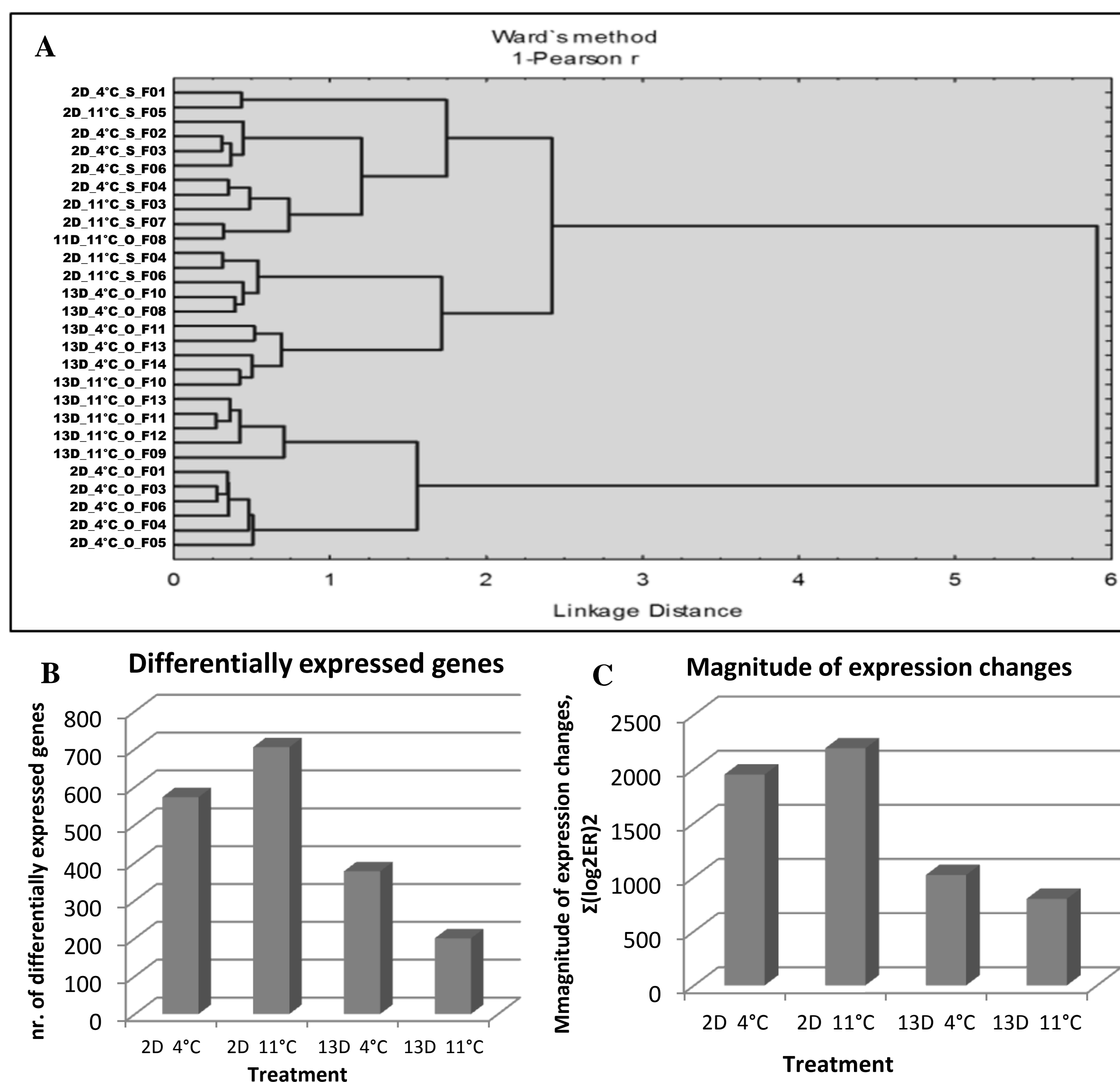

Figure 4: Alteration in the hepatic genetic expression in oil expressed polar cod at different temperatures. **A**: Hierarchical clustering of genes with more than 1.75-fold changes in their expression (3448 genes). **B**: Number of differentially expressed genes and **C:** Magnitude of expression changes by the 1517 genes displaying more than 2-fold change in expression. D – day, O – oil, S – Seawater, F – sample number.

3.2 Cellular response; biochemical pathways and physiological processes

3.2.1 Xenobiotic metabolism

The majority of the differentially expressed genes identified in the liver are involved in the xenobiotic metabolism, and almost all of them were up-regulated and had highest response on day 2 (Table 4, Supplementary table 3). One of the most responsive genes was the aryl-hydrocarbon receptor (AHR) repressor b (AHRR) with 14-fold induction on day 2 at 4 °C. Contradictory to most of the other genes that have a slightly higher induction at 11 °C, AHRR showed markedly lower response at 11 °C (5.7-fold). AHR is a ligand-activated transcription factor regulating a suite of genes involved in biotransformation such as the cytochrome P450 system. AHRR is involved in regulation of biotransformation by repressing the AHR signals (Evans et al. 2008). Another gene with markedly higher response at 4 °C than 11 °C was TCDD-inducible poly [ADP-ribose] polymerase (Tiparp), with respectively 10-fold and 6.4-fold induction on day 2 at 4 °C and 11 °C. In mouse (*Mus musculus*) Tiparp has been shown to also be a transcriptional repressor of AHR (MacPherson et al. 2013).

The identified differentially expressed genes are involved in a wide aspect of the xenobiotic metabolism. In phase I a number of CYPs involved in elimination of xenobiotics were induced, and CYP1A showed the highest induction with 7.6-fold up-regulation on day 2 at 11 °C. CYPs are known to be the major catalyst involved in the oxidation of xenobiotic chemicals (Guengerich 2001; 2008). Some of the major phase II enzymes were induced, such as UDP-glucuronosyltransferase and GST. They both detoxify reactive electrophiles so they cannot diffuse across membranes (Ritter 2000; Sherrat & Hayes 2002). Furthermore, one of the most responsive genes to oil and heat stress, the phase II gene 2 the 3-phosphoadenosine 5-phosphosulfate (PAPS) synthase (PAPSS) was respectively 20-fold and 8.2-fold up-regulated at 11°C and 4 °C on day 2. The synthesized PAPS was shown to be the universal sulfate donor in human sulfa conjugation (Venkatachalam 2003; Xu et al. 2000). Phase III genes involved in transporting detoxified xenobiotic out of the cell were induced, such as three members of the ATP-binding cassette family (ABC). ABCs catalyze the ATP-dependent transport of phase II modified xenobiotic products out of the cell (Jones & George 2004). The ABCs showed greatest induction at 11 °C.

Table 3: Xenobiotic genes displaying highly differentiated expression in polar cod liver after oil exposure at 4 °C or 11 °C. Polar cod were exposed to oil for 2 days followed by 11 days recovery. Numbers indicate fold-change to control expression

Gene	Day 2		Day 13	
	4 °C	**11 °C**	**4 °C**	**11 °C**
Aryl-hydrocarbon receptor repressor b	13.9	5.71	-1.08	-1.29
TCDD-inducible poly [ADP-ribose] polymerase	10.1	6.37	-1.37	-1.23
Cytochrome P450 1A1	1.80	7.56	-2.12	1.13
Cytochrome P450 1B1	4.30	5.22	-1.22	2.18
Cytochrome P450 2Y3	2.10	2.91	1.23	1.46
Cytochrome P450 11B2	2.15	2.78	1.50	1.26
3 -phosphoadenosine 5 -phosphosulfate synthase 2	8.23	20.6	1.18	1.13
Glutathione S-transferase M	2.65	1.31	1.56	-1.48
Glutathione S-transferase theta-4	2.44	2.58	-1.03	-1.16
ATP-binding cassette, sub-family G (WHITE), member 2	2.52	3.83	1.18	1.41
ATP-binding cassette transporter sub-family G member 2a	2.17	4.61	1.07	1.66
ATP-binding cassette sub-family C (CFTR/MRP) member 2	2.24	3.70	1.24	1.32

3.2.2 Metabolism of cofactors, aromatic and lipophylic compounds

Multiple genes involved in metabolisms of cofactors, aromatic and lipophylic compounds had significant up-regulated on day 2 (Table 5, Supplementary table 4). The most responsive genes were Mid1-interacting protein (MID1IP) and 6-pyruvoyl tetrahydrobiopterin synthase (PTS), which were up-regulated 13-fold at 4 °C and 17-fold at 11 °C on day 2. MID1IP plays a role in regulation of lipogenesis in the liver (Inoue et al. 2011; Tsatsos et al. 2008) while PTS is involved in the biosynthesis of tetrahydrobiopterin, which is a potential cofactor in a various processes e.g. natural cofactor of aromatic amino acid hydroxylases (Thony et al. 2000).

Table 4: Genes involved in metabolism of cofactors, aromatic and lipophilic compounds displaying highly differentiated expression in polar cod liver after oil exposure at 4 °C or 11 °C. Polar cod were exposed to oil for 2 days followed by 11 days recovery. Numbers indicate fold-change to control expression

Gene	Day 2		Day 13	
	4 °C	11 °C	4 °C	11 °C
Mid1-interacting protein 1-B	13.0	4.79	2.21	-1.19
6-pyruvoyl tetrahydrobiopterin synthase [PTP synthase]-1	2.48	16.8	1.26	2.36
6-pyruvoyl tetrahydrobiopterin synthase [PTP synthase]-2	2.44	13.26	1.22	2.41
Phosphoserine aminotransferase	4.63	8.05	-1.86	1.16
Aminolevulinate delta-synthetase 1	8.64	12.31	-1.98	-1.01
Enoyl Coenzyme A hydratase 1, peroxisomal	3.26	2.35	-1.13	1.75
Hydroxysteroid (17-beta) dehydrogenase 10	3.06	4.72	-1.09	-1.28
Tyrosine aminotransferase	5.48	3.07	1.13	-1.53
Oxysterol-binding protein -2	2.84	2.76	1.01	1.23
Tryptophan 2 3-dioxygenase A [TDO-A]	5.94	2.08	-1.18	-1.17
Progesterone receptor membrane component 1	1.38	2.88	1.47	1.66

3.2.3 Stress response and reparation

Most of the genes involved in stress and reparation were up-regulated (Fig. 6, Supplementary table 5). Several HSPs were induced on day 2 with up to 10-fold up-regulation at 4 °C and markedly lower expression at 11 °C. All HSP were back to normal on day 13. HSP functions as molecular chaperones with a diverse functions, including stabilizing stressed proteins (Becker & Craig 1994). Several crystalline gamma genes were induced at the combined treatment, but down- regulation at individual treatment. Crystalline gamma can interact with crystalline alpha to act as a chaperone to prevent heat-induced insolubilization (Wang & Spector 1994).

The genetic response to oxidative stress was relative small. One of the strongest responding genes to oxidative stress was mitochondrial uncoupling protein 3 (UCP3) with 7.9-fold down-regulation at day 2 at 4 °C. Down-regulating of UCP3 was shown to be involved in the protection from oxidative stress by reducing the number of free radicals (Brand et al. 2004; Saleh et al. 2002). A molecule with similar properties; solute carrier family 25 member 47-B, was also found to be down-regulated.

Reparation of the hepatic tissue could be seen in form of up-regulating of genes such as fibroblast growth factor 7 (FGF7), connective tissue growth factor, and xylosyltransferase 1 (XYLT1). FGF7 was the most responsive of these genes with an 18-fold up-regulation on day 2 at 11 °C. The gene cods for a mitogen involved in protection and reparation of many different types of epithelial cells, including reparation of toxically injured liver (Keller et al. 2004; Takase et al. 2013). XYLT1 was also strongly induced with a 10-fold up-regulated on day 2 at both temperatures, and the gene encodes an initiator of the biosynthesis of the glycosaminoglycan linkage in proteoglycans (Seo et al. 2005).

Table 5: Genes involved in stress response and reparation displaying highly differentiated expression after 2 days oil exposure at 4 °C or 11 °C followed by 11 days recovery. Numbers indicate fold-change to control expression

Gene	Day 2		Day 13	
	4 °C	11 °C	4 °C	11 °C
Heat shock cognate 70 kDa protein	10.1	2.91	-1.12	1.37
Heat shock protein 70-2	9.29	1.60	-1.22	-1.30
Heat shock protein 90-alpha 2	3.32	2.11	1.11	1.01
Crystallin gamma, gene 1	-3.27	10.4	-2.03	10.1
Crystallin gamma, gene 2	-2.16	15.7	-1.87	10.8
Crystallin gamma, gene 3	-1.85	9.01	-1.49	8.12
Mitochondrial uncoupling protein 3	-7.95	-2.68	-2.66	-1.24
Solute carrier family 25 member 47-B	-5.78	-3.62	1.71	-1.42
Fibroblast growth factor 7	6.45	17.9	1.18	0.810
Xylosyltransferase 1	10.4	9.65	1.09	1.20
Connective tissue growth factor	6.40	2.10	-1.24	-1.81

3.2.4 Immune genes

The majority of the differentially expressed genes associated with the immune system were down-regulated after 2 days of oil exposure (Table 7, Supplementary table 6). The majority of the differentially expressed genes showed lowest expression at 11 °C (day 2), but the most down regulated genes were found at 4 °C (day 2), such as cytokine receptor family member b17 (-14-fold and -3.3-fold at 4 °C and 11°C) and hepcidin precursor (-6.3-fold and -1.5-fold at 4 °C and 11°C).

Table 6: Immune genes displaying highly differentiated expression after oil exposure at 4 °C or 11 °C in polar cod sampled on day 2 and day 13. Numbers indicate fold-change to control expression.

Gene	Day 2		Day 13	
	4 °C	11 °C	4 °C	11 °C
Cytokine receptor family member b17	-13.7	-2.21	-2.04	-1.15
Hepcidin precursor	-6.28	-1.54	1.18	1.55
Major histocompatibility complex class I UDA	-2.96	-5.87	-2.93	-3.55
interferon regulatory factor 1	-2.61	-5.36	1.94	-1.31
C-type lectin	-1.44	-6.05	-4.10	-2.41
Immunoglobulin light chain	-2.15	-2.66	-2.07	-2.05
T-cell activation GTPase activating protein	-1.66	-2.08	1.21	-1.47
Toll-like receptor 21	-3.81	-2.46	-2.08	-2.51
Mannan-binding lectin	2.57	2.33	-1.05	-1.02
NF-kappa-B inhibitor alpha	1.79	2.31	-1.21	-1.02

3.3 qPCR as microarray verification

To validate the microarray results qPCR was performed on eleven genes selected based on their functional role and novelty (Fig. 5). Of the eleven genes were 7 of them found to have a significant altered expression by qPCR, these are marked with an asterisk in Figure 5. The results from qPCR and microarray showed the same trends, but qPCR is a more sensitive method than microarray and showed overall slightly higher values. However, the agreement between the methods was highly statistic significant [$\rho = 0.945$, $p > 2.2 \cdot 10^{-16}$] using the Spearmans's rho test.

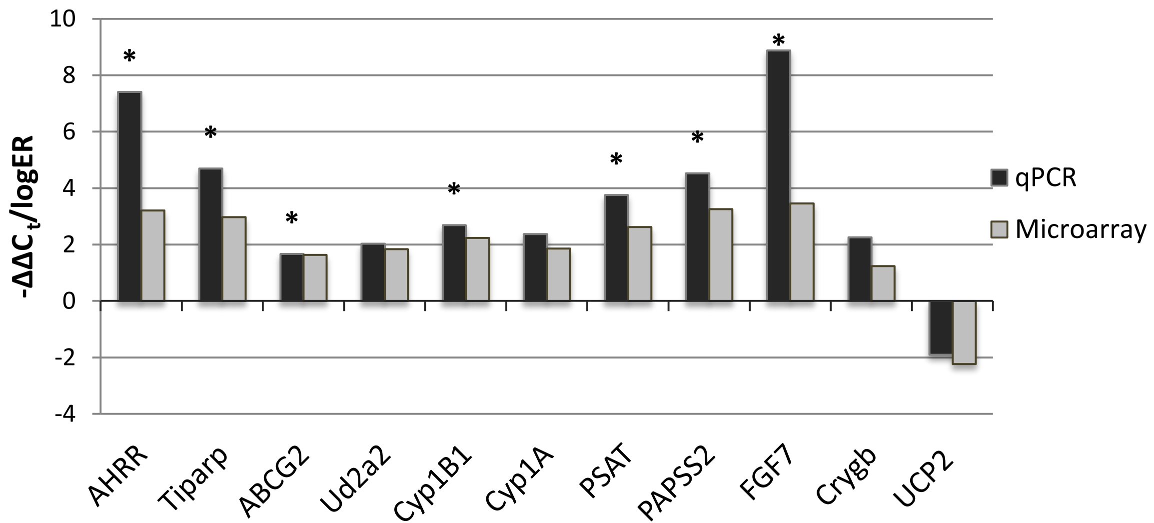

Figure 5: Validation of microarray data by qPCR. Pair wise comparison between microarray and qPCR on eleven genes of interest. Each bar represents the difference in gene expression between control and oil exposed individuals at day 2. For control and exposed, the mean value for all individuals (at both temperatures) were used. Genes showing highly significant ($p < 0.03$) difference gene expression between oil exposed and control groups measured by qPCR are marked with an asterisk.

4. Discussion

4.1 Combined effect of heat stress and dispersed oil exposure

It was hypothesized that the combined treatment of elevated temperature and oil exposure would trigger the most significant changes in gene expression levels. Indeed, the highest number of differentially expressed genes and the highest magnitude of expression were observed after 2 days oil exposure at 11 °C although the response was only slightly lower in the oil exposed fish at 4 °C. Whereas increased temperature has relative low effect on the oil exposed polar cod, synergistic effect of copper exposure and heat stress was found in fathead minnows with significant altered transcription levels (Lapointe et al. 2011). Such differences may be due to different stress response to heat in the two species, as polar cod has been observed to have some common features with the primary stress response in the cold water Antarctic fishes, such as the hormone response to heat stress (Whiteley et al. 2006). Antarctic fish appears to lack a common cellular defense mechanisms when subjected to elevated temperatures and polar cod exposed to acute heat stress are shown lack of effect on plasma cortisol level and increase in circulating catecholamines similar to Antarctic fish (Whiteley et al. 2006). Thus, the relative low response to elevated temperature might be due to restricted stress response to heat stress evolved in polar cod.

4.2 Depuration

To study the ability of polar cod to recovery after 48 hrs oil exposure, liver transcriptome changes were analyzed after 11 days in clean seawater. A markedly fall in numbers of differentially expressed genes and magnitude of expression was observed from day 2 to 13, suggesting depuration after the short time oil exposure. Indeed, no differences in expression of genes involved in toxicity and stress response was observed between oil exposed and control fish after 11 days of recovery. Furthermore, no observation of inducted apoptosis and inflammation was observed. Similar findings have been reported for antioxidant genes in polar cod exposed to a water soluble fraction of crude oil (40 μg L^{-1}) for 4 weeks followed by 2 weeks of recovery (Nahrgang et al. 2010a). Further, these polar cod studies are consistent with a study conducted on rainbow trout (*Oncorhynchus mykiss*) exposed to low dose of crude oil (0.4 – 2 mg L^{-1}) for 96 hrs followed by 96 hrs recovery (Hook et al. 2010) where recovery of genes in protein synthesis, xenobiotic metabolism, and oxidoreductase activity were observed. However, rainbow trout exposed to high dose of crude oil (10 mg L^{-1}), which was similar to the oil concentration in our polar cod study, showed a substantial number of genes with altered expression at 96 hrs exposure and after 96 hrs recovery (Hook et al. 2010).

Golden grey mullet (*Liza aurata*) exposed to dispersed oil (total petroleum hydrocarbons: 13.15 $\pm$ 2.6 mg L^{-1}) for 48 hrs followed by 14 days of depuration showed that enzymes induced by PAH were still present in the heart (Milinkovitch et al. 2013). This may indicate that the depuration in the heart is not as effective as in the liver after a short time exposure, and that also polar cod might be more affected than indicated in our study. The implications of even abrupt oil exposure is probably more serious at early developmental stages, since it has been showed that embryos exposed to oil have lifelong deformations in organs, such as the heart (Hicken et al. 2011).

4.3 Biochemical pathways and physiological the processes affected

4.3.1. PAH metabolism

The majority of the genes with altered expression in this study were found to be involved in a wide aspect of the xenobiotic metabolism or related biochemical processes indicating that the phase I – III was activated in response to oil exposure. For instance, several genes encoding cytochrome P450 enzymes involved in elimination of xenobiotics where induced in response to oil exposure in agreement with related studies (Levine & Oris 1999; Nahrgang et al. 2009a;

Rees et al. 2003). Notable, CYP1A had significant higher induction to oil exposure at 11 °C than 4 °C, contrary to the other cytochrome P450 enzymes. CYP1A has earlier been reported to have maximally inducted after 24 hrs exposure of B(a)P (1.23 $\pm$ 0.08 µg Ll^{-1}) and mRNA expression back to basal temperature after 72 hrs of exposure (Levine & Oris 1999). Our results may indicate a more rapid recovery of CYP1A at 4 °C than 11 °C.

Several other genes involved in xenobiotic metabolisms and related biochemical processes were also inducted in response to oil exposure, such as genes encoding regulators of the cytochrome P450 system (AHRR and Tiparp), cofactors (PSAT and PTS), and in regulation of lipogeneisis in the livere (MID1IP). Most of the genes showed the same expression pattern as the genes involved in the cytochrome P450 system and supporting the important role of the xenobiotic metabolisms to eliminate foreign substance in response to oil exposure. Some of the most induced genes are found in this group, making them sensitive biomarkers such as AHRR with almost 14-fold up-regulation.

4.3.2. Stress and immune response

In this study we found relatively low expression of stress- and immune genes as response to crude oil and heat stress. These results might also be explained by restricted primary stress response. For instance, heat stress is known to induce oxidative stress in fish (Heise et al. 2006), which had relatively low induction in this polar cod study. The strongest induced genes to oxidative stress, uncoupling protein 3 and solute carrier family 25 member 47, had strongest response at 11 °C. Polar cod and other polar species are unlikely to be subjected to high temperature in the Arctic and may therefore, as Antarctic notothenioids, not have evolved proper defense mechanisms to stress (Whiteley et al. 2006). However, decreased expression of genes involved in stress and immune response have been observed in rainbow trout exposed to high dose (0.5 mg L^{-1}) compared to lower doses (0.05 and 0.25 mg L^{-1}) of different contaminants (b-naphthoflavone, carbon tetrachloride, pyrene, and cobber) (Koskinen et al. 2004), However, decreased expression of genes involved in stress and immune response have been observed in rainbow trout exposed to high dose (0.5 mg L^{-1}) compared to lower doses (0.05 and 0.25 mg L^{-1}) of different contaminants (b-naphthoflavone, carbon tetrachloride, pyrene, and cobber) (Koskinen et al. 2004). Regardless of the cause for polar cods relative low transcrptomic stress response to heat stress has an earlier study reported severe physiological impairment in response to acute heat stress at 10 °C (Koskinen et al. 2004), suggesting that the polar cod is not adapting to elevated temperature.

Additionally, the most responsive gene to reparation of hepatic tissue, FGF7, was strongest induced at 11 °C and acts by preventing hepatocytes from accumulating toxic bile acid during liver injury (Sun et al. 2012). FGF7 is also a good biomarker candidate.

Protein stress showed greater response to the combined treatment than oxidative stress. Our finding on protein stress was in accordance with Williams et al. (2008), who showed early induction of chaperones and heat shock proteins in response to model environmental pollutants, such as cadmium chloride (50 µg kg^{-1}). Noteworthy is the low gene expression for heat shock protein in the oil exposed fish at 11 °C compared 4 °C. Anatarctic notothenioids lack heat shock response to heat stress (Koskinen et al. 2004) and it might it be that something similar is is impairing heat shock response at elevated temperature in polar cod. Concomitantly, crystallin gamma which functions as chaperone to prevent heat-induced insolubilization (Wang & Spector 1994) showds only significant induction at elevated treatment, indication some response to heat stress. Further studies are required to examine the heat shock response in polar cod to elevated temperature.

The down regulation of immune genes may indicate immunological disturbance in the polar cod. The immune system is a vulnerable system closely integrated with other organ system and which has shown to be depressed in both fish and mammals in response to pollutants such as PAHs and subsequently leading to increased susceptibility to pathogens infection (van der Oost et al. 2003). Noteworthy, an increase in protection of the fish against pathogens have been observed after pollution exposure (van der Oost et al. 2003; Williams et al. 2008). The depression of the immune system can be caused by the high dose of oil were the adaptive response is masked with general unspecific response to toxicity (Koskinen et al. 2004). However, this explanation will not explain the down-regulation of gene in the immune genes.

4.4 Methodological considerations

An Atlantic cod microarray chips were used successfully in this study to examine the hepatic transcriptional response in polar cod to dispersed oil and heat stress. The results were confirmed by qPCR, using primers designed for polar cod. The validity of the using microarray designed for Atlantic cod on polar cod is supported by Nahrgang et al. (2009a) study on polar cod where antibodies and primers designed to Atlantic cod were successfully used. It should however be noted that genes such as novel antifreeze protein genes in polar cod might not be detected by using the Atlantic cod microarray chip

The overall greater expression by qPCR than microarray can be explained by qPCR higher sensitivity. Microarray is less sensitive partly due to it finite amount of cDNA on the microarray chip and steric inhibition of binding of cDNA to the glass array (Hook et al. 2010). An achievement of finite amount of cDNA on the array may explain the higher difference in FGF7 and AHHR than the other genes showed by these two methods.

4.5 Conclusion and outlook

Polar cod exposed to mechanically dispersed oil shows rapidly response at transcriptional level by inducing genes involved in xenobiotic metabolisms to eliminate foreign compounds and this response was slightly enhanced by elevated temperature. The response was markedly decreased after 11 days of recovery indicating that polar cod is able to cope short oil exposure in relatively short time if the oil pollution is removed rapidly from the environment. Furthermore, the study identified sensitive and reliable biomarkers, such as AHRR and FGF7, to assess the impact of oil pollution on polar organisms and the study showed that this could be achieved by using microarray technology to analyze transcriptional responses to temperature and toxical stressor.

At the moment effective technologies for oil spill cleanup in the Arctic is still under development. During the Deepwater Horizon accident in the Gulf of Mexico in 2010 dispersants was extensively used to increase the amount of oil droplets in the seawater column. The theory was that the oil droplets will be more rapidly diluted and biodegraded in the water column than left on the surface (Olsvik et al. 2012). In the future polar cod might be exposed to oil droplets in addition to crude oil if this method is used in the Arctic. Therefore it would be interesting to investigate if oil droplets are more harmful to the polar species than crude oil only. A study comparing the effect of chemically dispersed oil to mechanically dispersed oil exposure is needed.

It should be kept in mind that our experiment was conducted under controlled laboratory conditions. In the Arctic polar cod is located in the ice edge and with climatic changes the ice will melt and polar cod will additionally need to adapt to a new habitat as well as cope with other compound present in the environment (Hop & Gjosaeter 2013).

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Supplementary tables

Supplementary table 1: Concentration of hydrocarbons used in the different treatment. Nominal oil concentration is the oil concentration added to the exposure tanks 2 days prior to time zero, THC is the total concentration of hydrocarbone in the exposure tanks measured, and the sum 26 PAH is the total concentration of 26 PAH compounds in the oil added to the exposure tanks. Both THC and PAH was measured on time zero (T_{0h}) and after 2 days (T_2).

Temperature	Treatment	Nominal oil conc. (mg L^{-1})	THC (mg L^{-1})		SUM 26 PAHs (µg L^{-1})	
			T_0	T_2	T_0	T_2
4˚C	Seawater	0	0.0	0.0	3.26	1.91
4˚C	MDO	66.7	9.6	6.8	323	134
11˚C	seawater	0	0.0	0.0	2.81	1.46
11˚C	MDO	66.7	13.5	0.7	284	15.6

Supplementary table 2: List over 26 PAH compounds in the dispersed oil used in this study. Concentration for each compound is measured in each of the exposure tanks on time zero (T_{0h}) and after 48 hrs exposure (T_{48h}).

	4 °C - control		4 °C - MDO		11 °C - control		11 °C - MDO	
PAH compound	T_0	T_{48}	T_0	T_{48}	T_0	T_{48}	T_0	T_{48}
Naftalen	0.496	0.178	8.92	1.99	0.306	0.063	2,49	0,313
Acenaftylen	< 0.002	< 0.002	0.027	0.012	< 0.002	< 0.002	0,016	< 0.002
Acenaften	< 0.015	< 0.015	0.813	0.223	< 0.015	< 0.015	0,670	0,067
Fluoren	< 0.069	< 0.069	2.19	0.629	< 0.069	< 0.069	2,14	0,197
Fenantren	< 0.049	< 0.049	6.61	2.49	< 0.049	< 0.049	7,29	0,426
Antracen	< 0.001	< 0.001	0.041	0.033	< 0.001	< 0.001	0,017	0,010
Fluoranten	< 0.03	< 0.03	0.512	0.296	< 0.03	< 0.03	0,555	0,030
Pyren	< 0.31	< 0.31	0.503	< 0.31	< 0.31	< 0.31	0,519	< 0.31
Benzo(a)antracen	< 0.007	< 0.007	0.091	0.060	< 0.007	< 0.007	0,102	< 0.007
Krysen	< 0.014	< 0.014	0.604	0.441	< 0.014	< 0.014	0,723	0,050
Benzo(b)fluoranten	< 0.031	< 0.031	0.209	0.153	< 0.031	< 0.031	0,220	< 0.031
Benzo(k)fluoranten	< 0.017	< 0.017	0.027	0.020	< 0.017	< 0.017	0,031	< 0.017
Benzo(a)pyren	< 0.01	< 0.01	0.104	0.079	< 0.01	< 0.01	0,110	< 0.01
Indeno(1.2.3-cd)pyren	< 0.023	< 0.023	0.042	0.037	< 0.023	< 0.023	0,053	< 0.023
Benzo(ghi)perylen	< 0.013	< 0.013	0.070	0.055	< 0.013	< 0.013	0,087	< 0.013
Dibenzo(a.h)antracen	< 0.009	< 0.009	0.018	0.015	< 0.009	< 0.009	0,019	< 0.009
Dibenzotiofen	0.016	< 0.006	0.928	0.352	0.009	< 0.006	1,05	0,068
C1-Naftalen	0.906	0.299	26.3	5.28	0.680	< 0.146	11,6	1,13
C2-Naftalen	0.879	< 0.731	69.7	17.0	0.853	< 0.731	49,2	2,65
C3-Naftalen	0.270	0.368	114	45.0	0.266	0.263	108	4,24
C1-Antr/Fenantren	< 0.182	< 0.182	15.6	8.77	< 0.182	< 0.182	17,7	0,930
C2-Antr/Fenantren	< 0.296	< 0.296	30.3	19.9	< 0.296	< 0.296	32,4	2,02
C3-Antr/Fenantren	< 0.165	< 0.165	23.0	17.0	< 0.165	< 0.165	25,7	1,76
C1-Dibenzotiofen	< 0.031	< 0.031	2.69	1.48	< 0.031	< 0.031	3,13	0,156
C2-dibenzotiofen	< 0.061	< 0.061	9.60	6.20	< 0.061	< 0.061	10,1	0,591
C3-dibenzotiofen	< 0.053	< 0.053	9.70	6.71	< 0.053	< 0.053	10,5	0,699

Supplementary table 3: Differentially expressed genes involved in xenobiotic metabolism in polar cod exposed to oil for 48 hrs at 4 or 11 °C. Samples were collected after end treatment and after 11 days of recovery Data are fold-changes to control.

Name	2D 4 °C	2D 11 °C	11D 4 °C	11D 11 °C
Xenobiotic metabolism: regulators, transporters and enzymes				
Aryl-hydrocarbon receptor repressor b[1]	13.97	5.71	-1.08	-1.29
Nuclear receptor coactivator 7	4.85	7.66	1.46	1.93
ATP-binding cassette, sub-family G (WHITE), member 2	2.52	3.83	1.18	1.41
ATP-binding cassette transporter sub-family G member 2a[1]	2.17	4.61	1.07	1.66
ATP-binding cassette sub-family C (CFTR/MRP) member 2	2.24	3.70	1.24	1.32
Cytochrome P450 2C33-like	2.02	2.50	1.25	1.24
Cytochrome P450 2Y3	2.10	2.91	1.23	1.46
Cytochrome P450 1A1[1]	1.80	7.56	-2.12	1.13
Cytochrome P450 2C33-1	2.11	3.21	1.39	1.51
Cytochrome P450 2C33-2	2.44	3.37	1.35	1.44
Cytochrome P450 1B1	4.30	5.22	-1.22	2.18
Cytochrome P450 2J2	1.72	2.89	2.18	-1.06
Cytochrome P450 27C1[1]	-5.05	1.58	-3.15	9.04
Cytochrome P450 11B2	2.15	2.78	1.50	1.26
Cytochrome P450 2J24-1[1]	1.13	2.23	1.41	1.28
Cytochrome P450 2J24-2[1]	-2.12	-1.31	-1.36	3.93
Cytochrome P450 7A1	-2.46	-1.65	1.54	-1.38
Cytochrome P450 19A1B, aromatase	1.38	-1.14	-1.64	-3.61
Alcohol dehydrogenase [NADP(+)] A	2.05	2.61	1.40	1.43
TCDD-inducible poly [ADP-ribose] polymerase	10.10	6.37	-1.37	-1.23
Uridine-cytidine kinase 2-A [UCK 2-A] [1]	1.84	6.68	-1.26	-1.08
Organic solute transporter subunit alpha [OST-alpha]	1.80	3.09	1.25	1.42
UDP glucuronosyltransferase 5 family polypeptide d1-1	1.96	2.71	1.50	1.83
Glutathione S-transferase M[1]	2.65	1.31	1.56	-1.48
Glutathione S-transferase theta-4	2.44	2.58	-1.03	-1.16
3 -phosphoadenosine 5 -phosphosulfate synthase 2[1]	8.23	20.60	1.18	1.13
Gamma-glutamyltransferase family	3.57	2.89	-1.02	-1.26
UDP glucuronosyltransferase 5 family polypeptide d1-2	2.32	5.39	1.79	1.67

[1]Significant difference between 4ºC and 11ºC at day 2 by t-test ($p < 0.05$)).

Supplementary table 4: Differentially expressed genes involved in xenobiotic metabolism related biochemical processes in polar cod exposed to oil for 48 hrs at 4 or 11 °C. Samples were collected after end treatment and after 11 days of recovery Data are fold-changes to control

Metabolism of cofactors, aromatic and lipophylic compounds				
5-methyltetrahydrofolate-homocysteine methyltransferase	2.03	2.20	-1.10	-1.22
6-pyruvoyl tetrahydrobiopterin synthase [PTP synthase]-2[1]	2.44	13.26	1.22	2.41
Phosphoserine aminotransferase	4.63	8.05	-1.86	1.16
Aminolevulinate delta-synthetase 1	8.64	12.31	-1.98	-1.01
Uroporphyrinogen decarboxylase [UPD] [1]	-1.04	2.45	1.71	2.44
6-pyruvoyl tetrahydrobiopterin synthase [PTP synthase]-1[1]	2.48	16.75	1.26	2.36
Tyrosine aminotransferase	5.48	3.07	1.13	-1.53
Tryptophan 2 3-dioxygenase A [TDO-A] [1]	5.94	2.08	-1.18	-1.17
3-hydroxyanthranilate 3 4-dioxygenase[1]	-1.22	2.14	-1.04	2.05
Enoyl Coenzyme A hydratase 1, peroxisomal	3.26	2.35	-1.13	1.75
Delta(3 5)-Delta(2 4)-dienoyl-CoA isomerase mitochondrial	2.24	2.68	1.04	1.40
Mid1-interacting protein 1-B[1]	13.00	4.79	2.21	-1.19
Oxysterol-binding protein-1[1]	-1.24	4.29	3.04	1.73
Hydroxysteroid dehydrogenase-like protein 2	1.68	2.68	1.57	1.26
Sterol-C5-desaturase	1.10	2.20	-1.15	1.42
Hydroxysteroid (17-beta) dehydrogenase 10[1]	3.06	4.72	-1.09	-1.28
Hydroxysteroid dehydrogenase-like protein 2	1.56	2.76	1.48	1.39
Progesterone receptor membrane component 1[1]	1.38	2.88	1.47	1.66
24-dehydrocholesterol reductase -	1.54	2.28	1.56	1.94
Oxysterol-binding protein-2	2.84	2.76	1.01	1.23

[1]Significant difference between 4ºC and 11ºC at day 2 by t-test ($p < 0.05$).

Supplementary table 5: Differentially expressed genes involved in stress responses and reparation in polar cod exposed to oil for 48hrs at 4 or 11 °C. Samples were collected after end treatment and after 11 days of recovery Data are fold-changes to control**.** Data are fold-changes to control.

Gene	2D 4 °C	2D 11 °C	11D 4 °C	11D 11 °C
Jun B	2.75	2.56	-2.74	-3.28
Jun C-1	4.30	2.10	-1.28	-2.42
Jun C-2	4.05	1.69	-1.01	-2.47
15 kDa selenoprotein	1.18	2.04	1.23	1.78
Crystallin gamma	-3.27	10.37	-2.03	10.08
Crystallin gamma	-1.62	5.76	-1.44	4.40
Crystallin gamma	-2.16	15.67	-1.87	10.84
Crystallin gamma	-2.20	3.72	-2.57	2.34
Crystallin gamma	-1.85	9.01	-1.49	8.12
DnaJ (Hsp40) homolog subfamily A member 3B	2.92	3.03	1.29	1.22
DnaJ (Hsp40) homolog subfamily B member 12	3.26	2.72	1.07	1.32
DnaJ (Hsp40) homolog subfamily B member 6	1.75	2.89	1.57	1.30
FK506 binding protein 5	3.49	5.43	-1.07	-1.08
Heat shock cognate 70 kDa protein	4.65	1.56	-1.05	1.06
Heat shock cognate 70 kDa protein	10.08	2.91	-1.12	1.37
Heat shock protein 70-1	8.57	2.57	-1.09	1.20
Heat shock protein 70-2	9.29	1.60	-1.22	-1.30
Heat shock protein 9	1.89	3.27	1.41	1.48
Heat shock protein 90-alpha 2	3.32	2.11	1.11	1.01
Transglutaminase 2 C polypeptide	6.12	15.04	1.17	2.19
Mitochondrial uncoupling protein 3	-7.95	-2.68	-2.66	-1.24
Solute carrier family 25 member 47-B	-5.78	-3.62	1.71	-1.42
Selenoprotein W 2a	1.42	2.39	1.17	1.14
Thioredoxin-like 1	2.23	2.10	1.29	1.17
Thioredoxin-like protein	1.46	2.45	1.47	1.74
Selenoprotein T1a	1.24	2.17	1.37	1.47
Glutaredoxin 3	3.55	2.13	1.69	-1.60
Connective tissue growth factor	6.40	2.10	-1.24	-1.81
Kruppel-like transcription factor 4a	2.74	4.27	-1.44	1.43
Krueppel-like factor 15	5.90	11.62	-2.40	1.02
Cysteine-rich angiogenic inducer 61 protein-like protein 1	7.43	1.95	-1.18	-1.16
Filamin A-interacting protein 1-like	6.43	17.90	1.18	-1.23
Iodothyronine deiodinase-1	5.32	10.67	-1.66	-1.18
Iodothyronine deiodinase-2	5.37	10.65	-1.76	-1.37
Fibroblast growth factor 7	6.45	17.88	1.18	0.81
Xylosyltransferase 1	10.42	9.65	1.09	1.20

Supplementary table 6: Differentially expressed immune genes in polar cod exposed to oil for 48hrs at 4 or 11 °C. Samples were collected after end treatment and after 11 days of recovery Data are fold-changes to control**.**Data are fold-changes to control.

Gene	2D 4 °C	2D 11 °C	11D 4 °C	11D 11 °C
Mannan-binding lectin	2.57	2.33	-1.05	-1.02
NF-kappa-B inhibitor alpha	1.79	2.31	-1.21	-1.02
MHC class I antigen, partial	1.43	3.35	1.19	1.77
Major histocompatibility complex class I UDA	-2.36	-2.57	-1.49	-1.36
Major histocompatibility complex class I UDA	-2.96	-5.87	-2.93	-3.55
Eosinophil chemotactic cytokine	-1.62	-1.88	-2.38	-3.28
Hepcidin precursor	-6.28	-1.54	1.18	1.55
Immunoglobulin light chain	-1.55	-2.62	-2.00	-2.13
Immunoglobulin light chain	-2.15	-2.66	-2.07	-2.05
Ig light chain - Atlantic cod (fragment)	-3.26	-3.06	-1.93	-1.69
C-type lectin	-1.44	-6.05	-4.10	-2.41
Toll-like receptor 21	-3.81	-2.46	-2.08	-2.51
Cytokine receptor family member b17	-13.74	-2.21	-2.04	-1.15
Suppressor of cytokine signaling 1b	1.14	-2.04	2.81	1.25
T-cell receptor beta chain	-1.68	-3.03	-1.54	-2.47
IL2-inducible T-cell kinase	1.11	-2.12	-1.24	-1.85
T-cell activation GTPase activating protein	-1.66	-2.08	1.21	-1.47
TNF receptor superfamily member 14	-2.24	-3.13	1.14	1.52
interferon regulatory factor 1	-2.61	-5.36	1.94	-1.31