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1 # Deciphering the Combined Effects of Environmental Stressors on Gene Transcription: a Conceptual Approach

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4 You Song*,†,‡, Jana Asselman§, Karel A. C. De Schamphelaere§, Brit Salbu‡, Knut Erik Tollefsen†,‡

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6 †Norwegian Institute for Water Research (NIVA), Section of Ecotoxicology and Risk Assessment,

7 Gaustadalléen 21, N-0349 Oslo, Norway

8 ‡Norwegian University of Life Sciences (NMBU), Centre for Environmental Radioactivity (CERAD). P.O.

9 Box 5003, N-1432 Ås, Norway

10 §Ghent University, Faculty of Bioscience Engineering, Laboratory of Environmental Toxicology and

11 Aquatic Ecology (GhEnToxLab). Campus Coupure Building F – 2nd Floor, Coupure Links 653, B9000

12 Ghent, Belgium.

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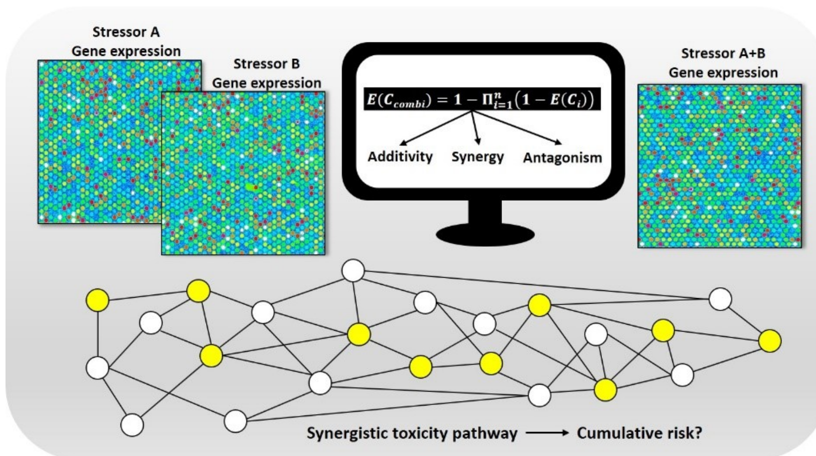
15 **TOC**



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■ ABSTRACT

Use of classical mixture toxicity models to predict the combined effects of environmental stressors based on toxicogenomics (OMICS) data is still in its infancy. Although several studies have made attempts to implement mixture modeling in OMICS analysis to understand the low-dose interactions of stressors, it is not clear how interactions occur at the molecular level and how results generated from such approaches can be better used to inform future studies and cumulative hazard assessment of multiple stressors. The present work was therefore conducted to propose a conceptual approach for combined effect assessment using global gene expression data, as illustrated by a case study on assessment of combined effects of gamma radiation and depleted uranium (DU) on Atlantic salmon (*Salmo salar*). Implementation of the independent action (IA) model in re-analysis of a previously published microarray gene expression data was performed to describe gene expression patterns of combined effects and identify key gene sets and pathways that were relevant for understanding the interactive effects of these stressors. By using this approach, 3120 differentially expressed genes (DEGs) were caused by additive effects, whereas 279 (273 synergistic, 6 antagonistic) were found to deviate from additivity. Functional analysis further revealed that multiple toxicity pathways, such as oxidative stress responses, cell cycle regulation, lipid metabolism and immune responses were enriched by DEGs showing synergistic gene expression. A key toxicity pathway of excessive reactive oxygen species (ROS) formation leading to enhanced tumorigenesis signaling is highlighted and discussed in detail as an example of how to take advance of the approach. Furthermore, a conceptual workflow describing the integration of combined effect modeling, OMICS analysis and bioinformatics is proposed. The present study presents a conceptual framework for utilizing OMICS data in combined effect assessment and may provide novel strategies for dealing with data analysis and interpretation of molecular responses of multiple stressors.

Key Words: Multiple stressor, Mixture modeling, Gene expression, Independent action, Synergy

■ INTRODUCTION

A multitude of environmental stressors (multiple stressors) may co-exist in the environment, thus creating complex exposure scenarios and potentially causing cumulative hazard and risk to organisms. Studies on multiple stressors have been increasing rapidly in the past decades (reviewed in ref[1-3]). Development of prediction models for combined (joint) toxicity has facilitated the assessment of multiple stressor effects, especially for mixtures of chemical contaminants.[4, 5] Prediction models such as concentration addition (CA), which often assumes two or more stressors having similar mode of action (MoA) and affecting common biological targets,[6, 7] or independent action (IA), which assumes dissimilar MoA of stressors, and multiplicative responses at the target sites,[8] have been successfully implemented in the hazard assessment of chemical mixtures utilizing both *in vitro* and *in vivo* experimental approaches.[9-11] The CA model often requires extensive data support derived from dose/concentration-response relationships, whereas the IA model can be applied based on effects observed from each single stressor without full knowledge on the dose/concentration-response relationships.[12] Therefore, the IA model is usually suitable for predicting the combined effects of stressors with distinct toxicological properties.

In the past decades, ecotoxicological research on multiple stressors and cumulative risk has shifted the focus more towards effects occurring at environmentally realistic low-exposure levels and long-term ecosystem impacts.[13] In concordance with this, inclusion of sensitive toxicological endpoints at lower levels of biological organization (e.g. molecular/cellular level) in routine toxicity testing and better mechanistic understanding are becoming increasingly important. Use of toxicogenomics (OMICS) approaches (e.g. transcriptomics, proteomics, metabolomics and epigenomics) in combination with advanced biostatistics/bioinformatics for identifying key molecular/cellular events and toxicity pathways fits this purpose well. Among all OMICS approaches, transcriptomics is the most frequently used in various multiple stressor studies and has proven to be a powerful tool for MoA characterization and toxicity pathway identification (e.g. ref[14, 15]). Altenburger and co-workers[12] critically reviewed the use of OMICS in 41 mixture toxicity studies in the period of 2002 to 2011 and reported that half of the studies employed transcriptomics for elucidating the combined toxicity at the molecular level. However, they[12] also pointed out that most of the studies only used qualitative assessment (i.e. comparison between single stressors and the mixture based on the presence or absence of a gene or pathway in order to demonstrate the differences in toxic mechanisms), whereas only a small portion of the studies attempted to apply quantitative mixture modeling (i.e. comparison based on a combined effect prediction model) to the OMICS data (e.g. ref[16-19]). It has become increasingly evident that lack of quantitative

70 assessment in such mixture studies are predominantly due to the high number of single data generated, the complexity
71 of the response patterns observed and the lack of ability to interpret the responses at the functional level. First, the
72 OMICS technologies typically generate thousands of data points, where the sheer handling of statistical treatment and
73 correction for potential errors (e.g. type I and II errors)[20] may introduce bias in identifying the relevance of single
74 responses. Second, difficulties in determining the maximal level of a molecular response, bi-directional regulation
75 (e.g. up- or down-regulation), and presence of non-monotonic concentration (dose)-response relationships may
76 challenge the generation of comparable thresholds across different molecular responses. Third, the integration and
77 interpretation of multiple responses into functional understanding with relevance to a given biological, biochemical
78 or toxicity pathway may not be straight forward to identify and is furthermore complicated by temporal changes often
79 occurring dramatically at the molecular level. Although several attempts have been made in recent years to address
80 these issues, such as critically evaluating different biostatistical approaches[21], developing high-throughput
81 concentration-response analysis of OMICS data[21], using various functional and pathway analyses[22] and performing
82 analyses using the IA model for predicting transcriptional changes after binary exposure to stressors,[18, 23] a clear
83 strategy to maximize the output from such types of studies to inform hazard assessment of multiple stressors is still
84 lacking.

85 The present work was therefore conducted as a case study to illustrate a conceptual approach for integrating mixture
86 modeling, transcriptomics and bioinformatics in combined effect assessment of multiple stressors. This study re-
87 analyzed the transcriptomic data generated from a previously published study on combined effects of gamma radiation
88 and depleted uranium (DU) in Atlantic salmon (*Salmo salar*).[14] The two stressors studied herein may co-occur in the
89 environment naturally or after anthropogenic activities such as uranium mining and nuclear accidents (e.g. nuclear
90 power plant accident in Chernobyl),[24] thus representing a realistic exposure scenario for combined effects of
91 radionuclides such as uranium (e.g. metal properties and alpha radiation) and external ionizing radiation. Gamma
92 radiation and uranium (i.e. DU in this case) are known to induce reactive oxygen species (ROS) and cause oxidative
93 damage to macromolecules as a common MoA.[14, 25-29] However, these stressors have distinct properties and display
94 differences in their response at the molecular scale. Previous studies also suggest that gamma radiation and DU may
95 have multiple MoAs and affect the same endpoint in salmon through dissimilar toxicity mechanisms.[14, 27-29] In addition,
96 transcriptomic analysis is a relatively untargeted analysis which investigates global gene expression responses without
97 presumption of the MoAs of a stressor. Therefore, the IA model is considered more appropriate in this case. The

98 objectives of the current study were to: 1) characterize different types of transcriptional responses as consequences of
99 additive, synergistic and antagonistic responses of the stressors using the IA prediction model; 2) identify key toxicity
100 pathways associated with differentially expressed genes (DEGs) displaying synergistic effects; 3) propose a
101 conceptual workflow for quantitative mixture modeling with the transcriptomic data.
102
103

104 ## ■ MATERIALS AND METHODS

105 **Design and Data Acquisition.** The detailed exposure experiment has been published elsewhere.[14] A simple
106 "a+b" design (i.e. same concentration/dose of single stressors as used in the mixture) was used in the binary exposure.
107 Briefly, juvenile (parr) Atlantic salmon were exposed to 14 mGy/h gamma radiation from a cobalt-60 source (FIGARO,
108 NMBU, Ås, Norway) for the first 5h (total dose: 70 mGy) of a 48h period (referred to as Gamma), 0.25 mg/L
109 waterborne DU (uptake: 5.5 µg U/kg in liver) for a continuous period of 48h (referred to as DU) and the combination
110 of these (referred to as Combined). Single-color microarray gene expression analysis was performed using total RNA
111 isolated from dissected fish liver (n=4), as previously described.[14] The microarray data was deposited in Gene
112 Expression Omnibus (GEO, accession number: GSE74012) and re-analyzed in the present study.

113 **Combined Effect Modeling.** The raw microarray data was downloaded from GEO and corrected for background
114 signal, flagged for low quality and missing features and log2 transformed for normalization (quantiles) using
115 GeneSpring GX v11.0 (Agilent Technologies) prior to combined effect modeling.

116 Differentially expressed genes were determined using the linear models implemented in the LIMMA package
117 (Bioconductor, R statistical environment),[30] with modifications.[31] Contrasts were defined over the linear model in the
118 statistical test to identify transcriptional responses as a consequence of single and/or combined exposure to the
119 stressors by two-way analysis of variance (two-way ANOVA), as previously described.[18, 23] The two-way ANOVA
120 examines the effect of each independent variable (Gamma and DU) and the interaction between them, on basis of
121 variance between treatment replicates. No multiple testing correction was applied to avoid loss of biologically relevant
122 genes for the functional analyses.

123 To assess the combined effects of Gamma and DU, the IA model[8, 32] was adapted to the gene expression data to
124 determine whether the observed transcriptional responses were in agreement or deviated from the assumption of
125 additivity, as previously described:[18, 23]

126

127 $$Y_{pred\ (Combined)} = \frac{Y_{obs\ (Gamma)} \times Y_{obs\ (DU)}}{Y_{obs\ (Ctrl)}} \quad (1)$$

128

129 Where $Y_{pred\ (Combined)}$ is the predicted absolute gene expression in Combined (i.e. Gamma + DU) under the
130 assumption of no interaction, $Y_{obs\ (Gamma)}$ is the measured absolute gene expression after exposure to Gamma alone,
131 $Y_{obs\ (DU)}$ is the measured absolute gene expression after exposure to DU alone. Gene expression is defined as an M-
132 value, in which a treatment is expressed relative to a control treatment, referring to up- or down-regulation. Therefore,
133 equation (1) can be transformed to (2), in which all observations are normalized relative to the control treatment, (i.e.,
134 $Y_{obs\ (Ctrl)}$, the measured absolute gene expression in the control). Equation (1) can be transformed to:

135

136 $$Log2\left(\frac{Y_{pred\ (Combined)}}{Y_{obs\ (Ctrl)}}\right) = \mathrm{Log2}\left(\frac{Y_{obs\ (Gamma)}}{Y_{obs\ (Ctrl)}} \times \frac{Y_{obs\ (DU)}}{Y_{obs\ (Ctrl)}}\right) = \mathrm{Log2}\left(\frac{Y_{obs\ (Gamma)}}{Y_{obs\ (Ctrl)}}\right) + \mathrm{Log2}\left(\frac{Y_{obs\ (DU)}}{Y_{obs\ (Ctrl)}}\right) \quad (2)$$

137

138 M-value is defined as the log2 value of the absolute gene expression in each treatment relative to the control.
139 Therefore, each component in equation can be rewritten as follows:

140

141 $$M_{pred\ (Combined)} = Log2\left(\frac{Y_{pred\ (Combined)}}{Y_{obs\ (Ctrl)}}\right)$$

142 $$M_{obs\ (Gamma)} = Log2\left(\frac{Y_{obs\ (Gamma)}}{Y_{obs\ (Ctrl)}}\right)$$

143 $$M_{obs\ (DU)} = Log2\left(\frac{Y_{obs\ (DU)}}{Y_{obs\ (Ctrl)}}\right)$$

144

145 Equation (2) can then be written as:

146 $$M_{pred\ (Combined)} = M_{obs\ (Gamma)} + M_{obs\ (DU)} \quad (3)$$

147

148 Therefore, if $M_{obs\,(Combined)} = M_{pred\,(Combined)} = M_{obs\,(Gamma)} + M_{obs\,(DU)}$, the combined effect on gene
149 transcription is considered additive. Then the transcriptional interactive effect (M_{Int}) that deviates from additivity can
150 be defined as:
151
152 $$M_{Int} = M_{pred\,(Combined)} - M_{obs\,(Combined)} = M_{obs\,(Gamma)} + M_{obs\,(DU)} - M_{obs\,(Combined)} \quad (4)$$
153
154 Based on equation (4), genes regulated as consequence of interaction (referred to as Interact) were defined as genes
155 whose M-values of interaction (M_{Int}) were significantly different from zero (p-value<0.05) and when no overlap of
156 the 95% confidence intervals of the predicted M-value ($M_{pred\,(Combined)}$) and observed M-value ($M_{obs\,(Combined)}$).
157 The expression of genes displaying synergistic (M_{Int} >0) or antagonistic (M_{Int} <0) patterns were considered the
158 consequence of interactions between the stressors. Venn diagram analysis was performed using Venny
159 (http://bioinfogp.cnb.csic.es/tools/venny/) to classify gene sets with different response patterns.
160 **Functional Enrichment Analysis.** To understand the toxicological functions of the gene sets, gene ontology
161 enrichment (GO, hypergeometric test, p<0.05) and pathway enrichment (Fisher's Exact test, p<0.05) analyses were
162 performed using Bingo v2.4[33] in Cytoscape v3[34] and Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City,
163 www.qiagen.com/ingenuity), respectively. No multiple testing correction was applied to avoid loss of biologically
164 relevant functions. As IPA is predominantly based on mammalian centric gene and pathway knowledge, ortholog
165 genes between Atlantic salmon and mammalian species were used for pathway analysis. Orthologs were identified
166 using a two-pass BLAST approach in Inparanoid 4.1,[35] as previously described.[14]
167
168
169 ## ■ RESULTS AND DISCUSSION
170 **Response Classification.** A total of 3460 (1484 up- and 1976 down-regulated) genes were identified as DEGs in
171 Atlantic salmon after combined exposure, of which 3124 were initially predicted as additive, 323 as synergistic and
172 13 as antagonistic by the IA model (SI, Table S1). To get more insight into different types of joint actions, DEGs were
173 categorized into two major groups on basis of the direction of transcriptional regulation compared to the control (i.e.
174 up- or down-regulation). Genes that were monotonically up-regulated or down-regulated in all groups (i.e. Gamma,

DU and Combined) were considered one-directional, whereas DEGs that were non-monotonically regulated (e.g. up-regulated by Gamma, down-regulated by DU, and up-regulated by Combined, etc.) were considered bi-directional. The one-directional group (Type 1) had a total of 2934 DEGs, of which 2847 were predicted to be consequences of additive, 82 as synergistic and 5 as antagonistic effects of the stressors (Table 1). The Type 1 joint actions are similar to that observed in combined effect assessment using conventional toxicological endpoints, such as survival, reproduction and growth. The bi-directional group (Type 2) had a total of 526 DEGs, of which 273 were predicted as consequences of additive, 191 as synergistic, 1 as antagonistic effects of the stressors (Table 1). It is also interesting to note that in the bi-directional group, the responses of 61 DEGs contradict the basic assumption of the IA prediction model (e.g. up-regulated in Gamma and DU but down-regulated in Combined, or vice versa) (SI, Table S1). The contradicting responses have also been frequently observed in multiple stressor studies based on individual (e.g. mortality and reproduction) and ecological endpoints.[36] It is not clear how this "two negatives make a positive" type of response (or vice versa) occurred. However, several known factors may potentially affect the model predictions as well as combined effect classification, such as appropriate mixture design (e.g. a+b, n×n, ray or surface design), types of OMICS technology employed (e.g. qPCR, microarray or RNA sequencing), statistical analysis (e.g. t-test, LIMMA, ANOVA, with or without multiple testing correction) and mechanistic understanding (e.g. gene functions and regulatory networks). In this case, the fourth type of joint action (i.e. contradicted) observed may likely be due to activation of feedback loops to upstream regulators upon exceeding certain gene transcription thresholds,[37] which ultimately cause modulation of downstream transcriptional regulation (e.g. from up-regulation to down-regulation, or vice versa). This is likely an adaptive response (compensatory mechanism) which has been commonly observed in organisms exposed to oxidative stressors.[38] If this is the case, the assumption of the IA model is breached and improvement of the IA model parametrization may therefore be required (e.g. by adding a random variable to the model to capture the variation of data that fails to meet the assumption of IA). Although many factors can affect the data quality and interpretation, the current case study has successfully demonstrated the usefulness of this conceptual approach for classification of gene sets according to the conventional types of joint action (e.g. majority of DEGs reasonably predicted as additive), and the ability to detect unexpected (or novel) types of combined effects (e.g. contradicted action).

Table 1. Types of combined effects on gene/pathway regulation.

Direction of transcriptional regulation	Type of joint action	Sub-type of joint action	Illustration	No. of DEG
One-directional (84.8%)	Type 1 Additivity (82.28%)	Additive up-regulation (34.74%)	(1)+(1)=2	1202
		Additive down-regulation (47.57%)	(-1)+(-1)=-2	1645
	Type 1 Synergy (2.37%)	Synergistic up-regulation (1.3%)	(1)+(1)>2	45
		Synergistic down-regulation (1.07%)	(-1)+(-1)<-2	37
	Type 1 Antagonism (0.14%)	Antagonistic up-regulation (0%)	0<(1)+(1)<2	0
		Antagonistic down-regulation (0.14%)	-2<(-1)+(-1)<0	5
Bi-directional (15.2%)	Type 2 Additivity (7.89%)	Counteracted up-regulation (4.45%)	(-1)+(2)=1	154
		Counteracted down-regulation (3.44%)	(-2)+(1)=-1	119
	Type 2 Synergy (5.52%)	Enhanced up-regulation (2.37%)	(-1)+(1)>1	82
		Enhanced down-regulation (3.15%)	(-1)+(1)<-1	109
	Type 2 Antagonism (0.03%)	Reduced up-regulation (0.03%)	0<(-1)+(1)<1	1
		Reduced down-regulation (0%)	-1<(-1)+(1)<0	0
	Contradicted (1.76%)	Reversed up-regulation (1.01%)	(-1)+(-1)>0	35
		Reversed down-regulation (0.75%)	(1)+(1)<0	26

Function Analysis. To further understand the toxicological functions of the DEGs displaying different types of joint actions, enrichment analyses were performed with the three DEG sets (Type 1 & 2 merged to avoid loss of biologically significant information) displaying additive, synergistic and antagonistic effects. Both GO (Figure 1A) and pathway (Figure 1B) analysis showed that the majority of the enriched functions were unique when comparing different types of interactions. A relatively lower number of GO functions and pathways were found to be common between different types of joint action, indicating that genes in the same functional cluster may have dissimilar patterns of response to combined exposure, possibly due to their multiple roles in toxicological responses to different types of

stressors and pathway cross-talks. For example, for the same GO function "cellular responses to oxidative stress", one set of supporting DEGs such as reactive oxygen species modulator 1 (*c20orf52/romo1*) and aryl hydrocarbon receptor nuclear translocator (arnt) were down-regulated and displayed Type 1 additivity, whereas another set of supporting DEGs such as peroxiredoxin 2 (prdx2) and Paxillin (pxn) were up-regulated by combined exposure and displayed Type 2 synergy. These findings suggest another level of gene set classification which may require substantial mechanistic understanding of individual gene functions and gene regulatory network.

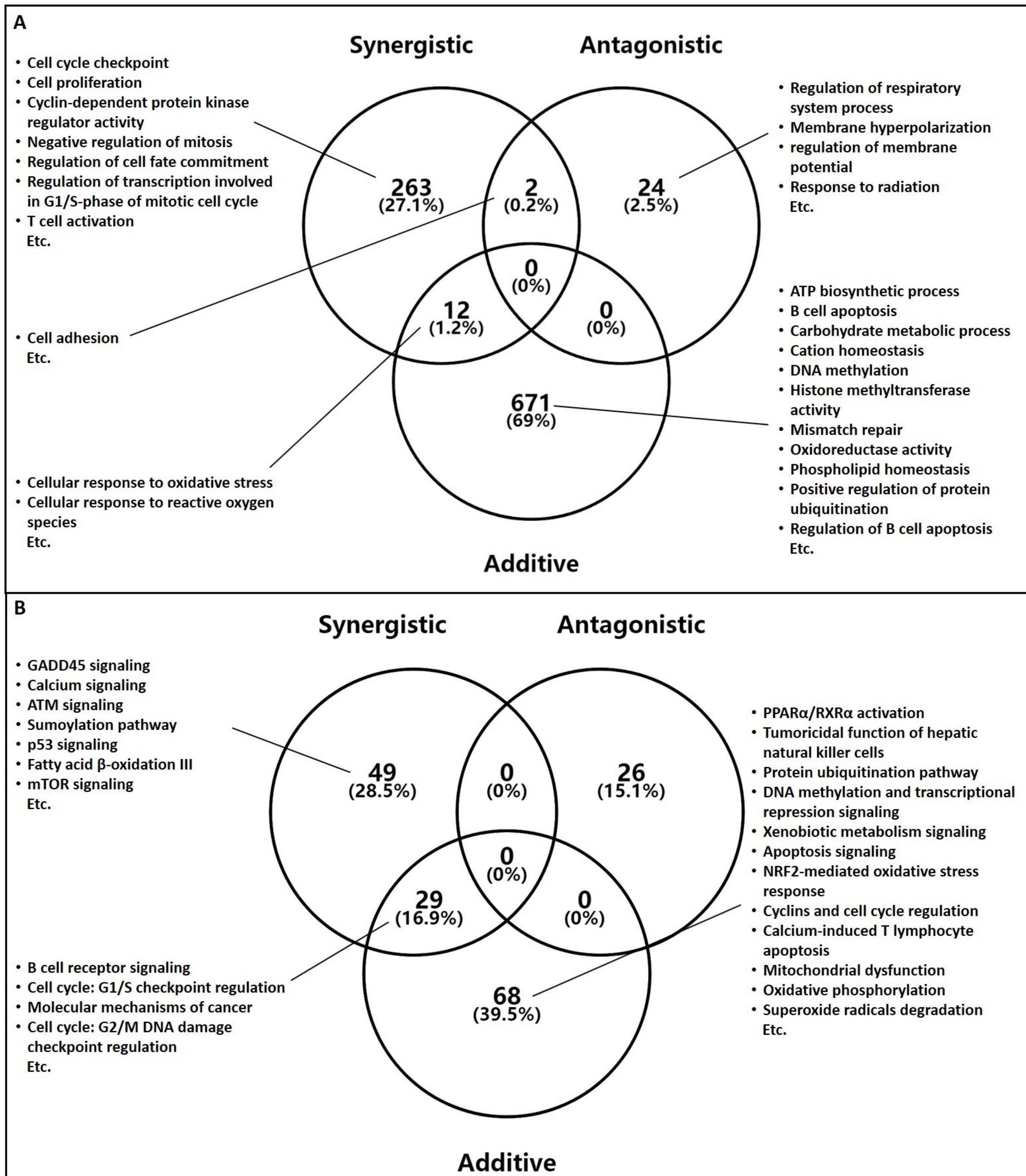
Differentially expressed genes displaying additive responses were mainly enriched in functions/pathways directly relevant for several main MoAs of Gamma and DU in salmon[14, 26-28] and zebrafish (*Danio rerio*),[25, 26] such as induction of oxidative stress responses, DNA damage responses, mitochondrial energetic dysfunctions and immune responses. Although similar pathways were also identified in the previous publication using MoA comparison-based qualitative approach, the comparative (qualitative) approach was not able to differentiate supporting DEGs displaying interactive or non-interactive (additive) actions of the stressors in the pathway.[14] The results obtained from the current quantitative approach thus clearly suggests added benefits of using the prediction model to classify gene sets with the same type of joint action without losing the resolution of mechanistic understanding.

The six DEGs displaying antagonistic effects were involved in a high number of functions mainly associated with metabolic processes, membrane integrity and DNA damage responses, which may also be relevant for the toxicity mechanisms of the stressors.[14, 28, 29] Genes such as GRIP and coiled-coil domain-containing protein 2 (*gcc2/gcc185,* Type 1 antagonism), PTPRF interacting protein binding protein 1 isoform 1 (*ppfibp1,* Type 1 antagonism), protein PXR1 (*pxr1,* Type 1 antagonism) were down-regulated by both single and combined stressors, whereas neuroligin 3 (*nlgn3,* Type 2 antagonism) was down-regulated by DU, up-regulated by Gamma and down-regulated by Combined. These are essential genes that are common for diverse types of biological functions in higher vertebrates, such as transmembrane protein activities, neuron development, cell organelle organization and nucleosome assembly.[39-42] Modulation of these genes by antagonistic action of Gamma and DU may potentially affect cellular signal transduction and development. However, due to the low number of DEGs in this category, it is difficult to obtain in-depth understanding of the MoAs and likely outcomes associated with the antagonistic action of the stressors.

The functional characterization was focused more on DEGs displaying apparent synergistic regulation, as these may potentially lead to synergistic responses along toxicity pathways relevant for adverse effects of the stressors. In line with this assumption, GO analysis revealed that these DEGs were mainly enriched in biological functions, such

as oxidative stress responses, cell cycle regulation and immune responses (SI, Table S2), all being demonstrated to have high relevance for the toxicity of both Gamma and DU.[14, 25, 26, 28, 29, 43] To further explore the toxicological functions based on curated pathways, the salmon DEGs were mapped to the mammalian orthologs (162 out of 275 mapped) and analyzed by IPA (SI, Table S1). Gene network analysis showed that these DEGs were grouped into 6 functional gene clusters, including 1) neurological disease, organismal injury and abnormalities, cancer; 2) developmental disorder, neurological disease, cell signaling; 3) cell death and survival, organ morphology, reproductive system development and function. These gene clusters are directly associated with the synergistic effects of the stressors as predicted by the IA model and highly relevant for the known effects of Gamma and DU in fish. [14, 25, 26, 28, 29, 43] Pathway analysis showed that DEGs displaying synergistic effects were exclusively involved in the ATM signaling, p53 signaling, GADD45 signaling, SUMOylation pathway, calcium signaling, mTOR signaling and fatty acid β-oxidation III, thus highlighting the modulation of two major functions, DNA damage responses and cellular energy homeostasis (SI, Table S3 & S4) by the synergistic effects of the stressors. These pathways are relevant for the major MoAs of Gamma and DU in Atlantic salmon [14, 28, 29] and zebrafish.[25, 26], indicating that the quantitative approach proposed herein is capable of capturing key mechanistic information based on small and highly related gene sets.

In addition, the 61 DEGs displaying apparent contradicting responses were mainly involved in the SUMOylation pathway and several biosynthetic processes of sugar derivatives, pyrimidine nucleotide and reductants. Although the roles of these pathways in Gamma- and DU-mediated toxicological responses in fish have not been well investigated, evidence from the mammalian studies suggests that several of these pathways are likely involved in certain feedback loops to regulate physiological processes. For example, the SUMO proteases are involved in a negative feedback loop to regulate cell survival in response to genotoxic stress.[44] The biosynthesis of nucleotides is also considered strictly regulated by certain feedback inhibition mechanisms.[45] Therefore, it is possible that genes displaying contradicting responses in this study were regulated by certain feedback loops in response to different levels of stress induced by single and combined stressors. However, whether this leads to functional changes of relevance still needs to be investigated.



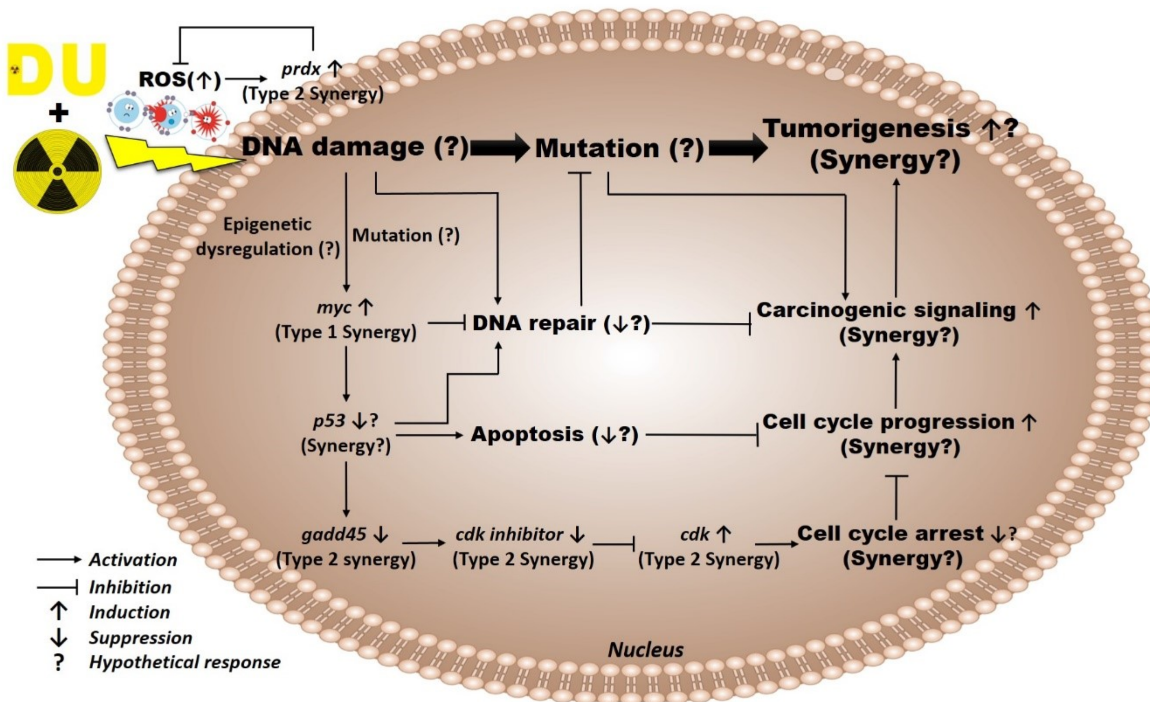
264

265 Figure 1. Venn diagram analysis of toxicologically relevant gene ontology (GO) functions (A) and canonical pathways (B) that
266 were enriched by differentially expressed genes (DEGs) displaying additive, synergistic and antagonistic effects in Atlantic salmon
267 (*Salmo salar*) after combined exposure to gamma radiation and depleted uranium.

268

269 **Putative Synergistic Pathway Characterization.** A number of molecular toxicity pathways were enriched
270 by DEGs displaying synergistic effects and highly relevant for the toxicity mechanisms of Gamma and DU in fish,
271 such as GADD45 signaling, nervous system and immune dysfunctions.[14, 28, 29] To illustrate the quantitative aspect and
272 novelty of the current approach, a putative synergistic toxicity pathway representing the major MoA of gamma
273 radiation and DU was characterized in detail: excessive DNA damage leading to promoted cell cycle progression and
274 carcinogenesis (Figure 2). This putative pathway was characterized as an illustration of using the results obtained from
275 the proposed quantitative approach to guide follow-up studies on anchoring the effects at higher levels of biological
276 organization. In contrast to the previous qualitative assessment which also identified this key toxicity pathway, the
277 new approach described herein allows quantification and understanding of the changes and patterns of gene expression
278 within the pathway. It is well-known that Gamma and DU can cause DNA damage in fish through direct actions, such
279 as excitation and ionization of DNA molecules (Gamma) and formation of U-DNA adducts (DU), or most likely
280 indirect actions such as induction of ROS and causing oxidative DNA damage.[46, 47] Peroxiredoxin-2 (*prdx2*), an
281 antioxidant encoding gene against oxidative stress, was synergistically up-regulated, potentially indicating excessive
282 ROS formation and subsequent DNA damage.[48] Between DNA damage and the activation of cancer signaling, the
283 oncogene *myc* plays a key role. The *myc* gene was found to be up-regulated due to the synergistic effect of Gamma
284 and DU in the present study. It is known that normal expression of this oncogene is involved in the cellular defensive
285 mechanisms against DNA damage and tumorigenesis, whereas abnormal regulation or mutation of this gene can lead
286 to completely opposite consequences.[49, 50] Overexpression of *myc* by gamma radiation has been reported to suppress
287 DNA repair, promote DNA damage and cell cycle progression from G1 to S phase, thus facilitating mutagenesis and
288 tumorigenesis in mammals.[51, 52] Studies on zebrafish (*Danio rerio*) also showed that overexpression of *myc* resulted
289 in increased proliferation of cancer cells, and induction of T-cell acute lymphoblastic leukemia and hepatoma.[53, 54]
290 Although detailed mechanism of *myc* overexpression leading to promoted cell cycle progression is not fully
291 understood, recent mammalian studies suggested that *myc* may impede the function of tumor protein P53 (*p53*), a
292 central transcription factor for activation of cell cycle arrest, DNA repair and programmed cell death, thus promoting
293 cell cycle progression.[55-57] The *p53* gene *per se* was not identified as a DEG after combined exposure, likely due to
294 large variations between individual replicates and limited induction potential.[58] However, its downstream target,
295 growth arrest and DNA-damage-inducible protein GADD45 gamma (*gadd45g*), an effector gene to mediate DNA
296 damage associated S and G2/M cell cycle arrest,[59] was highly down-regulated and displayed a synergistic response.

297 This transition from no effect to significant effect between upstream and downstream genes potentially shows a good
298 example that synergy may occur along a pathway. In addition, another downstream target of *p53*, tumor protein p53-
299 inducible nuclear protein 1 (*tp53inp1*) which triggers P53-dependent apoptosis,[60] was down-regulated but displaying
300 additive effect of the stressors. The evidence taken together suggest that *p53* was likely suppressed in salmon liver
301 after combined exposure to the two stressors. The *gadd45* gene is normally induced in response to low level of
302 genotoxic stress to control cell cycle progression, DNA repair and initiation of apoptosis to eliminate damaged cells.[61]
303 Repression of this gene promotes the expression of cyclin-dependent kinase inhibitors (e.g. *cdkn1b*), thus inhibiting
304 the expression of cyclin-dependent kinases (e.g. *cdkl1*), a gene responsible for progression of the cell cycle.[62] The
305 *cdkn1b* gene was found to be down-regulated, whereas *cdkl1* was up-regulated due to the combined effect in the
306 present study, thus suggesting that cell cycle progression was enhanced beyond the expectation of additivity by the
307 combined exposure. The key regulatory role of *gadd45* in this molecular pathway is likely dependent on the level of
308 stress. However, lack of temporal and dose-response data in the current study limits the possibility to investigate the
309 expression dynamics of this gene. In mammals, deficiency in the GADD45 pathway has been associated with
310 oncogenesis.[59] Collectively, impaired DNA repair, suppressed apoptosis and promoted cell cycle progression may
311 potentially facilitate the accumulation of mutated cells and activation of various carcinogenic signaling pathways,
312 which are highly associated with tumor formation (Figure 2). Although it was not clear if the adverse outcome(s) of
313 this toxicity pathway was also enhanced as result of combined exposure, due to lack of phenotypic anchoring, the
314 illustrative analysis conducted herein shows a strategy for extracting key information from the data and improved
315 interpretation of the results for guiding follow-up studies.
316



317

318 Figure 2. An example illustrating synergistic toxicity pathways of DNA damage leading to reduced cell cycle arrest and enhanced
319 carcinogenesis signaling in the liver of Atlantic salmon (*Salmo salar*) after combined exposure to gamma radiation and depleted
320 uranium (DU). ROS: reactive oxygen species; *prdx*: peroxiredoxin; *myc*: c-myc; *atm*: *p53*: tumor protein P53; *gadd45*: growth
321 arrest and DNA-damage-inducible protein GADD45; *cdk* inhibitor: cyclin-dependent kinase inhibitor; *cdk*: cyclin-dependent kinase.

322

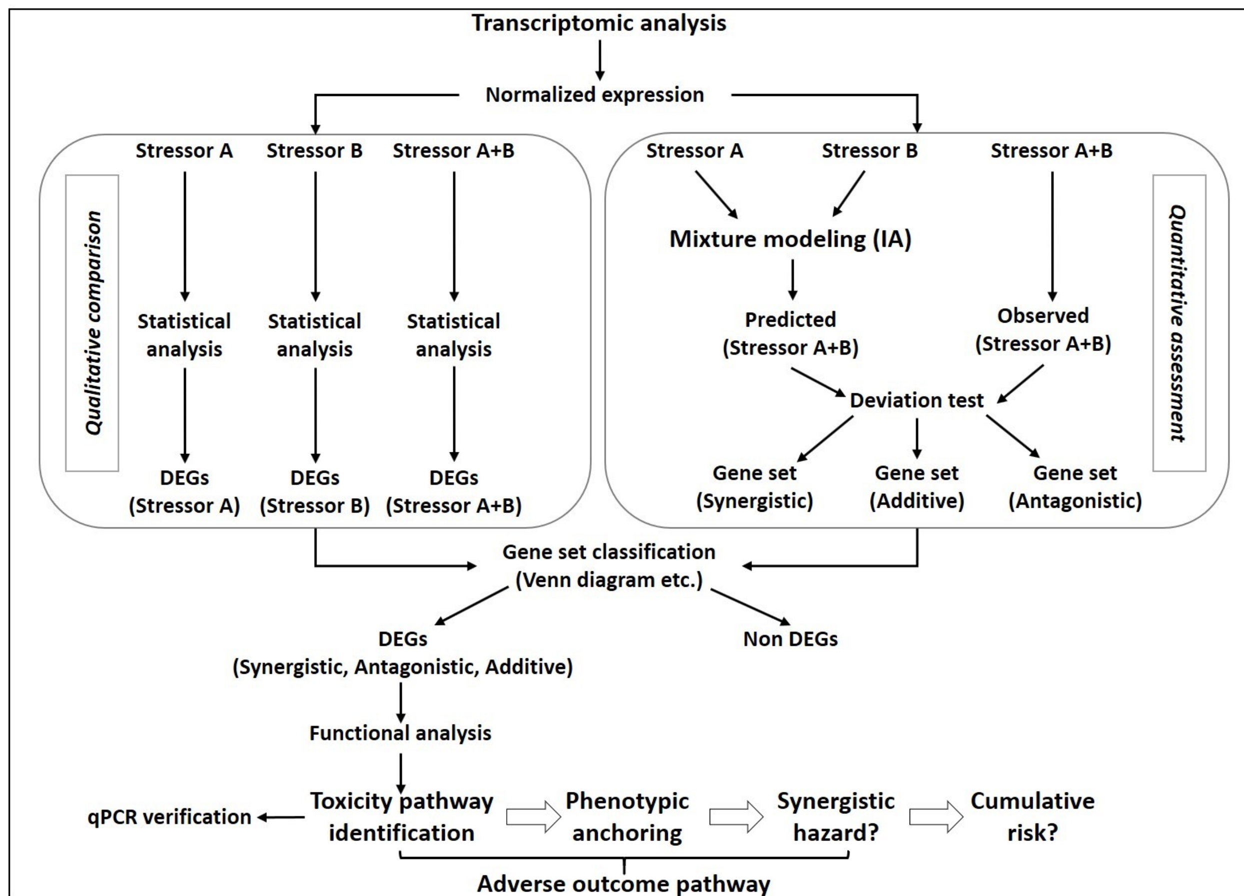
323 **Applications and limitations of the conceptual approach.** As illustrated by the case study, a conceptual
324 workflow for combined effect assessment using transcriptomic data is proposed (Figure 3). This conceptual approach
325 integrates mechanistically-based comparative analysis (qualitative/descriptive), expression-based mixture toxicity
326 modeling (quantitative) and biological pathway-based functional analysis (bioinformatics) to understand the
327 underlying mechanisms of combined effects in a toxicodynamics context and maximize the knowledge output from
328 such high-content OMICS analysis. This approach complies with the adverse outcome pathway (AOP) concept in
329 predictive ecotoxicology, which describes a conceptual framework that causally links the molecular initiating event
330 (MIE), a series of key events (KE) and the adverse outcome (AO) into a linear relationship that is relevant for risk
331 assessment.[63] By characterizing key molecular regulatory pathways, downstream KEs along an AOP potentially
332 leading to adversity relevant for cumulative risk can be targeted and anchored to well characterized toxicity pathways

using functional bioassays (tissue/organ level) or standardized toxicity tests (individual/population level). The IA prediction model used in this conceptual approach is suitable for quantitatively assessing the combined effects of environmental stressors with distinct toxicological profiles and multiple MoAs, such as a combination of chemical contaminants and natural stressors (e.g. pH, temperature, UV, ionizing radiation). The IA model is also considered appropriate for analyzing data generated from such high-content and hypothesis-generating OMICS analysis which may lacks temporal and dose-response relationships due to relatively high costs of these technologies. Nevertheless, this approach has both advantages and limitations. On one hand, classification of DEG sets by type of interaction (e.g. additivity, synergy, antagonism) can reduce the complexity of high-dimensional OMICS data, thus facilitating the identification of key gene sets relevant for understanding the joint actions of the stressors. On the other hand, grouping of genes according to the response (expression) patterns may potentially limit the characterization of their biological significance at the functional (e.g. gene clusters or pathways identified by the enrichment analyses) level of certain genes when classified into different types of interactions. Alternative to the currently proposed approach is to classify DEGs by their functional clusters (e.g. pathway functions) first, then group supporting DEGs in the same functional cluster (pathway) by type of interactions. However, complexity for interpretation may still exist, as one pathway may be enriched by DEGs displaying multiple types of joint actions (e.g. 50% DEGs showing synergy whereas the rest showing antagonism). Therefore, choice of classification approaches is highly dependent on a combination of whether the biological functions of DEG sets are relevant for the MoAs of the stressors and resulting perturbations of key toxic pathways, and whether DEGs in the same functional cluster uniformly display the same of type of joint action of the stressors. It would be interesting to try both approaches described above to capture all information needed in future assessments.

As clearly illustrated by the present case study, the proposed conceptual approach may also be limited by several key factors. First, mixture design is certainly an important aspect which may influence the overall conclusion. Although the simple "a+b" design employed in this case study has reasonably captured most patterns of combined effects, it has limitations to provide complete information due to lack of sufficient data points (e.g. dose-response relationships and temporal patterns of transcriptional responses) and may potentially introduce bias to the analysis. Altenburger and coworkers have reviewed appropriate mixture design for specific purposes and pointed out that use of dose-response and temporal gene expression data can refine the mixture design (e.g. by using appropriate concentration/dose in the mixture) and reduce uncertainties in combined effect modeling.[12] Second, the OMICS data

361 quality may also be highly dependent on the analytical technologies. The microarray analysis used in this case study
362 has been useful for identifying various types of transcriptional responses, but the technical limitations of this method
363 may potentially introduce experimental artefacts (e.g. cross-hybridization),[64] thus jeopardizing the identification of
364 true DEGs. Nevertheless, the previously published qualitative assessment[14] using the same dataset evaluated the
365 responses of six biomarkers genes by quantitative real-time reverse transcriptional polymerase chain reaction (qPCR)
366 and verified that results were in general consistent with that measured by microarray, thus suggesting that experimental
367 artefact due to the technology employed may not be the most important factor affecting the conclusions of this study.
368 To reduce potential experimental artefacts, use of state-of-the-art techniques (e.g. RNA sequencing) and inclusion of
369 multiple analytical approaches verifying the transcriptional changes may increase data confidence. Third, different
370 statistical analyses (e.g. t-test, LIMMA, ANOVA, with or without multiple testing correction) for determining DEGs
371 and data filtering methods (e.g. fold change cutoff, p-value cutoff) may lead to gain or loss of information on key
372 genes being highly relevant for key toxicity pathways. No multiple testing correction was applied in this study to
373 preserve the low-abundant transcripts and marginally regulated genes with potential biological significance. As a side-
374 effect, the chance of identifying false positives may also increase and affect data interpretation. Standardized
375 processing and reporting of OMICS data is therefore a prerequisite for reproducible output using the current approach
376 and highly required for regulatory applications.[65-68] Fourth, bioinformatics can also be a limiting factor for data
377 interpretation which is highly required by the current approach. Poor genome/transcriptome annotation (e.g. non-
378 model species such as Atlantic salmon) and lack of sufficient knowledge on gene co-expression networks at the
379 functional level (e.g. clusters and pathways) may thus become the bottlenecks for identification of key toxicity
380 pathways relevant for the combined toxicity of the stressors. Finally, lack of mechanistic knowledge at the molecular
381 and functional level may limit the understanding and interpretation of unexpected (or novel) responses which may be
382 highly relevant for assessing cumulative hazards. The IA model may also have limitations in capturing all types of
383 combined effects at the molecular level. For instance, if not being experimental artefacts or false positives, DEGs
384 displaying contradicted type of joint action may violate the assumptions of the IA model and should be interpreted on
385 a case-by-case basis. Although appropriate experimental design, biostatistics/bioinformatics, technology and
386 mechanistic knowledge are clearly required, the current case study has successfully demonstrated that a combination
387 of quantitative combined effects modeling and functional analyses may increase the ability to decipher and classify
388 relevant combined effects at the gene level and quantify combined effects relevant for key toxicity pathways.

389



390

391 Figure 3. Proposed workflow for mechanistically-based assessment of low-dose interactive effects of combined stressors using
392 transcriptomics data. Qualitative comparison: Mode of action (MoA)-based assessment; Quantitative assessment: Prediction
393 model-based assessment; DEG: differentially expressed gene; CA: concentration addition; IA: independent action. qPCR:
394 quantitative real-time reverse-transcription polymerase chain reaction.

395

396 **Future Perspectives.** A key question raised from the present study is whether additivity, synergism and
397 antagonism of gene expression and pathways at the molecular level can be used to predict the corresponding joint
398 action at the organismal or population level. Recent advance in gene co-expression network modeling showed that it
399 is possible to quantitatively predict adverse effects at the organismal level by using gene expression data,[19] which is a
400 first step of extrapolation between different levels of biological organization. This is especially important as future
401 regulatory toxicology requires reduced animal testing, better extrapolations from low to high biological levels (e.g. *in*
402 *vitro* to *in vivo*), and increased predictability across taxa and stressors[69] To answer this question, anchoring of

403 combined effects at multiple biological levels along a defined AOP or network of AOPs is needed. Anchoring of
404 relevant toxicity pathways being perturbed by a set of single and multiple stressor to key components in the AOP
405 continuum (i.e. the molecular initiating event and the key events) can help to identify more complex responses
406 involving multiple AOPs (i.e. network of AOPs) which may mutually interact to cause adverse outcomes of ecological
407 relevance.[12, 70] Another important question is whether the proposed approach can also be used for an increased number
408 of stressors. Although the principles outlined herein should ideally be applicable to an infinite number of stressors,
409 proof-of-concept studies to demonstrate the applicability and robustness for a number of stressors and extended dose-
410 rate/concentration ranges reflecting ecologically-relevant exposure scenarios is highly warranted. For different types
411 of studies, the choice of appropriate model is also important. A recent study by Schäfer and Piggott[71] proposed a
412 guideline for selecting the optimal null model (i.e. a prediction model assuming no interaction between the stressors)
413 for prediction of multiple-stressor effect on individuals or populations, which may also be adapted for modeling the
414 effects at the molecular level. Other modeling approaches in combination with the classical combined effect prediction
415 models, such as machine learning-based classification techniques[72] and advanced correlation/regression analysis[73]
416 may provide additional options for combined toxicity assessment of multiple stressors. Moreover, the complexity of
417 biological responses (i.e. directional responses) as observed in the present study as well as other studies (reviewed in
418 ref[36]) needs to be taken into account in the next generation of cumulative hazard assessment of multiple stressors.
419 Mechanistic knowledge on the MoAs of the stressors as well as molecular regulatory networks should be preferably
420 obtained prior to conducting complex multiple stressor studies using the OMICS tools. Reconceptualizing the
421 definitions for additivity, synergy and antagonism by considering more complex biological responses may be
422 required.[36]
423
424

425 ## ■ ASSOCIATED CONTENT

426 **Supporting Information (SI)**

427 The Supporting Information is available free of charge on the ACS Publications website at DOI:
428 Table S1: DEGs, Table S2: GOs, Table S3: Tox lists, Table S4: Canonical pathways (XLSX)
429

■ AUTHOR INFORMATION

Corresponding Author

*You Song, Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, NO-0349 Oslo, Norway. Tlf.: 02348, Fax: (+47) 22 18 52 00, E-mail: you.song@niva.no

Notes

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