Venezia2021

Programma di ricerca scientifica per una laguna “regolata”

Linea 2.1

Contaminanti emergenti in laguna, esposizione ed effetti

D2.1.7.1

Sviluppo della Linea di Evidenza (LoE) “trascrittomica”

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Sommario

Sintesi

La valutazione della qualità del sedimento in laguna di Venezia è fondamentale in virtù del ruolo essenziale che questo svolge nell'ecosistema acquatico lagunare, nella gestione dei canali navigabili e nella ricostruzione di strutture geomorfologiche. L'obiettivo primario della Tematica 2 all'interno del progetto Venezia2021 è pertanto quello di approfondire la conoscenza relativa alla contaminazione del sedimento lagunare, così da poter ottimizzare la sua gestione in un'ottica di sostenibilità e fornire un valido supporto alla classificazione normativa dei sedimenti tuttora in fase di revisione (il cosiddetto "Protocollo Fanghi" del 1993). Il presente lavoro si inserisce tra gli obiettivi fondamentali della Linea 2.1 nel suo tentativo di includere nuove evidenze scientifiche in un approccio integrato definito Weight of Evidence (WoE) per una migliore caratterizzazione del rischio associato ai sedimenti contaminati.

Tale approccio prevede l'utilizzo di informazioni eterogenee organizzate in "linee di evidenza" (Line of Evidence, LoE), siano esse analisi chimiche, studi di biodisponibilità, saggi ecotossicologici, caratterizzazione delle comunità bentoniche o biomarcatori cellulari, e la loro successiva integrazione al fine di derivare un quoziente di pericolo (HQ) per l'intera matrice sedimento. Con questo studio si vuole ampliare il ventaglio di risultati sperimentali finora integrabili in un approccio WoE quantitativo, presentando una nuova linea di evidenza basata su dati di alterazioni genomiche in organismi esposti a contaminanti. La linea di evidenza trascrittomica va pertanto a considerare le variazioni di espressione genetica in termini di RNA trascritto, aggiungendo ulteriori informazioni a supporto della caratterizzazione dei sedimenti lagunari.

Per fare questo, all'interno della task T2.1.2.7 si propone un metodo innovativo di elaborazione dei dati trascrittomici che permette la derivazione di un "indice di pericolo trascrittomico" ("Transcriptomic Hazard Index" - THI) quantitativo e integrabile, in un'ottica di WoE, con le altre linee di evidenza. La metodologia qui proposta utilizza dati trascrittomici da precedenti studi di laboratorio, alcuni dei quali ottenuti all'interno della linea 2.3 di Venezia2021, su vongole e mitili esposti a composti chimici rilevabili nell'ambiente lagunare: antiepilettici (carbamazepina), erbicidi (glifosato e AMPA), fragranze (amilsalicilato) e PFAS. I dati trascrittomici, elaborati con la Gene Set Enrichment Analysis (GSEA, Subramanian et al., 2005), sono stati tradotti tramite equazioni matematiche in un indice quantitativo che consente di valutare non solo la severità delle alterazioni a livello di regolazione nell'espressione genica, ma anche la rilevanza di tali alterazioni in termini di reazione fisiologica dell'organismo. Ne risulta quindi un indice numerico (THI) utile a classificare le situazioni di alterazione genomica con una delle cinque classi di pericolo definite: assente - *absent*, leggero - *slight*, moderato - *moderate*, rilevante - *major* o grave - *severe*.

Il THI così calcolato fornisce una valutazione a sé stante degli effetti dovuti a un'esposizione dell'organismo a composti chimici, nonché, una volta associato a evidenze raccolte con altri indagini, un quadro complessivo più accurato per la caratterizzazione dei sedimenti in ambiente acquatico. La metodologia qui proposta rappresenta un primo passo verso un approccio multidisciplinare che prevede l'inclusione delle analisi genomiche nei processi di valutazione del rischio ecologico (Ecological Risk Assessment, ERA). All'interno del progetto Venezia2021 i risultati di questa deliverable consentiranno l'inserimento delle indagini genomiche nell'applicazione dell'approccio WoE ai dati sperimentali raccolti per la caratterizzazione dei sedimenti nei canali navigabili (WP2.1.2) e per la valutazione degli impatti del MOSE sulla attività di molluschicoltura (WP 2.1.4).

1. Introduction

Understanding the interaction of water, sediment and organisms in complex environments, such as the Venice Lagoon, is of crucial importance for implementing sustainable managing solutions. This is particularly relevant in the management of sediment dredged from the lagoon waterways. The undergoing revision of the legislation regulating this aspect (the so-called “Protocollo Fanghi”, 1993) is believed to be better supported if an in-depth and multidisciplinary evaluation of the sediment quality is carried out.

In this context, Work Package (WP) 2.1.2 within Line 2.1 of the Venezia2021 project aims to expand the existing knowledge on the quality of the lagoon sediment by integrating different methods of investigation. A deeper knowledge can be elicited if data and information are gathered under different domains or “lines of evidence” (LoE). These results, once pooled together through a Weight of Evidence (WoE) approach, overcome the limitation of drawing conclusions on sediment quality from only a single analysis.

In the conceptual framework of Ecological Risk Assessment (ERA), WoE approaches have reached a broader evaluation of sediment quality by integrating so far evidence from studies concerning sediment chemistry (occurrence of chemical contaminants), contaminants’ bioavailability, ecotoxicological bioassays, local ecological communities and cellular biomarkers. According to the objectives of Task T2.1.2.7, to enrich this palette of investigations, a new LoE is proposed here that considers genomic-scale changes in RNA expression in organisms exposed to chemicals and/or environmental stressors. In this work, a methodology is presented to quantitatively elaborate into a quantitative index transcriptional data previously obtained from aquatic organisms exposed to chemicals.

The methodology makes use of results from the application of Gene Set Enrichment Analysis (GSEA, Subramanian et al., 2005) to recent studies investigating the response of *Mytilus galloprovincialis* and *Ruditapes philippinarum* exposed, under controlled laboratory conditions, to environmentally realistic concentrations of contaminants, i.e. pharmaceutics, herbicides, per-fluoroalkyl substances, and fragrances.

A set of mathematical algorithms was designed to translate the severity of transcriptional alterations in exposed organisms into a quantitative hazard index. The degree of de-regulation of gene sets organised into higher biological themes together with the physiological relevance of each biological theme contribute to the calculation of the hazard index. The outcome, expressed on a scale 0-100%, is classified according to five hazard classes (from absent to severe), providing an evaluation of the early individual effects of chemical exposure.

This methodology can serve as a proof of concept for the integration of “genomic tools” in ecological risk assessment based on multidisciplinary investigations. The proposed transcriptomics hazard index is intended to the creation of an additional LoE and its integration into the WoE model that will find application on the experimental data collected for the assessment of the lagoon sediment quality within WP2.1.2 and WP2.1.4.

2. Background and objectives

The rapid computational improvement and technological affordability in the application of -omics analyses has brought new insights into the biological outcomes of organisms when exposed to chemicals or environmental stressors even in complex environments (Milan et al., 2015; Wernesson et al., 2015; Sauer et al., 2017, Schmitz et al., 2022). These -omics studies are not merely functional to human health assessment; in fact, they find promising application in ecotoxicology and ecological risk assessment too and the study of gene and protein expression in non-model organisms in response to environmental toxicant exposures is known as "ecotoxicogenomics" (Snape et al., 2004; Ankley et al., 2006).

Among -omics technologies, of interest is transcriptomics which investigates genomic-scale changes in RNA expression, indicating a temporary and/or persistent alteration in the transcription of genes resulting in differentially expressed genes or set of genes (Subramanian et al., 2005; Dean et al. 2018). A major challenge for this analysis, like for other -omics tools, is the lack of functional annotations in non-model species (i.e., databases that link genes with their physiological functions). However, a great effort has been spent in the last decade expanding the gene expression library of aquatic species with the release of dozens of annotated genomes and transcriptomes in public repositories such as Ensembl or NCBI (Biales et al., 2016; Poynton et al., 2018; Toth et al., 2021).

As for biomarkers, transcriptomics can foster the understanding of perturbation due to environmental pollutants and/or stressors at whole-body level or in different cellular districts, thus predicting biological outcomes and clarifying the mechanisms or mode of action (MoA) of environmental pollutants (Ankley et al., 2006). Linking molecular changes to apical responses is one of the possible applications of transcriptomics and it comes at hand in addressing single chemical mechanism of action (e.g., Villeneuve et al., 2012; Pagé-Larivière et al., 2019; Bernardini et al., 2021). In addition, studying the correlations between gene expression profiles and pollutant exposure may allow the identification of specific molecular signatures of chemical stress that, in turn, may be employed as tools for site-specific ecotoxicological assessments (Milan et al., 2015).

Based on either laboratory or site-specific applications, it is with no doubts that transcriptomics could serve as an informative tool for ecological risk assessments (ERA). The biggest challenge, however, resides now in processing and interpreting transcriptomics data consistently under a common framework for application in the regulatory domain, as expressed in the ECETOX (European Centre for Ecotoxicology and Toxicology of Chemicals) workshop held in 2016 (Sauer et al., 2017; Gant et al., 2017) and in Verheijen et al. (2020). Drawbacks from the lack of technical and interpretative guidelines make it difficult to compare results obtained with different bioinformatics methods thus undermining the robustness of transcriptomics outputs, which constitute the largest source of -omics data available to date (Verheijen et al., 2020). This represents a missed opportunity for –omics data in general to be considered as potential effect-based tools in the Water Framework Directive (WFD) assessment scheme, which still lacks ecotoxicological cause-effect evidence (Solimini et al., 2009; Wernesson et al., 2015).

Studies of aquatic ecosystems have indeed moved in this direction by reviewing the WFD assessment scheme towards quantitative WoE (QWoE) approaches (among others: Gottardo et al., 2011a, b, Micheletti et al., 2011; Piva et al., 2011; Benedetti et al., 2012; Bebianno et al., 2015; Fan et al., 2015; Barjhoux et al., 2018; Lehtonem et al., 2019; Regoli et al. 2019). In a WoE approach, multiple and heterogeneous data from different domains are organized into distinct LoEs (Chapman et al., 2002, Suter, 2007), which, in case of quantitative WoE, are denoted by specific synthetic indices indicating the class of hazard, and then harmonized together using weighing or ranking (Linkov et al., 2009). Because of the modularity and flexibility of such models, the possibility of adding other relevant or site-specific LoEs to support management decision of polluted sites should be considered (Smith et al., 2002; Suter and Comier, 2011; Regoli et al., 2019). Transcriptomics could be one of these.

Among other goals, the ECETOX report wished that transcriptomics data could be part of QWoE approaches (Bridges et al., 2017), an objective not yet fully explored. While we can delve into the reasons behind this missed opportunity, the main limitations to the integration of transcriptomics results into QWoE stem primarily from the difficulty to interpret and integrate quantitative data into a unique hazard index. Consequently, also recently transcriptomics has served more as a qualitative tool in ERA schemes in support to evidence collected from other domains (Mezzelani et al., 2021, Schmitz et al., 2022).

Inspired by the methodological framework of Piva et al. (2011) and Regoli et al. (2019) and thanks to the insightful discussion with the research group of Marche Polytechnic University, a novel methodology is hereafter presented. It aims at translating transcriptional data into a synthetic hazard index that can reflect the relevance of molecular alterations in response to chemical exposure of aquatic organisms. Genes, grouped together into biologically outcome-wise categories called gene-sets (Liberzon et al., 2015), are analysed in terms of their relevant statistical expression with the Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005), as better explained in the methodological session. GSEA indicates, via a score, named Enrichment Score (ES), the degree of impairment of gene sets, which, in the proposed methodology, will then be coupled with the biological relevance of the transcriptomic alteration to calculate the class of effect and, eventually, the class of hazard derived from transcriptomics data. Calibration and validation of the conceptual model is performed with transcriptomics datasets collected in previous experiments and with data simulations aimed at creating more “extreme” transcriptomic alterations.

This methodology can serve as a proof of concept for the integration of -omics tools in ERA based on multidisciplinary investigations. To this end, the proposed quantitative “transcriptomic” hazard index can in future be incorporated in a QWoE approach for site-specific ERA, where the integration with results from other types of analyses (LoEs), such as chemistry, bioavailability, ecotoxicological bioassays, local ecological communities (e.g., macroinvertebrates) and cellular biomarkers, can contribute to elucidate the role of chemicals in giving rise to adverse biological and ecological effects.

3. Materials and methods

3.1 Analysis of transcriptomics data

By performing gene expression analysis, transcriptomics pools together huge amount of data to generate a list of differentially expressed genes (DEGs) by comparing samples kept in different conditions (e.g., treated, or polluted, versus a control). The expression of a gene is represented by its fold change (FC), a number indicating the magnitude of over-, or down-regulation, which is reflected by the different degree of transcription of mRNA across phenotypes of interest.

The expression profiling of tens of thousands of genes can pose big challenges in the following interpretation of transcriptional data. A "classic" way of defining interesting genes (i.e. genes differentially expressed, DE) is to set a threshold p-value (resulting from the differentially expression test) and a threshold fold-change, and deeming as DE those genes passing both thresholds. Although easy to implement, interpretation of results might not be straightforward as the *a priori* choice of the thresholds influences the number of significant genes and may not necessarily identify the relevant biological processes taking place in the organism (Liberzon et al., 2015). In light of this limitation, Gene Set Enrichment Analyses (GSEA) serves as a more appropriate tool in processing and interpreting gene expression data. GSEA is an analytical computational method that, giving an a priori gene set, known to be associated to a specific biological process, determines if there is a coordinated shift (e.g. up- or down-regulation) in the expression of the whole gene set between two conditions and if this change is statistically supported (Subramanian et al., 2005). Instead of focusing on individual genes significantly passing a pre-defined threshold(s), GSEA works with the full list of genes (i.e., not only the DE genes) producing results that pinpoint relevant biological responses. In light of the above considerations, in this work transcriptomics data is processed with GSEA. This method is no without challenges though. An important aspect in implementing the GSEA is indeed the organization of genes into gene sets.

Once contigs are annotated based on the most recently published findings and updated databases, genes responsible for a specific biological state or process are grouped together into gene sets. As a single gene can be accountable for multiple functions, redundancy in gene sets is thus likely to occur. Redundancy could affect gene sets, which may share a large number of genes, as well as their annotation, so that different groups eventually refer to very similar biological process (Liberzon et al., 2015). Potentially this can lead to an over-representation of some biological responses hindering the identification of others in the following statistical analysis. To overcome redundancy issues, in the present study the "hallmark" gene sets collection presented in Liberzon et al. (2015) and Dean et al. (2017) has been considered. Each hallmark gene set represents a distinct biological process that is then assigned to one of the eight higher "biological themes" or "categories", in accordance with Dean et al. (2017), i.e. cellular component, development, DNA damage, immune, metabolism, stress response, proliferation and signaling.

Hallmark gene sets, derived to reduce redundancy through experiments, computational procedure and expert curation, were named following the HUGO (Human Genome Organization) nomenclature which uses human gene symbols. In our study, where the exposed organisms are marine aquatic species (Manila clams *Ruditapes philippinarum* and mussels *Mytilus galloprovincialis*), the original collection had to be firstly adapted to account for the molecular heterogeneity. Initially the similarities between human genes and our species were investigated via BLASTx (Basic Local Alignment Search Tool, Altschul et al., 1990). Then, a first refinement to the hallmark database consisted of eliminating two gene sets that were never described in bivalves, i.e., HALLMARK_ANGIOGENESIS and HALLMARK HEDGEHOG SIGNALING. Afterwards hallmark sets were crosschecked to identify those sharing a great proportion of genes in common between gene sets. Accordingly, the immune response gene sets HALLMARK INTERFERON GAMMA RESPONSE and HALLMARK INTERFERON ALPHA RESPONSE, sharing 70 genes (corresponding to 36% and 75% of the entire HALLMARK INTERFERON GAMMA RESPONSE and HALLMARK INTERFERON ALPHA RESPONSE, respectively) were merged creating a unique inclusive hallmark representing both hallmark sets. The same approach was

applied to HALLMARK ESTROGEN RESPONSE EARLY and HALLMARK ESTROGEN RESPONSE LATE, characterized originally by an overlap of 50%.

Four more gene sets did not reach the sufficient number of genes to be statistically processed in the GSEA analysis, eventually leaving the hallmark list with 42 gene sets out of the original 50 gene sets. The confirmed hallmarks remain well populated with an average of about 70 genes ascribed to each gene set. The number of genes in each set ranges from a minimum of 9 to a maximum of 170, ensuring the recommended size for the GSEA application. Figure 1 shows the distribution of biological themes, along with annotated hallmark gene sets, in terms of gene size.

Figure 1. Wind rose plot depicting the hallmark gene sets selected for this study, grouped together in 8 higher biological themes. Percentages indicate the gene size of each biological theme.

From the DE test between two phenotypes (i.e. treated and control) performed with the R library edgeR (Robinson et al., 2010), all genes are ranked in decreasing order based on their fold change (FC). GSEA software then considers this ordered list, by looking at genes associated to a specific hallmark gene set. Walking down the list of N genes, a running sum S_i takes into account whether the encountered gene i belongs to the specific gene set g of size k:

$$S_i = \begin{cases} 0 & \text{If } i = 0 \\ S_{i-1} + \dfrac{|r_i|^p}{N_R} & \text{If } 1 \leq i \leq N \text{ and } i \in g \\ S_{i-1} - \dfrac{1}{N-k} & \text{If } 1 \leq i \leq N \text{ and } i \notin g \end{cases}$$

When a gene of the gene set is hit, the sum increases according to the gene's expression profile r_i, weighted by the total magnitude of change of the gene set, $N_R = \sum_{i \in g} |r_i|$. On the contrary, a missed gene leads to a decrease in the running sum, which is proportional to the number of genes not associated to the gene set of interest, i.e. $N - k$. When $p = 1$, every gene in set g contributes with its weighted expression, r_i indicates the fold change (Subramanian et al., 2005). The greater the fold change of gene i, the larger will be the increase in the running sum. By doing so, the magnitude of the increment reflects the correlation of the gene with the phenotype taking into account the degree of over- or under-regulation of each gene.

GSEA (run via the "clusterProfiler" R library; Wu et al., 2021) determines then whether the set of genes has a significance modification in expression by understanding how genes are located along the list. If the expression of the gene set relates to the phenotypic distinction, genes in the set are over-represented at the top (in case of over-regulation) or at the bottom of the list (for under-regulation). On the other hand, genes randomly distributed along the list indicate that the gene set associated to a specific biological function is not showing any statistical important modification in terms of expression. To define significant changes in investigated hallmark sets, a conservative *p-value* of 0.2 has been considered. The degree to which a gene set is over or under-represented at the extremes of the ranked list is denoted as the maximum deviation from zero of the above running sum. The Enrichment Score (ES) embodies such deviation and its quantification depends on the sub-group of genes that largely contribute with their fold change to the calculation of the running sum. This sub-group within the gene set is called leading-edge subset. Graphical illustration of the meaning of the ES is presented in Figure 2. Given its definition, the ES can vary between 1, when all genes belonging to the gene set are displayed at the top of the ranked list (up-regulated compared to the control group), and -1 when genes are all located at the bottom of the list (down-regulated compared to the control group).

By considering the ES, the transcriptional alteration elicited by the GSEA in exposed organisms can be more directly translated into a quantitative hazard index as explained in the next section.

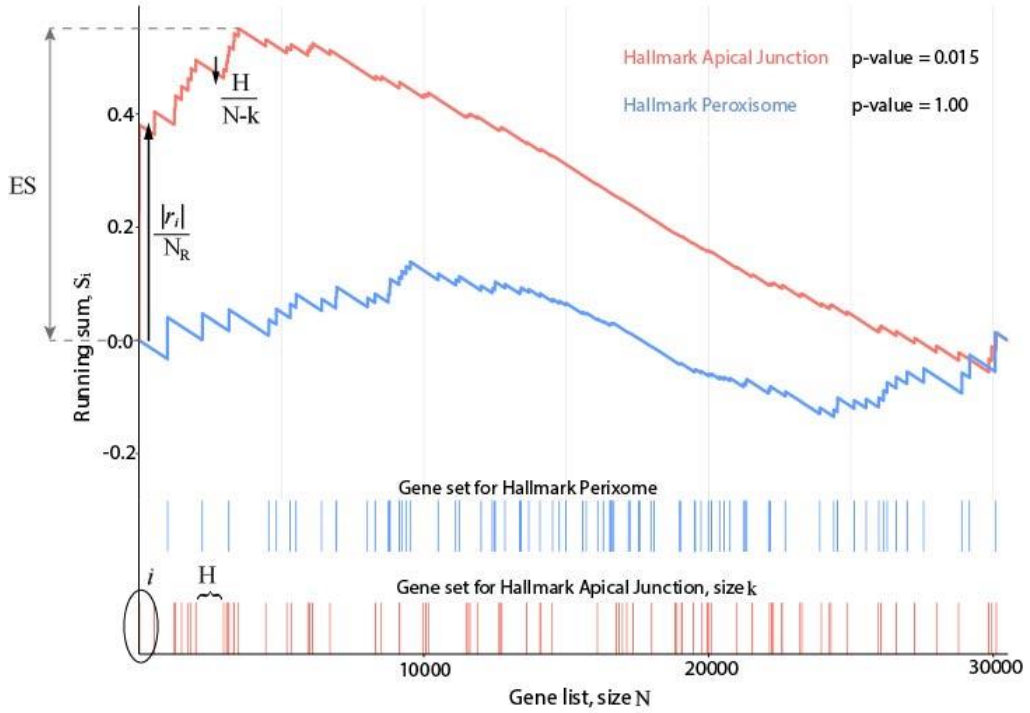


Figure 2. GSEA graphical explanation for two pathways, Hallmark Apical Junction and Hallmark Peroxisome. The latter is not statistically significant as its *p-value* is greater than 0.2 and genes are randomly distributed along the whole list of about 30000 genes. The continuous lines represent the running sum, which increases if walking down the list a gene belonging to the pathway is hit. The increase accounts for the normalized gene's fold change, r_i. The sum decreases by the number of missed genes accordingly to the number of genes not associated to the gene set. The enrichment score (ES) is represented as the maximum deviation of the sum from zero. Genes falling before the maximum value of the running sum add up to the leading edge subset for that particular pathway.

3.2 Elaboration of a transcriptomics quantitative index

With the objective of integrating GSEA results into ecological risk assessment, a quantitative index was developed to translate transcriptomics information into classes of hazard. The significant gene sets indicate which biological themes are subject to transcriptional alteration. To elucidate the severity of this alteration, specific algorithms were developed to account for both the relevance of each biological theme in terms of physiological reactions and the degree of its de-regulation. The conceptual framework finds inspiration in the mathematical elaboration of the line of evidence for biomarkers outlined in Piva et al (2011) and, as subsequently modified, in Regoli et al. (2019).

Firstly, each biological theme was assigned a "weight" between 1 and 3 based on the relevance of the biological endpoint triggered by the change in transcripts. The weighting 1 was assigned to exposures that not necessarily imply the onset of a toxicity state, while 1.5 to transcripts that preclude adverse effect; 2 was attributed to responses that are prognostic of impairment at higher levels of biological organization and 2.5 for hormonal, reproductive or cell cycle dysfunction. The weighting of 3 was assigned to alterations leading to cell death, apoptosis and cancer. Table 1 reports the biological themes along with the relative weights.

The relevance of de-regulation is instead derived by dividing the observed cumulative ES of significant gene sets by the maximum ES attainable within a specific biological theme. The maximum ES is obtained by adding together the highest level of de-regulation potentially achievable by each molecular pathway, i.e. |ES| = 1, contributing to the same biological theme. For example, since the biological theme "metabolic" consists of 6 gene sets, whose maximum attainable ES in absolute value is 1, the highest level of de-regulation is equal to 6.

Table 1. Biological themes with corresponding weight and maximum attainable ES.

Biological theme	Weight w	Maximum |ES|
Cellular component	1	3
Metabolic	1.5	6
DNA damage	2.5	3
Immune	1.5	5
Stress response	2	5
Development	2.5	5
Proliferation	3	6
Signaling	2.5	9

In the proposed approach, the transcriptional effect observed for a biological theme i is defined according to the following equation, where the theme's weight is normalized by the maximum value of 3 and the sum of the statistically significant ESs is divided by total ES attainable for the biological theme:

$$E_i = \frac{w_i}{w_{max}} \cdot \frac{\sum_{gene\ sets \in i} |ES|}{ES_{max,i}} \cdot 100$$

So defined, the transcriptomics effect for each biological theme can vary from 0% to 100%, with the latter representing the case of maximum deregulation achieved simultaneously by all the pathways contributing to a biological theme whose alterations can lead to cell death, apoptosis and cancer. Depending on the value of E_i, five classes for the "Effect" have been defined, namely: A - Absent ($E_i = 0\%$), B -Slight ($0\% < E_i \leq 7\%$), C- Moderate ($7\% < E_i \leq 25\%$), D- Major ($25\% < E_i \leq 40\%$), E- Severe ($40\% < E_i \leq 100\%$) (Figure 3).

Finally, the Transcriptomics Hazard Index (THI) associated with the overall transcriptomics results for each experiment (i.e., for each level of exposure) is calculated based on the percentages of biological themes falling within each class of effect:

$$THI = \%biol.themes_A \cdot a + \%biol.themes_B \cdot b + \%biol.themes_C \cdot c + \\ + \%biol.themes_D \cdot d + \%biol.themes_E \cdot e$$

Factors a to e find correspondence with the upper bound of each class of hazard, and their increasing values make so that larger importance is given to biological themes which are progressively more affected by transcriptomics variations (Figure 3). The value obtained by the THI can then range from $a \cdot 100\%$, when all biological themes are in class A-*absent*, to $e \cdot 100\%$ corresponding to all biological themes in class E denoted by severe effects.

Five classes for the final THI have been defined, namely: Absent, Slight, Moderate, Major, and Severe. As shown in Figure 3, the class of hazard results absent when THI is equal to zero. When THI varies between 0 and 70 (corresponding to all biological themes with slight effects), the hazard is slight; THI from 70 to 250 (when all biological themes have moderate effect) classifies the hazard as moderate, while from 250 to 400 it is considered major. Severe hazard index ranges between 400 and 1000, the latter caused by all biological themes with severe effects.

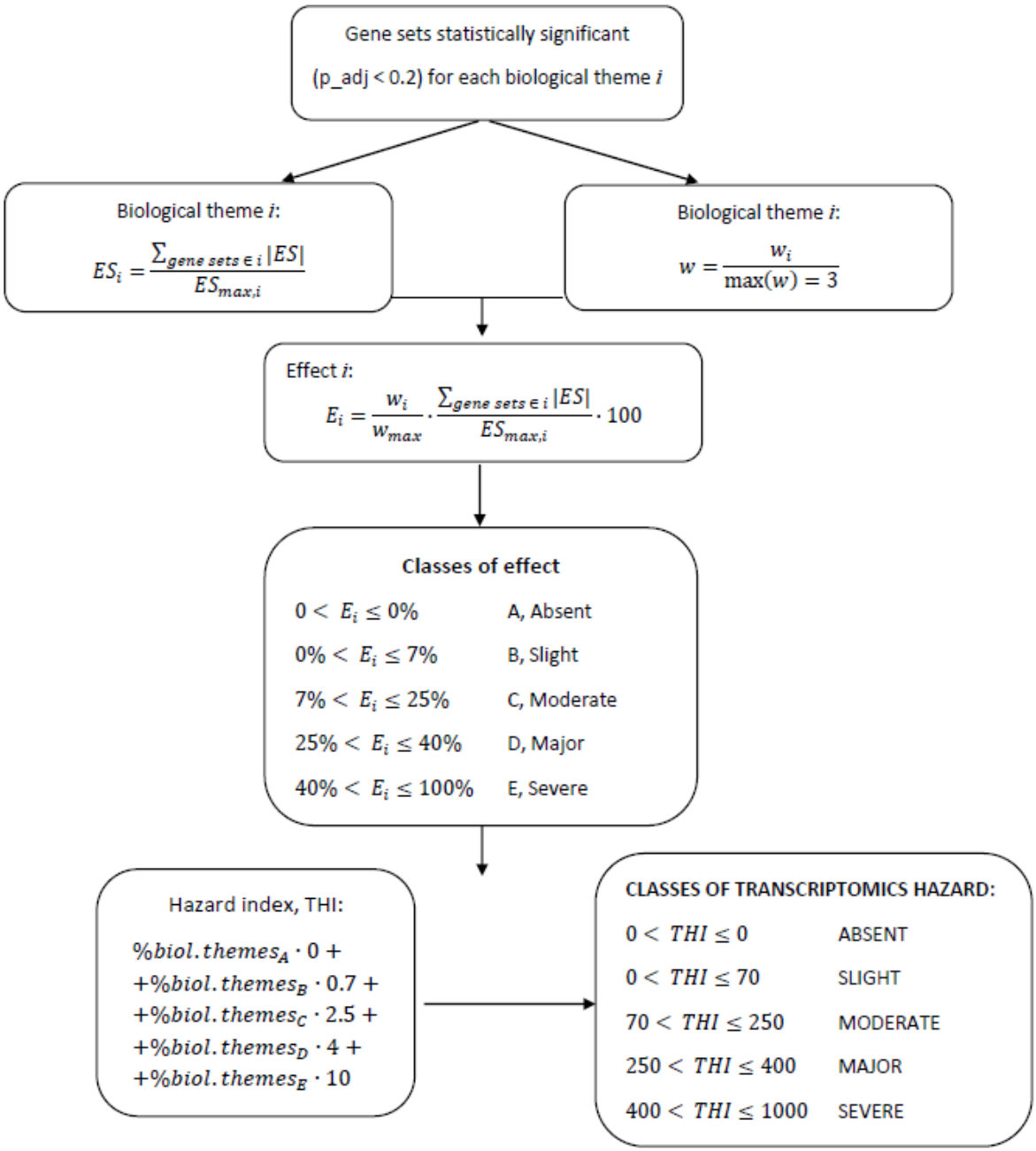


Figure 3. Flowchart and calculations of Transcriptomics Hazard Index.

The logical steps of the above procedure were tested, and then boundaries between classes of effects calibrated, using data from experimental studies along with transcriptional situations made up to simulate a vast range of genomic alterations. Experimental and simulated datasets supported the calibration of the boundaries between classes of effect for the single biological theme and, consequently, the determination of coefficients to derive the five classes of hazard. In both conditions, experimental and simulated ones, the calibration was continuously guided by expert judgment in order to align the THI output with the expert qualitative interpretation of transcriptomics altered profiles. Expert decisions were taken by considering the number of differentially expressed pathways, their degree of de-regulation and the significance of the alteration in the biological theme they contribute to in terms of physiological outcomes. All together, they supported the expert evaluation in assigning a qualitative class, from absent to severe (i.e., the same classes used for the evaluation of the THI, see Fig. 3), to each condition.

3.3 Experimental data

The proposed methodology for the estimation of a Transcriptomics Hazard Index (THI) was tested using datasets presented in Table 2 and hereafter briefly outlined. Data from previous studies consisted of transcriptional analysis for two types of bivalve species exposed, under controlled laboratory conditions, to emerging contaminants, i.e. per-fluoroalkyl substances, pharmaceutics, herbicides, and fragrances, and to environmental stressors, i.e. acidification. The two species, Manila clam *Ruditapes* philippinarum and mussel *Mytilus galloprovincialis*, are well known as sentinel species as well as for their ecological and economic importance. Bivalves are sedentary, filter-feeding organisms, which tend to accumulate metals and other pollutants in their tissues. In recent years, several studies worldwide investigated bivalve species to characterize the effects of emerging contaminants and the impact of anthropogenic activities in marine ecosystems including the Venice lagoon (e.g. Venier et al. 2006; Chapman et al., 2009; Milan et al., 2011; Milan et al., 2013; Milan et al., 2015).

All together, these datasets simulate realistic environmental conditions for the types of chemicals that can be nowadays found in marine water and for the concentration organisms are exposed to.

Table 2. Datasets with relative information about chemical, exposure dose and time, species and reference studies.

Chemical	Dose	Exposure time	Species	Reference study
C6O4	0.1 µg/l	7 days	*Ruditapes philippinarum*	Bernardini et al., 2021
C6O4	1 µg/l	7 days	*Ruditapes philippinarum*	Bernardini et al., 2021
PFOA	1 µg/l	7 days	*Ruditapes philippinarum*	Bernardini et al., 2021
C6O4	0.1 µg/l	21 days	*Ruditapes philippinarum*	Bernardini et al., 2021
C6O4	1 µg/l	21 days	*Ruditapes philippinarum*	Bernardini et al., 2021
PFOA	1 µg/l	21 days	*Ruditapes philippinarum*	Bernardini et al., 2021

Acidification and Carbamazepine (ACD + CBZ)	pH 7.6, 1 µg/l	28 days	*Mytilus galloprovincialis*	Mezzelani et al., 2021
Acidification (ACD)	pH 7.6	28 days	*Mytilus galloprovincialis*	Mezzelani et al., 2021
Carbamazepine (CBZ)	1 µg/l	28 days	*Mytilus galloprovincialis*	Mezzelani et al., 2021
Glyphosate (GLY)	100 µg/l	7 days	*Mytilus galloprovincialis*	Iori et al., 2020
AMPA	100 µg/l	7 days	*Mytilus galloprovincialis*	Iori et al., 2020
Glyphosate and AMPA	100 µg/l	7 days	*Mytilus galloprovincialis*	Iori et al., 2020
Glyphosate (GLY)	100 µg/l	21 days	*Mytilus galloprovincialis*	Iori et al., 2020
AMPA	100 µg/l	21 days	*Mytilus galloprovincialis*	Iori et al., 2020
Glyphosate and AMPA	100 µg/l	21 days	*Mytilus galloprovincialis*	Iori et al., 2020
Glyphosate	10 µg/l	21 days	*Mytilus galloprovincialis*	Milan et al., 2018
Glyphosate	100 µg/l	21 days	*Mytilus galloprovincialis*	Milan et al., 2018
Glyphosate	1000 µg/l	21 days	*Mytilus galloprovincialis*	Milan et al., 2018
Amyl Salicylate (AMY)	0.3 µg/l	7 days	*Mytilus galloprovincialis*	No published data
Amyl Salicylate	3 µg/l	7 days	*Mytilus galloprovincialis*	No published data
Amyl Salicylate	0.3 µg/l	14 days	*Mytilus galloprovincialis*	No published data
Amyl Salicylate	3 µg/l	14 days	*Mytilus galloprovincialis*	No published data

3.3.1 Perfluorooctanoic acid (PFOA) and Perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy) acetic acid (C6O4)Carbamazepine and water acidification

Perfluorooctanoic acid (PFOA) is a chemical belonging to the class of per- and poly-fluoroalkyl substances (PFAS). It has been found in surface and groundwater worldwide and detected globally in the tissues of fish, bird, and marine mammals (Fujii et al. 2007). Because of its properties, PFOA represents an intrinsic threat for aquatic organisms and human health leading to the industrial phase-out of this chemical. To cope with the need of a replacement, perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy) acetic acid ($C_6HF_9O_6$), commercially known as C6O4 or F-Diox acid, has been recently introduced as an alternative. C6O4 is a short chain perfluoropolyether substance, registered and patented by Solvay in 2014 to replace PFOA.

In the study of Bernardini et al. (2021), Manila clams (*Ruditapes philippinarum*) were used to perform controlled exposures to 0.1 and 1 µg/L C6O4 and 1 µg/L PFOA for 7 and 21 days. More details are reported in Bernardini et al. (2021).

3.3.2 Carbamazepine and water acidification

Carbamazepine (CBZ) is an aromatic anticonvulsant used as human antiepileptic pharmaceutical. Because of the massive use and limited removal by wastewater treatment plants, this compound has been detected in freshwater and costal ecosystems as well as in tissues of aquatic invertebrates (Mezzelani et al., 2020), raising concern in marine environment. Marine ecosystem is subject to a multitude of several environmental stressors, among others acidification (ACD) may drive the environmental fate, bioavailability and toxicity of such emerging compound.

In their study Mezzelani et al. (2021) exposed mussels (*Mytilus galloprovincialis*) to carbamazepine (1 µg/L, pH= 8.10/pCO_2= ~400 µatm), reduced pH/hypercapnia (pH = 7.6/pCO_2= ~1700 µatm) and to a mixture of stressors (pH = 7.6/pCO_2= ~1700 µatm, carbamazepine 1 µg/L). For further details, consider the published paper Mezzelani et al. (2021).

3.3.3 Glyphosate and Amino-methylphosphonic acid (AMPA)

Glyphosate (GLY) is the most widely used herbicide worldwide, targeting the 5-enolpyruvylshikimate-3-phosphate synthase enzyme in the shikimate pathway found in plants and some microorganisms. Amino-methylphosphonic acid (AMPA) is the product of the microbial degradation of glyphosate. Several studies highlighted a wide range of toxicological effects in non-target organisms, raising concerns about the potential risks to the aquatic compartment (Cavalcante et al., 2010; Modesto and Martinez, 2010; Uren Webster et al., 2014; Uren Webster and Santos, 2015; Matozzo et al., 2018; Matozzo et al., 2019). These compounds were tested in two laboratory experiments. In the first study by Iori et al. (2020), specimens of *M. galloprovincialis* were exposed to 100 µg/L of glyphosate, 100 µg/L of AMPA and a mixture of 100 µg/L of glyphosate + 100 µg/L of AMPA for 7 and 21 days. In the second work by Milan et al. (2018), mussels (M. galloprovincialis) were exposed for 21 days to 10, 100, and 1000 µg/L of glyphosate only. More details can be found in Iori et al. (2020) and Milan et al. (2018).

3.3.4 Amyl salicylate

Amyl salicylate ($C_{12}H_{16}O_3$) is one of the members of the salicylate family widely used as fragrance in perfumery and in other personal care products. Following the detection in costal environments, scientific community has classified fragrances as Contaminants of Emerging Concern (CECs).

For this chemical, the data used in the current work have not been published yet. In the study, mussels (*M. galloprovincialis*) were treated with 0.3 and 3 µg/L Amyl salicylate (AMY) for 7 and 14 days.

4. Results and discussion

The THI was calculated for transcriptomics GSEA outcomes for each experimental condition and each exposure dose and results were assigned to one of five classes of hazard, as described in Paragraph 3.2. Since in the selected studies (Paragraph 3.3) the experimental conditions induced modest hazard at transcriptomic level, that is hazard classes ranged from slight to moderate, scenarios of more adverse transcriptional alteration were simulated. Simulations consisted of ad-hoc created GSEA outputs where differentially expressed pathways aimed at reproducing major and severe transcriptional hazard. The list of statistically significant pathways denoting each experimental condition and simulated scenario is provided in the supplementary information.

Figure 4 illustrates an example of application to one of the experimental datasets and to the most severe simulated scenario.

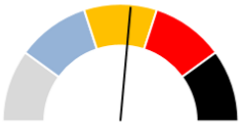
A

hallmark gene set	biological theme	w	ES
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	0.73
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2	0.71
HALLMARK_COMPLEMENT	IMMUNE	1.5	0.66
HALLMARK_HYPOXIA	STRESS RESPONSE	1.5	0.67
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	0.66
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	0.60
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2	0.61
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	0.57
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	0.57
HALLMARK_IL2_STAT5_SIGNALING	SIGNALING	2	0.57
HALLMARK_PI3K_AKT_MTOR_SIGNALING	SIGNALING	2.5	0.59
HALLMARK_COAGULATION	IMMUNE	1.5	-0.33
HALLMARK_IL6_JAK_STAT3_SIGNALING	IMMUNE	1.5	0.62

BIOLOGICAL THEME	Σ ES/ max(ES)	w/w_{max}	E_i	CLASS OF EFFECT
CELLULAR COMPONENT	0%	33%	0%	ABSENT
METABOLIC	0%	50%	0%	ABSENT
DNA DAMAGE	39%	83%	33%	MAJOR
IMMUNE	58%	50%	29%	MAJOR
STRESS RESPONSE	27%	67%	18%	MODERATE
DEVELOPMENT	0%	83%	0%	ABSENT
PROLIFERATION	10%	100%	10%	MODERATE
SIGNALING	21%	83%	17%	MODERATE

$THI = 194$

CLASS OF HAZARD: MODERATE



B

hallmark gene set	biological theme	w	ES
HALLMARK_ADIPOGENESIS	DEVELOPMENT	2.5	0.7
HALLMARK_MYOGENESIS	DEVELOPMENT	2.5	0.8
HALLMARK_SPERMATOGENESIS	DEVELOPMENT	2.5	0.8
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2.5	0.7
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2.5	0.8
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2.5	0.8
HALLMARK_IL2_STAT5_SIGNALING	SIGNALING	2.5	0.8
HALLMARK_PI3K_AKT_MTOR_SIGNALING	SIGNALING	2.5	0.7
HALLMARK_TGF_BETA_SIGNALING	SIGNALING	2.5	0.8
HALLMARK_HEDGEHOG_SIGNALING	SIGNALING	2.5	0.8
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	1	0.7
HALLMARK_APICAL_SURFACE	CELLULAR COMPONENT	1	0.6
HALLMARK_PEROXISOME	CELLULAR COMPONENT	1	0.6
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	DEVELOPMENT	2.5	0.7
HALLMARK_ANGIOGENESIS	DEVELOPMENT	2.5	0.7
HALLMARK_P53_PATHWAY	PROLIFERATION	3	0.6
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	3	0.7
HALLMARK_MYC_TARGETS_V1	PROLIFERATION	3	0.9
HALLMARK_E2F_TARGETS	PROLIFERATION	3	0.9
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	0.7
HALLMARK_COAGULATION	IMMUNE	1.5	0.7
HALLMARK_IL6_JAK_STAT3_SIGNALING	IMMUNE	1.5	0.9

BIOLOGICAL THEME	Σ ES/ max(ES)	w/w_{max}	E_i	CLASS OF EFFECT
CELLULAR COMPONENT	63%	33%	21%	MODERATE
METABOLIC	0%	50%	0%	ABSENT
DNA DAMAGE	50%	83%	42%	SEVERE
IMMUNE	46%	50%	23%	MODERATE
STRESS RESPONSE	0%	67%	0%	ABSENT
DEVELOPMENT	74%	83%	62%	SEVERE
PROLIFERATION	52%	100%	52%	SEVERE
SIGNALING	43%	83%	36%	MAJOR

$THI = 488$

CLASS OF HAZARD: SEVERE

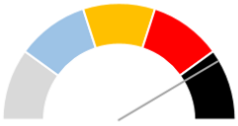


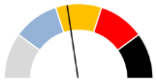
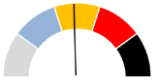
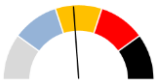
Figure 4. Calculation of the THI and relative class of hazard for (A) the experimental data of mussels exposed to carbamazepine (1 µg/L, pH= 8.10/pCO2= ~400 µatm), and for (B) an ad-hoc created GSEA outputs to obtain severe genomic alteration. W= weight; ES= Enrichment Score.

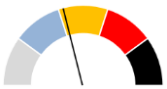
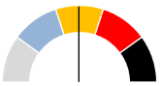
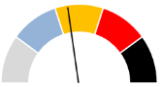
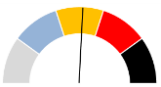
Similarly to the biomarkers LoE (Piva et al, 2011; Regoli et al., 2019), this methodology integrates the level of molecular alteration (corresponding to the enrichment score, ES) and the adverse biological effects on the organism's homeostasis (i.e. relative weight, w) into classes of hazard by considering only GSEA results that are statistically significant (*p-value* < 0.2). The derivation of the transcriptomics index differs though from the biomarkers index because of intrinsic differences between the two types of analysis. For example, the first class (*absent)* includes only cases where transcriptional effects are null, based on the consideration that the transcriptional alteration of just a pathway is caused by the differential expression of several genes populating the pathway, thus already deserving the class "slight". On the other opposite, considered the severity and the unlikeliness of a situation presenting biological themes with effect $E_i = 100\%$ (corresponding to the maximum severe effect, ES_i = 1 and w = 3), the class of hazard severe is here set to

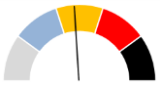
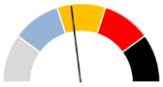
start for effects greater than 40%. Based on expert judgment, an effect larger than 40% indicates an already severe alteration at a transcriptional level anticipating an adverse biological outcome for the organism.

The results of an application of the proposed methodology to the experimental datasets are summarised in Table 3.

Table 3. Estimated THI as a numerical value and related hazard class for the experimental datasets considered in this study. Values of the THI are reported and visualized in a hazard-meter plot. Colours in plots identify the five classes of hazard: grey/absent, light blue/slight, yellow/moderate, red/major and black/severe.

Chemical	Species	Dose	Trascriptomics Hazard Index (THI): class and numerical value	Class of hazard
C6O4 (7 days)	*Ruditapes philippinarum*	0.1 µg/l	Moderate, 111	
C6O4 (7 days)	*Ruditapes philippinarum*	1 µg/l	Moderate, 80	
PFOA (7 days)	*Ruditapes philippinarum*	1 µg/l	Moderate, 89	
C6O4 (21 days)	*Ruditapes philippinarum*	0.1 µg/l	Moderate, 134	
C6O4 (21 days)	*Ruditapes philippinarum*	1 µg/l	Moderate, 71	
PFOA (21 days)	*Ruditapes philippinarum*	1 µg/l	Moderate, 153	
Acidification and Carbamazepine	*Mytilus galloprovincialis*	pH 7.6, 1 µg/l	Slight, 63	
Acidification	*Mytilus galloprovincialis*	pH 7.6	Moderate, 144	

Carbamazepine	*Mytilus galloprovincialis*	1 µg/l	Moderate, 194	
Glyphosate (7 days)	*Mytilus galloprovincialis*	100 µg/l	Moderate, 90	
AMPA (7 days)	*Mytilus galloprovincialis*	100 µg/l	Moderate, 71	
Glyphosate and AMPA (7 days)	*Mytilus galloprovincialis*	100 µg/l	Moderate, 89	
Glyphosate (21 days)	*Mytilus galloprovincialis*	100 µg/l	Slight, 49	
AMPA (21 days)	*Mytilus galloprovincialis*	100 µg/l	Slight, 26	
Glyphosate and AMPA (21 days)	*Mytilus galloprovincialis*	100 µg/l	Slight, 40	
Glyphosate (21 days)	*Mytilus galloprovincialis*	10 µg/l	Moderate, 163	
Glyphosate (21 days)	*Mytilus galloprovincialis*	100 µg/l	Moderate, 121	
Glyphosate (21 days)	*Mytilus galloprovincialis*	1000 µg/l	Moderate, 103	
Amyl Salicylate (7 days)	*Mytilus galloprovincialis*	0.3 µg/l	Moderate, 175	

Amyl Salicylate (7 days)	*Mytilus galloprovincialis*	3 µg/l	Moderate, 144	
Amyl Salicylate (14 days)	*Mytilus galloprovincialis*	0.3 µg/l	Slight, 40	
Amyl Salicylate (14 days)	*Mytilus galloprovincialis*	3 µg/l	Moderate, 125	

For modest alterations (slight and moderate), the index can capture the severity of the molecular variation with also an indication of the magnitude of the hazard. This is particularly evident in the case of 1 µg/l PFOA exposure (7 days) where the final score THI = 89 is slightly above the threshold between slight and moderate, thus reflecting the expert prediction which in that situation found it difficult to discriminate a slight from a moderate hazard. The quantitative hazard index works in conjunction with the class of hazard by complementing the indication of the transcriptomics hazard that would result in the simple use of a "label" (absent to severe). The THI helps with the interpretation of hazards falling in the proximity of the threshold between classes, where it is difficult to assign a single class. A next step towards a better interpretation of borderline transcriptomics conditions could provide the proposed methodology with a quantitative tool to estimate the uncertainty of the hazard output.

Where available, the obtained hazard has been compared with the corresponding results from previously published studies. Despite the use of different transcriptomics data analysis, the current methodology could well relate with the conclusions reached in the published studies.

In this work, the THI values calculated for two similar datasets do not apparently agree: glyphosate 100 µg/l 21 days (reference: Milan et al., 2018) was classified as moderate hazard and glyphosate 100 µg/l 21 days (reference: Iori et al., 2020) resulted classified as slight hazard. Such discrepancy stems from the different life stage mussels were exposed to in the two studies, with organisms in Milan et al. (2018) far from sexual maturity period to avoid spawning and additional stress.

Further evaluation was carried out by testing the methodology with 30 additional simulations covering absent to severe transcriptional hazards. The THI proved to be a good indicator of the different levels of adverse transcriptomic response finding confirmation in the expert judgment. The consistency of predicted and resulted hazard is promising, and it encourages further testing and refinement of the quantitative transcriptomics index proposed in this work to support ERA in aquatic ecosystems, something that has been sought for long (Schmitz et al., 2022). Within the multidisciplinary perspective adopted by WoE approaches, the construction of a supportive LoE based on transcriptional evidence could complement the interpretation and integration of results from other disciplines. Pooling together pieces of evidence from a broad spectrum of analyses, such as chemistry, bioavailability, ecotoxicological bioassays, conditions of local ecological communities (e.g. benthic invertebrates), cellular biomarkers, and now transcriptomics, would help to elucidate the adverse ecological effects of chemical or environmental stressors in aquatic ecosystems so that better-informed monitoring strategies can take place.

The framework presented in this work is the first step in this direction, but its transferability and reproducibility require further testing. The quantitative hazard tool has been applied to only laboratory-controlled exposures and the ability to interpret transcriptomics adverse outcome should be established also for *in situ* scenarios characterized by multiple stressors and mixture of chemicals. Given the flexibility

of the algorithms, the proposed methodology is not meant to be definitive leaving room for improvement both in terms of gene sets construction and thresholds between classes of effect, coefficients and class of hazard. However, it is a first attempt towards a quantitative interpretation of transcriptional data and the inclusion of -omics evidence in WoE approaches.

5. Conclusions

A methodology has been proposed to translate transcriptomics data into a hazard index with the ultimate goal of including gene expression information into quantitative Weight of Evidence approaches. By using previously collected genomic data of two aquatic species exposed to chemicals and environmental stressors, a set of algorithms allowed the derivation of a transcriptomics hazard index, and associated class of hazard (from absent to severe), for each experimental condition. The presented index accounts not only for the magnitude of genomic alteration but also for its biological importance in terms of organism's homeostasis. Experimental datasets supported the calibration of the designed method in delineating the five classes of transcriptomics hazard with the inclusion of simulations to investigate more adverse alterations. The application to the experimental datasets and simulated scenarios proved that the model can effectively discriminate different levels of adverse transcriptomic response when compared with expert judgment.

This work consists of the first attempt towards a quantitative interpretation of transcriptomics information and so it should not be considered in its final form. Its transferability and reproducibility require indeed further testing as the experimental datasets were all obtained in controlled laboratory environment. The next objective within Line 2.1 of Venezia2021 is the application of this methodology to experimental data collected in WP2.1.2 and WP2.1.4. By doing this, the ability of the index to reflect the transcriptional hazard posed by sediment from the lagoon waterways and by the MOSE activations could be further assessed. Ultimately, transcriptomics will be part of a multidisciplinary investigation on the quality of the lagoon sediment by constituting a stand-alone line of evidence to be integrated with other analyses under the framework of QWoE approaches.

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Annex: Supplementary information

ist of statistically significant hallmark gene sets obtained with the application of GSEA to the experimental conditions. Datasets are presented with relative biological theme, weight of the biological theme, gene set size and Enrichment Score (ES). For each dataset the transcriptional hazard class based on expert evaluation is reported. The class of hazard calculated with the Transcriptomics Hazard Index method can be found in the deliverable under section 4, Table 3.

C6O4, 0.1 µg/l, 7 days, *Ruditapes philippinarum*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	2	81	0.544569566
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	DEVELOPMENT	2.5	77	0.637398051
HALLMARK_COAGULATION	IMMUNE	1.5	51	0.551813219
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	56	0.546079231
HALLMARK_COMPLEMENT	IMMUNE	1.5	86	0.495785492
HALLMARK_TGF_BETA_SIGNALING	SIGNALING	2.5	32	0.621643869
HALLMARK_ANDROGEN_RESPONSE	SIGNALING	2.5	53	0.508208108
HALLMARK_NOTCH_SIGNALING	SIGNALING	2	20	0.632084752
HALLMARK_MTORC1_SIGNALING	SIGNALING	2.5	138	0.421050351
HALLMARK_PROTEIN_SECRETION	STRESS RESPONSE	1	71	0.496680367

C6O4, 1 µg/l, 7 days, *Ruditapes philippinarum*

HAZARD CLASS EXPECTED: SLIGHT

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	DEVELOPMENT	2.5	77	0.687356423
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	85	0.593379539
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	2	81	0.510945141
HALLMARK_PROTEIN_SECRETION	STRESS RESPONSE	1	71	0.53338526

PFOA, 1 µg/l, 7 days, *Ruditapes philippinarum*

HAZARD CLASS EXPECTED: SLIGHT/MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	DEVELOPMENT	2.5	77	0.654614695
HALLMARK_COAGULATION	IMMUNE	1.5	51	0.605312857
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	2	81	0.534009032
HALLMARK_GLYCOLYSIS	METABOLIC	2	105	0.525333615
HALLMARK_NOTCH_SIGNALING	SIGNALING	2	20	0.739648792
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	85	0.477229671

C6O4, 0.1 µg/l, 21 days, *Ruditapes philippinarum*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_MYOGENESIS	DEVELOPMENT	2	82	-0.50989942
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	STRESS RESPONSE	1.5	77	-0.459273739
HALLMARK_NOTCH_SIGNALING	SIGNALING	2	20	-0.709175157
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	83	-0.442749191
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	123	-0.396659359
HALLMARK_ESTROGEN_RESPONSE	SIGNALING	2.5	124	-0.371593724
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	130	-0.345289987
HALLMARK_P53_PATHWAY	PROLIFERATION	2.5	89	-0.369003877

C6O4, 1 µg/l, 21 days, *Ruditapes philippinarum*

HAZARD CLASS EXPECTED: SLIGHT

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_HYPOXIA	STRESS RESPONSE	1.5	84	0.414444603
HALLMARK_REACTIVE_OXYGEN_SPECIES _PATHWAY	STRESS RESPONSE	1.5	25	-0.617296765
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	85	-0.461911848
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	83	-0.431234886
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	STRESS RESPONSE	1.5	77	-0.419095296

PFOA, 1 µg/l, 21 days, *Ruditapes philippinarum*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_EPITHELIAL_MESENCHYMAL _TRANSITION	DEVELOPMENT	2.5	77	-0.647358646
HALLMARK_NOTCH_SIGNALING	SIGNALING	2	20	-0.818576305
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	85	-0.579616083
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	130	-0.521420835
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	123	-0.472788656
HALLMARK_P53_PATHWAY	PROLIFERATION	2.5	89	-0.50055799
HALLMARK_E2F_TARGETS	PROLIFERATION	3	140	-0.43738985
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	83	-0.490589835

Acidification and Carbamazepine, 28 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: SLIGHT

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	75	0.624083717
HALLMARK_COMPLEMENT	IMMUNE	1.5	79	0.572293355
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	72	0.549672959
HALLMARK_HYPOXIA	STRESS RESPONSE	1.5	83	0.537056639

Acidification, 28 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	75	0.714340328
HALLMARK_COMPLEMENT	IMMUNE	1.5	79	0.632359799
HALLMARK_HYPOXIA	STRESS RESPONSE	1.5	83	0.621262273
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	128	0.559907529
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	72	0.549737791
HALLMARK_COAGULATION	IMMUNE	1.5	47	-0.36224684
HALLMARK_IL6_JAK_STAT3_SIGNALING	IMMUNE	1.5	16	0.649051083
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	44	0.550901753
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2	76	0.539925404
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	121	0.502625553
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	STRESS RESPONSE	1.5	84	0.50280321

Carbamazepine, 28 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE/MAJOR

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	75	0.731354829
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2	68	0.710127709
HALLMARK_COMPLEMENT	IMMUNE	1.5	79	0.662244768
HALLMARK_HYPOXIA	STRESS RESPONSE	1.5	83	0.667217468
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	72	0.66137324
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	128	0.600496164
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2	76	0.612025646
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	79	0.571553391
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	44	0.573798317
HALLMARK_IL2_STAT5_SIGNALING	SIGNALING	2	67	0.567071862
HALLMARK_PI3K_AKT_MTOR_SIGNALING	SIGNALING	2.5	55	0.593548551
HALLMARK_COAGULATION	IMMUNE	1.5	47	-0.329143957
HALLMARK_IL6_JAK_STAT3_SIGNALING	IMMUNE	1.5	16	0.621325257

Glyphosate, 100 µg/l, 7 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_E2F_TARGETS	PROLIFERATION	3	153	0.418295936
HALLMARK_DNA_REPAIR	DNA DAMAGE	2	121	0.423068972
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	137	0.414726459
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	145	0.390958449
HALLMARK_IL6_JAK_STAT3_SIGNALING	IMMUNE	1.5	20	0.647417078
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	90	0.329078831
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2	89	0.351506029

AMPA, 100 µg/l, 7 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: SLIGHT

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	145	0.471654077
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	137	0.388619693
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	2	89	0.418376073
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	90	0.409592808

Glyphosate and AMPA, 100 µg/l, 7 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	145	0.460499326
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	137	0.427439751
HALLMARK_DNA_REPAIR	DNA DAMAGE	2	121	0.423838323
HALLMARK_E2F_TARGETS	PROLIFERATION	3	153	0.372903118
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	2	89	0.452576239
HALLMARK_GLYCOLYSIS	METABOLIC	2	116	0.410028196
HALLMARK_FATTY_ACID_METABOLISM	METABOLIC	2	115	0.394702669
HALLMARK_ESTROGEN_RESPONSE	SIGNALING	2.5	131	0.370789553

Glyphosate, 100 µg/l, 21 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: SLIGHT

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	91	0.443608831
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	137	-0.414234521
HALLMARK_E2F_TARGETS	PROLIFERATION	3	153	-0.390839172
HALLMARK_FATTY_ACID_METABOLISM	METABOLIC	2	115	0.376766098

AMPA, 100 µg/l, 21 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: SLIGHT

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_FATTY_ACID_METABOLISM	METABOLIC	2	115	0.458033296
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2	78	-0.509558676
HALLMARK_COMPLEMENT	IMMUNE	1.5	91	0.401690955

Glyphosate and AMPA, 100 µg/l, 21 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: SLIGHT

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_IL6_JAK_STAT3_SIGNALING	IMMUNE	1.5	20	0.633178384
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	91	0.410525318
HALLMARK_FATTY_ACID_METABOLISM	METABOLIC	2	115	0.386058546

Glyphosate, 10 µg/l, 21 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE/MAJOR

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	90	-0.502972263
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	97	-0.436127173
HALLMARK_FATTY_ACID_METABOLISM	METABOLIC	2	95	-0.450958958
HALLMARK_MYC_TARGETS_V1	PROLIFERATION	2	155	-0.373089984
HALLMARK_E2F_TARGETS	PROLIFERATION	3	106	-0.373269026
HALLMARK_DNA_REPAIR	DNA DAMAGE	2	89	-0.365269169
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	55	-0.392422478
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2	62	-0.406437348
HALLMARK_ESTROGEN_RESPONSE	SIGNALING	2.5	73	-0.41869073
HALLMARK_TGF_BETA_SIGNALING	SIGNALING	2.5	26	-0.52848036
HALLMARK_BILE_ACID_METABOLISM	METABOLIC	1	52	-0.419476841
HALLMARK_XENOBIOTIC_METABOLISM	METABOLIC	1.5	96	-0.37027371
HALLMARK_OXIDATIVE_PHOSPHORYLATION	METABOLIC	2	152	-0.324022006

Glyphosate, 100 µg/l, 21 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_E2F_TARGETS	PROLIFERATION	3	106	-0.563922648
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	97	-0.554824084
HALLMARK_P53_PATHWAY	PROLIFERATION	2.5	68	-0.577860486
HALLMARK_FATTY_ACID_METABOLISM	METABOLIC	2	95	-0.587164759
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	90	-0.481904106
HALLMARK_PANCREAS_BETA_CELLS	DEVELOPMENT	1	9	-0.84758327
HALLMARK_ESTROGEN_RESPONSE	SIGNALING	2.5	73	-0.574385631
HALLMARK_IL2_STAT5_SIGNALING	SIGNALING	2	50	-0.596422184

Glyphosate, 1000 µg/l, 21 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: SLIGHT/MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	97	-0.526489849
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2	54	-0.593629491
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	90	-0.461415605
HALLMARK_APICAL_SURFACE	CELLULAR COMPONENT	2.5	12	0.734270814
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	2	59	-0.456126898
HALLMARK_OXIDATIVE_PHOSPHORYLATION	METABOLIC	2	152	-0.408849739
HALLMARK_CHOLESTEROL_HOMEOSTASIS	METABOLIC	1.5	29	-0.575926199

Amyl Salicylate, 0.3 µg/l, 7 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	61	-0.605600693
HALLMARK_APICAL_SURFACE	CELLULAR COMPONENT	2.5	11	0.772424248
HALLMARK_PROTEIN_SECRETION	STRESS RESPONSE	1	63	-0.539690071
HALLMARK_MYC_TARGETS_V1	PROLIFERATION	2	144	-0.473746379
HALLMARK_ADIPOGENESIS	DEVELOPMENT	1	105	-0.468850291
HALLMARK_PANCREAS_BETA_CELLS	DEVELOPMENT	1	13	-0.702809657
HALLMARK_MYOGENESIS	DEVELOPMENT	2	75	-0.4588608
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2	61	-0.491975667

Amyl Salicylate, 3 µg/l, 7 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	75	-0.617604769
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	113	-0.569846616
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	2.5	97	-0.527503426
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	61	-0.557722671
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	39	-0.575517233
HALLMARK_COMPLEMENT	IMMUNE	1.5	69	-0.498292898
HALLMARK_MYOGENESIS	DEVELOPMENT	2	75	-0.526415903
HALLMARK_P53_PATHWAY	PROLIFERATION	2.5	75	-0.470890514
HALLMARK_PROTEIN_SECRETION	STRESS RESPONSE	1	63	-0.501924497

Amyl Salicylate, 0.3 µg/l, 14 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: SLIGHT

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_WNT_BETA_CATENIN_SIGNALING	SIGNALING	1	14	-0.740689925
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	69	0.460026575

Amyl Salicylate, 3 µg/l, 14 days, *Mytilus galloprovincialis*

HAZARD CLASS EXPECTED: MODERATE

Hallmark gene set	Biological theme	Weight	Set size	ES
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	1.5	113	0.532049015
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2	69	0.560429012
HALLMARK_EPITHELIAL_MESENCHYMAL _TRANSITION	DEVELOPMENT	2.5	63	0.573873845
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	39	0.540279954
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	75	-0.3260286
HALLMARK_MYOGENESIS	DEVELOPMENT	2	75	0.502301121
HALLMARK_COAGULATION	IMMUNE	1.5	43	0.518949054
HALLMARK_COMPLEMENT	IMMUNE	1.5	69	0.452571477
HALLMARK_SPERMATOGENESIS	DEVELOPMENT	2.5	40	-0.365698212

List of statistically significant hallmark gene sets created with artificial GSEA outputs. Hallmark pathways with their relative biological theme, weight and Enrichment Score (ES) are provided. Each simulation is presented with the transcriptional hazard class based on expert judgment and the hazard class calculated with the Transcriptomic Hazard Index (THI) method.

SIMULATION 1:

HAZARD CLASS EXPECTED: MAJOR

HAZARD CLASS CALCULATED: MAJOR

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2.5	0.8
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2.5	0.9
HALLMARK_DNA_REPAIR	DNA DAMAGE	2.5	0.7
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	1	0.5
HALLMARK_APICAL_SURFACE	CELLULAR COMPONENT	1	0.7
HALLMARK_PEROXISOME	CELLULAR COMPONENT	1	0.6
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	3	0.9
HALLMARK_P53_PATHWAY	PROLIFERATION	3	0.9
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	3	0.8
HALLMARK_MYC_TARGETS_V1	PROLIFERATION	3	0.7
HALLMARK_E2F_TARGETS	PROLIFERATION	3	0.6
HALLMARK_MYC_TARGETS_V2	PROLIFERATION	3	0.5
HALLMARK_COMPLEMENT	IMMUNE	1.5	0.6
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	0.6

SIMULATION 2:

HAZARD CLASS EXPECTED: SLIGHT/MODERATE

HAZARD CLASS CALCULATED: MODERATE

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	1	0.5
HALLMARK_APICAL_SURFACE	CELLULAR COMPONENT	1	0.6
HALLMARK_ANGIOGENESIS	DEVELOPMENT	2.5	0.4
HALLMARK_PANCREAS_BETA_CELLS	DEVELOPMENT	2.5	0.6
HALLMARK_ADIPOGENESIS	DEVELOPMENT	2.5	0.7
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	3	0.9
HALLMARK_P53_PATHWAY	PROLIFERATION	3	0.8
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2.5	0.9

SIMULATION 3:

HAZARD CLASS EXPECTED: SLIGHT/MODERATE

HAZARD CLASS CALCULATED: MODERATE

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_XENOBIOTIC_METABOLISM	METABOLIC	1.5	0.3
HALLMARK_BILE_ACID_METABOLISM	METABOLIC	1.5	0.5

HALLMARK_HYPOXIA	STRESS RESPONSE	2	0.7
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	0.4
HALLMARK_PROTEIN_SECRETION	STRESS RESPONSE	2	0.8
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2.5	0.5
HALLMARK_IL2_STAT5_SIGNALING	SIGNALING	2.5	0.6
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	0.8
HALLMARK_COMPLEMENT	IMMUNE	1.5	0.7

SIMULATION 4:

HAZARD CLASS EXPECTED: SLIGHT

HAZARD CLASS CALCULATED: SLIGHT

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_XENOBIOTIC_METABOLISM	METABOLIC	1.5	0.6
HALLMARK_BILE_ACID_METABOLISM	METABOLIC	1.5	0.6
HALLMARK_FATTY_ACID_METABOLISM	METABOLIC	1.5	0.6
HALLMARK_CHOLESTEROL_HOMEOSTASIS	METABOLIC	1.5	0.6

SIMULATION 5:

HAZARD CLASS EXPECTED: SEVERE

HAZARD CLASS CALCULATED: SEVERE

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_ADIPOGENESIS	DEVELOPMENT	2.5	0.7
HALLMARK_MYOGENESIS	DEVELOPMENT	2.5	0.8
HALLMARK_SPERMATOGENESIS	DEVELOPMENT	2.5	0.8
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2.5	0.7
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2.5	0.8
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2.5	0.8
HALLMARK_IL2_STAT5_SIGNALING	SIGNALING	2.5	0.8
HALLMARK_PI3K_AKT_MTOR_SIGNALING	SIGNALING	2.5	0.7
HALLMARK_TGF_BETA_SIGNALING	SIGNALING	2.5	0.8
HALLMARK_HEDGEHOG_SIGNALING	SIGNALING	2.5	0.8
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	1	0.7
HALLMARK_APICAL_SURFACE	CELLULAR COMPONENT	1	0.6
HALLMARK_PEROXISOME	CELLULAR COMPONENT	1	0.6
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	DEVELOPMENT	2.5	0.7
HALLMARK_ANGIOGENESIS	DEVELOPMENT	2.5	0.7
HALLMARK_P53_PATHWAY	PROLIFERATION	3	0.6
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	3	0.7
HALLMARK_MYC_TARGETS_V1	PROLIFERATION	3	0.9
HALLMARK_E2F_TARGETS	PROLIFERATION	3	0.9
HALLMARK_INFLAMMATORY_RESPONSE	IMMUNE	1.5	0.7
HALLMARK_COAGULATION	IMMUNE	1.5	0.7
HALLMARK_IL6_JAK_STAT3_SIGNALING	IMMUNE	1.5	0.9

SIMULATION 6:

HAZARD CLASS EXPECTED: MODERATE

HAZARD CLASS CALCULATED: MODERATE

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_APICAL_JUNCTION	CELLULAR COMPONENT	1	0.6
HALLMARK_ANGIOGENESIS	DEVELOPMENT	2.5	0.7
HALLMARK_COMPLEMENT	IMMUNE	1.5	0.6
HALLMARK_XENOBIOTIC_METABOLISM	METABOLIC	1.5	0.7
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	0.9
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	3	0.8
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2.5	0.7
HALLMARK_CHOLESTEROL_HOMEOSTASIS	METABOLIC	1.5	0.8

SIMULATION 7:

HAZARD CLASS EXPECTED: MAJOR

HAZARD CLASS CALCULATED: MAJOR

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2.5	0.8
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2.5	0.7
HALLMARK_DNA_REPAIR	DNA DAMAGE	2.5	0.8
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	0.8
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	3	0.8
HALLMARK_P53_PATHWAY	PROLIFERATION	3	0.7
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	3	0.8
HALLMARK_MYC_TARGETS_V1	PROLIFERATION	3	0.7
HALLMARK_E2F_TARGETS	PROLIFERATION	3	0.8
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	0.7
HALLMARK_PROTEIN_SECRETION	STRESS RESPONSE	2	0.8
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	STRESS RESPONSE	2	0.7
HALLMARK_TGF_BETA_SIGNALING	SIGNALING	2.5	0.8
HALLMARK_HEDGEHOG_SIGNALING	SIGNALING	2.5	0.7
HALLMARK_MTORC1_SIGNALING	SIGNALING	2.5	0.8

SIMULATION 8:

HAZARD CLASS EXPECTED: SLIGHT/MODERATE

HAZARD CLASS CALCULATED: MODERATE

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_FATTY_ACID_METABOLISM	METABOLIC	1.5	0.6
HALLMARK_CHOLESTEROL_HOMEOSTASIS	METABOLIC	1.5	0.6
HALLMARK_GLYCOLYSIS	METABOLIC	1.5	0.6
HALLMARK_TNFA_SIGNALING_VIA_NFKB	SIGNALING	2.5	0.6
HALLMARK_IL2_STAT5_SIGNALING	SIGNALING	2.5	0.6

HALLMARK_PI3K_AKT_MTOR_SIGNALING	SIGNALING	2.5	0.6
HALLMARK_DNA_REPAIR	DNA DAMAGE	2.5	0.6
HALLMARK_INTERFERON_RESPONSE	IMMUNE	1.5	0.6
HALLMARK_COMPLEMENT	IMMUNE	1.5	0.6

SIMULATION 9:

HAZARD CLASS EXPECTED: MAJOR/SEVERE

HAZARD CLASS CALCULATED: SEVERE

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_HYPOXIA	STRESS RESPONSE	2	0.7
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	0.7
HALLMARK_PROTEIN_SECRETION	STRESS RESPONSE	2	0.7
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	STRESS RESPONSE	2	0.7
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	STRESS RESPONSE	2	0.7
HALLMARK_MITOTIC_SPINDLE	PROLIFERATION	3	0.7
HALLMARK_P53_PATHWAY	PROLIFERATION	3	0.7
HALLMARK_G2M_CHECKPOINT	PROLIFERATION	3	0.7
HALLMARK_E2F_TARGETS	PROLIFERATION	3	0.7
HALLMARK_MYC_TARGETS_V2	PROLIFERATION	3	0.7
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2.5	0.7
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2.5	0.7
HALLMARK_DNA_REPAIR	DNA DAMAGE	2.5	0.7
HALLMARK_PEROXISOME	CELLULAR COMPONENT	1	0.7
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	DEVELOPMENT	2.5	0.7

SIMULATION 10:

HAZARD CLASS EXPECTED: MODERATE

HAZARD CLASS CALCULATED: MODERATE

Hallmark gene set	Biological theme	Weight	ES
HALLMARK_ADIPOGENESIS	DEVELOPMENT	2.5	0.7
HALLMARK_MYOGENESIS	DEVELOPMENT	2.5	0.7
HALLMARK_SPERMATOGENESIS	DEVELOPMENT	2.5	0.6
HALLMARK_E2F_TARGETS	PROLIFERATION	3	0.8
HALLMARK_MYC_TARGETS_V2	PROLIFERATION	3	0.7
HALLMARK_UV_RESPONSE_UP	DNA DAMAGE	2.5	0.7
HALLMARK_UV_RESPONSE_DN	DNA DAMAGE	2.5	0.6
HALLMARK_HYPOXIA	STRESS RESPONSE	2	0.8
HALLMARK_APOPTOSIS	STRESS RESPONSE	2	0.8