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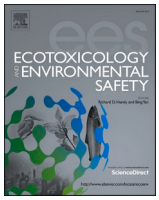

Differential expression of genes in *C. elegans* reveals transcriptional responses to indirect-acting xenobiotic compounds and insensitivity to 2,3,7,8-tetrachlorodibenzodioxin

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ABSTRACT

Caenorhabditis elegans is a well-established model organism for toxicity testing of chemical substances. We recently demonstrated its potential for bioanalysis of the toxic potency of chemical contaminants in water. While many detoxification genes are homologues to those in mammalians, *C. elegans* is reported to be deficient in cytochrome CYP1-like P450 metabolism and that its aryl hydrocarbon receptor (AhR) homolog encoded by *ahr-1* purportedly does not interact with dioxins or any other known xenobiotic ligand. This suggests that *C. elegans* is insensitive for compounds that require bioactivation (indirectly acting compounds) and for dioxins or dioxin-like compounds. This study analysed genome-wide gene expression of the nematode in response to 30 μM of aflatoxin B1 (AFB1), benzo(a)pyrene (B(a)P), Aroclor 1254 (PCB1254), and 10 μM of 2,3,7,8-tetrachlorodibenzodioxin (TCDD). After 24 h of exposure in the early L4 larval stage, microarray analysis revealed 182, 86, and 321 differentially expressed genes in the nematodes treated with 30 μM of AFB1, B(a)P, and PCB1254, respectively. Among these genes, many encode xenobiotic-metabolizing enzymes, and their transcription levels were among the highest-ranked fold-changed genes. Interestingly, only one gene (F59B1.8) was upregulated in the nematodes exposed to 10 μM TCDD. Genes related to metabolic processes and catalytic activity were the most induced by exposure to 30 μM of AFB1, B(a)P, and PCB1254. Despite the genotoxic nature of AFB1 and B(a)P, no differential expression was found in the genes encoding DNA repair and cell cycle checkpoint proteins. Analysis of concentration–response curves was performed to determine the Lowest Observed Transcriptomic Effect Levels (LOTEL) of AFB1, B(a)P, and PCB1254. The obtained LOTEL values showed that gene expression changes in *C. elegans* are more sensitive to toxicants than reproductive effects. Overall, transcriptional responses of metabolic enzymes suggest that the nematode does metabolize AFB1, B(a)P, and PCB1254. Our findings also support the assumption that the transcription factor AhR homolog in *C. elegans* does not bind typical xenobiotic ligands, rendering the nematode transcriptionally insensitive to TCDD effects.

1. Introduction

Biotransformation changes the chemical structure of xenobiotic compounds to reduce their toxicity and allow easier excretion of these compounds. Through this process, lipophilic chemicals are generally converted into more hydrophilic molecules by a series of chemical reactions. While xenobiotic biotransformation facilitates detoxification of compounds, it can occasionally generate toxic metabolites via a process known as bioactivation (Gu and Manautou, 2012; Murk et al., 1994). Several substances are categorized as “indirect-acting” in reference to the chemical agents with little or no toxicological activity, that become toxic upon metabolic activation (Barnes et al., 2018). Biotransformation machinery involves several protein components like the phase I monooxygenases (also referred to as cytochrome P450s), phase II conjugation enzymes and phase III xenobiotic transport proteins (Omiecinski et al., 2011). The expression of genes encoding these enzymes can be transcriptionally affected by exogenous conditions including the presence of a single compound or a mixture of xenobiotics (Baccarelli et al., 2004;

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Sen et al., 2007; Viñuela et al., 2010).

A recent study showed how the nematode *C. elegans* responds to direct-acting genotoxic model compounds (Karengera et al., 2021). In that study, the transcription of DNA damage repair and cell cycle checkpoints genes were not differentially affected by the selected toxicants, but several genes encoding biotransformation proteins were upregulated. Therefore, here we were interested in investigating genome-wide gene expression profiles of *C. elegans* exposed to the chemical agents that require metabolic conversion to become active toxicants.

C. elegans provides a suitable experimental model to study the effects of bioactive substances as it shares many gene functions with mammalians including those involved in xenobiotics biotransformation. For instance, the orthologs for many key mammalian redox systems have been reported in *C. elegans* including glutathione (GSH) and related systems, which are critical for detoxification of both xenobiotic and endogenous compounds in mammals (Ferguson and Bridge, 2019; Dancy et al., 2016). Furthermore, over 80 CYP genes encoding cytochrome P450 enzymes have been identified in the *C. elegans* genome (Menzel et al., 2005, 2001). Based on predicted amino acid sequences, the majority of *C. elegans* CYP genes were found to be closely related to the mammalian CYP2, CYP3, and CYP4 gene families (Gotoh, 1998). Interestingly, CYP1-like metabolism, which is indispensable for metabolizing numerous indirect-acting xenobiotics like polycyclic aromatic hydrocarbons (PAH) (Shimada and Fujii-Kuriyama, 2004), is reported not to be present in *C. elegans* (Leung et al., 2010). Furthermore, the mammalian aryl hydrocarbon receptor (AhR) (Hankinson, 1995; Okey et al., 1994) which plays a central role in the toxicity of many chemical agents like dioxins and dioxin-like compounds has a homologue (AHR-1) encoded by *ahr-1* gene in *C. elegans*. The nematode protein AHR-1 regulates several physiological processes such as neuronal development (Qin and Powell-Coffman, 2004), locomotion, egg laying, defecation behaviors, fatty acid synthesis, and others (Aarnio, 2014). Unlike its counterpart in mammalians (AhR), the nematode AHR-1 was demonstrated not to bind to its common activators such as TCDD or β-naphthoflavone (Powell-Coffman et al., 1998). This could mean that the nematode is not sensitive to transcriptional effects of dioxins.

In this paper, we therefore investigated to which extent C. *elegans* is responsive to indirect-acting model compounds and to dioxin. We analyzed genome-wide gene expression effects of three toxicants whose mode of action is dependent on cytochrome P450-mediated metabolic activation. We selected aflatoxin B1 (AFB1), benzo[*a*]pyrene (B(a)P), PCB mixture Aroclor 1254 (PCB1254) as representative compounds in the toxic classes of highly genotoxic mycotoxins, polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyl (PCB). As dioxin representative we selected 2,3,7,8-tetrachlorodibenzodioxin (TCDD). All four compounds are classified as aryl hydrocarbons containing one or more aromatic rings made of delocalized π electrons which are susceptible to oxidative reactions such as epoxidation, hydroxylation and others mostly mediated by cytochrome P450 enzymes. In humans, AFB1 is mainly metabolized in the liver by CYP1A2 and CYP3A4 isoenzymes to its metabolites including the genotoxic aflatoxin B1 exo-8,9-epoxide (Gallagher et al., 1996). B(a)P requires cytochrome P450 enzymes to form metabolites including the genotoxic B[*a*]P-7,8-diol-9,10-epoxide. In mice, the activation of B(a)P is mediated by hepatic CYP1 enzymes only, especially CYP1A1 and CYP1B1 (Leung et al., 2010; Arlt et al., 2008). PCB1254, a mixture of several polychlorinated biphenyls, is metabolized by humans or rodents: CYP2B, CYP2C, and CYP3A enzymes into 2,3,3′,4′,5-pentachloro-4-biphenylol as the major metabolite (Grimm et al., 2015; Reymann and Borlak, 2006). Like many dioxins and dioxin-like toxicants, the activity of TCDD is guided by the activation of the aryl hydrocarbon receptor (AhR) pathway (Baccarelli et al., 2004) and via the AhR TCDD also activates CYPs belonging to the CYP1 family such as CYP1A1 (Inui et al., 2014). Mammalian exposure to these four toxicants is linked to various effects such as immunotoxicity, oxidative stress, endocrine disruption, carcinogenicity, growth impairment, reproductive and developmental toxicity, and others (Baccarelli et al., 2004; Leung et al., 2010; Gallagher et al., 1996; Grimm et al., 2015).

The aims of this study were to investigate (1) genome-wide transcriptional effects of indirect-acting model toxicants and the CYP1 inducing dioxin in *C. elegans*, (2) to what extent the nematode can be used to detect the presence of the studied compounds, and (3) to relate the nematode responses to the expected modes of action of the compounds. More specifically, we investigated whether *C. elegans* indeed lacks the CYP1-like metabolism, what alternative enzymes could be used to metabolize CYP1 chemical substrates, and how the genes encoding these enzymes transcriptionally responded to the model toxicants. Additionally, we wondered whether *C. elegans* AHR-1 is not regulated by dioxins through receptor-ligand interactions, and how the nematode then transcriptionally responds to exposure to dioxins.

2. Materials and methods

2.1. C. elegans culture

The culture of wild-type N2 (Bristol) strain of *C. elegans* was prepared as described in (Karengera et al., 2021). Synchronized populations of nematodes were obtained using a modified version of the bleaching technique (Porta-de-la-Riva et al., 2012). Briefly, the first larval stage (L1) growth-arrested via starvation were obtained by hatching eggs in M9 buffer (Sulston and Hodgkin, 1988) overnight at 20 °C with gentle agitation. The fourth larval stage (L4) nematodes were obtained after 31 ± 0.5 h of development starting from L1 fed with *E-coli* OP50 at 20 °C.

2.2. Chemicals

Aflatoxin B1 from *Aspergillus flavus* (AFB1, ≥ 98% purity), Benzo[*a*] pyrene (B(a)P, ≥ 96% purity), Aroclor 1254 (PCB1254, analytical standards grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Compounds were dissolved in dimethyl sulfoxide (DMSO ≥ 99.9%, Ultra-Pure Grade) to prepare 20 mM stock solutions. Stock solution 2,3,7,8-tetrachlorodibenzodioxin (TCDD, 2 mM in DMSO) was prepared from TCDD compound (purity ≥ 98%) purchased from AccuStandard. Stock solutions were further diluted in M9 to make the required exposure concentrations with the final DMSO amount of 0.5% in each sample.

2.3. Chemical exposure

Non-lethal concentrations used in our experiments were selected according to the study of Leung and colleagues investigating AFB1 and B(a)P metabolic activation in *C. elegans* (Leung et al., 2010). Briefly, we first assessed whether the compound is soluble in the exposure medium. We then tested different concentrations of each compound to examine which non-lethal to the nematodes. The absence of mortality among the nematodes (after the exposure period) was confirmed by visual observation through a stereomicroscope. Twenty-four-hour exposure was carried out in Falcon™ 15-mL conical tubes at 20 °C. Each sample was made of 2885 μL M9 buffer and 15 μL stock solution compound (with a final DMSO concentration of 0.5%). The solvent (0.5% DMSO) has been previously reported not to influence *C. elegans* gene expression (Menzel et al., 2001) or its growth and reproduction at 24-hour exposure (Leung et al., 2010). For single-compound exposure, the nematodes were treated in quadruplicate with 30 μM for AFB1, B(a)P, and PCB1254). As TCDD is a very potent toxic compound with lowest effect levels in the pM range (see e.g. Murk et al., 1996 (Murk et al., 1996)). The relative toxic potency compared to PCB mixtures including PCB1254 (comparable to PCB A50) is $10^5 - 10^6$ higher (Murk et al., 1996), therefore we decided to use a higher non-lethal exposure concentration based on a previous study with *C. elegans* (Bao et al. in preparation). In that study exposure to 10 nM already resulted in significantly delayed larval developments and 10 μM still was non-lethal but the larval development

was halted. Therefore we chose to expose to 10 μM TCDD. Concentration-response experiments were run in triplicate with concentration ranging from 0.01 μM to 100 μM AFB1, 0.01 μM to 40 μM B(a)P or 0.1 μM to 100 μM PCB1254. Exposure with mixtures was performed in duplicate by combining toxicants (AFB1, B(a)P, or PCB1254) at the concentration of 0.1 μM, 1 μM, or 10 μM per each compound in the mixture. Approximately 10,000 nematodes were used for each sample, and there was no feeding during the exposure period. We chose to use starved L4 larvae to minimize any developmental effects. Our preliminary experiments (data not shown), resulted in better transcriptional responses in starved nematodes compared to the fed ones. Also, by not feeding the nematodes we expect less influence on the bioavailability or other kinetics of the toxicants as reported elsewhere (Spann et al., 2015). After exposure, the nematodes were immediately pelleted by spinning the exposure tubes in a centrifuge for 1 min, 400 x g (Beckman Coulter's Avanti J-15 centrifuge) at room temperature, followed by removal of the supernatants. Subsequently, pellets were transferred into 2 mL Safe-Lock micro test tubes and flash-frozen in liquid nitrogen for 1 min before storing them at − 80 °C until RNA extraction.

2.4. Microarray experiments

RNA template used in microarrays was isolated according to (Karengera et al., 2021). The mRNA expression profiles were measured using Agilent *C. elegans* (V2) Gene Expression Microarray 4 × 44 K slides. Microarray preparation, hybridization and scanning, and normalization and pre-processing of raw data were performed as described previously in (Karengera et al., 2021). The primary data were submitted to ArrayExpress (E-MTAB-11143). KEGG pathways, Gene Ontology (GO) and functional domains involving differentially expressed genes (DEGs) were analysed by DAVID software v6.8 (Huang et al., 2009). A threshold False Discovery Rate (FDR) ≤ 0.05 was considered as significantly enriched in the functional annotation categories.

2.5. RT-qPCR assays

RT-qPCR analyses were conducted for validating microarray data using RNA templates from the same batches as used in the microarray. Separate nematode exposure samples were prepared anew to analyze concentration-response curves of differential gene expression and to test transcriptional effects of the toxicants in mixtures. From these samples total RNA was isolated using TRIzol® Reagent combined with the PureLink® RNA Mini Kit and following the manufacturer's protocol (Thermo Fisher MAN0000406) with modifications. Briefly, the nematodes lysates were prepared by adding 1 mL of TRIzol® Reagent to the frozen pellets of nematodes and mixed well by pipetting up and down several times until fully resuspended. The lysates were then incubated for 5 min at room temperature to allow dissociation of nucleoproteins complexes. 0.2 mL chloroform (VWR, molecular biology grade) was added to each sample and the tubes were shaken vigorously by hand for 15 s followed by incubation for 2 min at room temperature. To obtain crude RNA extracts the samples were centrifuged at 12,000 x g (Eppendorf Centrifuge 5424) for 15 min at 4 °C. Approximately 550 μL of the colorless upper phase liquid containing RNA in each sample was carefully transferred to clean RNAase-free tube. An equal volume of 70% ethanol (Molecular Biology Grade, Fisher BioReagents™) was added and mixed by pipetting up and down to disperse any visible precipitate. After this we resumed the standard protocol including column-based RNA isolation through binding, washing, and elution steps. A NanoDrop spectrophotometer was used to measure RNA quantity and quality, where the purity was assessed by the ratio of absorbance at 260 nm and 280 nm. A260/A280 ratio of 1.8–2.0 was considered as pure enough for further use.

The synthesis of cDNA from RNA template, via reverse transcription (RT), was carried out using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme and following the manufacturer's guide with minor modifications as described in (Karengera et al., 2021). PCR primer design and PCR reactions were also performed as described in (Karengera et al., 2021). Primer sequences used for RT-PCR analysis are provided as supplementary information (Table S1). Raw data were analyzed in Bio-Rad CFX Manager™ Software v3.0 and normalized to *C. elegans* tubulin gamma chain (*tbg-1*) and 14–3–3-like protein (*par-5*) as housekeeping genes. The stability of expression levels of these genes was confirmed in our experimental conditions using GeNorm approach described in (Vandesompele et al., 2002).

2.6. Data analysis and statistics

Microarray data was statistically analyzed as described (Karengera et al., 2021). Linear model analysis was used to assess differentially expressed genes (DEG) per exposure condition whereby a threshold of p-value < 0.0001 was considered as statistically significant. Custom written scripts for the microarray analysis are available atNematology_published_papers / Karengera_2021_Indirect_acting_xenobiotics ·GitLab (wur.nl). RT-qPCR data obtained from concentration-response curves were used to calculate the "Lowest Observed Transcriptional Effect Level" (LOTEL) per gene target tested. LOTEL was considered as the lowest tested concentration that gave a statistically significant expression change for that gene transcript (p-value < 0.05). RT-qPCR data obtained with mixtures were analyzed by assuming additivity, so that the combined transcriptional effect on a particular gene equals to the sum of individual effects expected from each compound in the mixture. Experimentally obtained gene expression results (referred to as "actual effect") were then compared with its counterpart transcription level theoretically calculated by adding up the expected effect from individual compounds (referred to as "predicted effect") in that mixture. Pearson correlations were calculated between actual and predicted expressions for each compound mixture. Correlations were considered significant at p-value < 0.05. We then analysed the difference between predicted and measured values to determine additive or inhibitory effects on gene expressions. To validate microarray results, correlation between array and RT-qPCR data (presented as $\log_2$ fold changes) was determined per treatment condition using "*cor* function" in excel for computing the Pearson correlation coefficient "*R*".

3. Results

3.1. Transcriptome response to AFB1, B(a)P, PCB1254, and TCDD

Since non-lethal concentrations were chosen, the nematodes treated with toxicants did not show lethality at all tested concentrations (i.e., microarray or RT-qPCR data), as confirmed by visual observation through a stereomicroscope. We analyzed global gene transcription profiles of *C. elegans* exposed to 30 μM of AFB1, B(a)P, PCB1254, and 10 μM TCDD. Compared to the untreated nematodes, 182, 86, and 321 genes were significantly up- or downregulated in the nematodes treated with AFB1, B(a)P, and PCB1254, respectively. Of these genes, those with upregulated transcripts were remarkably predominant, as they accounted for around 87% of the total DEGs for AFB1 (159 genes) or PCB1254 (279 genes). For B(a)P treatment, all 86 DEGs were upregulated. Overlap among treatments by AFB1, B(a)P, and PCB1254 was found for 20 genes only, thus regulation of most genes was treatment-specific, especially for AFB1 and PCB1254 (Fig. 1A). Interestingly, 10 μM TCDD had 1 DEG, only F59B1.8 was 2.5 fold upregulated, and this gene expressed also in the nematodes treated with 30 μM AFB1 (1.3-fold upregulation) or 30 μM PCB1254 (2.5-fold upregulation). F59B1.8 is thought to be an innate immune regulator.

Gene transcripts with more than 10-fold change in expression (microarray data) represented about 13%, 15%, and 7% of the total genes regulated by 30 μM of AFB, B(a)P, and PCB1254, respectively (Fig. 1B). The expression levels of some of these genes were dramatically

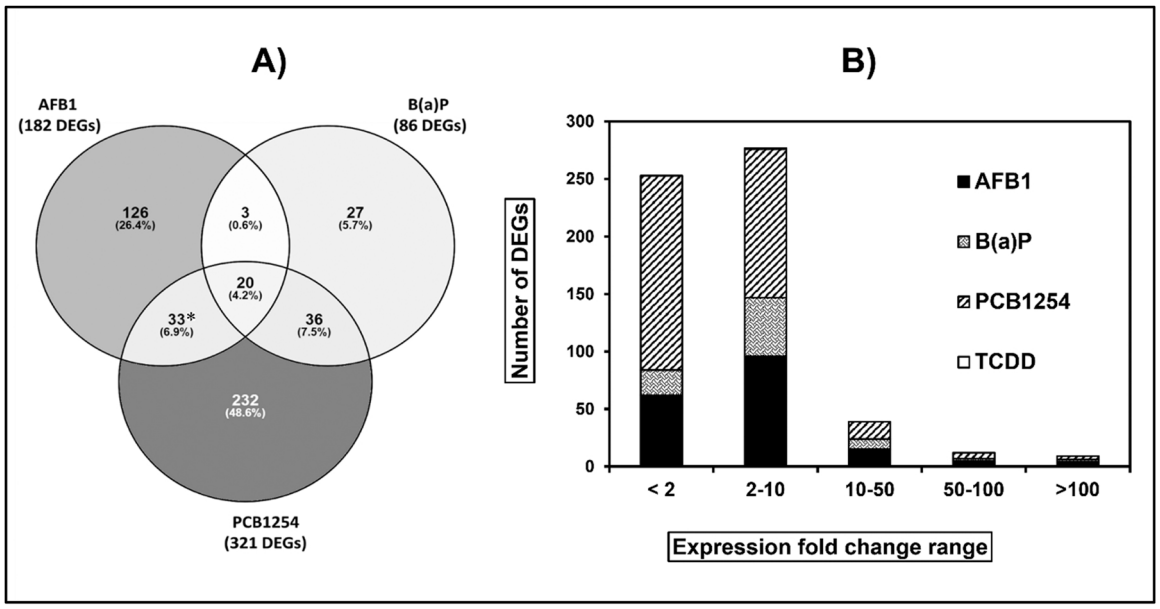

Fig. 1. Overlapping of differentially expressed genes (DEGs). The Venn diagram (A) shows the number of significantly regulated genes by 30 μM of AFB1, B(a)P, and PCB1254, and their overlaps. Asterix (*) in figure (A) symbolizes the only one gene (F59B1.8) affected by 10 μM TCDD and whose expression overlapped with AFB1 and PCB1254. Bar charts (B) displays the ranges of absolute fold-changes of the transcription levels induced by each treatment.

increased by more than 100-fold upregulation by AFB1 (*cyp-14A4, cdr-1,* F13H6.3*,* and B0205.14), B(a)P (*cyp-35C1* and *cyp-35D1*), or PCB1254 (*dhs-23*, R09E12.9, and F25D1.5). Most genes were mainly regulated in the range of 2- to 10-fold changes including 53%, 59%, or 40% of all DEG in AFB1, B(a)P and PCB1254, respectively. Genes regulated less than 2-fold change were found for 34%, 26%, and 53% of the DEGs in AFB1, B(a)P and PCB1254, respectively.

3.2. *Functional GO analysis of differentially expressed genes (DEGs)*

Functional analysis revealed that the main part of upregulated genes (microarray data) was involved in metabolism and detoxification mechanisms of the nematode. The top affected mechanisms for AFB1, B(a)P and PCB1254 were found in the molecular function category as catalytic and oxidoreductase activities as assessed by Gene Ontology (GO) analysis (Fig. 2 and Suppl. Excel Table). Further analysis in biological process (BP) category showed that metabolic process counted alone about 51%, 60%, and 40% of all upregulated genes by AFB1, B(a)P, and PCB1254, respectively (Fig. 2 and Suppl. Excel Table). These included genes encoding nuclear hormone receptors (NHRs), phase I metabolic enzymes (cytochrome P450s), and phase II conjugation enzymes such as glutathione-S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) (Table 1). Among cytochrome P450 genes, *cyp-14A4* and *cyp-35D1* were ranked in the top induced DEGs and

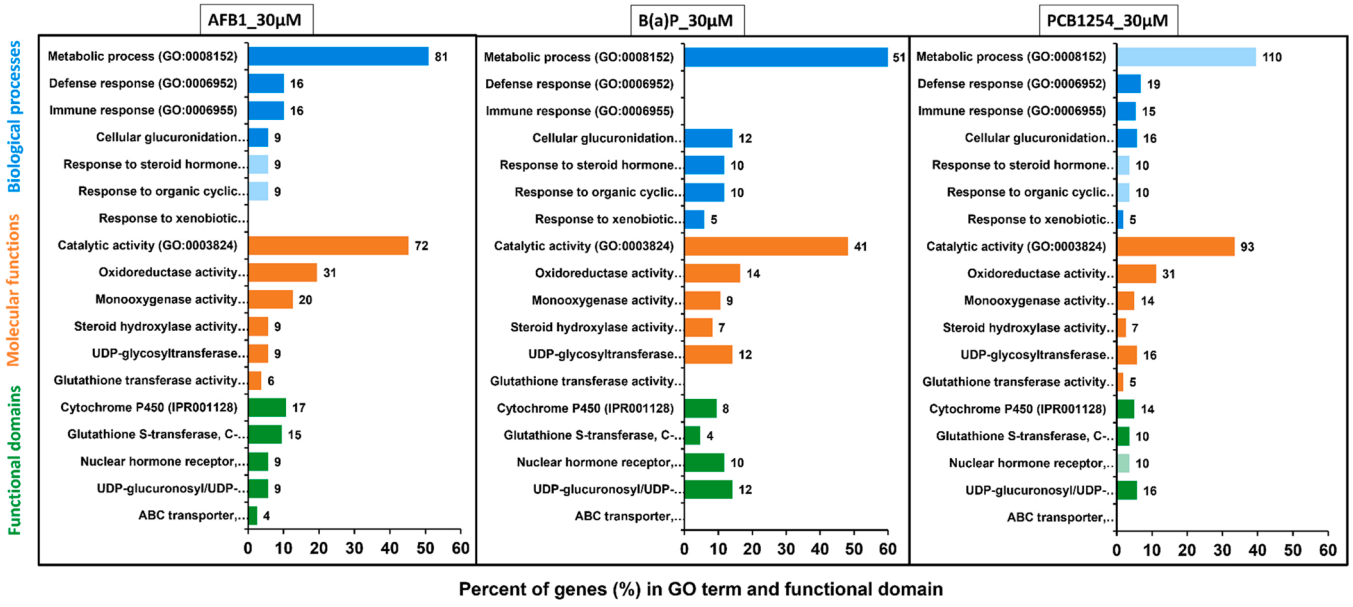

Fig. 2. Gene Ontology (GO) and domain enrichment analysis terms. Plotted are gene ontologies (in biological process and molecular function categories) and functional domains associated with upregulated genes following 24 h exposure to 30 μM AFB1, B(a)P, and PCB1254. The X-axis denotes percent of genes significantly enriched in a GO or domain term (False Discovery Rate, FDR < 0.05). The numbers at the end of each bar represents gene counts belonging to a corresponding GO term or domains. The light-coloured bars represent GO or domains for which the enrichment was not statistically significant (FDR > 0.05).

Table 1
Genes encoding nuclear receptors and biotransformation enzymes in *C. elegans*. Transcription of these genes were significantly differentially expressed after treatment with AFB1, B(a)P, and PCB1254. TCDD did not influence expression of any gene encoding nuclear receptors and biotransformation enzymes.

Treatment	Nuclear hormone receptor genes	Cytochrome P450 genes	Glutathione S-transferase genes	UDP-glucuronosyltransferase genes
AFB1	*nhr-62, nhr-106, nhr-112, nhr-130, nhr-142, nhr-178, nhr-196, nhr-235, nhr-237*	*cyp-14A4, cyp-35A5, cyp-33C2, cyp-14A1, cyp-33C1, cyp-13A6, cyp-13A7, cyp-25A2, cyp-33C5, cyp-33C4, cyp-33C7, cyp-34A9, cyp-13A10, cyp-33E2, cyp-34A5, cyp-13A3, cyp-13A1*	*gst-6, gst-7, gst-8, gst-12, gst-14, gst-21, gst-31, gst-33, gst-44, gsto-2*	*ugt-2, ugt-8, ugt-16, ugt-19, ugt-21, ugt-41, ugt-61, ugt-62*
B [*a*] P	*nhr-11, nhr-12, nhr-62, nhr-86, nhr-176, nhr-201, nhr-203, nhr-205, nhr-207, nhr-237*	*cyp-35A1, cyp-35A5, cyp-35A3, cyp-29A3, cyp-35B1, cyp-35A4, cyp-35C1, cyp-35D1*	*gst-21, gst-44*	*ugt-5, ugt-8, ugt-9, ugt-13, ugt-22, ugt-33, ugt-34, ugt-37, ugt-40, ugt-41, ugt-45*
PCB1254	*nhr-11, nhr-12, nhr-37, nhr-62, nhr-142, nhr-178, nhr-205, nhr-208, nhr-237, nhr-238*	*cyp-35A3, cyp-13A6, cyp-35C1, cyp-35A1, cyp-35A4, cyp-35A5, cyp-14A2, cyp-34A10, cyp-14A3, cyp-13A9, cyp-13A8, cyp-13A10, cyp-13A7, cyp-34A9, cyp-13A1, cyp-33B1, cyp-33C1*	*gst-5, gst-6, gst-9, gst-12, gst-14, gst-21, gsto-2*	*ugt-8, ugt-9, ugt-13, ugt-16, ugt-19, ugt-22, ugt-25, ugt-33, ugt-34, ugt-37, ugt-40, ugt-41, ugt-45, ugt-61*

were specifically upregulated by AFB1 (888 fold) and B(a)P (113 fold), respectively. Likewise, the transcription of *cyp-35A3* and *cyp-13A6* were the highest upregulated cytochromes by PCB1254 (71 and 57 expression fold, respectively), but their expression was not compound specific as they were also affected by AFB1 (*cyp-13A6)* and B(a)P (*cyp-35A3*). Furthermore, the induction of ATP-binding cassette (ABC) genes (*mrp-3, pgp-1, pgp-8, and pgp-9*), which are essential in xenobiotic detoxification, was found in the nematodes treated with AFB1.

Functional analysis of upregulated genes also showed the induction of defense and immune responses in the nematodes treated with AFB1 and PCB1254. The involved genes included those encoding *C. elegans* proteins like C-type lectins, P450, GSTs, NHRs, cadmium-responsive genes, and others. Treatment with B(a)P was linked with the nematode response to steroid hormones and organic cyclic compounds. These mechanisms were also found with AFB1 and PCB1254 treatments but were statistically not significant (FDR > 0.05). All the genes found to be related to the nematode response to steroid hormones and organic cyclic compounds were exclusively nuclear hormone receptor family members (NHRs) such as *nhr-62* and *nhr-237* (regulated by all compounds), *nhr-142* and *nhr-178* (by AFB1 and B(a)P), *nhr-12*, *nhr-11*, and *nhr-205* (by B(a)P and PCB1254), and others.

The annotations of individual DEGs showed some genes like *rpa-2, chk-1, ubql-1,* and *che-3* that can be linked to the genotoxic stress responses in *C. elegans*. Nevertheless, GO analysis did not reveal any of the known mechanisms associated with DNA damage responses (DDR) genes of *C. elegans* such as cell cycle checkpoints and DNA repair. For the downregulated transcripts, gene set enrichment analysis did not find any significantly affected cellular mechanism.

3.3. Validation of microarray data by RT-qPCR

To confirm gene expression results obtained from microarrays we used RT-qPCR for testing transcription of 24 gene targets selected from array data. The selected genes were among the top-ranked microarray transcripts expressed in AFB1, B(a)P, or PCB1254 treatment. Overall, significant correlation was observed between array and RT-qPCR results as shown by positive correlation coefficients $R_{(AFB1)} = 0.98$, $R_{(B(a)P)} = 0.96$, and $R_{(PCB1254)} = 0.89$ (Fig. S3).

3.4. Concentration-dependent differential gene expression

Using RT-qPCR we analysed concentration-response curves of mRNA expression of *gst-33, cyp-14A3, cyp-35A1, cyp-35A3, cyp-35A5*, and *cyp-35C1* (Fig. 3 and Fig. S1). These genes were among the top-ranked transcripts (microarray data) whose expression changes were validated using RT-qPCR. They were included in the analysis to enable the measurement of effects induced by the relatively low concentrations of AFB1, B(a)P, or PCB1254. For each compound, the "Lowest Observed Transcriptional Effect Level" (LOTEL) was determined. The lowest concentration inducing transcriptional effects (LOTEL) was 0.01 μM for AFB1, 0.1 μM for B(a)P, and 1 μM for PCB1254 (Table 2). At these concentrations, *gst-33* expressed in AFB1*, cyp-35A1, cyp-35A5,* and *cyp-35C1* in B(a)P, and *cyp-35A1, cyp-35A3, cyp-35A5*, and *cyp-35C1* in PCB1254 (Table 2).

3.5. Transcriptional effects of compounds in mixtures

We measured also joint transcriptional effects of AFB1, B(a)P, and PCB1254 tested in mixtures at the concentration of 0.1 μM, 1 μM, or 10 μM for each component. The RT-qPCR assay was used to assess the mRNA expression levels of *gst-33, cyp-14A3, cyp-35A1, cyp-35A3, cyp-35A5*, and *cyp-35C1*. Overall, significant positive correlations were found between actual and predicted expressions for compound mixtures, especially at 1 μM and 10 μM, as shown by positive correlation coefficients (Fig. 4). Nevertheless, some mixtures triggered either increase or reduction in the actual expression levels of the target genes compared to the predicted effects assuming additivity (Fig. S2), especially in the mixture containing AFB1. For instance, *gst-33* in the nematodes treated with 10-μM based mixtures was predicted to be upregulated by 22 fold (for AFB1 + B(a)P), 28 fold (for AFB1 + PCB1254), or 20 fold (for AFB1 + B(a)P + PCB1254). Instead, the actual *gst-33* expression was 20-fold, 7-fold, and 13-fold upregulation in respective aforementioned mixtures. Noteworthy, B(a)P and PCB1254 (individually or in mixture) did not have significant effect on *gst-33* expression. Based on single-compound exposure, only AFB1 induced *gst-33* expression with 29-fold upregulation in the 10 μM concentration.

4. Discussion

In this study, we determined transcriptional effects of indirect-acting model toxicants and TCDD in *C. elegans*. Several differentially expressed genes, especially those encoding biotransformation enzymes, were detected by microarrays for 30 μM AFB1, B(a)P, and PCB1254. For these three compounds, we also identified many genes whose expression is regulated by nuclear hormone receptor (NHR) transcription factors. Consistent with literature (Powell-Coffman et al., 1998), our findings using microarray showed that, even at the very high exposure concentration 10 μM used, *C. elegans* is insensitive to the transcriptional effects of TCDD whose mode of action is AhR-dependent.

Compounds tested in this study are known to be metabolically activated by mammalian cytochrome P450 (CYP) enzymes. AFB1 is mainly metabolized by human CYP1A2 and CYP3A4 (Gallagher et al., 1996), B(a)P by CYP1A1 and CYP1B1 in mice (Arlt et al., 2008), and PCB1254 by CYB2B, 2 C, and 3 A subfamilies in humans or rodents (Grimm et al., 2015; Reymann and Borlak, 2006). TCDD is metabolized in rats as well as in humans by CYP1A1, but it is very persistent (Inui et al., 2014; Inouye et al., 2002). The genes encoding phase I enzymes in *C. elegans* have been found to be closely related to the mammalian CYP2, 3, and 4

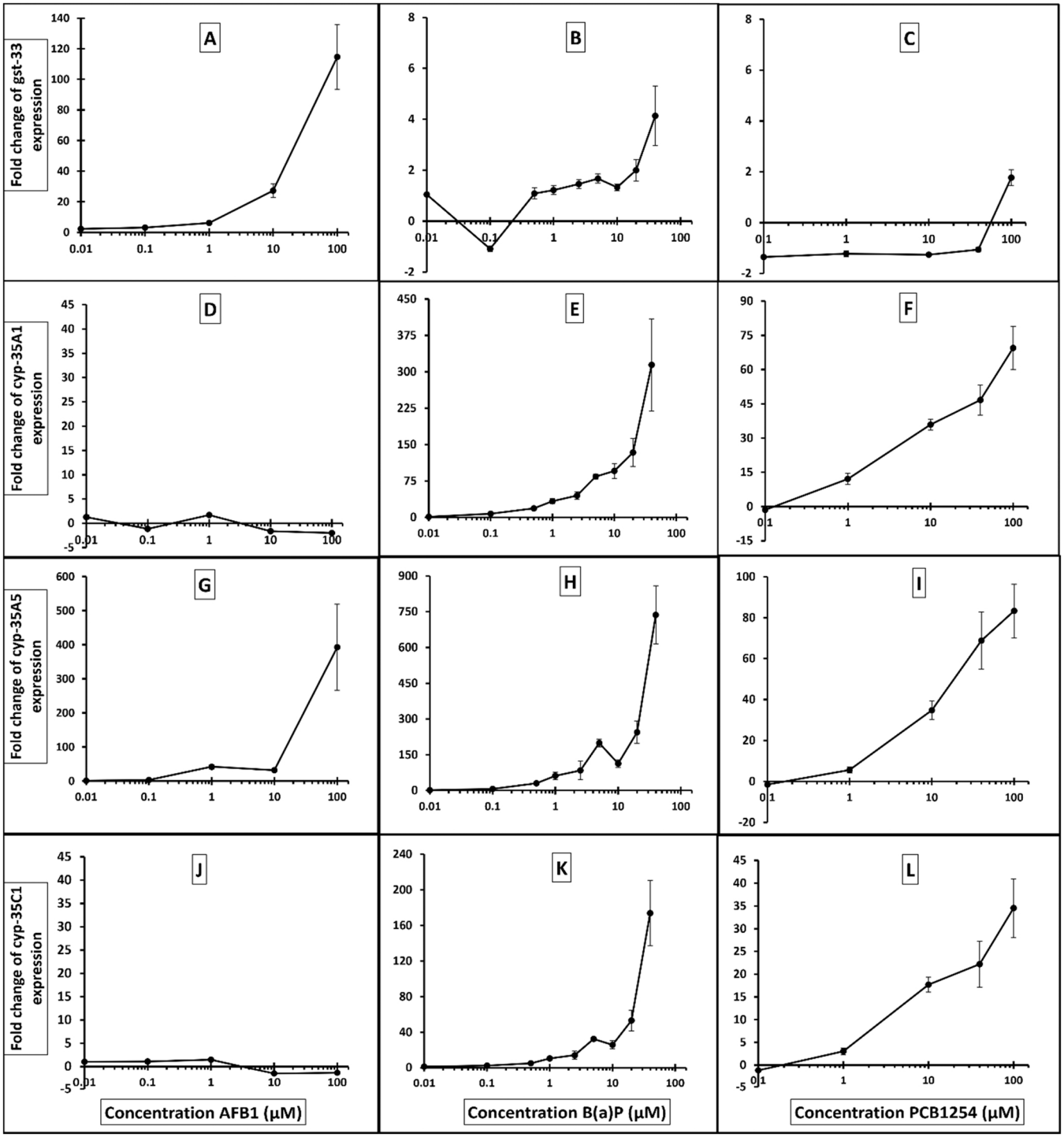

Fig. 3. Concentration-response curves of differential gene expression in *C. elegans*. L4 juveniles were treated with toxicants ranging from 0.01 µM to 100 µM AFB1, from 0.01 µM to 40 µM B(a)P or from 0.1 µM to 100 µM PCB1254 for 24 h. Concentration-dependent relative mRNA expression changes of *gst-33* (A, B & C), *cyp-35A1* (D, E, & F), *cyp-35A5* (G, H, & I), and *cyp-35C1* (J, K, & L) genes were determined by reverse transcription polymerase chain reaction (RT-qPCR) and normalized to *C. elegans* tubulin gamma chain (*tbg-1*) and 14–3–3-like protein (*par-5*) genes. Data represent the mean fold changes ± standard error of the mean (SEM) in three independent biological replicates (n).

families (Gotoh, 1998), whereas CYP1-like metabolism is absent in the nematode (Leung et al., 2010). In agreement with this literature, our findings revealed 17 CYP genes regulated by AFB1 that are related to the mammalian CYP2 (*C. elegans cyp-14A, 33C, 33E, 34A, 35A* subfamilies) and CYP3 (*C. elegans cyp-13A* subfamily and *25A2* gene) (Table S2). Among the eight CYP genes affected by B(a)P, seven are *C. elegans CYP35* family members *(cyp-35A, 35B, 35C, 35D* subfamilies) and *cyp-29A3*, which are related to the mammalian CYP2 and CYP4, respectively (Table S2). For the nematodes treated with PCB1254, we found 17 CYP genes related to the mammalian CYP2 (*C. elegans* cyp-*14A, 33B, 33C, 34A, 35A, 35C* subfamilies), and CYP3 (*C. elegans cyp-13A* subfamily) (Table S2).

Some human orthologues to *C. elegans* CYP genes, including those found in our study, have been previously reported (Hartman et al.,

Table 2
Lowest Observed Transcriptional Effect Levels (LOTEL) of toxicants per individual biotransformation-related gene target. The table shows LOTELs values selected from concentration-response curves of differential gene expression, as determined RT-qPCR. TCDD did not influence expression of these genes.

Gene name	AFB1		B (a)P		PCB1254	
	LOTEL (μM)	Fold change	LOTEL (μM)	Fold change	LOTEL (μM)	Fold change
gst-33	0.01	2.2	20	2.0	–	–
cyp-14A3	1	2.0	–	–	10	5.5
cyp-35A1	–	–	0.1	7.2	1	12.1
cyp-35A3	–	–	1	2.1	1	3.2
cyp-35A5	0.1	2.9	0.1	7.0	1	5.6
cyp-35C1	–	–	0.1	2.5	1	3.0

2021). The human *CYP4V2*, whose transcript is inducible by B(a)P in HepG2 human hepatocytes (Song et al., 2012), is an orthologue to the nematode *cyp-29A3* that in our study was upregulated (~5-fold increase) by only B(a)P. Our data also revealed that the transcripts of both *cyp-35A3* and *cyp-35A4* were increased by B(a)P and PCB1254. Previously, regulation of these genes was found to be restricted to the typical inducers of mammalian CYP1A such as β-naphthoflavone, PCB52, lansoprazole, and fluoranthene (Menzel et al., 2001). In human cell lines, cytochromes of CYP1A subfamily (*CYP1A1* and *CYP1A2*) are strongly inducible by B(a)P or PCB1254 (Song et al., 2012; Borlak and Zwadlo, 2003). We also found that *C. elegans cyp-35D1*, previously reported to not be regulated by the inducers of mammalian CYP1 (like B(a)P or others) (Menzel et al., 2001), was unexpectedly strongly upregulated in B(a)P exposure (~113-fold). Other nematode CYP35 regulated in our study by B(a)P or PCB1254 (e.g., *cyp-35A1, 35A5, 35B1,* and *35C1*) are reported to be orthologues to human *CYP2C18, CYP2D7,* and *CYP2E1* (Hartman et al., 2021). These human P450 proteins are not transcriptionally induced by B(a)P (Song et al., 2012) or PCB1254 (Reymann and Borlak, 2006). Further, our study also identified the regulation of the nematode CYP13A subfamily (*cyp-13A1, A7, A8,* and *A10*) by AFB1 or PCB1254. These genes are reported to be the orthologues to the mammalian *CYP3A4* and *CYP3A5* (Hartman et al., 2021). In comparison with the literature, *CYP3A4 can* indeed be upregulated by AFB1 in HepG2 cell line (Ratajewski et al., 2011), while *CYP3A5* is upregulated by PCB1254 in Caco-2 cells but not in HepG2 cell line

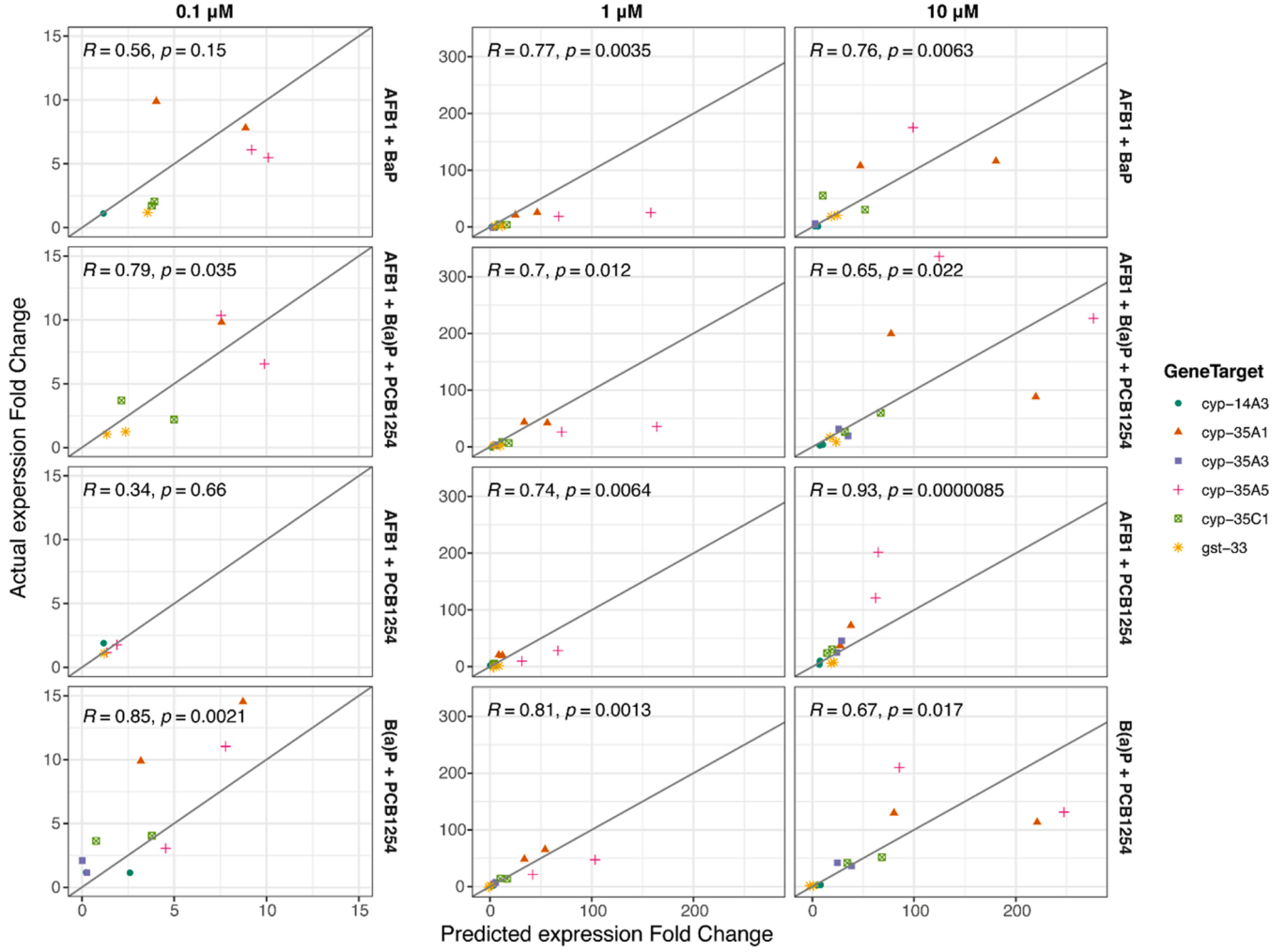

Fig. 4. Comparison between actual and predicted joint transcriptional effects of AFB1, B(a)P, and PCB1254 in mixtures. 24-hour exposure was started in *C. elegans* L4 larvae with combined toxicants (AFB1, B(a)P, or PCB1254) at the concentration of 0.1 μM, 1 μM, or 10 μM per each mixture component. The mRNA expression changes of *cyp-14A3, cyp-35A1, cyp-35A3, cyp-35C1,* and *gst-33* were determined by reverse transcription polymerase chain reaction (RT-qPCR) and normalized to *C. elegans* tubulin gamma chain (*tbg-1*) and 14–3–3-like protein (*par-5*) genes. Pearson correlations were calculated between actual and predicted expressions for each compound mixture. Correlations were considered significant at $p < 0.05$. Data represent the actual and predicted expression fold changes (not log-transformed values). Two independent biological replicates were carried out.

(Borlak and Zwadlo, 2003). Overall, our study showed that *C. elegans* biotransformation of xenobiotics is indeed transcriptionally inducible by the studied compounds (except TCDD) via phase I metabolism comparable to mammalians.

We also found many differential expressed genes linked to the phase II metabolism of xenobiotics. These included genes encoding glutathione-S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs), which are involved in *C. elegans* resistance against oxidative stress (Ferguson and Bridge, 2019; Hasegawa et al., 2010). Genes encoding P-glycoproteins (*pgp-8 and pgp-9*), multidrug resistance protein (*pgp-1*), and one hypothetical protein (*mrp-3)* were upregulated in the nematodes treated with AFB1. These four genes encode ATP-binding cassette (ABC) transporters that are involved in xenobiotic detoxification by facilitating the transport of toxicants across cell membranes (Lindblom and Dodd, 2006; Lincke et al., 1993) resulting in excretion. *C. elegans pgp-1* is homolog to the mammalian drug transporters such as *MDR1* and *MDR3* in humans or *Mdr1a* and *Mdr1b* in rodents (Lincke et al., 1992). Rat *Mdr1b* is transcriptionally inducible by genotoxic carcinogens including AFB1 (Ellinger-Ziegelbauer et al., 2004). In *C. elegans*, *pgp-1* is involved in the detoxification of heavy metals like cadmium (Cd) and arsenic (As) (Broeks et al., 1996). Compared to the Cd-regulated genes in *C. elegans* (Cui et al., 2007), many transcripts were similarly expressed in our study, including 40 and 27 genes in the nematodes treated with AFB1 and PCB1254, respectively. For AFB1-treated nematodes, the overlaps with Cd-induced genes included all four ABC transporter genes mentioned above together with the top three most expressed genes (*cyp-14A4, cdr-1,* and *cest-33*), eight cytochrome P450 genes, and five UGT genes. This suggests similar mechanisms of *C. elegans* detoxifying Cd and AFB1. The well-known cadmium-responsive gene *cdr-1 (*Liao et al., 2002*)* was also regulated by PCB1254 and B(a)P.

NHRs are ligand-activated transcription factors that regulate several vital functions in *C. elegans* (Antebi, 2015). There are 284 NHRs in *C. elegans* but only few of them has been well characterized (Peterson et al., 2019). In this study, we found differential expression of many genes which are regulated by nuclear hormone receptor (NHR) transcription factors NHR-8, NHR-86, and NHR-114. Receptor NHR-8, a homolog of mammalian liver X and vitamin D receptors, regulates *C. elegans* development, reproduction, and aging by controlling cholesterol and bile acid homeostasis (Magner et al., 2013). NHR-114 is required for nematode fertility and germline stem cell maintenance (Gracida and Eckmann, 2013), whereas NHR-86 regulates anti-pathogen responses (Peterson et al., 2019). These results suggest that the tested compounds can provoke the same responses maybe by acting as ligands to the above receptors.

The aryl hydrocarbon receptor (AhR) is another ligand-activated transcription factor which mediates biological and toxicological activity of many chemicals in mammalians including dioxins and related compounds (Hankinson, 1995; Okey et al., 1994). The AhR homolog (AHR-1) in *C. elegans* is encoded by the *ahr-1* gene, but the spectrum of its ligands (if there are any) is allegedly different from that of the mammalian AhR (Powell-Coffman et al., 1998). Indeed, to our knowledge, no exogenous ligand has ever been shown to directly bind and induce *C. elegans* AHR-1. AHR-1 possibly is sensitive to endogenous ligands (Wu et al., 2019; Guyot et al., 2013), and has been shown to regulate in *C. elegans* important physiological processes such as neuronal development (Qin and Powell-Coffman, 2004), locomotion, egg laying, defecation behaviors, and fatty acid synthesis (Aarnio, 2014). Our findings showed that only one gene (F59B1.8), involved in the nematode innate immune response (Shapira et al., 2006), was regulated by TCDD. This seems to be in line with the literature that *C. elegans* AHR-1 does not bind TCDD (Powell-Coffman et al., 1998), hence is transcriptionally insensitive. Nevertheless, a previous study showed that TCDD does delay the early larval development in *C. elegans* as shown by significant developmental delays for L3 larvae to reach L4 stage of larval growth, even in larvae that only were maternally exposed to levels as low as 10 nM of TCDD (Bao et al. in preparation). These effects could be explained by baseline toxicity (known as narcosis), a characteristic of many organic xenobiotics, which typically induces non-specific disruption of the integrity and functioning of cell membranes (Escher et al., 2002).

Our study also identified gene transcripts that can be linked to the toxicological effects of AFB1, B(a)P, and PCB1254. Among the affected genes, we found those regulated by transcription factors ELT-2, MDT-15, SKN-1, or DAF-16 in *C. elegans*. ELT-2 is presumably homolog to human GATA6 and regulates genes involved in the nematodes innate immune responses (Shapira et al., 2006). MDT-15 dependent genes are linked to *C. elegans* oxidative stress resistance and cyto-protection (Goh et al., 2014). SKN-1 is ortholog of mammalian Nrf proteins (Blackwell et al., 2015) and is a major regulator of the genes involved in oxidative stress response and longevity of *C. elegans* (Oliveira et al., 2009). A FOXO-family transcription factor (DAF-16) and its downstream genes are linked to *C. elegans* aging and stress responses via insulin/insulin-like growth factor 1 (IGF-I) signaling (Murphy et al., 2003; Rodriguez et al., 2013). Furthermore, we found overlap between our data and the transcriptional profiles of other compounds in literature, like cadmium (Cui et al., 2007) and deoxynivalenol (Di et al., 2018) known to be toxic to the *C. elegans* reproduction, development, and lifespan. Overall these findings suggest that the adverse effects expected from the tested toxicants were also represented by transcriptional profiles found in this study. Nonetheless, despite DNA-damaging properties (especially AFB1 and B(a)P), no differential expression was found among the genes encoding DNA repair and cell cycle checkpoint proteins, which was consistent with the findings with direct-acting genotoxic model compounds (Karengera et al., 2021).

In this study, we also analyzed concentration-dependent transcriptional effects of the toxicants and determined the Lowest Observed Transcriptional Effect Levels (LOTEL). This is a toxicological dose descriptor comparable to the Lowest Observed Adverse Effect Level (LOAEL) commonly used to relate the toxic effects of a chemical substance and the dose at which it takes place. Toxicogenomic studies in literature have previously advocated using threshold doses like LOTEL to evaluate toxicological profiles of chemicals (Gou et al., 2010; Poynton et al., 2008). From concentration-response curves obtained in our study, the lowest concentration inducing transcriptional effects (LOTEL) among the tested gene targets were 0.01 μM for AFB1, 0.1 μM for B(a)P, and 1 μM for PCB1254. In comparison with literature, these LOTEL values were about 541-fold for AFB1, 2-fold for B(a)P, or 48-fold for PCB1254 smaller than the median effective concentrations (EC_{50}) for toxic effects on *C. elegans* reproduction. For 72-hour exposure, EC_{50} that caused reproductive toxicity is equivalent to 5.41 μM for AFB1 (Yang et al., 2015), 0.23 μM for B(a)P (Sese et al., 2009), and 47.82 μM for PCB52 (Menzel et al., 2005). These results suggest that transcriptional effects in *C. elegans* are occurring at a concentration much lower than developmental effects, as is also the case in vertebrates like zebrafish embryotoxicity test (ZET) (Hermsen et al., 2012).

Furthermore, we assessed the joint transcriptional effects of the toxicants in mixtures by comparing the actual and predicted changes in gene expression. The findings suggest possible interactions between compounds in mixtures, as shown by increase or reduction in the actual measured expression levels compared to the predicted expression of the target genes. The observed potential interactions were more apparent for the mixtures containing AFB1. According to microarray results from this study, AFB1 regulated *cyp-35A5* only among the *C. elegans* CYP-35 family members known to be strongly inducible by many xenobiotics (Menzel et al., 2001). Despite this, AFB1 combined with either B(a)P or PCB1254 in mixtures seemed to influence the joint effects by either increasing or reducing the transcription levels of other CYP-35 genes (i.e., *cyp-35A1, cyp-35A3,* and *cyp-35C1)* tested in this study. Further research is needed to elaborate the possible mechanisms underlying such interactions between toxicants.

Overall, we identified transcriptional responses of *C. elegans* to toxic

substances requiring metabolic bioactivation. Several genes involved in xenobiotic biotransformation were regulated by AFB1, B(a)P, and PCB1254, suggesting that these compounds are metabolized in the nematode via phase I and II, or detoxified via transmembrane export as found for AFB1. These findings extend the knowledge on transcriptional inducibility of the nematode biotransformation enzymes in response to indirect-acting compounds. Moreover, this research adds important details about *C. elegans* gene expression profiles in response to prototypes of mycotoxins, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls contaminants. Importantly, this study revealed differential gene expressions which can be associated with toxicological activities of the tested compounds. We also found many candidate gene transcripts that can be used as transcriptional biomarkers for detecting the presence of these compounds. Whereas the mammalian aryl hydrocarbon receptor (AhR) mediates CYP1A1 induction and toxicological effects of dioxins and a multitude of dioxin-like compounds through ligand interaction, *C. elegans* did not respond to 10 μM TCDD in our study while already exposure to 10 nM was enough to induce developmental effects (Bao et al. in preparation). It is interesting to further assess the effects of TCDD at a broader range of concentrations as well as other dioxin-like compounds.

CRediT authorship contribution statement

Antoine Karengera, Albertinka J. Murk, and Inez J. T. Dinkla conceived the theoretical framework. Joost A. G. Riksen provided technical support on the microarray experiments, Mark G. Sterken performed statistical analyses on microarrays. Antoine Karengera planned and carried out the experiments, and analyzed RT-qPCR data. Antoine Karengera wrote the manuscript with input from Inez J. T. Dinkla, and in consultation with Mark G. Sterken, Albertinka J. Murk, and Jan E. Kammenga.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113344.

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