Accepted Manuscript

Hepatic transcriptional responses in Atlantic salmon (*Salmo salar*) exposed to gamma radiation and depleted uranium singly and in combination.

You Song[1,2,*], Brit Salbu[1], Hans-Christian Teien[1], Øystein Evensen[3], Ole Christian Lind[1], Bjørn Olav Rosseland[1,4], Knut Erik Tollefsen[1,2]

[1] Norwegian University of Life Sciences (NMBU), Faculty of Environmental Science and Technology, Department of Environmental Sciences (IMV), Centre for Environmental Radioactivity (CERAD). P.O. Box 5003, N-1432 Ås, Norway

[2] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway

[3] Norwegian University of Life Sciences (NMBU), Department of Basic Sciences and Aquatic Medicine, P.O. Box 8146 Dep., N-0033 Oslo, Norway

[4] Norwegian University of Life Sciences (NMBU), Department of Ecology and Natural Resource Management (INA). P.O. Box 5003, N-1432 Ås, Norway

Corresponding author:
Name: You Song
Address: Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway
Phone: + 47 02348
Fax: + 47 22185200
E-mail address: yso@niva.no

Abstract

Radionuclides are a special group of substances posing both radiological and chemical hazards to organisms. As a preliminary approach to understand the combined effects of radionuclides, exposure studies were designed using gamma radiation (Gamma) and depleted uranium (DU) as stressors, representing a combination of radiological (radiation) and chemical (metal) exposure. Juvenile Atlantic salmon (*Salmo salar*) were exposed to 70 mGy external Gamma dose delivered over the first 5h of a 48 h period (14 mGy/h), 0.25 mg/L DU were exposed continuously for 48 h and the combination of the two stressors (Combi). Water and tissue concentrations of U were determined to assess the exposure quality and DU bioaccumulation. Hepatic gene expression changes were determined using microarrays in combination with quantitative real-time reverse transcription polymerase chain reaction (qPCR). Effects at the higher physiological levels were determined as plasma glucose (general stress) and hepatic histological changes. The results show that bioaccumulation of DU was observed after both single DU and the combined exposure. Global transcriptional analysis showed that 3122, 2303 and 3460 differentially expressed genes (DEGs) were significantly regulated by exposure to gamma, DU and Combi, respectively. Among these, 349 genes were commonly regulated by all treatments, while the majority was found to be treatment-specific. Functional analysis of DEGs revealed that the stressors displayed similar mode of action (MoA) across treatments such as induction of oxidative stress, DNA damage and disturbance of oxidative phosphorylation, but also stressor-specific mechanisms such as cellular stress and injury, metabolic disorder, programmed cell death, immune response. No changes in plasma glucose level as an indicator of general stress and hepatic histological changes were observed. Although no direct linkage was successfully established between molecular responses and adverse effects at the organism level, the study has enhanced the understanding of the MoA of single radionuclides and mixtures of these.

Key words: Multiple stressor, depleted uranium, gamma radiation, Atlantic salmon, gene expression, mode of action

1 Introduction

Concern for combined effects has been raised in the recent decade due to exposure to multiple stressors in the aquatic environment (Holmstrup, 2010; Laskowski, 2010; Vanhoudt et al., 2012). Multiple stressors refer to co-exposure to fundamentally different stressors that influence the performance of an organism simultaneously, including abiotic stressors such as organic and inorganic chemicals, radiation, salinity, temperature, pH, and biotic stressors such as bacterial infection and food web relationships. Radionuclides may produce toxic effects in exposed organisms due to their chemical toxicity (e.g. heavy metal) and irradiation effects (e.g. alpha, beta, gamma radiation, x-ray). Thus, organisms exposed to a radionuclide are eventually facing a multiple stressor exposure situation, and effects associated with chemical and radiological toxicity are difficult to differentiate. Anthropogenically produced radionuclides such as cobalt (^{60}Co) are released to the environment from the nuclear weapon and fuel cycles, while naturally occurring radionuclides (NORM) such as uranium are enriched in minerals such as alum shales (Salbu, 2009). Global, regional and local deposition of radionuclides may potentially affect various environmental compartments, whereof the aquatic environmental is essential for the transport of radioactive substances. Fish are in this respect key aquatic species, not only because of their position in the food web structure and ecosystem functions, but also due to their high value as food sources for human. Therefore, fish have been commonly used as vertebrate models in various multiple stressor studies with radionuclides (Mothersill et al., 2007; Salbu et al., 2008; Olsvik et al., 2010; Vanhoudt et al., 2012; Bourrachot et al., 2014; Bucher et al., 2014; Gagnaire et al., 2014; Augustine et al., 2015; Lange et al., 2015).

Being a key NORM element, natural U includes the isotopes ^{234}U, ^{235}U and ^{238}U. To obtain high proportion of fissile ^{235}U for civil use in nuclear power reactors and military use in nuclear weapons, natural U undergoes an enrichment process. The remaining material after enrichment is depleted of ^{234}U and ^{235}U and is therefore referred to as depleted uranium (DU). The main isotope in DU is ^{238}U, a weak alpha emitter, which has much lower specific activity (approximately 1.47×10^4 Bq/g) and longer half-life (4.5×10^9 years) than the other U isotopes, and the chemical toxicity of DU is thus believed to be more important than its radiotoxicity (Sheppard et al., 2005). Depleted U is widely used in many applications, such as military use of armor penetrators, and civil use of counterweights for airplane construction and irradiation shielding. Gamma radiation, on the other hand, is ionizing radiation emitted from decay of high energy states of unstable nuclei such as ^{60}Co. The high energy gamma radiation from ^{60}Co (1173.23 and 1332.49 keV) is long ranged and widely used in many different fields such as sensors in the civil sector, medical treatment for cancer (e.g. gamma-knife), or used as a clean gamma radiation source in experimental irradiation facilities. In areas with extensive U mining, contamination by nuclear detonations or accidents and high NORM emission mixtures of radionuclides representing both internal and external exposure of ionizing radiation and as well as a number of non-radioactive metals toxic elements may pose an hazard to organism in the recipient (Lind et al., 2013).

The biological effects of DU and gamma radiation have been widely documented for mammalian species. It is well-known that both DU and gamma radiation induce cellular free radicals, such as reactive oxygen species (ROS), through Haber-Weiss and Fenton reactions (DU) or by excitation and ionization of water molecules (gamma radiation). The excessive formation of ROS may subsequently lead to cellular oxidative stress as a primary mode of

3

action (MoA) of these stressors, which may result in a number of cellular responses, such as DNA damage, apoptosis, inflammation and disturbance of cell signaling (Pourova et al., 2010). If present in the environment as bioavailable metal species, U may accumulate and distribute to tissues such liver and bone in aquatic species such as fish. Although DU excrete in a similar way as many other metals, this bioactive metal may affect several cellular targets and cause biological effects such as DNA damage and nuclear receptor signaling (Pereira et al., 2012; Song et al., 2012; Lerebours et al., 2013), interference with lipids and membrane-associated proteins and modulation of the functions of enzymes/proteins in the mitochondria (Lerebours et al., 2009; Lourenco et al., 2010; Song et al., 2012). In contrast to chemical stressors, radiation interacts with targets throughout the body and thereby exerting their toxicity directly (e.g. DNA breaks) or indirectly (via free H^+ and OH^- radicals) (Salbu et al., 2008). Therefore, the potential interactions between radiation and chemical stressors may be rather complex. Effects of gamma radiation has been amply demonstrated including induction of oxidative stress, DNA damage, apoptosis (Olsvik et al., 2010; Heier et al., 2013), perturbation of the mitochondrial respiratory chain (O'Dowd et al., 2009) and genomic instability (O'Dowd et al., 2006; Mothersill et al., 2007; Salbu et al., 2008).

To understand the combined toxicity of environmental stressors, substantial knowledge on their toxicological mechanisms is usually required. Vanhoudt and co-workers recently reviewed available multiple stressor studies, and pointed out there is an urgent need to improve systematic and mechanistic knowledge and quantitative assessments of multiple stressor effects and possible interactions (Vanhoudt et al., 2012). By understanding the MoA, which defines the critical molecular events leading to an adverse outcome, appropriate toxicological endpoints can be selected for use in the prediction of multiple-stressor effect. Toxicogenomics (OMICS) such as that provided by transcriptomics (DNA microarray, RNA sequencing), proteomics and metabolomics may assist the identification of potential MoA of a stressor, low-exposure-level stress responses and potential interactions between stressors in a genome-wide scale (Finne et al., 2007; Spurgeon et al., 2010; Altenburger et al., 2012).

As an initial approach to decipher the multiple stressor effect of radionuclides in aquatic vertebrates, the present study aimed to assess the hepatic molecular (global transcriptomics) and adverse effects (plasma glucose stress response and hepatic histopathology) in Atlantic salmon (*Salmo salar*) exposed to gamma radiation and DU singly and in combination. The objectives of this study were to: 1) characterize the short-term stress responses in fish exposed to gamma radiation and DU to characterize the MoAs of these stressors; 2) to assess how these two stressors act in combination to cause combined toxicity.

2 Materials and methods

2.1 Exposure and fish sampling

The experimental conditions and use of fish in this study strictly followed the Norwegian Welfare Act and research animal legislation by the local representative of the Norwegian Animal Research Authority (NARA ID: 3026). Briefly, Atlantic salmon juveniles (parr) were maintained at the Fish Laboratory of Norwegian University of Life Sciences (NMBU, Ås, Norway) and transported to the FIGARO gamma radiation experimental facility (NMBU, Ås, Norway). The test fish was acclimated to the experimental water (lake water from Maridalsvannet, Oslo, Norway)

conditions 7 days prior to the exposure studies. Feeding was stopped two days prior to the start of the exposure study Feeding was stopped two days prior to the start of the exposure study in order to avoid interaction of food on U speciation and minimize the influence of feces on U speciation.

Three exposure experiments were run simultaneously: 1) exposure to a single total dose of 70 mGy (12.2-15.8 mGy/h) gamma radiation (1173.23 and 1332.49 keV, representing two gamma ray lines emitted in each decay) delivered over the first 5 h of a 48 h experimental period; 2) exposure to a single nominal concentration of 0.25 mg/L DU ($UO_2(CH_3COO)_2 \cdot 2H_2O$, purity $\geq$ 98.0 %, specific activity 1.459×10^4 Bq/g, 57.3 % of the radioactivity from natural U, Fluka, Sigma-Aldrich, Buchs, Switzerland) continuously for 48 h; 3) exposure to a combination of gamma radiation and DU using the same exposure regimes as the single stressor studies. The chosen gamma dose and U concentration were both environmentally relevant (Simon et al., 2010; Salbu, 2011; Lind et al., 2013) and previously demonstrated to cause transcriptional effects in Atlantic salmon (Olsvik et al., 2010; Song et al., 2012; Heier et al., 2013; Song et al., 2014b), and thus considered optimal for studying the interaction of the two stressors. A ^{60}Co gamma source (63 GBq) was used for external irradiation of fish, and the field dosimetry (air kerma rates measured with an ionization chamber) was traceable to the Norwegian Secondary Standard Dosimetry Laboratory (Norwegian Radiation Protection Authority, NRPA, Oslo, Norway). The geometric center of the exposure boxes (W5 x L40 x H20 cm) was used as reference point and assumed to represent the best estimate of the dose. The maximum hypothetical variation in dose to fish was estimated to be $\pm$13 % by calculating the increased dose rate in the unlikely case that fish would stay in the box area closest to the source. Controls (no radiation, with or without DU) were placed in the same room, outside the beam cone and shielded by lead. For the controls, the external (background) dose rate to the fish was 0.2 µGy/h (Thermo Eberline FHT6020). Six fish in each treatment group were exposed in dark exposure chambers (30 L, fish loading approx. 3 g/L) in a closed flow-through system supplied with aerated lake water (Maridalsvannet, Norway). The dark exposure chambers were used to minimize additional stress to fish. The general water quality was monitored throughout the exposure, including logging of pH, temperature and conductivity. The sizes of the test fish (length 11.9 $\pm$ 0.3 cm, weight 14.95 $\pm$ 1.3 g, mean $\pm$ SD) were similar across groups.

After 48 h, fish were immediately sacrificed by cephalic concussion. Blood samples were collected for determination of plasma glucose level using the i-STAT® portable analyzer (Abbott Point of Care Inc., Princeton, NJ, USA) with EC8+ cassette (Abbot, East Windsor, USA), according to the manufacturer's instructions. Fish were then dissected according to the EMERGE protocol (Rosseland et al., 2001) to collect tissue samples for chemical analysis, gene expression analysis (snap-frozen in liquid nitrogen and stored at -80 ℃) and histopathological analysis (tissue submerged in 10 mL of 10% phosphate buffered formalin, pH = 7, and stored at 4 ℃).

2.2 Chemical analysis

The chemical analysis of variables in the control and DU containing test waters has been described previously (Song et al., 2012). Briefly, water samples were collected for measuring the concentrations of major cations (Ca, Mg, Na, K and Al) using inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer, Optima 5300

DV), and anions (NO_3^-, SO_4^{2-} and Cl^-) using Iachat IC5000 ion chromatograph. Total organic carbon (TOC) was determined using the total organic analyzer (Shimadzu TOC cpn, Kyoto, Japan).

Inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer Sciex ELAN 6000) was used to measure the concentrations of U in the exposure water and salmon liver. For measurement of U in the liver, samples were weighed and mixed with HNO_3, milliQ water and an internal standard. Samples were digested using an ultraclave (Milestone, Leutkirch, Germany) and diluted (10% ultrapure HNO_3 solution) prior to measurement of U concentrations using ICP-MS. The limits of detection (LD) for ICP-MS were 0.0056 µg/L and 0.0005 µg/g, for water and salmon liver samples, respectively.

2.3 Global transcriptomic analysis

Transcriptional changes were determined by a custom oligonucleotide microarray based on the consensus contingous (contig) mRNA and expressed sequence tags (EST) sequences of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*). Over 44,000 (44 k) unique probes were designed in Earray (Agilent Technologies, Santa Clara, USA) and printed on a 60 k oligoarray (Agilent Technologies, Santa Clara, CA, USA). For transcriptional analysis, total RNA was isolated from approximately 20-30 mg liver using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), as described previously (Song et al., 2012). The microarray analysis was performed according to Agilent's standard protocol "One-Color Microarray-Based Gene Expression Analysis, version 6.5", with small modifications (Song et al., 2014a).

Raw microarray data (signal intensity) was corrected for background signal, flagged for low quality and missing features and log_2 transformed for normalization (quantiles) using GeneSpring GX v11.0 (Agilent Technologies). Statistical analysis of the normalized microarray data was performed using the Bioconductor (http://www.bioconductor.org/) package LIMMA (Smyth, 2005) in the R statistical environment v3.1.2 (http://www.r-project.org/). Different contrasts were defined over the linear model in the statistical tests to identify transcriptional effects due to single and multiple stressor exposures, as described previously (De Coninck et al., 2014). No multiple testing correction was performed in order to avoid any loss of relevant data, as previously suggested (Song et al., 2014b). The raw and normalized microarray data has been deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, GEO accession number: GSE74012).

To understand the biological significance of the DEGs, functional enrichment analysis based on Gene Ontology (GO) was performed first using a hypergeometric test followed by a Benjamini–Hochberg (BH) false discovery rate (FDR) correction in Cytoscape v2.8 (Smoot et al., 2011) application Bingo v2.4 (Maere et al., 2005). Relationships between enriched GOs and related parent terms at higher functional levels were built using directed acyclic graph (DAG) and visualized in Cytoscape. For a more in-depth characterization of potential MoAs, salmon DEGs were mapped to their mammalian orthologs using a modified Inparanoid algorithm (Ostlund et al., 2010) as previously described (Song et al., 2014a) prior to pathway analysis using Ingenuity Pathway Analysis (IPA, Ingenuity®Systems, www.ingenuity.com). The enrichment of toxicity and canonical pathways were determined to get insight into the hepatic toxicological responses using curated mammalian models as proxies for fish. Venn

diagram analysis was performed using Venny (http://bioinfogp.cnb.csic.es/tools/venny/). A probability (p) value of 0.05 was applied to all statistical tests.

2.4 qPCR analysis

The quantitative real-time reverse transcription polymerase chain reaction (qPCR) was used to verify the microarray results. Briefly, complementary DNA (cDNA) was made from 2 μg total RNA by reverse transcription using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. The diluted cDNA template (5 ng), non-template control (NTC, i.e. nuclease-free water) and no-reverse-transcriptase control was then amplified in a 20 μL reaction containing 400 nM forward/reverse primers (Invitrogen™,Carlsbad, California, USA) and PerfeCTa® SYBR® Green FastMix® (Quanta BioSciencesTM, Gaithersburg, MD, USA) using a BioRad CFX 384 platform (Bio-Rad Laboratories, Hercules, CA, USA). An additional step of melting curve analysis was included to ensure the quality of amplification (i.e. valid amplification when amplicon had a unique melting peak). Standard curves were run with 50, 10, 2 and 0.4 ng of cDNA. Relative expressions were then determined from the standard curves based on threshold cycle (Cq) value by CFX Manager v3.0 (Bio-Rad) as described previously (Song et al., 2014a). Target gene expression was normalized to the geometric mean two reference genes, 18s ribosomal RNA (18s) and elongation factor 1 alpha b (ef1ab), as they were stably expressed cross treatments. All primer sequences used in the qPCR analysis (Table 1) were designed using Primer3 v0.4 (Rozen and Skaletsky, 2000) on basis of the contig sequences used for designing the microarray probes and putative *Salmo salar* mRNA sequences in GenBank (Benson et al., 2005).

Table 1. Primer sequences for real-time qPCR. Full descriptions of gene symbols can be found in Appendices (Table A3).

Gene symbol	GenBank accession	Forward (5'-3')	Reverse (5'-3')
18s	AJ427629	TGTGCCGCTAGAGGTGAAATT	GCAAATGCTTTCGCTTTCG
Ef1ab	AF321836	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG
Cox6b1	BT125515.1	GACAATGCTTGGCACATACG	TGTCAGCAGATGCAGAGTCC
Atp1a1	EG857701	ATAGCTGCACACCCTGGAAT	CTGGACACGCATAACACAGC
Myc	BT059112.1	GGACTCAATGCCAAACCACT	CGTGTCTATGTCCGAGAGCA
Prkcd	BT058908.1	GCTGGCAGAGTTGAAAGGTC	ACAGGTGTTCCTTGGACTGG
Camk2da	BT072752.1	GGATCTGTCAACGGTCCACT	TGACACCGTCAGCTTTCTTG
Xrcc5	XM_005800723.1	GGAGGATGAAGGAAACGTGA	TCGATCAACAAACCACCTGA

For qPCR verification, target DEGs representative of key regulators in toxicologically relevant canonical pathways were selected. Briefly, raw data was normalized to the geometric mean of the reference gene expression and checked for outlier using the ROUT test (Graphpad Software, Inc., San Diego, CA, USA). Differential gene expression was determined using one-way ANOVA followed by a Tukey HSD posthoc test. Data that failed to fulfil the assumptions of normal distribution and equal variance were analyzed using a Kruskal-Wallis non-parametric test followed by a Dunn's posthoc test. A probability (p) value of 0.05 was applied to all statistical tests. Statistical analysis was performed using Graphpad Prism v5.0 (Graphpad Software).

2.5 Histopathological analysis

Liver and gill histopathological analysis was also performed to examine potential changes at the light microscopic level. The collected tissue samples for histopathological analysis were submerged in 10 % phosphate buffered formalin and subsequently embedded in paraffin wax according to standard procedures. Sections were cut at 3-4 µm and stained with hematoxylin and eosin. Tissue sections were also stained with Periodic Acid Schiff (PAS) to visualize presence of glycogen (including amylase digestion as control) (Kiernan, 1999). Sections were examined using a Leica microscope equipped with a Leica DFC 420 digital camera (Leica Microsystems GmbH, Wetzlar, Germany).

3 Results

3.1 Quality control and exposure verification

The exposure media had average temperature of 9.7 ± 0.2 ºC (mean $\pm$ SD), conductivity of 3.4 mS/m and pH of 7.3 throughout the experiment. No significant changes of water TOC, ion and anion concentrations were observed throughout the study (Table 2). The measured concentrations of U in the media were 0.2 mg U/L (DU and Combi), and 0.41 µg U/L (Gamma and Control). The liver concentrations of U in Atlantic salmon after 48 h exposure were 5.5 ± 2.3 ng U/g w.w. (mean $\pm$ SD, n=6) and 5.8 ± 3.0 ng U/g w.w. in DU and Combi, respectively. Less than 0.5 ng/g ww U was found in the control and Gamma exposed fish. No mortality or visible morphological change was observed in test fish after the exposure. After 48 h, the blood glucose level was found to be slightly higher in Gamma (7.4 ± 5.8 mM, mean $\pm$ SD) and Combi (6.7 ± 4.2 mM) compared to DU (4.1 ± 0.9 mM) and Control (4.5 ± 1.6 mM).

Table 2. Uranium concentrations, total organic carbon (TOC), major cations and anions in the test media after 48 exposure. Gamma: gamma radiation; DU: depleted uranium; Combi: Gamma+DU.

Treatment group	U concentration (mg U/L)	TOC (mg/l)	Cl⁻ (mg/l)	NO₃⁻ (mg/l)	SO₄²⁻ (mg/l)	Na (mg/l)	Mg (mg/l)	Al (µg/l)	K (mg/l)	Ca (mg/l)
Control	0.00041	4.98	1.94	0.16	2.60	1.77	0.59	95	0.83	3.12
Gamma	0.00041	5.08	1.67	0.16	2.71	1.77	0.59	96	0.78	3.09
DU	0.2	5.20	1.76	0.15	2.68	1.77	0.60	101	0.78	3.27
Combi	0.2	5.21	1.69	0.15	2.69	1.74	0.59	101	0.74	3.08

3.2 Global transcriptional response

The microarray analysis identified a total of 3122 (1273 up-, 1849 down-regulated), 2303 (1004 up-, 1299 down-) and 3460 (1484 up-, 1976 down-) genes being differentially expressed in salmon liver after exposure to Gamma, DU and Combi, respectively (Figure 1). Among these DEGs, the majority was found to be treatment-specific by Venn diagram analysis, whereas 349 were identified as commonly regulated by all treatments. A higher number of DEGs were found to be commonly regulated by both Gamma and Combi (814) than that between DU and Combi (286). The complete DEG list can be found in Appendices (Table A1).

Gene Ontology-based functional enrichment analysis showed that 30 (Gamma) and 32 (Combi) GO functions were overrepresented after exposure, whereas no significant enrichment of GO was found for DU. Overrepresented GOs were linked to a range of biological functions, such as metabolic processes, oxidoreductase activity and membrane functions (Figure 1). The complete list of GO terms can be found in Appendices (Table A2).
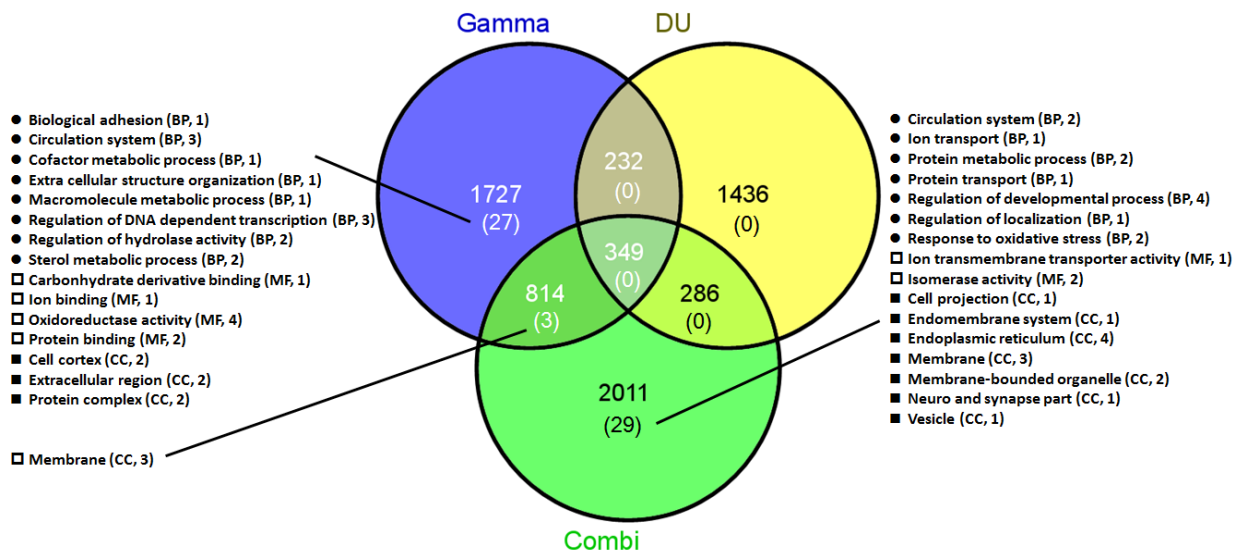

Figure 1. Venn diagram analysis of differentially expressed genes (DEGs) and associated overrepresented Gene Ontology (GO) functions (numbers in brackets) that were regulated in the liver of Atlantic salmon (*Salmo salar*) due to the effects of 70 mGy gamma radiation (Gamma, 5 h exposure), 0.25 mg/L depleted uranium (DU, 48 h exposure) and their combination (Combi) after short-term exposure. BP (solid circle): biological process; MF (open square): molecular function; CC (solid square): cellular component. The number of individual GOs for each category has been indicated for each term.

Ortholog mapping resulted in 59.4 % (Gamma), 56.9 % (DU) and 59.5 % (Combi) of Atlantic salmon DEGs being successfully mapped to mammalian orthologs and resulting mammalian orthologs used in Ingenuity Pathway Analysis (full list in Appendices, Table A3). The toxicity pathway analysis showed that 28 (Gamma), 19 (DU) and 31 (Combi) pathways were affected. Ten pathways were commonly affected by all treatments, while a considerable number of treatment-specific pathways were also identified (Figure 2-A). A higher number of toxicity pathways were found to be commonly affected by Gamma and Combi than that between DU and Combi. The complete list of toxicity pathways can be found in Appendices (Table A4).
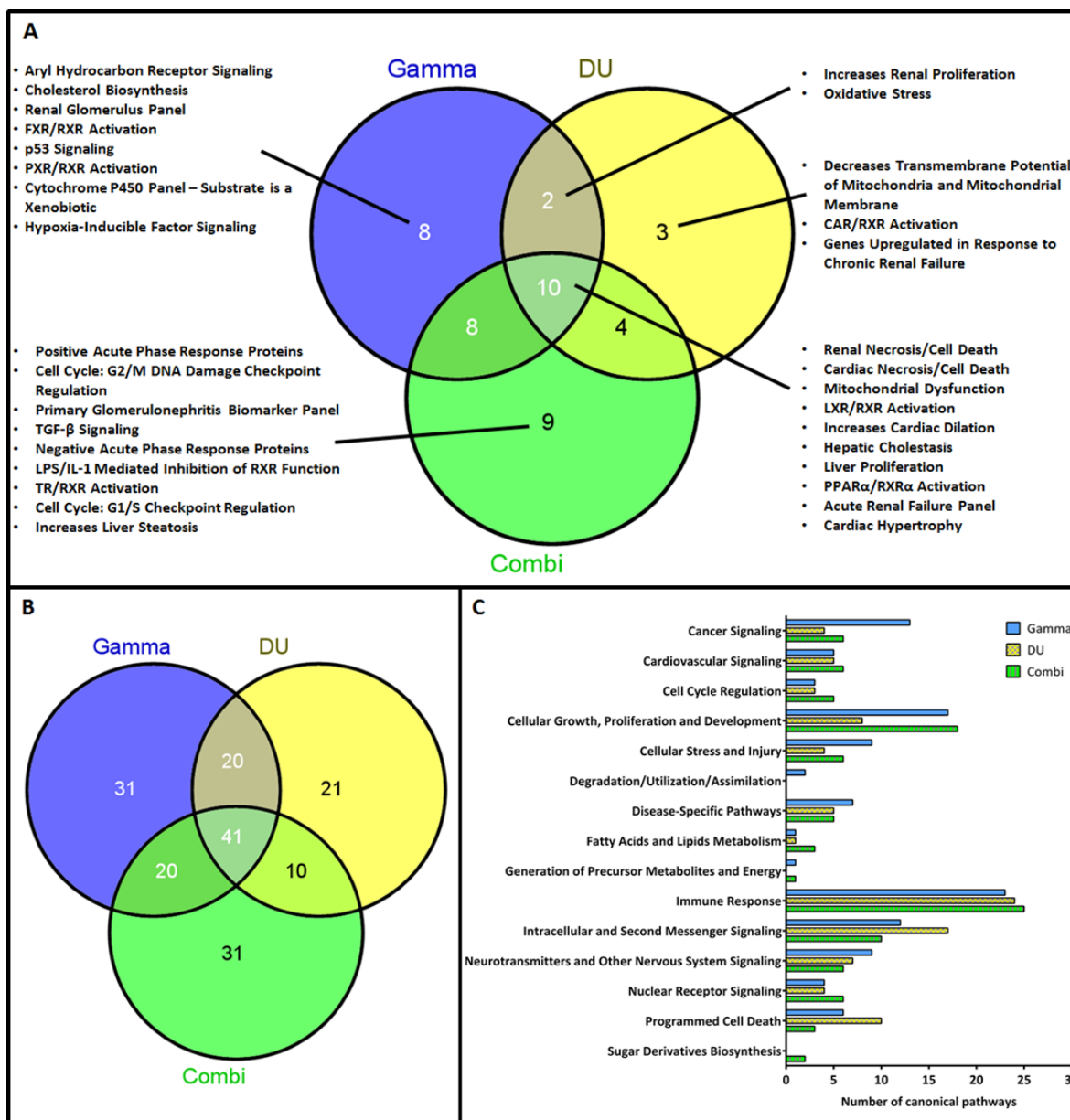
9

Figure 2. Venn diagram analysis of toxicity pathways (A), canonical pathways (B) and the main (apical) canonical pathway categories (C) that were affected by 70 mGy gamma radiation (Gamma, 5 h exposure), 0.25 mg/L depleted uranium (DU, 48 h exposure) and their combination (Combi) in the liver of Atlantic salmon (*Salmo salar*).

In addition to toxicity pathway analysis, the enrichment of well-curated canonical pathways was also determined in order to get more insight into the potential toxic mechanisms of the stressors. Differentially expressed genes involved in 112, 93 and 102 canonical pathways were found to be affected by Gamma, DU and Combi, respectively. Among these, 41 were found to be commonly regulated by both single and the combination of test stressors (Figure 2-B). A higher number of canonical pathways were found to be commonly affected by both Gamma and Combi than that between DU and Combi. Thirty-one (Gamma-specific), 21 (DU-specific) and 20 (common between Gamma and DU) pathways were only affected by the single stressors (i.e. Gamma or DU), while 31 were uniquely affected in

fish exposed to the combination of the two stressors. Grouping the pathways into higher functional categories to obtain an overall distribution of top pathway functions (Figure 2-C). A few functional categories revealed that immune response, intracellular and second messenger signaling and cell growth proliferation and development were affected by the stressors. Functional categories such as primary metabolic processes (degradation, utilization and assimilation), generation of precursor metabolites and energy and sugar derivatives biosynthesis were found to be stressor-specific,. The complete list of canonical pathways can be found in Appendices (Table A5).

3.3 qPCR verification

Genes for qPCR verification were selected on the basis of their representativeness of major pathways being affected by the stressors, including mitochondrial electron transport chain (cytochrome c oxidase subunit VIb polypeptide 1 (cox6b1), Na^+/K^+ ATPase alpha 1 (atp1a1)), DNA repair (X-ray repair complementing defective repair in Chinese hamster cells 5 (xrcc5)), calcium signaling (calcium/calmodulin-dependent protein kinase II delta (camk2da)) and apoptosis (v-myc avian myelocytomatosis viral oncogene (myc) and protein kinase C delta (prkcd)). Generally, the expression of most target genes tested using microarrays had good accordance (i.e. direction of regulation and magnitude of regulation) with that determined using qPCR (Figure 3), except for camk2da, which displayed higher fold changes by microarrays than that measured by qPCR.
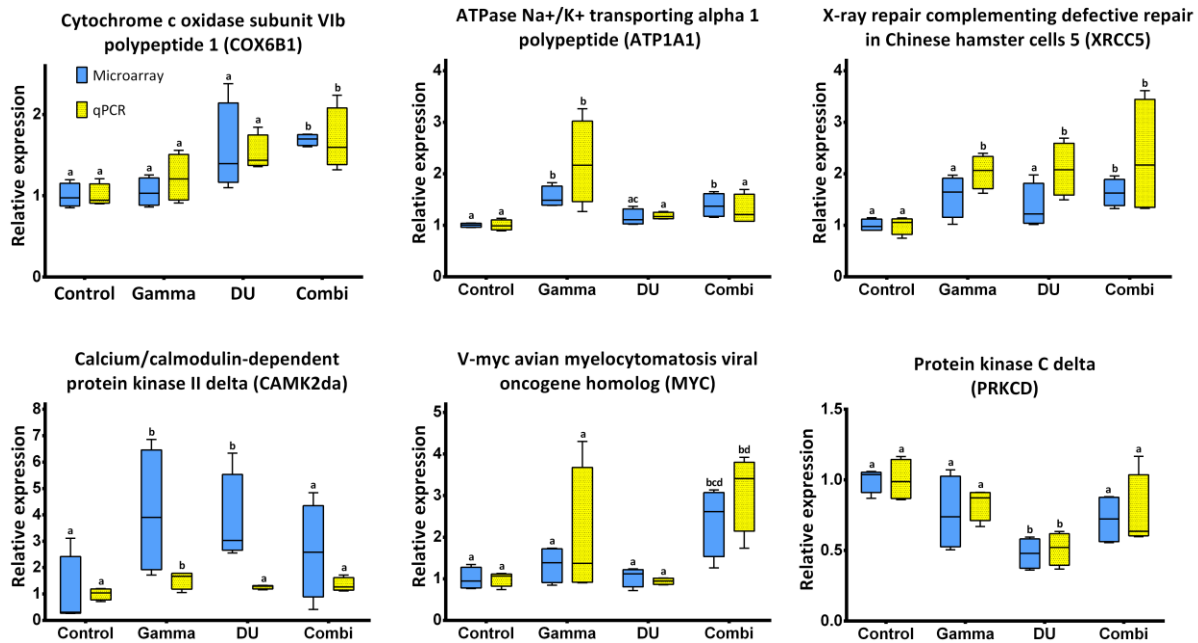

Figure 3. Expression of genes that were regulated by 70 mGy gamma radiation (Gamma), 0.25 mg/L depleted uranium (DU) and their combination (Combi) after short-term exposure in Atlantic salmon (*Salmo salar*) measured by microarray (blue, N=4) and real-time qPCR (yellow, N=4-6). Letter a: no significant difference from control; b: significantly different from control; c: significantly different from Gamma; d: significantly different from DU.

3.4 Histological effect

11

No adverse histological changes were observed in the liver following various treatments. Moderate vacuolization of the parenchymal liver tissue was considered to represent moderate glycogen storage (PAS positive, not shown) digested by amylase (Appendices, Figure A1). The morphology of the gills was not different in the treated groups compared to the controls and no specific pathological changes were observed in any of the groups (Appendices, Figure A1).

4. Discussion

The present work was designed to investigate effects induced in Atlantic salmon exposed to a combination of sublethal dose/concentration of gamma radiation and DU, representing radiation effects and metal toxicity of a radionuclide. The exposure regime was chosen based on the previous findings (Olsvik et al., 2010; Heier et al., 2013) by the current research group that Atlantic salmon exposed to high dose rate (12.2-15.8 mGy/h) for a short period had similar pattern of transcriptional regulation compared to 48h uranium exposure (Song et al., 2012; Song et al., 2014a). Toxic mechanism-focused analysis was used to evaluate the combined toxicity of Gamma and DU on global gene expression. The test organism used in this study, Atlantic salmon, is known to be one of the most sensitive fish species which has great values not only for the aquatic ecosystem, but also for human as important commercial fish in Northern Europe. Hypothetical accidents may indeed cause exposure of juvenile salmon to a range of radionuclides including gamma emitters and mg/L levels of U (runoff from NORM tailings) has been reported in freshwater systems in Norway (Popic et al., 2011).

The exposure experiment was performed in a highly controlled manner that caused no significant differences in general exposure conditions or water variables. Chemical analysis showed a clear increase in liver concentration of DU in salmon liver after single DU and Combi exposure. It should be noted that trace amount of DU was also detected in both Control and Gamma groups, due to the low natural background DU concentrations in the lake water (Maridalsvannet, Oslo, Norway) which was used to rear the test fish prior to the exposures (Song et al., 2012). The lack of differences between the two exposure groups (DU and Combi) indicated that the current exposure design was capable of maintaining stable exposure conditions over time and any differences observed between treatment groups were in fact related to the treatment effects. The bioconcentration factor (BCF) of U for liver (0.022 ± 0.009 L/kg) in this study was higher compared to the BCF (0.013 ± 0.007 L/kg) obtained by the same research group in a previous study, in which Atlantic salmon were also exposed to 0.25 mg/L DU for 48 h (Song et al., 2012; Song et al., 2014a). The difference in U bioaccumulation between the two exposure studies was likely due to the differences in U speciation, which is normally influenced by water quality and U concentrations and may affect the uptake of U. The exposure variables in the present study (i.e. actual U concentration 0.20 mg/L, pH 7.3, temperature 9.7 ± 0.2 °C, TOC, 5.1 ± 0.1 mg/L) were slightly different from the previous studies (i.e. actual U concentration 0.26 mg/L, pH 7.1-7.3, temperature 4.4 ± 0.2°C, TOC 4.6 ± 0.6 mg/L) by the same research group. Nevertheless, the BCFs in the present study were within the range of BCFs reported for U (0.001 to 149 L/kg) elsewhere (Sheppard et al., 2005; Goulet, 2011). The large differences in reported BCFs illustrate that it is essential to link toxic effects to both water and tissue concentrations of U to ensure comparability between studies. The bioaccumulation of DU in fish tissues

have been previously reported by a number of studies (Pyle et al., 2001; Barillet et al., 2007; Lerebours et al., 2009; Lerebours et al., 2010; Barillet et al., 2011; Kraemer and Evans, 2012; Lerebours et al., 2013). However, it appears that the current study is the first to report hepatic bioaccumulation of U in teleost fish. In the present study, fish were exposed to a sublethal dose of gamma radiation, DU and their combination. The test concentration (0.25 mg U/L) used in DU and combined exposure was within the range of the environmental U concentration of 0.02 μg/L to 3 mg/L (Salbu et al., 2013) and the reported median lethal concentration (LC50, 96 h) of approximately 3-6 mg/L in teleost fish (Sheppard et al., 2005). The reported median lethal dose (LD50) of gamma radiation in fish is approximately 11-56 Gy after 30 days exposure and 9-23 Gy after 40-50 days exposure (UNSCEAR, 1996), which were higher than the total dose (70 mGy) used in the present study. As a result, the exposures performed for both stressors were considered low and clearly sublethal to salmon.

In terms of assessing the combined toxicity at the gene level, microarray analysis was performed. Treatment-related DEGs were used to characterize and compare major MoAs of the single stressors with that obtained for the combination of the two. Comparative analysis of biological functions associated with DEGs showed that Gamma and DU may have a few common cellular targets, including water molecules, macromolecules (e.g. DNA and protein) and the enzymes in the mitochondria. Gamma and DU may induce ROS by interacting with water molecules to produce free radicals and subsequently induce oxidative stress. This primary MoA has been widely recognized for ionizing radiation and metals, and is well-documented in a number of fish studies (Barillet et al., 2007; Lerebours et al., 2009; O'Dowd et al., 2009; Olsvik et al., 2010; Song et al., 2012; Heier et al., 2013; Song et al., 2014a; Song et al., 2014b). Based on the current study, both Gamma, DU and their combination caused cellular responses indicative of oxidative stress in the salmon livers. This was deduced from the enrichment of the toxicity pathway related to oxidative stress after single Gamma and DU exposure (see (Song et al., 2014a; Song et al., 2014b) for details), and specifically the enrichment of DEGs in the nuclear factor erythroid 2-like 2 (nrf2)-mediated oxidative stress response pathway after exposure to Gamma and Combi. The nrf2-mediated oxidative stress response is usually activated by ionizing radiation in the post-exposure period (McDonald et al., 2010) as an adaptive response to the differential regulation of the antioxidant genes such as catalase (CAT), peroxiredoxin-1(prdx1) and thioredoxin (txn) (Crawford and Davies, 1994). Moreover, GO functional analysis also showed overrepresentation of biological processes such as ROS scavenging after exposure to Combi, but not in the single gamma or DU treatment, supporting the proposal that exposure to the two stressors caused oxidative stress. When focusing on the supporting DEGs in these pathways, different patterns of transcriptional response were found between treatments. It seems that Combi had more similar oxidative stress response to Gamma than to DU, as similar pattern of gene expression in the nrf2-mediated transcriptional regulation pathway was observed in Gamma and DU. This applies in particular to the up-regulation of ATP-binding cassette sub-family c member 2 (abcc2), heat shock protein 90kDa beta 1 (hsp90b1) and stress-induced-phosphoprotein 1 (stip1), and down-regulation of glutathione s-transferase theta 1 (gstt1) and glutathione s-transferase kappa 1 (gstk1) that were in agreement with proposed MoA of these stressors in the previous studies (Morel and Barouki, 1999; O'Dowd et al., 2009; Olsvik et al., 2010; Song et al., 2012; Heier et al., 2013; Song et al., 2014a; Song et al., 2014b). Repression of the antioxidant genes may be due to the decreased demand for antioxidant activities, or due to the interference of ROS with redox-based signaling pathways that

13

produced negative feedbacks to the normal antioxidant signaling (Pourova et al., 2010). Although no common pathway related to oxidative stress was found for DU and Combi, both treatments caused antioxidant responses by the induction of the cat gene, which encodes the cat enzyme for converting hydrogen peroxide to water and oxygen, indicating that antioxidant defense might be activated by these stressors as observed previously (Song et al., 2014a; Song et al., 2014b)

After short-term exposure, pathways related to DNA double strand break (DSB) repair were found to be significantly enriched in all treatment groups, indicating increased cellular signaling related to DNA damage. Gamma mainly regulated genes involved in the gadd45 signaling, DSB repair (by non-homologous end joining), and toxicity pathway such as p53 signaling. The p53 gene is normally activated following ionizing radiation-induced ataxia telangiectasia mutated (ATM) signaling and serves as an upstream regulator of various cellular responses, such as cell cycle regulation, DNA repair and apoptosis (Krauss, 2008). The gadd45 and DSB repair pathways are usually downstream processes of p53 and involved in the regulation of cell cycle progression and DNA repair mechanisms (Krauss, 2008). Depleted U mainly regulated two different canonical pathways in response to potential DNA damage, including ATM signaling and DSB repair (by homologous recombination). The ATM signaling was also enriched by DEGs in the Combi group, together with several canonical pathways involved in cell cycle arrest, such as cell cycle: G1/S checkpoint regulation, cell cycle: G2/M DNA damage checkpoint regulation, cyclins and cell cycle regulation. Interestingly, most of these pathways shared the gadd45g gene as a common responsive DEG. The gadd45g gene has previously been proposed as a biomarker representative of DNA damage after exposure to DU (Song et al., 2012) and ionizing radiation (Grace et al., 2002; Song et al., 2014b). Although different responses to DNA damage were observed, they were likely initiated by the same upstream transcriptional regulators, presumably the ATM signaling pathway. The effects of the two stressors on gene expression associated with DNA damage and repair pathways have been reported previously in fish (Lerebours et al., 2009; Song et al., 2012; Lerebours et al., 2013; Song et al., 2014a; Song et al., 2014b). DNA damage has also been considered as a key MoA of ionizing (gamma) radiation (Olsvik et al., 2010; Song et al., 2014b) and DU in fish (Lerebours et al., 2009; Lourenco et al., 2010; Song et al., 2012; Lerebours et al., 2013; Song et al., 2014a). DNA damage can be induced through direct or indirect actions by these stressors. Ionizing radiations may cause direct ionization of the DNA molecule and break the hydrogen bonds and/or oxygen-phosphate bonds (Razskazovskiy et al., 2000; Rak et al., 2011) thus leading to DNA strand breaks, whereas DU may affect the normal functions of DNA by binding to the DNA molecule and leading to the formation of U-DNA adduct (Stearns et al., 2005; Wilson et al., 2014). The indirect DNA damage is normally a result of oxidative damage caused by ROS (Miller et al., 2002; Miura, 2004). No matter which type of DNA damage mechanism is involved, the stress signals in most cases are detected by the phosphatidyl inositol 3-kinase-like family of serine/threonine protein kinases (PIKKs), including ATM (double-strand break responsive) and serine/threonine-protein kinase (ATR, single-strand break responsive), in most of the higher organisms (Abraham, 2001; Krauss, 2008). Therefore, the downstream events of DNA damage were essentially similar after exposure to Gamma, DU and Combi. The down-regulation of a few DNA damage associated genes found in the present study, such as gadd45g, breast cancer 1 (brca1) and double strand break repair protein RAD50 (rad50) supported the hypothesis that these stressors commonly affect the DNA damage and repair

signaling. The regulation of these genes have been observed previously in Atlantic salmon after exposure to gamma radiation and DU (Song et al., 2012; Song et al., 2014a; Song et al., 2014b). One of the key findings in the present study was that Gamma, DU and Combi all affected the functions of the mitochondria, and especially the electron transport chain (ETC). The mitochondrion has been widely considered as a key target of environmental stressors (Meyer et al., 2013). Disruption of mitochondrial functions such as DNA integrity and energy production has often been linked to many diseases such as cancer, Parkinson's disease and Alzheimer's disease in human (Meyer et al., 2013). Perturbations of the mitochondrial ETC have also been documented in fish exposed to gamma radiation (O'Dowd et al., 2009; Song et al., 2014b) and DU (Lerebours et al., 2009; Lerebours et al., 2010; Song et al., 2014a). Although a common cellular target for Gamma and DU was likely identified, DEGs supporting this hypothesis were still found to be different between the stressors. For example, Gamma generally repressed genes such as cytochrome c oxidase subunit VIIc (cox7c) and ATP synthase, H$^+$ transporting, mitochondrial F1 complex, delta subunit (atp5d) involved in all ETC protein complexes, whereas DU induced genes such as cytochrome c oxidase subunit VIb polypeptide 1 (cox6b1) and cox7c in ETC complex I, III and IV. These results were similar to the previously reported transcriptional changes in the mitochondrial ETC in fish after exposure to gamma radiation and DU (Lerebours et al., 2009; Song et al., 2014a; Song et al., 2014b). Exposure to Combi led to a mixture of up-regulated and down-regulated DEGs which were found in both Gamma and DU groups (Appendices, Figure A2). These evidences suggested that Gamma and DU may commonly affect the mitochondrial ETC, but likely with different MoAs. The repression of mitochondrial ETC genes by low-dose Gamma has not been well-understood, although similar findings have been previously reported (Song et al., 2014b). The induction of ROS by gamma radiation leading to oxidative stress in the mitochondrion may be one of the potential mechanisms involved in the repression of genes in the ETC (Kam and Banati, 2013). Besides ROS induced by external stressors, the mitochondrial ETC itself can also be a major source of intracellular ROS, as the ETC machinery is driven by multiple redox reactions (Drose and Brandt, 2012). As stress response to excessive ROS, a self-uncoupling mechanism in the mitochondria may be activated to reduce the ROS production from ETC (Mailloux and Harper, 2011; Shabalina, 2011; Mailloux and Harper, 2012). Another possible mechanism may be that Gamma-induced oxidative stress activated self-destructive programs in the cells, such as apoptosis that would inhibit downstream transcriptional activities involved in the ETC. The inhibition of mitochondrial ETC activity by ionizing radiation has been reported for several mammalian species (Kam and Banati, 2013), and only a limited number of studies has been performed on fish so far. A recent study on rainbow trout exposed to low doses (0.1-1 Gy) of gamma radiation for 2 h showed a general tendency of repression of mitochondrial ETC genes and enzymes in different tissues (O'Dowd et al., 2009). Depleted U seems to affect the mitochondrial ETC through different mechanisms. Uncoupling of OXPHOS is one potential MoA of DU in fish (Song et al., 2014a). Dissimilar to organic uncouplers, DU may potentially disturb the ETC by affecting the mitochondrial permeability transition pore (MPTP) and causing abnormal regulation of the mitochondrial osmolarity, which may eventually lead to disruption of proton gradient across the mitochondrial inner membrane (Song et al., 2014a). This mechanism has also been proposed for metals such as cadmium and mercury (Belyaeva et al., 2011). The activation of ETC genes by DU may therefore be a potential compensatory mechanism

in response to the loss of the mitochondrial membrane potential, abnormal osmoregulation in the mitochondrion and reduced ATP production.

Several downstream events may take place as consequences of oxidative stress, DNA damage and mitochondrial dysfunction, such as programmed cell death and immune response. Apoptosis is a type of programmed cell death and usually activated by oxidative stress (Chandra et al., 2000), DNA damage (Roos and Kaina, 2006) and mitochondrial dysfunction (Eckert et al., 2003). The apoptosis signaling pathway was significantly regulated by both single and multiple stressors in this study. Based on the supporting DEGs, different types of apoptosis signaling, such as intrinsic (mitochondrial), extrinsic (death receptor) and endoplasmic reticulum (ER) stress-induced apoptotic pathways were found to be regulated by all treatments. Genes associated with the extrinsic apoptosis seemed to be repressed by exposure to DU and Combi, potentially due to the down-regulation of nuclear factor kappa b (nf-κb). Interestingly, nf-κb was induced by Gamma. This transcriptional effect has not been reported previously in fish exposed to these stressors, and the differential regulation of the nf-κb gene by DU and Gamma may indicate counteracting effect of these two stressors on apoptotic signaling when in combination. Depleted U and Combi also affected a caspase-independent apoptotic pathway through the induction of apoptosis-inducible factor, mitochondrial 1 (aifm1). This gene has previously been observed to be induced in Atlantic salmon after exposure to DU (Song et al., 2014a). Induction of genes and enzymes involved in apoptosis, such as b-cell all/lymphoma 2 (bcl-2), caspase 3 (casp3), caspase 6 (casp6) and bcl2-associated x protein (bax) have been reported previously in fish exposed to gamma radiation (Salbu et al., 2008; Olsvik et al., 2010; Song et al., 2014b) and DU (Lerebours et al., 2009; Song et al., 2012; Song et al., 2014a).

The present study also identified multiple immune-related pathways related to Gamma, DU and Combi exposure. The activation of immune function is a common stress response in fish exposed to environmental stressors such as metals (Carlson and Zelikoff, 2008), and may also be a downstream event of oxidative stress (Al-Huseini et al., 2013; Gostner et al., 2013). A study on chronic exposure to a total dose of 4 Gy gamma radiation significantly reduced the antibody response in rainbow trout (Knowles, 1992), indicating an immune suppressive mechanism. Another study showed that after receiving a sublethal dose of 20 Gy gamma radiation, transfer of diseases such as T-cell leukemia to irradiated zebrafish was greatly facilitated due to the ablation of the immune response (Traver et al., 2004). Relatively more studies have documented the effect of DU on the immune system in fish. A study with zebrafish showed that genes involved in the inflammatory response such as interleukin-1b (IL-1b) were induced after a 28 d exposure to 23-130 μg/L DU (Lerebours et al., 2009). A wide range of immune-related hepatic histological effects, such as pigmented macrophage proliferation, haemorrhaging, glomerular lesions, tubules necrosis and inflammatory response were observed in Lake whitefish (*Coregonus clupeaformis*) after dietary exposure to 100-10000 μg/L DU for 10-100 days (Cooley et al., 2000). Another study measured the phenoloxidase-like (PO) activity as a biomarker of immune function in zebrafish exposed to 20 μg/L DU and observed increased PO activity after 48 h and repressed after 96 h (Gagnaire et al., 2013). Diverse immune-related pathways were significantly enriched in the present study, including major immune responses such as cellular immune response (e.g. production of nitric oxide and reactive oxygen species in macrophages), humoral immune response (e.g. B cell receptor signaling), cytokine signaling and inflammatory response (e.g. il-8 signaling), and pathogen-influenced

signaling (e.g. caveolar-mediated endocytosis signaling), as several interleukin (IL) genes (e.g. IL1R1) and major histocompatibility complex (HLA) genes (e.g. HLA-DRA) were found to be induced by the stressors. These findings were similar to the immune responses in fish after exposed to gamma radiation and DU (Lerebours et al., 2009; Song et al., 2014a; Song et al., 2014b). A few immune-related pathways showed suppression or potentiation response. For example, the IL-1 signaling pathway (inflammatory response) was found to be significantly enriched by Gamma or DU, but not the combination of the two, indicating potential counteraction of supporting genes in this pathway by the two stressors when present in combination. The primary immunodeficiency signaling pathway was found to be regulated only by Combi, thus suggesting that the two single stressors were affecting more DEGs associated with this cellular function when exposed in combination.

It needs to be noted that not all single DEGs identified by microarray analysis in this study were statistically significant due to lack of FDR correction. However, the findings at the GO and pathway level with multiple supporting DEGs increased the confidence for generating hypotheses on the potential MoAs of these stressors. In addition, qPCR verification of several key biomarker genes also supported that a selection of DEGs were in fact significantly expressed and thus supporting the identifying key MoAs. Although several transcriptional changes were identified, no adverse effect such as histological change at the organ level or changes in weight or condition index was identified. The exposure was not sufficiently high to cause damage observed as morphological changes at light microscopic level or in circulating glucose level, which has been proposed to be a biomarker for general stress (Roche and Boge, 1996; Pottinger and Carrick, 1999). This might be due to the low exposure level, short exposure duration, limited selections of apical endpoints to measure and potential equilibrium established between stressor-induced damage and rapid activation of corresponding compensatory mechanisms (e.g. antioxidant defense and DNA repair). Future work is required to include more endpoints at higher organismal levels in order to link stress responses at the molecular level to any functional or morphological changes at organ level.

5. Conclusions

The present study mainly focused on hepatic transcriptional responses to evaluate the combined effects of gamma radiation (Gamma) and depleted uranium (DU) on Atlantic salmon after short term exposure. Mechanistic evaluation of combined effects revealed that considerable discrepancies of global transcriptional changes and their associated toxicological pathways existed in fish between single and combined exposure, although many of the cellular responses could be grouped into a few common higher functional categories, such as immune response, growth and development and signal transduction. Three potential modes of action (MoAs) were proposed for Gamma, DU and the combination, including induction of oxidative stress, DNA damage and disturbance of oxidative phosphorylation. Due to the low exposure level and short duration, the present study was not able to link molecular responses to measurable effects at organ level. In conclusion, this study has characterized and compared the potential toxic mechanisms of Gamma, DU and their combination.

References

Abraham, R.T., 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. 15, 2177-2196.

Al-Huseini, L.M., Aw Yeang, H.X., Sethu, S., Alhumeed, N., Hamdam, J.M., Tingle, Y., Djouhri, L., Kitteringham, N., Park, B.K., Goldring, C.E., Sathish, J.G., 2013. Nuclear factor-erythroid 2 (NF-E2) p45-related factor-2 (Nrf2) modulates dendritic cell immune function through regulation of p38 MAPK-cAMP-responsive element binding protein/activating transcription factor 1 signaling. The Journal of biological chemistry 288, 22281-22288.

Altenburger, R., Scholz, S., Schmitt-Jansen, M., Busch, W., Escher, B.I., 2012. Mixture toxicity revisited from a toxicogenomic perspective. Environ Sci Technol 46, 2508-2522.

Augustine, S., Pereira, S., Floriani, M., Camilleri, V., Kooijman, S.A.L.M., Gagnaire, B., Adam-Guillermin, C., 2015. Effects of chronic exposure to environmentally relevant concentrations of waterborne depleted uranium on the digestive tract of zebrafish, Danio rerio. J. Environ. Radioact. 142, 45-53.

Barillet, S., Adam, C., Palluel, O., Devaux, A., 2007. Bioaccumulation, oxidative stress, and neurotoxicity in *Danio rerio* exposed to different isotopic compositions of uranium. Environmental toxicology and chemistry / SETAC 26, 497-505.

Belyaeva, E.A., Korotkov, S.M., Saris, N.E., 2011. In vitro modulation of heavy metal-induced rat liver mitochondria dysfunction: a comparison of copper and mercury with cadmium. Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements 25 Suppl 1, S63-73.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Wheeler, D.L., 2005. GenBank. Nucleic Acids Res. 33, D34-D38.

Bourrachot, S., Brion, F., Pereira, S., Floriani, M., Camilleri, V., Cavalie, I., Palluel, O., Adam-Guillermin, C., 2014. Effects of depleted uranium on the reproductive success and F1 generation survival of zebrafish (Danio rerio). Aquat. Toxicol. 154, 1-11.

Bucher, G., Mounicou, S., Simon, O., Floriani, M., Lobinski, R., Frelon, S., 2014. Different uranium distribution patterns in cytosolic protein pool of zebrafish gills after chronic and acute waterborne exposures. Chemosphere 111, 412-417.

Carlson, E., Zelikoff, J.T., 2008. The immune system of fish: a target organ of toxicity, in: Di Giulio, R.T., Hinton, D.E. (Eds.), The toxicology of fishes. CRC Press, Boca Raton, FL, USA, pp. 489-530.

Chandra, J., Samali, A., Orrenius, S., 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic. Biol. Med. 29, 323-333.

Cooley, H.M., Evans, R.E., Klaverkamp, J.F., 2000. Toxicology of dietary uranium in lake whitefish (*Coregonus clupeaformis*). Aquat Toxicol 48, 495-515.

Crawford, D.R., Davies, K.J., 1994. Adaptive response and oxidative stress. Environ Health Perspect 102 Suppl 10, 25-28.

De Coninck, D.I.M., Asselman, J., Glaholt, S., Janssen, C.R., Colhourne, J.K., Shaw, J.R., De Schamphelaere, K.A.C., 2014. Genome-Wide Transcription Profiles Reveal Genotype-Dependent Responses of Biological Pathways and Gene-Families in Daphnia Exposed to Single and Mixed Stressors. Environ. Sci. Technol. 48, 3513-3522.

Drose, S., Brandt, U., 2012. Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. Advances in experimental medicine and biology 748, 145-169.

Eckert, A., Keil, U., Marques, C.A., Bonert, A., Frey, C., Schussel, K., Muller, W.E., 2003. Mitochondrial dysfunction, apoptotic cell death, and Alzheimer's disease. Biochem Pharmacol 66, 1627-1634.

Finne, E.F., Cooper, G.A., Koop, B.F., Hylland, K., Tollefsen, K.E., 2007. Toxicogenomic responses in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to model chemicals and a synthetic mixture. Aquat Toxicol 81, 293-303.

Gagnaire, B., Bado-Nilles, A., Sanchez, W., 2014. Depleted Uranium Disturbs Immune Parameters in Zebrafish, Danio rerio: An Ex Vivo/In Vivo Experiment. Archives of Environmental Contamination and Toxicology 67, 426-435.

Gagnaire, B., Cavalie, I., Camilleri, V., Adam-Guillermin, C., 2013. Effects of depleted uranium on oxidative stress, detoxification, and defence parameters of zebrafish Danio rerio. Arch Environ Contam Toxicol 64, 140-150.

Gostner, J.M., Becker, K., Fuchs, D., Sucher, R., 2013. Redox regulation of the immune response. Redox report : communications in free radical research 18, 88-94.

Goulet, R., Fortin, C., Spry, DJ, 2011. Uranium, in: Chris M. Wood, A.P.F.a.C.J.B. (Ed.), Fish Physiology: Homeostasis and Toxicology of Non-Essential Metals. Academic Press, pp. 391-428.

Grace, M.B., McLeland, C.B., Blakely, W.F., 2002. Real-time quantitative RT-PCR assay of GADD45 gene expression changes as a biomarker for radiation biodosimetry. Int. J. Radiat. Biol. 78, 1011-1021.

Heier, L.S., Teien, H.C., Oughton, D., Tollefsen, K.E., Olsvik, P.A., Rosseland, B.O., Lind, O.C., Farmen, E., Skipperud, L., Salbu, B., 2013. Sublethal effects in Atlantic salmon (*Salmo salar*) exposed to mixtures of copper, aluminium and gamma radiation. J Environ Radioact 121, 33-42.

Holmstrup, M., Bindesbol, A. M., Oostingh, G. J., Duschl, A., Scheil, V., Kohler, H. R., Loureiro, S., Soares, A. M. V. M., Ferreira, A. L. G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P. E., Bayley, M., Svendsen, C., Spurgeon, D. J., 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Sci. Total Environ. 408, 3746-3762.

Kam, W.W., Banati, R.B., 2013. Effects of ionizing radiation on mitochondria. Free Radic. Biol. Med. 65, 607–619.

Kiernan, J.A., 1999. Histological and Histochemical Methods: Theory and Practice. Butterworth-Heinemann, Oxford, UK.

Knowles, J.F., 1992. The effect of chronic radiation on the humoral immune response of rainbow trout (*Onchorhynchus mykiss Walbaum*). Int J Radiat Biol 62, 239-248.

Krauss, G., 2008. Biochemistry of signal transduction and regulation, 4 ed. Wiley-VCH Verlag GmbH, Weinheim, Germany.

Lange, A., Sebire, M., Rostkowski, P., Mizutani, T., Miyagawa, S., Iguchi, T., Hill, E.M., Tyler, C.R., 2015. Environmental chemicals active as human antiandrogens do not activate a stickleback androgen receptor but enhance a feminising effect of oestrogen in roach. Aquatic toxicology 168, 48-59.

Laskowski, R., Bednarska, A. J., Kramarz, P. E., Loureiro, S., Scheil, V., Kudlek, J., Holmstrup, M., 2010. Interactions between toxic chemicals and natural environmental factors - A meta-analysis and case studies. Sci. Total Environ. 408, 3763-3774.

Lerebours, A., Adam-Guillermin, C., Brethes, D., Frelon, S., Floriani, M., Camilleri, V., Garnier-Laplace, J., Bourdineaud, J.P., 2010. Mitochondrial energetic metabolism perturbations in skeletal muscles and brain of zebrafish (*Danio rerio*) exposed to low concentrations of waterborne uranium. Aquat. Toxicol. 100, 66-74.

Lerebours, A., Cambier, S., Hislop, L., Adam-Guillermin, C., Bourdineaud, J.P., 2013. Genotoxic effects of exposure to waterborne uranium, dietary methylmercury and hyperoxia in zebrafish assessed by the quantitative RAPD-PCR method. Mutat Res 755, 55-60.

Lerebours, A., Gonzalez, P., Adam, C., Camilleri, V., Bourdineaud, J.P., Garnier-Laplace, J., 2009. Comparative Analysis of Gene Expression in Brain, Liver, Skeletal Muscles, and Gills of Zebrafish (Danio Rerio) Exposed to Environmentally Relevant Waterborne Uranium Concentrations. Environ. Toxicol. Chem. 28, 1271-1278.

Lind, O.C., Stegnar, P., Tolongutov, B., Rosseland, B.O., Stromman, G., Uralbekov, B., Usubalieva, A., Solomatina, A., Gwynn, J.P., Lespukh, E., Salbu, B., 2013. Environmental impact assessment of radionuclide and metal contamination at the former U site at Kadji Sai, Kyrgyzstan. J. Environ. Radioact. 123, 37-49.

Lourenco, J., Castro, B.B., Machado, R., Nunes, B., Mendo, S., Goncalves, F., Pereira, R., 2010. Genetic, Biochemical, and Individual Responses of the Teleost Fish Carassius auratus to Uranium. Archives of Environmental Contamination and Toxicology 58, 1023-1031.

Maere, S., Heymans, K., Kuiper, M., 2005. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics 21, 3448-3449.

Mailloux, R.J., Harper, M.E., 2011. Uncoupling proteins and the control of mitochondrial reactive oxygen species production. Free radical biology & medicine 51, 1106-1115.

Mailloux, R.J., Harper, M.E., 2012. Mitochondrial proticity and ROS signaling: lessons from the uncoupling proteins. Trends in endocrinology and metabolism: TEM 23, 451-458.

McDonald, J.T., Kim, K., Norris, A.J., Vlashi, E., Phillips, T.M., Lagadec, C., Della Donna, L., Ratikan, J., Szelag, H., Hlatky, L., McBride, W.H., 2010. Ionizing radiation activates the Nrf2 antioxidant response. Cancer Res 70, 8886-8895.

Meyer, J.N., Leung, M.C.K., Rooney, J.P., Sendoel, A., Hengartner, M.O., Kisby, G.E., Bess, A.S., 2013. Mitochondria as a Target of Environmental Toxicants. Toxicological Sciences 134, 1-17.

Miller, A.C., Stewart, M., Brooks, K., Shi, L., Page, N., 2002. Depleted uranium-catalyzed oxidative DNA damage: absence of significant alpha particle decay. J. Inorg. Biochem. 91, 246-252.

Miura, Y., 2004. Oxidative stress, radiation-adaptive responses, and aging. J Radiat Res 45, 357-372.

Morel, Y., Barouki, R., 1999. Repression of gene expression by oxidative stress. Biochem. J. 342, 481-496.

Mothersill, C., Salbu, B., Heier, L.S., Teien, H.C., Denbeigh, J., Oughton, D., Rosseland, B.O., Seymour, C.B., 2007. Multiple stressor effects of radiation and metals in salmon (*Salmo salar*). J Environ Radioact 96, 20-31.

O'Dowd, C., Mothersill, C.E., Cairns, M.T., Austin, B., Lyng, F.M., McClean, B., Talbot, A., Murphy, J.E.J., 2009. Gene Expression and Enzyme Activity of Mitochondrial Proteins in Irradiated Rainbow Trout (*Oncorhynchus Mykiss, Walbaum*) Tissues In Vitro. Radiat. Res. 171, 464-473.

O'Dowd, C., Mothersill, C.E., Cairns, M.T., Austin, B., McClean, B., Lyng, F.M., Murphy, J.E., 2006. The release of bystander factor(s) from tissue explant cultures of rainbow trout (*Onchorhynchus mykiss*) after exposure to gamma radiation. Radiat Res 166, 611-617.

Olsvik, P.A., Heier, L.S., Rosseland, B.O., Teien, H.C., Salbu, B., 2010. Effects of combined gamma-irradiation and metal (Al plus Cd) exposures in Atlantic salmon (Salmo salar L.). J. Environ. Radioact. 101, 230-236.

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.

Pereira, S., Camilleri, V., Floriani, M., Cavalie, I., Garnier-Laplace, J., Adam-Guillermin, C., 2012. Genotoxicity of uranium contamination in embryonic zebrafish cells. Aquat Toxicol 109, 11-16.

Popic, J.M., Salbu, B., Strand, T., Skipperud, L., 2011. Assessment of radionuclide and metal contamination in a thorium rich area in Norway. J. Environ. Monit. 13, 1730-1738.

Pottinger, T.G., Carrick, T.R., 1999. A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. Aquaculture 175, 351-363.

Pourova, J., Kottova, M., Voprsalova, M., Pour, M., 2010. Reactive oxygen and nitrogen species in normal physiological processes. Acta physiologica 198, 15-35.

Rak, J., Kobylecka, M., Storoniak, P., 2011. Single strand break in DNA coupled to the O-P bond cleavage. A computational study. J Phys Chem B 115, 1911-1917.

Razskazovskiy, Y., Debije, M.G., Bernhard, W.A., 2000. Direct radiation damage to crystalline DNA: What is the source of unaltered base release? Radiat. Res. 153, 436-441.

Roche, H., Boge, G., 1996. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. Mar. Environ. Res. 41, 27-43.

Roos, W.P., Kaina, B., 2006. DNA damage-induced cell death by apoptosis. Trends in molecular medicine 12, 440-450.

Rosseland, B.O., Massabuau, J.C., Grimalt, J.O., Hofer, R., Lackner, R., Raddum, G.G., Rognerud, S., Vives, I., 2001. Fish ecotoxicology. The EMERGE fish sampling manual for live fish., in: The EMERGE Project (European Mountain lake Ecosystems: Regionalisation, d.a.s.e.v. (Ed.).

Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for General Users and for Biologist Programmers, in: Misener, S., Krawetz, S.A. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press Inc., Totowa (NJ), pp. 365-386.

Salbu, B., 2009. Radioactive particles released from different nuclear sources, in: Oughton, D.H., Kashparov, V. (Eds.), Radioactive particles in the envrionment. Springer, The Netherlands, p. 279.

Salbu, B., Burkitbaev, M., Stromman, G., Shishkov, I., Kayukov, P., Uralbekov, B., Rosseland, B.O., 2013. Environmental impact assessment of radionuclides and trace elements at the Kurday U mining site, Kazakhstan. J. Environ. Radioact. 123, 14-27.

Salbu, B., Denbeigh, J., Smith, R.W., Heier, L.S., Teien, H.C., Rosseland, B.O., Oughton, D., Seymour, C.B., Mothersill, C., 2008. Environmentally relevant mixed exposures to radiation and heavy metals induce measurable stress responses in Atlantic salmon. Environ. Sci. Technol. 42, 3441-3446.

Salbu, B.S., P., Strømman, G., Skipperud, L., Rosseland, B.O., Heier, L.S.,, 2011. Legacy of uranium mining activitites in Central Asia — contamination, impact and risks. Salbu, B., Stegnar, P., editors. UMB Report 1. 2011.

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

Sheppard, S.C., Sheppard, M.I., Gallerand, M.O., Sanipelli, B., 2005. Derivation of ecotoxicity thresholds for uranium. J. Environ. Radioact. 79, 55-83.

Simon, O., Mottin, E., Geffroy, B., Hinton, T., 2010. Effects of dietary uranium on reproductive endpoints-fecundity, survival, reproductive success-of the fish *Danio rerio*. Environ. Toxicol. Chem. 30, 220-225.

Smoot, M.E., Ono, K., Ruscheinski, J., Wang, P.L., Ideker, T., 2011. Cytoscape 2.8: new features for data integration and network visualization. Bioinformatics 27, 431-432.

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, 62-71.

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

Song, Y., Salbu, B., Teien, H.C., Heier, L.S., Rosseland, B.O., Tollefsen, K.E., 2014b. Dose-dependent hepatic transcriptional responses in Atlantic salmon (Salmo salar) exposed to sublethal doses of gamma radiation. Aquat. Toxicol. 156, 52-64.

Spurgeon, D.J., Jones, O.A.H., Dorne, J.L.C.M., Svendsen, C., Swain, S., Sturzenbaum, S.R., 2010. Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. Sci. Total Environ. 408, 3725-3734.

Stearns, D.M., Yazzie, M., Bradley, A.S., Coryell, V.H., Shelley, J.T., Ashby, A., Asplund, C.S., Lantz, R.C., 2005. Uranyl acetate induces hprt mutations and uranium-DNA adducts in Chinese hamster ovary EM9 cells. Mutagenesis 20, 417-423.

Traver, D., Winzeler, A., Stern, H.M., Mayhall, E.A., Langenau, D.M., Kutok, J.L., Look, A.T., Zon, L.I., 2004. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood 104, 1298-1305.

UNSCEAR, 1996. Sources and effects of ionizing radiation, in: Radiation, U.N.S.C.o.t.E.o.A. (Ed.), New York.

Vanhoudt, N., Vandenhove, H., Real, A., Bradshaw, C., Stark, K., 2012. A review of multiple stressor studies that include ionising radiation. Environ. Pollut. 168, 177-192.

Wilson, J., Young, A., Civitello, E.R., Stearns, D.M., 2014. Analysis of heat-labile sites generated by reactions of depleted uranium and ascorbate in plasmid DNA. Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry 19, 45-57.