Title:

Mortality and transcriptional effects of inorganic mercury in the marine copepod *Calanus finmarchicus*

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WORD COUNT:

Abstract: 236, Text: 5812, References: 1987, Figure legends: 179.

Running head:

Toxicity of inorganic Hg to *C. finmarchicus*

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Abstract

Inorganic mercury is highly toxic to organisms including crustaceans and displays multiple toxic modes of action (MoA). The main aim of this work was to assess the acute and sublethal toxicity of mercury chloride ($HgCl_2$) in the marine copepod *Calanus finmarchicus*. A combination of short-term static studies to determine the acute toxicity and a transcriptional study to characterise the sublethal MoA of $HgCl_2$ were conducted with an in-house continuous culture of *C. finmarchicus*. Transcriptional changes were determined by a custom 6.6k *C. finmarchicus* Agilent oligonucleotide microarray and quantitative RTPCR analysis. The results demonstrate that $HgCl_2$ caused a concentration- and time-dependent reduction in survival ($NOEC_{48\ hrs}$= 6.9 μg/L[Hg^{2+}] and LC_{50} of 279, 73, 48 and 34 μg/L[Hg^{2+}] after 24, 48, 72 and 96 h, respectively) and that exposure to sublethal concentrations of $HgCl_2$ (5.0 μg/L [$Hg2^{+}$]) caused differential expression of 98 features (probes) on the microarray. Gene ontology (GO) and toxicological pathway analyses suggested that the main MOAs were 1) uncoupling of mitochondrial oxidative phosphorylation (OXPHOS) and ATP production, 2) oxidative stress and macromolecular damage, 3) inactivation of cellular enzymes, 4) induction of cellular apoptosis and autophagocytosis, 5) over-excitation of glutamate receptors (neurotoxicity), 6) disruption of calcium homeostasis and signalling, and 7) modulation of nuclear receptor activity involved in Vitamin D receptor signalling. Quantitative RTPCR analysis verified that the oligoarray performed well in terms of specificity and response, thus demonstrating that Hg^{2+} has multiple potential MoAs in *C. finmarchicus*.

Keywords: zooplankton, copepod, North Sea, Barents Sea, oil, mercury, climate change, pollution.

Introduction

Calanus finmarchicus (Crustacea: Copepoda) is among the most abundant marine zooplankton species in the North Atlantic Ocean and Barents Seas. Its high lipid content (up to 50%) makes the species a key element in the marine pelagic food web as it provides energy flow between primary producers and fish (Jaschnov 1970; Sakshaug et al. 1992). Its pelagic life style and impressive filtration rates facilitate exposure to and uptake of environmental contaminants that is increasingly being released into the marine environment by anthropogenic activities such as petroleum-related activities (Hansen et al. 2015) and mining-related activities (Farkas et al. 2017). Increasing water temperature and ocean acidification have also raised concern about the health and stability of *C. finmarchicus* populations due to natural and man-made perturbations of key ecosystem processes (Mayor et al. 2007; Pedersen et al. 2013). The high ecological relevance of *C. finmarchicus* for maintaining the integrity of the marine pelagic food web, and its potential sensitivity to pollutants and climate change, suggest that this copepod is a good biomonitoring species for the ecosystem health of the North Atlantic Ocean and the Barents Sea, and warrants development of targeted and robust methodologies for effect assessment and environmental monitoring.

Biological effects of natural and anthropogenic stressors may manifest itself at various levels of biological organization ranging from molecular responses (e.g. transcription of genes) to fitness-related effects disturbing survival, growth, development and reproduction. Most fitness-related adverse impacts are progressed over time through pollutant- or stressor-facilitated changes at the molecular (transcriptome) level, and such molecular changes can serve as early-warning indicators and biological markers (biomarkers) of an adverse effect. Whereas adverse (apical) effects are often tested in invertebrates using standardized acute and chronic ecotoxicological tests for risk regulatory purposes, tools for assessing sublethal molecular responses are less developed for invertebrate species such as *C. finmarchicus*. In recent years, successful extraction of high

quality RNA from copepods in combination with sequencing and *de novo* assembly of Calanoid transcriptomes (Lenz et al. 2014; Lenz et al. 2012; Ning et al. 2013; Yang et al. 2014; Asai et al. 2015; Tarrant et al. 2014; Tarrant et al. 2016) have facilitated studies on gene expression in response to a wide range of stressors. Such studies include transcriptional responses to increased temperature (Voznesensky et al. 2004), toxic algae (Lauritano et al. 2015), PAHs (Hansen et al. 2008), metals (Øverjordet et al. 2014) and industrial chemicals (Hansen et al. 2010; Hansen et al. 2014). In a study by Øverjordet et al. (2014), *C. finmarchicus* was found to be particularly susceptible to inorganic mercury, affecting transcription of glutathione S-transferase (*GST*), a key gene involved in the antioxidant defence mechanisms. The presence of as many as 40 *GST* genes in *C. finmarchicus* that display differential expression during development, by diet and other natural factors (Roncalli et al. 2015) suggest that high-content toxicogenomics approaches are required to improve the understanding of biochemical processes underlying the sensitivity of copepods to Hg.

Exposure to inorganic and organic mercury, common aquatic pollutants worldwide, may affect various biochemical processes relevant for the physiology and fitness of an organism and display considerable tissue-specificity (Stohs and Bagchi 1995). The mitochondria is frequently suggested as the main target for mercury toxicity in various organisms (Lund, Miller, and Woods 1993). Accumulation of mercury in the mitochondria is believed to uncouple oxidative phosphorylation (OXPHOS) at cytochrome C (CYCS) of complex III, to inhibit the transfer of electrons to complex IV and V of the electron transport chain (ETC) and reduce the transmembrane proton gradient as the driving force for cellular ATP production (Mieiro et al. 2015). The release of excessive electrons subsequently cause production of Reactive Oxygen Species (ROS), predominantly hydroxyl radicals, hydrogen peroxides and superoxide radicals, that can lead to oxidative damage of cellular proteins, enzymes, lipids and DNA if not detoxified by the cellular antioxidant defense (Lushchak 2011). This detrimental effect of ROS production is enhanced in many cells by a mercury-induced inactivation of thiol (-SH) and seleno (-SeH) containing enzymes

and proteins involved in the antioxidant defence (Farina, Rocha, and Aschner 2011). Mercury also binds covalently to glutathione (GSH) and by excretion of the aggregates from the cell deplete the mitochondrial GSH pool. The cells normally undergo apoptosis, necrosis and autophagy upon exposure to toxic agents such as Hg to disassemble damaged or dysfunctional cell components, where the ultimate effect is loss of cellular function and ultimately death. Although mercury has been extensively studied in aquatic vertebrates, determination of susceptibility and characterisation of molecular responses in marine copepods such as *C. finmarchicus* are still poorly characterised.

The aims of the present work was to characterise the toxicity of inorganic Hg (Hg^{2+}) to *C. finmarchicus* after short-term waterborne exposures. A combination of acute toxicity (mortality) assessment and determination of sublethal transcriptional changes were performed to characterise the toxic mode of action (MoA) of Hg^{2+}. A custom made oligonucleotide microarray (oligoarray) was designed for *C. finmarchicus* based on available ESTs sequences (NCBI), and the performance of the resulting oligoarray was assessed by parallel analysis by quantitative RTPCR. Differentially expressed genes were mapped to their mammalian orthologs and subjected to gene ontology (GO) functional enrichment and pathway analysis to identify potential molecular perturbations associated with known MoA in other species.

Materials and Methods

Calanus finmarchicus culture

The culture of *C. finmarchicus* was established from stage V copepodites (CV) collected locally in Trondheimsfjorden, Norway, with a Nansen zooplankton net (Ø 70 cm, 180 μm mesh, Hydro-Bios, Kiel, Germany). In the laboratory, the cultures were maintained in running natural seawater in polyester containers (280 L) at 8-10 °C and fed with a mixture of the unicellular algae *Rhodomonas baltica, Isochrysis galbana* and *Dunaliella tertiolecta*. The feeding regime was designed to maintain levels >150 μg of algal carbon L^{-1} in the cultures,

which supports normal growth and development of *C. finmarchicus* (Campbell et al. 2001).

Experiments

Two short-term (up to 96 h) static exposure experiments were set up at 10 °C; the initial acute toxicity test was performed according to ISO 14669:1999 (ISO 2000) to determine the No Observed Effect Concentration (NOEC), and the 50% Lethal effect Concentrations (LC_{50}) at different exposure times. In essence, a stock solution of 1000 mg/L Hg^{2+} was prepared by dissolving $HgCl_2$ (Pro analysis 99.5%, Riedel-de-Häen) in Milli-Q water and subsequently diluting it in filtered seawater to the following nominal concentrations: 7, 12, 19, 32, 54, 90 and 150 μg Hg^{2+}/L. The experiment was set up using triplicates of 2L polypropylene buckets each containing 1 L of spiked seawater and 10 specimens of *C. finmarchicus* copepod stage five (CV). Six replicates in seawater were used as negative controls. The number of survivors were recorded after 24, 48, 72 and 96h exposure and a sigmoidal concentration-response curve (variable slope) was calculated by Graphpad Prism 5.01 (GraphPad Software, La Jolla, Ca, USA).

The second experiment was set up to characterise the sublethal (transcriptional) effects of Hg^{2+}. Sets of 2 L glass bottles containing 25 specimens of *C. finmarchicus* (CV) in seawater were spiked with a sublethal concentration of 5 μg Hg^{2+}/L (<48h NOEC) and exposed in triplicate for 48h. Triplicate sets of exposure bottles with unspiked seawater and *C. finmarchicus* were used as negative controls. After 48h exposure, copepods were gently collected on a plankton mesh, preserved in RNAlater (1 ml) and frozen (-80°C) until RNA extraction (< 1 month later).

Mercury analysis

Mercury analyses were performed using a high performance inductively coupled plasma mass spectrometry (ICP-MS, Thermo Electronic Corporation, Waltham, MA, USA). All samples were diluted in ultrapure water (Q-option, Elga Labwater, Veolia Water Systems LTD, UK) and added HNO_3 (ScanPure grade, Chem Scan, Elverum, Norway), to a final concentration of 0.6 M prior to analysis. Sample injection was performed by the SC-FAST automated sample introduction system from Elemental Scientific (Omaha, USA), with an average sample flow of 0.25 mL/min. Argon was used as a carrier gas with a flow of 0.9 L/min. Low resolution detection were used for mercury.

RNA Isolation

Frozen, RNAlater preserved *Calanus finmarchicus* were thawed, pooled (n=25) and organism lysates obtained by homogenising the pooled samples (approx. 10-50 mg wet weight) in 1 mL TRIzol® reagent (Sigma-Aldrich, St. Louis, MO), using a Precellys orbital shaker bead mill (Bertin, Montigny-le-Bretonneux, France). Samples were homogenised for 3 x 10 sec at 6000 rpm with Precellys CK14 beads and cell debris were removed by centrifugation (8000g, 1 min). To permit complete dissociation of nucleoprotein complexes, the homogenized samples were incubated at room temperature (15 min.), 0.2 mL chloroform (Sigma-Aldrich) added, the tubes shaken vigorously (15 seconds) and incubated for 2-3 minutes (20°C). The samples were then centrifuged (12000g, 10 min., 4°C), the RNA precipitated and isolated by 0.5 ml isopropanol (Sigma-Aldrich). The resulting RNA pellet was washed in 1 mL 75 % ethanol, vortexed and centrifuged (7500g, 5 min, 4°C) before removing the ethanol by suction followed by air-drying the pellet for 5-10 min. The pellet was re-suspended in 50 µL nuclease free water and the residual ethanol allowed to evaporate off for 5 min. (72°C).

Genomic DNA was removed by DNase I treatment using Ambion Turbo DNA-free kit according to the vendor specifications (Applied Biosystems, Austin TX, USA). In essence, 10 µg RNA (50 µL) was added 0.1 volume 10x TURBO DNase buffer, 1 µL TURBO DNase and mixed gently prior to incubation (37°C, 20 min.). The DNase was inactivated by adding 0.1 volume inactivation reagent to the sample, followed by vortexing and incubation with gentle mixing (5 min., 20°C) before the RNA containing supernatant was obtained by centrifugation (10000g, 20°C, 90 seconds).

The RNA was quality and yield controlled by photometric analyses (260/230> 2.0, 260/280> 1.8, yield >500 ng) by Nanodrop (ND-1000, Nanodrop Technologies, Wilminton, Delaware, USA) and RNA integrity inspected by Bioanalyzer gelelectrophoresis with RNA 6000 nano chips (Agilent technologies, Santa Clara, California, USA).

Microarray gene expression analysis

The microarray analysis was performed by a 6.6k *C. finmarchicus* Agilent (60-mer) oligoarray array as described in the Agilent standard microarray protocol "One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) with Tecan HS Pro Hybridization, Version 5.7 May 2008" (Agilent Technologies). All chemicals used in the process were purchased from Agilent technologies, as parts of the kits: Agilent One-color RNA Spike-In Kit, Low RNA Input Linear Amplification Kit, PLUS, One-Color, Gene Expression Hybridization Kit, and Wash Buffer 1 and 2. Briefly, for each array, 200 ng (8.3 µL) of total RNA was mixed with 2 µL of the spike-in standard (Agilent One-color RNA Spike-In Kit), 1.2 µL oligo (d)T-T7 primer was then added and annealed to the RNA template (60ºC, 10 min.) before being cooled rapidly on ice. First strand cDNA was synthesized by incubating (2h, 40ºC) the template with first strand buffer containing 0.1 M dithiotreitol (DTT), 10 mM deoxyribose nucleotide mixture (dNTP mix), Moloney Murine Leukemia

Virus reverse transcriptase (MMLV-RT) enzyme and RNaseOut. The cDNA was then denatured (10 min., 65ºC), rapidly cooled to 4 ºC and the cRNA synthesised from the cDNA template using NTPs as well as Cyanine 3-creatine triphoshate (CTP) together with a T7 RNA Polymerase (2h, 40ºC). After this, the samples were kept at -80 ºC overnight. The following day, the labelled and amplified cRNA was purified using RNeasy Mini spin columns (Qiagen, Hilden, Germany), the eluate washed and cRNA yield (> 1.65 µg) and quality (specific activity: > 9.0 pmol Cy3/µg cRNA) deteremined by Nanodrop® spectrophotometer (ND-1000, Nanodrop Technologies, Wilminton, Delaware, USA). Samples passing the quality criteria were prepared for hybridisation by fragmentation of 1.65 µg labelled cRNA (30 min. 60ºC), and the reaction stopped by addition of 2x Gene Expression Hybridization Buffer. The hybridisation mix was hybridised to the oligoarray (65°C, 17h), then washed with Agilent Wash Buffer I and II before being dried with acetonitrile (Sigma-Aldrich) and scanned immediately at 5 µm resolution by a High Density microarray scanner (Agilent technologies). Details about the oligoarray design such as assembly of contigs, selection of probes, replication of probes and annotation of the *C. finmarchicus* 6.6k Agilent oligoarray are provided in the Supplementary Information – Oligoarray design.

Biostatistics and bioinformatics

Scanned images were analysed with Agilent feature extraction, Version 7.3 (Agilent Technologies). Resulting raw data were normalised (25 Quantile, median to baseline of all samples), features filtered on expression (20-100%), outlier flagged and differentially expressed genes (DEGs) across treatments identified by a moderated t-test using a Storey with Curve Fitting false discovery rate multiple testing correction ($P<0.1$) by GeneSpring GX 12.5 (Agilent Technologies). Significantly regulated genes (no fold cut-off threshold) were clustered (Euclidian, centroid) by treatment and gene regulation. Gene ontology (GO) functional enrichment analysis ($p<0.05$) was performed using

the BiNGO plugin (Maere, Heymans, and Kuiper 2005) in Cytoscape (Shannon et al. 2003; Bindea et al. 2009). The functional enrichment analyses were conducted with and without multiple testing (FDR-false discovery rate) correction to ensure that all relevant biological information was captured. Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/products/ipa) was conducted using the Inparanoid ortholog mapping to *Danio rerio*, *Homo sapiens*, *Mus musculus* and *Rattus norwegicus* as proxies for *C. finmarchicus* (see Supplementary Information – Oligoarray design, for details).

Quantitative RTPCR analysis

Total RNA (200 ng) was reversely transcribed to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. The quantitative real-time polymerase chain reaction was performed using CFX384™ detection system (Bio-Rad, Hercules, CA, USA) with total reaction volume of 20 µL containing cDNA from 5 ng of RNA, Quanta PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, Maryland, USA) and 400 nanomoles forward/reverse primer. For primer design, the Basic Local Alignment Search Tool for nucleotide 6-frame translation-protein (BlastX) was first used to align the *C. finmarchicus* full length sequences against NCBI non-redundant (nr) protein database to identify annotated *C. finmarchicus* sequences or conserved sequences among invertebrates (Table 1). Except for elongation factor 1-alpha (*EF1A*), which was derived from Hansen et al. (2010), all primer sequences were designed using Primer 3 (v0.4.0) software (http://frodo.wi.mit.edu/primer3) based on Genbank EST sequences for Calanus Sp. The primers were optimized for annealing temperatures prior to target gene amplification. Standard curves and amplification efficiencies were determined from 0.781, 3.12, 12.5 and 50 ng pooled cDNA from all samples. The relative expressions were calculated from the standard curves based on threshold cycle (Ct)

and normalized to the geometric mean of reference gene expression (Pfaffl 2001). The *EF1A*, mitochondrial 28s ribosomal protein s21 (*MRPS21*) and glucose 6 phosphate dehydrogenase (*G6PD*) were chosen as reference genes. Fold changes were calculated by normalizing the expressions to the control group. Original or Log_{10} transformed (where required) expression data with normal distribution and equal variance were subjected directly to unpaired t-test to assess the statistical differences between the control and treatments. All statistical analyses were performed in Graphpad Prism 5.01 (Graphpad Software, Inc., San Diego, CA, USA) with a probability (p) level of 0.05.

Table 1. NCBI BLASTx hits and primer sequences for quantitative real-time rtPCR.

Target gene	Genbank accession	BLASTx hit accesion	Putative protein	Species	Score (bits)	E-value	Forward primer (5'-3')/ Reverse primer (3'-5')	Annealing temp. (°C)
MMP9	ES237602.1	AAA51539.1	matrix metallopeptidase 9	*Homo sapiens*	88.2	2.00E-03	GTGACCAGCAGAGCAATGAA CCATCTGGGTCTCCATCTGT	59.1
MMP3	FG985888.1	NP_001116500.1	matrix metalloproteinase 1 isoform 2	*Bombyx mori*	168	2.00E-45	TGGCCTTAGTAGGCGAGATG AGTATGCGTGAGCCAAAACC	56
MMP1	FK041294.1	NP_001189002.1	matrix metalloproteinase 1, isoform H	*Drosophila melanogaster*	172	8.00E-47	TGTGGATGACGAAACCAGAA CAAAGTGAGCAGTTCCACCA	59.1
RHBG	ES237475.1	AAK50057.2	rh-like protein/rh50 glycoprotein	*Carcinus maenas*	156	3.00E-41	TTCTTTTGGGGTCATTCTGG TCCCAAAGTACCAGGCAAAC	52.3
EF1A	ES414812	AAD21847	elongation factor 1-alpha	*Eurytemora affinis*	328	3.00E-90	CTCCGACTCCAAGAACAAGC AATATGGGCGGTGTGACAAT	60
MRPS21	Microarray contig	NA	mitochondrial 28s ribosomal protein s21	NA	NA	NA	AACAGGTGCAATGGATTTCG TGCTCATGTCCTCGTCGTAG	56
G6PD	EL773753	CAE51229.1	glucose 6 phosphate dehydrogenase	*Adalia decempunctata*	199	5.00E-61	GCAGTGGAGAAAGGTCAAGC CCTTTCCCAGGTAGTGGTCA	56

Results

Mercury analysis

Analysis of the initial stock solution of mercury chloride after $10x10^6$ times dilution was calculated back to 1043 mg/L showing a recovery of 104.4 % compared to the nominal concentration. The exposure concentrations of the acute toxicity experiment had an average recovery of 41.3 ± 27.2 % compared to nominal values, showing the largest deviation at lower concentrations. The measured concentrations were used in all further calculations of acute effects giving accurate NOEC and LC_{50} values. The exposure solutions in the sublethal studies

were in the range 4.88–5.11 µg/L (mean 5.0 µg/L) with a recovery of 26.3 % of the nominal value.

Acute toxicity

Mercury chloride was toxic to *C. finmarchicus* at low µg/L concentrations. A concentration- and time-dependent decrease in survival was observed. The LC_{50}-values for 24, 48, 72 and 96 hrs were determined as 279 (95% confidence intervals: 207-376), 73 (67-79), 48 (42-55) and 34 (30-39) µg/L, respectively. At the lowest concentration used, no significant decrease in survival was observed after 48 hrs suggesting a 48h NOEC of 6.7 µg/L (Figure 1). No mortality was observed in the controls during the studies.

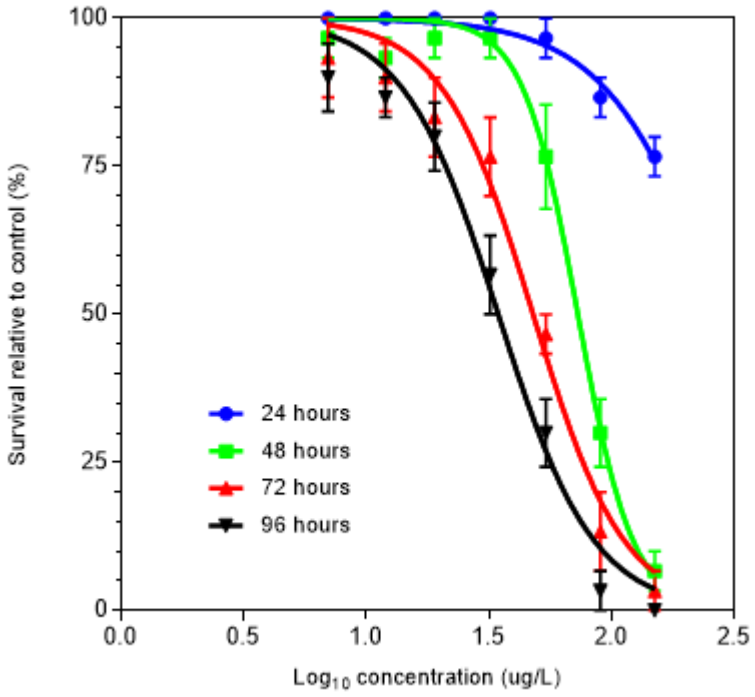

***Figure 1**: Survival (in % of controls) of Calanus finmarchicus after exposure to different concentrations of Hg^{2+} for 24, 48, 72 and 96 hrs. Data (mean±SEM, n=3) were characterised by a sigmoidal dose-response curve (to obtain the 50% lethal Concentration (LC50±SEM).*

Sublethal effects

A 48h static exposure study to 5.0 µg/L of Hg^{2+} was undertaken to determine the transcriptional responses of Hg^{2+} in *C. finmarchicus* by microarray and quantitative RTPCR analysis. No reduction in survival of *C. finmarchicus* was observed for the exposure to Hg^{2+} when compared to the negative controls.

Microarray analysis.

Of the total 6610 probes on the array (see suppl. Information – oligoarray design), 98 probes (50 up-regulated and 48 down-regulated), whereof 50 unique probes with high-quality BLAST hits, were identified as differentially expressed after the Hg^{2+} exposure (Figure 2). The most profound changes in transcription (Table 2) occurred for up-regulated genes (up to 23.5-fold compared to control), whereas down-regulated transcripts showed less regulation (up to 4.6-fold compared to control). While some of the differentially expressed features had unknown functions, a number of well-known markers of toxic effects such as microsomal glutathione s-transferase 3 (*MGST3*), aldehyde oxidase (*AOX1*), heat shock protein 70 (*HSP70*) and heat shock protein 90 (*HSP90*), juvenile hormone esterase (*JHE*), brain chitinase (*CHIA*), Rh family B glycoprotein (*RHBG*), oxidative stress protein/sequestosome 1 (*SQSTM1*), several matrix metalloproteinases (*MMP1, MMP3, and MMP9*), and phospholipase a1 (*PLA1A*) were affected by the treatment (Table 2). Interestingly, several transcripts for cellular transporters such as sodium chloride dependent amino acid transporter (*CPIJ015063-PA*), sodium-dependent phosphate transporter (*SLC34A1*), and solute carrier family 2 (facilitated glucose transporter) member 8 (*SLC2A8*) were also differentially expressed.

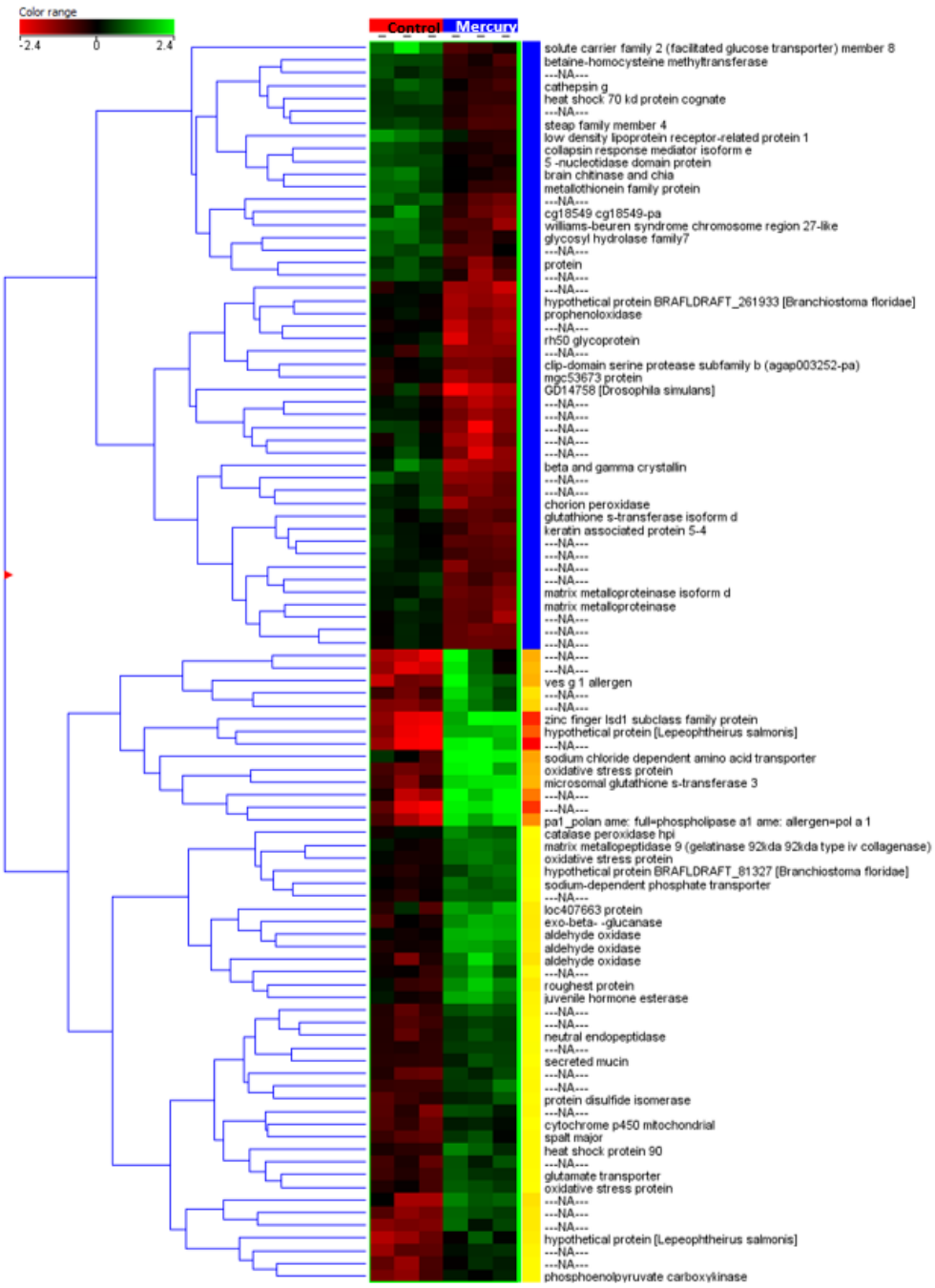

Figure 2. *Combined (genes and treatments) hierarchical clustering (Euclidian-Ward) of differentially regulated genes in Calanus finmarchicus exposed to clean sea water (Control) and 5.0 µg/L* Hg^{2+} *(mercury) for 48h.*

1

2

3

Table 2. Potentially toxicologically relevant genes differentially regulated in Calanus finmarchicus after 48h exposure to 5.0 µg/L Hg^{2+}.

Probe	Description	Gene Symbol	Acc. no./Ortholog source	eValue/ bit core	Fold
CF_138236068	*transmembrane serine 9*	*TMPS9*	Q7Z410	5.6E-04	23.5
CF_145881822	*proprotein convertase subtilisin kexin type 5*	*PCSK5*	XP_001705381, Q04592	9.4E-04	18.1
CF_193793009	*phospholipase a1*	*PLA1*	XP_001657378, Q9U6W0	1.3E-06	10.5
CF_Contig1200	*sodium/chloride dependent amino acid transporter*	*CPIJ015063-PA*	XP_001865227.1, EDS41438	1.4E-42	8.6
CF_Contig1740	*oxidative stress protein/Sequestosome 1*	*SQSTM1*	Q24629, XP_001608187	3.3E-10	7.7
CF_124246332	*ves g 1 allergen/pancreatic lipase-related protein 2*	PNLIPRP2	XP_002413330, Q6Q250	1.8E-08	7.6
CF_145881649	*microsomal glutathione s-transferase 3*	*MGST3*	Q9CPU4, XP_793267	1.1E-07	7.3
CF_Contig174	*nuclear receptor coactivator 2*	NCOA2	Ortholog (*h. sapiens*)	52	7.0
CF_124246101	*aldehyde oxidase*	*AOX1*	XP_001845789.1, EDS41696.1	3.6E-06	3.9
CF_Contig25	*moesin*	MSN	Ortholog (*h. sapiens*)	38	3.9
CF_190134167	*roughest protein*	*ROUGHEST*	XP_002416368, Q08180	1.0E-14	3.4
CF_132577273	*retinol dehydrogenase 13*	*RDH13*	XP_794540, Q8NBN7	5.7E-41	3.3
CF_193793001	*juvenile hormone esterase*	*JHE*	Q8VCU1, XP_001947547	2.6E-18	2.8
CF_Contig1572	*phosphoenolpyruvate carboxykinase*	*PCK*	XP_859670, P20007	8.5E-108	2.3
CF_145881707	*heat shock protein 90*	*HSP90*	P82995, ACF75907	6.6E-19	2.3
CF_125743378	*spalt major*	*SALM*	XP_002430173, P39770	3.2E-12	2.2
CF_Contig1394	*membrane metallo-endopeptidase-like 1*	*MMEL1*	XP_001950596, Q495T6	8.6E-20	2.2
CF_138236286	*matrix metallopeptidase 9*	*MMP9*	P14780, ACO12798	3.5E-05	2.1
CF_134036624	*protein disulfide isomerase*	*PDI*	XP_551775, P54399	1.8E-31	2.1
CF_Contig782	*beta-carotene oxygenase 2a*	*BCO2A*	NP_001035402.1, AAI15260.1	8.0E-11	2.1
CF_Contig1190	*excitatory amino acid transporter 3*	*EAAT3*	ACO12613, P51906	1.6E-32	2.1
CF_138236351	*sodium-dependent phosphate transporter*	*SLC34A1*	XP_001652004, Q8GX78	1.6E-22	2.0
CF_188486110	*cytochrome P450 12b1, mitochondrial*	*CYP12B1*	XP_001855581.1, EDS34539.1	3.1E-15	1.9
CF_Contig1890	*secreted mucin/mucin 5AC*	*MUC5AC*	Q6ZWJ8, XP_001122007	1.1E-05	1.9
CF_190134474	*5-nucleotidase domain protein*	*NT5E*	P21589, BAG82602	9.0E-16	-1.8
CF_Contig1058	*dihydropyrimidinase*	*DPYS*	XP_001658523.1, EAT40650.1	2,7-76	-1.9
CF_Contig71	*calcineurin binding protein 1*	*CABIN1*	Ortholog (*h. sapiens*)	35	-1.9
CF_188486614	*heat shock 70 kd protein cognate*	*HSP70*	NP_001036837, Q16956	1.4E-20	-2.0
CF_193793082	*glutathione s-transferase isoform d*	*GSTT1*	P20432, ACO12967	2.6E-12	-2.0
CF_Contig795	*brain chitinase*	*CHIA*	Q9W092, ACR23315	1.0E-21	-2.1
CF_EST_188486709	*tenascin*	*TENA*	P24821	8.6E-08	-2.1
CF_Contig842	*cathepsin g*	*CTSG*	P08311, AAK48894	5.3E-18	-2.2
CF_192823777	*acyl-CoA synthetase bubblegum family member 2*	*ACSBG2*	XP_624225.2	1.5e-27	-2.2
CF_190134531	*steap family member 4*	*STEAP4*	XP_002597199, Q923B6	1.2E-20	-2.2
CF_Contig1379	*matrix metalloproteinase isoform d*	*MMP1*	P22757, XP_001976657	2.9E-33	-2.3
CF_190887840	*matrix metalloproteinase 3*	*MMP3*	NP_001116500, P28862	7.0E-33	-2.4
CF_Contig1889	*low density lipoprotein receptor, putative*	*LRP1*	XP_002429721, EEB16983	4.1E-05	-2.5
CF_132578291	*phenoloxidase subunit 2*	*PRP2*	AAD45527, Q25519	4.0E-11	-2.5
CF_190888141	*broad-complex core protein isoform 6*	*BRC*	ACO11014.1	2.3e-05	-2.6
CF_145881534	*heme binding protein 2*	*HEBP2*	AAI58373.1	6.6E-18	-2.9
CF_190134017	*chorion peroxidase*	*CPIJ018105*	XP_001868291.1	3.7E-76	-2.9
CF_145881598	*cytochrome c, somatic*	*CYCS*	Ortholog (*h. sapiens*)	65	-3.0
CF_188486208	*major facilitator superfamily domain containing 11*	*MFSD11*	Q4R495, XP_001948589	6.2E-30	-3.2
CF_Contig1685	*rh50 glycoprotein*	*RHBG*	XP_002426946, EEB14208	3.8E-35	-3.3
CF_138236182	*solute carrier family 2 (facilitated glucose transporter) member 8*	*SLC2A8*	XP_002049252, Q9NY64	2.3E-17	-3.9

Table 3. Overrepresented gene ontology (GO) functions that were regulated in C. finmarchicus after 48h exposure to 5.0 µg/L Hg^{2+}. Only GO functions relevant for invertebrates and with >2 supporting genes were considered. A complete overview is found in supplementary table S2.

GO Cat	Biological endpoint	GO-ID	Description	p-value	DEG support
Biological process	Cell proliferation	GO:0050673	Epithelial cell proliferation	4.99E-02	2
		GO:0050679	Positive regulation of epithelial cell proliferation	1.16E-02	2
		GO:0050678	Regulation of epithelial cell proliferation	4.36E-02	2
	Cellular defensome	GO:0050896	Response to stimulus	2.77E-02	25
		GO:0006952	Defense response	4.12E-02	5
		GO:0001101	Response to acid	2.19E-02	2
		GO:0014075	Response to amine stimulus	2.93E-02	2
		GO:0043200	Response to amino acid stimulus	1.16E-02	2
		GO:0034097	Response to cytokine stimulus	3.76E-02	2
		GO:0009611	Response to wounding	3.82E-02	5
	Metabolism	GO:0008152	Metabolic process	1.71E-03	39
		GO:0019748	Secondary metabolic process	7.03E-03	3
		GO:0032963	Collagen metabolic process	1.54E-02	2
		GO:0006720	Isoprenoid metabolic process	2.19E-02	2
		GO:0044259	Multicellular organismal macromolecule metabolic process	2.68E-02	2
		GO:0044236	Multicellular organismal metabolic process	2.68E-02	2
		GO:0006721	Terpenoid metabolic process	1.35E-02	2
	Neurotransmission	GO:0001504	Neurotransmitter uptake	1.54E-02	2
	Regulation of biological quality	GO:0065008	Regulation of biological quality	3.20E-02	14
Molecular function	Binding	GO:0043167	Ion binding	4.02E-02	23
		GO:0043169	Cation binding	5.26E-03	19
		GO:0046872	Metal ion binding	8.42E-03	18
		GO:0031404	Chloride ion binding	1.85E-02	2
		GO:0005539	Glycosaminoglycan binding	3.67E-02	2
		GO:0008201	Heparin binding	1.85E-02	2
	Catalytic activity	GO:0003824	Catalytic activity	6.09E-03	35
		GO:0016787	Hydrolase activity	3.81E-02	18
		GO:0016491	Oxidoreductase activity	3.69E-02	10
		GO:0070011	Peptidase activity, acting on L-amino acid peptides	3.18E-02	7
		GO:0016798	Hydrolase activity, acting on glycosyl bonds	7.34E-03	4
		GO:0008237	Metallopeptidase activity	1.91E-02	4
		GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	3.25E-02	3
		GO:0004252	Serine-type endopeptidase activity	4.84E-02	3
		GO:0051213	Dioxygenase activity	3.67E-02	2
		GO:0016706	Oxidoreductase activity, acting on paired donors.	8.83E-03	2
		GO:0016684	Oxidoreductase activity, acting on peroxide as acceptor	4.28E-02	2
		GO:0031545	Peptidyl-proline 4-dioxygenase activity	2.51E-03	2
		GO:0031543	Peptidyl-proline dioxygenase activity	2.51E-03	2
		GO:0004601	Peroxidase activity	4.28E-02	2
		GO:0004656	Procollagen-proline 4-dioxygenase activity	2.51E-03	2
		GO:0019798	Procollagen-proline dioxygenase activity	2.51E-03	2
		GO:0004175	Endopeptidase activity	1.71E-03	7
	Transporter activity	GO:0015171	Amino acid transmembrane transporter activity	4.84E-02	3
		GO:0015179	L-amino acid transmembrane transporter activity	2.95E-02	3
		GO:0015172	Acidic amino acid transmembrane transporter activity	1.63E-02	2
		GO:0005310	Dicarboxylic acid transmembrane transporter activity	1.85E-02	2
		GO:0005313	L-glutamate transmembrane transporter activity	1.05E-02	2
		GO:0005283	Sodium:amino acid symporter activity	4.28E-02	2
Cellular component	Cell surface	GO:0009986	Cell surface	2.69E-02	5
	Extracellular region	GO:0005576	Extracellular region	1.10E-02	9
		GO:0044421	Extracellular region part	2.19E-03	7
		GO:0005615	Extracellular space	7.25E-03	5
	Intracellular organelle	GO:0005882	Intermediate filament	2.66E-02	2
		GO:0045111	Intermediate filament cytoskeleton	2.66E-02	2
		GO:0045095	Keratin filament	8.19E-03	2
	Membrane	GO:0016021	Integral to membrane	1.42E-02	14
		GO:0031224	Intrinsic to membrane	2.30E-02	14
		GO:0005887	Integral to plasma membrane	4.62E-02	6

A total of 112 GO functions were overrepresented among the DEGs when using data without FDR correction, of which 58 were supported by at least 2 DEGs (Table 3). Nineteen GO functions were associated with biological processes such as cell proliferation, cellular defensome, metabolic processes, neurotransmission and regulation of biological quality. Twenty-nine functions were related to molecular functions such as binding activity, catalytic activity, and transporter activity. Exposure to mercury also affected genes involved in cellular components such as cell surface (cell membrane), extracellular region and different intracellular organelles. No GO terms were enriched when using FDR corrected data (Suppl. Table S2_GOs).

Pathway analyses using enrichment by ortholog mapping to *D. melanogaster*, *D. rerio*, *H. sapiens*, *M. musculus* and *R. norwegicus* revealed significant enrichment of DEGs associated with relevant toxicity pathways (Table 4). These pathways, which provided detailed information on potential MoAs of Hg^{2+} from known MoA in mammals displayed considerable heterogeneity, although several DEGs were found to co-occur in different pathways. Several DEGs being associated with the cellular defensome (NRF2-mediated oxidative stress response, glutathione redox reactions I, glutathione depletion) such as *MGST3*, *SQSTM1*, and *AOX1* were up-regulated, whereas *GST1* were down-regulated. Significant enrichment of DEGs related to cellular growth, regulation and development was also observed, including down-regulation of matrix metallopeptidases (*MMP1* and *MMP3*), calcineuron binding protein 1 (*CABIN1*), 5-nucleotidase domain protein (*NT5E*) and up-regulation of *MMP9*, cytochrome P450, family 24 A1 (*CYP24A1*), and the nuclear receptor coactivator 2 (*NCOA2*).

Table 4. *Potential biological endpoints, toxicity pathways and genes (↓:down and ↑: up-regulation) affected in Calanus finmarchicus after 48h exposure to 5.0 µg/L Hg^{2+}. Only pathways considered relevant for invertebrates and with >2 supporting genes were considered.*

Biological endpoint	Pathway	P-value	Gene(s)
Cellular defensome	Glutathione depletion - Phase II Reactions	0.002	↓*GSTT1,*↑*MGST3*
	Glutathione redox reactions I	2.95E-03	↓*GSTT1,*↑*MGST3*
	NRF2-mediated oxidative stress response	4.47E-03	↓*GSTT1,*↑*SQSTM1,*↑*AOX1,*↑*MGST3*
Cellular growth, regulation and development	Cardiac hypertrophy	0.035	↓*NT5E,*↓*MMP1,*↑*MMP9,*↓*CABIN1*
	Hepatic fibrosis	0.047	↓*MMP1,*↑*MMP9*
	VDR/RXR activation	0.034	↑*CYP24A1,*↑*NCOA2*
	HIF1α signaling	6.17E-04	↓*MMP3,* ↑*MMP9,* ↓*MMP1*
	Inhibition of matrix metalloproteases	3.55E-07	↓*MMP3,* ↑*MMP9,* ↓*MMP1*
	Oncostatin M signaling	7.08E-03	↓*MMP3,*↓*MMP1*
Cellular inflammatory and immune response	Leukocyte extravasation signaling	8.32E-04	↓*MMP3,* ↑*MMP9,* ↓*MMP1,*↑*MSN*
	Nur77 signaling in T lymphocytes	1.82E-02	↓*CYCS,* ↓*CABIN1*
	IL-17 Signaling	2.95E-02	↓*MMP3,* ↑ *MUC5AC*
Nuclear Receptor Signaling	Estrogen receptor signaling	1.17E-02	↑*PCK,*↑*NCOA2*
	Aryl hydrocarbon receptor signaling	1.62E-02	↓*GSTT1,*↑*NCOA2,*↑*MGST3*
	VDR/RXR activation	3.47E-02	↑*CYP24A1,*↑*NCOA2*
	LPS/IL-1 mediated inhibition of RXR function	4.90E-02	↓*GSTT1,*↓*ACSBG2,*↑*MGST3*
Nucleotide and amino acid degradation	Urate biosynthesis/inosine 5'-phosphate degradation	3.24E-03	↓*NT5E,*↑*AOX1*
	Purine Nucleotides Degradation II (Aerobic)	7.41E-03	↓*NT5E,*↑*AOX1*
	Adenosine nucleotides degradation II	3.89E-03	↓*NT5E,*↑*AOX1*
	Guanosine nucleotides degradation III	2.95E-03	↓*NT5E,*↑*AOX1*

Gene symbols: ACSBG2 (acyl-CoA synthetase bubblegum family member 2), AOX1 (aldehyde oxidase), CABIN1 (calcineurin binding protein 1), CYCS (cytochrome c, somatic), CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1), GSST1 (glutathione s-transferase isoform d), LRP1 (low density lipoprotein receptor, putative), MGST3 (microsomal glutathione s-transferase, MMP1 (matrix metallopeptidase 1), MMP3 (matrix metallopeptidase 3), MMP9 (matrix metallopeptidase 9), MSN (Moesin), MUC5AC (secreted mucin/mucin 5AC), NCOA2 (nuclear receptor coactivator 2), NT5E (5-nucleotidase domain protein), PCK (phosphoenolpyruvate carboxykinase) and SQSTM1 (sequestosome 1).

Many of the same DEGs identified to be involved in cellular growth, regulation and development such as *MMPs* were also involved in cellular inflammatory and immune responses characterized in mammals (leukocyte extravasation signaling, Nur77 signaling in T lymphocytes and IL-17 signaling). Although the *MMPs* were consistently down-regulated, *MMP9* was up-regulated by Hg^{2+}. Down-regulation of somatic cytochrome C (*CYCS*) and

CABIN1 were exclusively associated with the pathway Nur77 signaling in T lymphocytes, whereas up-regulation of mucin 5AC (*MYC5AC*) were potentially associated with IL-17 signaling and leukocyte extravasation signaling.

Several mammalian pathways involving nuclear receptors signaling such as modulation of the estrogen (ER), arylhydrocarbon (AhR), vitamin D (VDR) and retinoid X (RXR) receptor signaling were apparently affected by the exposure to Hg^{2+}. Although no nuclear receptors (NR) were regulated directly by Hg^{2+} (Suppl. Table S1_DEGs), genes associated with NR activity such as phosphoenolpyruvate carboxykinase (*PCK*), *CYP24A1*, *NCOA2*, and *MGST3* were up-regulated, whereas acyl-CoA synthetase bubblegum family member 2 (*ACSBG2*), and GSTT1 were down-regulated by Hg^{2+} exposure, however.

Pathway analysis suggested that nucleotide and amino acid cycling were affected by the exposure to Hg^{2+}. The down-regulation of *NT5E* and up-regulation of *AOX1* were associated with a high number of pathways related to nucleotide (purine, adenosine, guanosine) degradation and urate biosynthesis/inosine 5'-phosphate degradation.

Quantitative RTPCR analysis

Quantitative RTPCR was performed to verify the microarray gene expression results. The results showed that no significant differences were found between control and Hg^{2+} for the reference genes *EF1A*, *MRPS21* and *G6PD*, albeit significant differences were observed for *MMP9*, *MMP3*, *MMP1* and *RHBG* by both microarray and quantitative RTPCR (Figure 3).

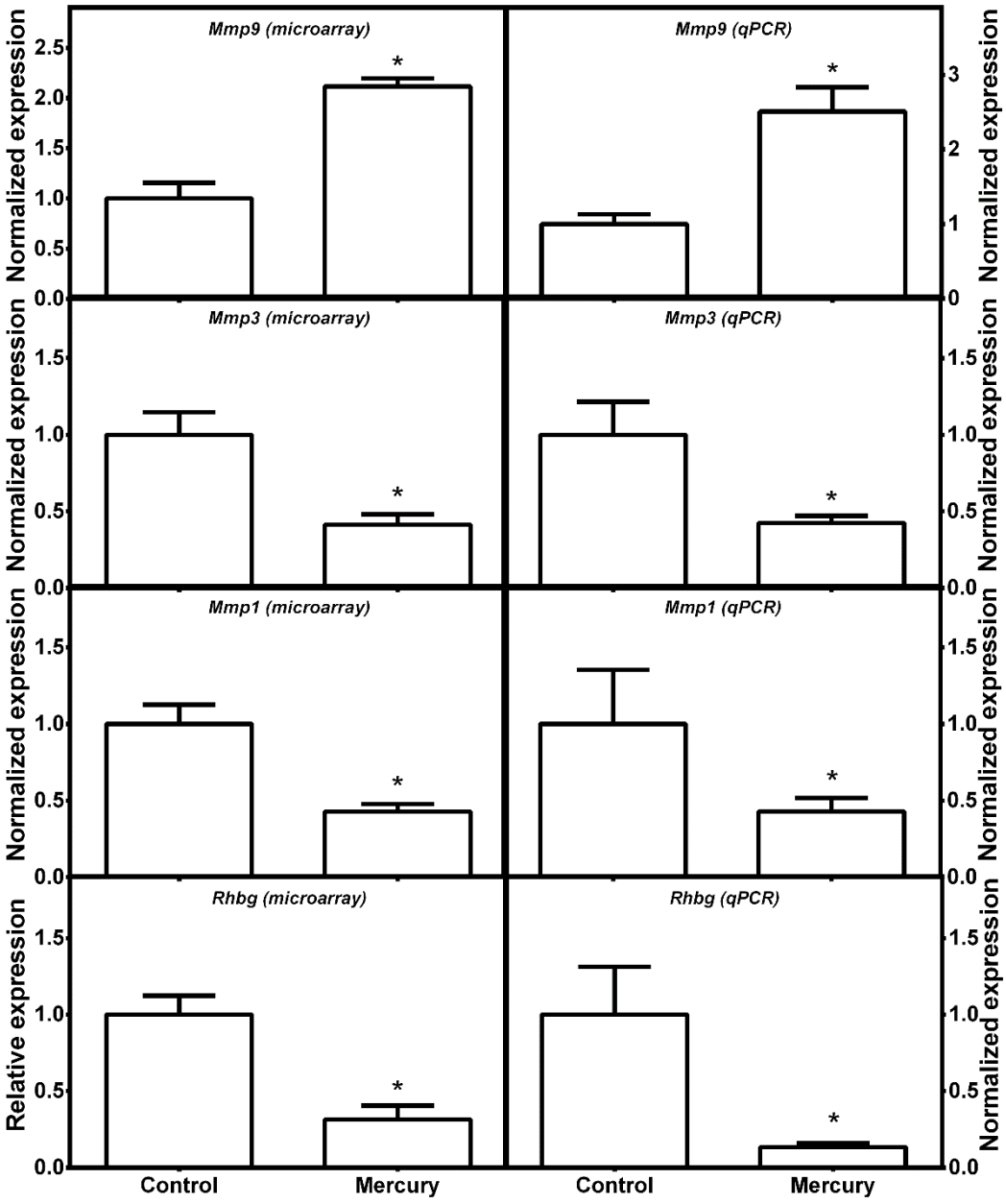

Figure 3. *A comparison of gene expression (microarray (N=3) and qPCR (N=4)) results (Mean ± SEM) in Calanus finmarchicus after 48h exposure to sea water (negative control) and 5.0 µg/L* Hg^{2+} *for matrix metallopeptidase 9 (MMP9), matrix metallopeptidase 3 (MMP3), matrix metalloproteinase isoform d (MMP1); and Rh family B glycoprotein (RHBG). Significant difference from control (t-test, p>0.05) indicated by *.*

Discussion

A continuous culture of *C. finmarchicus* has over the last 12 years successfully been kept under controlled laboratory conditions at the NTNU/SINTEF Sealab (Hansen et al. 2007). Because of this, homogenous specimens in terms of developmental stage and age are routinely available. This facilitates its use as a test species in ecotoxicology and ecotoxicogenomics. The culture has been used to study the effects of a high number of stressors including metals (Øverjordet et al. 2014), mine tailings (Farkas et al. 2017), prooxidants (Hansen et al. Submitted), organic chemicals (Hansen et al. 2014; Hansen et al. 2010; Hansen et al. 2008), and ocean acidification (Pedersen et al. 2013) using standard ecotoxicological endpoints such as acute toxicity and reproduction. The present work is the first to present a combination of acute toxicity assessment and characterization of broad-content sublethal transcriptional responses occurring after exposure to Hg^{2+} in this copepod. The oligoarray used, which was based on NCBI genbank sequences, contain 6.6K probes whereof 50% annotated and about one third was identified as orthologs to model-species such as *D. melanogaster*, *D. rerio*, *H. sapiens*, *M. musculus* and *R. norwegicus*. Standardised GO enrichment and toxicity pathway analyses were used successfully to interrogate the molecular MoA and provide suggestions of adverse effects and compensatory mechanisms occurring in *C. finmarchicus* after exposure to Hg^{2+}. The technical performance of the array was verified by quantitative RTPCR on 4 randomly chosen sequences, and demonstrated comparable results to that of the array. The current microarray design is complementing a growing molecular toolbox for calanoids, where *de novo* transcriptomes for *C. finmarchicus, C. helgolandicus* and *C. sinicus* have recently become available (Carotenuto et al. 2014; Lenz et al. 2014; Lenz et al. 2012; Ning et al. 2013; Tarrant et al. 2014; Tarrant et al. 2016; Yang et al. 2014). Although the current array contained gaps in transcriptome coverage compared to some of the RNA sequencing efforts undertaken elsewhere (see suppl. information – oligoarray design), the high-content approach used herein provided an exploratory and hypothesis generating initiative to assess putative effects of inorganic mercury in

marine copepods such as *C. finmarchicus*. The need for using functional enrichment analysis devoid of FDR correction and ortholog mapping to non-invertebrates for biological pathway analysis illustrate that the current high-content approach still needs to be improved to become fully descriptive, however.

Acute toxicity

Mercury, historically used in a high number of industrial products, is a well-known toxicant in humans and wildlife (Boening 2000). The divalent form of mercury is the most common oxidation state for Hg and is detected frequently in the environment (Kim and Zoh 2012). Mercury (II) is highly toxic to a range of aquatic organisms, and copepods appear to have a species-dependent sensitivity to Hg^{2+}. Lethality (e.g. LC_{50}) has been reported in the range of 10-600 µg/L (EPA 2013), and marine copepods have been particularly sensitive to the chemical as observed for the marine copepod *Acartia tonsa* (48h LC_{50}=18.3 µg/L and 96h LC_{50}=14.8 µg/L) (Sosnowski and Gentile 1978). Mercury (II) chloride displayed a slightly lower toxicity to *C. finmarchicus* with a 48h LC_{50} of 43.1 µg/L in this study, and seems to correspond well with LC_{50} values for other calanoid species (Øverjordet et al. 2014). However, the LC_{50} values from the present work were slightly lower than that reported by the ECETOX database (https://cfpub.epa.gov/ecotox/) for copepods such as *Pseudodiaptomus coronatus* and *Eurytemora affinis* (96h LC_{50} concentrations of 79 µg/L and 158 µg/L, respectively), and suggest that Hg^{2+} was highly toxic to *C. finmarchicus*.

Transcriptional changes

Exposure to mercury may affect various biochemical processes relevant for the physiology and fitness of an organism, and display considerable tissue-specificity. Several genes of potential relevance for the toxicity of inorganic mercury in eukaryotes were identified in the current study

and hypothetical MoA in *C. finmarchicus* proposed below (see Figure 4, for an overview).

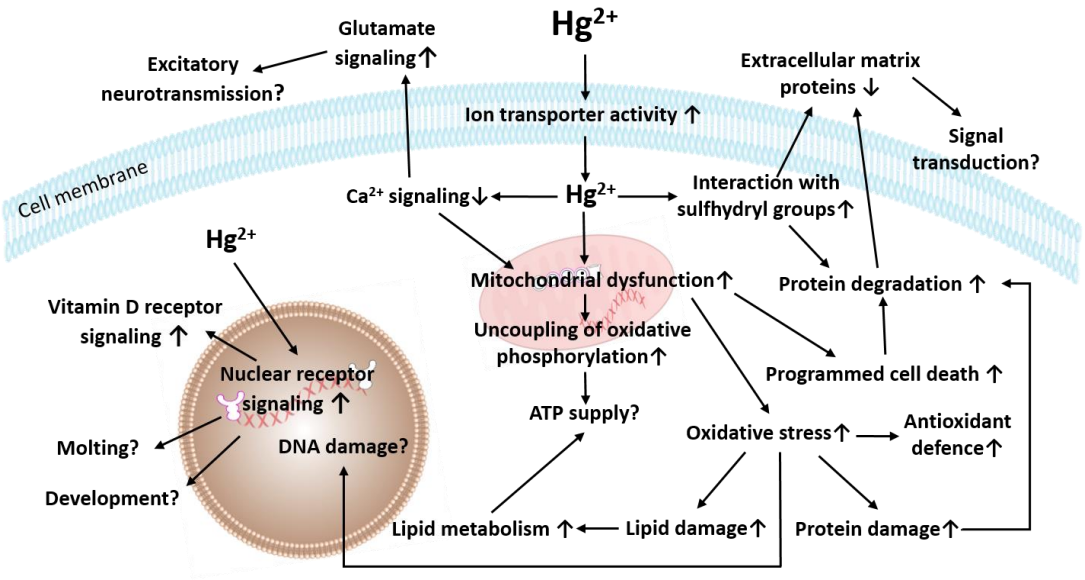

Figure 4. *Potential molecular modes of Action (MoA) of divalent mercury (Hg^{2+}) in Cala finmarchicus. The results depict hypothetical MoAs generated on basis of transcriptional char observed in Calanus finmarchicus after 48h exposure to 5.0 µg/L Hg^{2+} and review of known M of mercury in other eukaryotes.*

Cellular homeostasis and energetics

The transcriptional changes observed in the present study verified that *C. finmarchicus* was susceptible to sublethal Hg^{2+} exposure. A number of genes involved in the cellular antioxidant defence were induced at low ug/L concentrations to potentially compensate for both loss in radical scavenging proteins (e.g. GSH and TRX) and putative increase in oxidative stress occurring after Hg^{2+} exposure. A key mediator of the response to oxidative stress in eukaryotes is the activation of the nuclear factor-erythroid 2-related factor 2 (NRF2), which binds to the antioxidant response elements (ARE) and transactivates genes involved in repair and removal of damaged proteins, activate acute stress response proteins, increase detoxifying and antioxidant enzymes, and increase the total cellular antioxidant capacity in aquatic organisms (Lushchak 2011). Although not identified specifically herein, the main cellular

target of $Hg^{2+}$is often the mitochondria where it leads to uncoupling of OXPHOS by impairing the function of the protein CYCS, increasing ROS production, and directly depletes mitochondrial GSH (Menze et al. 2005; De Coen and Janssen 2003; Singaram et al. 2013). The current study demonstrated that genes encoding the protein AOX1, which is believed to catalyse the production of hydrogen peroxide (H_2O_2) from the superoxide radical in mitochondria (Valko, Morris, and Cronin 2005), was up-regulated as a potential indicator of mitochondrial ROS production. Retinol dehydrogenase 13 (*RDH13*), which protein products catalyzes the reduction and oxidation of retinoids in eukaryotes and is believed to protect the mitochondria against oxidative stress (Belyaeva et al. 2008), was also up-regulated. Increased expression of *MGST3,* suggested to be associated with glutathione-dependent peroxidase activity in several species, is causally related to the cellular detoxification of lipid hydroperoxides from cellular ROS (Chen et al. 2011). Although it has been demonstrated that mercury induces genes coding for the antioxidant enzymes GPX, GR, and TRXR in various organisms (De Coen and Janssen 2003; Singaram et al. 2013), these genes were not found to be differently regulated (*GPX*) or were not included on the current array design (*GR* and *TRXR*).

Mercury also displays high affinity for SH-groups and forms complexes with various thiol-compounds in eukaryotes, including GSH and selenoproteins such as thioredoxins (Farina et al. 2013). Up-regulation of protein disulfide isomerase (*PDI*), a gene encoding a member of the thioredoxin superfamily of redox proteins involved in cell redox homeostasis, protein folding and lipid metabolism (Wang and Tsou 1993), indicates that mercury may have depleted intracellular sulfhydryl-containing proteins in *C. finmarchicus*. Interestingly, glutathione-S-transferase (*GST*) that is involved in the conjugation of glutathione to hydrophobic and electrophilic compounds in eukaryotes (Eaton and Bammler 1999), was

down-regulated and contradict suggestions that *GST* are normally induced after exposure to mercury metals and oxidative agents (Lee et al. 2008; Hansen et al. 2008; Øverjordet et al. 2014). Limitation in transcriptome coverage for mercury-responsive *GSTs* on the current array design is likely explaining this discrepancy, as over 40 *GST* genes with considerable expression diversity and differential responsiveness to stressors have been identified in *C. finmarchicus* (Roncalli et al. 2015).

As OXPHOS is also dependent on products from glycolysis, the citric acid cycle, fatty acid and amino acid oxidation, interference with various metabolic processes may also affect the ATP production of animal cells (Mieiro et al. 2015; Nesci et al. 2016). The upregulation of DEGs involved in fatty acid synthesis from phospholipids (*PLA1*) and triglycerides (pancreatic lipase-related protein 2, *PNLIPRP2*), and down-regulation of DEGs involved in in long-chain fatty acyl CoA biosynthetic process (*ASBG2*) and fatty cell differentiation (steap family member 4, *STEAP4*) may indicate that mobilisation of short-chained fatty acids to compensate for reduction in ATP production occurred. Similar up-regulation of DEGs involved in gluconeogenesis (*PCK*) and downregulation of DEGs involved in carbohydrate metabolism (glucose) and transport (*SLC2A8*), suggest that compensatory mechanisms to increase free glucose may have been triggered to replenish energy after mercury exposure. Enhanced glycolysis to facilitate energy allocation for metal detoxification and increase tolerance suggest that these mechanisms are important in copepods after long term exposure to mercury (Xu, Shi, and Wang 2016).

Cellular growth, regulation and development

A potential key mechanism for mercury-enhanced intracellular ROS production is the destruction, inactivation and elimination of damaged cellular organelles and macromolecules (DNA, proteins,

and lipids). Apoptosis, necrosis and autophagy are all potentially involved in the elimination process and reconstruction of damaged cells and organelles after mercury exposure in eukaryotes (Menze et al. 2010). Several genes downstream key apoptotic regulatory genes, potentially indicative of cellular apoptosis and autophagy, were differentially regulated by mercury and provide insight into Hg^{2+} mediated toxicity in *C. finmarchicus*. The gene encoding SQSTM1, a ubiquitin binding protein that targets proteins for proteasomal degradation and regulates activation of the nuclear factor kappa-B (*NF-KB*)-mediated apoptotic signaling pathway in many organisms (Huang et al. 2013), was strongly up-regulated by Hg^{2+}. Concurrent up-regulation of *HSP90*, potentially involved in anti-apoptosis regulation and maintaining proteasome tertiary structure and cellular ATP content in eukaryotes (Lanneau et al. 2007; Imai et al. 2003), are supporting evidence for the potential activation of apoptosis and autophagosomal proteasome processes by Hg^{2+} in *C. finmarchicus.* Both apoptosis and autophagy involves lysosomal uptake and release of cathepsin proteases and activation of MMPs associated with degradation of extracellular matrixes (Menze et al. 2010). Interestingly, down-regulation of the genes cathepsin G (*CTSG*), *MMP1* and *MMP3* indicate triggering of molecular responses associated with cellular protection against tissue degradation associated with morphogenesis, angiogenesis, hematopoiesis, hypoxia, inflammatory and immune responses, or tumor formation in mammals. Up-regulation of *C. finmarchicus MGST3* (stimulate leukotrienes and prostaglandin E production) and moesin, *MSN* (regulation of lymphocyte migration and actin cytoskeleton organization), and down-regulation of *CHIA* (positive regulation of chemokine secretion and interleukin expression), suggest that Hg^{2+} may play a role in copepod inflammatory and immune responses, as observed for mammals elsewhere (Rice et al. 2014).

Neurotransmission.

Mercury has been proposed to cause neurotoxicity in mammals and vertebrates by interfering with

the normal function of the excitatory neurotransmitter glutamate (Aschner et al. 2000). The neurotoxic MoAs in arthropods and crustaceans are less characterized than in vertebrates, but many neurotoxic mechanisms are proposed to be conserved across taxa (Rousseaux 2008). The main neurotoxic MoAs of mercury in different organisms involve increased endogenous glutamate release and inhibition of glutamate re-uptake in the pre-synaptic ganglion leading to accumulation of glutamate and over-activation of N-methyl D-aspartate (NMDA)-type glutamate receptors. This over-excitation by Hg may cause increase in Na^+ and Ca^{2+} influx that leads to oxidative stress, damage to mitochondrial ATP production, loss of neuronal tissue function, and ultimately to neuronal death (Aschner et al. 2007). The present study suggest that the genes encoding the high-affinity glutamate transporter (EEAT3*)* and the neurotransmitter symporter (CPIJ015063-PA*)*, both believed to be involved in transport of glutamate from the synaptic cleft (Rousseaux 2008), were up-regulated to potentially increase removal of excessive glutamate from the synaptic cleft and ensure re-uptake in the pre-synaptic ganglia. Concurrent down-regulation of genes known to be involved in neurotransmitter secretion (*HSP70*) in vertebrates further support the hypothesis that Hg^{2+} increase the synaptic concentrations of neurotransmitters in *C. finmarchicus*. Increase in cellular Ca^{2+} would be expected to increase calmodulin-mediated activation of carbohydrate metabolism (glucose production) by up-regulating *PCK* and calcitonin-mediated increase in lipid metabolism (fatty acid synthesis) by activation of *PLA1* as seen herein. Down-regulation of the calcineurin repressor *CABIN1* and up-regulation of tenascin *TENA*, a gene encoding extracellular matrix proteins as observed herein could be taken as support for stimulation of inflammatory responses to remove damaged neuronal tissue and stimulate tissue remodeling.

Nuclear receptor signalling

Nuclear receptors (NR), a group of transcription factor found in all major metazoans, functions as sensors and transducer of various endogenous and exogenous signals (stressors and ligands) to

regulate expression of genes involved in development, homeostasis, and metabolism (Hwang et al. 2014; Evans and Mangelsdorf 2014). Several NR signalling pathways were apparently activated in *C. finmarchicus*, including that of the estrogen (ER), arylhydrocarbon (AhR) and vitamin D (VDR) receptor. A common feature of all these NR signalling pathways was the up-regulation of *NCOA2*, a co-activator for cytosolic (ER and AhR) and nuclear (VDR) NRs, and up-regulation of the NR co-repressor *Hsp90*, thus indicating that one or more of the NR signalling pathways were affected. As ER and AhR presence and function in crustaceans have been questioned (Kohler et al. 2007; Hahn 2002), modulation of VDR/RXR activity seems to be the most plausible explanation for this activity. A key factor in the activation of VDR/RXR is the up-regulation of the mitochondrial cytochrome P450 12b1 (*CYP12B1*) gene, an arthropod homologue to the vertebrate *CYP24A1* (Danielson and Fogleman 1997), which protein product catalyse the degradation of vitamin D3 and maintain calcium homeostasis by stimulating absorption and reduce release of Ca^{2+} in different tissues. Reduction of cellular vitamin D3 levels would also potentially reduce VDR/RXR activity and likely also release NCOA2 and RXR to engage as a co-activator with other NRs (Dawson and Xia 2012). Although the role of the *NCOA2* gene in arthropod NR-mediated processes is not entirely clear, interaction of nuclear coactivators with estrogen, retinoic and glucocorticoid signalling has been proposed (Baudino et al. 1998). Other key NRs that may potentially be modulated by co-regulators such as *NCOA2* and *HSP* gene isomers is the methylfarnesoate (MFR) receptor and ecdysone receptor (ECR) (Bernardo and Dubrovsky 2012; Tran et al. 2001). Although DEGs to support direct interaction with these receptors was not observed in the present study, up-regulation of *JHE* that encode enzymes responsible or catalysing the hydrolysis of juvenile hormone (i.e. methylfarnesoate in crustaceans), may potentially suggest that mercury directly or indirectly perturb MFR-mediated processes associated with life-stage dependent metamorphosis in *C. finmarchicus*.

Conclusion

The present study has clearly demonstrated that Hg^{2+} caused mortality at low microgram per liter concentrations in *C. finmarchicus.* A number of biological pathway were apparently perturbed at the molecular level at even lower exposure concentrations, whereof some were considered of larger toxicological relevance than others This applies in particular to transcriptional evidence supporting 1) uncoupling of OXPHOS in mitochondria leading to reduction of ATP-production, 2) induction of oxidative stress and cellular damage by overproduction of ROS and/or reduction of cellular antioxidant defenses, 3) direct interactions with sulfhydryl groups to inactivate key enzymes and proteins, 4) increase in apoptosis and autophagocytosis (protein degradation) of damaged organelles and tissues, 5) disruption of calcium homeostasis and signaling, 6) over-excitation of glutamate receptors (neurotoxicity), and 7) modulation of VDR/RXR activity (see Figure 4 for details). The current approach, which was exploratory and hypothesis-generating in principle, was based on a transcriptional profiling using an oligoarray with partial transcriptome coverage and were not specifically addressing temporal- nor concentration-dependent responses. Although useful, phenotypical anchoring to functional changes associated with the MoA proposed would be required to thoroughly assess if the transcriptional changes observed would lead to adverse effects of relevance in *C. finmarchicus*. Nevertheless, the present study has proposed a suite of biomarkers that can be used in combination with functional endpoints to assess potential toxic MoA of inorganic mercury in copepods.

Acknowledgements

Authors would like to thank Stine Kooyman (NIVA/NMBU, Norway) and Kenneth Macrae (NIVA/Norwegian Environment Agency, Norway) for RNA extraction, Mie L. Jareid (NIVA/NMBU) for running the microarray analysis and Ann M. Tarrant (Woods Hole Oceanographic Institution, USA) for providing annotation information on the *Calanus*

finmarchicus de novo transcriptome assembly. Authors gratefully acknowledge financial support from the Norwegian Research Council (project no. 196711, 268294, 223268), the Norwegian Institute for Water Research (NIVA) and SINTEF Materials and Chemistry (Calanomics project).

References

Asai, S., A. Ianora, C. Lauritano, P. K. Lindeque, and Y. Carotenuto. 2015. High-quality RNA extraction from copepods for Next Generation Sequencing: A comparative study. *Mar. Genomics* 24 Pt 1:115-8.

Aschner, M., T. Syversen, D. O. Souza, J. B. Rocha, and M. Farina. 2007. Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. *Braz. J. Med Biol Res* 40 (3):285-91.

Aschner, M., C. P. Yao, J. W. Allen, and K. H. Tan. 2000. Methylmercury alters glutamate transport in astrocytes. *Neurochem Int* 37 (2-3):199-206.

Baudino, T. A., D. M. Kraichely, S. C. Jefcoat, Jr., S. K. Winchester, N. C. Partridge, and P. N. MacDonald. 1998. Isolation and characterization of a novel coactivator protein, NCoA-62, involved in vitamin D-mediated transcription. *J Biol Chem* 273 (26):16434-41.

Belyaeva, O. V., O. V. Korkina, A. V. Stetsenko, and N. Y. Kedishvili. 2008. Human retinol dehydrogenase 13 (RDH13) is a mitochondrial short-chain dehydrogenase/reductase with a retinaldehyde reductase activity. *Febs J.* 275 (1):138-47.

Bernardo, T.J., and E.B. Dubrovsky. 2012. The Drosophila Juvenile Hormone Receptor Candidates Methoprene-tolerant (MET) and Germ Cell-expressed (GCE) Utilize a Conserved LIXXL Motif to Bind the FTZ-F1 Nuclear Receptor. *J Biol Chem* 287 (10):7821-7833.

Bindea, G., B. Mlecnik, H. Hackl, P. Charoentong, M. Tosolini, A. Kirilovsky, W. H. Fridman, F. Pages, Z. Trajanoski, and J. Galon. 2009. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 25 (8):1091-3.

Boening, D. W. 2000. Ecological effects, transport, and fate of mercury: a general review. *Chemosphere* 40 (12):1335-51.

Campbell, R.G., M.H. Wagner, G.J. Teegarden, C.A: Boudreau, and E.G. Durbin. 2001. Growth and development rates of the copepod *Calanus finmarchicus* reared in the laboratory. *Mar Ecol Prog Series* 221:161-183.

Carotenuto, Y., E. Dattolo, C. Lauritano, F. Pisano, R. Sanges, A. Miralto, G. Procaccini, and A. Ianora. 2014. Insights into the transcriptome of the marine copepod *Calanus helgolandicus* feeding on the oxylipin-producing diatom Skeletonema marinoi. *Harmful Algae* 31:153-162.

Chen, J., S. Xiao, Y. Deng, X. Du, and Z. Yu. 2011. Cloning of a novel glutathione S-transferase 3 (GST3) gene and expressionanalysis in pearl oyster, *Pinctada martensii*. *Fish Shellfish Immunol* 31 (6):823-30.

Danielson, P. B., and J. C. Fogleman. 1997. Isolation and sequence analysis of cytochrome P450 12B1: the first mitochondrial insect P450 with homology to 1 alpha,25 dihydroxy-D3 24-hydroxylase. *Insect Biochem Mol Biol* 27 (6):595-604.

Dawson, M.I., and Z. Xia. 2012. The retinoid X receptors and their ligands. *Biochim biophys acta* 1821 (1):21-56.

De Coen, W. M., and C. R. Janssen. 2003. A multivariate biomarker-based model predicting population-level responses of *Daphnia magna*. *Environ Toxicol Chem* 22 (9):2195-201.

Eaton, D. L., and T. K. Bammler. 1999. Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol Sci* 49 (2):156-64.

EPA, US. 2013. ECOTOX Database. http://cfpub.epa.gov/ecotox/. 28.03.2013.

Evans, R.M., and D.J. Mangelsdorf. 2014. Nuclear Receptors, RXR & the Big Bang. *Cell* 157 (1):255-266.

Farina, M., D. S. Avila, J. B. da Rocha, and M. Aschner. 2013. Metals, oxidative stress and neurodegeneration: a focus on iron, manganese and mercury. *Neurochem Int* 62 (5):575-94.

Farina, M., J. B. Rocha, and M. Aschner. 2011. Mechanisms of methylmercury-induced neurotoxicity: evidence from experimental studies. *Life Sci* 89 (15-16):555-63.
Farkas, J., D. Altin, K. M. Hammer, K. C. Hellstrom, A. M. Booth, and B. H. Hansen. 2017. Characterisation of fine-grained tailings from a marble processing plant and their acute effects on the copepod *Calanus finmarchicus*. *Chemosphere* 169:700-708.
Hahn, M. E. 2002. Aryl hydrocarbon receptors: diversity and evolution. *Chem Biol Interact* 141 (1-2):131-60.
Hansen, B. H., D. Altin, K. Bonaunet, and I. B. Overjordet. 2014. Acute toxicity of eight oil spill response chemicals to temperate, boreal, and Arctic species. *J Toxicol Environ Health A* 77 (9-11):495-505.
Hansen, B. H., D. Altin, A. Booth, S. H. Vang, M. Frenzel, K. R. Sorheim, O. G. Brakstad, and T. R. Storseth. 2010. Molecular effects of diethanolamine exposure on *Calanus finmarchicus* (Crustacea: Copepoda). *Aquat Toxicol* 99 (2):212-22.
Hansen, B. H., D. Altin, T. Nordtug, and A. J. Olsen. 2007. Suppression subtractive hybridization library prepared from the copepod *Calanus finmarchicus* exposed to a sublethal mixture of environmental stressors. *Comp Biochem Physiol Part D Genomics Proteomics* 2 (3):250-6.
Hansen, B. H., D. Altin, S. H. Vang, T. Nordtug, and A. J. Olsen. 2008. Effects of naphthalene on gene transcription in *Calanus finmarchicus* (Crustacea: Copepoda). *Aquat Toxicol* 86 (2):157-65.
Hansen, B. H., I. Salaberria, A. J. Olsen, K. E. Read, I. B. Overjordet, K. M. Hammer, D. Altin, and T. Nordtug. 2015. Reproduction dynamics in copepods following exposure to chemically and mechanically dispersed crude oil. *Environ Sci Technol* 49 (6):3822-9.
Hansen, B.H., Hallmann, D. Altin, B.M. Jenssen, and T. Ciesielski. Submitted. Acute hydrogen peroxide exposure does not cause oxidative stress in late-copepodite stage of *Calanus finmarchicus*. *J Toxicol Environ Health A* (Current Issue).
Huang, S., K. Okamoto, C. Yu, and F. A. Sinicrope. 2013. p62/sequestosome-1 up-regulation promotes ABT-263-induced caspase-8 aggregation/activation on the autophagosome. *J Biol Chem* 288 (47):33654-66.
Hwang, D.S., B.Y. Lee, H.S. Kim, M.C. Lee, D.H. Kyung, A.S. Om, J.S. Rhee, and J.S. Lee. 2014. Genome-wide identification of nuclear receptor (NR) superfamily genes in the copepod *Tigriopus japonicus*. *BMC Genomics* 15 (1):993.
Imai, J., M. Maruya, H. Yashiroda, I. Yahara, and K. Tanaka. 2003. The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. *EMBO J* 22 (14):3557-3567.
ISO. 2000. ISO 14669:1999 Water quality - Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea). International Organization for Standardization.
Jaschnov, W. A. 1970. Distribution of Calanus Species in the Seas of the Northern Hemisphere. *Int Rev Gesamt Hydrobiol Hydrographie* 55 (2):197-212.
Kim, M.K., and K.D. Zoh. 2012. Fate and Transport of Mercury in Environmental Media and Human Exposure. *J Prev Med Public Health* 45 (6):335-343.
Kohler, H. R., W. Kloas, M. Schirling, I. Lutz, A. L. Reye, J. S. Langen, R. Triebskorn, R. Nagel, and G. Schonfelder. 2007. Sex steroid receptor evolution and signalling in aquatic invertebrates. *Ecotoxicol* 16 (1):131-43.
Lanneau, D., A. de Thonel, S. Maurel, C. Didelot, and C. Garrido. 2007. Apoptosis Versus Cell Differentiation. *Prion* 1 (1):53-60.
Lauritano, C., Y. Carotenuto, V. Vitiello, I. Buttino, G. Romano, J. S. Hwang, and A. Ianora. 2015. Effects of the oxylipin-producing diatom *Skeletonema marinoi* on gene expression levels of the calanoid copepod *Calanus sinicus*. *Mar Genomics* 24 Pt 1:89-94.
Lee, K. W., S. Raisuddin, J. S. Rhee, D. S. Hwang, I. T. Yu, Y. M. Lee, H. G. Park, and J. S. Lee. 2008. Expression of glutathione S-transferase (GST) genes in the marine copepod *Tigriopus japonicus* exposed to trace metals. *Aquat Toxicol* 89 (3):158-66.
Lenz, P. H., V. Roncalli, R. P. Hassett, L. S. Wu, M. C. Cieslak, D. K. Hartline, and A. E. Christie. 2014. De novo assembly of a transcriptome for *Calanus finmarchicus* (Crustacea, Copepoda)-the dominant zooplankter of the North Atlantic Ocean. *PLoS One* 9 (2):e88589.
Lenz, P. H., E. Unal, R. P. Hassett, C. M. Smith, A. Bucklin, A. E. Christie, and D. W. Towle. 2012. Functional genomics resources for the North Atlantic copepod, *Calanus finmarchicus*: EST database and physiological microarray. *Comp Biochem Physiol Part D Genomics Proteomics* 7 (2):110-23.

Lund, B. O., D. M. Miller, and J. S. Woods. 1993. Studies on Hg(II)-induced H2O2 formation and oxidative stress in vivo and in vitro in rat kidney mitochondria. *Biochem Pharmacol* 45 (10):2017-24.
Lushchak, V. I. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol* 101 (1):13-30.
Maere, S., K. Heymans, and M. Kuiper. 2005. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* 21 (16):3448-9.
Mayor, D. J., C. Matthews, K. Cook, A. F. Zuur, and S. Hay. 2007. CO2-induced acidification affects hatching success in *Calanus finmarchicus*. *Mar Ecol Prog Series* 350:91-97.
Menze, M. A., G. Fortner, S. Nag, and S. C. Hand. 2010. Mechanisms of apoptosis in Crustacea: What conditions induce versus suppress cell death? *Apoptosis* 15 (3):293-312.
Menze, M. A., K. Hutchinson, S. M. Laborde, and S. C. Hand. 2005. Mitochondrial permeability transition in the crustacean *Artemia franciscana*: absence of a calcium-regulated pore in the face of profound calcium storage. *Am J Physiol Regul Integr Comp Physiol* 289 (1):R68-76.
Mieiro, C. L., M. Pardal, A. Duarte, E. Pereira, and C. M. Palmeira. 2015. Impairment of mitochondrial energy metabolism of two marine fish by in vitro mercuric chloride exposure. *Mar Pollut Bull* 97 (1-2):488-93.
Nesci, S., F. Trombetti, M. Pirini, V. Ventrella, and A. Pagliarani. 2016. Mercury and protein thiols: Stimulation of mitochondrial F1FO-ATPase and inhibition of respiration. *Chem Biol Interact* 260:42-49.
Ning, J., M. Wang, C. Li, and S. Sun. 2013. Transcriptome sequencing and de novo analysis of the copepod *Calanus sinicus* using 454 GS FLX. *PLoS One* 8 (5):e63741.
Pedersen, S. A., B. H. Hansen, D. Altin, and A. J. Olsen. 2013. Medium-term exposure of the North Atlantic copepod *Calanus finmarchicus* (Gunnerus, 1770) to CO_2-acidified seawater: effects on survival and development. *Biogeosciences* 10 (11):7481-7491.
Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29 (9):e45.
Rice, K. M., E. M. Walker, Jr., M. Wu, C. Gillette, and E. R. Blough. 2014. Environmental mercury and its toxic effects. *J Prev Med Public Health* 47 (2):74-83.
Roncalli, V., M. C. Cieslak, Y. Passamaneck, A. E. Christie, and P. H. Lenz. 2015. Glutathione S-Transferase (GST) Gene Diversity in the Crustacean *Calanus finmarchicus*-Contributors to Cellular Detoxification. *PLoS One* 10 (5):e0123322.
Rousseaux, C.G. 2008. A Review of Glutamate Receptors I: Current Understanding of Their Biology. *J Toxicol Pathol* 21 (1):25-51.
Sakshaug, E., A. Bjørge, B. Gulliksen, H. Loeng, and F. Mehlum. 1992. *Økosystem Barentshavet (in Norwegian). Norwegian Research Program for Marine Arctic Ecology (Promare). Universitetsforlaget, Oslo.*
Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 13 (11):2498-504.
Singaram, G., T. Harikrishnan, F. Y. Chen, J. Bo, and J. P. Giesy. 2013. Modulation of immune-associated parameters and antioxidant responses in the crab (*Scylla serrata*) exposed to mercury. *Chemosphere* 90 (3):917-28.
Sosnowski, S., and J. Gentile. 1978. Toxicological comparison of natural and cultured populations of *Acartia tonsa* to cadmium, copper and mercury. *J Fish Res Canada* 35:1366-1369.
Stohs, S. J., and D. Bagchi. 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med* 18 (2):321-36.
Tarrant, A. M., M. F. Baumgartner, B. H. Hansen, D. Altin, T. Nordtug, and A. J. Olsen. 2014. Transcriptional profiling of reproductive development, lipid storage and molting throughout the last juvenile stage of the marine copepod *Calanus finmarchicus*. *Front Zool* 11 (1):91.
Tarrant, A. M., M. F. Baumgartner, N. S. Lysiak, D. Altin, T. R. Storseth, and B. H. Hansen. 2016. Transcriptional Profiling of Metabolic Transitions during Development and Diapause Preparation in the Copepod *Calanus finmarchicus*. *Integr Comp Biol*.

Tran, H. T., H. B. Askari, S. Shaaban, L. Price, S. R. Palli, T. S. Dhadialla, G. R. Carlson, and T. R. Butt. 2001. Reconstruction of ligand-dependent transactivation of *Choristoneura fumiferana* ecdysone receptor in yeast. *Mol Endocrinol* 15 (7):1140-53.

Valko, M., H. Morris, and M. T. Cronin. 2005. Metals, toxicity and oxidative stress. *Curr Med Chem* 12 (10):1161-208.

Voznesensky, M., P. H. Lenz, C. Spanings-Pierrot, and D. W. Towle. 2004. Genomic approaches to detecting thermal stress in *Calanus finmarchicus* (Copepoda: Calanoida). *J Exp Mar Biol Ecol* 311:37-46.

Wang, C. C., and C. L. Tsou. 1993. Protein disulfide isomerase is both an enzyme and a chaperone. *Faseb J* 7 (15):1515-7.

Xu, X., L. Shi, and M. Wang. 2016. Comparative quantitative proteomics unveils putative mechanisms involved into mercury toxicity and tolerance in *Tigriopus japonicus* under multigenerational exposure scenario. *Environ Pollut* 218:1287-1297.

Yang, Q., F. Sun, Z. Yang, and H. Li. 2014. Comprehensive transcriptome study to develop molecular resources of the copepod *Calanus sinicus* for their potential ecological applications. *Biomed Res Int* 2014:493825.

Øverjordet, I.B., D. Altin, T. Berg, B.M. Jenssen, G.W. Gabrielsen, and B.H. Hansen. 2014. Acute and sub-lethal response to mercury in Arctic and boreal calanoid copepods. *Aquat Toxicol* 155:160-165.

Supplementary information – Oligoarray design

Mortality and transcriptional effects of inorganic mercury in the marine copepod *Calanus finmarchicus*.

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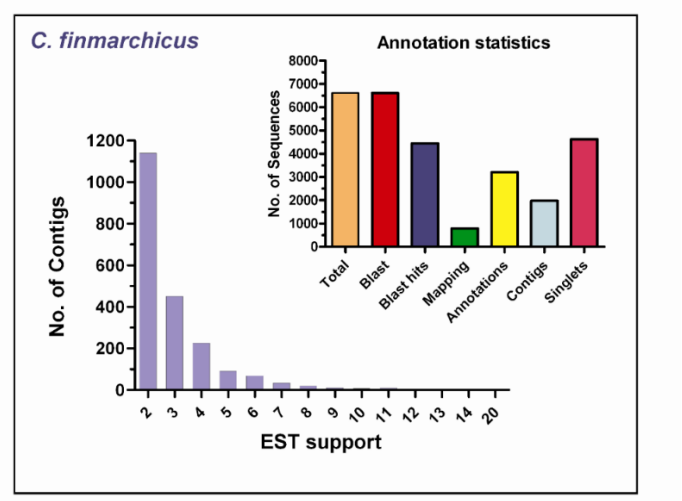
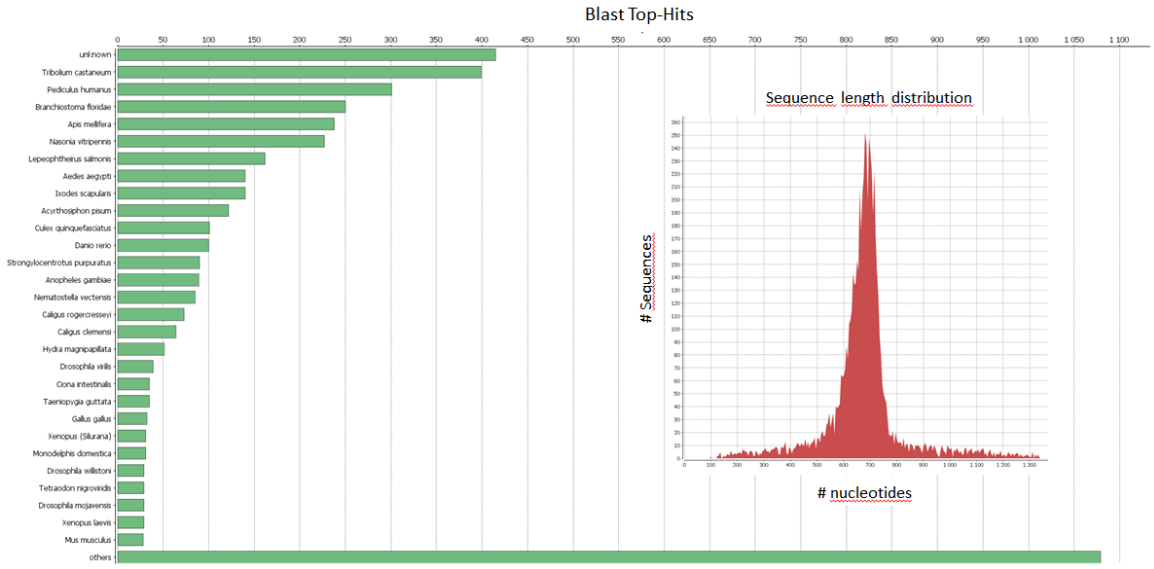
Oligoarray design

The 6.6k *Calanus finmarchicus* oligoarray (60-mer) were designed on basis of more than 11k Genbank ESTs, resulting into 1877 contigs and 4633 singletons (whereof 49% annotated) when subjected to ESTExplorer contig clustering and assembly followed by probe and sequence redundanct reduction (Supplementary Figure 1, insert). The majority of the contigs (~1800) were composed of 2-4 ESTs, whereas the last 400 were contigs constructed of 5-9 ESTs (Supplementary Figure 1, main). The probes were replicated at least in duplicate on the array.



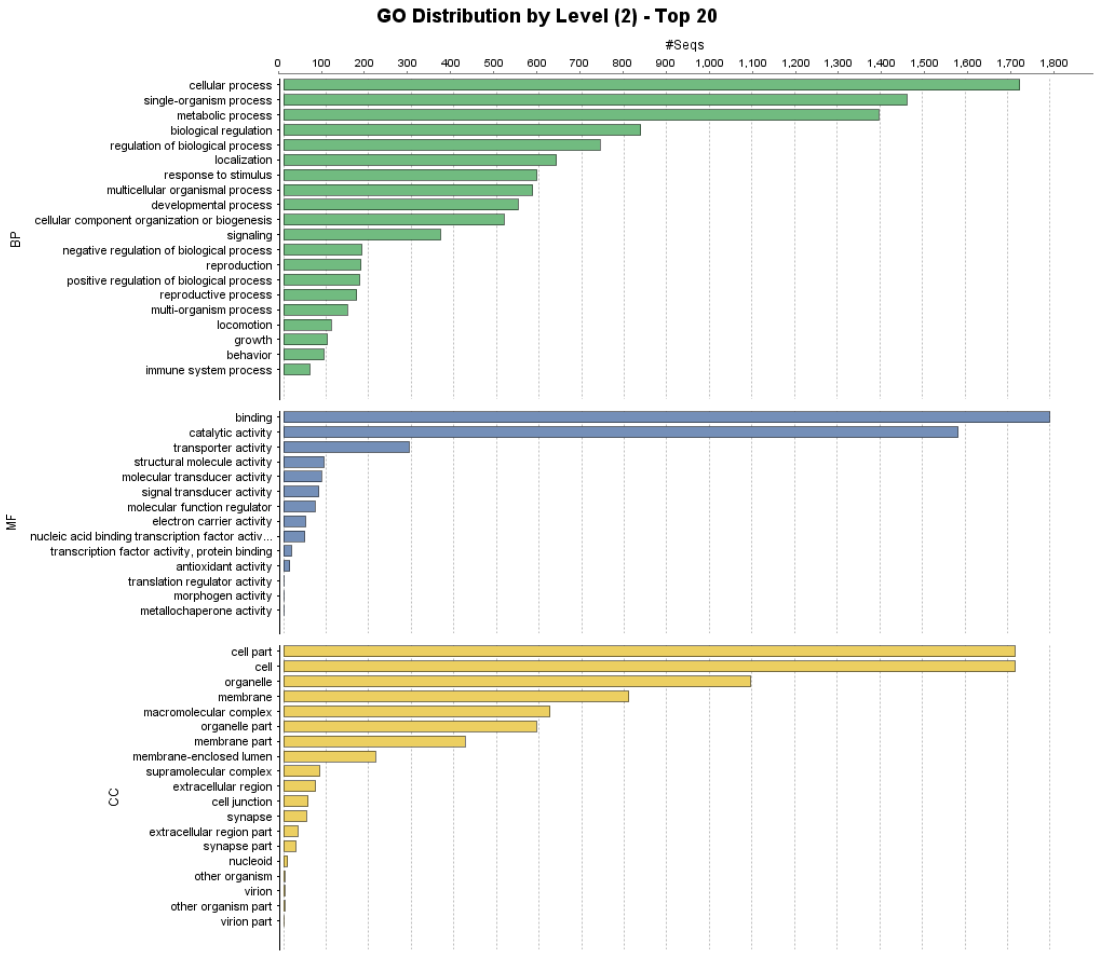
Supplementary Figure 1. *Contig assembly (main) and annotation statistics (insert) for the 6.6k Calanus finmarchicus oligonucleotide array.*

The length of resulting contigs and singletons showed that the majority of sequences were in the 500-750 nucleotide range (Supplementary Figure 2, insert). Blasting against the non-redundant and Swissprot database were successfully providing ~4200 sequences with high-quality blast hits, whereof a majority of the top hits were to crustaceans, insects and other invertebrates (Supplementary Figure 2, main). Top blast hits towards echinoideas (*strongylocentrotus purpuratus*), anemones (*Nematostella vectensis),* hydrozoa *(hydra magnipapilata),* tunicates (*Ciona intestinalis),* indicated that the calanus sequences displayed similarities to other aquatic invertebrates. Sequence similarity to fish (*Danio rerio, Tetraodon nigroviridis*), birds (*Taeniopygia guttata, Gallus gallus*), amphibians (*Xenopus* SP.), oppossums (*Monodelphis domestica*) and mice (*Mus musculus*) suggested that many of the sequences were also conserved between more evolutionary divergent species.



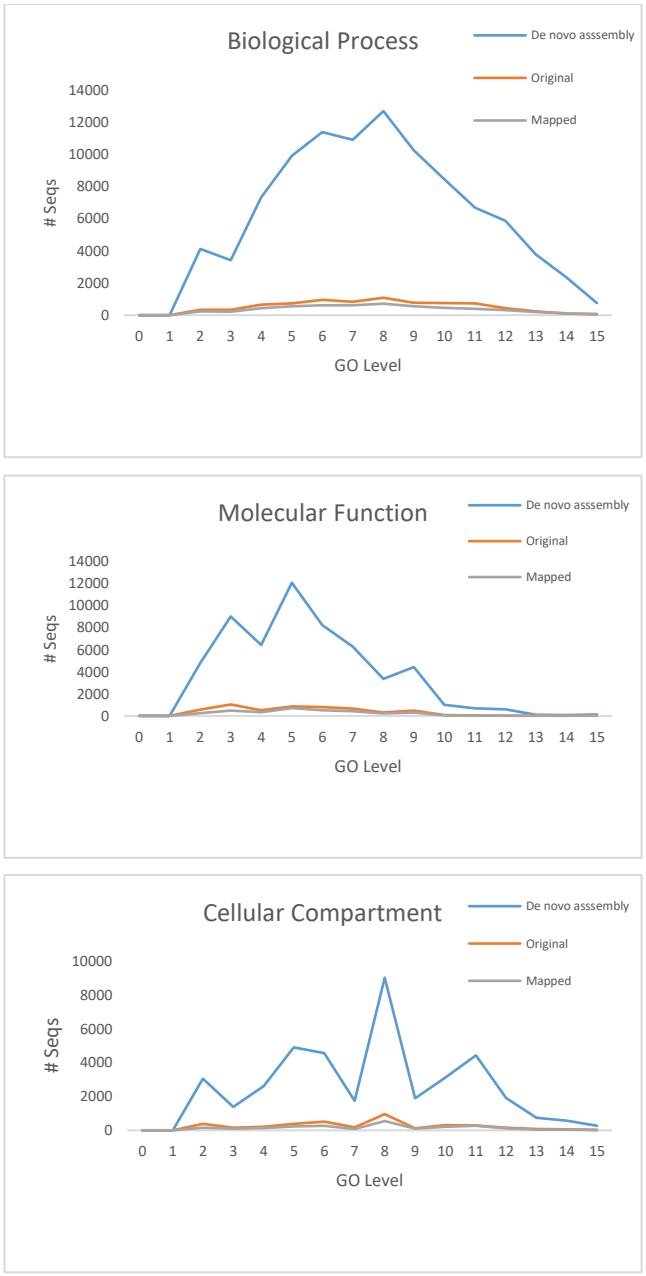
Supplementary Figure 2. *Blast Top-hits (main) and EST/Contig length distribution (insert) for sequences used for designing the 6.6k Calanus finmarchicus oligoarray probes.*

Mapping and annotation of the sequences to the Blast2Go database demonstrated that the array probes covered a wide range of genes representing 963 Gene ontologies (GOs) for molecular function, 2050 GOs for biological processes and 452 GOs for cellular compartments. Supplementary Figure 3 display the top twenty GO terms for GO categories biological processes (BP), molecular functions (MF) and cellular compartments (CC).



Supplementary Figure 3. *Top 20 Gene ontology (GO) annotation for the nucleotide sequences used for designing the 6k Calanus finmarchicus oligoarray.*

About 6.2k out of the 6.6k sequences (>94%), whereof about 3.6k (~55%) being annotated, were successfully mapped to the 200 k *de novo* transcriptome assembly by Tarrant et al. (2014) using Blast2GO (BlastX E-value: ≤1.0E-6, default blast2GO parameters). Supplementary Table S3_Blast Tarrant 2014 can be consulted for details about sequence alignments, species top hits and annotation information. The number of oligoarray sequences mapped to GO terms by Blast2GO were typically 4-35% of the total GO terms mapped to the *de novo* transcriptome, and were found to vary considerable with the GO level and GO categories (Supplementary Figure 4). The 3.6k oligoarray sequences successfully mapped to the *de novo* transcriptome assembly by Blast2GO represented 5.9-18% of the annotated GO terms for the de novo transcriptome assembly by Tarrant et al. (2014).



Supplementary Figure 4. *Comparison of sequences annotated to different Gene Ontology (GO) categories and GO levels of the oligoarray design (original), the oligoarray sequences mapped to the Tarrant et al. (2014) de novo transcriptome assembly (Mapped) and the Tarrant et al. (2014) de novo transcriptome assembly itself (De novo assembly).*

Reciprocal blast against model organisms suggested that *C. finmarchicus* exhibited considerable number of orthologs (33-39%) to a broad array of different taxa (Supplementary Table 1).

Supplementary table 1. *Identification of Drosophila melanogaster (D. melanogaster), Danio rerio (D. rerio), Homo sapiens (H. sapiens) Mus musculus (M. musculus), Rattus norvegicus (R. norvegicus) and Saccharomyces cerevisiae (S. cerevisiae) orthologs as proxies for Calanus finmarchicus gene sequences.*

	D. Melanogaster	*D. rerio*	*H. sapiens*	*M. musculus*	*R. norwevicu*	*S. cerevisiae*
Orthologs	*2341*	*2594*	*2493*	*2468*	*2437*	*2204*