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Using transcriptomics data and Adverse Outcome Pathway networks to explore endocrine disrupting properties of Cadmium and PCB-126



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ABSTRACT

Omics-technologies such as transcriptomics offer valuable insights into toxicity mechanisms. However, integrating this type of data into regulatory frameworks remains challenging due to uncertainties regarding toxicological relevance and links to adverse outcomes. Furthermore, current assessments of endocrine disruptors (EDs) relevant to human health require substantial amounts of data, and primarily rely on standardized animal studies. Identifying EDs is a high priority in the EU, but so are efforts to replace and reduce animal testing. Alternative methods to investigate EDs are needed, and so are health risk assessment methods that support uptake of novel mechanistic information.

This study aims to utilize Adverse Outcome Pathways (AOPs) to integrate transcriptomics data for identifying EDs, by establishing a link between molecular data and adverse outcomes. Cadmium (Cd) and 3,3',4,4',5-pentachlorobiphenyl (PCB126) were used as model compounds due to their observed effects on the endocrine system. An AOP network for the estrogen, androgen, thyroid, and steroidogenesis (EATS)-modalities was constructed. RNA sequencing (RNA-Seq) was conducted on zebrafish (*Danio rerio*) embryos exposed to Cd or PCB126 for 4 days. RNA-Seq data were then linked to the AOP network via Gene Ontology (GO) terms. Enrichment Maps in Cytoscape and the QIAGEN Ingenuity Pathway Analysis (IPA) software were also used to identify potential ED properties and to support the assessment.

Potentially EATS-related GO Biological Process (BP) terms were identified for both compounds. A lack of accurate standardized terms in KEs of the AOP network hindered a data-driven mapping approach. Instead, manual mapping of GO BP terms onto the AOP network revealed more connections, underscoring the need for harmonizing AOP development for regulatory use. Both the Enrichment Maps and the IPA results further supported potentially EATS-related effects of both compounds. While AOP networks show promise in integrating RNA-Seq data, several challenges remain.

Abbreviations: AhR, Aryl Hydrocarbon Receptor; AOPs, Adverse Outcome Pathways; AR, Androgen Receptor; BH, Benjamini-Hochberg; CAS, Chemical Abstracts Service Registry Number; Cd, Cadmium; ChEBI, Chemical Entities of Biological Interest; CLP, Classification, Labelling, and Packaging; CYP, Cytochrome P450; DEGs, Differentially Expressed Genes; dpf, Days post fertilization; EATS, Estrogen, Androgen, Thyroid, Steroidogenesis; ECHA, European Chemicals Agency; ED, Endocrine Disruptor; EFSA, European Food Safety Authority; ESRRA, Estrogen related receptor alpha; EU, European Union; EURION, European Cluster to Improve Identification of Endocrine Disruptors; FAIR, Findable, Accessible, Interoperable, Reusable; GO, Gene Ontology; GO BP, Gene Ontology Biological Process; GO MF, Gene Ontology Molecular Function; GPER1, G-protein coupled estrogen receptor 1; GSEA, Gene Set Enrichment Analysis; HGNC, HUGO gene nomenclature committee; hpf, Hours post fertilization; HPG, Hypothalamus, pituitary, gonadal axis; HPT, Hypothalamus, pituitary, thyroidal axis; IL, Interleukin; IPA, Ingenuity Pathway Analysis; IRS1, Insulin receptor substrate 1; KE, Key Event; KER, Key Event Relationship; MIE, Molecular Initiating Event; NAMs, New Approach Methodologies; NCOA, Nuclear receptor coactivator; NES, Normalized Enrichment Score; OECD, Organisation for Economic Co-operation and Development; ORA, Overrepresentation Analysis; PCA, Principal Component Analysis; PCB126, 3,3',4,4',5-pentachlorobiphenyl; PPAR, Peroxisome proliferator-activated receptor; PPARGC1A, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RNA-Seq, RNA Sequencing; WHO, World Health Organization.

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1. Introduction

Endocrine Disruptors (EDs) are a challenging group of chemicals, partly due to their complex toxicity that may manifest as a wide spectrum of effects in different organ systems and involves complex feedback mechanisms. The World Health Organization (WHO) defines EDs as substances that cause adverse health effects in an intact organism as a consequence of disrupting the normal functions of the endocrine system (WHO/IPCS, 2002). This definition provides the basis for the European Union's (EU) scientific criteria for identifying EDs and principles for ED assessment under the EU regulations for biocidal products and plant protection products, and the EU regulation for Classification, Labeling and Packaging (CLP, EC 2008). Consequently, the identification of EDs is highly dependent on data from *in vivo* animal studies that allow observations of adverse effects in apical endpoints.

Significant developments are ongoing to allow for the prediction of adversity based on mechanistic information from *in chemico*, *in vitro*, and *in silico* systems, where the link to an adverse outcome may be unclear. For this to be feasible, mechanistic information must be reliably linked to an adverse outcome. Adverse Outcome Pathways (AOPs) can provide a biologically plausible link between the mechanistic information from transcriptomics data and adverse outcomes. AOPs are pathways composed of several key events (KEs) that are causally connected through key event relationships (KERs), and the chain of events lead to an adverse outcome (Ankley et al., 2010). The AOP framework is used to construct simplified toxicity pathways across different levels of biological organization. KEs are also modular and can be included in multiple AOPs through different KERs. Based on shared KEs across AOPs, AOP networks can be generated, allowing for the investigation of how one or several molecular initiating events (MIEs) or early KEs, lead to one or several adverse outcomes through a series of intermediate KEs (Knapen et al., 2018; Spinu et al., 2019; Wiklund et al., 2023; Ziliacus et al., 2024).

During AOP development, KEs may include standardized terms, such as Gene Ontology (GO) terms, in their metadata. GO terms are a standardized ontology (Ashburner et al., 2000) and are indicative of e.g., a specific biological process (GO BP terms). Each GO term includes a set of genes. Based on the ratio of differentially expressed genes and whether they are up- or downregulated, the activation or inhibition of biological processes can be predicted. This functional analysis of differentially expressed genes can be performed, commonly with transcriptomics data. In transcriptomics experiments, the entire transcriptome is sequenced, which gives a more unbiased view of possible toxicological effects compared to choosing specific effects and genes to investigate beforehand.

The use of omics-technologies to identify toxicity mechanisms is prevalent within academia. In the past decade, the availability of omics and computational tools has increased, while the cost and time required for these experiments have decreased (Krewski et al., 2020). Omics also enable analyses of different toxicity mechanisms simultaneously, which can be helpful for identifying hazardous properties of chemicals. Despite the widespread use of omics, the regulatory acceptance of these methods remains low and such data are seldom used to inform chemical risk assessments (Harrill et al., 2021; JRC, 2024). Omics can be performed with either *in vivo* or *in vitro* models, though cell-based experiments are more specific to effects on a certain type of cell or tissue. Here, the *in vitro* zebrafish embryo model is a promising alternative, since RNA of the entire embryo can be sequenced to investigate effects on different organ systems.

In the EU, zebrafish embryos are considered an *in vitro* system until they are independently feeding larva (European Parliament and European Council, 2019), at approximately 5 days post fertilization. Compared to cell-based *in vitro* studies, zebrafish embryos allow investigations into effects on the Hypothalamic-Pituitary-Gonadal (HPG) or Hypothalamic-pituitary-thyroid (HPT) axes and endocrine feedback mechanisms. Moreover, both endocrine activity and developmental

toxicity mechanisms may be investigated in zebrafish embryos, and transcriptomics may provide information on both aspects at the same time. The estrogen, androgen, thyroid and steroidogenesis (EATS) endocrine pathways are highly conserved across vertebrate species and there is good empirical support for read-across between fish and mammals, especially for the HPG axis (McArdle et al., 2020). Taken together, this supports the potential of using zebrafish embryos to investigate effects of ED on human health.

The aim of this study was to develop and explore methodology for using transcriptomics data derived using the *in vitro* zebrafish embryo model for the identification of EDs. The work was based on the principles for ED assessment under the EU regulations for plant protection products and biocides and described in the guidance document (ECHA/EFSA guidance) by the European Chemicals Agency (ECHA), the European Food Safety Authority (EFSA), supported by the European Commission Joint Research Center (JRC) (ECHA/EFSA, 2018). The focus was thus on assessment of the EATS-modalities, since these modalities are in focus for regulatory ED assessment in the EU. Cadmium (Cd) and 3,3',4,4',5-pentachlorobiphenyl (PCB126) have both shown endocrine activity via the EATS-modalities *in vitro* and *in vivo* (Desaulniers, 1999; Jonsson et al., 2007; Margetaki et al., 2021; National Toxicology Program, 2006; Ronchetti et al., 2016, 2013; Tian et al., 2020), but they have not yet been identified as EDs in the EU. Therefore, they were used as model compounds to explore the new methodology and investigate potentially endocrine-related effects, and were tested in the zebrafish embryo model.

2. Materials and methods

2.1. AOP network generation

An EATS-related AOP network was constructed based on a previously published method (Wiklund et al., 2023). The AOP network was generated to anchor the transcriptomics data to specific KEs and to identify links between endocrine mechanisms and downstream apical effects. Briefly, a structured search in the AOP-Wiki (<https://aopwiki.org/>) was performed to identify relevant AOPs. Search terms, based on experimental parameters from the ECHA/EFSA guidance for the identification of EDs were used to identify relevant AOPs. The AOPs retrieved by the search were also manually screened for relevance, and a list of AOP IDs was constructed. Data were downloaded from the AOP-Wiki on 2024-09-19 (available in [supplementary material S1](#)). An R-script (<https://github.com/linuwi-ki/AOPN-Generation>) was used to format the data and filter it based on the list of relevant AOPs. Finally, the output of the R-script was imported into Cytoscape v3.9.1 (<https://cytoscape.org/>) to visualize the EATS-related AOP network.

A brief summary of the Key Event Components of KEs in the network was also constructed. The Key Event Components consists of structured ontology terms that describe the KE: a biological process (process term), a biological entity (object term), and an action. The biological process describes a specific function in a biological system that can be perturbed, like receptor signaling. The object is the biological entity which is subject to perturbation, such as a specific receptor. The action is simply the direction of change, like “decreased” or “increased”.

2.2. Chemicals and reagents

Embryo medium (E3; 0.33 mM MgSO₄, 0.17 mM KCl, 5.0 mM NaCl, 0.33 mM CaCl₂) was prepared by diluting a sterile-filtered 50x E3 medium stock solution with autoclaved purified water. Working solutions of 3,3',4,4',5-Pentachlorobiphenyl (PCB126, CAS 57465-28-8, AccuStandard) and Cadmium chloride (Cd, CAS 10108-64-2, Sigma-Aldrich) were prepared by diluting stock solutions of PCB126 (suspended in DMSO) and Cd (suspended in E3) in E3 medium. The final working solutions were 0.8, 0.4, and 0.08 nM PCB126 and 24, 12, and 2.4 µg/L Cd. The concentrations were selected based on prior toxicity screening, with

the highest concentration for each compound being the concentration causing 20 % cumulative effects on development (EC20), including uninflated swim bladder, scoliosis, edema, hatching rate, and lethality (data not shown). Three concentrations of each compound were included to ensure that relevant endocrine disrupting effects were captured.

2.3. Zebrafish husbandry, exposure and sampling

F0 zebrafish of the AB strain were housed in self-cleaning 3.5 l tanks with a density of five fish per liter in a centralized recirculatory aquatic system (Tecniplast, catalog# ZB30TK). Basic water parameters were continuously monitored and automatically adjusted to a temperature of 28 °C, a conductivity of 1200 µS/cm, and a pH 7.5. The light/dark cycle was 14 h light/10 h dark with a 20 min dawn and dusk period. Embryos were produced by mass-mating and kept in petri-dishes containing E3 medium in an incubator at 28.5 °C without light-cycle. Unfertilized eggs were removed after 6 h, and embryos with abnormal morphology or developmental stage (according to Kimmel et al., 1995) were removed prior to start of exposure.

At 24 h post fertilization (hpf), embryos were separated into 35 embryos per exposure group using glass petri dishes, including nominal external doses of 0.8, 0.4, 0.08 nM PCB126 or 24, 12, 2.4 µg/L Cd, and a control group receiving only E3. All exposures were prepared using fresh E3 media, and they were exposed to either Cd, PCB126, or only E3 media for 96 h (until 5 days post fertilization (dpf)) at a density of one embryo per ml exposure media, under the same conditions as previously stated. The embryos were monitored daily, and dead embryos were removed. At 5 dpf, embryos were sampled in four replicates in pools of 8 embryos per replicate and kept in RNAlater (Sigma-Aldrich) at 4 °C until further processing.

2.4. RNA preparation and sequencing

RNA-seq analyses were performed with embryos originating from one experiment. Prior to RNA extraction, RNA later was replaced with QIAzol Lysis Reagent (Qiagen, Hilden, Germany). 1 mm steel beads were added to each pool of embryos, and they were homogenized in a Bullet Blender (Next Advance Storm 24) for 4 min. RNA extraction was thereafter performed using the QIAGEN RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted RNA was then stored at -80 °C.

Quality control, library preparation, RNA-Seq and data pre-processing were performed by a third-party (Novogene, UK). Quality control of the samples was performed using a RNA Nano 6000 Assay Kit and a Bioanalyzer 2100 (Agilent Technologies, CA, USA), confirming a RIN value of ≥ 8.9 for all samples. Sequencing was performed using an Illumina Novaseq platform generating 150 bp paired-end reads. The *danio rerio* reference genome GRCz11 and the Hisat2 v2.0.5 package (Kim et al., 2019) were used for alignment. Quality control of raw reads was performed by investigating error rate distribution and GC content distribution. Low quality reads were filtered out based on the following criteria: adapter contamination, more than 10 % uncertain nucleotides, or an Illumina Qscore below 5 for more than 50 % of bases. Gene quantification from reads to counts was done with featureCounts v1.5.0 (Liao et al., 2014). Further quality control, filtering, normalization, differential gene expression analysis and data analysis was thereafter done by the authors using various packages in R as described below and the QIAGEN Ingenuity Pathway Analysis (IPA) software (Krämer et al., 2014).

Raw data is available at the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession ID GSE283372. The R-scripts used for data analysis, which packages were used, and package versions can be found on GitHub (https://github.com/linuwi-ki/RNA-Seq_AOPN_Processing). Quality control files with and without removal of outliers are available in supplementary material S2.

2.5. Transcriptomics data analysis

Prior to differential gene expression analysis, *danio rerio* Ensembl gene IDs were converted into HUGO gene nomenclature committee (HGNC) gene symbols using biomaRt v2.56.1 (Durinck et al., 2009) and release 105 of the human and *danio rerio* gene ensembl data sets (Harrison et al., 2024). Normalization and differential gene expression analysis was performed in R with DESeq2 v1.40.2 (Love et al., 2014). Genes were filtered for further analysis and kept only if they had at least five reads in three or more samples. Based on raw count distribution, filtered count distribution, and the Principal Component Analysis (PCA) of the differentially expressed genes (DEGs), two outliers were detected and removed from subsequent analysis (one control replicate and one low concentration Cd replicate). Venn-diagrams were generated using the R-package VennDiagram v1.7.3.

Different analyses were performed to determine the most appropriate type of output to link to the AOP network. GO BP terms and the IPA Upstream Regulators were chosen to connect data to the AOP network, since structured ontology terms for biological processes and objects can be included in KEs. Enrichment Maps were generated to provide an overview and easy interpretation of the data. Reactome terms, and 'Canonical Pathways' and 'Diseases & Functions' from IPA, also provided evidence that may be of relevance. However, these data were not connected to the network since the 'Canonical Pathways' and 'Diseases & Functions' data were mainly at a different level of detail compared to the KEs. GO BP terms were used instead of Reactome terms since they provided similar evidence and GO BP terms were more common in the AOP network.

2.5.1. Enrichment analysis

Enrichment analysis was performed in R using overrepresentation analysis (ORA) with enrichR v3.2 (Xie et al., 2021) and Gene Set Enrichment Analysis (GSEA) with fgsea v1.26.0 (Korotkevich et al., 2021). Enrichment was based on GO BP terms, GO molecular function (MF), and Reactome pathway terms for GSEA. DEGs with a Benjamini-Hochberg (BH) adjusted p-value ≤ 0.05 were included in the enrichment analyses. GO BP, GO MF and Reactome 2023 human gene sets were downloaded from the Molecular Signatures Database (Liberzon et al., 2011; Subramanian et al., 2005).

The Enrichment Maps app (v3.3.6) for Cytoscape was used to visualize networks of significant GO terms (Merico et al., 2010) for each compound with all concentrations together, to identify the overarching processes affected. Enrichment Maps connect GO terms based on the number of shared genes between any two gene sets, producing networks of GO terms involved in similar processes based on the gene set overlap. Only GO terms with a BH adjusted p-value ≤ 0.05 and an absolute normalized enrichment score (NES) ≥ 1.5 were included. The NES indicates if the predicted function is activated or inhibited. Standard settings were used when importing the GO terms, and an overlap ≥ 37.5 % of genes in the gene sets were required to connect two GO terms. GO terms that were connected to at least 2 other terms (making a group of ≥ 3 terms) were grouped in Cytoscape and categorized based on expert knowledge, where both the name and description of the GO terms in the cluster were considered.

2.5.2. Ingenuity pathway analysis

Data was also analyzed in the QIAGEN IPA software v01-23-01. A core analysis was followed by comparison analyses for both compounds separately, and together. Results were either filtered based on BH adjusted P-value ≤ 0.05 alone or in combination with an absolute bias-corrected Z-score ≥ 1.5. The Z-score indicates whether a biological function, pathway, or regulator is predicted to be activated (positive Z-score) or inhibited (negative Z-score) (Krämer et al., 2014). Upstream regulators, Canonical Pathways, and Diseases & Functions analyses were performed, and in all analyses, cancer-related terms were excluded through the IPA filter. Since certain zebrafish genes do not have a known

human ortholog in IPA, non-mapped genes from IPA were manually converted to human orthologs using biomaRt v2.56.1 to ensure that no genes of high importance were excluded from the IPA analysis.

2.6. Connecting transcriptomics data to endocrine activity and adversity

To connect the transcriptomics data to EATS-mediated adverse outcomes of regulatory relevance, data were connected to both KEs in the AOP network and to EATS-related parameters included in standardized toxicity tests listed in the ECHA/EFSA guidance (ECHA/EFSA, 2018). The data analysis workflow and which data were used to connect transcriptomics to the AOP network are shown in Fig. 1. Before GO terms could be linked to KEs and effect parameters, they were tagged and sorted by the GO term name, description, and NES. Terms were first tagged as: “EATS”, “Sensitive to EATS”, or “Unknown”. Then, terms received an additional, more specific tag: “EAS”, “T”, “Immunological”, “Metabolic”, “Cell metabolism”, “Neuro”, “Unknown”, “Organismal development”, or “Cell cycle / chromosome organization”. Tagging of terms was based on expert judgement and conducted by three independent researchers, and differences in tags were resolved by discussion. Finally, “EATS” and “Sensitive to EATS” tagged terms were included for subsequent connection to the ECHA/EFSA guidance parameters or the AOP network. The process of tagging of all significant GO terms, and all connections between GO terms and both the AOP network and ECHA/EFSA guidance parameters, is available in [supplementary material S3](#).

2.6.1. Connecting transcriptomics data to the AOP network

Connecting GO BP and GO MF terms to KEs in the AOP network was performed through both a data-driven and an expert-driven approach. In the data-driven approach, the accession ID of GO terms from all KEs in the network (for which this information was available), were extracted and compared to the accession IDs from the experimental data. The output was a list of GO terms that were present in both the AOP network

and the experimental data. In the expert-driven approach, GO terms were connected to KEs in the AOP network based on the name and description of both KEs and GO terms, through expert judgment. The connection between a KE and a GO term were judged as either “Directly relevant” or “Indirectly relevant”. For example, a GO term about hormone levels was considered relevant for several KEs on changes in specific hormone levels, but judged as indirectly relevant in all cases since the GO term does not specify a particular hormone. On the other hand, the GO term on “synapse assembly” with a negative NES was considered directly relevant to the KE “Decrease of synaptogenesis”, since they belong to the same process, direction of effect, and have a similar level of detail.

2.6.2. Connecting transcriptomics data to EATS-related parameters

Connecting GO terms to parameters from the ECHA/EFSA guidance was performed manually by connecting them to *in vitro* mechanistic parameters, *in vivo* mechanistic parameters, EATS-mediated parameters and parameters sensitive to, but not diagnostic of, EATS listed in tables 12–17 in the ECHA/EFSA guidance (ECHA/EFSA, 2018). This was done based on expert judgment and the name and description of the GO term was considered. The connection of GO terms and ECHA/EFSA guidance parameters was evaluated with an inclusive approach, since the GO terms were generally more specific than the broader ECHA/EFSA guidance parameters.

3. Results

3.1. AOP network generation

A total of 70 AOPs from the AOP-Wiki were identified as relevant for the EATS-modalities and were included in the network. One main network consisting of 237 KEs was formed and used for further analysis, while AOPs and KEs not connected to the main network (a total of 30

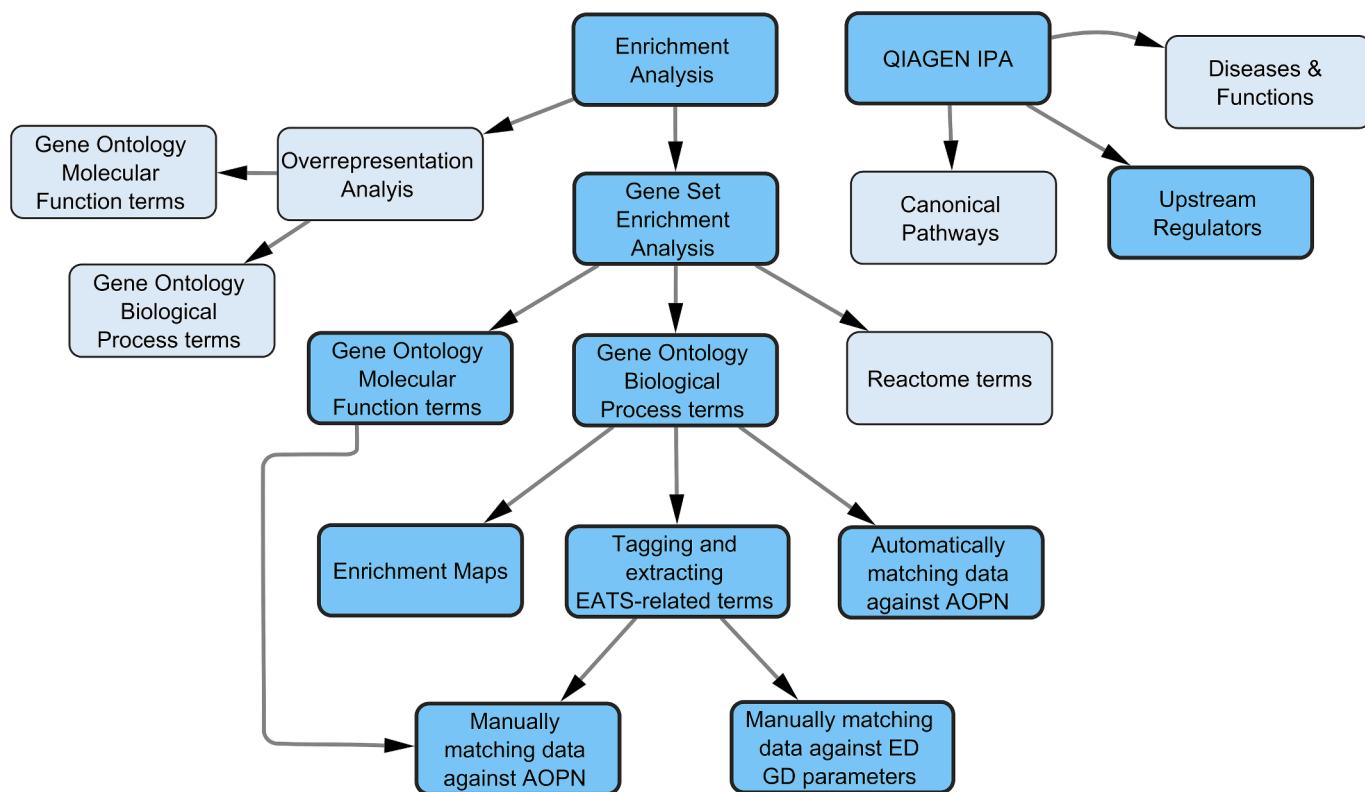


Fig. 1. Overall approach to transcriptomics data analysis and connecting data to the AOP network. The data analysis methods used to draw conclusions on endocrine disrupting properties of Cd and PCB126, and used to connect transcriptomics data to the AOP network or to EATS-related parameters from the ECHA/EFSA guidance, can be found in the darker blue squares with bold borders. The additional data is available in [supplementary materials S6 and S11](#).

KEs) were excluded. Unconnected AOPs and KEs may be AOPs in early stages of development or AOPs related to less established pathways. Moreover, some manual modifications to the network were made. Relevant KERs that were missing from some AOPs in the downloaded data could be identified in the information on the respective AOP-Wiki pages and were added manually. Duplicate KEs on the same biological process, but that existed as different KEs in the network due to differences in naming conventions, were merged into a single KE. All the manual modifications of the AOP network are available in [supplementary material S4](#). The full network (including unconnected KEs and AOPs) is included as a high-resolution image in [supplementary material S5](#).

Since object and process terms of KEs were to be used in further analyses, the completeness of this information in the AOP network was specifically analyzed ([Table 1](#)). In the main network, 57 % of KEs (134/237) had a standardized process term, but in only 43 % of KEs (101/237) the process term was a GO BP term. Similarly, 50 % of KEs (119/237) had a standardized object term included, but very few of the object terms were GO terms. Instead, Chemical Entities of Biological Interest (ChEBI) was the most frequent ontology used for object terms, making up 16 % (38/237) of all KEs or 32 % (38/119) of the object terms. While a majority of the process terms were GO terms, the object terms were more evenly divided across different ontologies.

3.2. Transcriptomics data analysis

The total number of DEGs per compound and concentration, as well as the ratio of up- and downregulated genes, are illustrated in Venn-diagrams ([Fig. 2](#)). In general, there were more DEGs for PCB126 than for Cd, and for both compounds, there were more upregulated than downregulated genes, except for the 0.08 nM PCB126. For Cd, the number of DEGs showed a concentration-dependent increase, while no concentration-dependent relationship was observed across the three concentrations for PCB126. The smallest overlap of DEGs between concentrations was observed between the low and middle concentration, being only 1 gene for Cd and 64 genes for PCB126. The biggest overlap was observed between the middle and high concentration for Cd

Table 1

Standardized process terms and object terms of KEs in the main AOP network and identification of the most frequently occurring types of standardized terms. The information is based on a snapshot of the AOPWiki from 2024 to 09-19, which is also available in [supplementary material S1](#).

Type of standardized term	Total number of KEs	KEs with a standardized term	The most common ontology	% of KEs with standardized terms and most common ontology terms
Process term	237	134	Gene Ontology terms: 101 KEs	<ul style="list-style-type: none"> • 57 % of KEs have a process term • 43 % of KEs have a GO term • 75 % of all process terms are GO terms
Object term	237	119	Chemical Entities of Biological Interest (ChEBI): 38 KEs	<ul style="list-style-type: none"> • 50 % of KEs have an object term • 16 % of KEs have a ChEBI term • 32 % of all object terms are ChEBI terms

with 160 common genes, and for PCB126 the biggest overlap of 285 genes was observed between all three concentrations.

3.2.1. Enrichment analysis

ORA was performed with GO BP and GO MF terms, while GSEA was performed with GO BP, GO MF, and Reactome terms (data not shown, figures and data available in [supplementary material S6](#)). Further analysis was focused on GO BP terms from GSEA, since this method takes expression fold change into account and GO BP terms are the most common standardized term of KEs in the AOP network ([Table 1](#)). As with the DEGs, there were more upregulated than downregulated GO BP terms after exposure to Cd or PCB126 for all concentrations except for the lowest concentration of PCB126. The significantly affected GO terms, based on a BH adjusted p-value ≤ 0.05 and NES $+/- 1.5$, were imported into Cytoscape to be used in the generation of Enrichment Maps ([Fig. 3](#)).

Cd exposure caused an upregulation of immune system related effects across all concentrations. Downregulation of terms related to neurodevelopmental and neuronal signaling were observed in the lowest and highest concentration, while cell metabolism changes, like effects on acetyl-CoA metabolism and the electron transport chain, were only observed in the low and middle concentration. Meanwhile, downregulation of metabolism, nutrient transport, and detoxification, as well as upregulation of organismal development and certain cell and tissue developments, were only observed in the highest concentration.

In PCB126-exposed embryos, effects on metabolism, immune system processes, and the development of certain cells and tissues, were observed in all concentrations, with concentration-dependent differences in direction of effects. Downregulation of neurodevelopment was observed in the low and middle concentration, but not the high concentration, while downregulation of neuronal signaling and organismal development was only observed in the low concentration. Cell metabolism was increased in the low and high concentration of PCB126, but not in the middle, and detoxification was only upregulated in the middle and high concentration. The effect that was observed only in the high concentration was downregulation of cell division processes.

Possible effects on endocrine activity were observed for both compounds, but these processes were not easily identifiable in the Enrichment maps. The endocrine processes were either present as a single GO term in the Enrichment Maps (for example regulation of hormone secretion in the high Cd concentration) thereby excluding it from the figure, or they were part of Metabolism-related clusters (for example as steroid metabolism or steroid biosynthesis). An example of an Enrichment Map for PCB 0.8 nM is shown in [Fig. 3](#), while the Enrichment Maps for all treatments are available in the [supplementary material \(supplementary material S7\)](#). A summary of the predicted functional effects for all treatments, as illustrated in the Enrichment Maps, is provided in [Table 2](#).

3.2.2. Upstream regulators

The upstream regulators from IPA provided further evidence indicating that the overall functional changes may be a consequence of endocrine perturbations. The IPA upstream regulators reveal compounds or endogenous mediators that were likely to have caused the observed changes in gene expression from the transcriptomics data. Several EATS and non-EATS endocrine-related entities were observed as statistically significant upstream regulators (BH adjusted p-value ≤ 0.05), and several also had significant Z-scores (absolute Z-score ≥ 1.5) for one or more concentrations. Non-EATS endocrine modalities include mechanisms that act via endocrine signaling, but that are not included in the EATS-modalities, for example the peroxisome proliferator-activated receptor (PPAR) or insulin signaling. EATS-related upstream regulators that were significant for both compounds included the androgen receptor (AR), beta-estradiol, estrogen related receptor alpha (ESRRA), G-protein coupled estrogen receptor (GPER1), nuclear receptor coactivator (NCOA) 1 and 3, and L-triiodothyronine. Other upstream

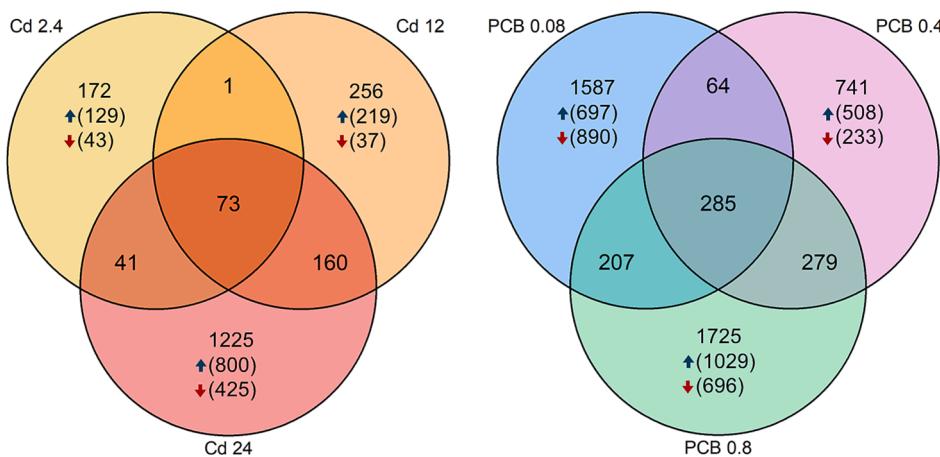


Fig. 2. Venn-diagram showing the number of differentially expressed genes (DEGs) per exposure and the number of DEGs that are shared between the different exposures. The number in parentheses following the blue up-arrow is the share of upregulated DEGs, while the number following the red down-arrow is the share of downregulated DEGs. Only DEGs with a BH adjusted p-value ≤ 0.05 were included.

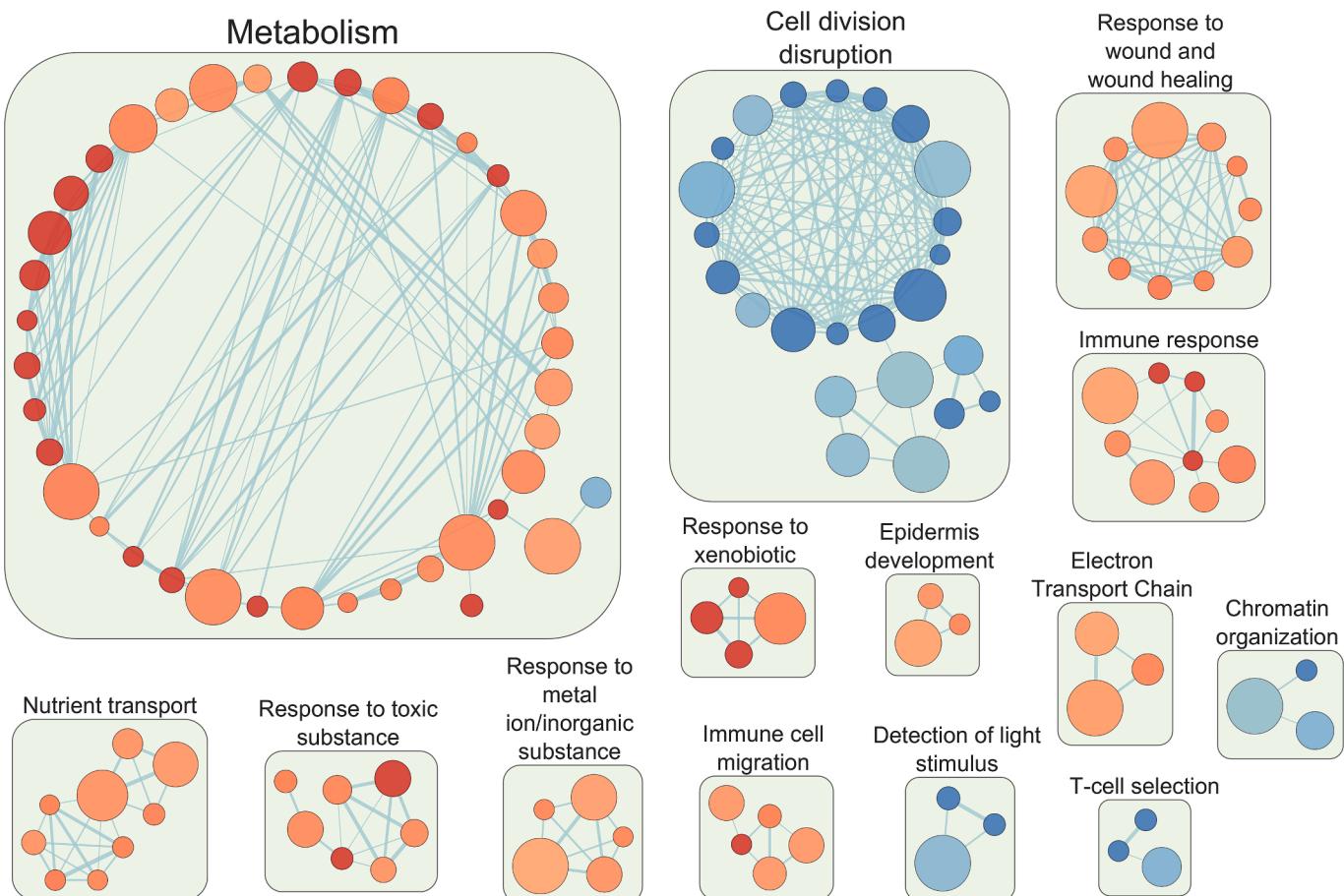


Fig. 3. Example of an Enrichment Map for PCB 0.8 nM. Each circle is a GO BP term, and terms are connected based on the overlap of their gene sets. The diameter of the circles indicates the number of genes included in that GO term's gene set. The thickness of the lines between circles indicates how much of an overlap exists between the two terms. A minimum of 37.5 % overlap is required for terms to be connected. Orange circles indicate that the GO term is upregulated ($NES \geq 1.5$), while blue circles indicate that the GO term is downregulated ($NES \leq -1.5$). Only GO terms with a BH adjusted p-value ≤ 0.05 and an absolute NES value ≥ 1.5 were included in the analysis, and only clusters with a minimum of 3 GO terms were visualized.

regulators of interest for non-EATS endocrine activity included 2-deoxyglucose, insulin, insulin receptor substrate 1 (IRS1) and PPAR gamma coactivator 1 alpha (PPARGC1A) for possible metabolic effects. Several immune system-related upstream regulators were also observed, such as lipopolysaccharide, interleukin (IL) 1, IL1B, IL2, IL4, dexamethasone and NFkB.

3.3. Connecting transcriptomics data to adverse outcomes

3.3.1. Connecting transcriptomics data to the AOP network

The data-driven approach to connect GO BP terms to the AOP network yielded few connections between GO BP terms and KEs (Table 3). Only 101/237 KEs included a GO term and were available for

Table 2

Overview of the major effect categories based on GO Enrichment Maps. Blue indicates downregulation of the process, while red indicates upregulation. Only GO terms with a BH adjusted p-value ≤ 0.05 and an absolute NES value ≥ 1.5 were included in the analysis. The list of affected biological functions is not exhaustive but includes the most frequently perturbed biological functions across the different exposures based on the Enrichment Maps.

Biological function	Cd 2.4 µg/L	Cd 12 µg/L	Cd 24 µg/L	PCB 0.08 nM	PCB 0.4 nM	PCB 0.8 nM
Neurodevelopment	↓		↓	↓	↓	
Neuronal signaling	↓		↓	↓		
Immune system effects	↑	↑	↑	↑	↓	↑
Metabolism			↑	↑	↑	↑
Nutrient transport		↑				↑
Organismal development (organogenesis)			↓	↓		
Chromatid/chromosome and cell division perturbation		↓	↓			↓
Cell metabolism	↑	↑		↑		↑
Cell/tissue development/differentiation			↓	↓	↓	↑
Response to xenobiotic (detoxification)			↑		↑	↑

Table 3

Results of the data-driven approach to connect transcriptomics data with KEs in the AOP network, based on GO terms. Only GO terms with a BH adjusted p-value ≤ 0.05 were included.

KE Description	KE ID	GO ID	GO BP term	Exposure
Decrease of synaptogenesis	385	GO:0007416	Synapse assembly	Cd 2.4 µg/L Cd 24 µg/L PCB 0.08 nM
Thyroxine (T4) in neuronal tissue, Decreased	280	GO:0010817	Regulation of hormone levels	PCB 0.08 nM PCB 0.4 nM PCB 0.8 nM
Reduction, Cholesterol transport in mitochondria	447	GO:0006839	Mitochondrial transport	Cd 24 µg/L
Increased, Intracellular Calcium overload	389	GO:0006816	Calcium ion transport	PCB 0.08 nM
Increased, Reactive oxygen species	1115	GO:1903409	Reactive oxygen species biosynthetic process	PCB 0.4 nM PCB 0.8 nM
Inadequate DNA repair	155	GO:0006281	DNA repair	PCB 0.8 nM

automatic connection to the transcriptomics data. Of these 101 KEs, only six unique KEs could be automatically connected to a GO BP term from the transcriptomics data. Moreover, the GO BP term used to connect the KE “Thyroxine (T4) in neuronal tissue, Decreased” was GO 0010817 “Regulation of hormone levels”, which does not necessarily reflect regulation of T4 specifically. Similarly, GO 0006839 “Mitochondrial transport” does not specifically state that cholesterol transport is affected, like in the KE “Reduction, Cholesterol transport in mitochondria”.

In the expert-driven approach to connect GO BP terms to the AOP network, more GO BP terms could be connected to KEs for both substances (Table 4). The entire expert-driven process for connecting GO terms to the AOP network is available in supplementary material S3. For Cd, 17 KEs were connected to at least one directly relevant GO BP term. When both directly and indirectly relevant GO BP terms were used, 55 KEs were connected to at least one GO BP term. For the PCB126 exposure, 22 KEs were connected to at least one GO BP term when only directly relevant connections were included, and 64 KEs when both directly and indirectly relevant GO BP terms were considered. Both exposures had some GO BP terms tagged as “EATS” or “Sensitive to EATS” that were not considered directly nor indirectly relevant to any KE in the AOP network, 8 GO BP terms for Cd and 17 for PCB126, indicating knowledge gaps in the AOP network.

GO MF terms were also manually connected to the AOP network, but only direct connections to KEs were made, and no indirect connections were included. Only 3 GO MF terms (GO 0,015,020 – glucuronosyltransferase activity, GO 008066 glutamate receptor activity, and GO 0046966 – nuclear thyroid hormone receptor binding) could be directly connected to any KE. Additionally, GO MF terms for UDP-glycosyltransferase activity (GO 0008194) were available for the low concentrations of both Cd and PCB126, but the NES was reversed compared to the action of the KE. Similarly, an increase in steroid hydroxylase activity (GO 0008395) was predicted in all concentrations of PCB126, but the only available KE for relevant for this GO terms was inhibition of 11β-hydroxylase.

When visualizing the KEs within the AOP network for which transcriptomics data indicated an effect, associations between affected KEs were revealed (Fig. 5 and Fig. 6). These figures are also provided as high-resolution images in supplementary material S9 and S10. For both substances, effects on brain development and function were observed through many GO terms and several KEs (Fig. 5 and Fig. 6). There were directly relevant GO terms for KEs on altered hippocampal gene expression, anatomy, and physiology, as well as a decrease of synaptogenesis, synaptic formation and plasticity, and decrease of neuronal network function, for both substances. Indirectly relevant GO terms

Table 4

Overview of GO BP terms that could be connected to KEs in the AOP network, separated by exposure. Only GO terms with a BH adjusted p-value ≤ 0.05 were included in the comparison.

Exposure	Significant GO BP terms	“EATS” or “Sensitive to EATS” tagged terms	Number of unique KEs with a GO BP term connected	GO BP terms not connected to any KE
PCB 0.08 nM	225	78 (34.6 %)	23 KEs with at least one directly relevant term.	17
PCB 0.4 nM	103	16 (15.5 %)	67 KEs with at least one directly or indirectly relevant term.	
PCB 0.8 nM	165	36 (21.8 %)		
Cd 2.4 µg/L	49	17 (34.7 %)	13 KEs with at least one directly relevant term.	12
Cd 12 µg/L	67	12 (17.9 %)	56 KEs with at least one directly or indirectly relevant term.	
Cd 24 µg/L	241	66 (27.4 %)		

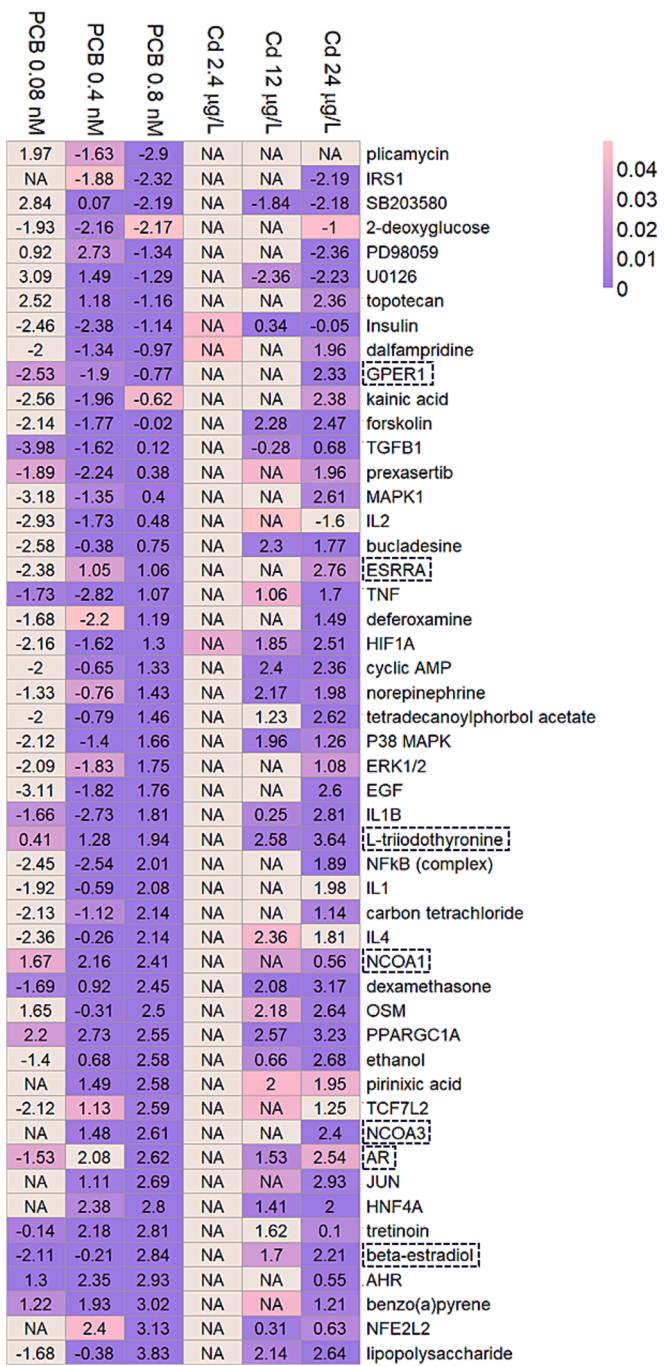


Fig. 4. Heatmap of upstream regulators from IPA. The number in each cell is the Z-score for that exposure and upstream regulator. The color indicates the BH adjusted p-value, and a darker shade of purple indicates a lower P-value. The boxed upstream regulators were judged as EATS-related. Only upstream regulators with a BH adjusted p-value ≤ 0.05 and an absolute Z-score ≥ 1.5 for at least one exposure were included. The figure is limited to the top 50 upstream regulators (based on BH adjusted p-value), and the full list of upstream regulators is available in [supplementary material S8](#).

were available for KEs upstream of these neurodevelopmental effects, mainly as effects on thyroid hormone changes. Additionally, there was one directly relevant GO term for a KE on inhibition of glutamate receptors and a few GO terms directly relevant for a decrease in neurotransmitter release for PCB126, upstream of the same neurodevelopmental effects or the downstream AO "Impairment, Learning and memory". Neurodevelopmental effects were also captured at different concentrations for both substances in the Enrichment Maps

(Table 2).

The rest of the KEs supported by directly relevant GO terms for Cd were spread out across different AOPs, and no single pathway perturbed from an MIE all the way to an AO could be clearly observed. Also, several early KEs related to hormone levels and hormone synthesis were indicated as affected due to a single GO term, "Regulation of hormone secretion" (GO ID 0046883). Nevertheless, KEs on an increase of developmental abnormalities, genomic instability, and chromosomal aberrations were supported by many different directly relevant GO terms, and it is biologically plausible that these effects are observed by endocrine activity, according to the AOP network. Moreover, induction of glucuronyltransferase activity was indicated by a directly relevant GO MF term, and could cause downstream effects on thyroid hormone levels.

The transcriptomics data for PCB126 could be directly and indirectly connected to more endocrine-related KEs compared to Cd. GO BP terms related to "estrogen metabolism" (GO 0008210), "hormone metabolism" (GO 0042445), "regulation of hormone levels" (GO 0010817), "regulation of hormone secretion" (GO 0046883), and "steroid metabolism" (GO 0008202) were observed across concentrations for PCB126. Although few of these GO terms were considered directly relevant for the KEs, since the GO terms did not mention specific hormones. Apart from the aforementioned effects on brain development and function, directly relevant GO terms were available for altered cholesterol metabolism, abnormal lipid metabolism, chromosomal aberrations and DNA damage, induction of CYP enzymes, an increase of developmental abnormalities, and alterations of the Wnt pathway. However, these KEs were spread out across the AOP network, and lacked directly relevant GO terms for KEs upstream and downstream of these KEs. Moreover, effects on antagonism of the thyroid hormone receptor are visible in the AOP network, but these KEs are supported by the same GO MF term.

Notably, almost no GO BP terms were predicted to be activated or inhibited in a direction contradictory to the KEs in the AOP network. For the few KEs with contradicting GO BP terms, there was either only one term available (and the KE was considered to have no relevant evidence) or there were many more GO BP terms that were aligned with the KE, and the contradictory GO BP term was excluded. However, as presented in section 3.3.1, there were fewer available terms and more contradictory evidence for the GO MF terms. The entire process of connecting GO terms to the AOP network is available in [supplementary material](#).

3.3.2. Connecting transcriptomics data to EATS-related parameters

Since the GO terms are often more specific than the EATS-related parameters in the ECHA/EFSA guidance, many GO terms were connected to only a few parameters (Table 5). For both exposures, most GO terms were connected to either fetal development or brain histopathology. Also, most terms were connected to parameters categorized as "Sensitive to, but not diagnostic of, EATS" (Fetal development, brain weight or histopathology, and brain morphometrics evaluation) and fewer GO terms were connected to parameters that are considered "EATS-mediated" (steroidogenesis, pituitary weight or histopathology, and hormone levels).

The connections between GO terms and EATS-related parameters were also very general and covered several levels of biological organization. For example, the GO terms connected to the fetal development parameter included both GO terms related to the development of organ systems, and changes in chromosome organization and separation. The GO terms connected to changes in hormone levels did not mention any specific hormones, but only hormone levels in general. On the other hand, the GO terms from the PCB126 exposure connected to steroidogenesis were specifically related to metabolism of steroids, hormones, and estrogen specifically. More detailed results are available in [supplementary material S3](#).

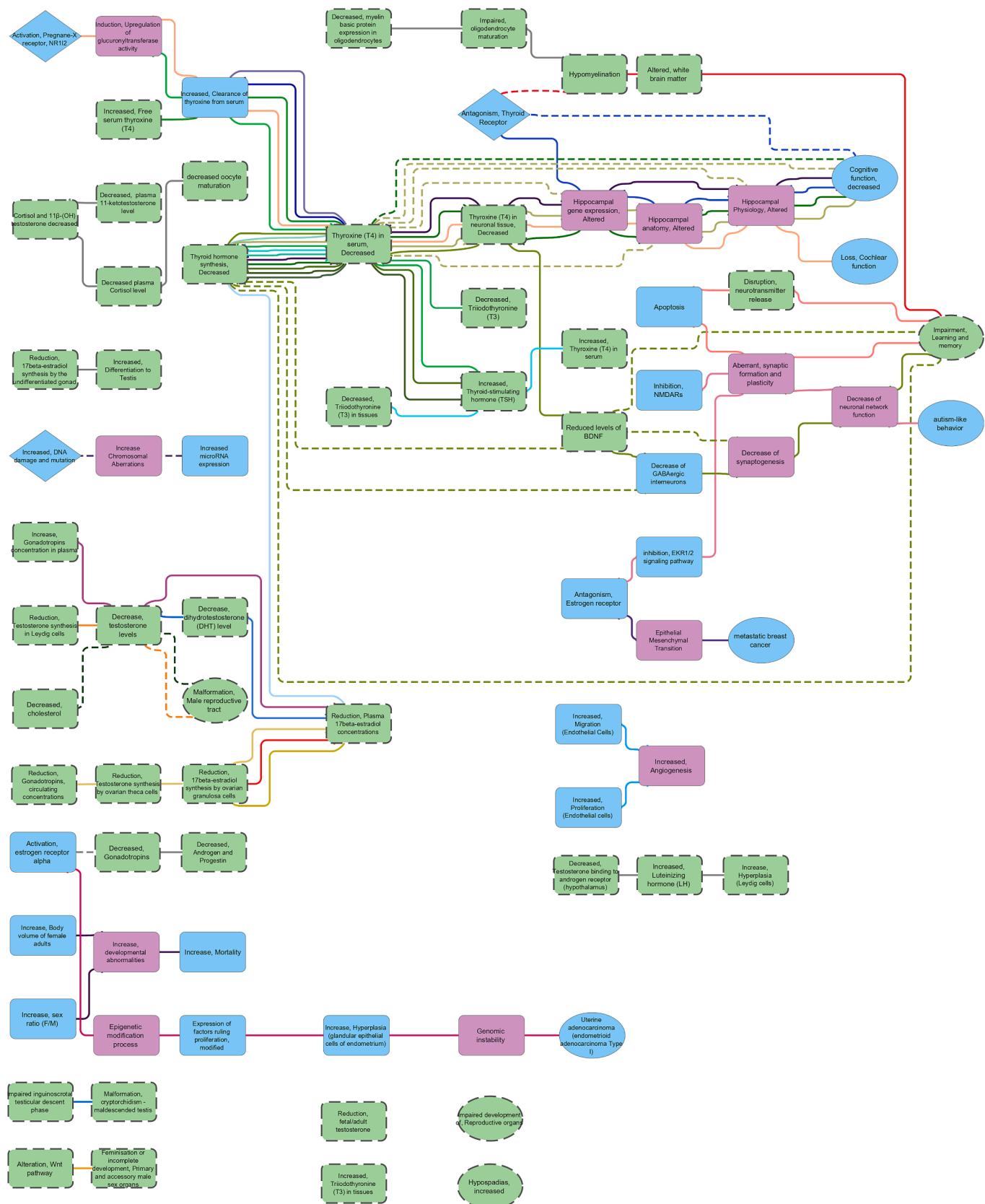


Fig. 5. A subset of the EATS-related AOP network after connecting GO BP terms for all concentrations of Cd against KEs in the network. This subnetwork was created by selecting KEs with directly relevant GO terms, their adjacent KEs ("first neighbors"), and then adding KEs with indirectly relevant GO terms. Purple nodes = KE with one or more directly relevant GO terms connected; Green nodes with dashed borders = KE with one or more indirectly relevant GO terms connected, but no directly relevant GO terms connected; Blue nodes = KE without GO term connected. KERs with the same color belong to the same AOP. A high-resolution image is available in [supplementary material S9](#).

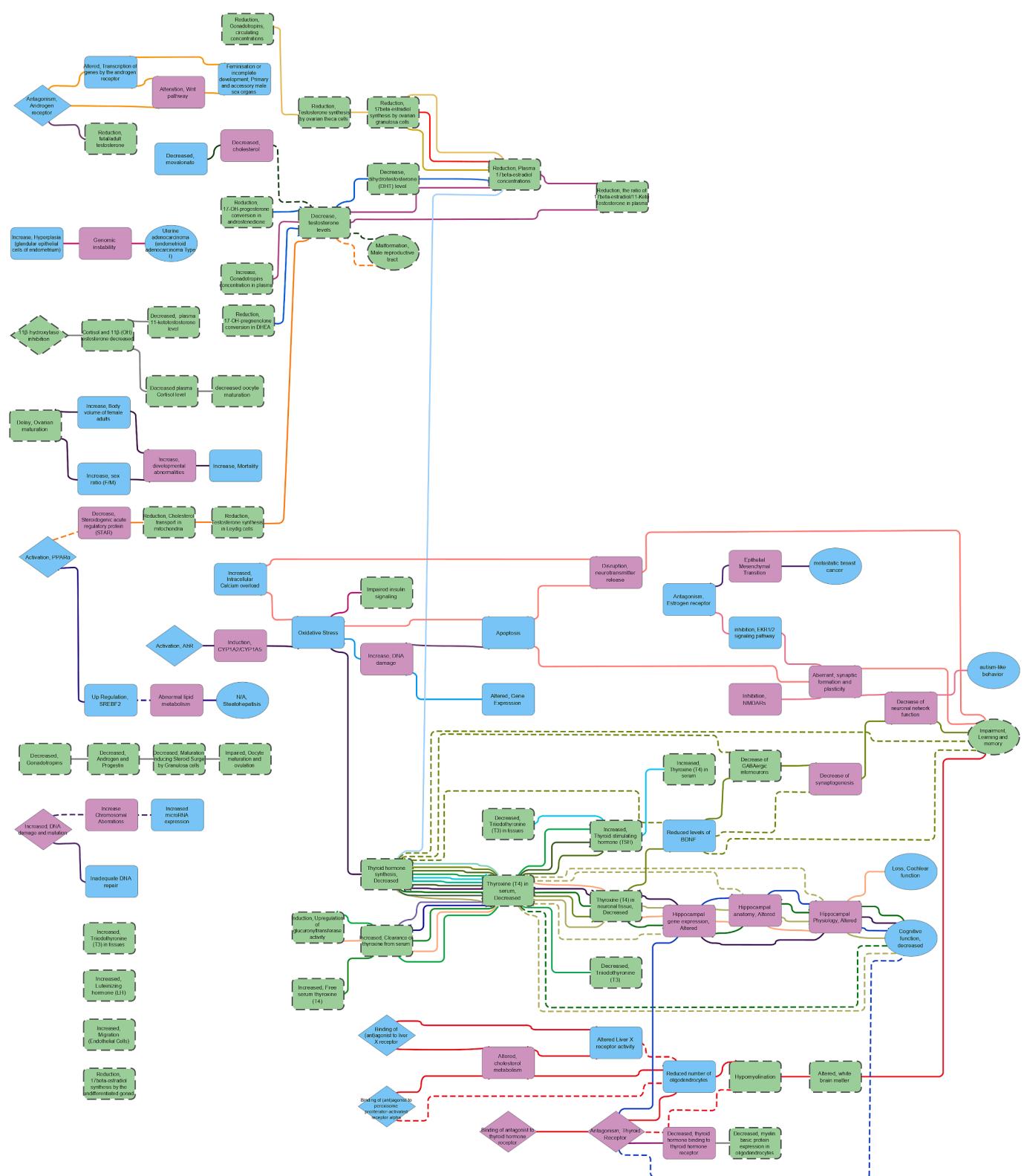


Fig. 6. A subset of the EATS-related AOP network after connecting GO BP terms for all concentrations of PCB126 to KEs in the network. This subnetwork was created by selecting KEs with directly relevant GO terms, their adjacent KEs (“first neighbors”), and then adding KEs with indirectly relevant GO terms. Purple nodes = KE with one or more directly relevant GO terms connected; Green nodes with dashed borders = KE with one or more indirectly relevant GO terms connected, but no directly relevant GO terms connected; Blue nodes = KE without GO term connected. KERs with the same color belong to the same AOP. A high-resolution image is available in [supplementary material S10](#).

Table 5

Overview of connecting the GO BP terms to endpoints from the ECHA/EFSA guidance.

ECHA/EFSA EATS-related parameter	Cd GO terms	PCB GO terms
Fetal development (or physical development of the fetuses)	51	57
Brain weight or histopathology	36	56
Brain morphometrics evaluation	5	1
Steroidogenesis	—	12
Pituitary weight or histopathology	1	—
Hormone levels (FSH, LH, E2, T, T3/T4, or TSH)	1	4
Total:	94	130

4. Discussion

In this work, RNA-Seq was performed on 5-day-old zebrafish embryos exposed to different concentrations of either Cd or PCB126. The aim was to explore how transcriptomics data could be utilized for the assessment of ED properties for these two compounds, using an AOP network approach. This was achieved by connecting transcriptomics data to KEs in an EATS-AOP network, using both a data-driven and an expert-driven approach. The data was also connected to established EATS-related parameters from the ECHA/EFSA guidance for ED assessment in the context of plant protection products and biocides. While our analyses revealed that Cd and PCB126 affect genes and pathways related to endocrine activity and processes sensitive to EATS perturbations, we also identified several aspects of AOP development with potential for improvement to enhance the integration of omics data for regulatory risk assessment.

The visualization of GO terms in the AOP network did not reveal any single pathway with perturbations spanning from the MIE to the adverse outcome for either compound. Several endocrine-related early KEs and some AOs were highlighted, while many intermediate KEs remained unconnected. This is not surprising, since many AOPs are still in early stages of development and there is a lack of KEs for many effects. Also, in endocrine-related AOPs, effects are mediated through changes in hormone levels, which is difficult to predict using gene expression data only. Early KEs were highlighted due to GO terms related to steroid biosynthesis or metabolism. However, MIEs commonly occur on a molecular level and are often related to single proteins. The GO BP terms were usually broader than the MIEs, and very few directly relevant GO MF terms were available in the experimental data. Alternatively, to target specific enzymes or receptors in MIEs, the approach presented in this work could be combined with *in chemico*, *in silico*, or *in vitro* experiments on MIEs and early KEs of interest, such as modulations of enzyme activity, binding to hormone transport proteins, or changes in the activity of specific hormone receptors. A higher number of directly relevant connections could be observed in late KEs and AOs where tissue- and organ-level perturbations exist, since many GO BP terms aim to predict processes at this level of biological organization.

In the AOP network-based analysis for Cd, several effects connected to KEs related to the thyroid system and developmental neurotoxicity were observed, as well as effects connected to KEs related to perturbations of EAS-mediated pathways leading to reproductive toxicity. These results are further supported by previous studies indicating that Cd affects thyroid hormone levels in both humans and zebrafish (Margetaki et al., 2021; Tian et al., 2020) and upregulate gene expression of deiodinases 1 and 2, and transthyretin, in zebrafish (Tian et al., 2020). Cd has also been shown to correlate with or cause developmental neurotoxicity in humans, rats, zebrafish, and mice (Pulido et al., 2019; Rodríguez-Barranco et al., 2014; Tian et al., 2021; Wang et al., 2022). In addition, Cd has been shown to affect estrogen signaling *in vitro* and *in vivo* (Ronchetti et al., 2016, 2013) and cause reproductive toxicity to both males and females of several species (Massányi et al., 2020; Tian et al., 2018), although the specific mechanisms are not clearly

understood. One review summarized effects of Cd on estrous cyclicity in female rats from one study (Nasiadek et al., 2019) and effects on spermatogenesis and male reproductive tract malformations in adult rats and mice from several studies (Han et al., 2020; Liu et al., 2020; Olaniyi et al., 2020; Ren et al., 2019, reviewed in Massányi et al., 2020). Although effects on organ- and endocrine system development were indicated by the transcriptomics data generated in this work, only indirectly relevant GO terms were available for the effects on EAS-mediated pathways.

Previous studies also support the KEs connected to the transcriptomics data for PCB126. Effects of PCB126 on thyroid hormone levels in rats (Alvarez-Lloret et al., 2009; National Toxicology Program, 2006) and developmental neurotoxicity in the form of behavioral changes and effects on learning and memory (Holene et al., 1998; Piedrafita et al., 2008) have been described previously. Interestingly, several KEs related to perturbations of brain function and development have many directly and indirectly relevant GO terms connected to them. These KEs occur upstream of KEs describing impairment of learning and memory and decreased cognitive function. The transcriptomics data also revealed effects on steroid metabolism and estrogen metabolism, which PCB126 is known to affect through altered expression of CYP-enzymes (Jonsson et al., 2007; Li, 2007), likely by activating the Aryl Hydrocarbon Receptor (AhR) (Shimada, 2002). The changes in steroid and estrogen metabolism may in turn lead to reproductive toxicity, which has been observed in both male and female rats exposed to the compound (Muto et al., 2003; Wakui et al., 2007). Many GO terms related to changes in hormone levels and the metabolism of hormones were available, however only indirect connections could be made to specific KEs in the network.

Although both Cd (Margetaki et al., 2021; Ronchetti et al., 2016, 2013; Tian et al., 2020) and PCB126 (Alvarez-Lloret et al., 2009; Jonsson et al., 2007; Li, 2007; National Toxicology Program, 2006) have been shown to affect the EATS-modalities, some uncertainties regarding their endocrine activity and ED potential remain. Future studies should focus on applying this AOP network-based approach to more well-characterized EDs to provide additional insights into using this approach to facilitate the use of omics data, and further development needs. For example, known EDs for different endocrine modalities and adverse effects should be used as model substances in addition to compounds without endocrine activity, to investigate the feasibility of the method to differentiate between EDs and non-EDs. Moreover, exploring how to use omics data and AOPs to provide quantitative information on KERs should be further investigated (Jin et al., 2022) as quantitative AOPs may be required for more reliable predictions of adverse effects based on mechanistic data (Paini et al., 2022; Spinu et al., 2020; Wiklund et al., 2024). The data-driven approach to automatically connect GO terms to KEs in the AOP network revealed some pitfalls, as many KEs in the AOP network lack standardized terms such as GO terms. Therefore, less than half of the KEs could be automatically connected to GO terms, though it is expected that KEs may lack GO terms when the AOP is under development. Attaching accurate standardized terms to KEs would allow for more time-efficient and objective application of AOP networks, but it would also make sure that AOPs are findable, accessible, interoperable, and reusable (FAIR, Wilkinson et al., 2016), which increases trust in the results (Wittwehr et al., 2024). Another observation was that certain KEs that did include a GO term showed highly variable levels of detail in the included term. For example, KE ID 286 “Altered, Transcription of genes by the androgen receptor” has the overly broad GO BP term “regulation of gene expression” (GO ID 0010468) attached. While this is correct, connecting experimental transcriptomics data using this term to the KE would provide very weak evidence that it is in fact the androgen receptor that caused the change in gene expression. Instead, more specific terms such as “androgen receptor signaling pathway” (GO ID 0030521) or “regulation of androgen receptor signaling pathway” (GO ID 0060765) would improve confidence that meaningful connections are made between transcriptomics data and the AOP

network. It should be noted though, that this aspect is further discussed in the “How It Is Measured or Detected” section of this specific KEs page, but it is not included in the KE component section, which impairs machine readability.

Two recent studies have illustrated how an AOP network with extensive information on genes or GO terms related to each KE can improve subsequent analyses (Jaylet et al., 2024; Saarimäki et al., 2023). One limitation of these approaches, as well as the current work presented here, is the uncertainty introduced by manually connecting GO BP terms and KEs. Jaylet et al. and Saarimäki et al. both used a combination of computational tools and manual verification/curation to annotate KEs with relevant genes and/or gene sets. The results of the case study by Saarimäki et al. align with the results presented herein, that well-annotated AOPs improve the results of mapping data onto an AOP network. However, the annotation should preferably be performed by the AOP authors to ensure that correct standardized terms are included in each KE.

The manual approach to connect GO terms with the AOP network revealed more connections between GO terms and KEs compared to the data-driven approach, but it was significantly more time-consuming. As the AOP-Wiki grows, manually connecting GO terms and KEs will become more time-consuming, and an automatic data-driven approach is preferred. A data-driven connection will also reduce the human bias introduced by using expert judgement to manually connect GO terms and KEs. Furthermore, there were some GO terms that could not be connected to any KE in the AOP network. This indicates that there may be knowledge gaps in the AOP network related to these processes. Identifying knowledge gaps was not a focus of this work, however it has been performed recently by others (Jaylet et al., 2024).

To explore another approach to using transcriptomics data in the process of ED assessment, we directly connected GO terms to the EATS-related parameters listed in the ECHA/EFSA guidance. This was done based on expert judgment of how relevant the GO terms were to each of the parameters in the guidance. This exercise was, however, largely inconclusive, primarily because many of the EATS-related parameters are significantly broader in scope than the GO terms, resulting in several different GO terms being connected to only a few EATS-related parameters. Most GO terms were categorized as either “Fetal development (or physical development of the fetuses)”, “Brain weight or histopathology”, or “Brain morphometrics evaluation”. For PCB126 specifically, several terms could also be connected to the parameter “Steroidogenesis” and to changes in hormone levels. “Changes in hormone levels” is not a single ED parameter, but a combination of different parameters on changes in specific hormone levels that exist in the ECHA/EFSA guidance (e.g. changes in estrogen, testosterone, or thyroid hormones). These specific hormone parameters were grouped together, since most GO terms for both compounds did not indicate any specific hormone, but only included changes in “hormone” or “steroid hormone” levels and/or metabolism. For Cd, a single GO term could be connected to “pituitary histopathology” and to changes in hormone levels, respectively. These results indicate that the effects observed on fetal development and the brain could be endocrine-mediated. Similar to the AOP Wiki recommendations, one action to facilitate the use of transcriptomics data in ED assessments could be to update the guidance with relevant GO BP terms for the EATS-related parameters, e.g. attach the GO term “Pituitary gland development” (GO:0021983) to the ECHA/EFSA guidance parameter “Pituitary weight or histopathology”. This would allow data generated from omics studies to be automatically connected to specific parameters, at least as a screening procedure to identify parameters for further investigation, or to be used as supporting evidence.

In the IPA software, several upstream regulators relevant to the EATS-modalities, with significant p-value and Z-score, were revealed. These regulators provide further support that the observed changes in gene expression caused by exposure to Cd or PCB126 are a consequence of endocrine activity. Data-driven connection of upstream regulators to the AOP network based on KE object terms is in theory possible but was

not attempted in this study. As several ontologies were used to describe the KE object terms (as mentioned in section 3.1), automatically connecting the IPA upstream regulators with these would be challenging. Nevertheless, upstream regulators such as beta-estradiol, L-triiodothyronine, GPER1, or the AR (Fig. 4), indicate that changes in hormone levels or direct interactions with hormone receptors may be responsible for the differential gene expression.

The enrichment analysis and the subsequent Enrichment Maps revealed that both compounds affect endocrine pathways and adverse outcomes that are considered EATS-mediated or sensitive to EATS. Enrichment Maps provide an overview of the results of the enrichment analysis in a manner that is easy to understand, and in Enrichment Maps, many different parameters can be visualized simultaneously. By comparing the Enrichment Maps for the different concentrations of the two compounds (Table 1) it was found that some observed effects were not necessarily concentration-dependent. Certain effects were only observed in the low and medium concentrations, such as effects on cell metabolism for Cd and effects on neurodevelopment, neuronal signaling and organismal development for PCB126. Other effects were observed only in the highest concentration group for each compound, for example, effects on metabolism, nutrient transport, organismal development and cell differentiation for Cd, and nutrient transport and cell division perturbations for PCB126. These patterns raise an important point in choosing relevant concentrations for both omics studies and studies on ED-related effects. However, certain effects were observed in the low and high concentration, but not in the middle concentration, namely effects on neurodevelopment for Cd and effects on cell metabolism for PCB126. A relatively high number of DEGs was observed in the lowest dose of PCB126, which may have contributed to this observation. Although non-monotonic dose-responses have been discussed in regard to EDs (e.g. Vandenberg et al., 2012), these effects warrant further investigation with additional concentrations. Important aspects when selecting a relevant dose range for *in vivo* transcriptomic studies aiming to identify a point of departure, were discussed in a recent publication (O'Brien et al., 2025). Certain study design principles from this article may also be relevant for experiments on zebrafish embryos.

The Enrichment Maps also revealed large networks of metabolic and immune system disruptions, which could be caused either by EATS-mediated or by non-EATS mechanisms. Several relevant upstream regulators for both immune function and metabolism were also predicted in IPA, such as interleukins IL1, IL1B, IL2, and IL4, for the immune system, and 2-deoxyglucose, insulin, and PPARGC1A for metabolism. Although it is known that both the immune system (Jaeger et al., 2021; Quatrini et al., 2021; Sabuz Vidal et al., 2021) and the metabolic system (Heindel et al., 2017; Nadal et al., 2017) are interlinked with the endocrine system, these types of effects are not currently established as EATS-related parameters in guidance by the Organisation for Economic Co-operation and Development (OECD) or ECHA/EFSA. Therefore, even though the effects of both compounds on these systems were clear, elucidating these mechanistic pathways was not a focus in this work. Metabolic disruption caused by EDs has been extensively studied, for example in projects in the European Cluster to Improve Identification of Endocrine Disruptors (EURION) funded under the EU Horizon 2020 framework (Audouze et al., 2020; Küblbeck et al., 2020; Legler et al., 2020). Effects of EDs on the immune system are currently significantly less established and described.

The zebrafish whole embryo homogenate is useful for RNA-Seq but also comes with some caveats. Zebrafish embryos up to approximately five days old are considered *in vitro* in the EU (European Parliament and European Council, 2019). Zebrafish are quick and cheap to both breed and house, and they are vertebrates with functional HPG and HPT axes, which is useful for identifying endocrine disrupting properties. For the purpose of RNA-Seq, zebrafish embryos allow for global transcriptomics to be investigated easily using whole embryo homogenates. However, whole embryos yield changes in gene expression from the entire organism. This could diminish effects that only occur in certain cell

populations or tissues. In this study, several significant GO terms related to organismal development, organogenesis, and the endocrine system, were observed in the transcriptomics data. However, these effects could only be indirectly linked to many KEs in the AOP network since there was a lack of information on which specific organ or tissue was affected. To investigate this, performing single cell RNA-Seq, or RNA-Seq of sorted cells or isolated tissues could provide the link needed between more broad GO BP terms and affected tissues or organs (Li and Wang, 2021). Alternatively, a computational deconvolution method could be used with bulk RNA data, e.g. data generated using whole zebrafish embryos, to predict gene expression profiles of specific cell types without the need to manually sort cells or tissues (Avila Cobos et al., 2018).

There are several explanations for the low regulatory uptake of transcriptomics studies. For example, the methodology and the type of data from transcriptomics experiments may be unfamiliar to many risk assessors (Sauer et al., 2017). Evaluating these data also requires both statistical and computational expertise, different to the expertise required for evaluation of standardized *in vivo* and *in vitro* experiments for traditional risk assessment. Moreover, the computational methods used to analyze transcriptomics data are constantly evolving and there is currently no standardized method to analyze the data (Harrill et al., 2021). There are also uncertainties regarding the biological significance of gene expression changes and their link to more apical adverse outcomes as changes in gene expression do not necessarily reflect changes in protein levels or activation/inhibition of events further downstream. There are several aspects to be considered to increase the regulatory uptake of transcriptomics data. For example, opportunities for risk assessors to gain knowledge on omics technologies through training and capacity building would be beneficial. There is a need for methodology that integrates transcriptomics data with regulatory risk assessments, and case studies on the application of New Approach Methodologies (NAMs) in chemical risk assessments (European Chemicals Agency, 2023). Also, standardization and validation of new methods would increase the confidence in their results and increase their regulatory uptake (van der Zalm et al., 2022). Lastly, there is ongoing work to develop a transcriptomics reporting framework to increase the uptake of transcriptomics studies in regulatory assessments (Harrill et al., 2021).

The lack of relevant AOPs for some EATS-related pathways was a limitation when conducting this work. For some effects there were GO terms, but no relevant AOPs available, for example metabolic disruption, cardiovascular system development, and the connection between EAS and brain development. Indeed, there is a need to develop more AOPs in general since important pathways are missing for many toxicological effects (Jaylet et al., 2024). In addition, many AOPs in the wiki are not yet fully developed or are incomplete, lacking information about intermediate KEs or KERs. It is also important to note that most AOPs included in the AOP network here have not yet undergone peer-review or endorsement by the OECD and many AOPs lack information in several sections of their respective AOP pages. In this case, we also identified several relevant AOPs and individual KEs in the AOP wiki that were not connected to the main EATS-network and consequently not part of linking the transcriptomics data. By combining similar KEs and adding missing KERs, some of these separate AOPs and KEs could be connected to the main network, as has been done in previous studies (Wiklund et al., 2023; Ziliacus et al., 2024).

5. Conclusions

The data-driven approach to connecting transcriptomics data to the AOP network was hindered by a lack of accurate standardized terms in KEs. The manual approach yielded many more connections compared to the data-driven approach, but it was more time-consuming and sensitive to subjective conclusions. By anchoring transcriptomics data to an AOP network, EATS-related mechanisms could be identified, though it remains uncertain which specific proteins are involved. Possible EATS-related adverse outcomes or late KEs could also be identified in both

the AOP network and Enrichment Maps. The findings also were supported by the upstream regulators from IPA and are in line with results from previous research on both compounds.

Transcriptomics data from zebrafish embryos can provide evidence on both endocrine activity and on adversity through enrichment analysis. To identify effects on MIEs related to specific receptors or enzymes, specific *in silico*, *in chemico*, or *in vitro* methods may be needed in addition to the RNA-Seq. The methodology presented herein could improve the regulatory uptake of transcriptomics data for the assessment of EDs, by connecting mechanistic data to adverse outcomes. However, work is needed on harmonizing AOP development and constructing additional AOPs, to improve the approach. Also, case studies on known EDs with different endocrine modalities and adverse effects, together with compounds lacking endocrine disrupting properties, should be conducted.

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CRediT authorship contribution statement

Linus Wiklund: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Emma Wincent:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Anna Beronius:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2025.109352>.

Data availability

The RNA-Seq data is available at NCBI GEO under accession ID GSE283372. The code used to analyse the data is uploaded to GitHub, and a link is available in the Materials & Methods section.

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