An Integrative Workflow to Prioritize Toxicity Initiation Events From Air-liquid-interface Exposure of Human Lung Cells to Combustion Aerosols: First Application to Emissions From a Heavy Fuel Oil or Diesel Fuel Operated Ship Engine

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Methodology

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**An integrative workflow to prioritize toxicity initiation events from air-liquid-interface exposure of human lung cells to combustion aerosols:
First application to emissions from a heavy fuel oil or diesel fuel operated ship engine**

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Abstract

Background

Advancement in the instrumentation techniques made it possible to identify the compositions and concentrations of various chemicals, such as organic compounds or metals in combustion aerosols. However, the prediction of combustion aerosol-induced toxicity end points in lung epithelium cells is difficult due to the large number of non-linear chemical-biological interactions. It is evident that some chemicals present in the combustion aerosols upregulate certain genes/ proteins while other chemicals downregulate them, making the prediction of toxicity of a mixture of chemicals a challenging task. Also, the presence of large numbers of feedback and feedforward regulatory loops makes the entire prediction process highly dynamic.

Methods

Here we present an integrative workflow to construct and analyze combustion aerosol-type specific chemical-gene regulatory network. For this, we develop an algorithm to estimate the combined regulatory effect of chemicals present in the combustion aerosol on each of the genes/proteins in the network. Further we rank the nodes by combining various network topological and non-topological parameters using a multi-objective optimization function. The top ranked nodes were used to identify aerosol-type specific key regulators that contributes toward the understanding of adverse outcomes due to the exposure of combustion aerosols.

Results

The integrative workflow is evaluated using transcriptomics analysis carried out on human bronchial epithelium cell line BEAS-2B exposed at the air liquid interface (ALI) to the combustion aerosols generated by ship engine running either on distillate Diesel Fuel (DF) or on Heavy Fuel Oil (HFO) along with chemometric analysis. Based on the prioritized chemicals, we prepared DF and HFO combustion aerosols-specific chemical-

66 gene regulatory network. Our study reveals the large differences in the observed
67 biological effects caused by operating the same ship engine on the different fuels.

68 **Conclusion**

69 The presented workflow can be used to investigate key regulatory processes associated
70 with the toxicity outcomes of mixture of chemicals and also for the hazard classification
71 and assessment of various combustion aerosols.

72 **Keywords**

73 Integrative workflow, Regulatory networks, Combustion aerosols, Mixture toxicity, air
74 liquid interface cell exposure

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Background

In most risk assessment studies, chemical entities are analyzed individually despite the fact that they are rarely present alone in a real life exposure matrix [1,2]. It is assumed that the behavior of chemicals in mixture is altogether different than the additive effect of individual compounds [3–5]. Previously designed theoretical models for predicting the toxicity of mixtures of chemicals present in an exposure matrix (e.g. combustion aerosols) are based on 1) concentration addition/ independent actions, when the overall toxicity of the chemicals in a mixture is the sum of the toxicity of individual chemicals; and 2) synergism/ antagonism, when the overall toxicity of chemicals in a mixture is either higher or lower than the simple addition of toxicity caused by the parent compounds [6]. Figure 1 highlights possible behavior and toxicological outcomes of two chemicals when present in an exposure matrix.

While there is increasing evidence that the cellular environment plays a crucial role in toxicity outcomes after exposure [7], advances in the omics technologies provide us with data that can be used to identify and characterize underlying mechanisms related to toxicity initiation events and progression [8–10]. Molecular mechanisms associated with the toxicological outcomes due to the exposure of xenobiotic are highly dynamic in nature, making the risk assessment of chemical mixture a difficult process [11]. It is well established that environmental chemicals can influence the expression profile of regulatory genes [12,13] involved in the mutagenesis. These chemicals also interfere in the physical interactions of the biological molecules and thus play a crucial role as etiological agents to make an organism susceptible for many chronic diseases [7,14]. Figure 2 highlights some of the dominant mechanisms associated with carcinogenesis,

cardiovascular and lung diseases due to combustion aerosol exposure. The combustion of fuels generates fine and ultrafine particles as well as reactive gas-phase species which are responsible for the production of reactive oxygen species (ROS), DNA stands breaks and initiation of signaling mechanisms inducing inflammations [15,16].

Combustion aerosol emissions from marine traffic have increasingly gained attention in the past two decades. Ships use large diesel engine without exhaust cleaning devices, that are common for road traffic vehicles and they burn fuel of low quality. In particular, the combustion of heavy fuel oil (HFO), a residue from crude oil vacuum distillation blended with middle distillate, releases substantial amounts of PM, including heavy metals and Black Carbon (BC), as well as SO_x and NO_x [17,18]. Epidemiological studies on different spatial scales found that ship traffic is significantly associated with the number of premature deaths and air quality in especially harbor cities [19,20]. Consequently, the International Maritime Organization decided to reduce the sulfur content of marine fuel in order to promote low-sulfur, i.e. “cleaner”, fuels or the use of sulfur scrubbing technology [21]. However, sulfur scrubbers operated in open-loop mode has been heavily criticized because of severe environmental threats [22–24]. Since 2020, ship owners have to comply with a mandatory global sulfur limit of 0.5% and 0.1% in so-called sulfur emission control areas (SECA). SECAs refer to sensitive ecosystems or areas with high population density and comprise Baltic Sea, North Sea, English Channel and most North American coastal waters. Although forecasts and assessments on consequences for human health suggest a beneficial effect from recent sulfur regulations [25–28], ship emissions refer to diesel exhaust without exhaust aftertreatment, for which the toxicity is well-known[29]. In fact, toxicological studies comparing several biological

endpoints and a multi-omics approach in *in vitro* exposures revealed that even the use of fossil diesel with the lowest sulfur content of available on the market leads to a rather different than lower toxicity[30,31].

In the last few years, many sophisticated instrumentation techniques were developed and used to study the overall chemical compositions from combustion aerosols of the marine traffic sector. For example, Rüger et al. used ultra-high-resolution mass spectrometry coupled to laser desorption/ionization for the detailed molecular level analysis of combustion aerosols from ship diesel engine [32]. Mueller et al. analyzed and compared the combustion aerosols generated from ship diesel engine running on heavy fuel oil (HFO) and diesel fuel (DF) using high resolution time-of-flight aerosol mass spectrometry in combination with aethalometer, particle sizer, online gas phase and filter measurement to conclude that average emission factors were higher for HFO than DF [33]. Kleeblatt et al. and Radischat et al. used resonance-enhanced multiphoton ionization time-of-flight mass spectrometry to analyze aromatic and polycyclic aromatic hydrocarbon from ship engine running on different fuel types [34,35]. Czech and colleagues carried on the their work by in-depth comparison of aromatic emission profiles of ships to other emissions arising from on-shore traffic or residential heating using a chemometric approach [36]. Corbin et al. focused on the trace metal content in different size fractions of HFO-PM by aerosol mass spectrometry and inductively-coupled plasma optical emission spectroscopy and revealed that vanadium is enriched in soot particles belonging to the fraction of ultrafine particles with aerodynamic diameters below 100 nm [18]. A comprehensive comparison of PM from HFO and DF combustion was conducted

by Streibel et al. and Sippula et al., serving as physico-chemical data pool for the merging with toxicological findings[17,37].

In order to investigate undesirable biological responses and toxicity endpoints from the aerosols exposure, researchers are now using realistic and comparable state-of-art *in vitro* experimental conditions (e.g. air-liquid interface) along with multi-omics profiling [30,38–40]. However, due to complex chemical compositions of combustion aerosols and highly dynamic interactions between the chemicals and biological molecules in the exposed cells, mechanistic understanding of toxicity initiation mechanisms is a real challenge [41]. In this study, we develop and present an integrative workflow for generating chemical-gene/protein interaction networks very specific to the combustion aerosols generated through a ship engine which runs on 1) Heavy Fuel Oil (HFO); and 2) Diesel Fuel (DF) and provide various steps to analyze these networks for the prediction of critical regulatory mechanisms responsible for the toxicity outcome after the aerosol exposure.

Methods and methodology

The measurements were performed within the HICE project (www.hice-vi.eu) four-stroke single-cylinder direct-injection diesel engine test bench with a nominal speed of 1500 rpm, maximum power of 80 kW and a total displacement of 3.18 L, located at Rostock University, see [17] for details. Briefly, the engine was operated either with clean distillate diesel fuel (DF, EN 590) or with Heavy Fuel Oil (HFO). The chemical composition and the aerosol particle properties of the engine emission were thoroughly characterized by off-line and on-line chemical and physical easements approaches. At the same time,

human epithelial lung cell cultures were exposed at the Air-Liquid-Interface (ALI) using as special at site mobile S2 biosafety laboratory and automated ALI-exposure system. The acute cytotoxicity, the multiomics response of the human, and response pathways were determined. This approach was published in great detail [30]. In this work, a new systems biology-based network approach is used to understand the aggregated effect of combustion aerosol by connecting the chemical data of the emissions to their potential biological targets.

1. Chemical exhaust aerosol characterization of the HFO- or DF-operated ship diesel engine

The aerosol from the exhaust pipe was first taken through a temperature insulated sampling probe to a pre-cyclone to remove coarse particles from the sample, and then diluted in two stages by applying a porous tube – ejector sampling system (Venacontra, DAS, Finland). The pre-cyclone and the sampling lines upstream of the diluters were heated to approximately 300°C.

The primary dilution was carried out using a porous tube diluter where compressed air flowed through a porous tube wall providing a sheath flow which prevented wall losses of vapours and particles. Thereafter, the aerosol was taken into an ejector diluter with a fixed dilution ratio of roughly 3. After this stage the sample stream was divided to several stainless-steel sampling lines and diluted further with ejector diluters (Palas, VKL 10 E, Germany) depending on the desired concentration range. The dilution was carried out with clean, dried and particle free compressed air at room temperature. The dilution ratios were calculated based on CO₂ measurements in the raw exhaust (AVL, CEB II) and after

189 dilution (Vaisala, Carbocap GMP343). The final applied dilution ratios were approximately
190 40 for the offline filter and gas-phase sampling, when the engine was operated on diesel
191 fuel (DF), and 100 for engine operation with HFO. Online photoionization mass
192 spectrometers for gas phase analysis were sampling directly from the undiluted raw gas.
193 PM_{2.5} filter samples for off-line analyses were taken with a modified speciation sampler
194 (Rupprecht & Patashnick 2300, Thermo Scientific, USA), which could sample four filters
195 in parallel at a flow of 10 L/min. PM samples were collected on quartz fibre filters (QFF,
196 T293, Munktell, Sweden) and PTFE membrane filters (Zefluor 1 µm, Pall, USA). The QFF
197 were conditioned before sampling by baking at 500°C for at least 12 hours. Immediately
198 after sampling the filters were frozen at -25°C. Gas phase carbonyls were collected by
199 derivatisation with 2,4-dinitrophenylhydrazine (DNPH) using commercially available
200 cartridges 'ORBO/555' (Sigma Aldrich, USA). Loaded cartridges were sealed immediately
201 after sampling with quick fit caps and stored in a refrigerator. Inorganic elements were
202 determined from PTFE filters using inductively coupled plasma atomic emission
203 spectroscopy. Analysis of elemental and organic carbon (EC/OC) was carried out from
204 QFF filters by a thermo/optical carbon analyzer (Desert Research Institute Model 2001A,
205 NV, USA) coupled to a time-of-flight mass spectrometer (Stefan Kaesdorf, Munich,
206 Germany) using single photon and resonance-enhanced multi-photon ionization (SPI and
207 REMPI, Diab 2015). Targeted analyses of semi- and low-volatile organic species,
208 including PAH and o-PAH for example, was done using in-situ derivatization and thermal
209 desorption - gas-chromatography - time-of-flight mass spectrometry (IDTD-GC-ToFMS,)
210 [42]. Carbonyls sampled in DNPH cartridges were quantified as carbonyl-hydrazones with
211 GC-MS [43]. Online analyses of organic gaseous engine emissions were carried out by

single-photon ionization mass spectrometry (SPI-MS) and resonance-enhanced multiphoton ionization mass spectrometry [44]. Black carbon (BC) was determined at 880 nm using a multi-wavelength aethalometer.

2. Air Liquid Exposure of BEAS-2B human lung cells to exhaust aerosol of a HFO- or DF-operated ship diesel engine and transcriptome analysis

Human bronchial epithelium cell cultures were exposed to diluted ship engine emission aerosols operated with HFO or DF. Detailed experimental settings for the exposure of cells and methodology for analysing transcriptomics data is provided in [38]. Briefly, confluent layers of human epithelial SV40-immortalised bronchial epithelial lung cells (BEAS-2B, purchased from ATCC, CRL-9609™; <http://www.lgcstandards-atcc.org/Products/All/CRL-9609.aspx>) were exposed to diluted (1:40 or 1:100, see (21)) engine exhaust for 4 hrs at the air-liquid-interface (ALI). The lung cells have direct contact to inhaled aerosol particles and gases and therefore are used as a model of aerosol inhalation. Cytotoxicity tests were performed to ensure a sub-cytotoxic exposure dose and the transcriptomics profile was analysed, the Omics data is stored in Gene Expression Omnibus (GSE63962). All experiments were performed in triplicate and referenced to clean air exposure.

3. Construction of combustion aerosol-type specific chemical-gene regulatory networks

In the following the construction of combustion aerosol-type specific chemical-gene regulatory network is explained step-by step:

Step 1: Construction of chemical-gene interaction networks

The information about chemical-gene/protein interaction was collected from Comprehensive Toxicogenomics Database (CTD) (<http://ctdbase.org>) [45]; chEMBL (<https://www.ebi.ac.uk/chembl>) [46]; PubChem (<https://pubchem.ncbi.nlm.nih.gov>) [47]; STITCH (<http://stitch.embl.de>) [48]; AOP KB (<http://aopkb.org>); ToxCast from US EPA (<https://www.epa.gov/chemical-research/toxicity-forecaster-toxcastm-data>) [49]; and other published literature. Many of these databases were designed based on the information extracted directly from experimental studies or through text mining algorithms. We first generated initial network by connecting targeted gene/proteins to the chemicals using basic expression i.e. “chemical X interacts with gene/protein Y” for majority of the chemicals present in combustion aerosol using in house script.

Step 2: Setting up of chemical gene/protein regulatory relationship

For the genes/proteins connected to a particular chemical in the initial interaction networks, we retrieved experimentally validated interactions among the connected genes/proteins using STRING (v9.1) [50], BioGRID (v3.4) and HPRD (release 9) [51] databases. These interactions were mapped over the initial networks to make them fully connected chemical gene regulatory networks. Wherever possible, we have manually curated each interaction by converting expression “chemical X interact with gene/protein Y” to “chemical X increase/decrease the expression of gene/protein Y” after careful reading of associated peer-reviewed publications. Database such as CTD, STITCH have hundreds to thousands of ambiguous chemical gene interactions where a chemical is shown to increase the expression of a gene in some studies while in others the expression is decreased after the exposure to the same chemical. For such ambiguous cases in our networks, we manually screened original publications and set the interaction type

(increase expression/decrease expression) which is represented by most of the publications specifically validated the chemical-gene association. Furthermore, we annotated gene/protein nodes present in the networks with HUGO name, HGNC ID, Entrez ID and UniProt ID. Finally we integrated all the information to prepare a knowledgebase (KB) containing annotated chemical-gene regulatory networks in Cytoscape [52] compatible XML files for network visualization and analysis.

Step 3: Identification of priority chemicals for HFO and DF combustion aerosols

In order to identify combustion aerosol-type specific chemicals, we first calculated concentration ($\mu\text{g}/\text{m}^3$) of various HFO and DF aerosol components, such as chemical compounds, elemental metals and gases in online/ offline measurements. As the aerosols are composed of hundreds of chemical compounds, metal elements and gases, we choose various concentration cut-off to filter out chemicals for the preparation of aerosol-type specific regulatory networks. In particular, we used concentration cut-off $1000 \mu\text{g}/\text{m}^3$ for the inorganic components and $1 \mu\text{g}/\text{m}^3$ for the organic components in the gas phase compounds of aerosols. For various organic compound classes and particles, the cut-off was set to 1 and $100 \mu\text{g}/\text{m}^3$ respectively. For polycyclic aromatic hydrocarbons, we set the concentration cutoff $0.005 \mu\text{g}/\text{m}^3$ due to the availability of high-quality toxicity data. For carbonyl compounds and elements, the concentration cut-off was set to 0.15 and $0.25 \mu\text{g}/\text{m}^3$ respectively. Furthermore, we assigned individual chemical component to a particular aerosol only if its concentration is more than one order of magnitude in comparison to another aerosol, if not, the chemical was assigned to both the aerosols.

Step 4: Generating combustion aerosol-type specific chemical-gene regulatory network

For the prioritized chemicals identified in step 3 from HFO and DF combustion aerosols, we selected and merged the respective chemical-gene interaction networks from our knowledgebase (Step 2) using ‘Advanced Network Merge’ application available in Cytoscape v. 3.7.1. In order to further augmenting regulatory interactions between the newly merged biological counterparts in the combined chemical-gene interaction network, we screened STRING [50], BioGrid [53], DIP [54], BIND [55] and HPRD [51] databases for the experimentally validated interactions.

All the steps in the construction of this knowledge-based network are summarized in Figure 3.

Mapping of gene expression data onto the regulatory network and Identification of key regulatory genes and network analyses

The whole transcriptomics profiles from human bronchial epithelium cell line (BEAS-2B) exposed to complete and filtered DF and HFO combustion aerosol were analyzed using R and the Bioconductor packages limma [56]. All the transcripts with log₂-expression fold change ≥ 1.5 and p-value < 0.05 were considered significant. The official gene symbols were used to map the log₂-expression fold change data on the respective combustion aerosol regulatory network for further analyses. With the aim to identify key regulatory genes in the HFO and DF biological response networks, first we measure the overall impact of chemicals present in the aerosol on the node (gene) and then used various network topological parameters along with the expression fold change of the gene

using a multi-objective optimization function as described in our previous publications [57–60]. The algorithm is summarized below:

1. For each chemical component $\mathcal{C}_i$ in the aerosol network where $i = 1, \dots, j$, we first count number of nodes $K_{\mathcal{C}_i}$ directly connected to it.
2. A scaling factor $\mathcal{S}_{\mathcal{C}_i}$ is then calculated of each chemical $\mathcal{C}_i$ by assuming that the concentration of a chemical $\mathcal{M}_{\mathcal{C}_i}$ is equally distributed in the regulation of directly connected genes.

$$\mathcal{S}_{\mathcal{C}_i} = \frac{\mathcal{M}_{\mathcal{C}_i}}{K_{\mathcal{C}_i}}$$

3. For each gene $\mathcal{K}$ where $\mathcal{K} = 1, \dots, \ell$ in the aerosol network, the overall impact of all the directly connected chemicals $\mathcal{IC}$ on to the gene is calculated as

$$\mathcal{IC}_{\mathcal{K}} = \sum_{i=1}^n (r_i \cdot \mathcal{S}_{\mathcal{C}_i})$$

n is the number of chemicals regulating node $\mathcal{K}$ and $r_i \in \{-1, +1\}$, where -1 refers to ‘inhibition/suppression’ and +1 refers to ‘activation’ of the gene by chemical i

4. Finally, the ranking score $\mathcal{R}$ for the node $\mathcal{K}$ is calculated as

$$\mathcal{R}_{\mathcal{K}} = w_{1x} \cdot \mathcal{BC}_{\mathcal{K}} + w_{2x} \cdot \mathcal{CC}_{\mathcal{K}} + w_{3x} \cdot \mathcal{CO}_{\mathcal{K}} + w_{4x} \cdot \mathcal{ND}_{\mathcal{K}} + w_{5x} \cdot |\mathcal{FC}_{\mathcal{K}}| + w_{6x} \cdot |\mathcal{IC}_{\mathcal{K}}| \quad (\text{Eq 1})$$

where w_{1-6} are weighting factors pouncing the importance of the associated parameter and x is the weighing scenario (1 to 7) as shown in Table 2. $\mathcal{BC}$ is the betweenness centrality, $\mathcal{CC}$ is the closeness centrality, $\mathcal{CO}$ is the clustering coefficient, $\mathcal{ND}$ is the node degree, $\mathcal{FC}$ is the log2 fold change of the gene in combustion aerosol compared to clean air control and $\mathcal{IC}$ is the overall impact of chemicals present in aerosol on node $\mathcal{K}$.

For the ranking of genes, we used seven different weighting scenarios iteratively by giving importance to one or other parameters as described above. The major goal was to remove any biasness in selecting top candidate genes responsible for overall dynamics of biological response network that arise due to the parameter values. These weighting scenarios are described in Table 2. After calculating ranking scores in each weighting scenario, top 25 genes were selected and analyzed for their role in regulating biological processes and pathways along with disease association. HFO and DF-specific combustion aerosol networks and top regulatory genes were analyzed using various apps available in Cytoscape v.3.7.1. For example, various topological properties of the networks were analyzed using NetworkAnalyzer release 2.7 [61]; network-disease associations were carried out using DisGeNET App v.4.0 [62,63]; gene-set enrichment analyses on the network were performed using ClueGO [64] and EnricherR [65]. All the statistical analyses were performed using R version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

The overall study design comprising experimental and bioinformatics methods is described in Figure 4.

Results

A total of 100 unique chemicals and 15 broad chemical classes were identified in the online and offline measurements from the HFO and DF exhaust aerosols in various experimental techniques. A comprehensive list of these chemicals and chemical classes along with their concentrations in $\mu\text{g}/\text{m}^3$ and respective concentration ratios in HFO and DF aerosol is shown in supplementary figures 1-6 [see Additional file 1].

All the chemicals and chemical classes were screened for their potential biological targets in human using various state-of-art chemical-gene interaction databases. We could identify biological targets for 52 compounds for which chemical-gene interaction networks were prepared in the standardized Cytoscape compatible format. Further, each of the networks was converted into a fully connected regulatory network by assigning the possible regulatory and functional interactions between the biological partners (gene/protein) using various interaction databases. In particular, the information was collected from interaction databases such as BIND, DIP, HPRD, BIOGRID and STRING. The list of chemicals is provided in table 1.

To assign chemicals to a specific combustion aerosol, we set concentration cutoffs for various chemical classes as described in the material and methods section.

After prioritization of the aerosol-type specific chemicals based on the concentration cutoff, we could assign 46 chemicals to the HFO and 26 to the DF exhaust aerosol (Table 1).

After assigning chemicals to a specific aerosol based on concentration cutoff, we merged individual chemical-gene/protein networks together to construct a comprehensive aerosol-type specific biological response network using merge network app of Cytoscape version 3.7.1. Furthermore, new regulatory connections between biological counterparts were identified and mapped to the network using Cytoscape Bisogenet apps. In case of HFO biological response network, there were in total 780 nodes and 5393 interactions while in DF biological response network, number of nodes and interactions were 512 and 2631 respectively (Figure 5).

On aerosols specific biological response networks, we mapped the log₂-fold change transcriptomics data from our previously published HFO and DF exposure studies on human bronchial epithelium cell line (BEAS-2B) compared to the respective clean air control[30]. In HFO aerosol-specific biological response network, total 68 genes are found to be differentially regulated candidates (absolute fold change >1.5 in comparison to clean air control; 57 genes upregulated and only 11 genes downregulated). In DF biological response network, although less chemicals are present in the aerosol in comparison to HFO-PM, a total of 95 genes were differentially regulated (50 upregulated and 45 downregulated) with absolute fold change >1.5 in comparison to clean air control.

Transcription factors (TFs) play an important role in the regulation of downstream target genes. Biological processes and functions are largely modulated when TFs genes are differentially regulated or TFs are bound to various chemical ligands. Considering this fact, we further identified TFs that are directly regulated by the chemicals present in the aerosols. In HFO biological response network, we found a total of 91 nodes which are known TFs, among them 13 were upregulated while only three were downregulated (absolute FC > 1.5) in comparison to clean air control. Among these TFs, FOS, SNAI1, MYC, JUN, FOSL1 and EGR1 were highly upregulated (log₂ FC > 1.0); while MDM4, NFIB and MDM2 were downregulated. The DF biological response network contains only 58 TFs which are directly connected and regulated by various chemicals present in the aerosol. Among them 26 TFs were differentially expressed (absolute FC > 1.5) after the exposure in comparison to clean air control. FOS, SNAI1, MXD1, KLF4, ATF3, EGR1, DDIT3, FOSL1 and NR4A1 were among the highly upregulated TFs (log₂ FC > 1.0);

386 while E2F2, NR3C1, NFE2L1 and HIVEP2 were among the highly downregulated ($\log(2)$
387 $FC < -1.0$) TFs.

388 From the comprehensive aerosol-type specific biological response networks, we
389 identified key nodes responsible for overall network dynamics. For this, we used multi-
390 objective optimization function as described in Equation 1 using predefined weighting
391 factors assigned to node's topological and non-topological parameters in an iterative
392 manner. From each of the seven weighting scenarios, top 25 genes were selected.
393 Finally, a non-redundant set of nodes was prepared from the matrix of 7x25 nodes for
394 both HFO (75 genes) and DF (70 genes) biological response networks. Parameters
395 values, ranking scores in seven different weighting scenarios and top ranked candidates
396 are provided in Additional file 2 and 3 for HFO and DF aerosols. Among the top regulatory
397 genes from both HFO and DF biological response network, 37 are shared by both the
398 aerosols.

399 For the differentially regulated nodes connected to aerosol type-specific network, we
400 performed GO analysis for the biological processes and the disease association. We used
401 Cytoscape ClueGO v.2.5.5 for this purpose. Pathways only with $p\text{-value} \leq 0.05$ were
402 analysed for the enrichment purpose. We consider only those biological processes which
403 have minimum 5 genes or 5% of the genes among top regulators for HFO and DF
404 respectively. We merged redundant biological processes with >50% overlap. Figure 6
405 indicates the overrepresented biological processes by top nodes identified through multi-
406 objective optimization approach in HFO and DF biological response network.
407 Interestingly, large numbers of immune related processes are overrepresented by the DF
408 network in comparison to HFO. Top regulatory genes in case of DF aerosol exposure are

associated with neuroinflammatory responses, neuronal apoptosis, cell cycle regulation, fibroblast proliferation, production and responses to reactive oxygen species. In case of HFO exposure, top regulatory genes are mainly related to learning processes, neuron death, neurotransmitter receptor activity, placenta development along with the response to reactive oxygen species and oxidative stress.

To identify enriched disease terms associated with the top ranked genes in DF and HFO combustion aerosol networks, we used web-based enrichment analysis tool Enrichr [65]. More specifically, we used the DisGeNet library which provides curated data of genes and variants associated with human disease. Figure 7 highlights top 20 disease terms enriched for top ranked genes identified in DF and HFO biological response network. Fibroid tumor is the top enriched disease in case of DF aerosol network with 30 associated genes out of 70 top ranked genes. In case of HFO aerosol network, reperfusion injury is the top enriched disease and represented by 24 out of 75 top ranked genes. Apart from several tissue neoplasms, asthma is only enriched in case of DF aerosol network.

Discussion

To identify combustion aerosol-type specific chemicals, we performed chemometric analysis of ship engine exhaust generated from HFO and DF in the online and offline measurements using various experimental techniques described in [32–34,66,67]. Identification of chemicals is highly sensitive to the detection limits of various instrumentation facilities.

The chemicals present in the combustion aerosols interact with biological counterparts (genes, proteins) after exposure and responsible of the perturbation of biological processes and pathways. For this, the information is collected from literatures and public databases which is highly context specific. For a large number of chemical gene interactions in various networks, there were discrepancies on the type of regulations (e.g. activation, suppression, modification, secretion etc.) in biochemical databases. For those ambiguous cases, we manually referred to the original literature and select the most appropriate regulation based on 1) number of studies confirming a specific type of regulation; and 2) the experimental techniques used to predict the interaction type (activation/ suppression).

We hypothesized that the biological responses from the exposure of fuel type-specific ship engine combustion aerosol is due to the chemical entities present in high concentration and their interactions with the biological counterparts in a highly non-linear fashion. For this, we assigned chemicals to either one or both the HFO and DF combustion aerosol depending on their concentration identified in various online/offline chemometric analyses. We understand that the prioritization of chemicals to a particular aerosol simply based on concentration is not the best strategy as some chemicals, when present even in very small quantities, can influence biological systems due to their target specificity and high toxicity. The most appropriate approach to prioritize the chemicals in a combustion aerosol would be based on their permissible exposure limit (PEL) or on other concentration thresholds such as LD50 or EC50 values. However, for many of the chemicals, value of various toxic concentrations (e.g. PEL, LD50 and EC50) is not known and generation of such information has many methodological and technological

challenges. This is the reason why we simply used concentration cut-off for constructing aerosol type-specific biological response networks.

As shown in Figure 5, aerosol-specific biological response networks provide a platform to map and visualize experimental data for generating data-driven hypotheses. In this case, we mapped transcriptomics data from BEAS-2B human lung cells exposed to HFO- or DF-operated ship diesel engine exhaust compared to clean air control at air-liquid interface. The mapping of the data on the aerosol-specific biological response network not only indicated which genes are highly differentially regulated but also hints at the possible regulatory mechanisms responsible for this change. For example, the FOS gene is highly upregulated in both the HFO and DF combustion aerosol. FOS is a proto-oncogen, plays an important role in many cellular functions and is upregulated in many types of cancer. In case of HFO aerosol-specific biological response network, FOS is directly regulated by 5 chemicals (zinc, chrysene, sulfur dioxide, nitrogen oxide and manganese). While manganese downregulate FOS, other 4 chemicals upregulate it. This suggests that the combined effect of 4 chemicals upregulating FOS is much higher than the manganese. In case of DF aerosol-specific biological response network, FOS is upregulated and connected with only two chemicals (chrysene and nitrogen oxide). Interestingly, the expression of FOS is much higher in DF than HFO when both the aerosols are individually compared to clean air control. It may be due to the presence of one negative regulator for FOS (manganese) in HFO aerosol. The BBOX1 was the most significantly downregulated gene in HFO biological response network due to the presence of Aluminium which is known for the downregulation of this gene expression [68]. These analyses indicate that aerosol-specific biological response networks as generated in this

work could help to decipher mechanisms associated with combined effect of chemical entities present in aerosols. The mapping of the transcriptomics data onto the network also indicate that large number of TFs are differentially regulated due to the exposure of DF aerosol (n=26) in comparison to clean air control, while in case of HFO, only 16 TFs are differentially regulated. These observations also suggest that exposure to DF aerosol may have more intense biological responses in comparison to HFO aerosol even though large number of chemicals were identified exclusively in HFO aerosol.

We also ranked genes/proteins present in the aerosol-specific biological response network using various topological and non-topological parameters. Among the topological parameters, we mainly considered node degree, betweenness centrality and clustering coefficient as these parameters points towards the hub node, the gate keeper for information flow and the probability of forming clusters respectively. For non-topological parameters, we considered aerosol-type specific expression fold change compared to clean air control. Expression profile of a gene is highly dynamic in nature which largely depends on the context and concentration of its regulators. In case of HFO and DF combustion aerosols, for many chemicals the value significantly varies between two aerosols despite concentrations distinct above the cut-off. To consider the concentration of chemicals into the account for analyzing biological responses, we calculated “Overall impact of chemicals” on each of the genes/proteins present in aerosol-type specific network considering total number of genes/ proteins regulated by a chemical. Amount of chemical was then equally distributed among the connected genes/proteins to calculate a scaling factor for each chemical. Finally, for each of the gene/protein combined impact of all the chemicals was calculated by summing up of scaling factors of each of the

connected chemicals along with their regulation directions (for detail, please see the algorithms described in the material and method section). Combining “Overall impact of chemicals” with topological and non-topological parameters along with various weighting scenarios, we ranked nodes using the equation – 1 iteratively. Furthermore, selecting top 25 nodes in each of the weighting scenarios, we identified key regulators that influence to overall network dynamics were identified. Considering different weight to different parameters (Table 2), we removed any biasness associated with topological / non-topological parameters in selecting top ranked nodes.

Conclusion

Identification and assessment of biological responses from the exposure to complex mixtures of chemicals such as in combustion aerosols is challenging due to multiple nonlinear regulations among the genes and proteins. The combined effect may be evaluated by mapping all the possible known regulations (up or down regulation) of individual chemicals among the connected nodes. In this manuscript, we provide a workflow to construct aerosol-type specific biological response networks (Figure 3) using the chemical fingerprint of the aerosols as input. This workflow integrates various steps to design a comprehensive chemical-biological association networks in a systematic way to reveal potential molecular mechanisms associated with the exposure of mixture of chemicals. We provide an exemplary algorithm to estimate the combined regulatory effect of chemicals on genes/protein present in the network. The knowledgebase generated in this manuscript for the example of the emission of a ship engine operated either with diesel fuel or heavy fuel oil provides 52 chemical-gene/protein regulatory networks which can be used for preparation and analyses of biological response network associated with

a chemical or mixture of chemicals. The here developed strategy can be used to evaluate the health impact of different aerosols based on their chemical fingerprint and chemical fingerprint.

If we consider the investigated ship engine emission aerosols, the study reveals the large differences in the observed biological effects caused by operating of the ship engine on the different fuels. This motivates to thoroughly investigate the impact of the chemical composition of the ship fuel on the potential health effects because the current IMO legislation has a tremendous impact on the composition of future shipping fuels. Since 2020, the so called IMO “Sulfur Cap” legislation bans fuels with more than 0.5 % sulphur. These conventional residual fuels (i.e. HFO’s) with typical sulfur contents between 1.5 and 3.5% can only be used if sulphur emission control technologies (i.e. so called “scrubbers”), which however are rarely implemented yet, are used. Thus it is expected that new fuels (desulfurized HFO’s, sulfur lean but highly aromatic refining fractions etc.) will come to the market [69]. The here presented approach might be utilized for the evaluation of the relative health risk of the emission associated with the use of the new shipping fuels.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The transcriptomics datasets analysed during the current study are available in the Gene Expression Omnibus ([GSE63962](#)). All other data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RZ, OW, SSG, SG and TS conceptualized the workplan. SSG constructed and analyzed aerosol-specific biological response network. SSG, SG and OW prepared the algorithm for prioritization of nodes in the network. SSG, RZ and TS wrote the first draft. SO, HC, JP, MS, OP, JJ, MH, GD, TK, JB, BS, HH, MD, CS, KH, SS and RZ performed offline and online chemometric analysis of combustion aerosols. All authors reviewed and finalized the manuscript.

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Figures & Tables

Figure 1: Schematic representation of possible toxicity outcomes of two chemicals (A & B) alone and in a mixture.

Chemicals when present together in an exposure matrix can show the synergism, addition, antagonism or inhibition in the overall toxicity outcomes. The simplest assumption is the concentration addition where the overall toxicity of the mixture can be calculated by addition of toxicity caused by chemicals individually. However, due to the complex regulatory events in a biological system, chemicals in an exposure matrix mostly show the synergism or antagonism where the overall toxicity is either more or less than the concentration addition. Sometime chemicals, when present together in an exposure matrix, inhibit each other or form a composite which is less toxic than the parent compounds.

Figure 2: Possible mechanisms for combustion aerosols exposure induced carcinogenesis, cardiovascular and lung pathogenesis.

Chemical, metals, fibers and particulate matter released from ship/automobile exhausts, industrial exhaust or wood combustion can generate oxidative stress in the exposed cells, which may lead to various chronic conditions including carcinogenesis, cardiovascular and lung diseases. Other possible effects might be through frustrated phagocytosis in macrophages (more relevant for fibers) which may induce chronic inflammations. Chemicals present in combustion aerosols may also cause genotoxicity by interfering in DNA methylation, histone modification or DNA strand breakage. Chemical entities present in combustion aerosols also form complexes with altogether different toxicity profiles than the parent compounds. Most often these complexes increase the bioavailability of toxic chemicals by slow and sustain release inside the cell.

Figure 3: Workflow for constructing aerosol-type specific chemical – gene interaction regulatory network

Figure 4: Overall experimental and bioinformatic flowchart of this study. blue shaded: – experimental workflow aspects, green shaded: big data/bioinformatic workflow aspects)

Figure 5: Aerosol-type specific biological response networks.

(a) HFO-combustion aerosol; (b) DF-combustion aerosol. Chemicals are shown as red color triangles, biological components (gene, protein, mRNA etc.) are shown as blue color nodes. Size of the node reflects node degree (i.e. number of connections from a node to other nodes). For the readability, nodes with node degree >25 are labeled. Red lines are inhibition and green ones are activation from source to target nodes while blue color lines are protein-protein interaction without the regulatory directions.

Figure 6: Biological processes associated with top regulators in (a) HFO; and (b) DF biological response network. Nodes depict over-represented terms; colors reflect the label of the most significant term per group

Figure 7: Enriched disease terms for top ranked genes in (A) DF and (B) HFO aerosol network. The gene-disease interaction matrices are created using the Enrichr tool. Rows are top ranked genes in DF and HFO network, while columns are enriched disease terms arranged based on the combined score which is a combination of p-value and z-score to rank enriched disease terms. The length of the colored bar with disease name represents the significance of disease terms with respect to the associated genes. The colored cells in the matrix indicates that the corresponding gene-disease association.

Table 1: List of compounds having molecular interaction network and their aerosol-type specificity.

Table 2: Weighting scenarios for the identification of prioritized nodes in biological response network

Figures

Figure 1

Schematic representation of possible toxicity outcomes of two chemicals (A & B) alone and in a mixture. Chemicals when present together in an exposure matrix can show the synergism, addition, antagonism or inhibition in the overall toxicity outcomes. The simplest assumption is the concentration addition where the overall toxicity of the mixture can be calculated by addition of toxicity caused by chemicals individually. However, due to the complex regulatory events in a biological system, chemicals in an exposure matrix mostly show the synergism or antagonism where the overall toxicity is either more or less than the concentration addition. Sometime chemicals, when present together in an exposure matrix, inhibit each other or form a composite which is less toxic than the parent compounds.

Figure 2

Possible mechanisms for combustion aerosols exposure induced carcinogenesis, cardiovascular and lung pathogenesis. Chemical, metals, fibers and particulate matter released from ship/automobile exhausts, industrial exhaust or wood combustion can generate oxidative stress in the exposed cells, which may lead to various chronic conditions including carcinogenesis, cardiovascular and lung diseases. Other possible effects might be through frustrated phagocytosis in macrophages (more relevant for fibers) which may induce chronic inflammations. Chemicals present in combustion aerosols may also cause genotoxicity by interfering in DNA methylation, histone modification or DNA strand breakage. Chemical entities present in combustion aerosols also form complexes with altogether

different toxicity profiles than the parent compounds. Most often these complexes increase the bioavailability of toxic chemicals by slow and sustain release inside the cell.

Figure 3

Workflow for constructing aerosol-type specific chemical – gene interaction regulatory network

Figure 4

Overall experimental and bioinformatic flowchart of this study. blue shaded: – experimental workflow aspects, green shaded: big data/bioinformatic workflow aspects)

(a) HFO

(b) DF

Figure 5

Aerosol-type specific biological response networks. (a) HFO-combustion aerosol; (b) DF-combustion aerosol. Chemicals are shown as red color triangles, biological components (gene, protein, mRNA etc.) are shown as blue color nodes. Size of the node reflects node degree (i.e. number of connections from a node to other nodes). For the readability, nodes with node degree >25 are labeled. Red lines are inhibition and green ones are activation from source to target nodes while blue color lines are protein-protein interaction without the regulatory directions.

Figure 6

Biological processes associated with top regulators in (a) HFO; and (b) DF biological response network. Nodes depict over-represented terms; colors reflect the label of the most significant term per group

Figure 7

Enriched disease terms for top ranked genes in (A) DF and (B) HFO aerosol network. The gene-disease interaction matrices are created using the Enrichr tool. Rows are top ranked genes in DF and HFO network, while columns are enriched disease terms arranged based on the combined score which is a combination of p-value and z-score to rank enriched disease terms. The length of the colored bar with disease name represents the significance of disease terms with respect to the associated genes. The colored cells in the matrix indicates that the corresponding gene-disease association.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AdditionalFile1.docx](#)
- [Additionalfile2.xlsx](#)
- [Additionalfile3.xlsx](#)
- [Table1.docx](#)
- [Table2.docx](#)