Exploring mode of action for acute toxicity of primary aromatic amines with *Daphnia magna* using differential gene expression analysis

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Abstract

The diverse structures and modes of action (MOAs) of organic pollutants present a challenge in forecasting their environmental toxicity. Aromatic amines, widely used in industrial applications, are of particular interest due to their broad structural variation. Although they have been classified as polar narcotics, due to their narcotic MOA in fish, certain substitution patterns of the aromatically bound amino group exert excess toxicity towards species such as *Daphnia magna*. The underlying mechanisms bebind this phenomenon remain unclear. Therefore, we investigated how substitution patterns affect the MOA in this aquatic invertebrate. We used transcriptomics to study how three primary aromatic amines (PAAs), namely 4,4'-methylenedianiline (4,4'-MDA), 2,2'-methylenedianiline (2,2'-MDA) and 2,4-toluenediamine (2,4-TDA), affect *D. magna*. Our experimental design also included two reference compounds, 1-octanol (a non-polar narcotic) and aniline (a polar narcotic in fish, but excess toxicant in *D. magna*). Our analysis suggests very distinct biological MOAs for the three PAAs. While the *para*-substituted 4,4'-MDA treatment showed similar effects as the excess toxicant aniline, its *ortho*-substituted isomer 2,2'-MDA showed similar effects as the narcotic 1-octanol. Our results indicate a specific influence of substituent positioning in PAAs on their toxicity and MOA towards *D. magna*. This study illustrates the use of transcriptomics and its associated

functional analysis to guide industrial chemical development towards more environmentally safe alternatives.

Keywords: Primary aromatic amines, Ecotoxicity, Transcriptomics, Mode of action, *Daphnia magna*

1. Introduction

Organic pollutants, with their diverse structures and modes of action (MOAs), pose a significant challenge in understanding and predicting their environmental toxicity. Assuming that structurally similar pollutants induce toxicity through analogous MOAs, pollutants are categorized into four MOA classes: non-polar narcotics (inert chemicals), polar narcotics (less inert chemicals), reactive chemicals, and specifically acting chemicals [18, 13]. Non-polar narcotics act via a non-specific MOA and are considered baseline toxicants [18, 13]. Narcotic toxicity is assumed to be induced by perturbation of the structure and functioning of biological membranes [2]. Therefore, their toxicity is linked to hydrophobicity (log Kow) and predictable through quantitative structure-activity relationship (QSAR) models [18, 13]. Examples include chlorobenzenes, alcohols, ethers and chlorinated alkanes [18, 13]. Polar narcotics are slightly more toxic, with effect concentrations 5-10 times lower than predicted from baseline toxicity QSARs, probably due to their hydrogen bond capacities [18]. This class includes (substituted) phenols and (substituted) anilines [18]. Reactive and specifically acting chemicals exert excess toxicity with effect concentrations at least 10 times lower than predicted by baseline toxicity QSAR. Reactive chemicals have electrophilic properties and react in a specific way with nucleophile groups in macromolecules, while specifically acting chemicals target a specific cellular receptor [18].

The MOA of a chemical is associated with its interspecies variation in toxicity, where higher toxicity correlates with greater interspecies differences [17]. While structurally analogous narcotics typically exhibit limited interspecies variations, chemicals with a specific or reactive MOA display elevated interspecies variation, primarily attributed to the specific MOA in exceptionally sensitive species [17]. Such chemicals are of particular interest for further study in terms of MOA to improve our understanding of the observed elevated interspecies variation. In that respect, aromatic amines exhibit distinctive toxicity characteristics. Primary aromatic

amines (PAAs), extensively utilized as raw materials and intermediates in the production of dyes, rubber, and pharmaceuticals, are prevalent environmental contaminants, dispersing into the atmosphere, water, and soil [4]. Despite being classified as polar narcotics and in contrast to other organic pollutants, aromatic amines exhibit notable interspecies variation in their toxicity, due to the extreme sensitivity of daphnids towards them. Anilines act as polar narcotics in fish during acute exposure, yet certain substitution patterns of the amino group show excess toxicity towards *Daphnia magna* and other water fleas [5, 17, 16, 10]. The underlying mechanisms behind this phenomenon remain unclear. Possibly, certain metabolism reactions are involved, such as ring hydroxylation, N-hydroxylation or N-acetylation. Metabolic pathways, including the site of aryl hydroxylation, exhibit substantial variations among different species and daphnids might possess a unique capability to oxidize aniline metabolites into toxic reaction products compared to other species [12, 16]. Remarkably, the substitution pattern on aromatic amines affects their toxicity. Ramos et al. (2002) [12] investigated the sensitivity of *D. magna* to anilines that were substituted and/or non-substituted in the ortho-, meta- and para-positions at life history level [12]. No significant differences were found between meta- and para-substituted and non-substituted aromatic amines. All the log acute excess toxicity (log Te) values were close to the general average of 1.73 ± 0.78. However, the excess toxicity of *ortho*-substituted anilines was significantly lower than the non-substituted ones [12]. As *ortho*-substituted anilines are less toxic than their non-substituted counterparts, it is suggested that the process involved in the toxicity of aromatic amines to daphnids is sterically hindered by these substitutions [12]. Moreover, the effect of ortho-, meta- and para-substituents, on the carcinogenic potency of aromatic amines in rats and mice has been investigated [1]. It was found that in mice, substitutions in the *ortho* and *meta* positions to the amino group tend to decrease the carcinogenic potency [1].

Their wide usage makes PAAs a significant source of environmental pollutants [4], raising concerns about their environmental and health impacts. As highlighted above, their substituent positioning profoundly influences their mode of action. Therefore, it is critical to understand the relationship between their chemical structure and biological effects. Such knowledge is essential to inform the development of "safe-by-design" products that minimize ecological harm while retaining functional utility. Therefore, this study focuses on evaluating how underlying biological mechanisms of three

widely used PAAs containing different substitution patterns, i.e. 2,2'-methylenedianiline (2,2'-MDA), 4,4'-methylenedianiline (4,4'-MDA) and 2,4-diaminotoluene (2,4-TDA), affect *D. magna*. First, 4,4'-MDA is a large-scale production commodity chemical predominantly utilized as a precursor for the synthesis of 4,4'-methylene diphenyl diisocyanate (MDI). It also finds applications in the production of epoxy resins and adhesives. It is manufactured as technical-grade MDA, which comprises approximately 60% w/w of 4,4'-MDA, along with smaller quantities of its isomers, 2,4'-MDA and 2,2'-MDA, and other polynuclear amines [15]. The toxicity mechanism of 4,4'-MDA remains not fully understood, but information on structurally similar compounds suggests the involvement of a metabolic intermediate, N-hydroxymethylenedianiline [8]. The related aromatic amine toluene diamine (TDA) serves as an intermediate in the production of toluene diisocyanate (TDI) [15]. Moreover, in the recycling of polyurethane (PU), typical degradation products resulting from depolymerization include 4,4'-MDA and 2,4-TDA, which can be reused as building blocks for the production of virgin PU [3]. Acute exposure experiments were conducted using the model organism *Daphnia magna*, a widely recognized indicator species for freshwater ecosystems. The experiments included acute toxicity testing and gene expression profiling, a robust approach for identifying molecular pathways and mechanisms impacted by toxicants [2].

This research aims to provide critical insights into the MOA and associated ecological risks of PAA's to support informed decision-making regarding the use of specific isomers, paving the way for safer industrial practices and the design of environmentally sustainable products. Moreover, the study highlights the broader significance of integrating molecular biology tools into ecotoxicological assessments to enhance our understanding of pollutant impacts at the organismal and ecosystem levels.

2. Materials and methods

2.1. Chemicals

Tests were performed with three PAAs (Figure 1), namely 4,4'-MDA (CAS 101-77-9), 2,2'-MDA (CAS 6582-52-1), and 2,4-TDA (CAS 95-80-7), alongside two reference compounds, namely aniline (CAS 62-53-3) and 1-octanol (CAS 111-87-5). The compounds 4,4'-MDA (product number 31640), 2,4-TDA (product number 442311), aniline (product number 242284), and

1-octanol (product number 293245) were purchased from Sigma-Aldrich. The compound 2,2'-MDA was kindly supplied by Covestro.

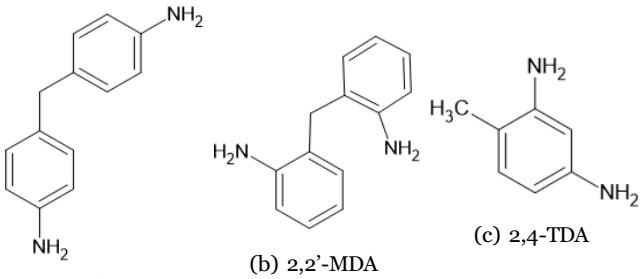


Figure 1: Chemical structures of the three primary aromatic amines investigated in this research, drawn using ChemSketch.

2.2. D. magna culture and maintenance

The *Daphnia magna* Straus K6 clone used in this study was sampled in a pond in Kallo (Antwerp, Belgium) in 1983 and has been cultivated in house ever since. It has been parthenogenetically cultured in a modified COMBO [6] medium, adapted to represent more environmentally relevant conditions (55 mg $L^{-1}CaCl_2.2H_2O$, 55.5 mg L^{-1} $MgSO_4.7H_2O$, 1 mg L^{-1} H_3BO_3), and without the addition of nitrogen and phosphorus. Culture media were aerated and allowed to equilibrate for at least 24 h before use. Animals were transferred to clean medium twice a week. Animals were kept in a climate-controlled room at 20 °C and a 16:8-h light:dark cycle at a density of 50 daphnids L^{-1}. Daphnids from this stock were daily fed an algae mix composed of *Pseudokirchneriella subcapitata* (Korshikov) F.Hindák, 1990 and *Chlamydomonas reinhardtii* P.A.Dangeard, 1888 at a 3 to 1 cell number ratio. This algae mix was given at concentration of $3.75 * 10^6$ cells daphnid^{-1} day^{-1} from day 0 to day 8 and $7.5 * 10^6$ cells daphnid^{-1} day^{-1} from day 9 to day 21 of their life.

2.3. Determination of effect concentrations

2.3.1. ECOlogical Structure-Activity Relationships (ECOSAR) modeling

Quantitative structure-activity relationships (QSARs) were employed to predict the aquatic toxicity of the chemicals using the ECOlogical Structure-Activity Relationship (ECOSAR) Model Class Program (v2.2), accessible from the Environmental Protection Agency's (EPA) website

(https://www.epa.gov/tsca-screening-tools/ecological-structure-activity-relationships-ecosar-predictive-mode). The program determines the relevant ECOSAR class(es) and calculates ecotoxicity using a log Kow value. We reported the predicted EC50 values at 48 h for daphnids. Moreover, an excess toxicity factor (Te) was computed for each chemical as the ratio of the EC50 in the "Neutral Organics" class predicted by ECOSAR and the EC50 values in the other classes.

2.3.2. Experimental assays

Prior to the gene expression experiment, a series of acute immobilisation tests were performed with the PAAs and reference compounds. A standard 48-h *Daphnia magna* immobilisation test was conducted according to OECD guideline No. 202 [9] in constant incubation conditions (Temperature of 20°C ± 1°C; 16-h light and 8-h dark cycle). At the start of the test, daphnids were less than 24 h old and, to reduce variability, the animals were third brood offspring. The test was conducted in COMBO medium, with the same composition as the culture medium, in glass vessels of 25 mL. The daphnids were not fed during the test. Temperature, pH and dissolved oxygen of the medium were measured at the beginning and the end of the test. A control (i.e. blank, 3 replicates) and five test concentrations in triplicate for each compound were tested with 10 juveniles added in each glass vessel. The stock solution of two compounds, i.e. 2,2'-MDA and 4,4'-MDA, was made in ethanol, from which a serial dilution series in COMBO medium was made before exposure. As the highest concentration of ethanol exposed during the test was 0.2% and 0.008% for 2,2'-MDA and 4,4'-MDA, respectively, it was considered as negligibly low. However, an additional ethanol control was still added to account for potential solvent effects. For all compounds, tested concentrations and full experimental design can be found in Table S1. Immobility of the daphnids was registered at 24 and 48 h. At the end of the test, the observed mortality data provided insight into the toxic concentrations and whether *D. magna* showed comparable sensitivity to the toxicity data obtained with ECOSAR, aiding in the design of the subsequent gene expression experiment.

2.4. Experimental design for gene expression analysis

In the gene expression experiment, each time 50 third brood juveniles, less than 24 h old, were exposed to 4,4'-MDA (at two different concentrations), 2,2'-MDA, 2,4-TDA, the reference compounds 1-octanol and aniline and

a negative control. The stock solutions of 4,4'-MDA and 2,2'-MDA were prepared in ethanol, resulting in ethanol concentrations in the final exposure solutions of less than 0.1%. Given these low ethanol concentrations, we anticipated minimal impact on gene expression. However, to confirm this assumption, we included an additional solvent control containing 0.1% ethanol.

For each treatment, nine glass Weck jars, filled with 250 mL of COMBO medium, were set up, containing 50 individuals each. This resulted in a total of 72 experimental units. The same incubation conditions as described in the range-finding test were used for this experiment, except that here the animals were fed daily with $2 * 10^5$ cells of *P. subcapitata* per mL of medium. Animals were specifically fed during exposure to avoid gene expression signatures of starvation stress as confounding factors in the design. After 24, 48 and 96 h of exposure, each time three Weck jars were taken out of the experiment to use for RNA extraction. Surviving animals in each jar were counted and pooled (per jar) for RNA extraction.

Exposure concentrations of the test chemicals were analytically verified by EHS Analytics (Table S2). The analytical procedure for the aromatic amines involved extraction, derivatization with pentafluoropropionic anhydride, and subsequent analysis using Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LC-MS-MS) according to Analytica Chimica Acta 510 (2004) 109-119, with modification for aqueous matrix without prior hydrolysis. The limit of quantification (LOQ) was 0.05 µg/mL for MDA and TDA, and 0.02 µg/mL for aniline. The analysis of 1-octanol was subcontracted to RPS analyse B.V. (Breda, The Netherlands). In brief, 1-octanol was analyzed using Gas-Chromatography coupled with Mass spectrometry with an LOQ of 5 µg/L. As the analytically measured concentrations were significantly lower than our nominal concentrations, the reported concentrations in this paper are based on the analytical measurements. Concentrations were measured at the start of the experiment and after 48 h of exposure. For all compounds, except 1-octanol, the measured concentrations after 48 h were maintained within ± 20 percent of the initial concentration. As 1-octanol decays over the 48 h time period, we decided to calculate and report all measured concentrations in terms of the time-weighted mean over these 48 h, as specified in OECD guideline 211, ANNEX 6. Thereby, a time-weighted mean in our case is calculated as:

Table 1: The measured concentrations of the primary aromatic amines and 1-octanol exposed to the daphnids during the gene expression experiment. The concentrations are based on analytical measurements at the start and after 48 h of our gene expression experiment and calculated as a time-weighted mean.

Compound	Concentration (mg/L)
4,4'-MDA	0.27
4,4'-MDA	0.14
2,2'-MDA	7.96
2,4-TDA	1.20
aniline	0.11
1-octanol	0.08

$$concentration = \frac{conc0 - conc1}{ln(conc0) - ln(conc1)}$$

The calculated concentrations of the PAA's and 1-octanol exposed to the daphnids during the gene expression experiment are summarized in Table 1.

2.5. RNA extraction, library preparation and sequencing

Total RNA was extracted from the (pooled) surviving daphnids from each Weck jar using the Qiagen RNeasy kit and Qiashredder according to the manufacturer's instructions (Qiagen, Antwerp, Belgium). The RNA quantity and quality was assessed using the NanoDrop™ spectrophotometer (Version 2000c, Thermo Scientific™, Thermo Fisher Scientific) with quality criteria of 260/280 ratio between 1.8 and 2.0, and 260/230 ratio between 2.0 and 2.2. Of 65 samples with sufficient quality, 10 µL of RNA from each sample was delivered to the NXTGNT lab (Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium) for library preparation and next-generation sequencing. For library preparation, 250 ng of RNA was utilized, and the QuantSeq 3' mRNA-Seq Library Prep Kits (Lexogen, Vienna, Austria) were employed following the manufacturer's protocol, with 14 PCR cycles during library preparation. Library quantification was performed by qPCR, following Illumina's 'Sequencing Library qPCR Quantification protocol guide' from February 2011. The resulting libraries' size distribution, purity (absence of free adaptors), and quality were assessed using a High Sensitivity DNA chip (Agilent Technologies, Santa Clara,

CA, USA). Finally, sequencing was carried out on a high-throughput Illumina NextSeq500 flow cell, generating 75 bp single reads. All sequencing data was deposited in NCBI database, available under accession number PRJNA1032585. Treatment with 4,4'-MDA and 2,2'-MDA caused significant mortality of *D. magna* at 96 h, resulting in fewer replicas for RNA extraction and sequencing. Therefore, these treatments were not considered at 96 h, as they might be too biased given the limited number of surviving individuals (Table S3).

2.6. Bioinformatics and data analysis

Figure S1 schematically presents the overall mRNA-Seq data analysis workflow.

2.6.1. Trimming and mapping of the RNA sequencing reads

Reads were adapter and quality trimmed with Trimmomatic (version 0.38-Java-1.8). To verify the removal of adapter-derived and low-quality sequences, the fastq files were inspected with FastQC (version 0.11.9-Java-11) both before and after trimming. The resulting reports were summarized using multiQC (version 1.14-foss-2022a). Reads were aligned to the *D. magna* reference transcriptome [11] using Salmon (version 1.4.0-gompi-2020b with parameters -l U –validateMappings –useVBOpt –numBootstraps 100 –seqBias –gcBias) to produce estimated counts per transcript. The reference transcriptome was indexed with a k-mer size of 25.

2.6.2. Transcript-level analysis

A transcript-level analysis between groups of samples was done in R (version 4.3.1) with edgeR (version 3.42.4). To obtain the transcript-level counts, the Salmon output was read directly into edgeR with the catchSalmon function, using the bootstrap replicates to calculate and apply an overdispersion correction for each count.

Transcripts with very low counts across all libraries were filtered out using the edgeR function filterByExpr with its default arguments. Briefly, this function retains transcripts if their counts per million (CPM) meet or exceed a calculated threshold in at least a minimum number of samples. Thereby, the CPM cutoff is defined as $\frac{10}{\text{median of the library size}} * 10^6$, and the minimum sample count corresponds to the smallest group size, which was three in this study. Additionally, each transcript requires a minimum total count of 15 reads across all samples.

The data was then split into three subsets of data, one for each time point. For each subset of data, the design matrix was constructed with the negative control as the reference group at that time point. The read counts were normalized using the trimmed mean of M-values (TMM) method. Exploratory unsupervised clustering analysis of gene expression based on logFC was done by construction of multidimensional scaling (MDS) plots at every time point. Differential transcript expression (DTE) analysis was performed using the edgeR quasi-likelihood pipeline. Significant differentially expressed (DE) transcripts were called at an adjusted p-value below 0.05 and without a log2 fold change cutoff.

2.6.3. Analysis of transcript lists

The annotation information on each gene was obtained from the published *Daphnia magna* transcriptome [11].

Heatmaps were constructed with the heatmap.2 function from the R package gplots (version 3.1.3). Venn diagrams were constructed with the venn.diagram function from the R package VennDiagram (version 1.6.0).

Gene set enrichment analysis (GSEA) was done with the mroast function from the limma R/Bioconductor package (version 3.34.2). Enrichment was considered significant when false discovery rates were below 0.05.

3. Results

3.1. Ecotoxicity of the tested primary aromatic amines

The ECOSAR modeling results are summarized in Table 2. Several chemicals exhibited structural features aligning with multiple ECOSAR classes. The excess toxicity factor (Te) (Table 2) surpassed 10 for 4,4'-MDA, 2,4-TDA, and aniline, suggesting an excess toxicity [18]. Conversely, for 2,2'-MDA and 1-octanol, the Te was below 10, indicative of a narcotic MOA [18]. These results are in line with our immobilization experiment in Figure 2, where immobility of the daphnids was observed after 24 and 48 h (Figure 2). Aniline and 4,4'-MDA were the most toxic, followed by 2,4-TDA, with 2,2'-MDA and 1-octanol being least toxic.

Table 2: Toxicity values for the chemicals tested in this study obtained through ECOSAR modeling. Morever, the table includes the log Kow (given by ECOSAR) and the calculated excess toxcicity factors (Te) (EC50 in the "Neutral organics" class predicted by ECOSAR divided by the EC50 in the other classes) for each chemical. NA, not available

Chemical	log Kow estimated	log Kow measured	EC50 ECOSAR				Te
			Neutral organics	Anilines (Hindered)	Anilines (Unhindered	Anilines (Amino meta)	
4,4'-MDA	2.18	1.59	64.6		1.85		34.92
2,2'-MDA	2.18	NA	64.6		19.6		3.30
2,4-TDA	0.16	0.14	2180	215	3.77	3.45	10.14 / 578.25 / 632
Aniline	1.08	0.9	270		1.67		161.68
1-Octanol	2.81	3	12.4				

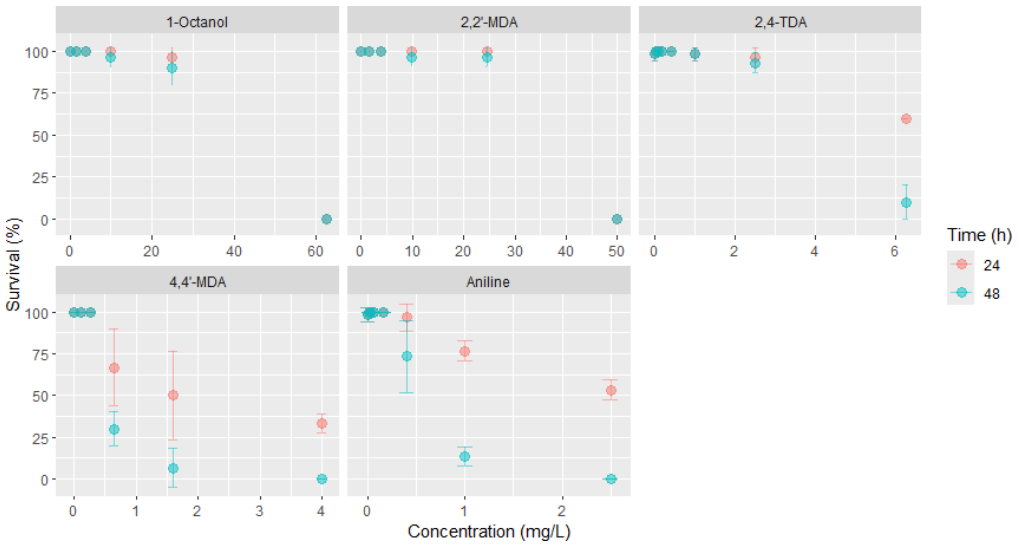


Figure 2: Survival of *Daphnia magna* after 24 and 48 h of exposure to the primary aromatic amines and reference compounds. Error bars represent standard deviation.

3.2. Differential gene expression

To elucidate the molecular mechanisms underlying toxicity through RNA sequencing, *D. magna* were exposed to the PAAs and reference compounds at concentrations summarized in Table 1, as well as a solvent control (0.1% ethanol) and a negative control containing medium only. The initial objective was to expose the daphnids to concentrations approximating the EC10 for all PAAs and both the EC10 and EC50 for 4,4'-MDA. However, the analytically

determined concentrations deviated substantially from the nominal values, likely due to factors such as insolubility. Consequently, only the measured concentrations, representing the actual exposure levels, are reported in Table 1. Additionally, the life-history traits of the samples were monitored (Table S3, Figure 3). Survival remained above 75% across all treatments after 48 hours, except at the highest test concentration of 4,4'-MDA, as anticipated.

RNA was extracted after 24, 48 and 96 h of incubation. After 96 hours, 4,4'-MDA and 2,2'-MDA resulted in mortality rates that were too high to be used for further RNA sequencing, and were therefore excluded from consideration. On average, $7.6 * 10^6 \pm 1.5 * 10^6$ reads were generated per sample with an average read length of 75 base pairs.

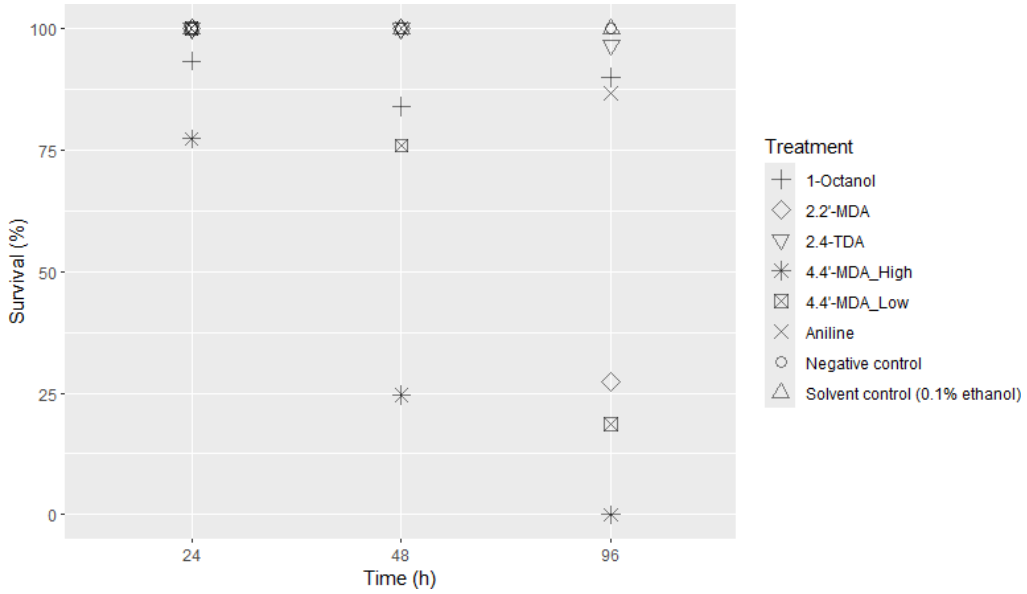


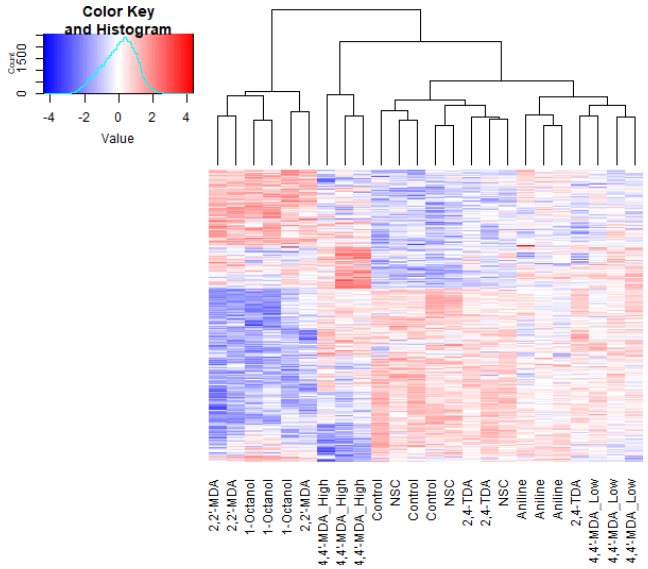
Figure 3: Survival of *Daphnia magna* during the gene expression experiments.

After trimming and mapping, a filter to remove low counts resulted in removal and conservation of 14422 and 14702 transcripts, respectively. As there were no DE transcripts between the solvent negative control and the negative control at any time point, we concluded that the low solvent concentrations did not affect the gene expression.

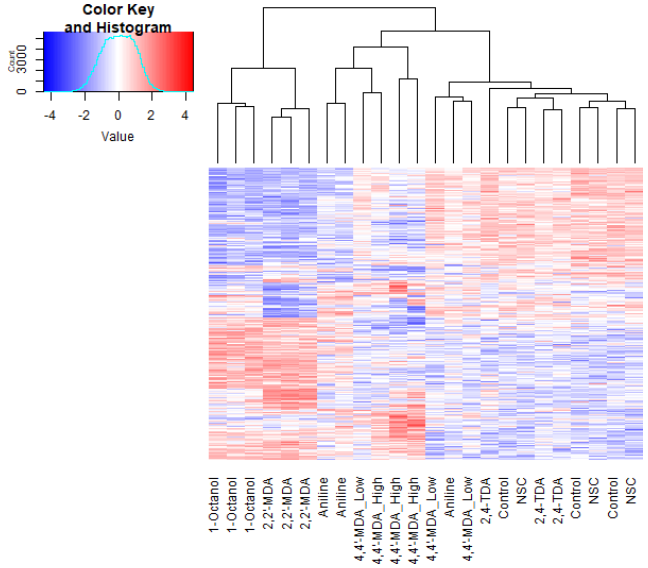
All RNA samples were clustered in two dimensions using multi-dimensional scaling (MDS) plots (Figure S2) for each time point. At 48 h, 2,2'-MDA and 1-octanol treatment samples each formed a distinct cluster,

separated from the other treatments. At 96 h, aniline and 1-octanol samples formed distinct clusters, while the negative control, solvent control and 2,4-TDA samples clustered together.

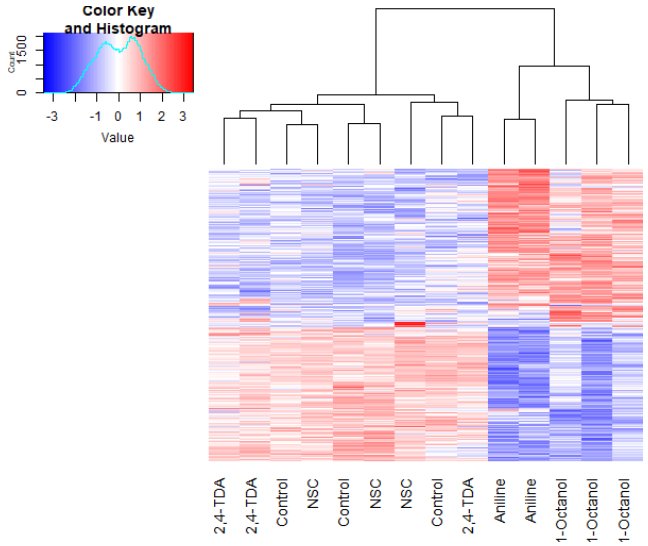
At time point 24, 48 and 96 h, a total of 2633, 6997 and 5183 transcripts, respectively, out of the 14702 transcripts, were identified as DE in at least one of the chemical treatments. These DE transcripts were sorted by their p values and used to construct heatmaps (Figure 4). Treatments 2,2'-MDA and 1-octanol clustered together at 24 and 48 h, as did 2,4-TDA and the negative solvent control at each time point. Moreover, 4,4'-MDA, at both concentrations, and aniline clustered together at time point 48 h.



(a) 24 hours



(b) 48 hours



(c) 96 hours

Figure 4: Heatmaps illustrating the differentially expressed genes across all treatments for the three tested time points, i.e. 24 (a), 48 (b) and 96 (c) h of exposure. Each row represents the relative expression (Z-score) of that transcript for each sample. Sample names are denoted by the chemical treatment, followed by a replicate number. MDA, methylenedianiline; TDA, diaminotoluene; NSC, negative solvent control

3.2.1. Pairwise differences: exposure groups vs their control

As 4,4'-MDA and 2,2'-MDA treatments contained ethanol as solvent, they were compared to the negative solvent control, while the other treatments were compared to the negative control containing medium only. Figure 5 presents an overview of the DE transcripts observed for each treatment in comparison to their control across all time points. With the exception of 2,4-TDA, all treatments induce DE transcripts relative to their control at each time point. 2,2'-MDA and 1-octanol emerge as the most potent inducers of DE transcripts.

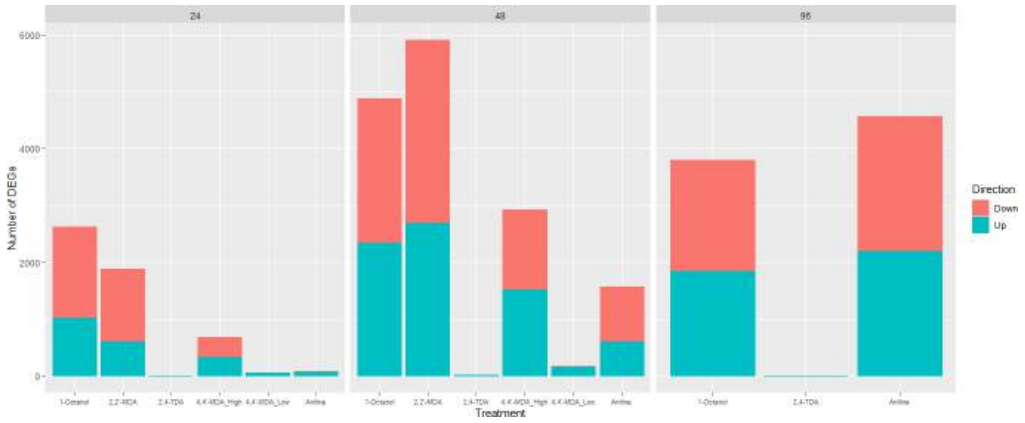
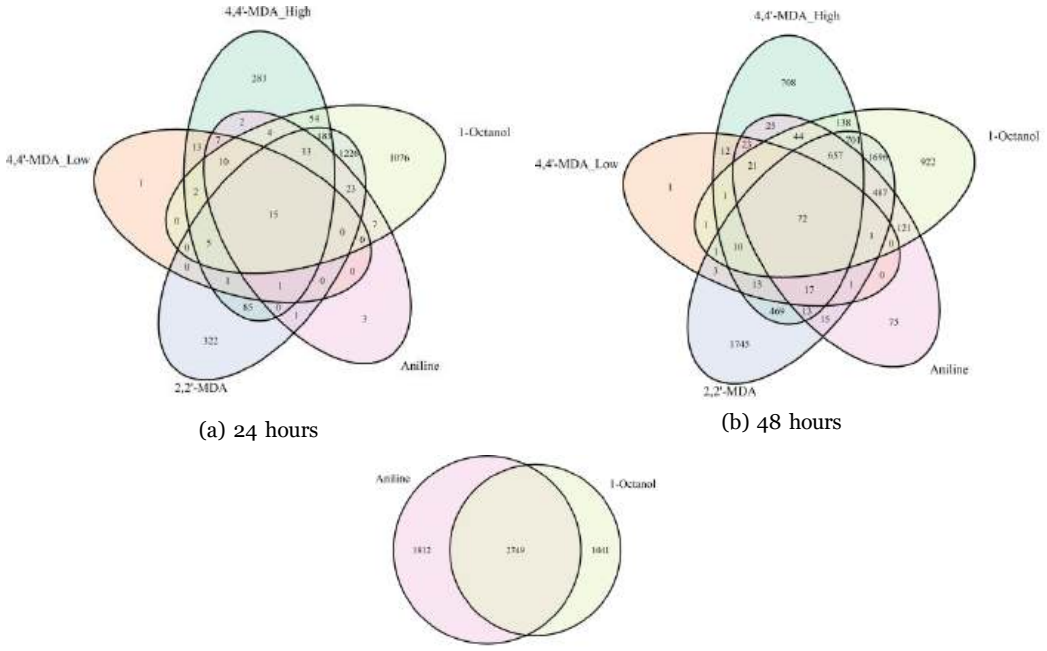


Figure 5: Number of differentially expressed transcripts induced by each treatment compared to their respective negative control at each time point (24, 48, and 96 hours).

Figure 6 displays Venn diagrams of the DE transcripts of each treatment compared to their control at every time point. At 24 h (Figure 6a), 4,4'-MDA at its low concentration and aniline share almost all their DE transcripts with at least one of the other treatments and their intersection is 0. At 48h (Figure 6b), there is only one treatment-specific DE transcripts for 4,4'-MDA at EC10 and the intersection with aniline is 0.



(a) 24 hours

(b) 48 hours

(c) 96 hours

Figure 6: Venn diagrams illustrating the number of shared differentially expressed transcripts between the treatments compared to the solvent control after 24 (a), 48 (b) and 96 (c) h of exposure. Note that 2,4-TDA is not included in any of the Venn diagrams, as it barely induces any differential expression.

3.2.2. Pairwise differences between 4,4'-MDA and the polar narcotic aniline, and between 2,2'-MDA and the non-polar narcotic 1-octanol

Based on the heatmaps, 4,4'-MDA and aniline elicit analogous gene expression effects in *D. magna*, as do 2,2'-MDA and 1-octanol. Therefore, the differential expression between 4,4'-MDA and aniline on the one hand, and 2,2'-MDA and 1-octanol on the other hand was determined. There were no DE transcripts between 4,4'-MDA at its low concentration and aniline at 24 h of exposure, and only 23 (8 down- and 15 upregulated) DE transcripts at 48 h. For 2,2'-MDA versus 1-octanol, there was one DE transcript at 24 h, but 639 (285 down- and 354 upregulated) at 48 h.

3.3. Functional analyses: KEGG pathways, KEGG orthologies and gene families

A gene set enrichment analysis (GSEA) of KEGG pathways and gene families of each treatment versus the negative solvent control was done at every time point (significantly enriched gene sets (FDR < 0.05) in Supporting information 2). Many families and KEGG pathways were significantly enriched. The KEGG pathways were subdivided in categories according to the KEGG pathway database. As the 48 h time point displayed the most DEGs, we only present the results at this time point in Figure 7.

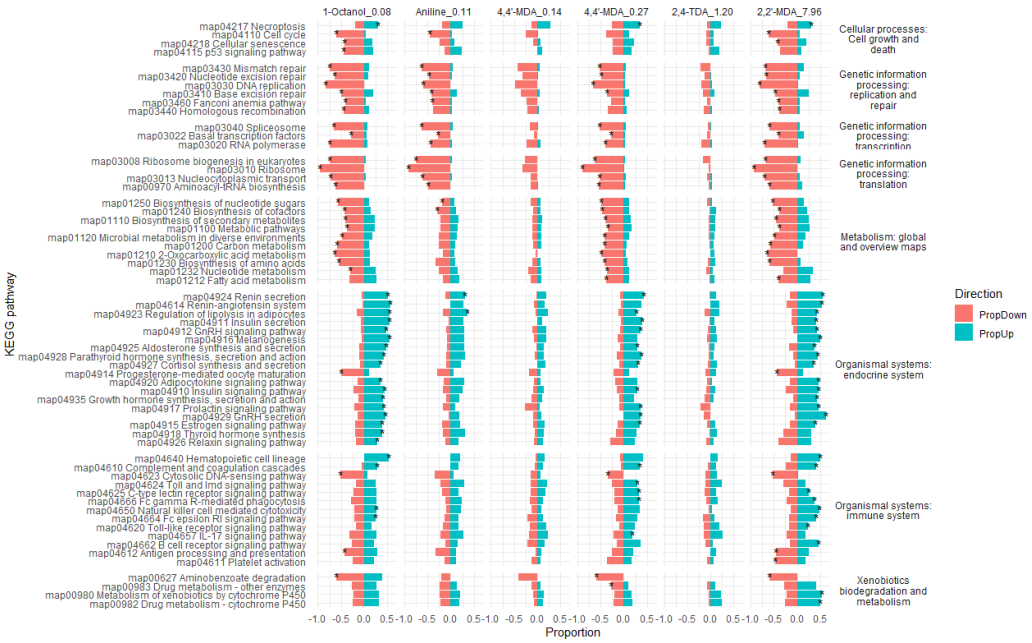


Figure 7: KEGG pathways significantly up- or downregulated (FDR < 0.05) within the specified categories by at least one treatment are shown. The proportion of up- and downregulated genes ($z > \sqrt{2}$ and $z < -\sqrt{2}$, respectively) for each pathway and treatment is visualized. If the pathway up- or downregulation was significant for a specific treatments, it is marked with an asterisk.

Overall, the enrichment profiles of 1-octanol, aniline, high-concentration 4,4'-MDA, and 2,2'-MDA show substantial similarity (Figure 7 for a subset of the KEGG pathways and Venn diagram in Figure 8). Notably, 4,4'-MDA demonstrates a clear concentration-dependent effect. The low concentration shows non-significant enrichment, albeit with a trend resembling that of the

higher concentration, whereas the high concentration results in significant enrichment comparable to 1-octanol and 2,2'-MDA (Figure 7).

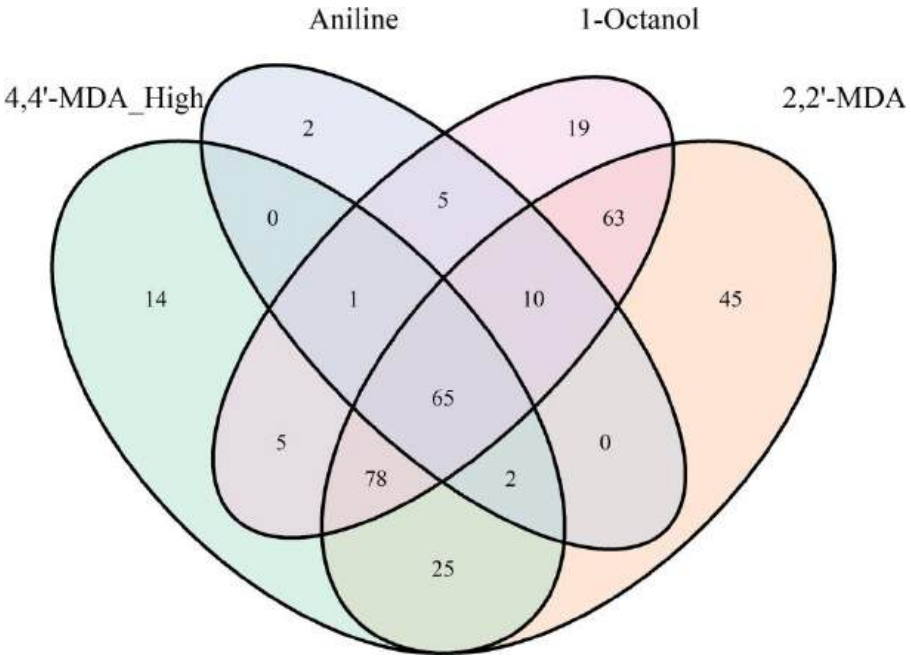


Figure 8: Venn diagram for the KEGG pathways significantly up- or downregulated (FDR < 0.05) by the treatments at 48 h.

At 48 h, 2,2'-MDA, 4,4'-MDA at its high concentration and 1-octanol induce a global suppression of metabolic processes, as reflected by the significant downregulation of KEGG pathways such as *Metabolic pathways* and *Biosynthesis of nucleotide sugars, cofactors, secondary metabolites* etc. Moreover, transcription and translation pathways are downregulated by 1-octanol, aniline, 4,4'-MDA at its high concentration and 2,2'-MDA, as are pathways involved in genetic information processing.

2,2'-MDA is the only treatment that significantly upregulates the genes in the pathways *Metabolism of xenobiotics by cytochrome P450* and *Drug metabolism - cytochrome P450*. The upregulation of metabolism of xenobiotics by cytochrome P450 suggests that the chemical is recognized as a foreign compound, triggering detoxification pathways to process and eliminate it. The cytochrome P450 system plays a key role in metabolizing

xenobiotics and may also generate reactive metabolites that contribute to oxidative stress.

Looking at the *Cellular processes: cell growth and death* pathways, we see a downregulation of the *Cell cycle* pathway by 1-octanol, aniline, 4,4'-MDA at its high concentration and 2,2'-MDA. Simultaneously, the *Necroptosis* pathway is upregulated by 1-octanol, 4,4'-MDA at its high concentration and 2,2'-MDA. Necroptosis is a programmed form of necrosis.

4. Discussion

We investigated how the substituent positioning of three PAAs, namely 4,4'-MDA, 2,2'-MDA and 2,4-TDA, drives toxicity and MOA in *Daphnia magna*. Despite their structural similarities, they exerted a distinct toxic potency.

Toxicity effect concentrations were estimated with ECOSAR and experimentally verified. ECOSAR calculated the baseline toxicity, in which it is assumed that the chemical acts via a narcotic MOA ("Neutral organics" class), but was also able to classify some of the chemicals into other toxicity classes, thereby calculating adjusted EC50 values. The latter outcomes more closely aligned with our experimental findings. In our computation of the excess toxicity factor (Te) (the EC50 obtained with ECOSAR modeling for the "Neutral organics" class divided by the EC50 in the other classes), distinct excess toxicity was evident for 4,4'-MDA, 2,4-TDA, and aniline, whereas 2,2'-MDA and 1-octanol exhibited no such excess toxicity.

The chemical 4,4'-MDA, substituted in the *para* positions, demonstrated a comparable EC50 value to the excess toxicant aniline, along with a parallel impact on gene expression. Conversely, its isomer, 2,2'-MDA, substituted in the *ortho* positions, exhibited significantly lower toxicity, sharing a similar EC50 and gene expression profile with the nonpolar narcotic 1-octanol. Finally, the chemical 2,4-TDA, characterized by a singular aromatic ring and substitution in both the *ortho* and *para* positions, demonstrated an experimental toxicity intermediate to those of 4,4'-MDA and 2,2'-MDA.

The heightened susceptibility of daphnids to specific aromatic amines may be attributed to bioactivation reactions, resulting in the formation of more toxic reaction products compared to other species [12]. Aromatic amines became recognized as procarcinogens that undergo competing bioactivation and/or detoxification reactions [19]. Figure 9 shows a general proposed model of aromatic amine bioactivation and detoxification [19]. The

bioactivation reactions include enzymatically catalyzed N-hydroxylation and O-esterification of the exocyclic amine group [19, 14]. The resulting ester conjugate is unstable and readily decomposes in a reactive electrophile, the arylnitrenium ion, which is the critical metabolite implicated in toxicity and DNA damage [19, 14].

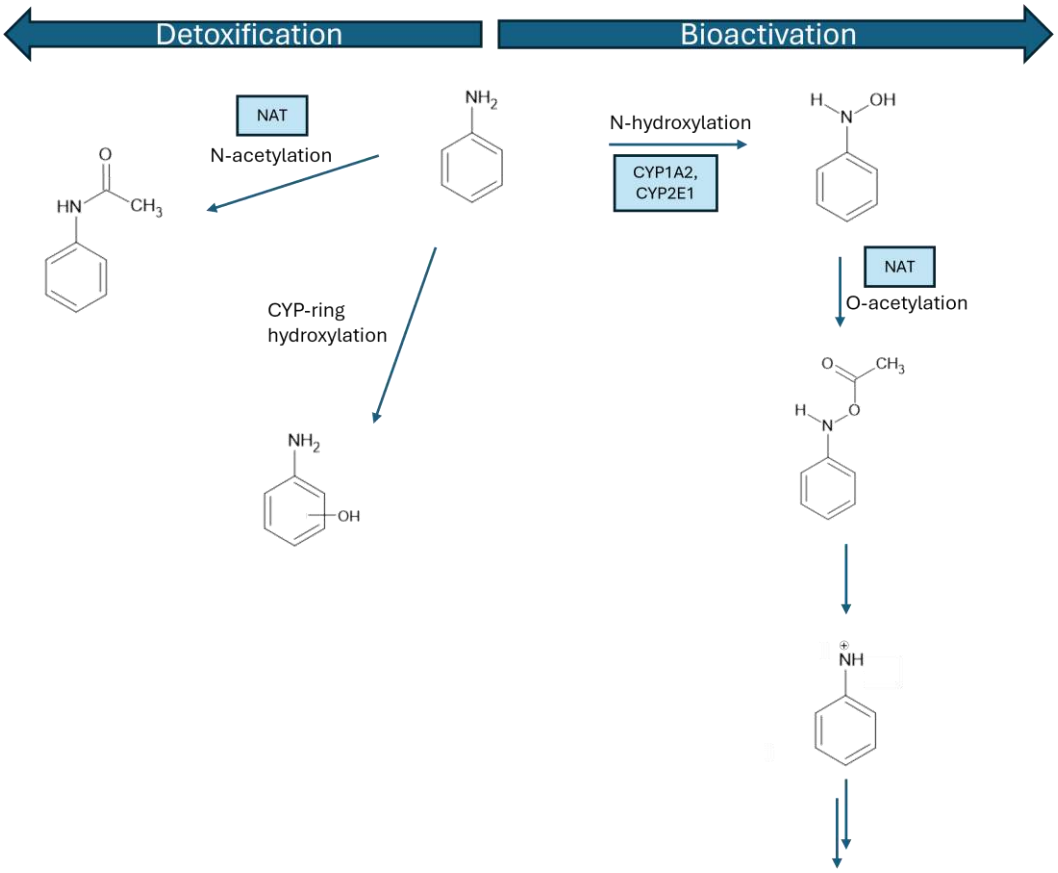


Figure 9: Reactions of biological activation and detoxification of aniline. Not that only a subset of the known metabolic pathways are shown, since other enzymes may also play a role. Adapted from Wang et al. 2019 [19].

Ramos et al. (2002) [12] investigated the impact of substituent positioning in anilines, encompassing various chlorinated, bromo, trifluoro, alkyl, chloromethyl, and chloronitroanilines, on excess toxicity towards *D. magna*. Their findings indicated lower excess toxicity in cases of *ortho* substitutions [12], aligning with our observations. 2,4-TDA, possessing one

aromatic ring and both *ortho* and *para* substitutions, demonstrated lower toxicity than aniline (Table 2), a characteristic that may be attributed to its *ortho*-substituent. It is pertinent to note that Ramos et al. (2002) [12] exclusively examined amines with a single aromatic ring, whereas our study encompassed aromatic amines with both one and two (unconjugated) aromatic rings. In the context of two-aromatic ring compounds, the *ortho*-substituted 2,2'-MDA, containing two *ortho*-substitutions, even exhibited no discernible excess toxicity in *Daphnia magna*. Considering our results, the results of Ramos et al. (2002) [12] and Figure 9, we could explain the order of toxic potencies observed for our aromatic amines in Figure 10.

Regarding the KEGG pathway gene set enrichment analyses, we found that the observed effects depend on the concentration, as was clearly indicated for 4,4'-MDA. Including a well-known narcotic chemical, 1-octanol, allowed us to see the similarities in enriched KEGG pathways between the PAAs and 1-octanol. This indicates that the PAAs are not specifically acting chemicals, targeting a specific cellular receptor, typically for Verhaar class 4 chemicals [18]. However, we conclude that their substituent positioning affects bioactivation reactions, in which highly reactive compounds can be more easily formed for 4,4'-MDA, but not for 2,2'-MDA. These bioactivation reactions produce reactive chemicals (Verhaar class 3 [18]), with electrophilic properties that react in a specific way with nucleophile groups in macromolecules. The substituent positioning significantly affects formation and reactivity of intermediates and therefore the toxic potency of the compounds. Bioactivation reactions are catalyzed by various enzymes (Figure 9) and variations in cytochrome P450 enzyme distribution and function between animal groups results in a differential metabolism and elimination kinetics of PAAs and other compounds. Koenig et al. (2012) [7] have proven a marked difference in the presence and activities of CYP isoforms between fish and the crustacean *Aristeus antennatus*. There is a need to further investigate quantitative and qualitative differences in xenobiotic metabolism among animal groups.

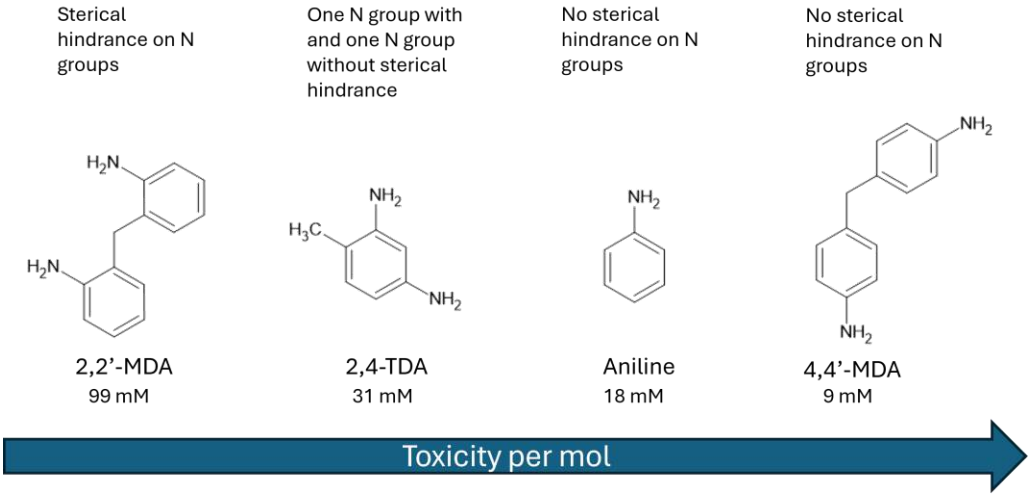


Figure 10: The toxicity per mol of the molecules examined in this study.

5. Conclusion

This study provides a comprehensive investigation into the MOA of PAAs using transcriptomics, demonstrating that environmental toxicity and MOA are influenced not only by chemical class but also by the type and precise positioning of substituents.

Our findings reveal that PAAs can exhibit either narcotic effects or excess toxicity (reactive chemicals), depending on substituent arrangement. Specifically, 2,2'-MDA, with *ortho* substituents, displayed narcotic toxicity, whereas its isomer 4,4'-MDA, with *para* substituents, as well as 2,4-TDA and aniline, exhibited an excess toxicity.

Based on literature search, our toxicity experiments and transcriptomic data, we classify 4,4'-MDA, aniline and 2,4-TDA as Verhaar class 3 reactive chemicals, due to bioctivation reactions, in *Daphnia magna*. We propose that their toxic potency can be predicted by how easily they can become bioactivated, which depends on their type and positioning of substituents. This work underscores the potential of transcriptomic studies to support safe and sustainable design approaches in chemistry.

6. Acknowledgements

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and methylenediphenyl diisocyanate. Any opinions expressed herein are those of the authors, not necessarily of the Institute or its member companies. The authors are grateful to Jolien Depecker, Emmy Pequeur, Sylvie Decraene, and Ellen De Meester for their technical contributions. The authors extend their gratitude to Marianne Van den Hove for the administrative support. Computational resources, facilitated through the Stevin Supercomputer Infrastructure, and services used in this study were made possible by the VSC (Flemish Supercomputer Center), which receives funding from Ghent University, FWO, and the Flemish Government's department of EWI.

7. Supporting Information

The following files are available free of charge.

- Supporting information: Additional figures and tables, including detailed experimental results, gene set enrichment analysis results, gene expression patterns etc
- Supporting information 2: Overrepresentation analyses of KEGG pathways, KEGG Orthology numbers and families

References

[1] Romualdo Benigni et al. "Structural motifs modulating the carcinogenic risk of aromatic amines". In: Environmental and molecular mutagenesis 50.2 (2009), pp. 152–161.

[2] Nathalie Dom et al. "Physiological and molecular effect assessment versus physico-chemistry based mode of action schemes: Daphnia magna exposed to narcotics and polar narcotics". In: Environmental science & technology 46.1 (2012), pp. 10–18.

[3] Jörg Eberz et al. "Selective separation of 4, 4'-Methylenedianiline, Isophoronediamine and 2, 4-Toluenediamine from enzymatic hydrolysis solutions of polyurethane". In: Solvent Extraction and Ion Exchange 41.3 (2023), pp. 358–373.

[4] Saba A Gheni et al. "Toxicity, hazards, and safe handling of primary aromatic amines". In: ACS Chemical Health & Safety 31.1 (2023), pp. 8–21.

[5] Gary W Holcombe et al. “Simultaneous multiple species testing: acute toxicity of 13 chemicals to 12 diverse freshwater amphibian, fish, and invertebrate families”. In: Archives of Environmental Contamination and Toxicology 16 (1987), pp. 697–710.

[6] Susan S Kilham et al. “COMBO: a defined freshwater culture medium for algae and zooplankton”. In: Hydrobiologia 377.1-3 (1998), pp. 147–159.

[7] Samuel Koenig, Pilar Fernández, and Montserrat Solé. “Differences in cytochrome P450 enzyme activities between fish and crustacea: relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs)”. In: Aquatic toxicology 108 (2012), pp. 11–17.

[8] Fernando Llados and Zemoria A Rosemond. “Toxicological profile for methylenedianiline”. In: (1998).

[9] Test No OECD. “202: Daphnia sp”. In: Acute Immobilisation Test 619 (2004).

[10] Peter C von der Ohe et al. “Structural alerts a new classification model to discriminate excess toxicity from narcotic effect levels of organic compounds in the acute daphnid assay”. In: Chemical research in toxicology 18.3 (2005), pp. 536–555.

[11] Luisa Orsini et al. “Daphnia magna transcriptome by RNA-Seq across 12 environmental stressors”. In: Scientific data 3.1 (2016), pp. 1–16.

[12] Eñaut Urrestarazu Ramos, Manon A Vaal, and Joop LM Hermens. “Interspecies sensitivity in the aquatic toxicity of aromatic amines”. In: Environmental toxicology and pharmacology 11.3-4 (2002), pp. 149–158.

[13] Erwin WM Roex et al. “Ratios between acute aquatic toxicity and effects on population growth rates in relation to toxicant mode of action”. In: Environmental Toxicology and Chemistry: An International Journal 19.3 (2000), pp. 685–693.

[14] Robert J Turesky and Loic Le Marchand. “Metabolism and biomarkers of heterocyclic aromatic amines in molecular epidemiology studies: lessons learned from aromatic amines”. In: Chemical research in toxicology 24.8 (2011), pp. 1169–1214.

[15] Elif Unterberger-Henig. “Comparative evaluation of three methylene dianiline isomers in the bacterial reverse mutation assay, the in vitro gene mutation test, and the in vitro chromosomal aberration test”. In: Toxicology and Industrial Health 38.9 (2022), pp. 529–543.

[16] Manon Vaal et al. “Pattern analysis of the variation in the sensitivity of aquatic species to toxicants”. In: Chemosphere 35.6 (1997), pp. 1291–1309.

[17] Manon Vaal et al. “Variation in the sensitivity of aquatic species in relation to the classification of environmental pollutants”. In: Chemosphere 35.6 (1997), pp. 1311–1327.

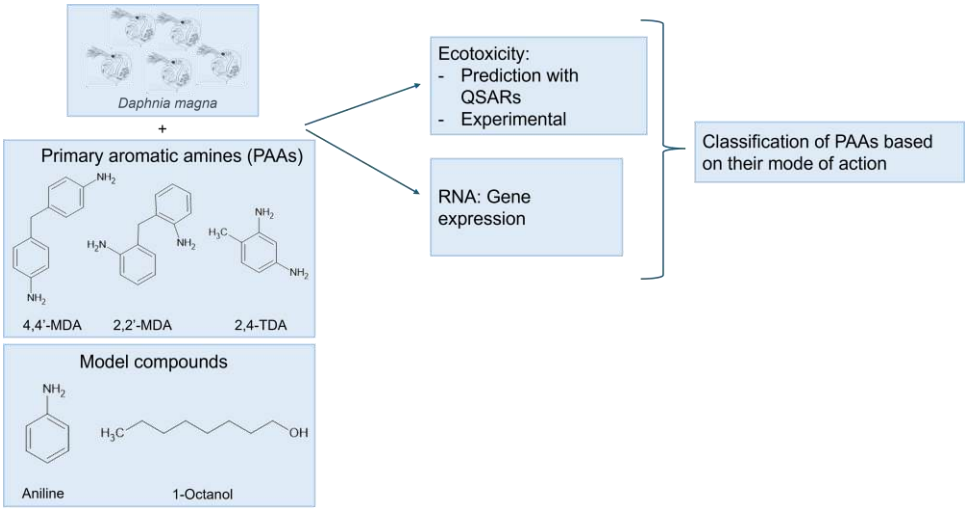
[18] Henk JM Verhaar, Cees J Van Leeuwen, and Joop LM Hermens. “Classifying environmental pollutants”. In: Chemosphere 25.4 (1992), pp. 471–491.

[19] Shuang Wang et al. “Primary aromatic amines and cancer: Novel mechanistic insights using 4-aminobiphenyl as a model carcinogen”. In: Pharmacology & Therapeutics 200 (2019), pp. 179–189.

Graphical Abstract

Exploring mode of action for acute toxicity of primary aromatic amines with *Daphnia magna* using differential gene expression analysis

Friedel Dewulf, Ilias Semmouri, Filip Van Nieuwerburgh, Karel De Schamphelaere, Jana Asselman



Highlights

Exploring mode of action for acute toxicity of primary aromatic amines with *Daphnia magna* using differential gene expression analysis

Friedel Dewulf, Ilias Semmouri, Filip Van Nieuwerburgh, Karel De Schamphelaere, Jana Asselman

- Substituent positioning of aromatic amines affects their toxicity in *D. magna*
- The structural isomers 4,4'-MDA and 2,2'-MDA exhibit distinct modes of actions
- 4,4'-MDA mimics excess toxicity effects in *D. magna*
- 2,2'-MDA exhibits narcotic behaviour in *D. magna*
- Acute immobilization and gene expression analysis reveal distinct modes of action