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

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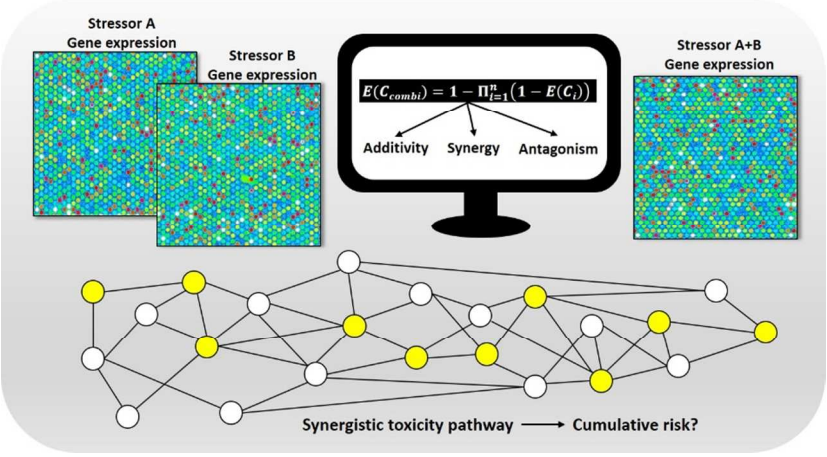

■ ABSTRACT

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

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

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

■ MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

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

Equation (2) can then be written as:

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

Therefore, if $M_{obs\ (Combined)} = M_{pred\ (Combined)} = M_{obs\ (Gamma)} + M_{obs\ (DU)}$, the combined effect on gene transcription is considered additive. Then the transcriptional interactive effect (M_{Int}) that deviates from additivity can be defined as:

$$M_{Int} = M_{pred\ (Combined)} - M_{obs\ (Combined)} = M_{obs\ (Gamma)} + M_{obs\ (DU)} - M_{obs\ (Combined)} \quad (4)$$

Based on equation (4), genes regulated as consequence of interaction (referred to as Interact) were defined as genes whose M-values of interaction (M_{Int}) were significantly different from zero (p-value<0.05) and when no overlap of the 95% confidence intervals of the predicted M-value ($M_{pred\ (Combined)}$) and observed M-value ($M_{obs\ (Combined)}$). The expression of genes displaying synergistic (M_{Int} >0) or antagonistic (M_{Int} <0) patterns were considered the consequence of interactions between the stressors. Venn diagram analysis was performed using Venny (http://bioinfogp.cnb.csic.es/tools/venny/) to classify gene sets with different response patterns.

Functional Enrichment Analysis. To understand the toxicological functions of the gene sets, gene ontology enrichment (GO, hypergeometric test, p<0.05) and pathway enrichment (Fisher's Exact test, p<0.05) analyses were performed using Bingo v2.4[33] in Cytoscape v3[34] and Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), respectively. No multiple testing correction was applied to avoid loss of biologically relevant functions. As IPA is predominantly based on mammalian centric gene and pathway knowledge, ortholog genes between Atlantic salmon and mammalian species were used for pathway analysis. Orthologs were identified using a two-pass BLAST approach in Inparanoid 4.1,[35] as previously described.[14]

■ RESULTS AND DISCUSSION

Response Classification. A total of 3460 (1484 up- and 1976 down-regulated) genes were identified as DEGs in Atlantic salmon after combined exposure, of which 3124 were initially predicted as additive, 323 as synergistic and 13 as antagonistic by the IA model (SI, Table S1). To get more insight into different types of joint actions,

DEGs were categorized into two major groups on basis of the direction of transcriptional regulation compared to the control (i.e. 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).

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197

198 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

199

200 **Function Analysis.** To further understand the toxicological functions of the DEGs displaying different types of
201 joint actions, enrichment analyses were performed with the three DEG sets (Type 1 & 2 merged to avoid loss of
202 biologically significant information) displaying additive, synergistic and antagonistic effects. Both GO (Figure 1A)
203 and pathway (Figure 1B) analysis showed that the majority of the enriched functions were unique when comparing
204 different types of interactions. A relatively lower number of GO functions and pathways were found to be common
205 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.

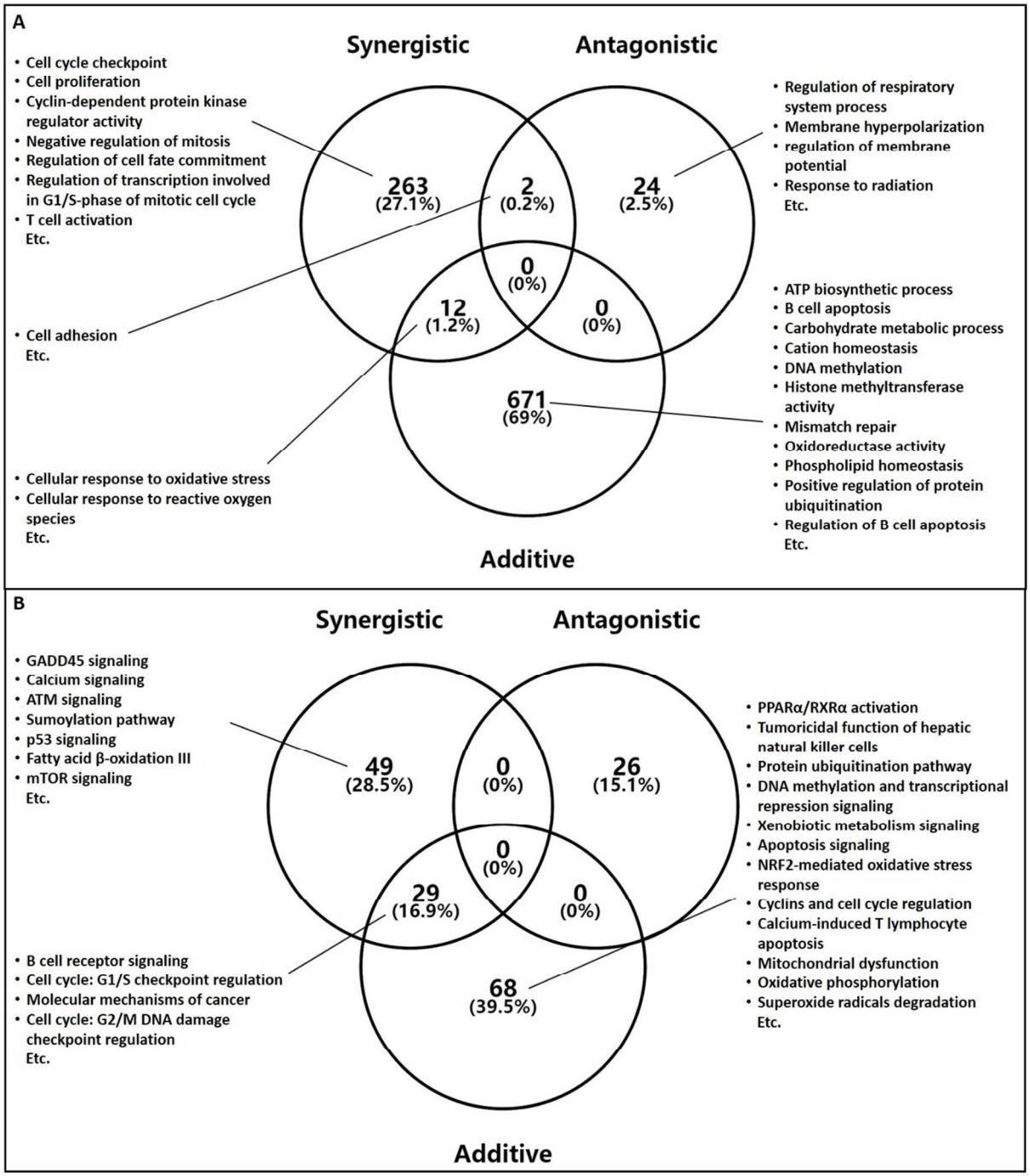

Figure 1. Venn diagram analysis of toxicologically relevant gene ontology (GO) functions (A) and canonical pathways (B) that were enriched by differentially expressed genes (DEGs) displaying additive, synergistic and antagonistic effects in Atlantic salmon (*Salmo salar*) after combined exposure to gamma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

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

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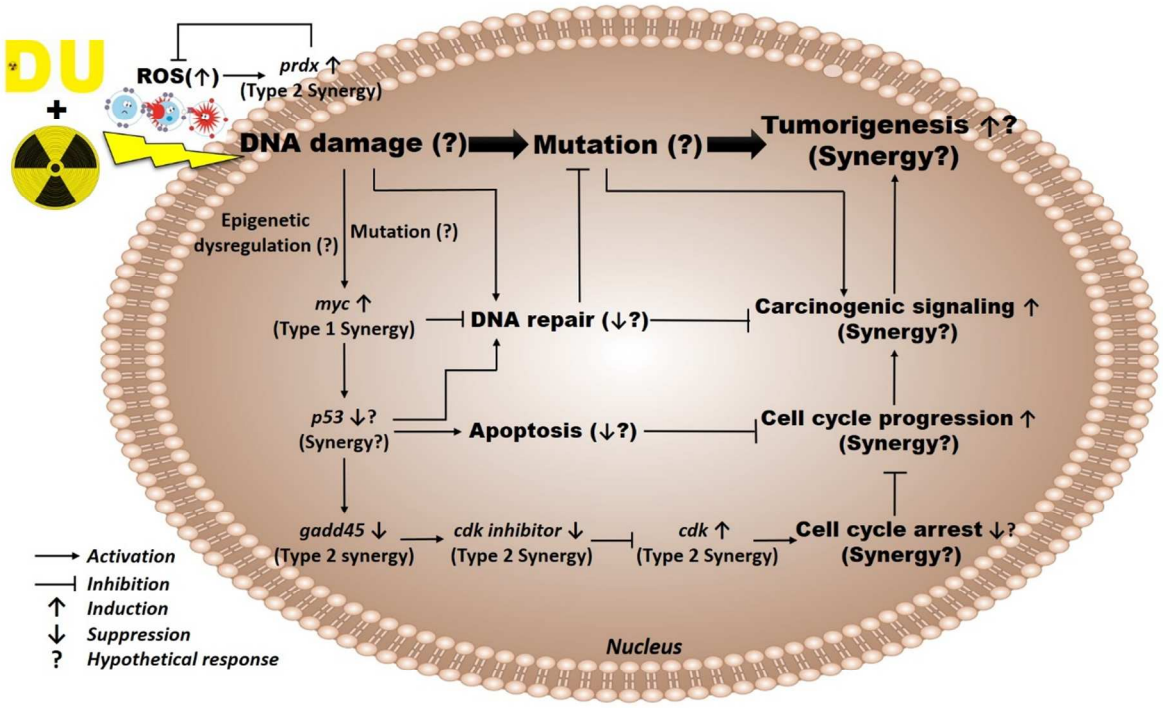


Figure 2. An example illustrating synergistic toxicity pathways of DNA damage leading to reduced cell cycle arrest and enhanced carcinogenesis signaling in the liver of Atlantic salmon (*Salmo salar*) after combined exposure to gamma radiation and depleted uranium (DU). ROS: reactive oxygen species;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

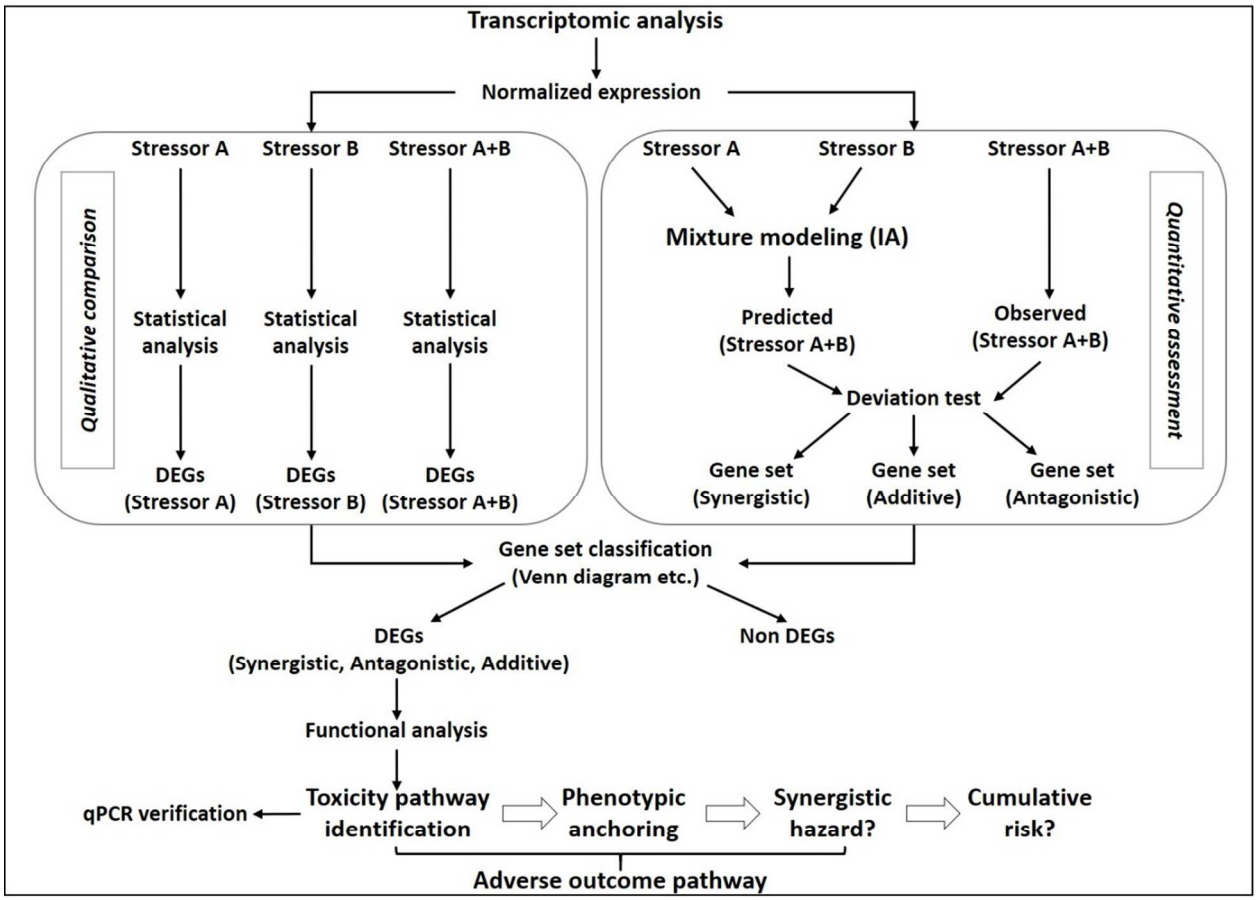
Applications and limitations of the conceptual approach. As illustrated by the case study, a conceptual workflow for combined effect assessment using transcriptomic data is proposed (Figure 3). This conceptual approach integrates mechanistically-based comparative analysis (qualitative/descriptive), expression-based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

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

388 analyses may increase the ability to decipher and classify relevant combined effects at the gene level and quantify
389 combined effects relevant for key toxicity pathways.
390



391

392 Figure 3. Proposed workflow for mechanistically-based assessment of low-dose interactive effects of combined stressors using
393 transcriptomics data. Qualitative comparison: Mode of action (MoA)-based assessment; Quantitative assessment: Prediction
394 model-based assessment; DEG: differentially expressed gene; CA: concentration addition; IA: independent action. qPCR:
395 quantitative real-time reverse-transcription polymerase chain reaction.
396

397 **Future Perspectives.** A key question raised from the present study is whether additivity, synergism and
398 antagonism of gene expression and pathways at the molecular level can be used to predict the corresponding joint
399 action at the organismal or population level. Recent advance in gene co-expression network modeling showed that it
400 is possible to quantitatively predict adverse effects at the organismal level by using gene expression data,[19] which is
401 a first step of extrapolation between different levels of biological organization. This is especially important as future

regulatory toxicology requires reduced animal testing, better extrapolations from low to high biological levels (e.g. *in vitro* to *in vivo*), and increased predictability across taxa and stressors[69] To answer this question, anchoring of combined effects at multiple biological levels along a defined AOP or network of AOPs is needed. Anchoring of relevant toxicity pathways being perturbed by a set of single and multiple stressor to key components in the AOP continuum (i.e. the molecular initiating event and the key events) can help to identify more complex responses involving multiple AOPs (i.e. network of AOPs) which may mutually interact to cause adverse outcomes of ecological relevance.[12, 70] Another important question is whether the proposed approach can also be used for an increased number of stressors. Although the principles outlined herein should ideally be applicable to an infinite number of stressors, proof-of-concept studies to demonstrate the applicability and robustness for a number of stressors and extended dose-rate/concentration ranges reflecting ecologically-relevant exposure scenarios is highly warranted. For different types of studies, the choice of appropriate model is also important. A recent study by Schäfer and Piggott[71] proposed a guideline for selecting the optimal null model (i.e. a prediction model assuming no interaction between the stressors) for prediction of multiple-stressor effect on individuals or populations, which may also be adapted for modeling the effects at the molecular level. Other modeling approaches in combination with the classical combined effect prediction models, such as machine learning-based classification techniques[72] and advanced correlation/regression analysis[73] may provide additional options for combined toxicity assessment of multiple stressors. Moreover, the complexity of biological responses (i.e. directional responses) as observed in the present study as well as other studies (reviewed in ref[36]) needs to be taken into account in the next generation of cumulative hazard assessment of multiple stressors. Mechanistic knowledge on the MoAs of the stressors as well as molecular regulatory networks should be preferably obtained prior to conducting complex multiple stressor studies using the OMICS tools. Reconceptualizing the definitions for additivity, synergy and antagonism by considering more complex biological responses may be required.[36]

■ ASSOCIATED CONTENT

Supporting Information (SI)

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Table S1: DEGs, Table S2: GOs, Table S3: Tox lists, Table S4: Canonical pathways (XLSX)

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Notes

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