Transcriptional changes in Atlantic salmon (*Salmo salar*) after embryonic exposure to road salt

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Highlights:

- Exposure to 5000 mg/L road salt (NaCl) the first 24h after fertilization caused global transcriptional changes detected at the eyed egg stage in Atlantic salmon.
- Main transcriptional changes indicate interference with osmoregulation, ionregulation, oxidative stress, metabolism, renal function and development in the embryos.
- Effects in selected biomarker for exposure and effect occurred as low as 100 mg/L road salt (NOTEL 50 mg/L)

Key words:

Road salt, Atlantic salmon, transcriptional effects, osmoregulation, ionregulation, oxidative stress, metabolism, renal function, development.

Abstract

Road salt is extensively used as a deicing chemical in road maintenance during winter and has in certain areas of the world led to density stratifications in lakes and ponds, and adversely impacted aquatic organisms in the recipients of the road run-off. Aquatic vertebrates such as fish have been particularly sensitive during fertilisation, as the fertilisation of eggs involves rapid uptake of the surrounding water, reduction in egg swelling and in ovo exposure to high road salt concentrations. The present study aimed to identify the persistent molecular changes occurring in Atlantic salmon (*Salmo salar*) eggs after 24h exposure to high concentrations (5000 mg/L) of road salt at fertilisation. The global transcriptional changes were monitored by a 60k salmonid microarray at the eyed egg stage (Clarvay stage, 255 degree days after fertilisation) and identified a high number of transcripts being differentially regulated. Functional enrichment, pathway and gene-gene interaction analysis identified that the differentially expressed genes (DEGs) were mainly associated with toxiciologically relevant processes involved in osmoregulation, ionregulation, oxidative stress, metabolism (energy turnover), renal function and developmental in the embryos. Quantitative rtPCR analysis of selected biomarkers, identified by global transcriptomics, were monitored in the eggs for an extended range of road salt concentrations (0, 50, 100, 500 and 5000 mg/L) and revealed a positive concentration-dependent increase in Cyp4A14, a gene involved in lipid turnover and renal function, and Nav1, a gene involved in neuraxonal development. Biomarkers for osmoregulatory responses (ATP1A2), the main sodium/potassium ATP-fueled transporter for chlorid ions and oxidative stress (Txndc9), a gene involved in regulation of cell redox homeostasis, displayed apparent concentration-dependency with exposure, although large variance in the control group precluded robust statistical discrimination between the groups. A No Transcriptional Effect Level (NOTEL) of 50

mg/L road salt was found to be several orders of magnitude lower than the adverse effects documented in developing fish embryos elsewhere, albeit at concentrations realistic in lotic systems receiving run-off from road salt. It remains to be determined whether these transcriptional changes may cause adverse effects in fish at ecologically relevant exposure concentrations of road salt.

1 Introduction

In northern North America and Europe, road salt (mainly NaCl with minor contributions of other salts scuh as $Ca2^{+}$, $Mg2^{+}$, $SO42^{+}$, anticaking agents and trace impurities) is extensively used as a deicing chemical for road maintenance during winter (Kelly et al., 2008). Sodium chloride (NaCl) is by far the most the preffered deicing chemical (Canada, 2001). For example, 99.5% of the annual applied deicing chemical on Norwegian roads is NaCl (Sivertsen, 2012). In Norway there has been a significant increase in the amount of annually applied road salt, and since year 2000 this amount has more than tripled (Kronvall, 2013). This is of concern as prolonged application of road salt may cause negative effects on the aquatic environment (Sanzo and Hecnar, 2006). For example, increase in road salt due to deicing operations has led to density stratification in lakes and ponds, reduced water circulation and subsequently oxygen depletion in the hypolimnion (Kjensmo, 1997; Novotny et al., 2008). Although road salt concentrations are quickly diluted in running waters, it is now evident that also streams may be severly affected (Corsi et al., 2015; Ramakrishna and Viraraghavan, 2005). Road salt concentrations can vary considerably in different waterbodies, and concentrations in the range of 150-5000 mg/L NaCl have been reported in rural lakes and urban impoundment lakes (Canada, 2001). Such elevated concentrations of NaCl have been reported to cause negative impact on survival of rainbow trout at concentrations ranging from 2000-4560 mg/L (Vosylienė et al., 2006), reduction in biodiversity in water bodies and wetland areas by affecting primary producers and invertebrate communities at concentrations above 220 mg Cl^{-}/L (Canada, 2001), and enhanced the toxicity of other stressors such as Cu, Zn, Ni, Pb, Cr, Cd, and Hg (Anderson et al., 1995; Ramakrishna and Viraraghavan, 2005). Early life stages of different aquatic organisms such as Atlantic salmon are particularily susceptible to road salt (Mahrosh et al., 2014) and are at risk during periods of the

year when emissions are large. Increased mortality, reduced weight, reduced activity, and increased physical abnormalities are some of the effects observed by high road salt concentrations (2636-5109 mg/L) in other aquatic vertebrates such as wood frog (*Rana sylvatica*) larvae (Sanzo and Hecnar, 2006).

In Norway, the initial need for deicing of roads often co-occurs with sensitive periods such as spawning of salmonids. Atlantic salmon (*Salmo salar* L.) is highly sensitive to road salt, especially during the early developmental stages, from fertilization until hatching (Mahrosh et al., 2014). Episodic salting activities and run-off to nearby rivers and streams are thus expected to affect the water quality at critical windows of salmon development. Road salt has been reported to reduce egg swelling and hence inhibit the formation of the perivitelline space (Li et al., 1989) that ultimately may lead to egg mortality (Mahrosh et al., 2014; Yang and Chen, 2006). Although not studied for road salt specifically, reduction in embryo viability due to osmoregulatory stress has been demonstrated in Nile tilapia (*Oreochromis niloticus*) at salinites ranging from 15,000-32,000 mg/L (Fridman et al., 2012), and thus introduce the possibility that road salt may also display different toxic modes of action (MOA) in fish. Road salts are believed to also influence hatching of fish eggs by affecting the buoyancy in water (Murashige et al., 1991), and cause deformities when exposed during fertilization and swelling (Mahrosh et al., 2014). Different abnormalities such as spinal curvature and tail flexure have also been reported after road salt and NaCl exposure (Haddy and Pankhurst, 2000; Mahrosh et al., 2014). As developing fish embryo undergoes a number of different processes involved in organ development and differentiation, these organisms may be susceptible to external stressors during critical windows of development. Perturbations of any of these processes beyond the boundaries

of homeostasis may potentially lead to adverse effects such as loss of viability, delayed maturation, malformations and non-functional organs (Dave and Xiu, 1991; Johnson et al., 2007).

Although adverse effects are often directly associated with regulatory-relevant endpoints such as survival, growth, development and reproduction, initial interactions between the stressors and their biological targets normally occur at the molecular level (Miracle and Ankley, 2005). The current study aimed to characterize the transcriptional changes occurring in Atlantic salmon eggs at the eyed egg stage (Clarvay stage, typically at 255 degree days after fertilisation) after a 24h exposure to environmentally relevant road salt concentrations during fertilization. Global transcriptomics analysis by a 60k oligonucleotide salmonid microarray was used to characterize the MoA and assemble information about toxicity pathways affected by the exposure. Concentration-response curves for a selection of biomarker identified by the microarray were monitored by quantitative rtPCR to derive the No Observed Transcriptional Effect Level (NOTEL) as an indicator of the no effect concentration (NOEC) in the embryos.

2 Materials and methods

2.1 Experimental setup

Dry stripped Atlantic salmon eggs and sperm were obtained from the Aquagen hatchery (Sunndalsøra, Norway) and subjected to *in vitro* dry fertilization according to method described in Mahrosh et al. (2014). Eggs were exposed from fertilization to 24h post fertilization to Lake Maridalsvannet water (Oslo Norway) as control, and 50, 100, 500, and 5000 mg/L road salt (Isbryter'n rock salt, kindly donated from GC Rieber Salt AS, www.gcrieber-salt.no). The road salt (containing 98.5 % NaCl, 0.30 % Ca and Mg, 0.70 % SO_4, 0.3 % H_2O and 70-100 mg/kg

anticaking agent E 535-sodium ferrocyanide) was used in the studies as considered to be more ecologically relevant than pure NaCl. Temperature-controlled flow-through exposures (40-60 mL/min) with control and road salt-spiked water were conducted in duplicate with specially designed exposure boxes (155×106×45mm, 739 cm^3) containing 200 eggs in each. Following 24h exposure during the fertilization and swelling stage (Gorodilov, 1996), all groups received control water under the same experimental conditions as described above until the end of the experimental period. The temperature and water qualities were monitored throughout the study. After 255 degree days (i.e. the product of temperature and number of days to reach a certain developmental stage), when the eggs reached the eyed embryo stage, eggs were sampled, snap-frozen in liquid nitrogen and stored at -80 ºC for subsequent RNA extraction and gene expression analysis.

2.2 Water quality paremetres

Temperature, pH and conductivity were logged continuously using Campbell CR200 data logger (Cambell Scientific, Logan, UT, USA) and measured weekly by the handheld multimeter WTW 340i (Xylem, White Plains, NY, USA), equipped with SenTix® 41 glass electrode and TetraCon® 325 conductivity probe. Dissolved oxygen was measured by using the optic probe WTW 4301. Concentrations of major cations in unfiltered acidified (2% HNO_3) water samples were measured using ICP-OES. Total organic carbon (TOC) and major anions were determined in unfiltered samples by using Shimadzu TOC cpn Total organic analyzer (Shimadzu, Kyoto, Japan) and Lachat IC5000 Ion Chromatography (Hach Company, Loveland, CO, USA), respectively.

2.3 RNA isolation

Six Atlantic salmon eggs with an average individual wet weight of 0.146±0.020g were used for gene expression analysis (n=6). Total RNA was extracted using Direct-zol™ RNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA) according the manufacturer's instructions. Briefly, the whole egg was lysed with TRIzol® reagent (Sigma-Aldrich, St. Louis, MO, 100 mg tissue/1 mL TRIzol) and homogenized (3 x 10 sec at 6000 rpm) using ZR BashingBead™ (Zymo) in a Precellys orbital shaker bead mill (Bertin, Montigny-le-Bretonneux, France). The homogenate was centrifuged (12,000 g, 1 min) and 500 µL supernatant was carefully transferred to a 1.5 mL RNase-free centrifuge tube. The remaining homogenate was kept in a -80℃ ultrafreezer for further analysis. One volume of ethanol (95%-100%) was added directly to the supernatant, vortexed well, and transferred into a Zymo-Spin™ IIC Column in a collection tube. The column was centrifuged (12,000 g, 1 min) and transferred into a new collection tube. To remove genomic DNA, the column was first washed once with 400 µL RNA wash buffer (12000 g, 1 min), and added with a mixture of 3 µL RNase-Free DNase I (1 U/µL), 3 µl 10X reaction buffer and 24 µL RNA wash buffer, incubated at 37℃ for 20 min and centrifuged (12,000 g for 30 s). The DNase treated RNA was then washed once with 400 µL RNA Prep Buffer (12,000 g, 1 min), once with 800µL RNA wash buffer (12000 g, 30 s) and a final wash with 400 µL RNA wash buffer (12,000 g, 2 min). Nuclease-free water (12 µL) was added to the column and centrifuged (10,000 g, 30 s) to elute RNA into a DNase/RNase-Free tube. The RNA yield and purity were quantified by photometric analyses (260/280 and 230/260 ratios> 1.8, yield >50 ng/µL) using Nanodrop® spectrophotometer (ND-1000, Nanodrop Technologies, Wilminton, Delaware, USA) and RNA integrity determined by Bioanalyzer gelelectrophoresis with RNA 6000 Nano chips (Agilent technologies, Santa Clara, California, USA). All samples with exception of the individuals

exposed to 50 mg/L road salt (RIN =6.1), passed the quality criterias set (RIN>7.5, 260/280 ratio >1.8). The purified RNA samples were stored in -80℃ freezer for further analysis.

2.4 Microarray analysis

A 60k high density custom salmonid oligonucleotide microarray was manufactured by Agilent Technologies (Santa Clara, CA, USA). The microarray probes were designed based on Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) consensus sequences (contigs) and single ESTs from the cGRASP project (http://web.uvic.ca/grasp) and NCBI Unigenes (http://www.ncbi.nlm.nih.gov/unigene) for the two species (*S. salar*: build 31 and *O. mykiss*: build 27) as described in Song et. al. (2014). The array design can be accessed at Gene Expression Omnibus (GEO, accession number: GPL18864).

The global gene expression was determined in the control (n=4) and the highest exposure group (5000 mg road salt/L, n=4) using 50 ng RNA as starting template by a standard one-color protocol (Agilent Technologies, v6.5, Santa Clara, CA, USA) using commercially available kit and kit components from Agilent Technologies, as described previously in Song et al. (2014). After hybridization, the slides were washed and scanned using an Agilent Technologies C scanner (scan region 61x21.6 mm, resolution 3 μm, Tiff image 20 bit) and raw expression data was extracted using Agilent Feature Extraction (v10.6) software.

2.5 Quantitative rtPCR

Quantitative rtPCR was performed using RNA from the control and all road salt exposed eggs (50, 100, 500 and 5000 mg/L road salt, n=5-6), essentially as described by Song et al. (2014). In brief, the complementary DNA (cDNA) was reverse transcribed from 2 μg total RNA

using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions. A total reaction volume of 10 µL containing 10 ng cDNA template, Quanta PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, Maryland, USA) and 300 nanomoles forward/reverse primer was used for quantitative rtPCR using a CFX384™ detection system (Bio-Rad, Hercules, CA, USA). The *Salmo salar* primer sequences (Table 1) were designed using Primer 3 (v0.4.0, http://frodo.wi.mit.edu/primer3), based on the cGRASP contigs, Genbank ESTs or Unigene sequences. The starting quantity of cDNA template and the amplification efficiency were determined based on a standard curve made from 0.6, 3, 15 and 75 ng input cDNA. The expression of target gene was normalised against total RNA and fold change was then calculated by comparing the normalised treatment gene expression to the control samples. This normalization approach has been successfully used previously to obtain reliable results (Song et al., 2012).

------[Insert Table 1 here]----------

2.6 Statistics and Bioinformatics

Scanned images were analysed with Agilent Feature Extraction, Ver 7.3 (Agilent Technologies). Resulting raw data were normalised (25 Quantile, median to baseline of all samples), features filtered on expression (20-100%), outlier (non-uniform and saturated features) flagged and significantly regulated genes across treatments identified by a moderated T-test (Storey with Curve Fitting FDR multiple testing correction $p \leq 0.1$, two-fold cut-off) by GeneSpring version 12.5 (Agilent Technologies). Significantly regulated genes were clustered (Squared Euclidean,

Ward's linkage rule) by treatment and gene regulation, and subjected to functional gene ontology (GO) enrichment analysis (Fisher's exact test/hypergeometric test, FDR $p \leq 0.1$). Protein-protein networks, canonical pathways and relationships to well-characterised toxicological processes were identified by Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/products/ipa) using ortholog mapping to *Danio rerio*, *Homo sapiens*, *Mus musculens* and *Rattus norwegicus* as proxies for *S. salar.* Orthologs were identified by a reciprocal two-pass slightly modified blast (BLAST+ binaries instead of BLASTALL) to the RefSeq database (http://www.ncbi.nlm.nih.gov/refseq) using an Inparanoid algorithm (http://inparanoid.sbc.su.se/cgi-bin/index.cgi) according to specification provided by the developer (Ostlund et al., 2010).

Statistical analysis of the quantitative rtPCR data were performed using JMP Pro, Ver. 10.0.0 (SAS Institute Inc, Cary, NC, USA). A one-way ANOVA test was conducted followed by a Tukey-Kramer post-hoc test to identify significantly regulated single gene responses ($p \leq 0.05$). The assumptions of normally distributed data with equal variance were met in all data with the exception of one gene (Cyp4A14) which were log transformed using Johnsons transformation available in Minitab16 (Minitab Inc., State College, PA, USA). Regression analysis (GraphPad prism 5.0, Graphpad Software, Inc., San Diego, CA, USA) was applied to study the significant effect of road salt in combination with water quality parameters such as pH, conductivity and TOC. Significant effect was defined by using the criterion $p \leq 0.05$ as level of significance.

3 Results

3.1 Water chemistry

The water quality was different between groups during the experimental 24 hrs exposure period (Table 2). Thereafter, all groups were placed in the control lakewater until the end of the experimental period. During the experimental 24 h exposure periode a significant increase in conductivity (R^2=0.98, P=0.0001) was observed by road salt addition, mainly due to increased concentration of Na^+ and Cl^-. Post exposure and swelling, all eggs recived the same low ionic water (48±6 µS/cm) with pH 7.2±0.2 The pH was in range 6.9–7.5 and dissolved oxygen was 12.6±0.6 mg/L, and unaffected by road salt addition. During the experimental period the temperature was in average 5.4±0.6 °C, similar for all groups and eyed egg stage was reached after 47 days, about 255 degree days (not significantly different between the treatments), and all fish reached the Clarvay stage within 4.6 to 7.1 degree days.

------[Insert Table 2 here]----------

3.2 Global gene expression analysis

The microarray analysis showed that 1002 of the total 60k features on the array were significantly regulated (721 up-regulated and 281 down-regulated) after exposure to 5000 mg/L Road salt (Fig. 1), whereof the majority of the features were identified to have high-quality BLAST hits and about 50% were successfully identified as orthologs to zebrafish, humans, rats or mice. Supplementary Table S1 displays the differentially expressed genes in detail.

------[Insert fig 1 here]----------

Gene ontology (GO) functional enrichment analysis identified a number of biological processes and molecular functions being particularly enriched among the selection of differentially expressed genes (DEGs). A total of 16 GOs were identified as significant when considering both up- and down-regulated genes, whereas differentiation between directions of regulation led to the unique enrichment of 13 GOs associated with up-regulated transcripts (Supplementary Table S2). Of the GOs identified, a majority of the GOs were associated with general development processes and development of the sensory system in particular (Table 3). A few genes associated with the response to superoxide (PRDX2, APOA4, UCP3), were identified being relevant when considering only the up-regulated transcripts.

------[Insert Table 3 here]----------

Mapping to mammalian orthologs and analysis of enrichment in well-annotated (curated) toxicological (toxicity) pathways revealed that the exposure to high road salt concentrations caused differential regulation of genes associated with a number of biological processes (Table 4, Supplementary Table S3). Genes associated with renal function/cell death and acute renal failure, mitochondrial dysfunction (depolarization of mitochondria and mitochondrial membrane), oxidative stress and nuclear receptor signaling (LXR/RXR and TR/RXR activation) were clearly up-regulated by the episodic exposure to 5000 mg/L road salt.

------[Insert Table 4 here]----------

A number of canonical pathways were identified as being enriched among the differentially expressed transcripts (Table 5, Supplementary Table S4). Of the 17 pathways affected, nuclear receptor signaling (LXR/RXR activation, TR/RXR activation, and MIF-mediated glucocorticoid regulation), cellular immune response (clathrin-mediated endocytosis signaling, agranylocyte adhension and diapedesis, MIF-mediated glucocorticoid regulation and granzyme B signaling), neurosignaling (regulation of actin-based motility by Rho, neurotransmitter and other nervous system signaling), cellular growth, proliferation and development (epithelial adherens junction signaling, ILK and Notch signaling), human disease-specific pathways (Parkinson's and artherosclerosis signaling), intercellular and second messenger signaling (calcium signaling), apoptosis (April-mediated signaling) and cancer (Notch signaling) were affected.

------[Insert Table 5 here]----------

Protein-protein (gene-gene) interaction network analysis revealed a number of biological functions being affected by treatment to road salt (Supplementary Table S5). A selection of toxicological relevant gene-interactions and corresponding DEGs are shown in Supplementary Fig. 1, including gene-networks related to energy production and lipid metabolism, organ and embryonic development, cell signaling and interaction, and nervous system development and function.

3.3 Quantitative rtPCR verification

Quantitative rtPCR (qrtPCR) analysis of gene expression was performed to establish a concentration-response relationship and determine the NOTEL for a selection of differentially expressed genes that were central in the toxicological pathways and gene-interaction networks. Significant differences between the exposed groups and the control were detected in two out of the four genes, Cyp4A14 and Nav1 (Fig. 3). In both cases, the groups exposed to 100 and 500 mg/L displayed higher expression than the control group and an apparent concentration-dependent relationship was observed. An apparent concentration-response was observed also for ATPase1A2 and Txndc9, albeit the high variance in the control group precluded any statistical differences to be detected. The qrtPCR analysis showed that the overall NOTEL of the selected DEGs, based on the induction of Cyp4A14 and Nav1, were 50 mg/L road salt.

-----[Insert Fig. 2 here]----------

4. Discussion

The use of road salt has great societal benefits allowing deicing of roads, reducing car accidents, human damage and casualties due to traffic accidents (Bjørnskau, 2011). Although having an advantageous role in this respect, use of high amounts of road salt has led to extensive run-off of road salt components to nearby land and surface waters. Although the composition of road salt is complex and different types are often used (e.g. MgCl2, CaCl2 and various organic salts such as formates and acetates), the main salt applied on roads and that ultimately is emitted to the environment is NaCl (Canada, 2001; Lysbakken, 2013

). Chloride concentrations in urban impoundment lakes and snow clearings from streets can reach as high as 5000 mg/L, whereas Cl-concentrations in ponds, stream and rivers may range up to 4300 mg/L (Canada, 2001). In addition, chloride concentrations up to 18,000 mg/L have been reported in various runoff waters due to increased use of road salt (Sanzo and Hecnar, 2006). These large increases in aquatic salt concentrations, mainly due to the increase in NaCl but also minor contributions from Ca^{2+}, Mg^{2+}, SO_4^{2+}, anticaking agents such as E535-sodium ferrocyanide (<2% w.w) and trace impurities, are potential stressors to aquatic organisms living in the receiving fresh waters. Observations of high mortality, reduced weight and activity, and increased physiological abnormalitites in larval wood frogs exposed to road deicing salt at concentrations ranging from 2636 to 5109 mg/L (Sanzo and Hecnar, 2006) suggest that high concentrations of road salt may be hazardous to aquatic organisms. Reduced salmon egg swelling during fertilization and reduction in egg survival confirm that exposure to high concentrations (5000 mg/L) of road salt also affect fish (Mahrosh et al., 2014). Increase in salmon larvae deformities after exposure to high road concentrations suggest that exposure during critical windows of salmon embryo development may also give rise to adverse effects later in life (Li et al., 1989; Mahrosh et al., 2014). Although not measured directly, results may indicate that some of these effects may be related to higher uptake of Na and Cl in the egg fluid during swelling (Mahrosh et al., unpublished). But reduced swelling also causes a reduction in the periviteline space (PVS), the space between the zona pellucida and the cell membrane of the fertilized ovum (Li et al., 1989). Addition of saltwater with salt concentrations around 1000 mg/L during swelling have been shown to reduce the PVS by as much as 50% (Li et al., 1989), and exposure to other stressors such as low pHs and Al^{3+}, Zn^{2+}, Mg^{2+} and SO_4^{2-} may also reduce the PVS (Eddy and Talbot, 1983). Despite the asumptions that NaCl is the main cause for these effects, contribution from others salts such as Ca^{2+}, Mg^{2+}, SO_4^{2+}, anticaking agents and trace

impurities cannot be ignored. For example, Vosyline et al (2006) observed that NaCl-based road salt (96hr LC_{50} = 18.25 g/L) appared to be slightly more acute toxic to juvenile rainbow trout (*Oncorhynchus mykiss*) than pure NaCl (96hr LC_{50} = 20.38 g/L), although the differences were not statistically significant. The apparent difference was attributed to trace elements such as vanadium, strontium, arcenic, zinc, manganeese etc. and ferrocyanide present in the road salt. A recent study utilizing early life stages of the freshwater mussel *Villosa iris* was in fact launched to determine whether cyanide compounds increased the road salt toxicity (Pandolfo et al., 2012). The main conclusion from that study was that the cyanides present in road salt were most likely a lower risk to aqutic organisms than the chlorides. This because the cyanides in the road salt are present only in small amounts and as very stable metal-cyanide complexes with low toxic potential. As the present study has been conducted with the complete road salt formulation, and not single components, the assessments performed on basis of the results should therefore reflect the toxicity both from NaCl, other ions and any relevant additives. Effort to decipher the contribution from other components including the anti-caking agents and trace impurities are certainly worthy investigation, but was beyond the scope of this study.

The results from the present study verify that road salt exposure affected the developing salmon egg by modulating the gene expression of a high number of genes. As many as 721 genes were up-regulated, whereas 281 were down-regulated by the exposure to 5000 mg/L road salt. Although a selection of single DEGs could be associated with the direct response to road salt exposure, functional (GO) enrichment and pathway analysis identified a number of potential mechanisms that may provide more indepth understanding of the toxic MoA of road salt in the developing embryos. The potential major toxic MoA has been reviewed in detail below and is supported by supplementary information (Supplementary Tables S1-S5).

4.1 Ion- and acid-base regulation: Fertilisation of salmon eggs involve uptake of considerable amount of sourrounding water (nearly 25 to 30% of the original volume of the egg) through the permeable chorion within the first hours (Davenport et al., 1981) and the internal environment will thus resemble the major composition of the surrounding water (Li et al., 1989). However, in environments with increased salinities up to 1 %, reduced PVS has been observed in Atlantic salmon eggs during swelling, thus leading to a limited space for the developing embryos (Li et al., 1989). It should therefore be expected that the developing embryos exposed to high concentrations of road salt were experiencing osmotic stress due to a combination of accumulation of water with high road salt concentrations in the PVS and reduced swelling of the eggs themselves. To cope with this high electrolyte challenge, developing fish eggs and larvae regulate the salt balance (Lasker and Theilacker, 1962), perhaps as early as the blastula stage in certain fish species (Holliday and Jones, 1965). The present data suggest that developing salmon embryos exposed to 5000 mg/L road salt were indeed experiencing osmotic stress, and that they were not able to successfully excrete the various ions of road salt efficiently across the chorion in the period up to the eyed egg stage. As seen for freshwater fish exposed to high salinity (Hoar, 1988), the salmon eggs exhibited physiological (transcriptional) changes consistent with hypo-osmoregulation, e.g. the regulation of internal plasma and tissue ions to concentrations being lower than that of the sourroundings. Genes associated with well-known osmoregulatory functions in freshwater fish including ATP-fueled active transport of Cl^- across the gill and skin epithelium by Na^+/K^+ATPase (ATP1A2) was clearly upregulated. Interestingly, several genes associated with regulation of the embryo acid-base balance (Genz et al., 2011) were affected in a similar manner (up-regulated) including carbonic anhydrase 4 (CA4) and solute carrier family proteins (SLC43A3, SLC22A7, SLC9A3 and SLC9A1). A number of additional DEGs, primary related to ion transport (SLC39A9, SLC39A14, and SLC30A1) were also up-regulated, although their

primary roles are more clearly associated with the transport of sugars, bile salts, organic acids, amines and metals such as zink (Guh et al., 2015). Reports that specific blockers of Na^+/K^+ATPase activity and transmembrane transport of solutes and ions enhance the adverse effect of high salt exposure in zebrafish embryos suggest that osmoregulation is a key mechanism also in road salt toxicity (Lahnsteiner, 2009). Interestingly, dechorionation of the zebrafish embryos did not eliviate the adverse effects observed by exposure to hyper-saline solutions (Lahnsteiner, 2009), thus indicating that the ion transport from the embryo out to the PVS is likely the crucial step in reducing osmotic stress in developing embryos. Quantitative rtPCR measurements of transcriptional changes in ATP1A2 identified a concentration-dependent increase in expression, albeit high variance in the control group precluded determination of whether these changes were significantly different from the control group.

4.2 Metabolism and energy turnover: A number of DEGs, associated with the activation of nuclear receptors pathways (e.g. the LXR/RXR and TR/RXR) were differentially expressed and the majority of these seemed to be associated with increasing the cellular turnover of energy. As stress responses, such as increase in regulation of ions and acid-base balance, may cause increased energy demand to maintain physiological homeostasis, these transcriptional changes are likely associated with activation of cellular pathways leading to increased lipid, steroid, amino acid, and carbohydrate metabolism (Martinez-Alvarez et al., 2002; Tseng and Hwang, 2008). Although not studied specifically in this work, modulation of different nuclear receptor pathways (Table 4 and 5) should be expected to affect the mobilization of numerous macromolecules as source for increasing cellular energy turnover. Up-regulation of DEGs such as APOE, APOA4, NCOR1, ACACA and MMP9 are all associated with the action of LXR/RXR by increasing the transport and ensuring homeostasis of lipids,

lipopolysaccarides and cholesterol during stress-induced energy depletion (Cordier et al., 2002; Lu et al., 2010; Prunet et al., 2007). Modulation of the DEGs COL6A3, SLC16A2 in the TR/RXR pathway indicate that mobilization of amino acids is additionally altered to accommodate an increased energy demand, whereas upregulation of SLC2A6 and GLUT1 indicate that glucose transport were also activated. Modulation of a number of DEGs in the Clathrin-mediated Endocytosis Signaling suggest that internalization of nutrients, hormones and other signaling molecules over the cellular plasma membrane into intracellular compartments were induced to accomodate a more rapid cellular metabolic turnover. Although not determined directly, increased metabolic turnover was coherent with the observation of up-regulation of CA4 that catalyses the reversible reaction of CO_2 and water to facilitate CO_2 excretion or HCO_3^- transformation due to increased CO_2 production during cellular metabolism (Gilmour et al., 2009).

Up-regulation of TF and IL1RAPL1, which is associated with the mobilization of divalent ions such as iron and calcium during acute-phase responses to stress, suggest that other pathways involving in more broadly acting intracellular signaling were also affected. In fact, differential expression of a number of genes coding for Ca^{2+} pumps (e.g. calcium-binding mitochondrial carrier protein s-1 (SLC25A24), Ca^{2+} exchangers (e.g. anoctamin calcium activated chloride channel (ANO1) and transmebrane Ca-ion channels (e.g. calcium homeostasis endoplasmic reticulum protein (CHERP), orai calcium release-activated calcium modulator 1 (ORAI1), soluble calcium binding proteins such as Striatin (STRN) involved in maintaining intracellular calcium levels and regulating the activities of different proteins, enzymes and transcription factors (e.g. HDAC10, HDAC4, and calmodulin-dependent protein kinases), support the hypothesis that modulation of Ca-signaling were likely occurring (Boeuf and Payan, 2001; Evans et al., 2005). Interestingly, up-regulation of UCP3 has also

been associated with Ca-signalling and is additionally linked to regulation of cellular export of fatty acids, mitochondrial oxidation capacity and uncoupling of the transmembrane proton transport in the mitochondria (Shabalina and Nedergaard, 2011). The potential depolarization of the mitochondria by UCP3 was found to be consistent with the up-regulation of DEGs such as HTT, CYR61 and GZMB (Table 3) that is associated with the mitochondrial membrane permeability, membrane potential and incorporation of proteins in the mitochondrial membrane (Ismailoglu et al., 2014; Jacquemin et al., 2015; van Waveren et al., 2006). Mitochondrial depolarization has frequently been reported as the primary cause for cell injury or death for a number of stressors including natural toxins and environmental pollutants (Song et al., 2014; Tiano et al., 2001).

4.3 Oxidative stress: Formation of Reactive Oxygen Species (ROS) or other free radicals may result in direct damage of cellular components such as protein, lipids and DNA, or may affect other biological pathways which might not necessarily be linked directly to oxidative stress (Lushchak, 2011). Although the aforementioned up-regulation of UCP3 is suggested to provide some level of protection towards mitochondrial oxidative stress (Cannon et al., 2006), several cellular antioxidant defences were activated after exposure to high concentrations of road salt. The up-regulation of GGT1 and PRDX2 to increase the antioxidant capacity were consistent with increase in antioxidant responses in vertebrates exposed to high salinity (Martinez-Alvarez et al., 2002). However, down-regulation of NFE2L1 and NQO1 seemed to contrast existing knowledge of ROS-induced antioxidant defence in fish (Mukhopadhyay et al., 2015; Sarkar et al., 2014; Wang and Gallagher, 2013). The rationale for salt-induced oxidative stress responces in fish is not entirely clear, but it has been reported in other organisms that high concentrations of NaCl cause oxidative stress (Carlstrom et al., 2009; Zhang et al., 2004; Zhou et al., 2005) and cellular damage (Burg et al., 2007). Quantitative

rtPCR analysis of thioredoxin domain containing genes (Txndc9), which is belived to play an important role in the defense against oxidative stress by directly reducing hydrogen peroxide and certain radicals and by serving as a reductant for peroxiredoxins (Palanisamy et al., 2014; Wei et al., 2012), displayed an apparent concentration-dependent increase in expression. Although a modest up-regulation (approximately 2-fold) was observed for Txndc9 by qrtPCR, as much as a 25-fold upregulation was observed for Txndc9 when reviewing the microarray data (Supplementary Table S1). Indepth studies to characterize whether high road salt concentrations cause oxidative stress beyond the protective antioxidant capacity of the developing salmon embryo and whether adverse cellular effects were occurring, is clearly warranted to determine if exposure to the developing embryo may lead to adverse effects later in life.

4.4 Renal function: Exposure of developing salmon embryos to high salt concentrations were associated with differential regulation of a number of DEGs involved in renal necrosis, cell death and acute renal failure. Lack of proper regulation of a number of these DEGs may ultimately lead to apoptosis and necrosis of renal tissues. Modulation of a number of genes (e.g. APOE, MAP2K7, BGN, HBEGF, TNFAIP3, C1QA, PRSS1, SDHC, P2RX7, TP53BP2, VOPP, RHOG, HTT, BNIP3L, SNCA, and PLAT) predominantly involved in stimulation, but also to a limited degree negative regulation of apoptosis suggest a large recruitment of genes involved in termination of damaged cells (Table 4). Observation of enriched DEGs in well-known apoptotic pathways such as April-mediated signaling and Granzyme B signaling (Table 4) confirm that cells were potentially adversely affected. Several DEGs (e.g. TNFAIP3, HBEGF, CIQA, CD68) involved in removal of cell debries as well as inflammatory and immune responses support that some level of tissue damage were likely occurring after exposure to high road salt concentrations. Differential regulation of genes involved in the

migration of leukocytes from the vascular system to sites of pathogenic exposure (e.g. the Agranulocyte Adhesion and Diapedesis signaling pathway) and induction of ROS production to degrade damaged tissues, additionally suggest that cellular responses to injury through activating inflammatory responses were taking place. Concentration-dependent increase in Cyp4A14, which is proposed involved in arachidonic acid metabolism, icosanoid biosynthesis and kidney development in response to endogenous and exogenous stimuls (Capdevila et al., 2015), suggest that renal function may be affected even at lower concentrations than 5000 mg/L.

4.5 Cellular growth, proliferation and development: The analysis performed clearly identified that many of the DEGs were associated with cellular growth, proliferation and development. This applied in particular to organ and embryonic development such as the sensory system (eye morphogenesis and eye development), the cardiovascular system (Trehalose degradation II, GDP-glucose biosynthesis, NOTCH signaling, MIF-mediated glucoroticoid regulation), the hematological system (Agranulocyte adhension and diapesis, MIF-mediated glucoroticoid regulation, atherosclerosis signaling, April-mediated signaling) and the reproductive system (Serotonin and melatonin biosynthesis, TR/RXR activation). Although the interference with these developmental processes were not studied in detail in the present paper, observations that a number of DEGs such as TCF4, MYL3, MYL6, MYL7, SSX2IP, IQGAP1, NOTCH1, HEY2, RhoG, and RhoH are involved in the maturation, polarization and migration of cells in organ development and morphogenesis (Table 4), may suggest that prolonged hypertension due to road salt exposure may interfere with normal development of organs involved in hepatic and hematological processes (Vosylienė et al., 2006), sensory processes (Sanzo and Hecnar, 2006), acid-base balance (Bentley and Schmidt-Nielsen, 1971), hepatic and neural development (Klein

et al., 2011; Yonkers and Ribera, 2009). Concentration-dependent up-regulation of the neuron navigator (Nav1), a gene expressed predominantly in the nervous system and believed associated with axonal guidance (Novak et al., 2006) by qrtPCR suggest that neuronal development and regeneration in the developing embryos may be affected by high road salt concentrations.

4.6 No Transcriptional Effect Level (NOTEL): The knowledge of adverse effects of road salt exposure is limited and biomarkers to determine potential adverse effects are poorly developed. The present work have developed a better characterization of potential genes being candidate markers for more thorough assessment, including proposing a suite of genes to determine the NOTEL for osmotic stress (*ATP1A2*), oxidative stress (*Txndc9*), changes in lipid processing and renal function (*Cyp4A14*) and neuroaxonal development (*Nav1*). Although all of these DEGs showed a concentration-response relationship, NOTEL values were only obtained for *Cyp4A14* and *Nav1* due to the high variance in the control group for the other genes determined. The current assessment suggests that concentrations lower than 100 mg/L (NOTEL=50 mg/L) were not causing any transcriptional responses in the embryos and were found to be several orders of magnitude lower than the concentrations causing adverse effects in salmonids and amphibians (Mahrosh et al., 2014; Sanzo and Hecnar, 2006). A more thorough assessment to identify additional biomarker candidates for exposure, cellular response and adverse effects of road salt based on the existing data is expected to yield a better basis for future hazard and risk assessments.

4.7 Ecological consequence

The fertilization stage is very sensitive for pollutant uptake as the egg may accumulate stressors such as salt and pollutants from the ambient environment (Nakano, 1956). As seen herein, substantial transcriptional responses were identified at high road salt concentrations, whereof some may be of relevance for adverse outcomes important for environmental risk. A NOTEL of 50 mg/L road salt were established, based on changes in marker genes for osmotic stress and neuroaxonal developmental changes, and were found to be in the low range of typial road salt concentrations in surface waters receiving run-off from roads where deiceing salts have been used. However, such concentrations are likely to occur in small streams that receive diluted run-off from road maintenance operations. For example, Winter et al. (2011) reported Cl^- concentrations much greater than 50 mg/L in tributaries to lake Simcoe (Canada) in the years 2004-2007 and Betts et al (2014) reported annual Cl^- concentrations ranging from 118-765 mg/L in urban rivers within the City of Toronto (Canada). Despite lack of data, we believe that such concentrations may also be common in small Norwegian streams. In Norway, small streams along the coast line are known to be important for salmonids. For example, in Østfold county approximately 40 small streams, some having discharge on an average level less than 0.04 m^3/sec, are inhabited by brown trout (Karlsen, 2012). The current GIS-based environmental impact assessment method for road salt in Norway is so far only considering chemical stratification and biological effects in lentic systems (Kronvall, 2013). However, the present results indicate that fish inhabiting lotic systems close to roads may be affected by road salt exposures, at least on the molecular level, although potential adverse effects of such transcriptional changes are still unknown. Future initiatives should therefore aim to provide better links between mechanistical data obtained at the molecular level with functional changes that may cause adverse effects relevant for regulatory decissions, and by doing so supporting identification of vulnerable recipients according to the intention of the EU Water Framework Directive.

5. Conclusions

Large transcriptional changes were observed in eyed egg stage embryos exposed to 5000 mg/L road salt during the first 24h after fertilisation. These stranscriptional changes were mainly associated with a number of potentially toxicity mechanisms including osmoregulation, ionregulation, oxidative stress, metabolism, renal function and developmental in the embryos. A number of DEGs were identified to be affected by the exposure and selection of these were used to derive the NOTEL for osmoregulation (ATP1A2), oxidative stress (Txndc9), renal function (Cyp14A4) and organ development (Nav1). Although all of the genes displayed a concentration-dependacy, only expression of Cyp14A4 and Nav1 (100 mg/L and higher) were significantly regulated due to large data variation in the control group. The present data propose that road salt concentrations below 100 mg/L road should not be expected to affect developing embryos when exposed during fertilisation and these concentrations were found to be several orders of magnitude lower than the adverse effects reported elsewhere for Atlantic salmon and other aquatic vertebrates. The concentrations causing transcriptional responses were typically in the concentrations range of that expected to occur in lotic systems receiving dilute run-off from roads using road salt, and suggest using biomarker approaches to assist the risk assessment and risk management process of these types of exposure scenarios. Although transcriptional changes were observed at fairly low road salt concentrations, it's still unresolved whether these transcriptional changes may cause adverse effects at higher level of organization. Further research should aim to provide better links between mechanistical data obtained at the molecular level with functional changes that may affect regulatory relevant chronic endpoints such as effects on growth, development and reproduction.

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Availability of supporting data

The raw data sets supporting the results of this article are available in the Gene Expression Omnibus (GEO) repository, accession number: GSE71714 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE71714.

7. References

Anderson, B.S., Hunt, J.W., Piekarski, W.J., Phillips, B.M., Englund, M.A., Tjeerdema, R.S., Goetzl, J.D., 1995. Influence of salinity on copper and azide toxicity to larval topsmelt Atherinops affinis (Ayres). Arch Environ Contam Toxicol 29, 366-372.

Bentley, P.J., Schmidt-Nielsen, K., 1971. Acute effects of sea water on frogs (Rana pipiens). Comparative biochemistry and physiology. A, Comparative physiology 40, 547-548.

Betts, A.R., Gharabaghi, B., McBean, E.A., 2014. Salt vulnerability assessment methodology for urban streams. Journal of Hydrology 517, 877-888.

Bjørnskau, T., 2011. Safety effects of road salting in winter maintenance [in Norwegian]. Insitute for Transport Economics, Oslo.

Boeuf, G., Payan, P., 2001. How should salinity influence fish growth? Comp Biochem Physiol C Toxicol Pharmacol 130, 411-423.

Burg, M.B., Ferraris, J.D., Dmitrieva, N.I., 2007. Cellular response to hyperosmotic stresses. Physiological reviews 87, 1441-1474.

Canada, E., 2001. Canadian Environmental Protection Act, 1999, Priority Substances List Assessment Report – Road Salt Hull, Quebec, Canada, p. 167.

Cannon, B., Shabalina, I.G., Kramarova, T.V., Petrovic, N., Nedergaard, J., 2006. Uncoupling proteins: a role in protection against reactive oxygen species--or not? Biochim Biophys Acta 1757, 449-458.

Capdevila, J.H., Wang, W., Falck, J.R., 2015. Arachidonic acid monooxygenase: Genetic and biochemical approaches to physiological/pathophysiological relevance. Prostaglandins Other Lipid Mediat.

Carlstrom, M., Brown, R.D., Sallstrom, J., Larsson, E., Zilmer, M., Zabihi, S., Eriksson, U.J., Persson, A.E., 2009. SOD1 deficiency causes salt sensitivity and aggravates hypertension in hydronephrosis. Am J Physiol Regul Integr Comp Physiol 297, R82-92.

Cordier, M., Brichon, G., Weber, J.-M., Zwingelstein, G., 2002. Changes in the fatty acid composition of phospholipids in tissues of farmed sea bass (Dicentrarchus labrax) during an annual cycle. Roles of environmental temperature and salinity. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 133, 281-288.

Corsi, S.R., De Cicco, L.A., Lutz, M.A., Hirsch, R.M., 2015. River chloride trends in snow-affected urban watersheds: increasing concentrations outpace urban growth rate and are common among all seasons. Sci. Total Environ. 508, 488-497.

Dave, G., Xiu, R., 1991. Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish,Brachydanio rerio. Arch. Environ. Contam. Toxicol. 21, 126-134.

Davenport, J., Lønning, S., Kjørsvik, E., 1981. Osmotic and structural changes during early development of eggs and larvae of the cod, *Gadus morhua* L. J. Fish Biol. 19, 317-331.

Eddy, F.B., Talbot, C., 1983. Formation of the perivitelline fluid in atlantic salmon eggs (salmo salar) in fresh water and in solutions of metal ions. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 75, 1-4.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiological reviews 85, 97-177.

Fridman, S., Bron, J., Rana, K., 2012. Influence of salinity on embryogenesis, survival, growth and oxygen consumption in embryos and yolk-sac larvae of the Nile tilapia. Aquaculture 334–337, 182-190.

Genz, J., Esbaugh, A.J., Grosell, M., 2011. Intestinal transport following transfer to increased salinity in an anadromous fish (Oncorhynchus mykiss). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 159, 150-158.

Gilmour, K.M., Thomas, K., Esbaugh, A.J., Perry, S.F., 2009. Carbonic anhydrase expression and CO2 excretion during early development in zebrafish Danio rerio. J Exp Biol 212, 3837-3845.

Gorodilov, Y., 1996. Description of the early ontogeny of the Atlantic salmon, Salmo salar, with a novel system of interval (state) identification. Environ Biol Fish 47, 109-127.

Guh, Y.J., Lin, C.H., Hwang, P.P., 2015. Ion transport mechanisms and functional regulation. EXCLI Journal 14, 627-659.

Haddy, J.A., Pankhurst, N.W., 2000. The effects of salinity on reproductive development, plasma steroid levels, fertilisation and egg survival in black bream Acanthopagrus butcheri. Aquaculture 188, 115-131.

Hoar, W.S., 1988. The Physiology of Smolting Salmonid.

Holliday, F.G.T., Jones, M.P., 1965. Osmotic regulation in the embryo of herring (Clupea harengus). Journal of Marine Biological Association of U.K. 45, 305-311.

Ismailoglu, I., Chen, Q., Popowski, M., Yang, L., Gross, S.S., Brivanlou, A.H., 2014. Huntingtin protein is essential for mitochondrial metabolism, bioenergetics and structure in murine embryonic stem cells. Dev. Biol. 391, 230-240.

Jacquemin, G., Margiotta, D., Kasahara, A., Bassoy, E.Y., Walch, M., Thiery, J., Lieberman, J., Martinvalet, D., 2015. Granzyme B-induced mitochondrial ROS are required for apoptosis. Cell Death Differ 22, 862-874.

Johnson, A., Carew, E., Sloman, K.A., 2007. The effects of copper on the morphological and functional development of zebrafish embryos. Aquat Toxicol 84, 431-438.

Karlsen, L., 2012. Vulnerable streams (In Norwegian). Vann 47, 386-392.

Kelly, V.R., Lovett, G.M., Weathers, K.C., Findlay, S.E., Strayer, D.L., Burns, D.I., Likens, G.E., 2008. Long-term sodium chloride retention in a rural watershed: legacy effects of road salt on streamwater concentration. Environ Sci Technol 42, 410-415.

Kjensmo, J., 1997. The influence of road salts on the salinity and the meromictic stability of Lake Svinsjoen, southeastern Norway. Hydrobiologia 347, 151-158.

Klein, C., Mikutta, J., Krueger, J., Scholz, K., Brinkmann, J., Liu, D., Veerkamp, J., Siegel, D., Abdelilah-Seyfried, S., le Noble, F., 2011. Neuron navigator 3a regulates liver organogenesis during zebrafish embryogenesis. Development 138, 1935-1945.

Kronvall, K.W., 2013. Road Salt and Environmental Hazards—Identification of Vulnerable Water Resources, in: Rauch, S., Morrison, G., Norra, S., Schleicher, N. (Eds.), Urban Environment. Springer Netherlands, pp. 465-474.

Lahnsteiner, F., 2009. The effect of different kinds of electrolyte and non-electrolyte solutions on the survival rate and morphology of zebrafish Danio rerio embryos. J Fish Biol 75, 1542-1559.

Lasker, R., Theilacker, G.H., 1962. Oxygen Consumption and Osmoregulation by Single Pacific Sardine Eggs and Larvae (Sardinops caerulea Girard). ICES J. Mar. Sci. 27, 25-33.

Li, X., Jenssen, E., Fyhn, H.J., 1989. Effects of salinity on egg swelling in Atlantic salmon (Salmo salar). Aquaculture 76, 317-334.

Lu, X.J., Chen, J., Huang, Z.A., Shi, Y.H., Wang, F., 2010. Proteomic analysis on the alteration of protein expression in gills of ayu (Plecoglossus altivelis) associated with salinity change. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 5, 185-189.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 101, 13-30.

Lysbakken, K.R., 2013

Salting of Winter Roads: The Quantity of Salt on Road Surfaces after Application.

, Faculty of Engineering Science and Technology, Department of Civil and Transport Engineering. Norwegian University of Science and Technology (NTNU), Trondhein, Norway, p. 140.

Mahrosh, U., Kleiven, M., Meland, S., Rosseland, B.O., Salbu, B., Teien, H.C., 2014. Toxicity of road deicing salt (NaCl) and copper (Cu) to fertilization and early developmental stages of Atlantic salmon (Salmo salar). J Hazard Mater 280, 331-339.

Martinez-Alvarez, R.M., Hidalgo, M.C., Domezain, A., Morales, A.E., Garcia-Gallego, M., Sanz, A., 2002. Physiological changes of sturgeon Acipenser naccarii caused by increasing environmental salinity. J Exp Biol 205, 3699-3706.

Miracle, A.L., Ankley, G.T., 2005. Ecotoxicogenomics: linkages between exposure and effects in assessing risks of aquatic contaminants to fish. Reproductive Toxicology 19, 321-326.

Mukhopadhyay, D., Srivastava, R., Chattopadhyay, A., 2015. Sodium fluoride generates ROS and alters transcription of genes for xenobiotic metabolizing enzymes in adult zebrafish (Danio rerio) liver: expression pattern of Nrf2/Keap1 (INrf2). Toxicol Mech Methods, 1-10.

Murashige, R., Bass, P., Wallace, L., Molnar, A., Eastham, B., Sato, V., Tamaru, C., Lee, C.S., 1991. The effect of salinity on the survival and growth of striped mullet (Mugil cephalus) larvae in the hatchery. Aquaculture 96, 249-254.

Nakano, E., 1956. Changes in the egg membrane of the fish egg during fertilization. Embryologia 3, 89-103.

Novak, A.E., Taylor, A.D., Pineda, R.H., Lasda, E.L., Wright, M.A., Ribera, A.B., 2006. Embryonic and larval expression of zebrafish voltage-gated sodium channel alpha-subunit genes. Dev Dyn 235, 1962-1973.

Novotny, E.V., Murphy, D., Stefan, H.G., 2008. Increase of urban lake salinity by road deicing salt. Sci Total Environ 406, 131-144.

Ostlund, G., Schmitt, T., Forslund, K., Kostler, T., Messina, D.N., Roopra, S., Frings, O., Sonnhammer, E.L., 2010. InParanoid 7: new algorithms and tools for eukaryotic orthology analysis. Nucleic Acids Res 38, D196-203.

Palanisamy, R., Bhatt, P., Kumaresan, V., Chaurasia, M.K., Gnanam, A.J., Pasupuleti, M., Kasi, M., Arockiaraj, J., 2014. A redox active site containing murrel cytosolic thioredoxin: analysis of immunological properties. Fish Shellfish Immunol 36, 141-150.

Pandolfo, T.J., Cope, W.G., Young, G.B., Jones, J.W., Hua, D., Lingenfelser, S.F., 2012. Acute effects of road salts and associated cyanide compounds on the early life stages of the unionid mussel Villosa iris. Environ Toxicol Chem 31, 1801-1806.

Prunet, P., Auperin, B., Mativet, S., Leborgne, G., Lepage, O., 2007. Gene expression profile analysis to detect confinement and salinity stress-related genes in rainbow trout (Oncorhynchus mykiss). Aquaculture 272, Supplement 1, S302.

Ramakrishna, D.M., Viraraghavan, T., 2005. Environmental Impact of Chemical Deicers – A Review. Water Air. Soil. Poll. 166, 49-63.

Sanzo, D., Hecnar, S.J., 2006. Effects of road de-icing salt (NaCl) on larval wood frogs (Rana sylvatica). Environ Pollut 140, 247-256.

Sarkar, S., Mukherjee, S., Chattopadhyay, A., Bhattacharya, S., 2014. Low dose of arsenic trioxide triggers oxidative stress in zebrafish brain: expression of antioxidant genes. Ecotoxicol Environ Saf 107, 1-8.

Shabalina, I.G., Nedergaard, J., 2011. Mitochondrial ('mild') uncoupling and ROS production: physiologically relevant or not? Biochem Soc Trans 39, 1305-1309.

Sivertsen, Å., 2012. Salt SMART. Norwegian Public Roads Administration (NPRA), Oslo, Norway, p. 101.

Song, Y., Salbu, B., Heier, L.S., Teien, H.C., Lind, O.C., Oughton, D., Petersen, K., Rosseland, B.O., Skipperud, L., Tollefsen, K.E., 2012. Early stress responses in Atlantic salmon (Salmo

salar) exposed to environmentally relevant concentrations of uranium. Aquat Toxicol 112-113, 62-71.

Song, Y., Salbu, B., Teien, H.C., Sorlie Heier, L., Rosseland, B.O., Hogasen, T., Tollefsen, K.E., 2014. Hepatic transcriptomic profiling reveals early toxicological mechanisms of uranium in Atlantic salmon (Salmo salar). BMC Genomics 15, 694.

Tiano, L., Fedeli, D., Ballarini, P., Santoni, G., Falcioni, G., 2001. Mitochondrial membrane potential in density-separated trout erythrocytes exposed to oxidative stress in vitro. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1505, 226-237.

Tseng, Y.-C., Hwang, P.-P., 2008. Some insights into energy metabolism for osmoregulation in fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 148, 419-429.

van Waveren, C., Sun, Y., Cheung, H.S., Moraes, C.T., 2006. Oxidative phosphorylation dysfunction modulates expression of extracellular matrix--remodeling genes and invasion. Carcinogenesis 27, 409-418.

Vosylienė, M.Z., Baltrėnas, P., Kazlauskienė, A., 2006. Toxicity of road maintenance salts to rainbow trout Oncorhynchus mykiss. Ekologija 2, 15-20.

Wang, L., Gallagher, E.P., 2013. Role of Nrf2 antioxidant defense in mitigating cadmium-induced oxidative stress in the olfactory system of zebrafish. Toxicol Appl Pharmacol 266, 177-186.

Wei, J., Ji, H., Guo, M., Qin, Q., 2012. Isolation and characterization of a thioredoxin domain-containing protein 12 from orange-spotted grouper, Epinephelus coioides. Fish Shellfish Immunol 33, 667-673.

Winter, J.G., Landre, A., Lembcke, D., O'Connor, E.M., Young, J.D., 2011. Increasing chloride concentrations in Lake Simcoe and its tributaries. WATER QUAL. RES. J. CAN. 46, 157-165.

Yang, Z., Chen, Y., 2006. Salinity tolerance of embryos of obscure puffer Takifugu obscurus. Aquaculture 253, 393-397.

Yonkers, M.A., Ribera, A.B., 2009. Molecular components underlying nongenomic thyroid hormone signaling in embryonic zebrafish neurons. Neural development 4, 20.

Zhang, Z., Dmitrieva, N.I., Park, J.H., Levine, R.L., Burg, M.B., 2004. High urea and NaCl carbonylate proteins in renal cells in culture and in vivo, and high urea causes 8-oxoguanine lesions in their DNA. Proc Natl Acad Sci U S A 101, 9491-9496.

Zhou, X., Ferraris, J.D., Cai, Q., Agarwal, A., Burg, M.B., 2005. Increased reactive oxygen species contribute to high NaCl-induced activation of the osmoregulatory transcription factor TonEBP/OREBP. American journal of physiology. Renal physiology 289, F377-385.

Figure caption

Figure 1. Combined (genes and treatments) hierarchical clustering (Squared Euclidian, Ward's linkage) of differentially regulated features in Atlantic salmon (S. salar) embryos after exposure to control water (left columns) and 5000 mg/L Road salt (right columns) from fertilization to 24h post-fertilization. The data (n=4) represent differential gene expression determined at the eyed egg stage. Extended list of differentially expressed genes is provided in supplementary Table S1.

Figure 2. Expression of selected genes in eyed eggs of Atlantic salmon (S. salar) after exposure to lake water (control) and lake water added different concentrations of road salt from fertilization to 24h post-fertilisation. Asterics denote groups being significantly different from control ($p \leq 0.05$). Data represent minimum 5 individual replicates per group: cytochrome P450 4A14 precursor (Cyp4A14), thioredoxin domain-containing protein 9 (Txndc9), sodium potassium-transporting atpase subunit alpha-2 precursor (ATP1A2) and Neuron navigator 1 isoform 2 (Nav1).

Table(s)

Table 1. Atlantic salmon primer sequences (5'-3') for the quantitative rtPCR analysis

Gene	Gene name	GenBank Acc.	Forward	Reverse
Stmn3	stathmin-like 3	DY700722	CTCACAACCACATCCCAACA	GCCTCCTGAGACTTCCTCCT
Cyp4A14	cytochrome P450, family 4, subfamily a, polypeptide 14	BT058728	TGATCAGGAAAAACCCAAGG	GTAGGGAAAGATCCGGAAGC
Txndc9	thioredoxin domain containing 9	BT043664	GGAGCAGTTGGACAAAGAGC	CACACGATTGCTCTCCTTCA
Atp1A2	ATPase, Na+/K+ transporting	EG826654	TGTGATCCTTGCTGAGAACG	AGATCGGCCCACTGTACAAC
Nav1	neuron navigator 1	GT297797	AATTAGCTGCTCAGCCCGTA	GCACTGGCAAGACCTACCTC

Table 2. *Physical and chemical parameters of the rearing water in the 24 hrs exposure period (during swelling) and in the post-swelling period until the eyed egg stage (Clarvay stage to 255 degree days).*

	During swelling stage					Post-swelling
		Road salt concentration (mg/L)				
Parameter	Control	50	100	500	5000	All groups
Temperature	7.1	5.5	4.6	5	5.5	5.4±1.6
Conductivity (µS/cm)	35	145	240	850	1870	48±6.5
pH	6.9	7.2	7.5	7.3	7.2	7.2±0.2
Na (mg/L)	3.0	22	34	190	NA	3.0±1.5
Ca (mg/L)	6.0	6.0	6.0	8.4	9.0	4.8±0.1
Mg (mg/L)	1.2	1.0	1.0	1.0	5.0	1.2±1.0
K (mg/L)	2.1	2.0	2.8	5.0	12	2.1
TOC (mg/L)	4.25	4.04	4.03	3.94	4.01	4.25±1.0
F^- (mg/L)	< 0.1	< 0.1	< 0.1	NA	NA	< 0.1
Cl^- (mg/L)	5.46	32	59	450	NA	5.46
SO_4^{2-} (mg/L)	2.43	2.57	2.65	NA	NA	2.43
NO_3^- (mg/L)	0.21	0.45	0.10	NA	NA	0.21

NA = not analysed,

Table 3. *Biological processes associated with up-regulated genes in Atlantic salmon (S. salar) embryos after exposure to 5000 mg/L road salt from fertilization to 24h post-fertilisation. The data represent differential gene expression determined at the eyed egg stage. A complete compilation of GO terms can be found in Supplementary data S2.*

GO ID	Term	Genes
GO:0048592	eye morphogenesis	SIX3, AXIN1, DSCAM, GS-1, GM2, CRYGB, CRYGN, CRYGNB
GO:0000303	response to superoxide	PRDX2, APOA4, UCP3
GO:0070309	lens fiber cell morphogenesis	CRYGNB, GS-1, CRYGN, CRYGB, GM2
GO:0001654	eye development	SIX3, JMJD6, AXIN1, CRYGNB, CRYBA1, GS-1, CRYBA4, DSCAM, CRYGN, CRYGB, GM2

Abbreviations: SIX3: Homeobox protein SIX3, AXIN1: Axin-1, DSCAM: Down syndrome cell adhension molecule, GS-1: β-crystallin S-1, GM2: γ-crystallin M2, CRYGB: γ-crystallin B, CRYGN: γ-crystallin N, CRYGNB: γ-crystallin N-B, PRDX2: peroxiredoxin 2, APOA4: Apoplipoprotein A-IV precursor, UCP3: Mitochondrial uncoupling protein, JMJD6: Histone arginine demethylase, GRYBA1: β-crystallin A-2, and GRYBA4: β-crystallin A-4.

Table 4. *Toxicity pathways associated with differentially expressed genes in Atlantic salmon (S. salar) embryos after exposure to 5000 mg/L road salt from fertilization to 24h post-fertilization. The arrows indicate direction of gene regulation at the eyed egg stage (↑=up-regulation, ↓= down-regulation). Extended list of toxicity pathways is provided in Supplementary Table S3.*

Toxicity Pathway	**p-value**	**Ratio**	**Molecules**
Renal Necrosis/Cell Death	0.002	0.044	↑APOE,↑TCF4,↓MAP2K7,↑CA4,↑BGN,↑HBEGF,↑MST1, ↑TNFAIP3,↑C1QA,↓PRSS1,↑SDHC,↑P2RX7,↑TP53BP2, ↓HK1,↑VOPP1,↑RHOG,↑HTT,↑BNIP3L,↑SNCA, ↓PLAT
LXR/RXR Activation	0.005	0.065	↑APOE,↑APOA4,↑TF,↓NCOR1,↑ACACA,↑IL1RAPL1, ↑MMP9,↑IL36B
Increases Depolarization of Mitochondria and Mitochondrial Membrane	0.005	0.176	↑HTT,↑CYR61, ↑GZMB
Oxidative Stress	0.033	0.070	↓NQO1, ↑GGT1, ↓NFE2L1, ↑PRDX2
TR/RXR Activation	0.036	0.058	↑UCP3,↑COL6A3,↓SLC16A2,↓NCOR1,↑ACACA
Acute Renal Failure Panel (Rat)	0.043	0.065	↑CD68,↑SLC9A3,↑Cyp4a14,↑SLC30A1

Abbreviations: LXR: liver X receptor; RXR: retinoic X Receptor; TR: Thyroid hormone receptor;
Genes: *ACACA: acetyl-CoA carboxylase alpha; APOE: apolipoprotein E; BGN: biglycan; BNIP3L: BCL2/adenovirus E1B 19kDa interacting protein 3-like; C1QA: complement component 1, q subcomponent, A chain; CA4: carbonic anhydrase IV; CD68: CD68 molecule; Cyp4a14: cytochrome P450, family 4, subfamily a, polypeptide 14; CYR61: Cysteine-rich, angiogenic inducer, 61; GGT1: Gamma-glutamyltransferase 1, GZMB: granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1); HBEGF: heparin-binding EGF-like growth factor; HTT: huntingtin; HK1: hexokinase 1; IL1RAPL1: interleukin 1 receptor accessory protein-like 1; IL36B: interleukin 36, beta, MAP2K7: mitogen-activated protein kinase kinase 7; MMP9: matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase); MST1: macrophage stimulating 1 (hepatocyte growth factor-like); NCOR: nuclear receptor corepressor 1; NFE2L1: nuclear factor, erythroid 2-like 1; NQO1: NAD(P)H dehydrogenase, quinone 1; P2RX7: purinergic receptor P2X, ligand-gated ion channel, 7; PLAT: plasminogen activator, tissue; PRDX2: peroxiredoxin 2; PRSS1: protease, serine, 1 (trypsin 1); RHOG: ras homolog family member G; SDHC: succinate dehydrogenase complex, subunit C; SLC16A2: solute carrier family 16, member 2 (thyroid hormone transporter); SLC30A1: solute carrier family 30 (zinc transporter), member 1; SLC9A3: solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3), member 3; SNCA: synuclein, alpha (non A4 component of amyloid precursor); TCF4: transcription factor 4; TNFAIP3: tumor necrosis factor, alpha-induced protein 3; TF: transferrin; TP53BP2: tumor protein p53 binding protein, 2; UCP3: uncoupling protein 3 (mitochondrial, proton carrier), COL6A3: collagen, type VI, alpha 3; VOPP1: vesicular, overexpressed in cancer, prosurvival protein 1*

Table 5. *Canonical pathways being affected in Atlantic salmon (S. salar) embryos after exposure to 5000 mg/L road salt from fertilization to 24h post fertilisation. The FDR-corrected p-value and ratio of genes identified being differentially expressed versus total number of genes on the pathway is depicted in detail. The arrows indicate direction of gene regulation at the eyed egg stage (↑=up-regulation, ↓= down-regulation). Extended list of canonical pathways is provided in Supplementary Table S4.*

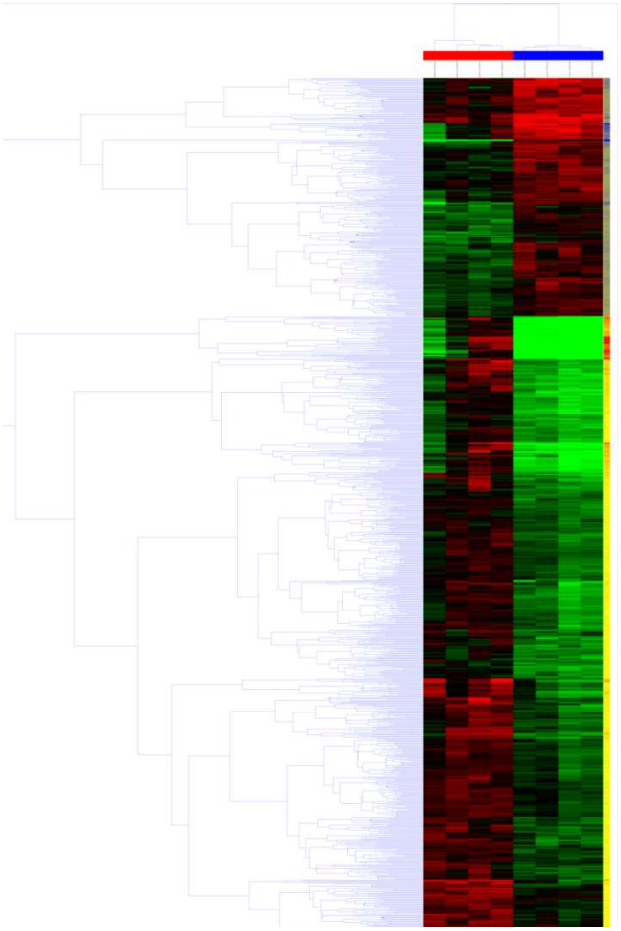
Canonical Pathways	Signalling pathway category	Top functions and disease	p-value	Ratio	Genes
LXR/RXR Activation	Nuclear Receptor Signaling	Lipid Metabolism; Small Molecule Biochemistry; Molecular Transport	0.006	0.059	↑APOE,↑APOA4,↑TF,↓NCOR1,↑ACACA, ↑IL1RAPL1,↑MMP9,↑IL36B
Clathrin-mediated Endocytosis Signaling	Cellular Immune Response; Organismal Growth and Development; Pathogen-Influenced Signaling	Cellular Function and Maintenance; Infectious Disease; Cellular Assembly and Organization	0.007	0.051	↑AP2B1,↑APOE,↑APOA4,↓SNX9,↓EPS1, ↑TF,↑CSNK2A1,↓SH3GL2,↑ITGB7, ↓HIP1R
Trehalose Degradation II (Trehalase)	Trehalose Degradation	Cardiovascular System Development and Function; Cell Morphology; Embryonic Development	0.012	0.250	↓HK1,↑GCK
Agranulocyte Adhesion and Diapedesis	Cellular Immune Response	Cell-To-Cell Signaling and Interaction; Cellular Movement; Hematological System Development and Function	0.016	0.048	↑PODXL,↑CLDN8,↑MYL6,↑CXCL6, ↑ITGB7,↑MMP9,↓MYL3,↑IL36B,↓MYL7
Regulation of Actin-based Motility by Rho	Neurotransmitters and Other Nervous System Signaling	Cell Signaling; Cellular Assembly and Organization; Cellular Function and Maintenance	0.034	0.056	↑RHOG,↑MYL6,↑RHOH,↓MYL3,↓MYL7
MIF-mediated Glucocorticoid Regulation	Cellular Immune Response; Nuclear Receptor Signaling	Hematological Disease; Infectious Disease; Organismal Injury and Abnormalities	0.038	0.071	↑NFKBIE,↑PLA2G4F,↓CD74
Epithelial Adherens Junction Signaling	Cellular Growth, Proliferation and Development	Cellular Assembly and Organization; Cellular Function and Maintenance; Cell Morphology	0.038	0.048	↑TCF4,↑MYL6,↓SSX2IP,↓IQGAP1, ↓NOTCH1,↓MYL3,↓MYL7
GDP-glucose Biosynthesis	Sugar Nucleotides Biosynthesis	Molecular Transport; Protein Trafficking; Cardiovascular System Development and Function	0.040	0.118	↓HK1,↑GCK
Granzyme B Signaling	Cellular Immune Response	Cell Death and Survival; DNA Replication, Recombination, and Repair; Cell Morphology	0.045	0.125	↑PRF1,↑GZMB
Serotonin and Melatonin Biosynthesis	Hormones Biosynthesis	Reproductive System Development and Function; Endocrine System Development and Function; Small Molecule Biochemistry	0.045	0.125	↑TPH1,↑ASMT
Parkinson's Signaling	Disease-Specific Pathways; Neurotransmitters and Other Nervous System Signaling	Cell Death and Survival; Hereditary Disorder; Neurological Disease	0.045	0.125	↓PARK2,↑SNCA
Atherosclerosis Signaling	Cardiovascular Signaling; Disease-Specific Pathways	Cell-To-Cell Signaling and Interaction; Tissue Development; Hematological System Development and Function	0.045	0.044	↑APOE,↑APOA4,↑PLA2G4F,↑ALOXE3, ↑MMP9,↑IL36B
April Mediated Signaling	Apoptosis	Cellular Development; Cellular Growth and Proliferation; Hematological System Development and Function	0.047	0.070	↓MAP2K7,↑TNFSF13,↑NFKBIE
Notch Signaling	Cancer; Organismal Growth and Development	Hair and Skin Development and Function; Organ Development; Organ Morphology	0.047	0.070	↑HEY2,↓NOTCH1,↑DTX2
TR/RXR Activation	Nuclear Receptor Signaling	Amino Acid Metabolism; Drug Metabolism; Endocrine System Development and Function	0.047	0.052	↑UCP3,↑COL6A3,↓SLC16A2,↓NCOR1, ↑ACACA
ILK Signaling	Cellular Growth, Proliferation and Development	Cellular Movement; Cellular Development; Cellular Growth and Proliferation	0.047	0.042	↑RHOG,↑MYL6,↑PPP1R14B,↑RHOH, ↑ITGB7,↑MMP9,↓MYL3,↓MYL7
Calcium Signaling	Intracellular and Second Messenger Signaling	Cell Signaling; Molecular Transport; Vitamin and Mineral Metabolism	0.047	0.038	↑HDAC4,↑MYL6,↓TNNC1,↑RYR3, ↑HDAC10,↓MCU,↓MYL3,↓MYL7

Abbreviations: LXR: liver X receptor; RXR: retinoic X Receptor; TR: Thyroid hormone receptor; MIF: Macrophage migration inhibitory factor; GDP-glucose: Guanosine 5-diphosphoglucose; ILK: Integrin-linked kinase;

Genes: *ACACA: acetyl-CoA carboxylase alpha; ALOXE3: arachidonate lipoxygenase 3; AP2B1: adaptor-related protein complex 2, beta 1 subunit; APOA4: apolipoprotein A-IV; APOE: apolipoprotein E; ASMT: acetylserotonin O-methyltransferase; CD74: CD74 molecule, major histocompatibility complex, class II invariant chain; CLDN8: claudin 8; COL6A3: collagen, type VI, alpha 3; CSNK2A1: casein kinase 2, alpha 1 polypeptide; DTX2: deltex homolog 2 (Drosophila); EPS15: epidermal growth factor receptor pathway substrate 15; GCK: glucokinase (hexokinase 4), GZMB: granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1); HDAC10: histone deacetylase 10; HDAC4: histone deacetylase 4; HEY2: hes-related family bHLH transcription factor with YRPW motif 2; HIP1R: huntingtin interacting protein 1 related; HK1: Hexokinase 1; IL1RAPL1: interleukin 1 receptor accessory protein-like 1; IL36B: interleukin 36, beta; IQGAP1: IQ motif containing GTPase activating protein 1; ITGB7: integrin, beta 7; MAP2K7: mitogen-activated protein kinase kinase 7; MCU: mitochondrial calcium uniporter; MMP9: matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase); MYL3: myosin, light chain 3, alkali; ventricular, skeletal, slow; MYL6: myosin, light chain 6, alkali, smooth muscle and non-muscle; MYL7: myosin, light chain 7, regulatory; NCOR1: nuclear receptor corepressor 1; NFKBIE: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon; NOTCH1: notch 1; PARK2: parkin RBR E3 ubiquitin protein ligase; PLA2G4F: phospholipase A2, group IVF; PODXL: podocalyxin-like; PPP1R14B: protein phosphatase 1, regulatory (inhibitor) subunit 14B; PRF1: perforin 1 (pore forming protein); RHOG: ras homolog family member G; RHOH: ras homolog family member H; RYR3: ryanodine receptor 3; SH3GL2: SH3-domain GRB2-like 2; SLC16A2: solute carrier family 16, member 2 (thyroid hormone transporter); SNCA: synuclein, alpha (non A4 component of amyloid precursor); SNX9: sorting nexin 9; SSX2IP: synovial sarcoma, X breakpoint 2 interacting protein; TCF4: transcription factor 4; TF: transferrin; TNFSF13: tumor necrosis factor (ligand) superfamily, member 13; TNNC1: troponin C type 1 (slow); TPH1: tryptophan hydroxylase 1; UCP3: uncoupling protein 3 (mitochondrial, proton carrier).*

Figure(s)

Figure 1_Tollefsen et al.



Down-regulated transcripts
Features: 281 (Suppl. Table S1)

All transcripts
Features: 1002 (Suppl. Table S1)
GOs: 16 (Table 3, Suppl. Table S2)
Toxicity pathways: 6 (Table 4, Suppl. Table S3)
Canonical pathways: 17 (Table 5, Suppl. Table S4)
Gene-gene interaction networks: 25 (Suppl. Fig. 1, Suppl. Table S5)

UP-regulated transcripts
Features: 721 (Suppl. Table S1)

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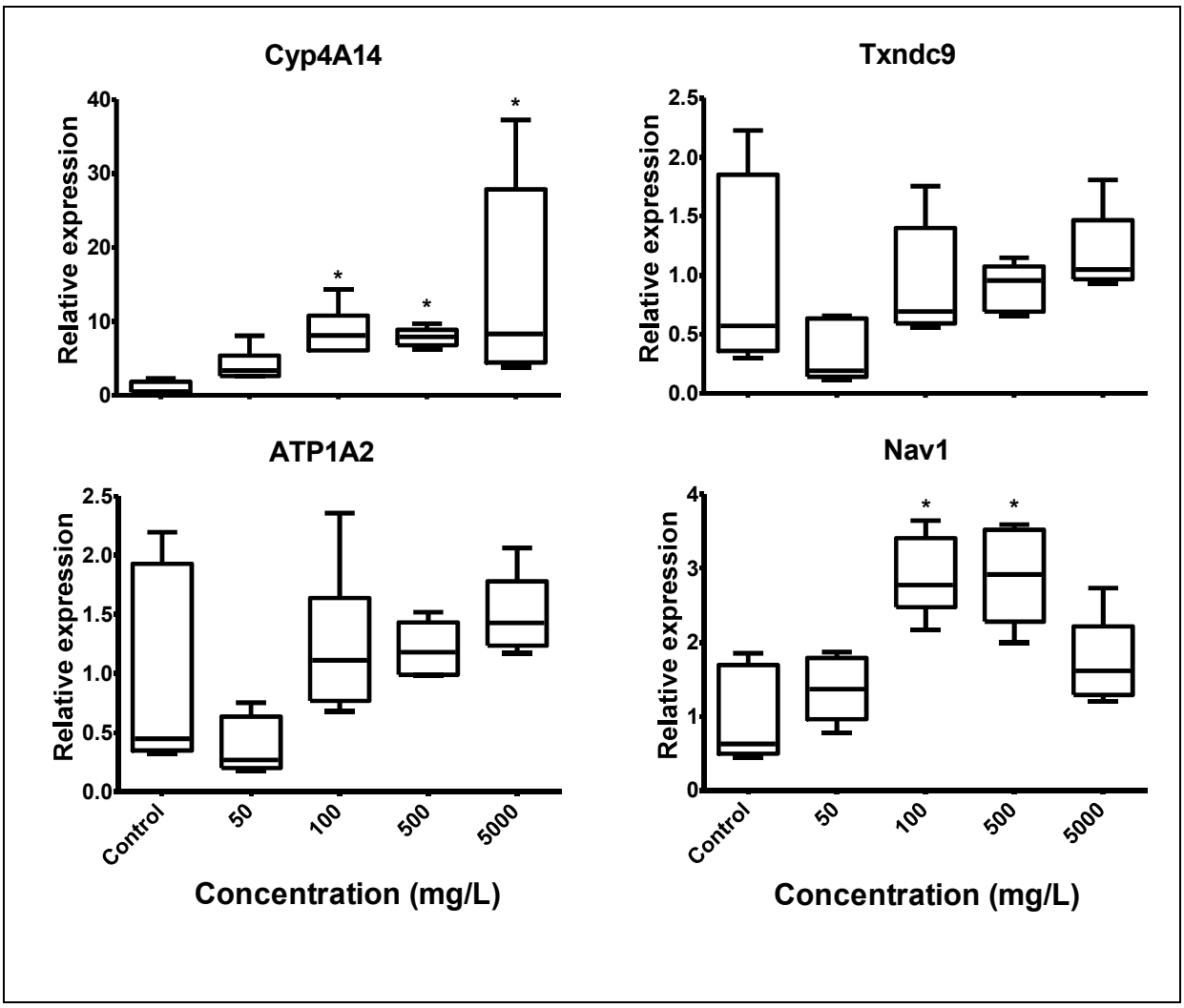
Figure 2_Tollefsen et al.



Supplementary Material

Click here to download Supplementary Material: Tollefsen et al. Supplementary Tables_YSO14092015.xls

- Exposure to 5000 mg/L road salt the first 24h after fertilization caused global transcriptional changes detected at the eyed egg stage in Atlantic salmon.
- Main transcriptional changes indicate interference with osmoregulation, ion regulation, oxidative stress, metabolism, renal function and development in the embryos.
- Effects in selected biomarker for exposure and effect occurred as low as 100 mg/L road salt (NOTEL 50 mg/L)

Transcriptional changes in Atlantic salmon (*Salmo salar*) after embryonic exposure to road salt

Knut Erik Tollefsen, You Song, Merethe Kleiven, Urma Mahrosh, Sondre Meland, Bjørn Olav Rosseland and Hans-Christian Teien.

Supplementary figures

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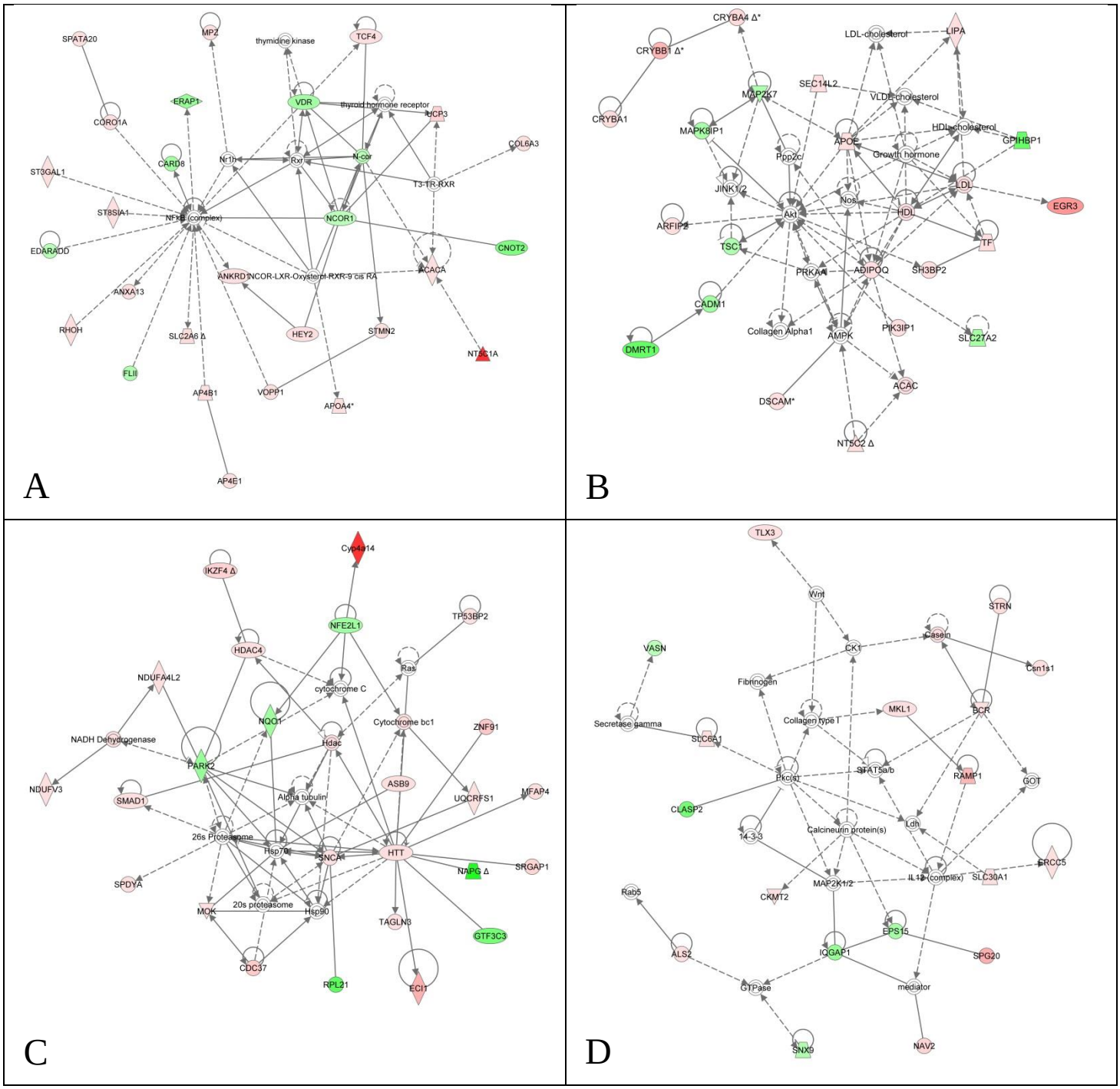

Suppl. Fig. 1. Gene-gene interaction networks in Atlantic salmon (*S. salar*) eyed embryos affected by exposure to 5000 mg/L road salt from fertilization to 24h post fertilisation: A) energy production and lipid metabolism; B) organ and embryonic development, C) cell signaling and interaction and D) nervous system development and function. Color intensity indicates fold regulation of the genes at the eyed egg stage. (red=up, green=down, white=not applicable). Extended list of Regulatory networks is provided in supplementary Table S5.