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Andreas Schüttler | Modeling and Predicting Toxicogenomic Effect Profiles...

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NOT FOR SALE.

Andreas Schüttler

Modeling and Predicting Toxicogenomic Effect Profiles of Environmental Chemicals and Their Mixtures

Towards Non-Target Bioanalytics for Environmental Monitoring

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HELMHOLTZ
CENTRE FOR
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RESEARCH – UFZ

MODELING AND PREDICTING TOXICOGENOMIC EFFECT PROFILES OF ENVIRONMENTAL CHEMICALS AND THEIR MIXTURES

Towards Non-Target Bioanalytics for Environmental Monitoring

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigte Dissertation

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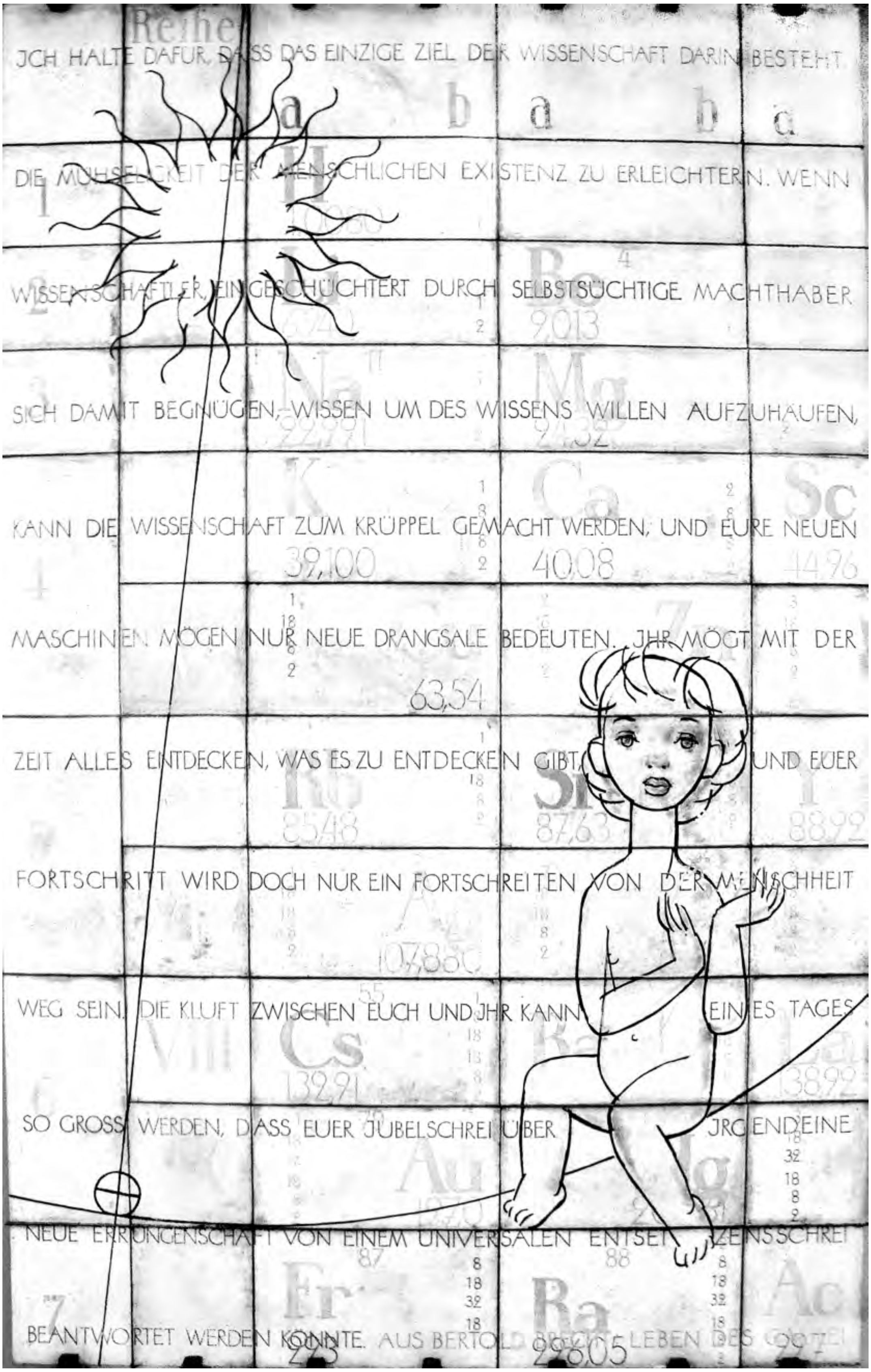

Wandbild im ehemaligen Institut für stabile Isotope Leipzig, Bert Heller (um 1964)

ABSTRACT

While many water bodies are found to be polluted by a mixture of numerous chemicals, suitable tools which allow a comprehensive diagnosis of the resulting biological effects are lacking. However, such effect information would be needed in water body management to facilitate measures which counteract decreasing ecological quality. Toxicogenomic methods (i.e., the application of *omics* technology in toxicology) could fill this gap by providing non-target bioanalytical approaches. Therefore, this dissertation aims at advancing the applicability of toxicogenomics in environmental monitoring.

It has been shown that exposing organisms to chemicals may induce compound specific changes on the transcriptome, i.e. the entire composition of RNA transcripts in a cell or tissue. Transcripts are copies of genes on the DNA, which may have regulatory functions or encode the assembly of proteins. The analysis of transcriptome profiles, which can be conducted with the help of cDNA-microarrays, may indicate specific compound exposure, and at the same time provide insights about the cellular or physiological responses in the organism (e.g., biotransformation, proliferation, cell death). These properties could be used to apply transcriptomics for comprehensive diagnoses of biological effects and the respective responsible substances in environmental samples.

However, the inference of compound profiles is still challenging. The responses are dependent on exposure settings, such as exposure concentration or duration. Next to compound specific transcript regulations, there are also more general responses to be expected, which appear in response to several chemicals. This complicates the interpretation and comparison of transcriptome profiles, especially when evaluating mixture profiles. Additionally, it has not been resolved yet how the transcriptome profiles of single compounds combine in a mixture.

Therefore, this work first of all strived for identifying general responses towards chemical exposure. To achieve this, a meta-analysis was conducted, which compiled published transcriptome data of zebrafish embryos (*Danio rerio*) exposed to chemicals (chapter 2). Herein, transcripts could be identified, showing a common trend in response to certain groups of compounds or in specific exposure settings. The meta-analysis also revealed a large heterogeneity in experimental design, though. Additionally, missing time and concentration dependent investigations made extrapolation of effects infeasible and complicated

thorough comparisons between different compounds or exposure settings.

To allow for extrapolation and enhance comparability of transcriptome profiles, an experimental design and analysis strategy was developed to infer time and concentration resolved transcriptome profiles (chapter 3). With the help of a *self-organizing map* of the previously compiled meta-analysis data the profiles could be aggregated to "toxicogenomic landscapes". This allowed visual representations and facilitated comparisons between compounds and exposure settings. To allow for quantitative description of the profiles, a regression model was developed, capturing the time and concentration dependence of the toxicogenomic effects. The analysis strategy, which included landscape projection and regression modeling, was applied in a case study on the exposure of zebrafish embryos to three selected model compounds. The developed strategy allowed to infer compound specific toxicogenomic landscapes, suitable for extrapolation and comparison between compounds and exposure settings. Since two of the model compounds were known to inhibit the enzyme cyclooxygenase, characteristic patterns for this mode of action could be identified from the landscapes.

Next, to advance our understanding of mixture effects on transcriptome level, the toxicogenomic profile was investigated which was induced by a mixture of the three previously characterized model compounds (chapter 4). Careful experimental design allowed to show for the first time that qualitative as well as quantitative combination effects (i.e., effects induced by more than one compound) occur on transcriptome level. Additionally, it was found that the mixture concept of concentration addition performed well in predicting the combination effects, outperforming other concepts, such as effect addition, boolean mixture, or independent action. Furthermore, the patterns of cyclooxygenase inhibition could be recovered in the mixture.

In summary, this dissertation offers strategies for retrieving and modeling time and concentration resolved toxicogenomic profiles of environmental chemicals. Additionally, it advances our understanding of how these toxicogenomic effect profiles of single substances combine in a mixture exposure and facilitates the prediction of mixture profiles. This can advance the applicability of toxicogenomics for non-target bioanalytical effect diagnosis.

ZUSAMMENFASSUNG

Während viele Gewässer mit Mischungen zahlreicher Chemikalien belastet sind, fehlen derzeit Methoden, die eine umfassende Diagnose der dadurch verursachten biologischen Effekte ermöglichen. Solche Effektinformationen werden jedoch im Gewässermanagement benötigt, um geeignete Maßnahmen gegen das Absinken der ökologischen Qualität treffen zu können. Toxikogenomische Methoden (d.h. die Anwendung von *omics* Technologien in der Toxikologie) könnten diese Lücke füllen und als ungerichtete ("Non-Target") bioanalytische Werkzeuge dienen. Deshalb verfolgt diese Dissertation das Ziel, die Anwendbarkeit toxikogenomischer Methoden im Umweltmonitoring zu verbessern.

Vorangegangene Studien haben gezeigt, dass die Exposition von Organismen gegenüber Chemikalien stoffspezifische Änderungen im Transkriptom, also in der Gesamtheit aller RNA Transkripte in einer Zelle oder einem Gewebe, hervorrufen kann. Transkripte sind Kopien von Genen auf der DNA, die regulatorische Funktion haben können oder als Bauanleitung für Proteine dienen. Die Analyse von Transkriptomprofilen ist z.B. mit Hilfe von cDNA-Microarrays möglich und kann auf die Exposition gegenüber bestimmten Stoffklassen hinweisen und gleichzeitig Informationen bezüglich der zellulären oder physiologischen Reaktion der Organismen liefern (z.B. Biotransformation, Proliferation, Zelltod). Deshalb könnten Transkriptomanalysen für eine umfassende Diagnose biologischer Effekte und der dafür verantwortlichen Substanzen in Umweltproben genutzt werden.

Jedoch bleibt die Erstellung toxikogenomischer Stoffprofile schwierig, da sich diese abhängig von den Expositionsbedingungen (z.B. Expositionskonzentration oder Dauer) verändern können. Neben stoffspezifischen Mustern sind auch generellere Antworten zu erwarten, die als Reaktion auf verschiedene Stoffe auftreten. Dies erschwert die Interpretation von Transkriptomprofilen, insbesondere bei der Analyse von Chemikalienmischungen. Des Weiteren ist bisher nicht geklärt, wie sich die Transkriptomprofile von Einzelstoffen bei einer Mischungsexposition zusammenfügen bzw. wiederfinden lassen.

Deshalb war es in dieser Arbeit zunächst das Ziel, generelle Änderungen im Transkriptom nach Chemikalienexposition zu identifizieren. Dafür wurden in einer Meta-Analyse veröffentlichte Transkriptomdaten von Embryonen des Zebrabärblings (*Danio rerio*) zusammengestellt (Kapitel 2). Es konnten Transkripte identifiziert werden, die einen generellen Trend in ihrer Reaktion auf Chemikaliengruppen oder spezifische Expositionsbedingungen zeigen. Die Meta-Analyse offenbarte jedoch

auch eine ausgeprägte Heterogenität in den Expositionsbedingungen. Gleichzeitig wurde innerhalb der Studien in der Regel keine Zeit- oder Konzentrationsabhängigkeit der Effekte untersucht. Daher lassen sich Ergebnisse kaum auf andere Bedingungen übertragen, was den Vergleich zwischen Substanzprofilen erschwert.

Für die Verbesserung der Übertragbarkeit und Vergleichbarkeit toxikogenomischer Profile wurden ein experimentelles Design und eine Analysestrategie entwickelt, welche die Erstellung zeit- und konzentrationsabhängiger toxikogenomischer Profile von Chemikalien ermöglichen (Kapitel 3). Die Profile wurden mit Hilfe einer *self-organizing map* der vorher kompilierten Daten der Meta-Analyse zu „toxikogenomischen Landschaften“ aggregiert. Diese können visuell dargestellt werden und erleichtern den Vergleich zwischen Stoffen und Expositionsbedingungen. Für eine quantitative Beschreibung der Profile wurde ein Regressionsmodell entwickelt, welches die zeit- und konzentrationsabhängigen Effekte erfassen kann. Landschaftsprojektion sowie Regressionsmodellierung wurden an einem Fallbeispiel angewendet, bei dem Zebrabärblingsembryonen gegenüber drei ausgewählten Stoffen exponiert wurden. Die entwickelte Strategie erlaubte die Erstellung von stoffspezifischen toxikogenomischen Landschaften, die sich für die Extrapolation und den Vergleich zwischen den Stoffen eigneten. Da es sich bei zwei der ausgewählten Stoffe um Cyclooxygenase-Hemmer handelte, konnte ein charakteristisches Effekt-Muster für diese Wirkweise identifiziert werden.

Als nächstes wurde die Ausweitung unseres Mischungsverständnisses auf Transkriptomebene angestrebt. Dafür wurde das toxikogenomische Profil genauer untersucht, welches durch die Mischung aus den drei Modellchemikalien der vorherigen Untersuchung hervorgerufen wurde (Kapitel 4). Sorgfältige Versuchsplanung ermöglichte erstmals auf Transkriptomebene den Nachweis qualitativer wie quantitativer Kombinationseffekte (d.h. Effekte, die von mehr als einer Komponente hervorgerufen werden). Es wurde gezeigt, dass das Mischungskonzept der Konzentrationsadditivität im Vergleich mit anderen Konzepten (wie Effektadditivität, Boolesche Mischung, Unabhängige Wirkung) die besten Vorhersagen für Kombinationseffekte liefern kann. Des Weiteren konnte das Effekt-Muster für Cyclooxygenase-Hemmung in der Mischung wiedergefunden werden.

Zusammenfassend, enthält diese Dissertation Strategien für die Erstellung und Modellierung zeit- und konzentrationsaufgelöster toxikogenomischer Profile von Umweltchemikalien. Zusätzlich wird unser Verständnis darüber erweitert, wie sich toxikogenomische Profile in Mischungen zusammenfügen, und damit die Vorhersage von Mischungsprofilen erleichtert. Dies dient der Weiterentwicklung und Implementierung toxikogenomischer Methoden für die Non-Target bioanalytische Effektdiagnose.

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LIST OF ABBREVIATIONS

AH arylhydrocarbon

AIC_c small sample Akaike information criterion

AO adverse outcome

AOP adverse outcome pathway

BO boolean mixture

CA concentration addition

CDNA complementary desoxyribonucleic acid

CI confidence interval

COX cyclooxygenase

CTD Comparitive Toxicogenomics Database

DEG differentially expressed gene

DNA desoxyribonucleic acid

EA effect addition

GCA generalized concentration addition

GEO Gene Expression Omnibus

GO Gene Ontology

GSEA gene set enrichment analysis

HPE hours post exposure

HPF hours post fertilization

IA independent action

KE key event

LC lethal concentration

logFC log_2(fold-change)

MCMC Markov Chain Monte Carlo

MOA mode of action

MSIGDB Molecular Signatures Database

NOAEL no observed adverse effect level

NOEC no observed effect concentration

NSAID non-steroidal anti-inflammatory drug

PAH polycyclic aromatic hydrocarbon

PDR prediction deviation ratio

PGE(2) prostaglandin E-2

PGH(2) prostaglandin H-2

RNA ribonucleic acid

SCE shuffled complex evolution

SOM self-organizing map

ZETAC Zebrafish Embryo Toxicogenomic trAnscriptome Coordinate system

ZETU Zebrafish Embryo Toxicogenomic Universe

ZFE zebrafish embryo

1 BACKGROUND AND MOTIVATION

Problem: Contamination of aquatic ecosystems with mixtures of chemicals with unknown effect.

There is increasing evidence for complex chemical contamination in European water bodies (Loos et al., 2013; Moschet et al., 2014). As analytical chemistry is improving and analysis of more compounds with lower detection limits is becoming feasible, an increasing number of chemicals is being detected in the aquatic environment. However, in most cases routine surveillance measures cannot resolve how these chemical mixtures effect aquatic organisms and ecosystems (Altenburger et al., 2015).

At the same time, we observe that a only a small part of European water bodies are in good ecological status (European Environment Agency, 2012) and biodiversity in aquatic ecosystems is decreasing (Dudgeon et al., 2006). There are several stressors, such as increasing temperature, hydromorphology, invasive species or increasing nutrient level which probably play a role in this development, and contamination by organic chemicals is discussed to be one of them (Beketov et al., 2013; Malaj et al., 2014; Schäfer et al., 2016).

Biological effects of chemicals and their mixtures need to be investigated.

It is the declared goal of the European Union to improve the ecological status of a majority of water bodies towards a good shape (*Council Directive 2000/60/EC* 2000). To be able to take the right steps to achieve this goal, it is necessary to elucidate if and how chemicals have impact on aquatic ecosystems. This includes determining whether adverse effects can be assigned to main drivers out of the multitude of detected chemicals (Brack et al., 2007). Additionally, it should be investigated to what extent combination effects play a role in the effects of complex contamination (Altenburger et al., 2015).

1.1 MEASUREMENT AND PREDICTION OF BIOLOGICAL EFFECTS CAUSED BY ENVIRONMENTAL CHEMICALS

There are different approaches to elucidate the biological effects of environmental chemicals. One way is to predict the biological effects from chemical monitoring (termed "bio-effect prediction" by Schroeder et al., 2016). A complementary approach applies bioassays to evaluate biological effects of environmental extracts (termed "bio-effect surveillance by Schroeder et al., 2016).

1.1.1 *Predicting biological effects from chemical monitoring*

To estimate the risk posed by (routinely) measured chemicals in the water bodies across Europe, Malaj et al. (2014) compiled toxicity parameters (i.e., LC_{50}) of detected compounds and related them to the respective environmental concentrations. The study indicated high risk for mainly chronic effects on organisms in many water bodies. However, the approach was biased by the number of compounds targeted in the respective monitoring efforts (more compounds measured at a site were leading to a higher risk). Combination effects could not be considered, and toxicity drivers were not identified. Additionally, due to often incomplete toxicity data, such as lack of chronic effect data, safety factors were applied, which makes the results of such kind of approaches imprecise.

Predicting effects from chemical monitoring data is biased by quality of chemical analytics and knowledge about detected substances.

Another study dealing with the prediction of biological effects from chemical monitoring data was a study by Busch et al. (2016), which focused on the modes of action (MOAs) of detected chemicals. The study showed that there is a wide range of compounds with different MOAs to be expected from the organic chemicals present in the water bodies. However, this picture might still be rather incomplete; Again, results were heavily dependent on the monitored chemicals on the one hand, and on the quality and comprehensiveness of effect information about the detected chemicals on the other hand.

1.1.2 *Measuring biological effects*

An approach which does not depend on chemical analytics was taken by Neale et al. (2017), who used a bioassay battery to asses biological effects of environmental samples. The study also compared the measured effects with predicted effects for analytically detected substances. It became clear that the measured biological effects could not be sufficiently explained by the predictions based on the detected chemicals. This demonstrates the need for bioanalytical testing when comprehensively assessing environmental health. However, even a bioassay battery used in the discussed study could only cover a reduced number of selected effects.

Assessing biological effects directly is necessary but only possible for selected endpoints with conventional methods.

Another approach combining bioanalytical testing, chemical fractionation, and analytics is effect-directed-analysis (EDA). Here, an environmental sample is fractionated, and the fractions tested for biological effects. Fractions showing biological effects are subsequently analyzed for their ingredients. This can help in identifying toxicity drivers in environmental extracts (Brack et al., 2007; Brack, 2003). However, biological testing is limited to a few selected endpoints, possibly overlooking important biological effects. Additionally, there might be an underestimation of combination effects.

1.2 (ECO)TOXICOGENOMICS

The brief overview over approaches to assess biological effects from environmental samples shows that both effect prediction from chemical monitoring as well as effect measurement with the help of bioassays may provide incomplete information about exposure and effects of environmental chemicals. However, while in chemical monitoring the chemical universe is nowadays being tackled with non-targeted analytics, comparable approaches assessing a comprehensive set of *biological* effects are yet lacking. Here, *omics* methods could come into play, which aim to get a system wide view of molecular changes occurring in cells, tissues and organisms. They enable comprehensively measuring the abundance of cellular constituents, such as gene transcripts (*transcriptome*), proteins (*proteome*), or metabolites (*metabolome*). *Omics* methods are discussed for offering solutions for a range of (eco)toxicological challenges (Afshari et al., 2011; Bahamonde et al., 2016; Brinke and Buchinger, 2017). The field tackling toxicological questions with *omics* methods has been termed *toxicogenomics* (Nuwaysir et al., 1999) and was extended to *ecotoxicogenomics*, describing the involvement of ecotoxicological topics (Snape et al., 2004).

Toxicogenomics could offer the possibility for a comprehensive biological effect assessment.

The basic idea of toxicogenomic approaches was framed by Nuwaysir et al. (1999) who stated that almost all toxic effects should be reflected in a change of gene expression at some point. Changes could either be direct effects of the contaminant (e.g., receptor activation leading to increased transcription of a gene), or indirect effects (e.g., a contaminant has neurotoxic effects, which influences breathing, which is then reflected on molecular level by a change in metabolism and gene expression). Soon after the first development of *omics* techniques, it was envisioned by Nuwaysir et al. (1999), Hamadeh et al. (2002), and Snape et al. (2004) to use characteristic toxicogenomic profiles of substances (i.e., distinct molecular changes induced by a contaminant) to categorize substances according to their molecular mode of action (MOA), such as estrogenic activity, cell cycle inhibitor, or similar.

This idea has been extended to the detailed elucidation of adverse outcome pathways (AOPs), respectively modes of action (MOAs) of compounds (Perkins et al., 2011; Woo et al., 2015) since one would expect that there are always some important key events (KEs) of a toxicants AOP which are expected to have an impact on the molecular constituents of the cells (i.e. the transcriptome, proteome or metabolome). The key events of an AOP can either be constituted by a change in gene expression or a higher level key event may be followed by a change in gene expression via feedback mechanisms (compare Figure 1.1). By conducting time and concentration dependent measurements it may be possible to resolve key events of different levels and their causality in an AOP (compare figure Figure 1.3).

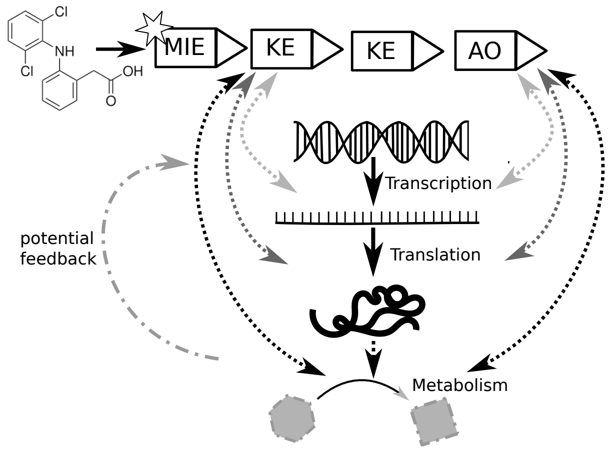


Figure 1.1: Representation of adverse outcome pathway in gene expression and metabolism. The key events (KEs) and adverse outcome (AO) of an AOP can be propagated by changes in gene expression (i.e., transcription, translation) or metabolism; or KEs/AO are followed by a change in gene expression/metabolism. Feedback mechanisms may lead to changes in transcription even if translation or metabolism are mainly affected (inspiration taken from Brinke and Buchinger, 2017).

Other applications of *omics* in environmental toxicology are the identification of new biomarkers (e.g., Fetter et al., 2015; Wang et al., 2008) or estimating the degree of pollution of specific environmental sites of interest (Boer et al., 2015). More recently, toxicogenomic analyses have also been proposed for a comprehensive effect assessment of contaminated sites by relating toxicogenomic profiles induced by environmental samples with known chemical-gene interactions (e.g., Perkins et al., 2017; Schroeder et al., 2016).

1.2.1 *Transcriptomics*

Toxicogenomic effects can be measured on different molecular biological levels (compare Figure 1.1) with each level having its own specific benefits, regarding its experimental accessibility, information content, or the possibility to extrapolate to different species, for example (Brinke and Buchinger, 2017). In this work the focus will be on the toxicogenomic effects on the transcriptome measured with the help of cDNA-microarrays. Transcriptomic methods focus on the change in gene transcription and measure the differential abundance of specific RNA molecules (Manzoni et al., 2016).

Transcriptional activity is highly regulated in eukaryotic cells to assure tissue specific gene expression as well as respond and adopt to external stimuli (Latchman, 1997). In response to toxicant exposure, transcriptional regulation may be part of a "controlled" adaption processes (e.g., regulation of metabolic enzymes) or sign of cellular malfunction (e.g., ectopic cell proliferation or hormone secretion, cell death). Regulation takes place via the modulation of chromatin structure (Berger, 2007) or the recruitment of transcription factors (proteins or RNA molecules) to the DNA region of specific genes (Hobert, 2008; Latchman, 1997). Transcription factor activity can itself be regulated, e.g. via phopsphorylation (Hunter and Karin, 1992). In many cases external stimuli activate specific receptors, which initiate a signaling cascade resulting in the activation of transcription factors. For example, one of the most prominent signaling pathways in response to chemical exposure is the arylhydrocarbon (Ah) receptor mediated signaling pathway (Schmidt and Bradfield, 1996). This receptor gets activated by certain polycyclic aromatic hydrocarbons (PAHs), for example, which allows the receptor to dimerize with the Ah receptor nuclear translocator. Subsequently the complex moves into the nucleus and binds to target regions on the DNA. If one observes increased abundance of Ah receptor target gene transcripts (such as cyp1a, for example) one can deduce an exposure to Ah receptor ligands and activation of PAH metabolic enzymes.

Transcriptional activity is highly regulated, i.a. in response to external stimuli.

Certainly, other levels of molecular regulation exist in the cell which are not captured by transcriptome analyses, such as post-translational modifications. Additionally, regulation on transcriptome level is not in every case translated into higher level effects. Anyhow, the transcriptome can be assessed in a more comprehensive manner than the proteome or metabolome (Brinke and Buchinger, 2017). This is because gene transcripts only vary in their specific nucleotide sequence but not in their biochemical properties as proteins or metabolites do. The comprehensiveness of biological effect information, which is a major focus in this work, may therefore be achieved most effectively using transcriptome analyses.

Due to the uniform biochemical properties of RNA, transcriptional abundance can be assessed most comprehensively

1.2.2 *The zebrafish embryo in ecotoxicology and toxicogenomics*

Zebrafish embryos (ZFEs) as well as adult zebrafish are increasingly used in biomedical (Lieschke and Currie, 2007; Lin et al., 2016) and environmental research (Scholz et al., 2008) due to the advantageous combination of biological complexity of a whole organism and the potential for high throughput handling (Driessen et al., 2014). Hermsen et al. (2011) and Driessen et al. (2015) showed that ZFE transcriptome analyses could be applied in human or environmental toxicology testing by providing information about hepatotoxicity or embryotoxicity, for example. Several studies have analyzed the embryo's transcriptome after it was subjected to chemical exposure (Williams et al., 2014). The application of toxicogenomics using the ZFE provides the advantages of a whole organism apical assay, which is able to capture and integrate a wide range of effects (Altenburger et al., submitted). Additionally, the approach provides diagnostic power by facilitating the dissection of induced effects on molecular level.

1.3 VISION: ECOTOXICOGENOMICS AS NON-TARGET BIOASSAY

A major contribution of toxicogenomics to ecotoxicological research could be the application as a non-target bioanalytical tool in environmental surveillance. Complementary to non-target chemical analytics such an analysis could give a comprehensive summary about the type and extent of biological effects expected from the ingredients of environmental extracts.

Comparison of environmental toxicogenomic profile with single compound profiles could give information about effects and drivers.

In Figure 1.2 it is schematically shown how such a non-target tool could potentially be used: The toxicogenomic profile of an environmental site of interest can be derived from exposure of a model organism to an environmental extract retrieved from the respective site. This profile can then be compared to known toxicogenomic profiles of single substance exposures. This would already allow hypotheses about a biological effect category (e.g., if the profile resembles profiles of neurotoxic compounds), adverse outcome (e.g., apoptosis, heart failure, decreased growth) and main effect drivers in the extract. Since we usually expect several compounds to exert an effect, we will expect the environmental toxicogenomic profile to be a combination of single compound profiles. An iterative procedure could be envisioned trying to reproduce the environmental toxicogenomic profiles from the single compound profiles. The procedure could optionally be supported by chemical analytics.

1.4 AIMS AND OBJECTIVES

In order to be able to predict mixture toxicogenomic profiles, as envisioned in Section 1.3, some challenges have to be overcome. First, we

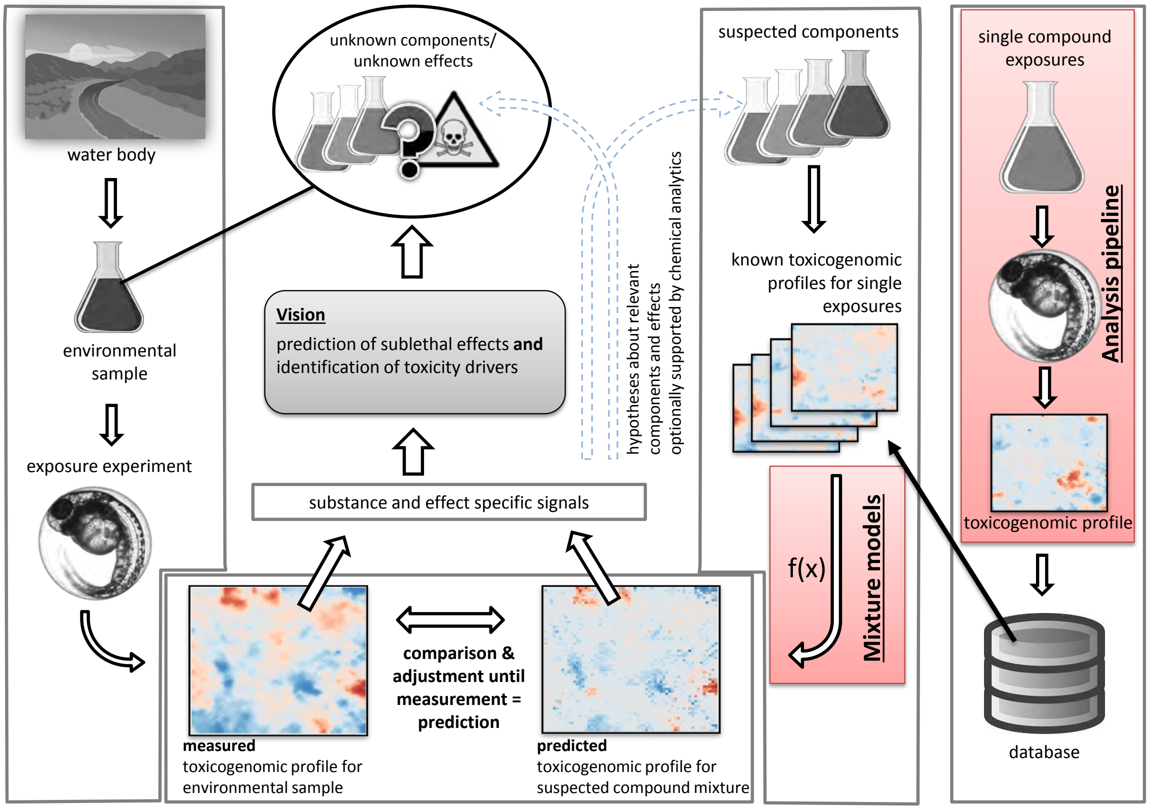


Figure 1.2: Scheme of envisioned application of toxicogenomic methods in environmental surveillance: A toxicogenomic profile of an environmental site is compared with a predicted combination of toxicogenomic profiles of single substances. Challenges addressed in this dissertation are shaded in red and include the development of an analysis pipeline to derive extrapolated toxicogenomic profiles, and the development of mixture models for toxicogenomic profiles.

need to validate our expectation that similar compounds, respectively similar adverse outcomes, are inducing similar toxicogenomic profiles (see Section 1.4.1 and Chapter 2).

Aims: Develop strategy to infer extrapolatable toxicogenomic profiles and elucidate predictability of mixture effects.

Additionally, it is not without complication to compare the toxicogenomic profile induced by an environmental sample with profiles which are induced by single compounds. This is because the compounds in an environmental sample will probably occur in other concentrations than in the single compound exposures, from which we derived the "database" profiles. Therefore, we need to develop a strategy and analysis pipeline to infer robust compound profiles which allow extrapolation to other exposure settings (see Section 1.4.2 and Chapter 3).

Last but not least, for the envisioned approach we need to be able to predict how toxicogenomic profiles combine in a mixture (see Section 1.4.3 and Chapter 4).

1.4.1 *Molecular profiles for effect categories and adversity - Which genes are responding?*

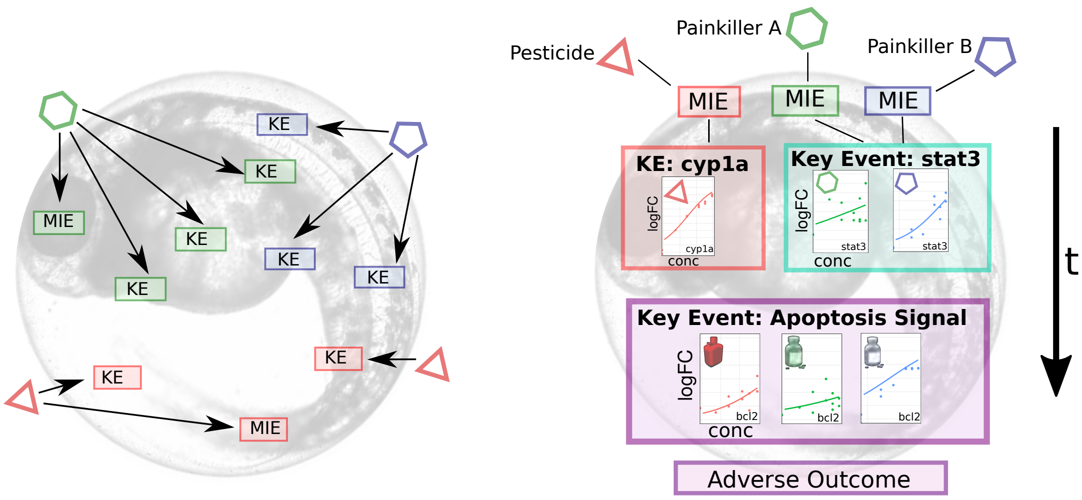
Numerous studies analyzed the ZFE transcriptome after chemical exposure.

There is a substantial number of studies, dealing with toxicogenomic effects of single compounds (e.g., for teleost fish in aquatic toxicology, compare Williams et al., 2014). They usually compare genome expression in tissues or whole model organisms between treatment and control conditions, resulting in ranked list of differentially expressed genes, proteins or metabolites. The functional interpretation of the toxicogenomic profiles is then achieved by integrating knowledge about protein function from public databases (such as Gene Ontology, Reactome, KEGG). This can be supported by over-representation analyses or gene set enrichment analysis (GSEA). The extraction of biological functions from toxicogenomic profiles is an important first step and many studies could demonstrate the suitability of toxicogenomics to reveal biological effects of toxicants and elucidate (parts) of their AOPs. For example, Klüver et al. (2011) could identify the regulation of the small heat shock protein *hspb11* as key transcriptional response towards neurotoxic compounds in the early developmental stages of the ZFE. Perkins et al. (2011) could identify potential key events of the AOP of flutamide in the ZFE using a reverse engineering approach. Hermsen et al. (2013) identified compound specific regulated pathways in the ZFE after exposure to different embryotoxicants. Those pathways were indicative of the MOA of the respective compounds. Additionally, they found some commonly regulated pathways indicative of embryotoxicity. Schiller et al. (2013) found gene set regulation of different endocrine disruptors to be indicative of estrogenic, respectively anti-androgenic activity. Integrating transcriptome data of *Daphnia magna* exposed to different chemicals, Antczak et al. (2015) identified altered calcium homeostasis as a potential key event of narcotic compounds. Woo et al. (2015) applied network perturbation analysis to identify proteins directly targeted by anti-cancer drugs.

Toxicogenomic studies demonstrate potential of methodoly, however they have provided mostly anecdotal evidence, so far.

This is just to give a few examples of the potentials of toxicogenomics to elucidate molecular responses, i.e., AOPs of chemicals. Most of the mentioned studies have examined toxicogenomic effects or AOPs for single substances in isolation, while the connection between the different AOPs is still unclear (compare scheme in figure Figure 1.3a). However, it is expected that "many distinct [adverse outcome] paths will [...] converge on a relatively finite number of terminal adverse outcomes" (Villeneuve et al., 2014). Therefore, we expect some of the toxicogenomic patterns to be of a compound specific nature, while patterns directly related to adverse outcome should be similar for a wider range of different compounds. For example, in the ZFE this has been shown for the case of crystallin transcript regulation, being suspected to indicate an unspecific developmental delay (Hermsen et al., 2013); similarly Antczak et al. (2015) identified calcium homeostasis as key event for

We expect the convergence of AOPs to be reflected in converging toxicogenomic profiles.



(a) current toxicogenomic AOP knowledge (b) envisioned future toxicogenomic AOP knowledge

Figure 1.3: Scheme of current and envisioned AOP knowledge.

a range of different narcotic compounds (see above). In combination with the resolution of time and concentration dependency, information about specificity of regulation could enable the integration of multiple AOPs (compare scheme in figure Figure 1.3b).

If we want to define robust toxicogenomic profiles, reflecting a certain MOA/AOP, which can be recovered in an environmental extract later on, we need to be able to distinguish between specific and general responses. Thus, as a first step in this work, I strived to identify general toxicogenomic responses in the ZFE by aggregating and comparing results from many different single compound exposures. Most toxicogenomic studies published so far have focused on the isolated analysis of single chemicals (Schroeder et al., 2016), while an aggregation of these results is missing so far. Therefore, I conducted a meta-analysis of toxicogenomic transcriptome data in order to get a qualitative overview about toxicogenomic effects in the zebrafish embryo. The main questions were:

A meta-analysis was conducted to identify general responses in the ZFE transcriptome.

- Which experimental factors influence the type and extent of gene regulation in ZFE?
- Can genes be identified which generally respond towards any kind of chemical exposure?
- Can genes be identified generally responding in groups of common treatments (e.g., exposure time, MOA of chemical, effect concentration)?

These questions are addressed in Chapter 2.

1.4.2 *Concentration- and time dependence - How do the genes respond?*

Concentration or time dependence is rarely considered in gene expression studies.

As it was outlined above, we need profiles which can be extrapolated across varying exposure concentrations for the application of toxicogenomic profiling as non-target bioanalytical tool. However, most toxicogenomic studies (implicitly) assume a binary type of molecular response, i.e. the assumption is that a compound induces/represses the expression of a gene or protein or not. This is reflected in the experimental design, where the comparison of one experimental treatment versus control condition is standard and not exception (an approach possibly adopted from biomedical studies like in cancer research, where one can only compare diseased versus healthy samples). However, when comparing the toxicogenomic profiles of two compounds, this binary assumption becomes highly problematic and is conflicting with a fundamental principle of toxicology, namely "the dose makes the poison" (paraphrasing Paracelsus, 1493-1541). One could add to this that also the time makes the poison and both statements have been demonstrated to be true for toxicogenomic profiles (e.g., Hamadeh et al., 2002). While it may not be the most pressing need for the analysis of single compound related questions, for the comparison of single compounds and finally interpreting the toxicogenomic effects of a mixture, it is not only important to determine *if* a gene is responding but it is also necessary to find a quantitative description of *how* those genes respond.
As already mentioned, the notion of concentration and time dependence of toxicogenomic profiles is not new. However, there are only few studies considering those variables. Thomas et al. (2007) took an approach to calculate benchmark doses for Gene Ontology (GO) categories. However, the authors were using linear and polynomial models, which might not be the best representation of toxicological responses. Gündel et al. (2012) and Smetanová et al. (2015) extended the description of concentration response relationships to proteomics and metabolomics and also used sigmoidal models to describe the concentration response relationships. Gao et al. (2015) is one of the few studies measuring the signal over dose *and* time. However, the signals are integrated into effect levels, which leads to a loss of information, the model parameters are limited in their explanatory power, and inter- or extrapolation to different exposure settings is hardly feasible.

Here, the objective was to extend the previous work on time- and concentration dependence, and develop an experimental design and data analysis strategy to derive robust toxicogenomic profiles, which may be extrapolated across exposure duration and concentration, and are comparable between different compounds. This included to find a general regression model for gene regulation. It should be able to capture transcript dynamics after exposure to different compounds. The model should resolve time- and concentration dependence, and include model parameters which lend themselves for biological interpretation. This should enable us to quantitatively describe how a gene is reacting and inter-/extrapolating to different exposure settings, which is crucial for mixture modeling. I exemplarily applied the newly developed experimental and analysis pipeline on three model substances. The retrieved toxicogenomic profiles should later on be recovered in an artificial mixture. The analysis strategy is described and evaluated in detail in Chapter 3.

Objective: develop experimental design and analysis pipeline for deriving robust toxicogenomic profiles

1.4.3 *Combination effects*

Environmental extracts will in many cases comprise of a multitude of compounds (Loos et al., 2009, 2013). They might be partly similarly acting and partly dissimilarly acting (Altenburger et al., 2004). For a meaningful interpretation of the resulting toxicogenomic "mixture-profile" in a way as it is envisioned in Figure 1.2 we need to get an idea how single compound profiles combine in mixtures.

To predict the combined action of non-interacting components in a mixture based on biological activity of the components, mainly two conceptual models have been established (reviewed in e.g., Altenburger et al., 2013; Cedergreen et al., 2013). These concepts are called concentration addition (CA, also known as Loewe additivity) and independent action (IA, also known as Bliss independence). The model of CA adds up the concentrations of the individual components scaled by an effectiveness factor. The model of IA multiplies the fractional effects of the individual components (for details see Chapter 4). In ecotoxicology, these models have been successfully used to predict combination effects of biological effects such as lethality (Altenburger et al., 2013).

MIXTURE TOXICOLOGY AND TOXICOGENOMICS In the field of toxicogenomics, conceptual models like CA or IA are rarely used (Altenburger et al., 2012). Instead many times a boolean combination is implicitly assumed, i.e. if a gene is regulated by one of the mixture components at any concentration, it is expected to be regulated in the mixture (at any concentration). This approach might partly be due to the predominating experimental design of contrasting treatment versus control conditions and the reluctance to establish concentration response relationships needed for a quantitative prediction of combination effects. Additionally, genome regulation might in some cases be deemed too complex (e.g. including redundancy or feedback-loops) to be sufficiently described and predicted by established "simple" regression models such as the hill model, or concepts like CA or IA in the case of mixture investigations. Experiments based on a boolean combination assumption usually result in identifying high numbers of genes either

CA and IA are well established mixture concepts in ecotoxicology, but have not been thoroughly evaluated in toxicogenomics studies so far.

unexpectedly regulated in the mixture or unexpectedly unregulated in the mixture - a result which is difficult to interpret without knowing about concentration response relationships (Altenburger et al., 2012).

There are first indications that established mixture concepts can be transferred to the field of toxicogenomics. Labib et al. (2017) showed that combined effects of PAHs on the mean expression of genes of a few selected pathways could be well predicted using the independent action model. De Coninck et al. (2014) also used the independent action model to identify interacting mixture components.

While they provide a good starting point for evaluating mixture toxicology on transcriptome level, the mentioned studies relied on a very limited number of applied concentrations (three for Labib et al. (2017) and one for De Coninck et al. (2014)) and a thorough evaluation of the occurrence and predictability of combination effects on genome scale has not been performed. Therefore, in this work, I strived for a genome wide view of toxicogenomic combination effects, based on robust concentration and time resolved single compound profiles (compare Chapter 3). I mainly focused on the following questions:

- Is it possible to identify toxicogenomic combination effects (i.e., effects which are stronger than would be expected by any of the individual components)
- Are the combination effects predictable using established mixture concepts such as concentration addition (CA), independent action (IA) or effect addition (EA)?
- How well can the whole toxicogenomic profile of a mixture be predicted from the profiles of the mixtures components?
- Can effects from single substances be recovered in the mixture exposure?

These questions are addressed in Chapter 4.

REFERENCES

Council Directive 2000/60/EC (2000). Directive of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal of the European Communities. L327.

Afshari, C. A., H. K. Hamadeh, and P. R. Bushel (2011). “The evolution of bioinformatics in toxicology: Advancing toxicogenomics.” In: *Toxicological Sciences* 120.SUPPL.1. DOI: 10.1093/toxsci/kfq373.

Altenburger, R., H. Walter, and M. Grote (Dec. 2004). “What Contributes to the Combined Effect of a Complex Mixture?” In: *Environmental Science & Technology* 38.23, pp. 6353–6362. DOI: 10.1021/es049528k.

Altenburger, R., S. Scholz, M. Schmitt-Jansen, W. Busch, and B. I. Escher (Mar. 2012). “Mixture Toxicity Revisited from a Toxicogenomic Perspective.” In: *Environmental Science & Technology* 46.5, pp. 2508–2522. DOI: 10.1021/es2038036.

Altenburger, R., T. Backhaus, W. Boedeker, M. Faust, and M. Scholze (2013). “Simplifying complexity: Mixture toxicity assessment in the last 20 years.” In: *Environmental Toxicology and Chemistry* 32.8, pp. 1685–1687. DOI: 10.1002/etc.2294.

Altenburger, R., S. Ait-Aissa, P. Antczak, T. Backhaus, D. Barceló, T.-B. Seiler, F. Brion, W. Busch, K. Chipman, M. L. de Alda, et al. (Apr. 2015). “Future water quality monitoring — Adapting tools to deal with mixtures of pollutants in water resource management.” In: *Science of The Total Environment* 512-513, pp. 540–551. DOI: 10.1016/j.scitotenv.2014.12.057.

Altenburger, R., W. Busch, B. I. Escher, G. Jakobs, M. Krauss, P. A. Neale, A. C. Almeida, H. Hollert, R. Schlichting, Y. Shao, et al. (submitted). “Mixture effect detection in complex contaminated samples – an interlaboratory comparative study using bioassays.”

Antczak, P., T. a. White, A. Giri, F. Michelangeli, M. R. Viant, M. T. D. Cronin, C. Vulpe, and F. Falciani (2015). “Systems Biology Approach Reveals a Calcium-Dependent Mechanism for Basal Toxicity in Daphnia magna.” In: *Environmental Science & Technology* 49.18, pp. 11132–11140. DOI: 10.1021/acs.est.5b02707.

Bahamonde, P. A., A. Feswick, M. A. Isaacs, K. R. Munkittrick, and C. J. Martyniuk (2016). “Defining the role of omics in assessing ecosystem health: Perspectives from the Canadian environmental monitoring program.” In: *Environmental Toxicology and Chemistry* 35.1, pp. 20–35. DOI: 10.1002/etc.3218.

Beketov, M. A., B. J. Kefford, R. B. Schäfer, and M. Liess (July 2013). “Pesticides reduce regional biodiversity of stream invertebrates.” In: *Proceedings of the National Academy of Sciences of the United States of America* 110.27, pp. 11039–43. DOI: 10.1073/pnas.1305618110.

Berger, S. L. (2007). “The complex language of chromatin regulation during transcription.” In: *Nature* 447.7143, pp. 407–412. DOI: 10.1038/nature05915.

Boer, T. E. de, T. K. S. Janssens, J. Legler, N. M. van Straalen, and D. Roelofs (2015). “Combined Transcriptomics Analysis for Classification of Adverse Effects As a Potential End Point in Effect Based Screening.” In: *Environmental Science & Technology* 49.24, acs.est.5b03443. DOI: 10.1021/acs.est.5b03443.

Brack, W., H. J. C. Klamer, M. López de Alda, and D. Barceló (Jan. 2007). “Effect-directed analysis of key toxicants in European river basins a review.” In: *Environmental Science and Pollution Research International* 14.1, pp. 30–38. DOI: 10.1065/espr2006.08.329.

Brack, W. (Oct. 2003). "Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures?" In: *Analytical and bioanalytical chemistry* 377.3, pp. 397–407. DOI: 10.1007/s00216-003-2139-z.

Brinke, A. and S. Buchinger (2017). "Toxicogenomics in Environmental Science." In: *In vitro Environmental Toxicology - Concepts, Application and Assessment*. Ed. by G. Reifferscheid and S. Buchinger. Cham: Springer International Publishing, pp. 159–186. DOI: 10.1007/10_2016_15.

Busch, W., S. Schmidt, R. Kühne, T. Schulze, M. Krauss, and R. Altenburger (Aug. 2016). "Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring." In: *Environmental Toxicology and Chemistry* 35.8, pp. 1887–1899. DOI: 10.1002/etc.3460.

Cedergreen, N., C. Svendsen, and T. Backhaus (2013). "Chemical Mixtures : Concepts for Predicting Toxicity." In: *Encyclopedia of Environmental Management*. Ed. by S. E. Jorgensen. New York: Taylor and Francis, pp. 2572–2581. DOI: 10.1081/E-EEM-120046684.

De Coninck, D. I. M., J. Asselman, S. Glaholt, C. R. Janssen, J. K. Colbourne, J. R. Shaw, and K. a. C. De Schamphelaere (Mar. 2014). "Genome-wide transcription profiles reveal genotype-dependent responses of biological pathways and gene-families in Daphnia exposed to single and mixed stressors." In: *Environmental science & technology* 48.6, pp. 3513–22. DOI: 10.1021/es4053363.

Driessen, M., A. S. Kienhuis, A. P. Vitins, J. L. a. Pennings, T. E. Pronk, E. J. V. D. Brandhof, M. Roodbergen, B. V. D. Water, and L. T. M. V. D. Ven (2014). "Gene expression markers in the zebrafish embryo reflect a hepatotoxic response in animal models and humans." In: *Toxicology Letters* 230.1, pp. 48–56. DOI: 10.1016/j.toxlet.2014.06.844.

Driessen, M., A. P. Vitins, J. L. A. Pennings, A. S. Kienhuis, B. V. D. Water, and L. T. M. van der Ven (2015). "A transcriptomics-based hepatotoxicity comparison between the zebrafish embryo and established human and rodent in vitro and in vivo models using cyclosporine A, amiodarone and acetaminophen." In: *Toxicology Letters* 232.2, pp. 403–412. DOI: 10.1016/j.toxlet.2014.11.020.

Dudgeon, D., A. H. Arthington, M. O. Gessner, Z.-I. Kawabata, D. J. Knowler, C. Lévêque, R. J. Naiman, A.-H. Prieur-Richard, D. Soto, M. L. J. Stiassny, et al. (2006). "Freshwater biodiversity: importance, threats, status and conservation challenges." In: *Biological Reviews* 81.02, p. 163. DOI: 10.1017/S1464793105006950.

European Environment Agency (2012). *European waters — assessment of status and pressures. European Environment Agency*. Tech. rep. Copenhagen: European Environment Agency. DOI: 10.2800/63266.

Fetter, E., S. Smetanová, L. Baldauf, A. Lidzba, R. Altenburger, A. Schüttler, and S. Scholz (Oct. 2015). "Identification and Characterization of Androgen-Responsive Genes in Zebrafish Embryos." In: *Environmental Science & Technology* 49.19, pp. 11789–11798. DOI: 10.1021/acs.est.5b01034.

Gao, C., D. Weisman, J. Lan, N. Gou, and A. Z. Gu (Apr. 2015). "Toxicity Mechanisms Identification via Gene Set Enrichment Analysis of Time-Series Toxicogenomics Data: Impact of Time and Concentration." In: *Environmental Science & Technology* 49.7, pp. 4618–4626. DOI: 10.1021/es505199f.

Gündel, U., S. Kalkhof, D. Zitzkat, M. von Bergen, R. Altenburger, and E. Küster (Feb. 2012). "Concentration–response concept in ecotoxicoproteomics: Effects of different phenanthrene concentrations to the zebrafish (Danio rerio) embryo proteome." In: *Ecotoxicology and Environmental Safety* 76, pp. 11–22. DOI: 10.1016/j.ecoenv.2011.10.010.

Hamadeh, H. K., P. R. Bushel, S. Jayadev, K. Martin, O. DiSorbo, S. Sieber, L. Bennett, R. Tennant, R. Stoll, J. C. Barrett, et al. (2002). "Gene expression analysis reveals chemical-specific profiles." In: *Toxicological sciences : an official journal of the Society of Toxicology* 67.2, pp. 219–31. DOI: 10.1093/TOXSCI/67.2.219.

Hermsen, S. A. B., T. E. Pronk, E. J. van den Brandhof, L. T. M. van der Ven, and A. H. Piersma (2011). "Chemical class-specific gene expression changes in the zebrafish embryo after exposure to glycol ether alkoxy acids and 1,2,4-triazole antifungals." In: *Reproductive Toxicology* 32.2, pp. 245–252. DOI: 10.1016/j.reprotox.2011.05.010.

Hermsen, S. A. B., T. E. Pronk, E.-J. van den Brandhof, L. T. M. van der Ven, and A. H. Piersma (Oct. 2013). "Transcriptomic analysis in the developing zebrafish embryo after compound exposure: individual gene expression and pathway regulation." In: *Toxicology and applied pharmacology* 272.1, pp. 161–71. DOI: 10.1016/j.taap.2013.05.037.

Hobert, O. (2008). "Gene Regulation by Transcription Factors and MicroRNAs." In: *Science* 319.5871, pp. 1785–1786. DOI: 10.1126/science.1151651.

Hunter, T. and M. Karin (Aug. 1992). "The regulation of transcription by phosphorylation." In: *Cell* 70.3, pp. 375–387. DOI: 10.1016/0092-8674(92)90162-6.

Klüver, N., L. Yang, W. Busch, K. Scheffler, P. Renner, U. Strähle, and S. Scholz (Jan. 2011). "Transcriptional response of zebrafish embryos exposed to neurotoxic compounds reveals a muscle activity dependent hspb11 expression." In: *PLoS ONE* 6.12, e29063. DOI: 10.1371/journal.pone.0029063.

Labib, S., A. Williams, B. Kuo, C. L. Yauk, P. A. White, and S. Halappanavar (July 2017). "A framework for the use of single-chemical transcriptomics data in predicting the hazards associated with complex mixtures of polycyclic aromatic hydrocarbons." In: *Archives of Toxicology* 91.7, pp. 2599–2616. DOI: 10.1007/s00204-016-1891-8.

Latchman, D. S. (1997). "Transcription factors: An overview." In: *International Journal of Biochemistry and Cell Biology* 29.12, pp. 1305–1312. DOI: 10.1016/S1357-2725(97)00085-X.

Lieschke, G. J. and P. D. Currie (May 2007). "Animal models of human disease: zebrafish swim into view." In: *Nature Reviews Genetics* 8.5, pp. 353–367. DOI: 10.1038/nrg2091.

Lin, C.-Y., C.-Y. Chiang, and H.-J. Tsai (2016). "Zebrafish and Medaka: new model organisms for modern biomedical research." In: *Journal of Biomedical Science*, pp. 1–11. DOI: 10.1186/s12929-016-0236-5.

Loos, R., B. M. Gawlik, G. Locoro, E. Rimaviciute, S. Contini, and G. Bidoglio (2009). "EU-wide survey of polar organic persistent pollutants in European river waters." In: *Environmental Pollution* 157.2, pp. 561–568. DOI: 10.1016/j.envpol.2008.09.020.

Loos, R., R. Carvalho, D. C. António, S. Comero, G. Locoro, S. Tavazzi, B. Paracchini, M. Ghiani, T. Lettieri, L. Blaha, et al. (2013). "EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents." In: *Water Research* 47.17, pp. 6475–6487. DOI: 10.1016/j.watres.2013.08.024.

Malaj, E., P. C. von der Ohe, M. Grote, R. Kühne, C. P. Mondy, P. Usseglio-Polatera, W. Brack, and R. B. Schäfer (July 2014). "Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale." In: *Proceedings of the National Academy of Sciences* 111.26, pp. 9549–9554. DOI: 10.1073/pnas.1321082111.

Manzoni, C., D. A. Kia, J. Vandrovcova, J. Hardy, N. W. Wood, P. A. Lewis, and R. Ferrari (Nov. 2016). "Genome, transcriptome and proteome: the rise of omics data and their integration in biomedical sciences." In: *Briefings in Bioinformatics* 70.3, pp. 375–87. DOI: 10.1093/bib/bbw114.

Moschet, C., I. Wittmer, J. Simovic, M. Junghans, A. Piazzoli, H. Singer, C. Stamm, C. Leu, and J. Hollender (May 2014). "How a Complete Pesticide Screening Changes the Assessment of Surface Water Quality." In: *Environmental Science & Technology* 48.10, pp. 5423–5432. DOI: 10.1021/es500371t.

Neale, P. A., R. Altenburger, S. Ait-Aissa, F. Brion, W. Busch, G. d. A. Umbuzeiro, M. S. Denison, D. Du Pasquier, K. Hilscherová, H. Hollert, et al. (2017). "Development of a bioanalytical test battery for water quality monitoring: Fingerprinting identified micropollutants and their contribution to effects in surface water." In: *Water Research* 123. DOI: 10.1016/j.watres.2017.07.016.

Nuwaysir, E. F., M. Bittner, J. Trent, J. C. Barrett, and C. A. Afshari (Mar. 1999). “Microarrays and toxicology: The advent of toxicogenomics.” In: *Molecular Carcinogenesis* 24.3, pp. 153–159. DOI: 10.1002/(SICI)1098-2744(199903)24:3<153::AID-MC1>3.0.CO;2-P.

Perkins, E. J., J. K. Chipman, S. Edwards, T. Habib, F. Falciani, R. Taylor, G. Van Aggelen, C. Vulpe, P. Antczak, and A. Loguinov (Jan. 2011). “Reverse engineering adverse outcome pathways.” In: *Environmental Toxicology and Chemistry* 30.1, pp. 22–38. DOI: 10.1002/etc.374.

Perkins, E. J., T. Habib, B. L. Escalon, J. E. Cavallin, L. Thomas, M. Weberg, M. N. Hughes, K. M. Jensen, M. D. Kahl, D. L. Villeneuve, et al. (Aug. 2017). “Prioritization of Contaminants of Emerging Concern in Wastewater Treatment Plant Discharges Using Chemical:Gene Interactions in Caged Fish.” In: *Environmental Science & Technology* 51.15, pp. 8701–8712. DOI: 10.1021/acs.est.7b01567.

Scholz, S., S. Fischer, U. Gündel, E. Küster, T. Luckenbach, and D. Voelker (July 2008). “The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing.” In: *Environmental Science and Pollution Research* 15.5, pp. 394–404. DOI: 10.1007/s11356-008-0018-z.

Schiller, V., A. Wichmann, R. Kriehuber, C. Schäfers, R. Fischer, and M. Fenske (Dec. 2013). “Transcriptome alterations in zebrafish embryos after exposure to environmental estrogens and anti-androgens can reveal endocrine disruption.” In: *Reproductive Toxicology* 42, pp. 210–223. DOI: 10.1016/j.reprotox.2013.09.003.

Schäfer, R. B., B. Kühn, E. Malaj, A. König, and R. Gergs (2016). “Contribution of organic toxicants to multiple stress in river ecosystems.” In: *Freshwater Biology* 61.12, pp. 2116–2128. DOI: 10.1111/fwb.12811.

Schroeder, A. L., G. T. Ankley, A. H. Keith, and D. L.Villeneuve (2016). “Environmental Surveillance and Monitoring - The Next Frontiers for High-Throughput Toxicology.” In: *Environmental Toxicology and Chemistry* 35.3, pp. 513–525. DOI: 10.1002/etc.3309.

Schmidt, J. V. and C. A. Bradfield (1996). “Ah Receptor Signaling Pathways.” In: *Annual Review of Cell and Developmental Biology* 12.1, pp. 55–89. DOI: 10.1146/annurev.cellbio.12.1.55.

Smetanová, S., J. Riedl, D. Zitzkat, R. Altenburger, and W. Busch (Sept. 2015). “High-throughput concentration-response analysis for omics datasets.” In: *Environmental Toxicology and Chemistry* 34.9, pp. 2167–2180. DOI: 10.1002/etc.3025.

Snape, J. R., S. J. Maund, D. B. Pickford, and T. H. Hutchinson (Apr. 2004). “Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology.” In: *Aquatic Toxicology* 67.2, pp. 143–154. DOI: 10.1016/j.aquatox.2003.11.011.

Thomas, R. S., B. C. Allen, A. Nong, L. Yang, E. Bermudez, H. J. Clewell, and M. E. Andersen (July 2007). "A Method to Integrate Benchmark Dose Estimates with Genomic Data to Assess the Functional Effects of Chemical Exposure." In: *Toxicological Sciences* 98.1, pp. 240–248. DOI: 10.1093/toxsci/kfm092.

Villeneuve, D. L., D. Crump, N. Garcia-Reyero, M. Hecker, T. H. Hutchinson, C. A. LaLone, B. Landesmann, T. Lettieri, S. Munn, M. Nepelska, et al. (2014). "Adverse outcome pathway development II: Best practices." In: *Toxicological Sciences* 142.2, pp. 321–330. DOI: 10.1093/toxsci/kfu200.

Wang, R.-L., D. Bencic, A. Biales, D. Lattier, M. Kostich, D. Villeneuve, G. T. Ankley, J. Lazorchak, and G. Toth (Mar. 2008). "DNA microarray-based ecotoxicological biomarker discovery in a small fish model species." In: *Environmental Toxicology and Chemistry* 27.3, pp. 664–75. DOI: 10.1897/07-192.

Williams, T. D., L. Mirbahai, and J. K. Chipman (Mar. 2014). "The toxicological application of transcriptomics and epigenomics in zebrafish and other teleosts." In: *Briefings in Functional Genomics* 13.2, pp. 157–171. DOI: 10.1093/bfgp/elt053.

Woo, J. H., Y. Shimoni, W. S. Yang, P. Subramaniam, A. Iyer, P. Nicoletti, M. Rodríguez Martínez, G. López, M. Mattioli, R. Realubit, et al. (July 2015). "Elucidating Compound Mechanism of Action by Network Perturbation Analysis." In: *Cell* 162.2, pp. 441–451. DOI: 10.1016/j.cell.2015.05.056.

2 META-ANALYSIS OF TRANSCRIPTOME DATA

2.1 INTRODUCTION

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Technologies measuring the entirety of gene transcripts, proteins or metabolites in a sample ("omics") are increasingly used in toxicological research and are discussed to be included into chemical regulatory assessments in the future (Marx-Stoelting et al., 2015). In toxicology, these methods are primarily used to obtain insight into a compound's mode of action, to group similar acting compounds or to define new biomarkers (Afshari et al., 2011). Several studies have been published demonstrating responses to chemical exposure on transcriptome, proteome, metabolome or epigenome level in different organisms. Here, we review the achievements made with the ZFE in the field of toxicogenomics (measuring gene transcript abundance, often also termed "gene expression"). ZFEs as well as adult zebrafish are increasingly used in biomedical (Lieschke and Currie, 2007; Lin et al., 2016) and environmental research (Scholz et al., 2008) due to the advantageous combination of biological complexity of a whole organism and the potential for high throughput handling (Driessen et al., 2014). The added value of ZFE transcriptome analyses for human toxicology testing has been discussed before (Driessen et al., 2015; Hermsen et al., 2011) and several studies have analyzed the embryo's transcriptome after it was subjected to chemical exposure (Williams et al., 2014). However, to our knowledge, results of ZFE toxicogenomics studies have not been aggregated or compared so far. In many studies it could not be resolved, which of the observed effects were specific for the compound or compound class studied and which would be expected to be of a more general nature, e.g., related to disturbance of embryonic development or global homeostasis. We assume that compounds mostly act via a range of different molecular mechanisms (e.g., different target molecules), but we also expect effect cascades to converge into smaller and more general sets of toxicity pathways (Villeneuve et al., 2014). This should also be reflected on the transcriptome level so that chemical induced differential transcription of at least some genes or gene sets should be independent of compound or embryonic stage. To be able to derive a meaningful and reproducible grouping of compounds and later on elucidate connections of molecular effects with higher level effects, which are of interest not only for regulatory issues, such knowledge about common responses is of utter importance.

Thus, in this meta-analysis we aimed at the identification of genes or gene sets showing a general response towards chemical exposure. In the study presented here, we compared 33 microarray studies including exposures of the ZFE to 60 different compounds (948 arrays in total) and aimed at identifying common transcriptional regulation across all experiments as well as across meaningful subgroups. We followed two complementary approaches (see Figure 2.1 and Figure A.1). First, we re-

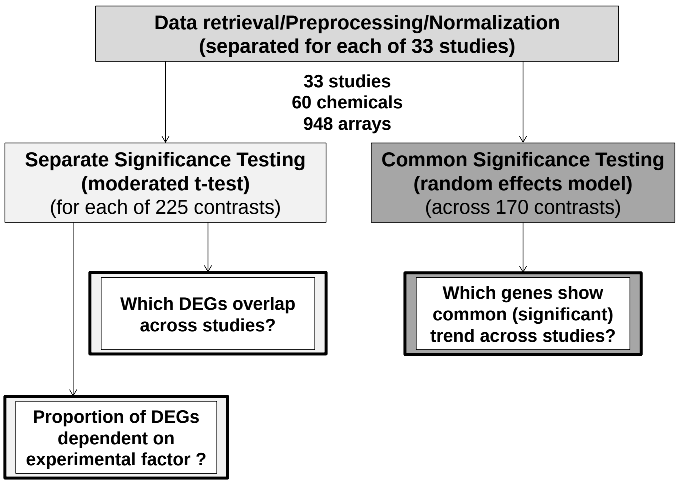


Figure 2.1: Approach of the meta-analysis of transcriptome studies of the zebrafish embryo after chemical exposure. For details also see Figure A.1.

analyzed each experimental dataset separately, identified differentially expressed genes (DEGs) using moderated t-statistics and then determined the overlap of DEGs across datasets. In a second approach, we aggregated normalized data from all studies and determined a summary effect size using a random effects model. Functional enrichment analysis allowed us to derive conclusions about some general stress responses. Finally, we discuss, what is needed for future experiments, in order to allow for more powerful interpretations of omics profiles after chemical exposure.

2.2 METHODS

For our analysis we followed a strategy similar to that proposed by (Ramasamy et al., 2008). Studies were selected for the meta-analysis in which microarray measurements of global gene transcription changes in the ZFE after exposure to chemical compounds were performed (gene knock-down studies were not included). A database query was conducted in Gene Expression Omnibus and ArrayExpress (no search term, Filters: Organism: Danio rerio, Data type: expression profiling by array, one-color array or two color common reference) and all studies satisfying above restrictions were manually selected. This resulted in 33 studies reporting on 60 compounds tested in 948 arrays (see table 2.1).

Table 2.1: List of chemicals included in the meta-analysis together with assigned mode of action, study ID and corresponding references.

Compound	**MoA**	**Reference(s)**	**ID(s)**
2,3,7,8-tetrachlorodibenzo-p-dioxin	A	Alexeyenko et al. (2010) and Hahn et al. (2014)	22; 40
All-trans-retinoic acid	A	Hermsen et al. (2013) and Weicksel et al. (2013)	17*; 27
Benz(a)anthracene	A	Goodale et al. (2013)	10
Decabromodiphenylether	A	Garcia-Reyero et al. (2014)	29*
Dimethoxybenzene	A	Klüver et al. (2011)	5
Dinitrophenol	A	Klüver et al. (2011)	5
Ethanol	A	Sarmah et al. (2013) and Tal et al. (2012)	23; 26*
Paraquat	A	Driessen et al. (2015)	24*
Pentachlorophenol	A	Xu et al. (2014)	13
Perchloroethylene	A	Smetanová et al. (2015)	6*
Tert-butylhydroquinone	A	Hahn et al. (2014)	40
Thioacetamide	A	Driessen et al. (2015)	24*
Azinphos-methyl	B	Klüver et al. (2011)	5
Caffeine	B	Hermsen et al. (2013)	17*
Carbamazepine	B	Hermsen et al. (2013)	17*
Chlorpromazine	B	Driessen et al. (2015)	24*
Cyanopeptolin	B	Faltermann et al. (2014)	35*

Table 2.1: List of chemicals included in the meta-analysis together with assigned mode of action, study ID and corresponding references.

Compound	**MoA**	**Reference(s)**	**ID(s)**
Fluoxetine	B	Park et al. (2012)	11
Lithium carbonate	B	Driessen et al. (2015)	24*
Morphine	B	Herrero-Turrión et al. (2014)	21
Sertraline	B	Park et al. (2012)	11
Valproic acid	B	Driessen et al. (2015) and Hermsen et al. (2013)	24*; 17*
17-alpha ethinylestradiol	C	Driessen et al. (2015) and Schiller et al. (2013b)	24*; 2*
17-beta estradiol	C	Hao et al. (2013) and Saili et al. (2013)	25*; 14*
Beclomethasone	C	Prykhozhij et al. (2013)	37*
Bisphenol A	C	Lam et al. (2011), Saili et al. (2013), and Schiller et al. (2013b)	16; 14*; 2*
Flutamide	C	Schiller et al. (2013b)	2*
Genistein	C	Schiller et al. (2013a)	1*
GSK4716	C	Saili et al. (2013)	14*
Linuron	C	Schiller et al. (2013b)	2*
Methylparaben	C	Schiller et al. (2013b)	2*
Propanil	C	Schiller et al. (2013b)	2*
Triiodothyronine	C	Pelayo et al. (2012)	30
1-Naphthylisothiocyanate	NA	Driessen et al. (2015)	24*
2-mercaptoethanesulfonic acid functionalized gold nanoparticle	NA	Truong et al. (2013)	38*
Acetaminophen	NA	Driessen et al. (2015)	24*
Adefovir	NA	Driessen et al. (2015)	24*
Amiodarone	NA	Driessen et al. (2015)	24*
Cyclopamine	NA	Büttner et al. (2012)	9
Cyclosporin A	NA	Driessen et al. (2015)	24*
Dibenzothiophene	NA	Goodale et al. (2013)	10
D-Mannitol	NA	Driessen et al. (2015) and Hermsen et al. (2013)	24*;17*
Epoxyeicosatrienoic acid	NA	DiBiase et al. (2012)	31
Flusilazole	NA	Hermsen et al. (2012)	4*
G3-Polyamidoamine	NA	Oliveira et al. (2013)	18*
G4-Polyamidoamine	NA	Oliveira et al. (2013)	18*
GANT-61	NA	Büttner et al. (2012)	9
Isoniazid	NA	Driessen et al. (2015)	24*

Table 2.1: List of chemicals included in the meta-analysis together with assigned mode of action, study ID and corresponding references.

Compound	MoA	Reference(s)	ID(s)
Leflunomide	NA	White et al. (2011)	32
Methyl tert-butyl ether	NA	Bonventre et al. (2013)	12
Midostaurin	NA	Oggier et al. (2011)	39*
N,N,N-trimethylammoniumethanethiol functionalized gold nanoparticle	NA	Truong et al. (2013)	38*
Oil emulsion	NA	Penn et al. (2013)	36
Prochloraz	NA	Schiller et al. (2013b)	2*
Pyrene	NA	Goodale et al. (2013)	10
Saccharin	NA	Hermsen et al. (2013)	17*
SANT-2	NA	Büttner et al. (2012)	9
Sorafenib	NA	Kawabata et al. (2015)	34*
Tetracycline	NA	Driessen et al. (2015)	24*
Trimethyltin chloride	NA	Tanguay et al. (2011)	28*

2.2.1 *Data import, quality control, normalization and cleaning*

Raw data of each study were downloaded from Gene Expression Omnibus or Array Express and imported into R (version 3.2.2, R Core Team, 2015), which was used together with packages from the Bioconductor repository (Huber et al., 2015) for all subsequent analysis. For each dataset quality control and normalization were conducted as described in Appendix A.

ANNOTATION Since the microarrays used for the different studies have been designed and annotated using different genome-versions (and possibly different annotation strategies) it was necessary to renew the annotation for all arrays. All array probes were mapped against the recent Danio rerio genome (DanRer10, September 2014) and annotated using the Ensembl Database (Ensembl Release 80, May 2015). The annotation strategy was based on (Arnold et al., 2014) and is described in Appendix A.

2.2.2 *Grouping of contrasts*

To be able to derive biologically meaningful information from the large number of different treatments included in the analysis, treatments were grouped according to experimental factors. Those factors were:

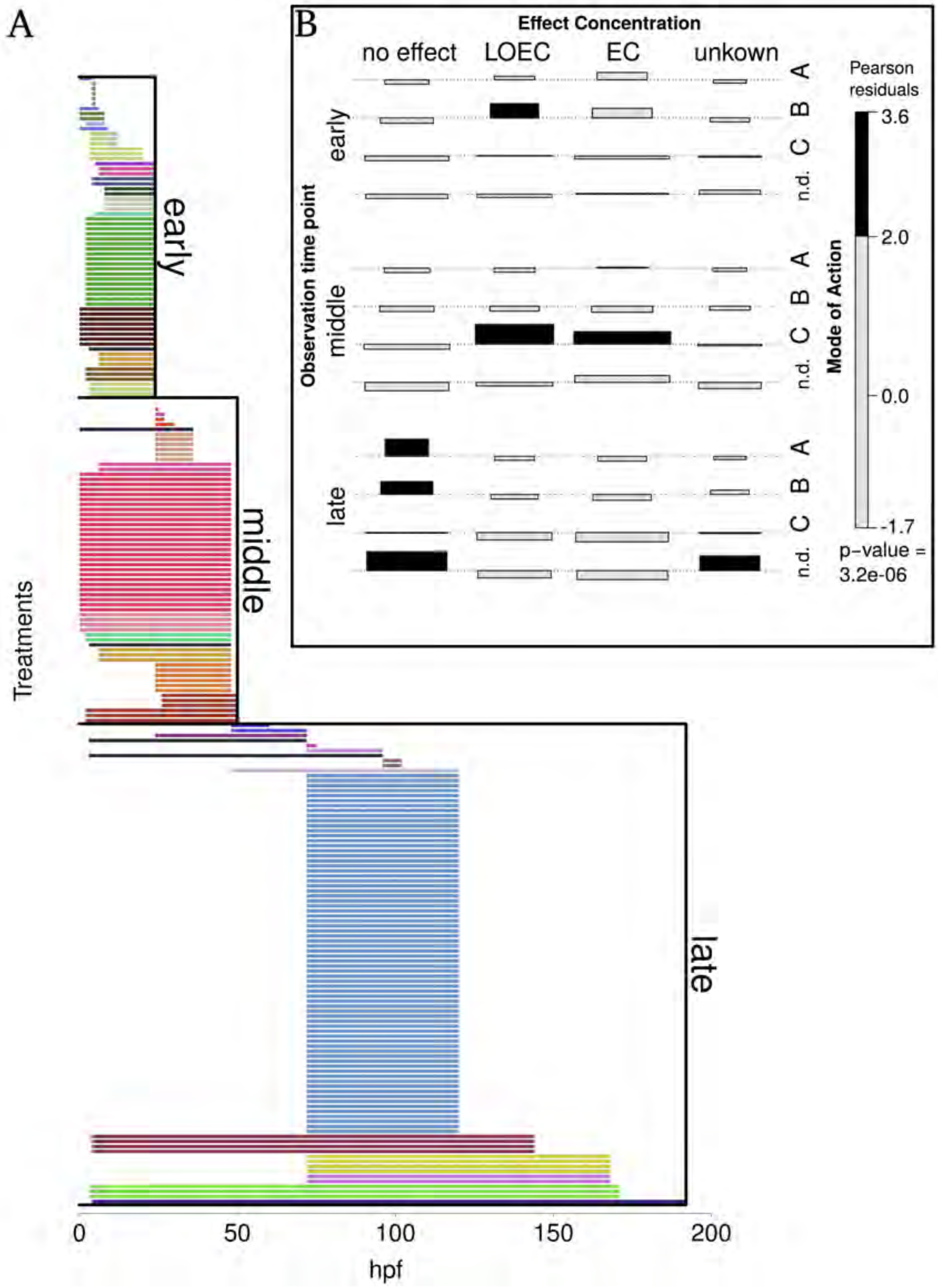


Figure 2.2: Metadata of experiments included in the meta-analysis. (A) Onset and duration of chemical exposure, each bar represents exposure window of one experiment, bar colors indicate different studies, experiments are grouped as in meta-analysis into early (exposure end before 24 hpf), middle (exposure end before 50 hpf) and late exposures (exposure end after 50 hpf). (B) Association plot of experimental subgroups. Width of bar proportional to expected counts, height of bar proportional to Pearson residuals. Black bars indicate significant dependence. This plot shows, that some of the experimental subgroups are not independent, e.g. early experiments were often conducted with LOEC concentrations of neuroactive substances. Mode of Action: A = reactive, teratogenic, B = neuroactive, C = endocrine. Effect Concentration : no effect = applied concentration had no reported phenotypic effect in the experiment, LOEC = applied concentration was lowest observed effect concentration or some not precisely defined low effects up to EC_{10}. EC = applied concentration reported to induce visible or lethal effects.

a) observation time points, b) modes of action of compounds and c) exposure concentration. The groups were assigned using a rather broad perspective. This way groups included enough different treatments and studies to be able to detect general patterns and not just specific results of one treatment:

a) Observation time point: the diverse exposure windows (compare Figure 2.2a) were grouped into three categories according to observation time point in the ZFE (which was the exposure end in most cases) with early exposures ending at latest at 24 hpf, intermediate exposures ending after 24 hpf and before 50 hpf and late exposures ending later than 50 hpf.

b) Modes of action: modes of action or effect categories were retrieved from literature for the 60 chemicals used in the different studies. Three groups were analyzed in more detail, namely reactive, teratogenic or carcinogenic substances ("A"), neuroactive substances ("B") and endocrine disrupting chemicals ("C"). To achieve maximum consistence, chemicals were only assigned to a group if strong evidence for the assignment existed. See Table 2.1 for the assignments.

c) Chemical concentration: all considered studies reported the molar concentrations of the applied exposure solution. However, for being able to compare the exposure concentrations of different substances in a quantitative way, it is necessary to relate the exposure concentration to a comprehensive effect scale (such as lethal concentration). Since this was only available for a few studies, experiments were grouped into three sets with respect to effect concentrations on the ZFE phenotype: the "no effect" group included all treatments using arbitrarily chosen no effect concentrations and treatments using no observed effect concentration (NOEC), no observed adverse effect level (NOAEL) or fractions of NOEC or NOAEL for exposure; the "LOEC" group contained all treatments using exposure concentration reported as LOEC, treatments leading to not precisely defined low effects, as well as treatments with exposure concentrations of EC_{10} and lower as well as $BMC_{GMS}1$-$BMC_{GMS}10$ (as defined by Hermsen et al. (2011)). Finally, the "EC" group contained all treatments with exposure concentrations reported as inducing visible effects, if quantified larger than EC_{10} as well as BMC_{GMS} >=10 and all reported lethal concentrations (min.=LC_5).

2.2.3 *Analysis*

The analyzed studies highly varied with regard to the number of measured time points and applied concentrations. Studies with many concentrations or time points measured would therefore have a biased impact on the analysis. This is why in each subgroup only the highest concentration and latest time point of each study and compound was included (suspected to represent the treatment showing the strongest and most general toxicity profile). The meta-analysis was performed with two complementary approaches (Figure 2.1).

(i) For separate significance testing each dataset was quantile normalized, and differentially expressed genes were identified with a moderated t-test using the R-package "limma" (Ritchie et al., 2015). Cutoff criteria for DEGs were $|log\mathrm{FC}|>1$ and adjusted p-value (adj.p.val)<0.05. The proportion of DEGs for each treatment was determined and related to experimental factors. Additionally, those genes were determined which were identified as DEG in most treatments (across all treatments and subgroups).

(ii) In order to identify genes with a common trend of differential transcription, common significance testing was applied: Each array was normalized by cumulative proportion transformation using the R-package "YuGene", a normalization method specifically designed for cross-platform normalization considering different dynamic ranges for different platforms (Lê Cao et al., 2014). Then, random effect models were used to determine a summary effect size for each gene based on the fold-change and significance was estimated using a permutation analysis (1000 permutations) based on Significance Analysis of Microarrays (Tusher et al., 2001) applying the R-packages "MAMA" (Ihnatova, 2013) in combination with "GeneMeta" (Choi et al., 2003; Lusa et al., 2015). Cutoff criteria for genes with a significant summary effect size ("meta-genes") were |effect size|>1 and adj.p.val<0.05. This method is described in detail by (Choi et al., 2003).

To derive a mechanistic understanding from the lists of genes identified as meta-genes, functional annotation was performed using the Bioconductor package "clusterProfiler" (Yu et al., 2012). A manually combined library of zebrafish gene sets from KEGG (Release 75.0, Kanehisa (2000)), ZFIN (April 2015, Sprague (2006)), GeneOntology (Ensembl release 80,Gene Ontology Consortium (2015)), WikiPathways (Ensembl release 80, Kelder et al. (2012)) and Interpro-domains (Ensembl release 80, Mitchell et al. (2015)) was created for enrichment analysis (for details see Appendix A).

2.3 RESULTS

This meta-analysis aggregated microarray data from 33 studies in which transcriptome responses in ZFE upon exposures to an overall number of

60 different chemicals were investigated. We systematically compared experimental settings with regard to exposure time, concentration and microarray platform. Since the number of DEGs can give a first hint on the extent of molecular disturbance in the embryo (Hermsen et al., 2012), we compared the proportion of DEGs between the studies. We also investigated the influence of different experimental factors on the extent of gene regulation. In the last step we strived to identify commonly regulated genes in the ZFE after chemical exposure.

2.3.1 *Heterogeneous exposure settings*

A first important finding is that exposure settings across studies are quite heterogeneous with respect to exposure time, concentration and the applied microarray platform.

EXPOSURE TIMES A summary of exposure onset and durations of all treatments is depicted in Figure 2.2a. Studies include very early exposures immediately after fertilization (e.g., Hermsen et al., 2012; Schiller et al., 2013a,b), as well as exposures of stages after the hatching of the embryo (e.g., Driessen et al., 2015; Faltermann et al., 2014; Hahn et al., 2014; Kawabata et al., 2015). The exposure durations cover a range between one hour (Alexeyenko et al., 2010) to several days (e.g., Garcia-Reyero et al., 2014; Lam et al., 2011; Park et al., 2012). RNA extraction for microarray analysis usually took place immediately after the end of exposure, with only one exception: Alexeyenko et al. (2010) analyzed molecular responses to dioxin at several time points during depuration after a short exposure time.

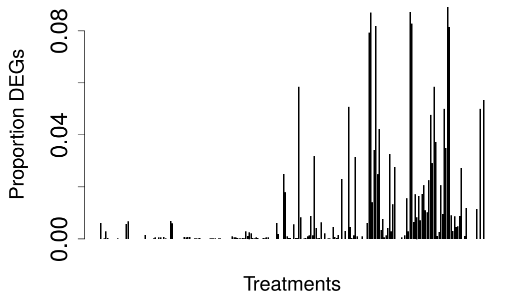
EXPOSURE CONCENTRATIONS Since studies are based on different motivations and assumptions, the experimental strategies also differed with respect to exposure concentrations. Five of 33 studies used an exposure concentration showing no phenotypic effects in the embryo (e.g., Bonventre et al., 2013; Driessen et al., 2015; Hao et al., 2013), three studies used the lowest concentration leading to a sublethal phenotypic or lethal effect (e.g., Saili et al., 2013) and eight studies used concentrations causing death of 5-20 percent of all embryos (e.g., Klüver et al., 2011; Lam et al., 2011; Oliveira et al., 2013). Five studies recorded phenotypic effects for the applied exposure concentrations but did not quantify the effects (e.g., Alexeyenko et al., 2010; Tal et al., 2012; White et al., 2011). Three studies used an exposure concentration, which was related to concentrations found in the environment (e.g., Garcia-Reyero et al., 2014; Park et al., 2012) and eight studies did not explicitly relate their applied concentration to a reference concentration or phenotypic effect (e.g., Hahn et al., 2014; Kawabata et al., 2015; Xu et al., 2014).

MICROARRAY PLATFORMS The studies considered in this meta-analysis made use of different microarray platforms. Commercially available microarrays manufactured by Agilent or Affymetrix are used most frequently. Besides general differences in array design between those companies, the different generations of arrays also appear to differ substantially in terms of transcriptome coverage as depicted in Figure A.3a. Here, a summary of transcriptome coverage of all used arrays shows that only few platforms achieve to cover most known *Danio rerio* genes. Roughly only 20% (5,000) of known genes are covered across all platforms and can be compared across all conditions. However, if genes are grouped into gene sets using ontology and pathway information from databases, most gene sets are covered with at least 30% of their genes on all arrays (see Figure A.3b). For our effect size analysis we used a reduced dataset from fewer studies with the intention to increase the overlap in covered genes across studies and thus the number of genes that could be analyzed. The number of genes could be increased to a coverage of about 45% (12,000 genes). We included data from different platforms since a limitation to only one platform would have reduced the analysis to too few compounds to derive any general conclusions.

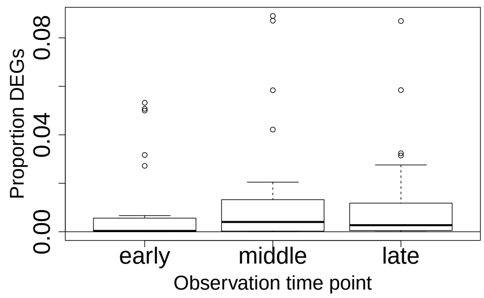
2.3.2 *Association of experimental factors*

Figure 2.2b shows that in this meta-analysis not all experimental groups can be considered to be independent. Shaded bars show a significant correlation between experimental factors. For example, substances in MoA group A ("reactive, teratogenic") were often investigated in no effect concentrations at late time-points.

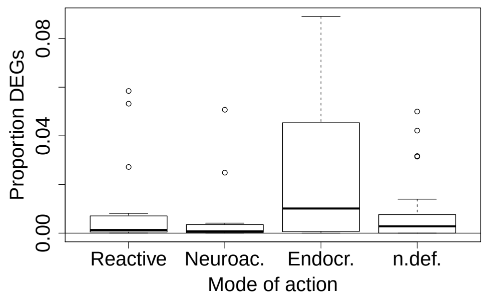
EXTENT OF GENE REGULATION DEPENDS ON EXPOSURE CONCENTRATION As a first analysis step the proportion of DEGs among all measured genes was determined for all treatments (adjusted p-Value <0.05, $|log\mathrm{FC}|>1$). The proportion of DEGs ranged from no genes in 77 of 225 contrasts (treatment-control) up to a maximum of roughly 9% DEGs (Figure 2.3a). Treatments ending at time points between 24 hpf and 50 hpf seem to induce a slightly higher number of genes than those ending at earlier or later time points (see Figure 2.3b). Additionally, experiments using no-effect concentrations showed fewer significantly regulated genes than experiments using a concentration reported to cause visible effects on the phenotype (Figure 2.3d). The fact that some contrasts do not show any significantly regulated genes goes in hand with the published studies about the corresponding datasets, since some of these studies chose not to correct their statistical test for multiple hypothesis testing for deriving a list of regulated genes (Bonventre et al., 2013; Garcia-Reyero et al., 2014; Saili et al., 2013; Xu et al., 2014).



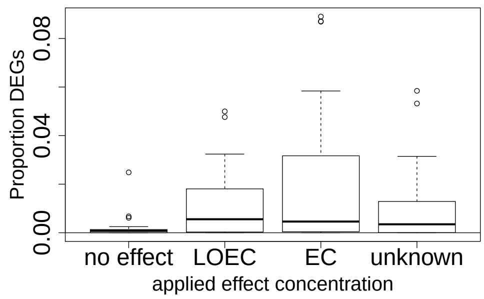
(a) Individual experiments, each bar represents one individual treatment



(b) Subgroups according to observation time points



(c) Mode of action subgroups



(d) Subgroups according to the applied effect concentrations

Figure 2.3: Proportion of DEGs among all measured genes in respective subgroups.

Other studies tested a range of concentrations with the lower concentrations not always yielding significant transcriptional changes. Additionally, the mode of action seems to have an effect on the extent of gene regulation. In Figure 2.3c it is illustrated that endocrine acting substances seem to induce a comparatively larger response. However, this might also be due to the fact, that endocrine substances were mostly tested at higher effect concentrations (Figure 2.2b). We grouped the proportion of regulated genes according to concentration and time (Figure A.4a) and according to concentration and mode of action (Figure A.4b) and it appears that both, mode of action as well as concentration play a role in the extent of gene regulation. Therefore and as explained above, it is hardly possible to consider the groups independently. Additionally, it should be noted that some of the treatments in all considered groups show none or only few DEGs. This explains the increased variability for all groups with an increased median proportion of DEGs (leading to "higher" boxes in Figures 2.3b-d).

2.3.3 *Low overlap in differentially expressed genes between studies*

In the next analysis step we strived to identify genes commonly differentially expressed in the embryo in response to chemical exposure in

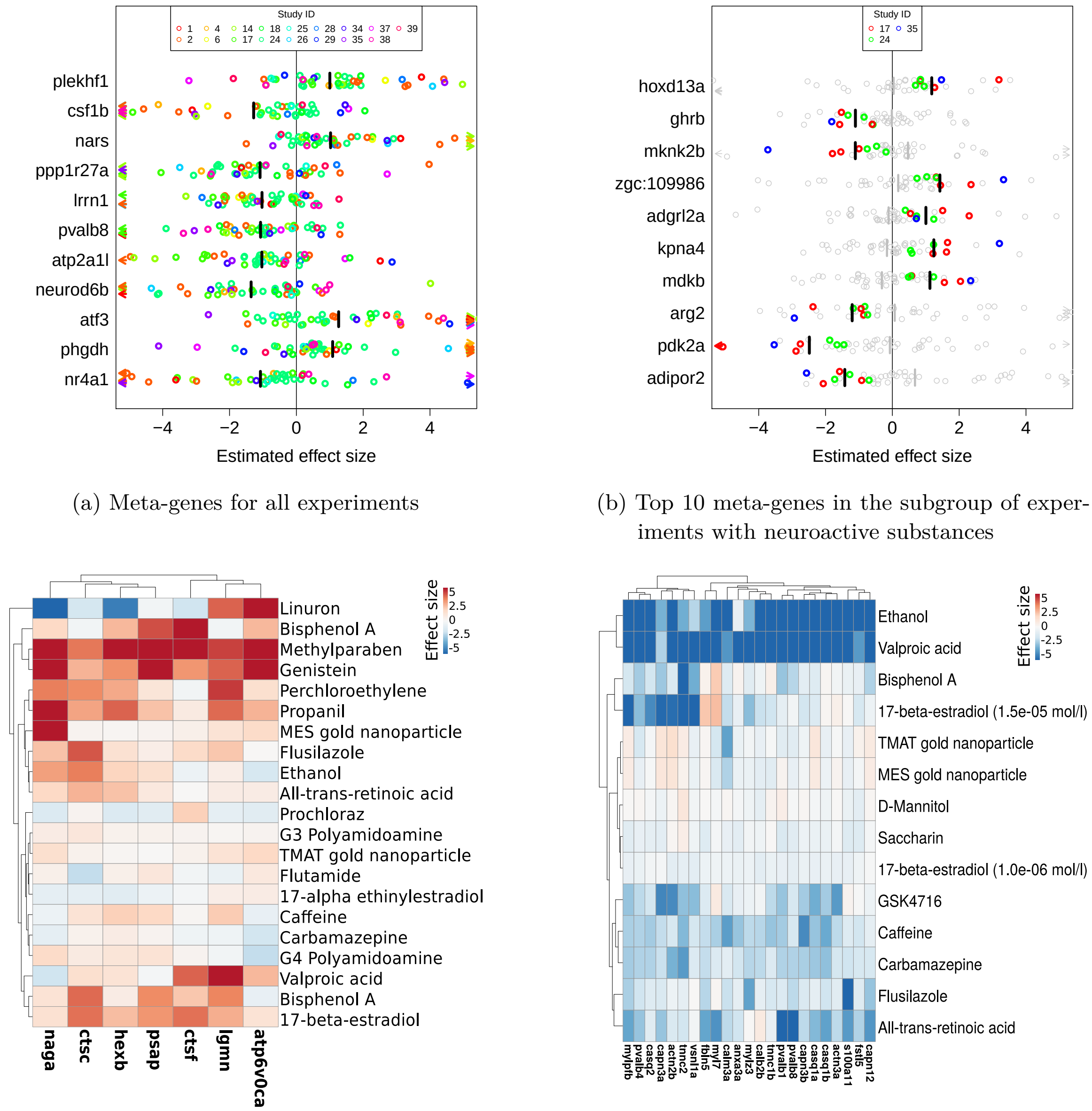
Table 2.2: Number of "meta-genes" and enriched gene sets in experimental subgroups and number of contrasts and studies contributing to the subgroup.

Category	Group	Genes		Gene sets		Contrasts	Studies
		Up	Down	Up	Down		
Time	early	164	413	75	62	14	6
	middle	177	209	4	7	15	7
	late	12	12	0	0	21	8
Mode of action	reactive	28	15	5	1	6	5
	endocrine	126	249	0	1	13	6
	neuroactive	120	168	4	32	7	3
Concentration	noeffect	11	14	2	0	16	5
	LOEC	411	531	0	21	15	5
	EC	151	327	11	35	21	9
	all	4	7	17	1	45	17

general. In a first approach, DEGs were identified for each treatment and subsequently overlaps of DEGs were determined across all treatments as well as across subgroups of treatments. In order to reduce study bias, only those treatments with at least one DEG and only the highest concentration and latest time point investigated per substance and study were considered (resulting in a maximum of 60 contrasts/33 studies). Across all treatments and in all subgroups some overlapping DEGs could be identified with an overlap higher than would be expected by chance ($p<0.05$). However, even the most commonly differentially transcribed genes appeared in less than 50% of analyzed treatments and regularly showed a study bias, if several compounds of one study were included in the dataset (see Figures A.5-A.14). Each list of DEGs (if there were any detected) for each treatment was also analyzed for functional enrichment using the R-package clusterProfiler (Yu et al., 2012). Again, the overlap of enriched terms was determined, which was found to be in the same range as for genes - with no gene set appearing in more than 50% of the treatments. Since the evidence for general regulation of the genes or gene sets with the highest overlap was weak, no further biological conclusions were derived from the findings of this analysis step. Rather, in a second step genes were searched for that show a common trend of regulation irrespective of statistical significance in the single studies.

2.3.4 *Effect size analysis – common trends of gene regulation*

Our expectation to find some genes identified to be differentially expressed in all treatments (and accordingly in experimental subgroups)



(a) Meta-genes for all experiments

(b) Top 10 meta-genes in the subgroup of experiments with neuroactive substances

(c) Meta-genes associated with the Lysosome pathway (KEGG) in the EC-subgroup (exposures with concentrations causing apical effects)

(d) Meta-genes associated with calcium binding (GeneOntology annotation) in the subgroup of early exposures

Figure 2.4: Estimated effect sizes for selected meta-genes (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis). Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. (a)/(b) Circles represent single effect sizes, arrows single effect sizes smaller -5 or larger 5, colors represent study affiliation, bars summary effect sizes, grey circles and bars single effect sizes and summary effect sizes of experiments not in the group, Study IDs as given in Table 1. (c)/(d) values clipped below -5 and above 5.

as evidence for common gene regulation was not met. We reasoned that there might still be genes showing a common trend of differential expression across treatments, without being identified as significantly differentially expressed in each individual treatment. Those genes would not be identified using the overlap method described above. Thus, in a second approach we obtained a summary effect size for each gene across all contrasts before determining its significance by permutation analysis. The summary effect size (bars in Figure 2.4) can be regarded similarly to a weighted average of the effect sizes of individual treatments, while the single effect sizes of each treatment and gene (circles in Figure 2.4) were obtained from the fold-changes (treatment vs. respective controls) and normalized by the standard deviation. This method only allowed those genes to be included in the analysis which were tested in all treatments. Therefore, only studies with at least 15,000 investigated genes were included in the analysis (this cutoff was chosen as a trade-off between number of analyzable studies (17 studies containing 176 treatments) and number of analyzable genes (11,679/ 45% coverage)). Again, to reduce study bias the dataset was reduced to those treatments with highest concentration and latest time point for each compound and study. The results of this analysis are summarized in Table 2.2, where the number of genes with a summary effect size deviating significantly from zero ($FDR < 0.05$, $|\text{effect size}| > 1$, here called "meta-genes") is given for each group as well as the number of enriched functional terms within this group of meta-genes. Summary effect sizes across all contrasts were significant for 11 genes, including four genes with positive (plekhf1, nars, atf3, phgdh) and seven genes with negative summary effect size (csf1b, ppp1r27a, lrrn1, pvalb8, atp2a1l, neuro6b, nr4a1). In spite of statistical significant summary effect sizes, single effect sizes of each contrast still show large variation between treatments (Figure 2.4a). Several treatments even show a reverse effect size in comparison to the summary effect size, meaning for example that the gene is upregulated in some studies whereas the summary effect size shows a significant repression.

2.3.5 *Meta-genes for experimental subgroups*

As mentioned above, we also calculated the summary effect sizes for all included genes across different experimental subgroups. For 6 of the 9 subgroups we identified more than 100 genes showing a common trend of increased or decreased expression (see Table 2.2, $FDR<0.05$, $|\text{effect size}|>1$). The distribution of effect size and FDR was different between subgroups, as depicted in the volcano plots in Figure A.15. All genes with a significant summary effect size and respectively enriched gene sets are summarized in Table S1 and S2 (`https://academic.oup.com/toxsci/article/157/2/291/3055845#supplementary-data`) for all experimental subgroups. Heatmaps for each subgroup summarizing the single effect sizes of up to 100 of the respective "meta-genes" are provided as supplemental material (Figure A.16-Figure A.24). Figure 2.4b illustrates exemplary for the top 10 genes responding to neuroactive substances, that meta-genes identified in the subgroups at least partially show a consistent trend (all individual treatments show either increased or decreased expression compared to control). Furthermore, it demonstrates that summary effect sizes for experimental subgroups may substantially differ from the summary effect size for all treatments (light grey bars in Figure 2.4b indicating no effect across all studies) which may indicate a group specific response.

OBSERVATION TIME POINT For early observation time points (up to an embryo age of 24 hpf) 164 genes had an estimated summary effect size larger than 1 (increased expression, FDR < 0.05) and 413 genes had an effect size smaller than -1 (decreased expression, FDR< 0.05). In contrast to other time related subgroups, numerous functional terms were enriched for those genes. Among genes with increased expression, terms related to mesoderm development as well as several T-box transcription factors (tbx6, tbx6l, tbx16, ta) were predominantly affected. Two signaling pathways were overrepresented, namely the Wnt-pathway (e.g., fibronectin 1b, wnt inhibitory factor 1, wnt11r, dact2) and the Fgf-pathway (e.g., fgf receptor 1a, fgf 17, fgf receptor like 1b). Both pathways play an important role in ZFE development (Ota et al., 2009; Verkade and Heath, 2009). Additionally, ten of the genes with increased expression were coding for proteins with EGF-like domains. The motif enrichment performed with human orthologue gene annotations indicated potential upstream regulation for ten of the induced genes by the vitamin D receptor (VDR) and the hepatic transcription factor 1 (TCF1). Among the terms enriched for genes with decreased expression one cluster contained genes for beta- and gamma-crystallins. Another cluster could be linked to neuron expression, myotome and muscle development. An effect on muscle development is also indicated by the enriched transcription factor motifs of MEF2A (Myocyte Enhancer Factor 2A) and MYOD (myogenic differentiation 1). Besides myogenesis and muscle cell differentiation those potential upstream regulators are involved in neuronal differentiation, cell growth control, apoptosis and cell cycle arrest (Berkes and Tapscott, 2005; McKinsey et al., 2002; Naya and Olson, 1999; Weintraub et al., 1991). Additionally, there were clusters of genes enriched for calcium ion binding proteins (e.g., calpains, parvalbumins, calsequestrins) and for metabolic processes like glycolysis. Furthermore, a cluster of genes coding for homeobox-domain containing proteins was found to show decreased expression in early treatments (e.g., hoxb13a, lhx1b, lhx9, nkx2.1). This protein class is highly conserved and plays an essential role in morphogenesis and early development (Kimmel, 1989; Mallo et al., 2010). A clusterplot showing the downregulated terms of the early exposure group is shown in Figure A.25. In observations between 24 hpf and 50 hpf (group: "middle") 177 genes showed an average increase in expression but only the terms blood, leucine zipper domain, steroid biosynthesis and cellular amino acid metabolic process were enriched for this group of genes. Among the 209 genes with decreased expression only a small group of six genes could be annotated to be connected with dentary expression. In late measurements twelve genes showed an average increase and twelve an average decrease in expression, but no functional terms were enriched.

CONCENTRATION Next to the consideration of observation time points we also grouped the experiments according to the applied exposure concentrations. We built three groups of experiments, namely concentrations with no reported apical effect ("no effect"), concentrations in the LOEC range ("LOEC") and concentrations with a reported apical effect ("EC"). Eleven genes showed an average expression increase and 14 an average decrease in the "no effect" concentration group. Enrichment analysis for this group resulted in only two terms connected to the fin bud, of which two genes were among the genes with increased expression. Experiments of the "LOEC" group showed 410 genes with increased and 531 genes with decreased expression. However, in spite of the large number of genes showing increased expression, only genes with decreased expression could be functionally annotated. Here, diverse anatomical terms were enriched, including musculature system, heart tube, dentary, blood and fin bud. Additionally, genes coding for proteins involved in glycolysis (e.g., fbp2, pfkma, pfkmb, gapdh) were decreased in expression as well as genes coding for globin like and EF-hand domain containing proteins (e.g., parvalbumins). Experiments with reported apical effects of EC10 and higher, showed 151 genes with increased expression. These were enriched among others for steroid biosynthesis, the protein domain of ABC transporters and a group of lysosome genes including a range of peptidases (cathepsins, legumain) and glycosidases (hexb, naga). The expression increase of these genes indicates a raise in degradation of damaged proteins and macromolecules as a response to cell damage. In Figure 2.4c the single effect sizes of significantly regulated lysosomal genes in "EC" experiments are exemplary illustrated in a heatmap. Here it can be seen that not all compounds applied in a concentration causing an apical effect induced lysosomal genes in a similar way, however an overall trend of induction is obvious. The 327 genes with decreased expression in the "EC" group showed enrichment for muscle, dentary and heart expression as well as the troponin domain (similar to the "LOEC" group). Additionally, the terms focal adhesion and ECM-receptor interaction were enriched, possibly indicating destabilized tissue structures. Transcription factor motif enrichment is similar to the "early" group of genes with decreased expression.

MODE OF ACTION The studies included into the effect size analysis investigated 40 chemicals of which 22 were grouped into three broad groups with similar modes of action, namely reactive, carcinogenic or teratogenic compounds (group “A”); neuroactive compounds (group “B”); and compounds known to act on the endocrine system (“C”). We identified 28 genes with increased and 15 genes with decreased expression in the group of reactive substances (“A”) of which two genes (nfkb2, tnfrsfa) are coding for proteins containing the death domain and three genes are coding for transporters of the major facilitator superfamily (slc37a2, slc17a9b, svolp). Among the genes with increased expression we also found acy3, a gene coding for aminoacylase 3, an enzyme which is known to deacetylate mercapturic acids (Newman et al., 2007), which are common transformation products of reactive/electrophile chemicals (Vermeulen, 1989). Neuroactive compounds (“B”) induced an increased expression of 120 genes, of which 8 were found to be related to the neural rod (wnt4a, gdf6a, cxcl12a, hspa4b, greb1, heyl, hoxd13a, mdkb) and 3 are connected to neuron migration (prickle1b, cxcl12a, hdac1). The 168 genes with decreased expression showed enrichment for muscle and heart expression, glycolysis, the insulin signaling pathway and fatty acid degradation. The third group of endocrine acting chemicals (“C”) showed common trends of increased expression of 126 genes and decreased expression of 249 genes but no functional term was enriched besides Glycolysis for decreased genes.

2.4 DISCUSSION

n our meta-analysis we revealed that overlap of gene expression profiles is generally low between analyzed studies. However, common trends of differential expression, especially in meaningful subgroups, hint at important stress response mechanisms in the zebrafish embryo which we will discuss below. At the same time we also identified limitations in experimental design which we will briefly discuss.

The studies compared here all sought to use global gene transcription analysis to analyze the molecular response provoked in the zebrafish embryo by exposure to specific compounds. Some studies were interested in substance related effects at a specific embryonic stage like gastrulation, others looked at further developed stages, e.g., with developed hepatocytes. Some studies aimed to identify the mode of action of a specific chemical, others aimed to demonstrate the suitability of the microarray technique for toxicological studies using well known model chemicals. Those different specific research interests resulted in many different experimental designs. This heterogeneity in experimental factors is a challenge when comparing the data. Since the setup does not give a balanced design regarding the analyzed subgroups (see Figure 2.2b), it is almost impossible to trace back differences between experiments to a single factor. Another confounding factor was the difference in array design with only a small set of genes being jointly observed on all arrays. Moreover, to obtain comparable data, all data were "forced" into one analysis design here which was restricted to one treatment per chemical and study in order to reduce study bias. This however reduced power in our analysis for those studies, which chose to investigate more concentrations or time points and less replicates, for example, which is actually a favorable and strong study design.

In the analysis of overlap of regulated genes between treatments none of the most frequently significantly affected genes was found in more than 50% of the treatments. Also in smaller groups of studies no larger overlap was detected. This finding goes in line with earlier meta-analyses of microarray data of tissue samples of ovarian cancer, also showing low overlap between observed expression profiles (Györffy et al., 2008) as well as earlier studies in ecotoxicology indicating, that the number of commonly regulated genes gets smaller as the group of experiments is getting larger (Hermsen et al., 2013). In a meta-analysis of changes within zebrafish proteomes after chemical exposure overlap of regulated proteins was even smaller and found to be below 30% (Groh and Tollefsen, 2015). A recent study by (Vidal-Dorsch et al., 2016) showed a lower than expected overlap in an inter-laboratory comparison of ecotoxicological microarray analyses. All these results indicate, that analyses of top-lists are always prone to bias and especially when evaluating single studies, stronger proof of biological regulation e.g., by enrichment analysis (as performed by Driessen et al., 2014) or molecular interaction network analyses are needed. However, a functional enrichment analysis as performed here also revealed a maximum overlap of only 50% in the subgroups, which might be expected to be larger for more consistent experimental designs.

In spite of a generally low overlap of significantly regulated genes between studies we could detect common trends of gene regulation using an effect-size based approach and found indications for certain molecular responses to chemical stress in the ZFE. Molecular responses were assigned to a range of anatomical regions, biological processes and signaling pathways. The eleven genes showing a significant trend across all treatments already hint at a few general molecular processes in response to chemical stress. The gene atf3 (activating transcription factor 3) plays a role in cell cycle arrest and apoptosis (Lu et al., 2006) and shows a trend of upregulation across all treatments (Figure 2.4a). The same is true for plekhf1 (pleckstrin homology and FYVE domain containing 1) which is connected to lysosome dependent apoptosis (Chen et al., 2005). In the “EC” group the importance of lysosomes in stress response is even more pronounced with several lysosomal genes upregulated (Figure 2.4c).

Among the seven genes with a trend for downregulation across all treatments three genes are connected to calcium homeostasis. The gene pvalb8 (parvalbumin 8) is the zebrafish orthologue of the human ONCOMODULIN 2 coding for a high-affinity calcium ion-binding protein that belongs to the superfamiliy of calmodulin proteins containing an EF-hand protein domain. Also, the expression of the gene for the orphan nuclear receptor nr4a1 showed a trend of downregulation across all studies. It has been shown that the transcriptional regulation of this endocrine receptor is dependent on calcium level (Abdou et al., 2016; Medzikovic et al., 2015). Additionally, the zebrafish gene atp2a1l is an orthologue of the SERCA gene, coding for an intracellular Ca2+ transporting ATPase in the sarcoplasmatic reticulum of muscle cells. This pump has recently been shown to be inhibited by lipophilic compounds in *Daphnia magna* and it was hypothesized that intracellular calcium level plays an essential role in basal toxicity (Antczak et al., 2015). Finally, the ryanodine receptors (RYR2 and RYR3) functioning to release calcium from the sarcoplasmic reticulum, e.g. in the context of muscle contraction (Pessah et al., 2010), had a significant negative effect size in four of the nine subgroups. Indeed, repression of genes connected to calcium binding or transport was a recurring observation in our analysis and most prominent in early exposures. Downregulation of genes of calcium binding proteins in the early exposure group is displayed in a heatmap in Figure 2.4d. Here, it can also be seen that negative control compounds like saccharin or mannitol but also nanoparticles do not have an effect on this group of genes, while all other compounds lead to differing degrees of downregulation in early exposed ZFE. A downregulation of calcium binding proteins as response to chemical stress was also described as potential key event in mouse embryonic stem cells after progesterone exposure (Kang et al., 2016) and in ZFE exposed to Vitamin D3 (Zhang, 2015). Furthermore, in a proteomics study calcium signaling proteins were found among the most frequently differentially expressed proteins besides actins, myosins, crystallins and metabolic enzymes in a meta-analysis of zebrafish proteome data obtained after chemical stress (Groh and Tollefsen, 2015). Brette et al. (2014) showed that the decrease of intracellular calcium current and calcium cycling leading to disruption of excitation-contraction coupling in fish cardiomyocytes after crude oil exposure is a key mechanism in crude oil caused cardiotoxicity in fish. Calcium has many different regulatory functions, one of which is its role during cell cycle progression (e.g., Kahl and Means, 2003; Roderick and Cook, 2008). In a study with *Saccharyomyces cerevisiae* O'Duibhir et al. (2014) detected cell cycle arrest as a general stress response towards environmental perturbations. A cell cycle arrest would also go in line with our finding that in the subgroups related to observation time many downregulated genes are connected to those anatomical regions, which develop during the respective developmental stage. This is true for heart, musculature system and parts of the brain (including the eyes) all of which start to develop before 24 hpf (Kimmel et al., 1995). Those regions are all connected to downregulated genes in early treatments (ending before 24 hpf). In the second group (exposure ending between 24 hpf and 50 hpf) the dentary system seems to be affected, which is starting to develop just before 2 dpf (Heyden and Huysseune, 2000). In the late exposure group (measurement later than 50 hpf), when most of the zebrafish morphogenesis is completed, no anatomical terms were enriched among the meta-genes. Overall, this could indicate a general delay in development, but also a reduction in growth and metabolism (with the mentioned anatomical regions mostly affected). This is further supported by a significant downregulation of many genes involved in glucose metabolism in five of the nine subgroups. In summary, it might be hypothesized that the repression of calcium binding proteins indicates an early stress response which induces a cell cycle arrest and suppression of differentiation (as shown by Kang et al., 2016). However, it remains to be elucidated whether the downregulation of calcium binding transcripts and proteins is really the cause or rather the result of a perturbation of development and differentiation. Most calcium binding genes are cell type specific and might occur as repressed just because the cell type is not as developed as in the respective control.

Additionally, genes can be identified showing the same trend of differential expression for compounds, which are expected to act in a similar way. Induced genes in the group of neuroactive compounds could partly be functionally annotated with the terms neural rod and neuron migration. For endocrine disruptors and reactive compounds the meta-genes could not be functionally annotated, which indicates a knowledge gap in functional annotation of signaling pathways and gene-gene interconnections during zebrafish development. Missing knowledge about relevant sets of genes involved in responses towards toxicants is also indicated by the fact, that more downregulated meta-genes are annotated in gene sets (representing known metabolic and homeostatic functions repressed by chemical stress) than upregulated meta-genes which might represent specific but unknown responses to chemical-biomolecule interactions during development. However, a detailed functional analysis of single affected genes might also give evidence for a specific response as shown for the gene acy3 (aminoacylase 3), which was exclusively affected in the group of reactive compounds and identified as a potential biomarker indicating biotransformation of electrophiles (Newman et al., 2007; Vermeulen, 1989).

Apart from mechanistic evidence, the findings of our study should help to improve the design of toxicogenomic experiments in the future. The analysis of proportions of regulated genes revealed that experiments using concentrations which did not induce visible effects in the embryo showed a low proportion of genes differentially transcribed. This should be considered in toxicogenomics experiments as well as in discussions about the sensitivity of responsive transcriptomes. To foster the interpretation of toxicogenomics studies the separation between general responses versus specific responses as well as primary versus secondary and early versus late responses induced by individual substances is important. This is especially true, if such data should become useful for risk assessment in the framework of the AOP concept (Ankley et al., 2010). This might become much easier in the future if studies are designed to advance from "snapshot" experimental designs covering only single concentrations and time points to a design including a range of concentrations and time points for each study as illustrated in Figure 2.5. A minimum number of five time points and five different

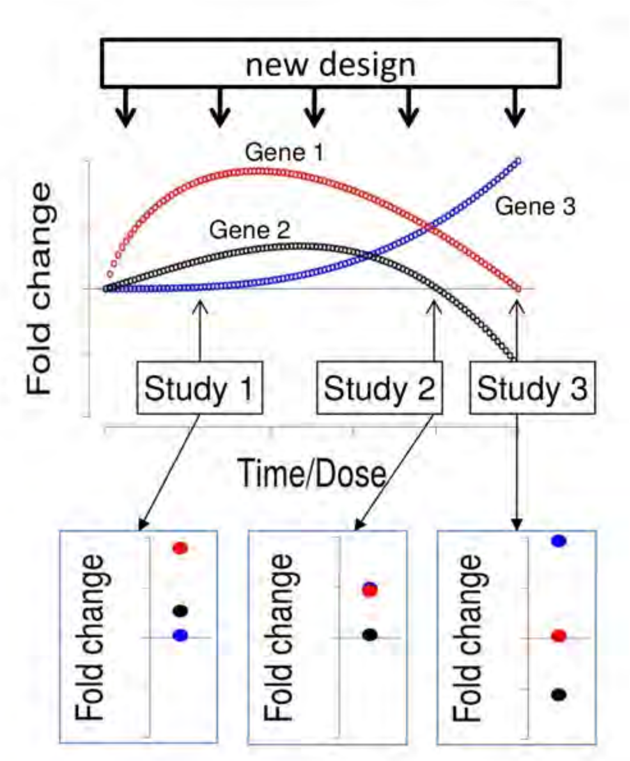


Figure 2.5: Schematic representation of concentration and time dependent gene expression. Current experimental designs ("Study 1-Study 3") only give snapshot information and can lead to seemingly contradicting results in toxicological experiments. Concentration and time variation measurements ("new design") would allow establishing systematic relationships.

exposure concentrations would be recommended as this would allow for simple descriptive modeling approaches. We recommend equal spacing of time points and concentrations on a logarithmic scale. The exposure time frame should be chosen according to the scientific question. Only if one is specifically interested in disturbance of early embryonic development, exposure should start immediately after fertilization. Otherwise we recommend to start not before 24 hpf , when the embryo has already developed to its phylotypic appearance. Furthermore, exposure concentrations should be anchored to a phenotypic effect (e.g., LC_X).

This will foster the establishment of causal relationships between gene expression and adverse outcome in the future.

2.5 CONCLUSION

In a meta-analysis of 33 toxicogenomic ZFE studies we could not identify a general uniform stress response. We found that the analyzed transcriptome studies show great heterogeneity in experimental settings that we take responsible for heterogeneous results. However, in selected experimental subgroups we could identify common trends of gene regulation. We identified gene sets connected to anatomical development, metabolism or calcium homeostasis as downregulated in different subgroups whereas upregulation of gene sets seemed to be more diverse and thus more specific. Induced genes could among others be linked to signaling pathways (e.g. Wnt, Fgf) as well as lysosomal structures and apoptosis. This analysis shows some methodological constraints of existing ZFE transcriptome studies but at the same time it provides a starting point to substantially increase our understanding of toxic effects of chemicals and stress responses in the developing zebrafish embryo.

REFERENCES

Abdou, H. S., N. M. Robert, and J. J. Tremblay (Apr. 2016). "Calcium-dependent Nr4a1 expression in mouse Leydig cells requires distinct AP1/CRE and MEF2 elements." In: *Journal of Molecular Endocrinology* 56.3, pp. 151–161. DOI: 10.1530/JME-15-0202.

Afshari, C. A., H. K. Hamadeh, and P. R. Bushel (2011). "The evolution of bioinformatics in toxicology: Advancing toxicogenomics." In: *Toxicological Sciences* 120.SUPPL.1. DOI: 10.1093/toxsci/kfq373.

Alexeyenko, A., D. M. Wassenberg, E. K. Lobenhofer, J. Yen, E. Linney, E. L. L. Sonnhammer, and J. N. Meyer (May 2010). "Dynamic Zebrafish Interactome Reveals Transcriptional Mechanisms of Dioxin Toxicity." In: *PLoS ONE* 5.5. Ed. by S. Vollmer, e10465. DOI: 10.1371/journal.pone.0010465.

Ankley, G. T., R. S. Bennett, R. J. Erickson, D. J. Hoff, M. W. Hornung, R. D. Johnson, D. R. Mount, J. W. Nichols, C. L. Russom, P. K. Schmieder, et al. (2010). "Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment." In: *Environmental Toxicology and Chemistry* 29.3, pp. 730–741. DOI: 10.1002/etc.34.

Antczak, P., T. a. White, A. Giri, F. Michelangeli, M. R. Viant, M. T. D. Cronin, C. Vulpe, and F. Falciani (2015). "Systems Biology Approach Reveals a Calcium-Dependent Mechanism for Basal Toxicity in Daphnia magna." In: *Environmental Science & Technology* 49.18, pp. 11132–11140. DOI: 10.1021/acs.est.5b02707.

Arnold, C., F. Externbrink, J. Hackermüller, and K. Reiche (Nov. 2014). "CEM-Designer: Design of custom expression microarrays in the post-ENCODE Era." In: *Journal of Biotechnology* 189, pp. 154–156. DOI: 10.1016/j.jbiotec.2014.09.012.

Berkes, C. A. and S. J. Tapscott (Aug. 2005). "MyoD and the transcriptional control of myogenesis." In: *Seminars in Cell & Developmental Biology* 16.4-5, pp. 585–595. DOI: 10.1016/j.semcdb.2005.07.006.

Bonventre, J. A., T. S. Kung, L. A. White, and K. R. Cooper (2013). "Manipulation of the HIF-Vegf pathway rescues methyl tert-butyl ether (MTBE)-induced vascular lesions." In: *Toxicology and Applied Pharmacology* 273.3, pp. 623–634. DOI: 10.1016/j.taap.2013.10.008.

Brette, F., B. Machado, C. Cros, J. P. Incardona, N. L. Scholz, and B. A. Block (Feb. 2014). "Crude Oil Impairs Cardiac Excitation-Contraction Coupling in Fish." In: *Science* 343.6172, pp. 772–776. DOI: 10.1126/science.1242747.

Büttner, A., W. Busch, N. Klüver, A. Giannis, and S. Scholz (Apr. 2012). "Transcriptional responses of zebrafish embryos exposed to potential sonic hedgehog pathway interfering compounds deviate from expression profiles of cyclopamine." In: *Reproductive Toxicology* 33.2, pp. 254–263. DOI: 10.1016/j.reprotox.2011.12.010.

Chen, W., N. Li, T. Chen, Y. Han, C. Li, Y. Wang, W. He, L. Zhang, T. Wan, and X. Cao (2005). "The lysosome-associated apoptosis-inducing protein containing the pleckstrin homology (PH) and FYVE domains (LAPF), representative of a novel family of PH and FYVE domain-containing proteins, induces caspase-independent apoptosis via the lysosomal-mitocho." In: *Journal of Biological Chemistry* 280.49, pp. 40985–40995. DOI: 10.1074/jbc.M502190200.

Choi, J. K., U. Yu, S. Kim, and O. J. Yoo (2003). "Combining multiple microarray studies and modeling interstudy variation." In: *Bioinformatics* 19.Suppl 1, pp. i84–i90. DOI: 10.1093/bioinformatics/btg1010.

DiBiase, A., P. Li, and Y. Zhou (2012). *Zebrafish whole embryos at 36 hpf treated with DMSO or 5 um 11,12-EET*. ArrayExpress Dataset, E-GEOD-39707.

Driessen, M., A. S. Kienhuis, A. P. Vitins, J. L. a. Pennings, T. E. Pronk, E. J. V. D. Brandhof, M. Roodbergen, B. V. D. Water, and L. T. M. V. D. Ven (2014). "Gene expression markers in the zebrafish embryo reflect a hepatotoxic response in animal models and humans." In: *Toxicology Letters* 230.1, pp. 48–56. DOI: 10.1016/j.toxlet.2014.06.844.

Driessen, M., A. P. Vitins, J. L. A. Pennings, A. S. Kienhuis, B. V. D. Water, and L. T. M. van der Ven (2015). "A transcriptomics-based hepatotoxicity comparison between the zebrafish embryo and established human and rodent in vitro and in vivo models using cyclosporine A, amiodarone and acetaminophen." In: *Toxicology Letters* 232.2, pp. 403–412. DOI: 10.1016/j.toxlet.2014.11.020.

Faltermann, S., S. Zucchi, E. Kohler, J. F. Blom, J. Pernthaler, and K. Fent (2014). "Molecular effects of the cyanobacterial toxin cyanopeptolin (CP1020) occurring in algal blooms: Global transcriptome analysis in zebrafish embryos." In: *Aquatic Toxicology* 149, pp. 33–39. DOI: 10.1016/j.aquatox.2014.01.018.

Garcia-Reyero, N., B. L. Escalon, E. Prats, J. K. Stanley, B. Thienpont, N. L. Melby, E. Barón, E. Eljarrat, D. Barceló, J. Mestres, et al. (2014). "Effects of BDE-209 contaminated sediments on zebrafish development and potential implications to human health." In: *Environment International* 63, pp. 216–223. DOI: 10.1016/j.envint.2013.11.012.

Gene Ontology Consortium (Jan. 2015). "Gene Ontology Consortium: going forward." In: *Nucleic Acids Research* 43.D1, pp. D1049–D1056. DOI: 10.1093/nar/gku1179.

Goodale, B. C., S. C. Tilton, M. M. Corvi, G. R. Wilson, D. B. Janszen, K. A. Anderson, K. M. Waters, and R. L. Tanguay (2013). "Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish." In: *Toxicology and Applied Pharmacology* 272.3, pp. 656–670. DOI: 10.1016/j.taap.2013.04.024.

Groh, K. J. and K. E. Tollefsen (2015). "The Challenge: Adverse outcome pathways in research and regulation-Current status and future perspectives." In: *Environmental Toxicology and Chemistry* 34.9, p. 1935. DOI: 10.1002/etc.3042.

Győrffy, B., M. Dietel, T. Fekete, and H. Lage (Nov. 2008). "A snapshot of microarray-generated gene expression signatures associated with ovarian carcinoma." In: *International Journal of Gynecological Cancer* 18.6, pp. 1215–1233. DOI: 10.1111/j.1525-1438.2007.01169.x.

Hahn, M. E., A. G. McArthur, S. I. Karchner, D. G. Franks, M. J. Jenny, A. R. Timme-Laragy, J. J. Stegeman, B. R. Woodin, M. J. Cipriano, and E. Linney (2014). "The Transcriptional Response to Oxidative Stress during Vertebrate Development: Effects of tert-Butylhydroquinone and 2,3,7,8-Tetrachlorodibenzo-p-Dioxin." In: *PLoS ONE* 9.11, e113158. DOI: 10.1371/journal.pone.0113158.

Hao, R., M. Bondesson, A. V. Singh, A. Riu, C. W. McCollum, T. B. Knudsen, D. a. Gorelick, and J. Å. Gustafsson (2013). "Identification of estrogen target genes during zebrafish embryonic development through transcriptomic analysis." In: *PLoS ONE* 8.11, pp. 1–18. DOI: 10.1371/journal.pone.0079020.

Hermsen, S. A. B., T. E. Pronk, E. J. van den Brandhof, L. T. M. van der Ven, and A. H. Piersma (2011). "Chemical class-specific gene expression changes in the zebrafish embryo after exposure to glycol ether alkoxy acids and 1,2,4-triazole antifungals." In: *Reproductive Toxicology* 32.2, pp. 245–252. DOI: 10.1016/j.reprotox.2011.05.010.

Hermsen, S. a. B., T. E. Pronk, E.-J. van den Brandhof, L. T. M. van der Ven, and A. H. Piersma (2012). "Triazole-induced gene expression changes in the zebrafish embryo." In: *Reproductive toxicology (Elmsford, N.Y.)* 34.2, pp. 216–24. DOI: 10.1016/j.reprotox.2012.05.093.

Hermsen, S. A. B., T. E. Pronk, E.-J. van den Brandhof, L. T. M. van der Ven, and A. H. Piersma (Oct. 2013). "Transcriptomic analysis in the developing zebrafish embryo after compound exposure: individual gene expression and pathway regulation." In: *Toxicology and applied pharmacology* 272.1, pp. 161–71. DOI: 10.1016/j.taap.2013.05.037.

Herrero-Turrión, M., I. Rodríguez-Martín, R. López-Bellido, and R. E. Rodríguez (2014). "Whole-genome expression profile in zebrafish embryos after chronic exposure to morphine: identification of new genes associated with neuronal function and mu opioid receptor expression." In: *BMC Genomics* 15.1, p. 874. DOI: 10.1186/1471-2164-15-874.

Heyden, C. van der and A. Huysseune (Dec. 2000). "Dynamics of tooth formation and replacement in the zebrafish (Danio rerio) (Teleostei, Cyprinidae)." In: *Developmental Dynamics* 219.4, pp. 486–496. DOI: 10.1002/1097-0177(2000)9999:9999<::AID-DVDY1069>3.0.CO;2-Z.

Huber, W., V. J. Carey, R. Gentleman, S. Anders, M. Carlson, B. S. Carvalho, H. C. Bravo, S. Davis, L. Gatto, T. Girke, et al. (Jan. 2015). "Orchestrating high-throughput genomic analysis with Bioconductor." In: *Nature Methods* 12.2, pp. 115–121. DOI: 10.1038/nmeth.3252.

Ihnatova, I. (2013). "MAMA: an R package for Meta-Analysis of MicroArray." In:

Kahl, C. R. and A. R. Means (Dec. 2003). "Regulation of cell cycle progression by calcium/calmodulin-dependent pathways." In: *Endocrine reviews* 24.6, pp. 719–36. DOI: 10.1210/er.2003-0008.

Kang, H. Y., Y.-K. Choi, and E.-B. Jeung (Sept. 2016). "Inhibitory effect of progesterone during early embryonic development: Suppression of myocardial differentiation and calcium-related transcriptome by progesterone in mESCs: Progesterone disturb cardiac differentiation of mESCs through lower cytosolic Ca2+." In: *Reproductive Toxicology* 64, pp. 169–179. DOI: 10.1016/j.reprotox.2016.06.001.

Kanehisa, M. (Jan. 2000). "KEGG: Kyoto Encyclopedia of Genes and Genomes." In: *Nucleic Acids Research* 28.1, pp. 27–30. DOI: 10.1093/nar/28.1.27.

Kawabata, M., N. Umemoto, Y. Shimada, Y. Nishimura, B. Zhang, J. Kuroyanagi, M. Miyabe, and T. Tanaka (Feb. 2015). "Downregulation of Stanniocalcin 1 Is Responsible for Sorafenib-Induced Cardiotoxicity." In: *Toxicological Sciences* 143.2, pp. 374–384. DOI: 10.1093/toxsci/kfu235.

Kelder, T., M. P. van Iersel, K. Hanspers, M. Kutmon, B. R. Conklin, C. T. Evelo, and A. R. Pico (Jan. 2012). "WikiPathways: building research communities on biological pathways." In: *Nucleic Acids Research* 40.D1, pp. D1301–D1307. DOI: 10.1093/nar/gkr1074.

Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann, and T. F. Schilling (1995). "Stages of Embryonic Development of the Zebrafish." In: *Developmental Dynamics* 203, pp. 253–310. DOI: 10.1002/aja.1002030302.

Kimmel, C. B. (Aug. 1989). "Genetics and early development of zebrafish." In: *Trends in Genetics* 5.8, pp. 283–288. DOI: 10.1016/0168-9525(89)90103-0.

Klüver, N., L. Yang, W. Busch, K. Scheffler, P. Renner, U. Strähle, and S. Scholz (Jan. 2011). "Transcriptional response of zebrafish embryos exposed to neurotoxic compounds reveals a muscle activity dependent hspb11 expression." In: *PLoS ONE* 6.12, e29063. DOI: 10.1371/journal.pone.0029063.

Lam, S. H., M. M. Hlaing, X. Zhang, C. Yan, Z. Duan, L. Zhu, C. Y. Ung, S. Mathavan, C. N. Ong, and Z. Gong (Dec. 2011). "Toxicogenomic and Phenotypic Analyses of Bisphenol-A Early-Life Exposure Toxicity in Zebrafish." In: *PLoS ONE* 6.12. Ed. by F. Mueller, e28273. DOI: 10.1371/journal.pone.0028273.

Lê Cao, K. A., F. Rohart, L. McHugh, O. Korn, and C. a. Wells (2014). "YuGene: A simple approach to scale gene expression data derived from different platforms for integrated analyses." In: *Genomics* 103.4, pp. 239–251. DOI: 10.1016/j.ygeno.2014.03.001.

Lieschke, G. J. and P. D. Currie (May 2007). "Animal models of human disease: zebrafish swim into view." In: *Nature Reviews Genetics* 8.5, pp. 353–367. DOI: 10.1038/nrg2091.

Lin, C.-Y., C.-Y. Chiang, and H.-J. Tsai (2016). "Zebrafish and Medaka: new model organisms for modern biomedical research." In: *Journal* *of Biomedical Science*, pp. 1–11. DOI: 10.1186/s12929-016-0236-5.

Lu, D., C. D. Wolfgang, and T. Hai (Apr. 2006). "Activating Transcription Factor 3, a Stress-inducible Gene, Suppresses Ras-stimulated Tumorigenesis." In: *Journal of Biological Chemistry* 281.15, pp. 10473–10481. DOI: 10.1074/jbc.M509278200.

Lusa, L., R. Gentleman, and M. Ruschhaupt (2015). *GeneMeta: Meta-Analysis for High Throughput Experiments.* R package version 1.44.0.

Mallo, M., D. M. Wellik, J. Deschamps, and A. Manuscript (2010). "Hox genes and regional patterning of the vertebrate body plan." In: *Developmental Biology* 344.1, pp. 7–15. DOI: 10.1016/j.ydbio.2010.04.024.

Marx-Stoelting, P., A. Braeuning, T. Buhrke, A. Lampen, L. Niemann, M. Oelgeschlaeger, S. Rieke, F. Schmidt, T. Heise, R. Pfeil, et al. (2015). "Application of omics data in regulatory toxicology: report of an international BfR expert workshop." In: *Archives of Toxicology* 89.11, pp. 2177–2184. DOI: 10.1007/s00204-015-1602-x.

McKinsey, T. A., C. L. Zhang, and E. N. Olson (Jan. 2002). "MEF2: a calcium-dependent regulator of cell division, differentiation and death." In: *Trends in Biochemical Sciences* 27.1, pp. 40–47. DOI: 10.1016/S0968-0004(01)02031-X.

Medzikovic, L., C. A. Schumacher, A. O. Verkerk, E. D. van Deel, R. Wolswinkel, I. van der Made, N. Bleeker, D. Cakici, M. M. G. van den Hoogenhof, F. Meggouh, et al. (Oct. 2015). "Orphan nuclear receptor Nur77 affects cardiomyocyte calcium homeostasis and adverse cardiac remodelling." In: *Scientific Reports* 5, p. 15404. DOI: 10.1038/srep15404.

Mitchell, A., H.-Y. Chang, L. Daugherty, M. Fraser, S. Hunter, R. Lopez, C. McAnulla, C. McMenamin, G. Nuka, S. Pesseat, et al. (Jan. 2015). "The InterPro protein families database: the classification resource after 15 years." In: *Nucleic Acids Research* 43.D1, pp. D213–D221. DOI: 10.1093/nar/gku1243.

Naya, F. J. and E. Olson (Dec. 1999). "MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation." In: *Current Opinion in Cell Biology* 11.6, pp. 683–688. DOI: 10.1016/S0955-0674(99)00036-8.

Newman, D., N. Abuladze, K. Scholz, W. Dekant, V. Tsuprun, S. Ryazantsev, G. Bondar, P. Sassani, I. Kurtz, and A. Pushkin (2007). "Specificity of aminoacylase III-mediated deacetylation of mercapturic acids." In: *Drug Metabolism and Disposition* 35.1, pp. 43–50. DOI: 10.1124/dmd.106.012062.

O'Duibhir, E., P. Lijnzaad, J. J. Benschop, T. L. Lenstra, D. van Leenen, M. J. Groot Koerkamp, T. Margaritis, M. O. Brok, P. Kemmeren, and F. C. Holstege (June 2014). "Cell cycle population effects in perturbation studies." In: *Molecular Systems Biology* 10.6, pp. 732–732. DOI: 10.15252/msb.20145172.

Oggier, D. M., A. Lenard, M. Küry, B. Hoeger, M. Affolter, and K. Fent (2011). “Effects of the protein kinase inhibitor PKC412 on gene expression and link to physiological effects in zebrafish Danio rerio eleuthero-embryos.” In: *Toxicological Sciences* 119.1, pp. 104–115. DOI: 10.1093/toxsci/kfq330.

Oliveira, E., M. Casado, M. Faria, A. M. V. M. Soares, J. M. Navas, C. Barata, and B. Piña (Aug. 2013). “Transcriptomic response of zebrafish embryos to polyaminoamine (PAMAM) dendrimers.” In: *Nanotoxicology* 5390, pp. 92–9. DOI: 10.3109/17435390.2013.858376.

Ota, S., N. Tonou-Fujimori, and K. Yamasu (2009). “The roles of the FGF signal in zebrafish embryos analyzed using constitutive activation and dominant-negative suppression of different FGF receptors.” In: *Mechanisms of Development* 126.1-2, pp. 1–17. DOI: 10.1016/j.mod.2008.10.008.

Park, J.-W., T. P. Heah, J. S. Gouffon, T. B. Henry, and G. S. Sayler (2012). “Global gene expression in larval zebrafish (Danio rerio) exposed to selective serotonin reuptake inhibitors (fluoxetine and sertraline) reveals unique expression profiles and potential biomarkers of exposure.” In: *Environmental Pollution* 167, pp. 163–170. DOI: 10.1016/j.envpol.2012.03.039.

Pelayo, S., E. Oliveira, B. Thienpont, P. J. Babin, D. Raldúa, M. André, and B. Piña (2012). “Triiodothyronine-induced changes in the zebrafish transcriptome during the eleutheroembryonic stage: Implications for bisphenol A developmental toxicity.” In: *Aquatic Toxicology* 110-111, pp. 114–122. DOI: 10.1016/j.aquatox.2011.12.016.

Penn, A., K. Kleinow, F. Xin, A. Bui, R. Xiao, Z. Perveen, and P. Polk (2013). *Microarray data from zebrafish embryos (ZFE) exposed for 48-hr periods to oil emulsions collected from GoM shoreline sites following the Deepwater Horizon blowout.* Gene Expression Omnibus Dataset, GSE52873.

Pessah, I. N., G. Cherednichenko, and P. J. Lein (Feb. 2010). “Minding the calcium store: Ryanodine receptor activation as a convergent mechanism of PCB toxicity.” In: *Pharmacology & Therapeutics* 125.2, pp. 260–285. DOI: 10.1016/j.pharmthera.2009.10.009.

Prykhozhij, S. V., A. Marsico, and S. H. Meijsing (Sept. 2013). “Zebrafish Expression Ontology of Gene Sets (ZEOGS): a tool to analyze enrichment of zebrafish anatomical terms in large gene sets.” In: *Zebrafish* 10.3, pp. 303–15. DOI: 10.1089/zeb.2012.0865.

R Core Team (2015). *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing. Vienna, Austria.

Ramasamy, A., A. Mondry, C. C. Holmes, and D. G. Altman (2008). "Key issues in conducting a meta-analysis of gene expression microarray datasets." In: *PLoS Medicine* 5.9, pp. 1320–1332. DOI: 10.1371/journal.pmed.0050184.

Ritchie, M. E., B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi, and G. K. Smyth (Apr. 2015). "limma powers differential expression analyses for RNA-sequencing and microarray studies." In: *Nucleic Acids Research* 43.7, e47–e47. DOI: 10.1093/nar/gkv007.

Roderick, H. L. and S. J. Cook (May 2008). "Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival." In: *Nature Reviews Cancer* 8.5, pp. 361–375. DOI: 10.1038/nrc2374.

Saili, K. S., S. C. Tilton, K. M. Waters, and R. L. Tanguay (July 2013). "Global gene expression analysis reveals pathway differences between teratogenic and non-teratogenic exposure concentrations of bisphenol A and 17β-estradiol in embryonic zebrafish." In: *Reproductive Toxicology* 38, pp. 89–101. DOI: 10.1016/j.reprotox.2013.03.009.

Sarmah, S., P. Muralidharan, C. L. Curtis, J. N. McClintick, B. B. Buente, D. J. Holdgrafer, O. Ogbeifun, O. C. Olorungbounmi, L. Patino, R. Lucas, et al. (2013). "Ethanol exposure disrupts extraembryonic microtubule cytoskeleton and embryonic blastomere cell adhesion, producing epiboly and gastrulation defects." In: *Biology Open* 2.10, pp. 1013–21. DOI: 10.1242/bio.20135546.

Scholz, S., S. Fischer, U. Gündel, E. Küster, T. Luckenbach, and D. Voelker (July 2008). "The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing." In: *Environmental Science and Pollution Research* 15.5, pp. 394–404. DOI: 10.1007/s11356-008-0018-z.

Schiller, V., A. Wichmann, R. Kriehuber, E. Muth-Köhne, J. P. Giesy, M. Hecker, and M. Fenske (Jan. 2013a). "Studying the effects of genistein on gene expression of fish embryos as an alternative testing approach for endocrine disruption." In: *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 157.1, pp. 41–53. DOI: 10.1016/j.cbpc.2012.09.005.

Schiller, V., A. Wichmann, R. Kriehuber, C. Schäfers, R. Fischer, and M. Fenske (Dec. 2013b). "Transcriptome alterations in zebrafish embryos after exposure to environmental estrogens and anti-androgens can reveal endocrine disruption." In: *Reproductive Toxicology* 42, pp. 210–223. DOI: 10.1016/j.reprotox.2013.09.003.

Smetanová, S., J. Riedl, D. Zitzkat, R. Altenburger, and W. Busch (Sept. 2015). "High-throughput concentration-response analysis for omics datasets." In: *Environmental Toxicology and Chemistry* 34.9, pp. 2167–2180. DOI: 10.1002/etc.3025.

Sprague, J. (Jan. 2006). “The Zebrafish Information Network: the zebrafish model organism database.” In: *Nucleic Acids Research* 34.90001, pp. D581–D585. DOI: 10.1093/nar/gkj086.

Tal, T. L., J. A. Franzosa, S. C. Tilton, K. A. Philbrick, U. T. Iwaniec, R. T. Turner, K. M. Waters, and R. L. Tanguay (Apr. 2012). “MicroRNAs control neurobehavioral development and function in zebrafish.” In: *The FASEB Journal* 26.4, pp. 1452–1461. DOI: 10.1096/fj.11-194464.

Tanguay, R. L., C. Huang, J. Chen, J. La Du, K. Lin, K. M. Waters, L. Truong, Q. Dong, and S. C. Tilton (2011). *Expression Analysis of Trimethyltin Chlorides (TMT) Impact on Embryonic Development.* ArrayExpress Dataset, E-GEOD-28131.

Truong, L., S. C. Tilton, T. Zaikova, E. Richman, K. M. Waters, J. E. Hutchison, and R. L. Tanguay (Mar. 2013). “Surface functionalities of gold nanoparticles impact embryonic gene expression responses.” In: *Nanotoxicology* 7.2, pp. 192–201. DOI: 10.3109/17435390.2011.648225.

Tusher, V. G., R. Tibshirani, and G. Chu (2001). “Significance analysis of microarrays applied to the ionizing radiation response.” In: *Proceedings of the National Academy of Sciences of the United States of America* 98.9, pp. 5116–21. DOI: 10.1073/pnas.091062498.

Verkade, H. and J. K. Heath (2009). “Wnt Signaling Mediates Diverse Developmental Processes in Zebrafish.” In: *Wnt Signaling.* Ed. by E. Vincan. Totowa, NJ: Humana Press, pp. 225–251. DOI: 10.1007/978-1-60327-469-2_17.

Vermeulen, N. P. E. (1989). “Analysis of mercapturic acids as a tool in biotransformation, biomonitoring and toxicological studies.” In: *Trends in Pharmacological Sciences* 10.5, pp. 177–181. DOI: 10.1016/0165-6147(89)90232-0.

Vidal-Dorsch, D. E., S. M. Bay, S. Moore, B. Layton, A. C. Mehinto, C. D. Vulpe, M. Brown-Augustine, A. Loguinov, H. Poynton, N. Garcia-Reyero, et al. (2016). “Ecotoxicogenomics: Microarray interlaboratory comparability.” In: *Chemosphere* 144, pp. 193–200. DOI: 10.1016/j.chemosphere.2015.08.019.

Villeneuve, D. L., D. Crump, N. Garcia-Reyero, M. Hecker, T. H. Hutchinson, C. A. LaLone, B. Landesmann, T. Lettieri, S. Munn, M. Nepelska, et al. (2014). “Adverse outcome pathway development II: Best practices.” In: *Toxicological Sciences* 142.2, pp. 321–330. DOI: 10.1093/toxsci/kfu200.

Weicksel, S. E., J. Xu, and C. G. Sagerström (2013). “Dynamic Nucleosome Organization at hox Promoters during Zebrafish Embryogenesis.” In: *PLoS ONE* 8.5, pp. 1–14. DOI: 10.1371/journal.pone.0063175.

Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, T. Blackwell, D. Turner, R. Rupp, S. Hollenberg, et al. (Feb. 1991). “The myoD gene family: nodal point during specification of the muscle cell lineage." In: *Science* 251.4995, pp. 761–766. DOI: 10.1126/science.1846704.

White, R. M., J. Cech, S. Ratanasirintrawoot, C. Y. Lin, P. B. Rahl, C. J. Burke, E. Langdon, M. L. Tomlinson, J. Mosher, C. Kaufman, et al. (2011). "DHODH modulates transcriptional elongation in the neural crest and melanoma." In: *Nature* 471.7339, pp. 518–522. DOI: 10.1038/nature09882.

Williams, T. D., L. Mirbahai, and J. K. Chipman (Mar. 2014). "The toxicological application of transcriptomics and epigenomics in zebrafish and other teleosts." In: *Briefings in Functional Genomics* 13.2, pp. 157–171. DOI: 10.1093/bfgp/elt053.

Xu, T., J. Zhao, P. Hu, Z. Dong, J. Li, H. Zhang, D. Yin, and Q. Zhao (2014). "Pentachlorophenol exposure causes Warburg-like effects in zebrafish embryos at gastrulation stage." In: *Toxicology and Applied Pharmacology* 277.2, pp. 183–191. DOI: 10.1016/j.taap.2014.03.004.

Yu, G., L.-G. Wang, Y. Han, and Q.-Y. He (2012). "clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters." In: *OMICS: A Journal of Integrative Biology* 16.5, pp. 284–287. DOI: 10.1089/omi.2011.0118.

Zhang, Y. (June 2015). "Network analysis reveals stage-specific changes in zebrafish embryo development using time course whole transcriptome profiling and prior biological knowledge." In: *BioData Mining* 8.1, p. 26. DOI: 10.1186/s13040-015-0057-1.

3 TOXICOGENOMIC LANDSCAPES – CONCENTRATION AND TIME-DEPENDENT RESPONSES IN THE TOXICOGENOMIC UNIVERSE

3.1 INTRODUCTION

Toxicogenomic studies offer great potential to give insight into molecular processes. This can help tackling problems and questions in the field of environmental toxicology (Brinke and Buchinger, 2017; Snape et al., 2004), e.g., in the assessment of environmental health (Bahamonde

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et al., 2016), which will be our focus here. At the same time, toxicogenomic data bear the challenge of analyzing and interpreting highly complex molecular profiles, even when considering only one exposure condition. The integration of time or concentration dependent measurements or different molecular levels (such as transcriptome, proteome and metabolome) even adds another layer of complexity. The seemingly endless number of potential gene expression combinations makes every exposure study outcome an individual case and a new challenge for functional interpretation. This is why a lot of effort has been put into developing strategies to analyze and interpret genome scale molecular profiles and discriminate important subtle changes from statistical noise. Approaches in toxico- or pharmacogenomic studies include multiple hypothesis testing combined with functional enrichment analyses (e.g., Schiller et al., 2013), the study of benchmark doses for genes or gene sets (e.g., Dean et al., 2017; Hermsen et al., 2013; Thomas et al., 2007), inference of networks integrating knowledge about molecular responses to chemicals (e.g., Perkins et al., 2011, 2017; Schroeder et al., 2017), or the study of network perturbation (Woo et al., 2015).

However, as has been laid out in Chapter 2, most toxicogenomics datasets are limited to a few exposure scenarios. At the same time, these scenarios differ greatly between studies. This makes most *omics* results hard to compare, and it is difficult or even impossible to judge the generality or specificity of the respective findings and derive conclusions for exposure settings different from the ones measured (compare Figure 2.5). However, when we want to move to the application field of environmental monitoring, comparability of profiles as well as the ability to extrapolate toxicogenomic profiles on concentration and time scale will be essential. This is because on the one hand, when categorizing certain patterns in toxicogenomic profiles (similar as it was already envisioned by Nuwaysir et al., 1999) with only anecdotal exposure information available, there is a high risk of deriving false conclusions due to spurious correlations or multi-functionality of gene sets. On the other hand, environmental concentrations of toxicants in environmental extracts will differ from concentrations used in a single substance exposure. For that reason, we will have to obtain concentration resolved toxicogenomic profiles of environmental toxicants and find ways to extrapolate to concentrations which have not been measured. Assuming that toxicogenomic profiles of environmental extracts will resemble the profiles of its components, it should then be possible to compare profiles of environmental extracts with those of single compounds to identify major effects (compare Figure 1.2).

Therefore, heading towards the application of transcriptomics as tool for non-target bioanalytics, our goal was to develop an experimental and bioinformatic pipeline, being generally applicable for the generation of toxicogenomic profiles of any chemical of interest. It should

Terminology Box 3.1: SOM terminology

SELF ORGANIZING MAP/KOHONEN MAP Unsupervised machine learning algorithm belonging to the class of neural networks. Data is clustered onto a two-dimensional grid according to similarity. It was first developed by Kohonen (1982).

TOXNODE coordinate of the →*zebrafish embryo toxicogenomic universe* with a definite number of genes assigned to it.

ZEBRAFISH EMBRYO TOXICOGENOMIC UNIVERSE (ZETU) Available datasets from zebrafish embryo single compound exposures clustered by the →*self organizing map* method. Forms the backbone dataset for the →*ZETAC*.

ZEBRAFISH EMBRYO TOXICOGENOMIC TRANSCRIPTOME COORDINATE SYSTEM (ZETAC) Association grid of the →*zebrafish embryo toxicogenomic universe*. Assigns each zebrafish gene a specific position on a two-dimensional map (here 60×60 →*toxnodes*).

COMPOUND TOXICOGENOMIC LANDSCAPE map showing average *log*FCs, or regression parameter estimates of →*toxnodes* (arranged according to →*zebrafish embryo toxicogenomic compass*) for a specific exposure condition or compound.

result in compound profiles which can be extrapolated across exposure duration and concentration, and which are comparable between different compounds.

On experimental side this required an experimental design covering a set of different exposure durations and a range of carefully selected contaminant concentrations (compare Section 3.2.2).

SELF-ORGANIZING MAPS Regarding data analysis the retrieved high-dimensional data set had to be aggregated into a digestible and comparable format. With the help of the established machine learning method of self-organizing maps (SOMs) (Kohonen, 1982; Wirth et al., 2011) the toxicogenomic responses in the zebrafish embryo were clustered by compiling public datasets from the meta-analysis (Chapter 2) and the profiles of our model compounds. We call the resulting database *Zebrafish Embryo Toxicogenomic Universe* (ZETU) (compare terminology box 3.1). It consists of 3600 *"toxnodes"*, each node containing several transcripts, showing similar behavior in response to chemical exposure ("co-expression"). The backbone of the Zebrafish Embryo Toxicogenomic Universe (ZETU) is a two dimensional coordinate system, assigning each toxnode (and thereby each transcript) a defined coordinate. The coordinate system we call *Zebrafish Embryo Toxicogenomic trAnscriptome Coordinate system* (ZETAC). When the average *log*FC of each toxnode, considering a specific treatment, is projected on this coordinate system, we obtain a treatment specific *toxicogenomic landscape*. If there is concentration and time resolved data available, we obtain a set of landscapes, of which the behavior of each toxnode can be described by regression modeling. The obtained landscapes or landscape sets are easily comparable between compounds, since the underlying toxnodes are always arranged in the same way based on the *ZETAC*. This way the landscape can in a first step be visually inspected.

To allow for extrapolation of the toxicogenomic profiles, two different regression models were developed and applied to describe the toxnode behavior. They describe the *log*FC of transcript levels (compared to control conditions) in an exposure duration and concentration dependent manner. Besides extrapolation, the fitted model parameters can also serve as descriptors of the toxicodynamic landscape.

CASE STUDY The whole pipeline was applied on three exemplarily selected environmental contaminants. The quality of the toxicogenomic landscapes and the ability of the regression models were assessed to describe the behavior of toxnodes. Additionally, we analyzed and interpreted the most prominently affected toxnodes in order to elucidate, what can be concluded from the toxicogenomic landscapes about the effect of the substances and if this does represent our current knowledge about the compounds. Additionally, we asked whether compounds, which are expected to act similarly, actually lead to similar toxicogenomic landscapes. Recurring patterns might be applicable for detecting a certain mode of action in a chemical mixture (e.g., an environmental extract).

The experimental setup and analysis pipeline developed and described here could serve as a blueprint for creating informative and comparable toxicogenomic profiles of environmental toxicants. The application of the proposed *Zebrafish Embryo Toxicogenomic Universe* (ZETU) allows to incorporate all/most toxicogenomic data already created in the past and thus can already be used to retrieve important toxicological information. The quality of the toxicogenomic universe can be further improved, by the integration of more data in the future.

In the following the pipeline is explained in more detail. A flowchart of the whole procedure is shown in Figure 3.1.

3.2 EXPERIMENTAL SETUP

The experimental procedure is described in detail in Appendix B. In the following, the experimental design and the selection of the model compounds will be motivated.

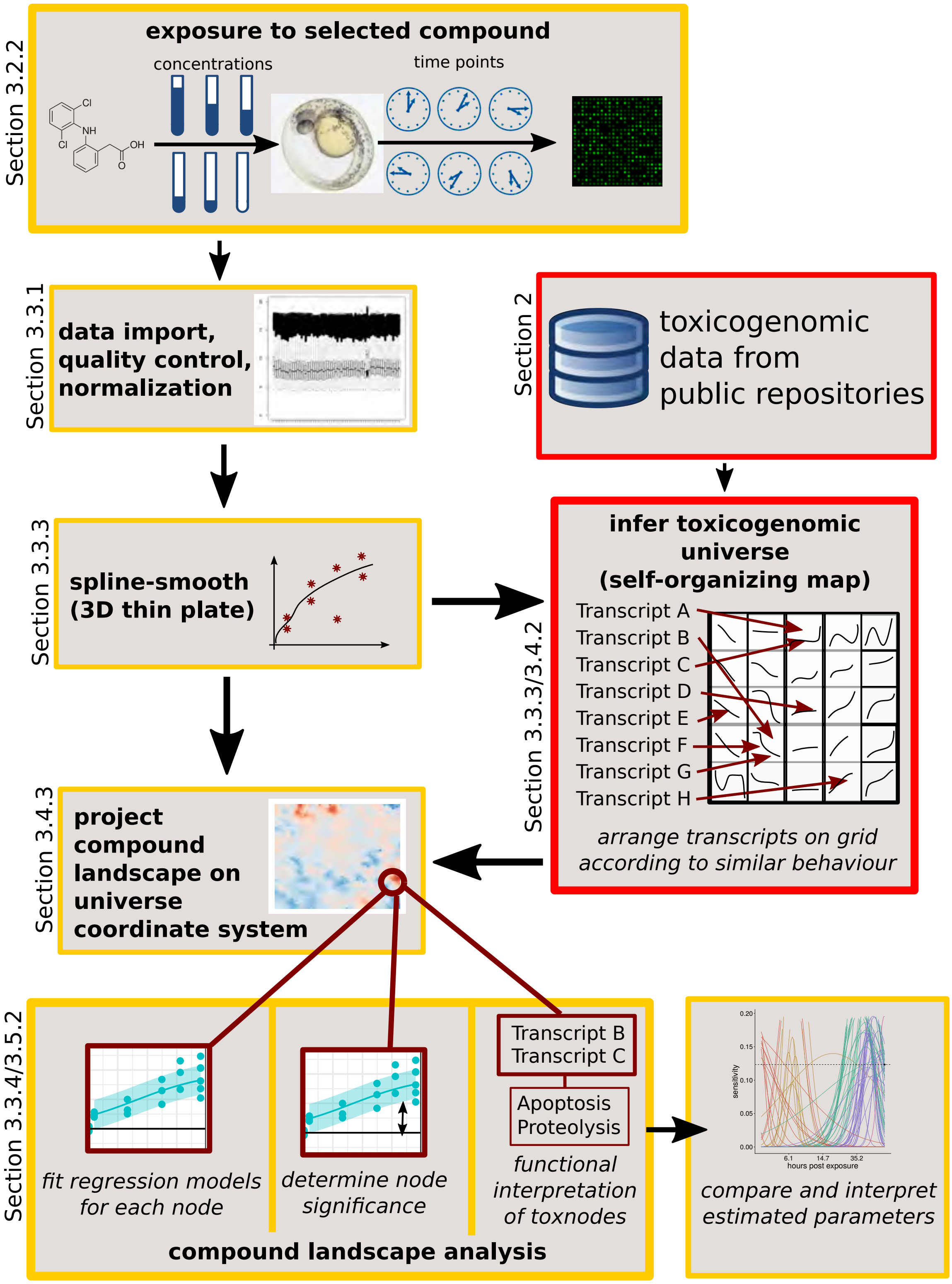


Figure 3.1: Flowchart of developed toxicogenomic analysis pipeline.

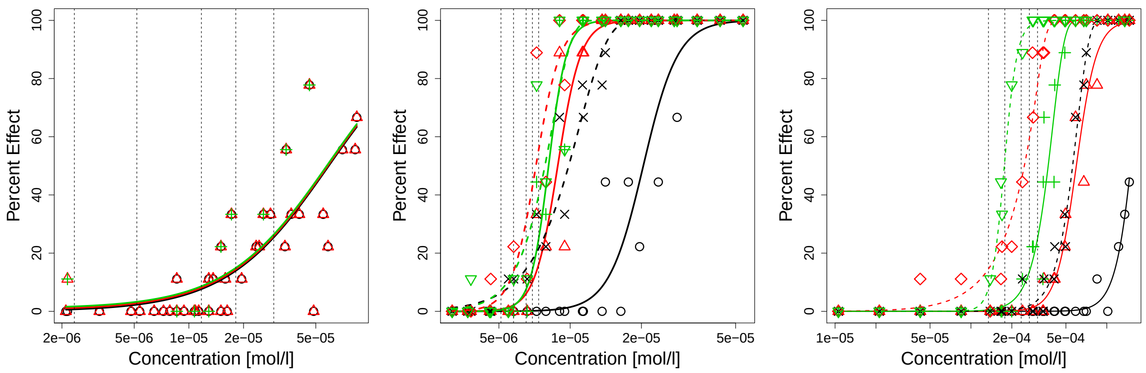
3.2.1 *Model compound selection*

Three model compounds, namely diuron, diclofenac and naproxen were chosen for exposure due to the following criteria: 1) The compounds should have an environmental relevance; 2) Two of the compounds should be expected to act similarly, while one should be acting dissimilar to the other two; 3) Compounds should show excess toxicity in the zebrafish embryo test (i.e., lethality occurs at lower concentration than would be predicted from the liposome-water partitioning coefficient of the compound K_{lipw}, compare Klüver et al., 2016). This was to increase the chance of detecting specific molecular effects and not solely baseline toxicity.

DICLOFENAC Diclofenac is used as a pharmaceutical substance, often applied as pain killer and to reduce inflammation. It belongs to the group of non-steroidal anti-inflammatory drugs (NSAIDs) and is a known inhibitor of both variants of the COX enyzme. COX produces prostaglandins, which act as inflammatory signaling molecules (reviewed in Ricciotti and FitzGerald, 2011). By inhibiting COX an inflammatory response is repressed. As environmental toxicant diclofenac first gained attention due to its toxicity in vultures, which has lead to a significant decline in the vulture population in Pakistan (Oaks et al., 2004). But also in aquatic toxicology it was identified as priority environmental pollutant (e.g., Busch et al., 2016). There have been numerous toxicological studies in aquatic organisms (reviewed in Lonappan et al., 2016). In fish, there have been reports about adverse effects of diclofenac on gill, liver, kidney and the gastrointestinal tract, as well as reduced egg growth and delay in hatching. Jin et al. (2014) analyzed prostaglandin signaling in developing zebrafish embryos and found that prostaglandin as a product of COX, affects ciliogenesis in developing zebrafish.

NAPROXEN Naproxen is, like diclofenac, widely applied as COX-inhibitor of the NSAID group. It can be detected in surface waters (Busch et al., 2016; Tixier et al., 2003; Verenitch et al., 2006), and it has been shown to lead to histopathological liver damage and pericardial edema in zebrafish embryos (Li et al., 2016).

DIURON Diuron is a compound with herbicidal properties, identified as priority pollutant in European water bodies (Busch et al., 2016). In plants, it is specifically inhibiting the electron transfer from photosystem II. Thus, there is no specific effect expected on animals like the zebrafish. With this we expect diuron to act differently compared to diclofenac and naproxen. In zebrafish embryos, diuron was found to have sublethal effects on heartbeat and spontaneous movements (Velki et al., 2017).



(a) Diuron (b) Diclofenac (c) Naproxen

Figure 3.2: Concentration response relationships for apical effects induced by (a) diuron (b) diclofenac and (c) naproxen. Vertical lines illustrate selected exposure concentrations for transcriptome experiments. Curves illustrate best fit model, solid lines for lethality, dashed lines for sublethal+lethal effects, colours indicate different exposure durations. For diuron, no time dependent lethality was observed after 24 hpe. Sublethal effects: Exposure time 24-48hpf (×); 24-72hpf (◇); 24-96hpf (▽). Lethal effects: Exposure time 24-48hpf (○); 24-72hpf (△); 24-96hpf (+).

3.2.2 *Exposure design*

Exposure settings for our transcriptome measurements were designed to meet several requirements. We wanted to be able to follow compound specific toxicodynamic processes but also account for differences in toxicokinetics. Most importantly, results were supposed to be comparable between the compounds.

The exposure for a standard zebrafish embryo toxicity test starts immediately after fertilization (OECD, 2013). However, there might be many unspecific developmental effects in the first hours of development. Therefore, we decided for an exposure period between 24 and 96 hpf. Time points of RNA-extraction during the exposure were 3, 6, 12, 24, 48, and 72 hours post exposure (hpe). The exposure concentrations were phenotypically anchored to the lethal concentration (LC) at 96 hpf/72 hpe. The LC_{25}, modeled from experimental observations (see Figure 3.2), served as highest and the $LC_{0.5}$ as lowest exposure concentration with 6 equal dilution steps in between, with dilution steps 1,2,4 and 6 chosen for exposure (see Equation 3.1, Equation 3.2, and Figure 3.3). The selected concentrations for transcriptome experiments are summarized in Table 3.1.

$$\text{Dilution factor (DF)} = \left(\frac{LC_{25}}{LC_{0.5}}\right)^{1/6} \tag{3.1}$$

$$\text{Exposure concentrations} = \frac{LC_{25}}{DF^x}; x = 0, 1, 2, 4, 6 \tag{3.2}$$

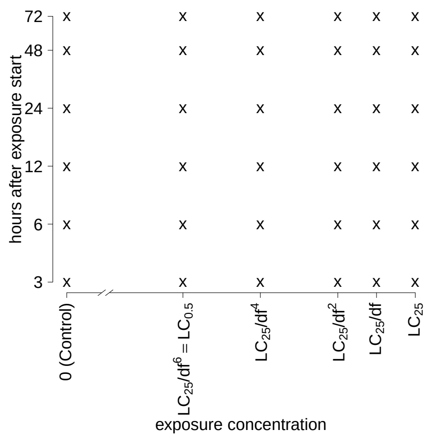


Figure 3.3: General experimental design for transcriptome experiments. Each "x" indicates one sampling condition, df = compound specific dilution factor (compare Table 3.1)

Table 3.1: Exposure concentrations applied for transcriptome experiment.

substance	**dilution factor**	**applied concentrations** (µmol L^{-1})
diuron	1.57	1.9; 4.7; 11.7; 18.4; 28.9;
diclofenac	1.06	5.1; 5.8; 6.5; 6.9; 7.4
naproxen	1.15	134.6; 177.6; 234.3; 269.1; 309.1

MODELING OF CONCENTRATION RESPONSE RELATIONSHIPS

To determine an accurate value for LC_{25} and $LC_{0.5}$, concentration response relationships were modeled from experimental data using established toxicological models (Scholze et al., 2001). All analyses were conducted using the statistical software R (version 3.3.1, R Core Team, 2016), together with the package *bbmle* (Bolker and Team, 2017). Three models, namely *logit*, *weibull* and *generalized logit* were fit to experimental data using maximum likelihood estimation. Afterwards the best fitting model was selected by comparing the AIC_c. The best fitting model for each substance was used to determine the LC_{25} and $LC_{0.5}$.

3.3 DATA ANALYSIS PIPELINE

The toxicogenomic transcriptome experiments conducted in this study included the exposure to the compounds diuron, diclofenac, and naproxen, each substance applied at five concentrations (plus control) and the transcriptome measured at six time points. Since each measurement includes abundance data for $\sim 20,000$ genes, the whole dataset sums up to more than 2×10^6 data points. We strived for establishing an analysis pipeline, extracting the most relevant dynamics from the data in a digestible and comparable format. The pipeline includes several steps from data normalization up to the projection of model parameters on a coordinate system, which we termed *Zebrafish Embryo Toxicogenomic trAnscriptome Coordinate system* (ZETAC) here. In the following, each step of the pipeline is motivated and explained in detail. All data analyses were performed using the software R (version 3.3.1, R Core Team, 2016). The data discussed in this chapter have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE109496 (`https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109496`). All scripts used for analyses and figures in this chapter have been deposited in GitHub and are accessible in the GitHub repository *ZFEmixtomics* (`https://github.com/anschue/ZFEmixtomics`).

3.3.1 *Import, quality control and normalization*

Before actually analyzing the data, results were normalized and data from flawed measurements were detected by quality control measures and removed. To account for differences in data distribution between the samples regarding amounts of total RNA content (which can arise during the extraction, labeling or hybridization processes, for example), data has to be normalized between samples.

DATA HANDLING The median fluorescence for each array spot was extracted by the Agilent Feature Extraction Software (Version 11.5.1.1, compare Appendix B). Those values were imported into R using the package *limma* (Ritchie et al., 2015). Quality control was performed by checking density distributions and signal intensities of spike in probes. Samples that showed irregular density distributions were removed from further analysis. Processed intensity values were normalized using the *cyclic loess* method. This method takes into account information from all datasets for its normalization relation, which has been shown to give better results than using a baseline array (Bolstad et al., 2003). Thereby, the method is comparable to the commonly used quantile normalization, though not as "aggressive" in its normalization (Ballman et al., 2004). The method is based on normalizing the relationship between the difference in log expression values and the average of log expression values of two arrays and was first applied to normalize two-color arrays (Dudoit et al., 2002).

After normalization all data was transformed by log_2. Subsequently, the median of replicate probes was calculated. Only replicates which had not been flagged for poor quality during the feature extraction process (due to inhomogeneous spots or background) were considered. Laboratory batch effects in the diclofenac experiment were removed using the R-package "sva" (Leek et al., 2012).

3.3.2 *Time normalization*

Transcript abundance is changing quite drastically for many transcripts during the course of embryo development, even without exposure to a chemical (compare Section 3.4 and Yang et al., 2013). At this point the effect of the chemical was of main interest. Therefore, the developmental effect on the transcriptome was removed by normalizing all transcript level values against the control of the respective time point. This resulted in log_2(fold-change) (logFC) data for all experimental conditions.

3.3.3 *Self-organizing maps*

A major challenge when analyzing *omics* data is to find a way to represent the important properties of the data in a way feasible to grasp, but without loosing important information at the same time. When dealing with a multitude of different conditions (like exposure to a variety of compounds) this becomes even more challenging since any gene might be important under some conditions. At the same time, transcript levels of single genes might not be a consistent measure across different biological or technical replications (Feswick et al., 2017). The machine learning method of SOMs has the potential to deal with these challenges. The algorithm has been developed by Kohonen (1982), was first applied to gene expression data by Törönen et al. (1999) and Tamayo et al. (1999), and has been further developed and tested for tissue expression as well as toxicogenomic data by Wirth et al. (2011, 2012). This method clusters transcripts into groups of co-regulated or co-expressed transcripts. Those groups are arranged on a two-dimensional grid in a way that similar behaving groups end up in the same regions. Each coordinate on the map – called "toxnode" here ("node" in general SOM terminology)– therefore gets assigned a distinct group of genes. The resulting map can subsequently be used to plot the representative responses of each group (termed "metagene" by Wirth et al. (2011)) under certain conditions, e.g., the exposure to a specific compound. Alternatively, quantitative descriptors of each toxnode can be retrieved and projected. This way one can inspect the responses of the whole transcriptome at the same time. Additional surveying of the map and the member genes of certain toxnodes or map-regions allows to draw functional conclusions. For example, a common regulator or common function can be suspected for member-genes of the same toxnode or map region (Wirth et al., 2012). Furthermore, the clustering of genes into toxnodes (for which SOMs represent one among many available methods) allows to derive quantitative summaries for each node. In this way, regression models can be derived from groups of transcripts rather than from single transcripts. When treating all genes belonging to one toxnode as replicates for this toxnode, one can infer much more robust parameter estimates. Thereby, one may dampen the influence of biological variability on the results, often inherent to *omics* data.

THE ZEBRAFISH EMBRYO TOXICOGENOMIC UNIVERSE With regard to the application of *omics* methods to environmental surveillance we aimed to create one universal SOM from all toxicogenomic responses known so far in our model organism. This SOM represents the "toxicogenomic universe" of this organism and the results of all (preceding or subsequent) toxicogenomic experiments can be projected on the coordinate system of this universe (*Zebrafish Embryo Toxicogenomic trAnscriptome Coordinate system* (ZETAC)), thereby creating toxicogenomic landscapes for particular exposure settings. Compound landscapes can either be represented by the average *log*FCs of each toxnode for defined exposure settings. Or, if time and concentration dependent data are available, estimated parameter values of a regression model for each toxnode can be projected on the coordinate system. Here, the extensive dataset from the meta-analysis (compare Chapter 2) was combined with results from our exposures to infer the *Zebrafish Embryo Toxicogenomic Universe* (ZETU) based on all retrievable current toxicogenomic zebrafish embryo transcriptome data.

A major advantage of the SOM at this point is the ability to deal with missing values. In the meta-analysis in Chapter 2, many datasets were included with only a limited number of transcripts (e.g, due to older microarray generations). In the SOMs, one can include the information of all those arrays, and thus retrieve as much information as possible.

One disadvantage of the self-organizing maps (as of most kinds of unsupervised analysis/clustering methods) is that there is no proper way of dealing with replicates (Wirth et al., 2012). Our experimental design has few replicates but many treatments along continuous variables, which is unfavorable since the machine learning algorithm might prefer to cluster genes with "common outliers". Therefore, to reduce the influence of biological noise and measurement outliers on the machine learning process, the experimental data was spline smoothed with a thin plate spline. The *log*FCs which resulted from the fitted spline were used for further analysis. For experiments aggregated in the meta-analysis mostly no continuous data were available, and thus a spline fit was not feasible (compare Chapter 2). Therefore, the "raw" *log*FCs between the means of treatment and control of each exposure condition were taken as input from the meta-analysis data for SOM inference.

DATA HANDLING The spline smoothed *log*FC data from our experiments were combined with the *log*FC data from the meta-analysis to one dataset.

Before smoothing the data, the Grubb's test (Grubbs, 1950, implemented in R-package *outliers*) was used iteratively to remove outliers from the group of data points (points were removed until $p >= 0.001$).

Table 3.2: Properties of SOM learning

parameter	**value**
learning rate	0.99 – 0.001
neighborhood radius	40 – (-40)
neighborhood function	gaussian
epochs	1000
distance function	manhattan distance

Then, a thin plate spline was fitted to the treatment conditions of each probe using the R-package *mgcv* (Wood, 2003), and *log*FC values for each measurement condition were extracted. The R-package *kohonen* (Wehrens and Buydens, 2007) was used to train the self-organizing map on a 60×60 rectangular grid. The properties of the map and the learning algorithm are summarized in Table 3.2. The outcome of this step is one 60×60 grid of 3600 "toxnodes". Each gene present in our dataset is "permanently" assigned to one toxnode, while each toxnode contains genes which behave similarly across all exposure conditions. This grid, we call the *Zebrafish Embryo Toxicogenomic trAnscriptome Coordinate system* (ZETAC).

3.3.4 *Regression models for time and concentration dependent toxicogenomic response*

On the way to a more mechanistic understanding of toxicogenomic responses, an important step is the ability to quantitatively describe the responses. Most useful with regard to the possibility of connecting to other biological levels (e.g., lethality) or toxicokinetics would be a mechanistic model, e.g., described by a set of differential equations. However, this is still a challenge especially on *omics* scale and a general understanding of the responses is deemed necessary, at first. Therefore, we decided for implementing a regression model, capturing the toxicogenomic responses of different substances as general and comparable as possible. This should enable us to inter- and extrapolate to exposure conditions not measured (essential for mixture predictions) and to describe the responses with the help of meaningful model parameters.

Description of regression models

Different regression models have been proposed for concentration dependent toxicogenomic responses, e.g., polynomials, splines, linear, exponential, gaussian and sigmoidal models (e.g., Smetanová et al., 2015; Thomas et al., 2007). Usually, the strategy is to fit different models and afterwards select the one with the best fit. This, however, impedes the comparison of fitted parameters for a responding gene between different compounds or the comparison between different genes or toxnodes. Therefore, we strived for a model, capturing the responses as general as possible. Two similarly structured models were set up to capture the response of transcripts (respectively toxnodes), the *Hill-Gauss* model for monotonic responses (on the concentration scale) and the *Gauss-Gauss* model for biphasic responses.

HILL-GAUSS MODEL The Hill-Gauss model describes a sustained response on the concentration scale and a temporal response on the time scale. It is based on the "Hill equation", a 3-parameter non-linear model, originally describing the binding of hemoglobin to oxygen dependent on oxygen saturation (Hill, 1910). Due to its flexibility on the one hand, and physiological meaningfulness on the other hand, it was later on used in many applications (reviewed in Goutelle et al., 2008) and also proposed for pharmacological dose response modeling (Wagner, 1968). One representation of the Hill-equation is Equation 3.3. It is defined by the parameters $logFC_{max}$, $slope$, and X_{50}. The parameter $logFC_{max}$ is the maximum logarithmic fold change observed for the respective transcript, the *slope* defines the steepness of the curve and X_{50} defines the concentration, where the response (i.e., logFC) reaches half-maximum.

We observed that on time scale most responses showed a biphasic response. This progression can be captured by a time dependency of the parameter X_{50} in Equation 3.3. Empirically, we discovered that the dynamics of the reciprocal of X_{50} is in many cases accurately captured by the logarithmic gaussian function (Equation 3.4). We call the reciprocal of X_{50} "Sensitivity", since a small X_{50} indicates a sensitive response (i.e., the half-maximum is already reached at low concentrations). When inserting Equation 3.4 into Equation 3.3, we get a full regression model describing the time *and* concentration dependent logFC after compound exposure (Equation 3.5):

$$logFC(c) = \frac{logFC_{max}}{1 + e^{-slope*(\log(concentration)-\log(X_{50}))}} \tag{3.3}$$

$$Sensitivity(t) = \frac{1}{X_{50}(t)} = maxSen * e^{-0.5*(\frac{log(time)-log(\mu)}{sigma})^2} \tag{3.4}$$

$$logFC(c,t) = \frac{logFC_{max}}{1 + e^{-slope*(\log(concentration)-\log(\frac{1}{maxSen*e^{-0.5*(\frac{log(time)-log(\mu)}{sigma})^2}}))}} + \epsilon \tag{3.5}$$

$$\epsilon \sim \mathcal{N}(0, \sigma^2),$$

where $logFC_{max}$ corresponds to the maximum fold change of the respective gene across all conditions, $maxSen$ is the maximum sensitivity ($1/EC_{50}$) of the gene, μ is the time-point of maximum sensitivity, and $sigma$ represents a measure of duration of the sensitivity interval.

GAUSS-GAUSS MODEL The Gauss-Gauss model (Equation 3.6) is similar to the Hill-Gauss model, only that the concentration dependent response is also described by a log-Gaussian function. This results in a three dimensional Gauss-function:

$$\begin{aligned} logFC(c,t) = \\ & logFC_{max} * exp\left[-\left(\frac{(log(c)-log(mconc))^2}{2*sconc^2}+\right.\right. \\ & \left.\left.\frac{(log(t)-log(\mu))^2}{2*sigma^2}\right)\right]+\epsilon \\ & \epsilon \sim \mathcal{N}(0,\,\sigma^2), \end{aligned} \tag{3.6}$$

where $logFC_{max}$ is the maximum fold change of the respective gene across all conditions, μ is the time-point of maximum *log*FC, *sigma* a measure of duration of change, *mconc* the concentration of maximum fold change, and *sconc* is a measure of concentration range with change.

PARAMETER ESTIMATION OF REGRESSION MODELS Parameter estimation of non-linear regression models as described above is a common challenge in many fields of natural and life sciences (Bates and Watts, 1988). Since no explicit solution exists, parameters have to be estimated iteratively to optimize the function value. Several challenges arise with this. For example, initial parameter guesses are required, which might not be straight forward to select and possibly have a strong impact on the outcome. This is primarily because there might be local optima of the objective function, which iterations can converge to. Additional problems may arise, if the objective function surface or the derivative is not smooth or discontinuous. Numerous parameter estimation methods exist, trying to deal with those challenges in various ways. Especially, when working with *omics* data, which means that a regression model has to be fit thousands of times to different data points, an estimation method should be rather independent from the choice of initial parameter guesses. Otherwise, initial parameters have to be estimated first from the data points, which might be infeasible in many cases and lead to a high number of false results.

An estimation method which was developed to optimize parameter values while taking into consideration the complete feasible parameter space is called shuffled complex evolution (SCE, described in Duan et al., 1993). Until now, it has mostly been used in hydrological modeling. It is based on the Nelder-Mead-Algorithm (Nelder and Mead, 1965), but includes the generation and shuffling of several simplices generated from random points across the parameter space. This makes the algorithm robust and independent from starting values, which is why we selected it for parameter estimation of our transcript level models. We used the R implementation of the algorithm in the package *hydromad* (Andrews et al., 2011). To estimate parameter uncertainty we applied Markov

Chain Monte Carlo (MCMC) sampling using the R-implementation of the statistics software *stan* (Carpenter et al., 2017).

PARAMETER BOUNDARIES While the shuffled complex evolution (SCE) algorithm is not sensitive to parameter starting values, it is - like other global parameter estimation methods - sensitive to the parameter boundaries, which therefore have to be defined carefully. To limit the fitted parameter values to a range that make sense in the context of the model, boundaries were set as summarized in Table B.1 in Appendix B. The motivations for the parameter boundaries are summarized in Section B.5.

DATA HANDLING Normalized *log*FC data (derived in the previous step) were used as input data for the model. Measured data from all probes assigned to one node of the SOM were used to estimate one parameter set for each node and substance (i.e., experimental replicates and transcriptional replicates/groups of transcripts were treated as belonging to one distribution here). The Grubb's test (Grubbs, 1950, implemented in R-package *outliers*) was used iteratively to remove outliers from the group of data points (points are removed until $p>=0.001$). The extreme values across all samples and experimental conditions were determined for each node. Then, the dataset for each node was used to estimate parameters for the Hill-Gauss and the Gauss-Gauss model using the SCE algorithm assuming up-regulation. This estimation procedure was repeated 6 times with 6 different random seeds and the best model afterwards selected using AIC_c. The same procedure was repeated assuming down-regulation. The best up-regulation model and the best down-regulation model were again compared using AIC_c and the best fit model subsequently used for a quantitative description of the node.

3.3.5 *Selecting significantly affected toxnodes*

Most known genes of the zebrafish genome (26,143 of 34,738 coding and non-coding genes currently referenced in the ensembl database, release 90, Yates et al., 2016) are represented on our map in 3600 toxnodes, but we expect only a minority of the nodes to be affected by exposure to a single compound. To be able to extract meaningful information from the map, we need a measure defining the confidence/significance for each node (and treatment), that the signal we see is not just random noise but a systematic change in transcription. Therefore, we calculated a significant effect level for each toxnode. The significant effect level is given by the summed differences between the 95% confidence interval (CI) for the fitted regression model and the 2.5% respectively 97.5% quantile of all controls of the respective toxnode (compare Figure 3.4).

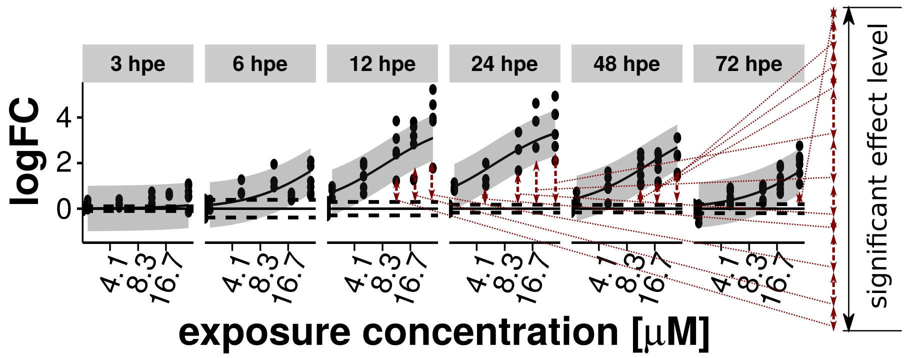


Figure 3.4: Demonstration of calculation of significant effect level for a toxnode.

In detail, for each substance and condition we used the estimated parameters and best-fit model (Hill-Gauss or Gauss-Gauss) to calculate an estimated *log*FC. The estimated error term of the model determined the confidence interval of the treatment. We then summed up the distances between the CI and the control quantiles. All toxnodes with a summed CI-distance above zero were selected as significantly affected.

3.4 RESULTS

Our central goal was to develop an experimental and data analysis pipeline, which allows to infer time- and concentration dependent toxicogenomic profiles of chemical substances acting on the ZFE as model species. We exemplarily applied our pipeline on three selected environmental contaminants, namely diuron, diclofenac and naproxen and measured the transcriptome of ZFEs after exposure to increasing concentrations of these compounds. We show here that the application of self-organizing maps (SOMs) on spline smoothed data and subsequent regression modeling allows to establish robust toxicogenomic landscapes. The established landscapes may be used to elucidate adverse outcome pathways (AOPs) of the respective compounds or gain functional knowledge about gene products or signaling pathways in the zebrafish embryo (ZFE). Here, we focused on extracting patterns in the landscapes which appeared to be characteristic for the effect of a cyclooxygenase (COX)-inhibitor (i.e., common effects of diclofenac and naproxen not appearing after diuron exposure). These structures may be used later on to detect COX-inhibition in chemical mixtures such as environmental extracts.

3.4.1 *Data overview*

To get an overview over the structure and quality of the high-dimensional transcriptome data, they were condensed to a two dimensional plot using multidimensional scaling. Here, each sample is represented on a two-dimensional plot by one data point. The data points are arranged in a way that the distance on the plot represents the distance between the samples. Figure 3.5 shows a multidimensional scaling plot for the three normalized datasets. It shows that samples of one embryo age (of all experiments) cluster together. This means that the highest proportion of variation in transcript abundance is due to the age of the embryo. It also shows that the transcriptome measurements are reproducible with respect to the stage of embryo development, since no batch effect can be seen between the three datasets, which stem from experiments performed several months apart. In the age-related cluster some of the exposed samples diverge from the control samples, for the 12 hpe and 6 hpe samples they are more similar to the controls of the preceding time point indicating a developmental delay induced by the chemical. Since the main interest lies in the effect of the compound exposure here, the dataset was normalized to the respective time point controls.

3.4.2 *The toxicogenomic universe*

To reduce complexity in the transcriptome data and make it more easily digestible, results are structured by a self-organizing map (SOM). On a SOM similar behaving transcripts are clustered together in nodes and those nodes are arranged on a two dimensional grid. Our goal was to make the results of our model substances comparable to each other as well as to existing exposure studies and include as much information as possible in the layout of the map. Therefore we inferred a SOM from our experimental (spline-smoothed) *log*FC transcriptome data of our three model substances. Additionally, we added the *log*FCs of all studies compiled in the meta-analysis (compare Chapter 2). This resulted in a dataset of 358 different conditions and 32210 unique genes (A few more studies could be used in this design than in the original meta-analysis, since the SOM-learning algorithm can deal with missing values). Of those 32210 genes, 26143 genes had enough information to be clustered on the map.

The first question we asked is, if it is feasible to create such a map at all, since it relies on the assumption that a majority of transcripts forms consistently co-expressed clusters. If this was not the case, the nodes of such a map would show inconsistent profiles and the average response of most nodes will be low. Diagnostic plots (Figure 3.6) show that the training process converges. The quality of the map appears to be good, since genes are evenly distributed across the map and within distances only show a few outliers. The number of transcripts per node range between 0 and 28 with a median of 7. The average manhattan distance of transcripts within a node ranges from 0.3 to 211 with a median of 88. In Figure 3.7 a)/c)/e) the distribution of *log*FCs of transcripts in three exemplary nodes are shown, one with a small average within distance, one with medium, and one with high average

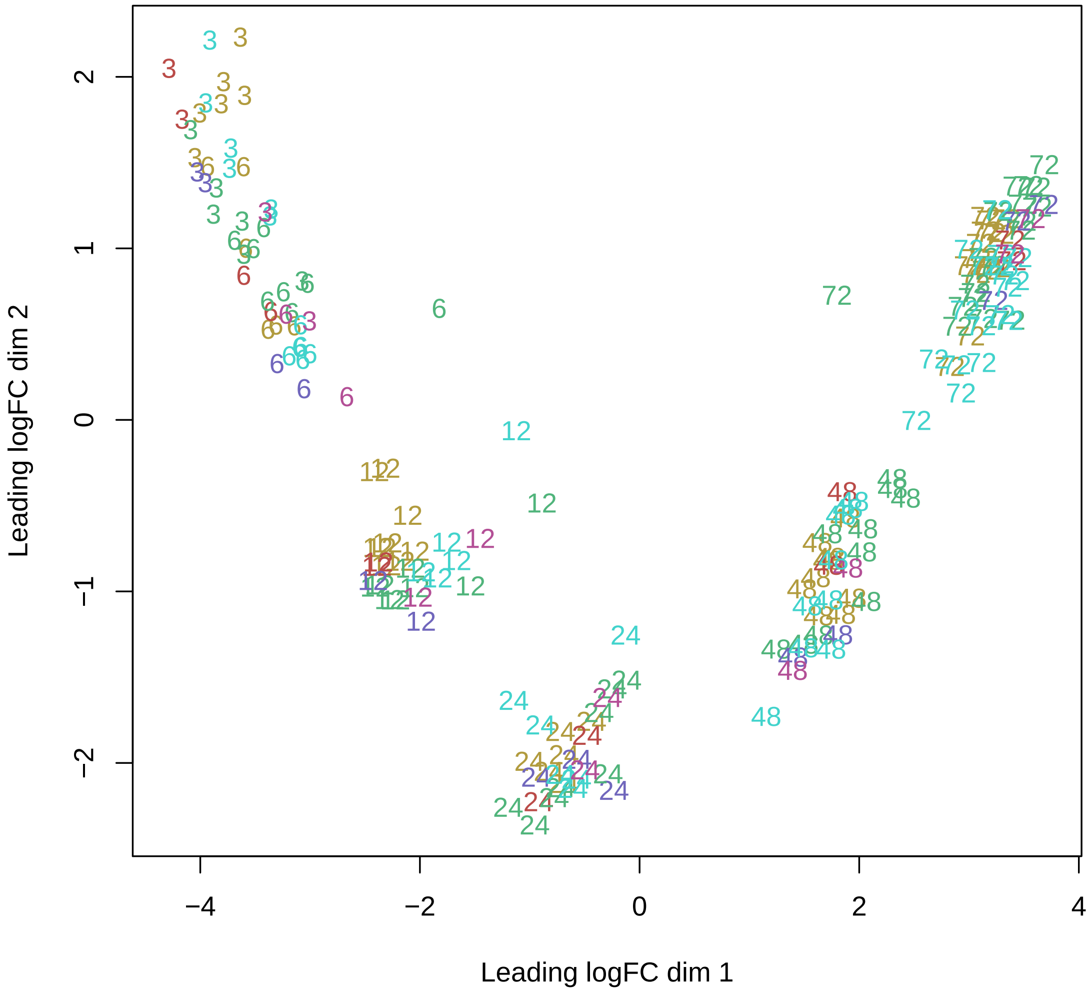
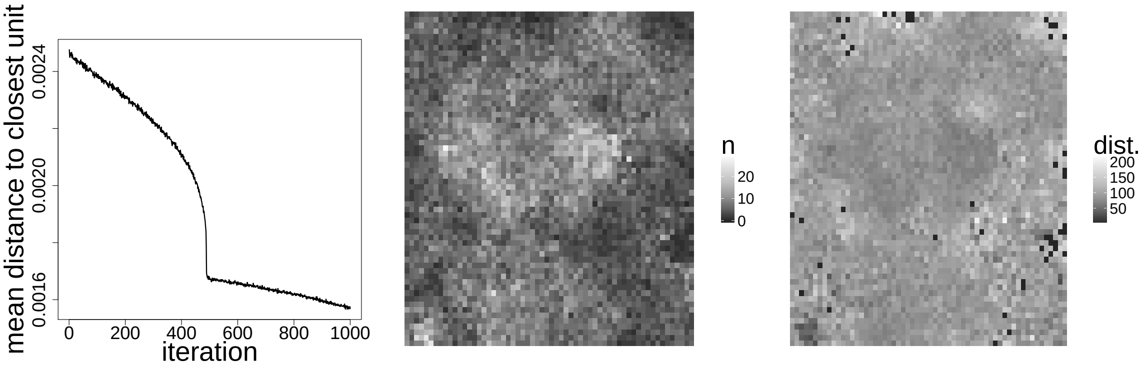


Figure 3.5: Multidimensional scaling plot of all transcriptome samples for diuron, diclofenac and naproxen exposure between 24 and 96 hpf. Numbers: exposure duration in hours; diuron controls (■); diuron treatments (■); diclofenac controls (■); diclofenac treatments (■); naproxen controls (■); naproxen treatments (■); the plot shows the relation between the samples and illustrates that samples cluster according to embryonic stage.



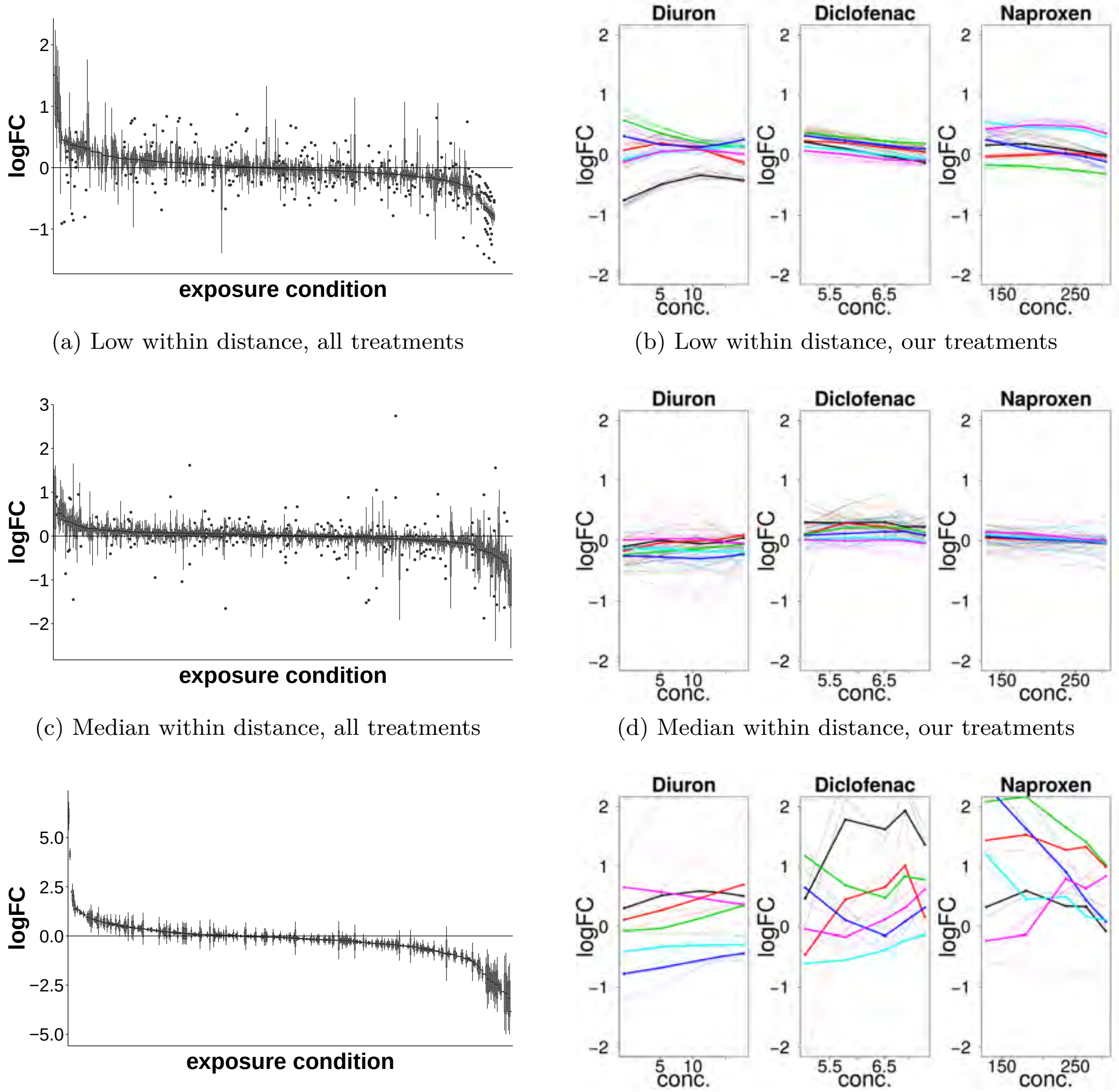
(a) training progress (b) counts (c) within distance

Figure 3.6: Diagnostic plots of toxicogenomic universe. In (a) the progress of the machine-learning process is illustrated with the mean-distance of all genes to their assigned toxnode. The distance decreases by reassignment of the genes and a decreasing neighborhood size. After 500 iterations the neighborhood size equals to one and the learning process converges. In (b) the number of genes assigned to each toxnode are plotted and show some higher populated areas in the middle part. In (c) the medium within distance of each node is shown.

within distance. The plots show that transcripts within nodes, even those with a higher within distance, show similar expression profiles across different exposure settings (i.e., a narrow distribution for most exposure settings). This indicates that co-expression seems to be quite consistent across the 358 experiments. This becomes even more clear, when evaluating the time and concentration resolved response of toxnodes showing small/medium/high within distances for exposures to our model substances (Figure 3.7 b)/d)/f)). Here the within distances are smaller in average (manhattan distance ranging from 0.2 to 74 with a median of 18) and distinct co-regulation between member genes across the different concentrations and time-points can be seen.

3.4.3 *Visual inspection of molecular toxicodynamic maps*

From qualitatively evaluating and comparing the model compounds' *log*FC landscapes projected on the toxicogenomic coordinate system, we already get an impression of the molecular action of the compounds (Figures 3.8-3.10). The landscapes show a time and concentration dependent regulation in both directions (up- and down-regulation). For naproxen the response is strongest in the latest two time points, while for diclofenac and diuron there are also strong effects visible in earlier time points. In general, exposure duration seems to play a more pronounced role for node regulation than exposure concentration. Unlike expected, diclofenac and naproxen –both inhibiting the same enzyme–



(a) Low within distance, all treatments

(b) Low within distance, our treatments

(c) Median within distance, all treatments

(d) Median within distance, our treatments

(e) High within distance, all treatments

(f) High within distance, our treatments

Figure 3.7: Transcript *logFC*s within different example nodes. For (a)/(c)/(e), each box represents the distribution of the member transcripts in one experimental condition. (a) node with minimum within distance (node 65, medium distance 27.7); (c) node with medium within distance (node 2533, medium distance 88.1); (e) node with maximal within distance (node 3560, medium distance 211.1). A trend of co-regulation (i.e., narrow distributions) can be seen also in the toxnode of high within distance. For (b)/(d)/(f) the horizontal axis represents the exposure concentration from $LC_{0.5}$ to LC_{25}. 3 hpe (▬); 6 hpe (▬); 12 hpe (▬); 24 hpe (▬); 48 hpe (▬); 72 hpe (▬). Facets = Exposure compounds. (b) node with minimum within distance (node 719, medium distance 7.0); (d) node with medium within distance (node 451, distance 18.4); (f) node with maximal within distance (node 1441, distance 74.3).

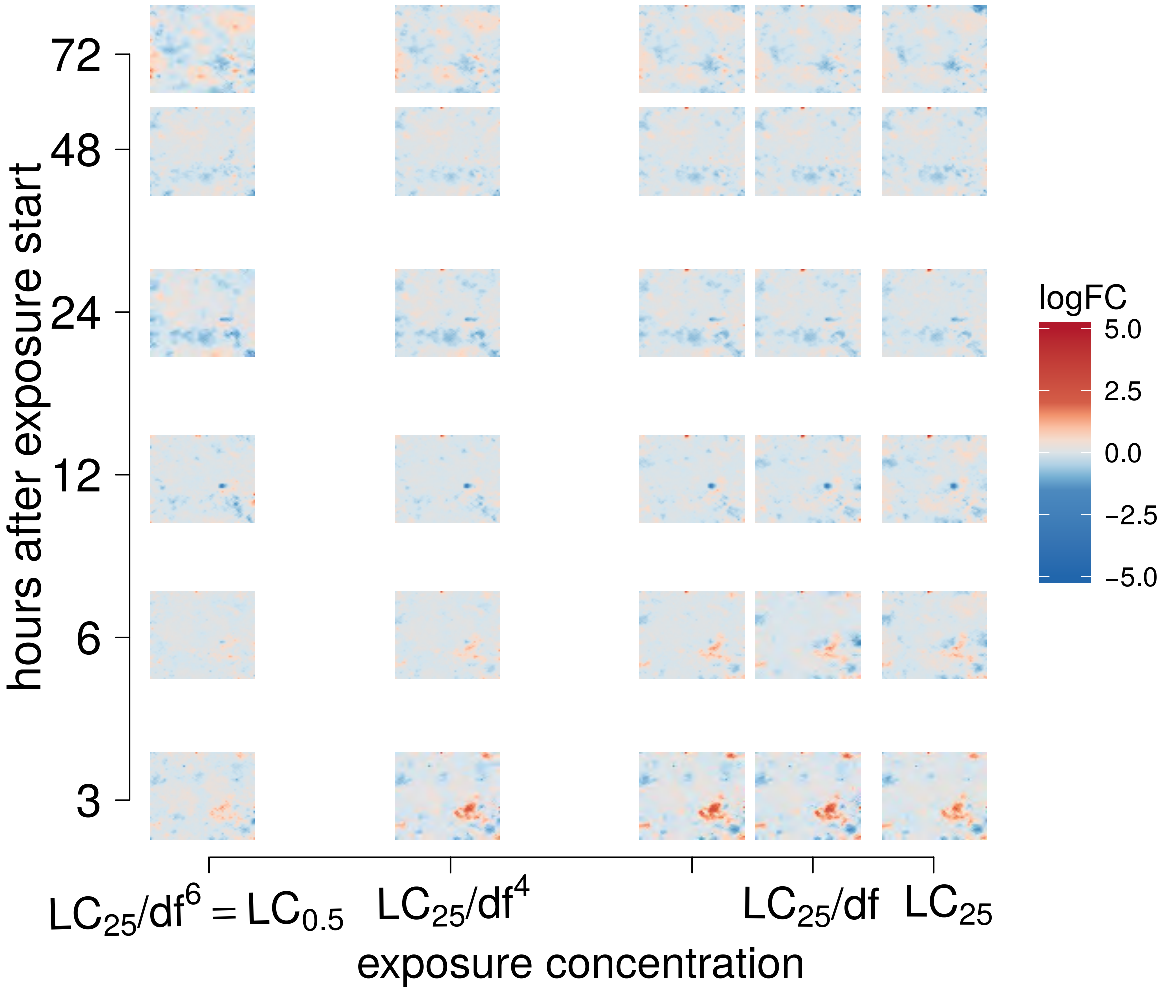


Figure 3.8: Toxicodynamic landscape for diuron projected on the ZETAC. Shown is the representative *log*FC landscape for each sampled time point/exposure concentration.

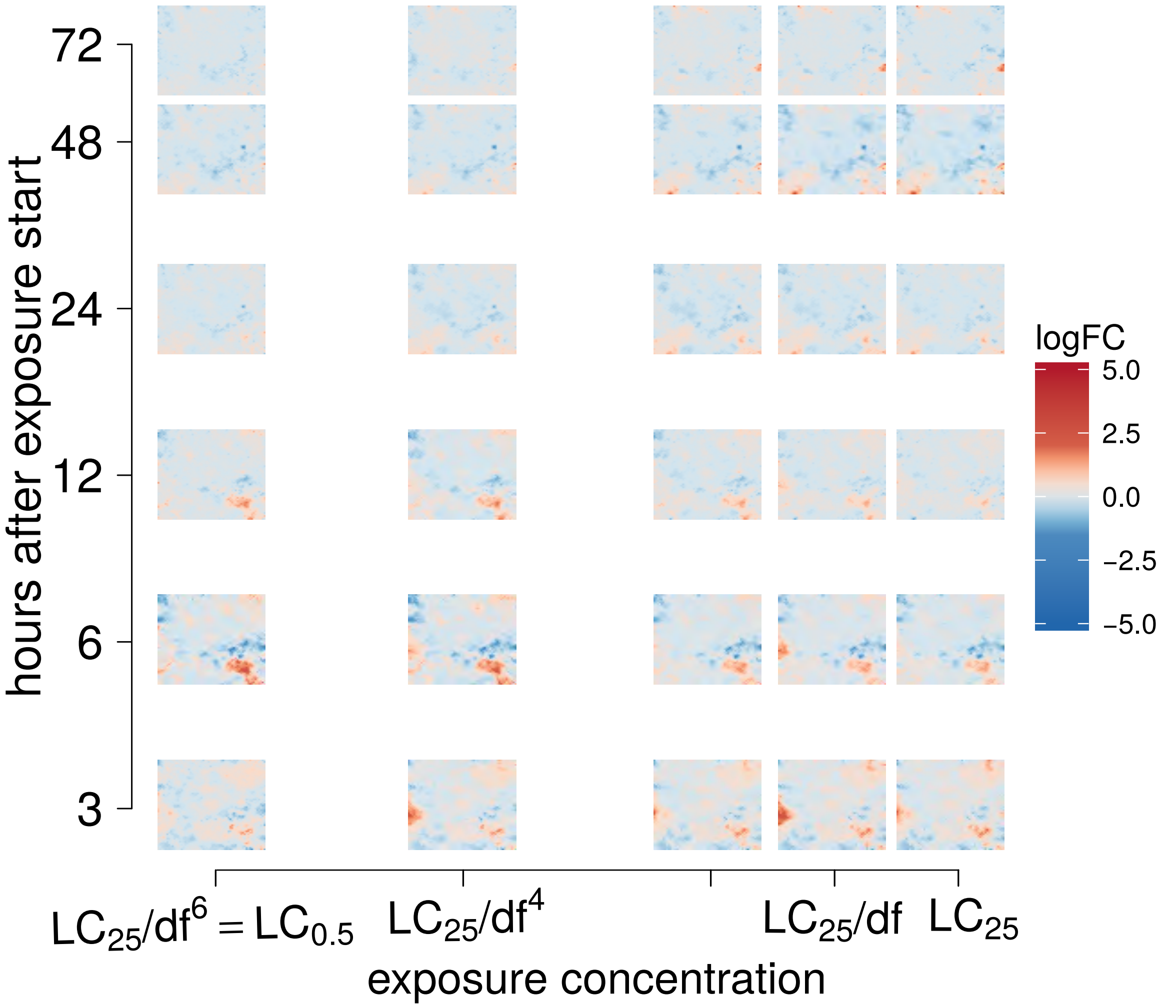


Figure 3.9: Toxicodynamic landscape for diclofenac projected on the ZETAC. Shown is the representative *logFC* landscape for each sampled time point/exposure concentration.

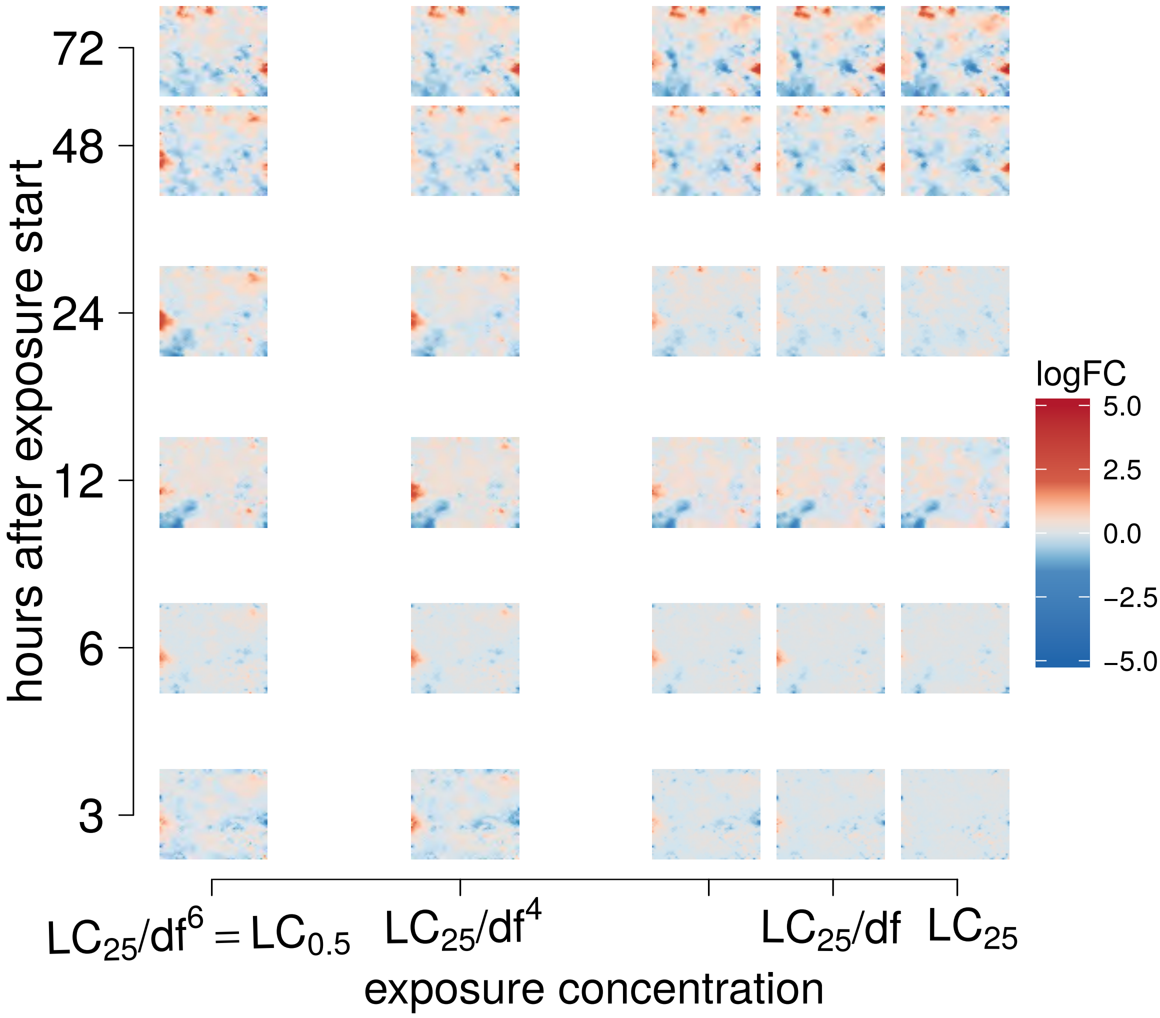


Figure 3.10: Toxicodynamic landscape for naproxen projected on the ZETAC. Shown is the representative *log*FC landscape for each sampled time point/exposure concentration.

show quite different profiles in their toxicogenomic landscapes. The profiles show that naproxen clearly induces the strongest transcriptional response of the three substances.

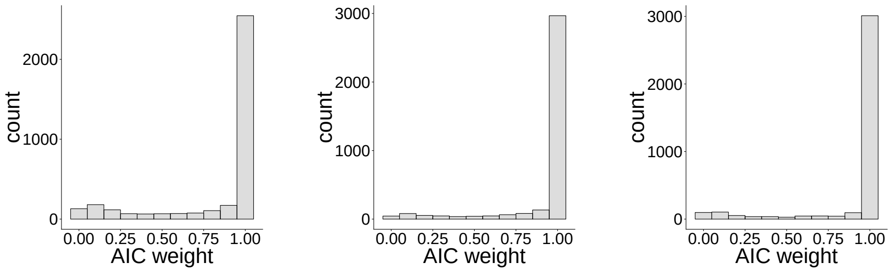
3.4.4 *Quantitative description of toxnode levels by regression models*

In a next step, we strived for a quantitative description of the toxnode-dynamics in order to derive more substantial and comparable information about the landscapes, as well as to deal with noise and uncertainty, and to be able to extrapolate to other conditions. Therefore, we developed and fitted two different regression models (compare Section 3.3.4), the Hill-Gauss and Gauss-Gauss model, to describe time- and concentration-dependent behavior of each toxnode for each substance. Since these models have not been applied so far, the first step was to determine the model quality. In this context it is important to determine how well the model can describe the data and how reliable the parameter estimates are.

QUALITY OF DATA DESCRIPTION BY FITTED MODEL The model fitting algorithm converged for fitting all nodes. There is no trivial measure for goodness of fit for non-linear models (such as R^2 for linear models, compare Spiess and Neumeyer, 2010). Therefore, the quality of data description by the model was determined using the AIC$_c$-weight compared to a null model. In Figure 3.11 and Figure 3.12 the AIC$_c$-weights for the Hill-Gauss respectively Gauss-Gauss model versus the null model are summarized. In the vast majority of cases the regression models are preferred over the null model. When comparing the regression models to the more flexible spline fit (compare Figures B.1 and B.2 in Appendix B) which is assumed to be the "optimal fit" here, there are (as could be expected) many toxnodes, where the spline fit is preferred. However, for roughly 20% of the nodes the Hill-Gauss or Gauss-Gauss models are even preferred over a spline fit.

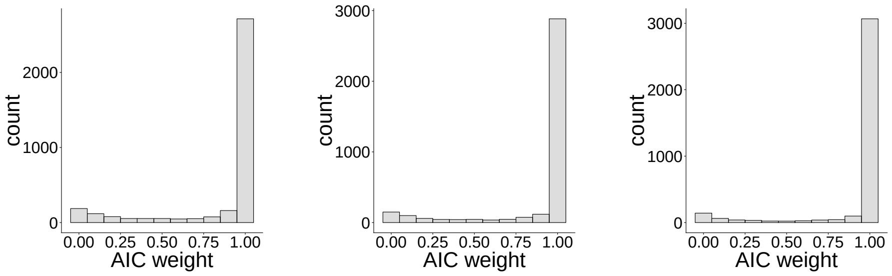
COMPARISON HILL-GAUSS VS. GAUSS-GAUSS MODEL The Hill-Gauss model can accurately describe the response of many nodes. For a majority of nodes the Hill-Gauss model is at least as good as the Gauss-Gauss model (Figure 3.13). However, for a substantial amount (~1/3) of nodes, the Gauss-Gauss model can capture the node response better than the Hill-Gauss model. One example for such a case is shown in Figure 3.14.

QUALITY OF PARAMETER ESTIMATES When we want to use the fitted parameter values as descriptors of the toxnodes, we need to be aware of the respective reliability of the values. Therefore, we applied a Bayesian analysis using Markov-Chain Monte Carlo sampling to obtain an estimate of parameter uncertainty. The sampling revealed that not



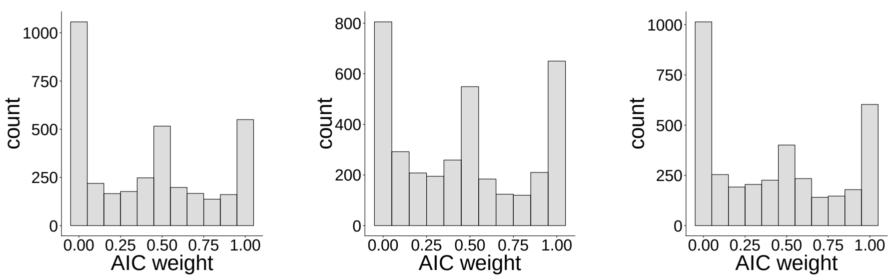
(a) Diuron (b) Diclofenac (c) Naproxen

Figure 3.11: Histogram of AIC$_c$-weights for Hill-Gauss model compared to null model for all toxnodes. The best fitting model is preferred over the null model for most of the toxnodes.



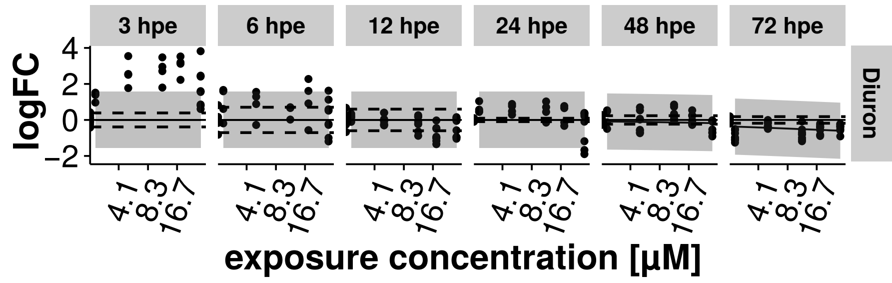
(a) Diuron (b) Diclofenac (c) Naproxen

Figure 3.12: Histogram of AIC$_c$-weights for Gauss-Gauss model compared to null model for all toxnodes. The best fitting model is preferred over the null model for most of the toxnodes.

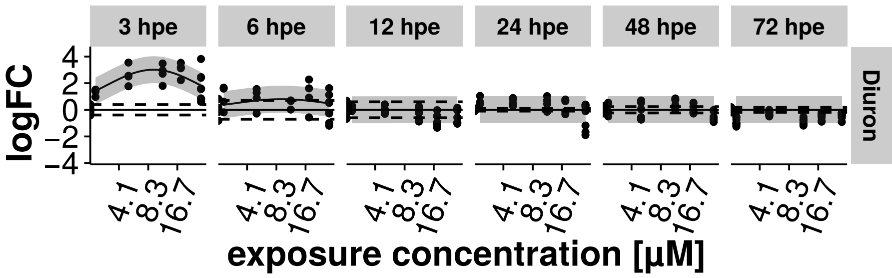


(a) Diuron (b) Diclofenac (c) Naproxen

Figure 3.13: Histogram of AIC$_c$-weights Hill-Gauss model compared to Gauss-Gauss model. For about a third of the nodes, the Gauss-Gauss model would be preferred, however it should only be used if the Hill-Gauss model cannot describe the behavior, since Hill-Gauss can be interpreted easier.



(a) Hill-Gauss model



(b) Gauss-Gauss model

Figure 3.14: Comparison of Hill-Gauss and Gauss-Gauss fit for the response of example node #1362 towards diuron exposure. Shaded area represent 95% CI. The response is captured better by the Gauss-Gauss model.

in all cases the parameter estimates could be well defined. However, a rough classification based on the parameter estimates of a node should be feasible. It is obvious that the time parameter (μ) could be much better defined than the concentration parameter (minEC50/mconc). The confidence ranges for parameter estimates are summarized in Figures B.3-B.8 in Appendix B.

SELECTION OF SIGNIFICANTLY AFFECTED TOXNODES Certainly, we only expect a small fraction of the toxnodes to show a relevant response after exposure to a specific compound. To judge whether a node shows a significant regulation in our exposure scenario, we compared the 95% confidence interval for the regression models with the 2.5% respectively 97.5% quantiles of control measurements. We selected those nodes with a sum of differences above or below zero. This resulted in 75 nodes for diuron, 67 nodes for diclofenac, and 328 nodes for naproxen exposure, meeting this criterion. Five nodes are regulated in both diuron and diclofenac exposures, 27 nodes

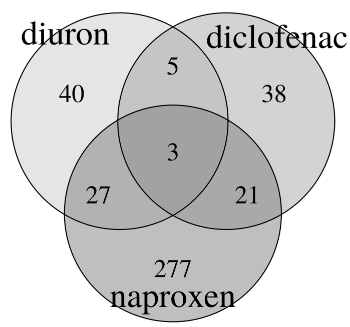


Figure 3.15: Venn Diagram of significantly affected toxnodes

in diuron and naproxen, and 21 nodes in diclofenac and naproxen exposures, three nodes are regulated in exposures of all three compounds (compare Figure 3.15).

These top toxnodes for each substance (respectively substance group) can now be analyzed in detail, by retrieving parameter estimates and determining under which circumstances those toxnodes are affected in the toxicogenomic universe.

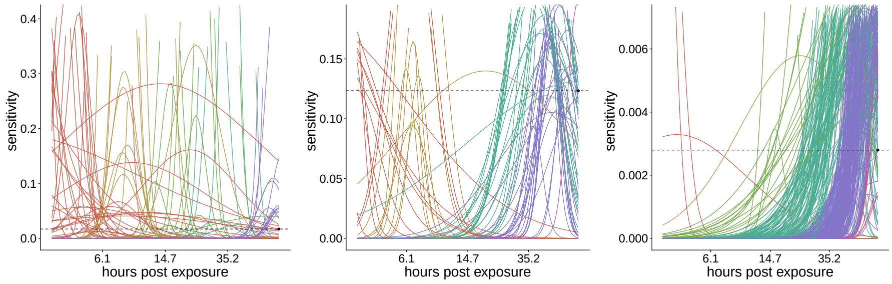
SENSITIVITY DYNAMICS OF SELECTED TOXNODES The dynamics of toxnode sensitivity (i.e., $1/EC_{50}$) can be assessed by evaluating the estimated parameter values for $maxS50$, μ and $sigma$ of the Hill-Gauss model. In Figure 3.16 the sensitivities for significantly affected toxnodes are plotted over time. There are clearly more toxnodes sensitive early after the beginning of diuron exposure compared to naproxen exposure, for which most toxnodes are sensitive not before 24 hours post exposure (HPE). In Figure 3.17 the respective first time points of toxnode regulation after compound exposure are plotted on the ZETAC.

3.4.5 *Summary of toxicogenomic landscapes of model substances*

From the inferred toxicogenomic landscapes we can deduce informations about molecular effects of the tested compounds on the ZFE. Transcripts clustered together in one toxnode are found co-regulated across many exposure settings. This may indicate a common biological function or process they are involved in or a common upstream regulator. In this way, the member-genes of significantly regulated toxnodes can indicate the type of biological processes affected in response to a compound exposure. At the same time, the estimated model parameters can give quantitative information about the dynamics (i.e., μ, $sigma$) and the concentration dependence (i.e, $maxS50$, $hillslope$). The ratio of $\frac{min(EC_{50})_{apical}}{min(EC_{50})_{Toxnode}}$ indicates how much earlier (on the concentration scale) a toxnode is affected, before apical effects are visible.

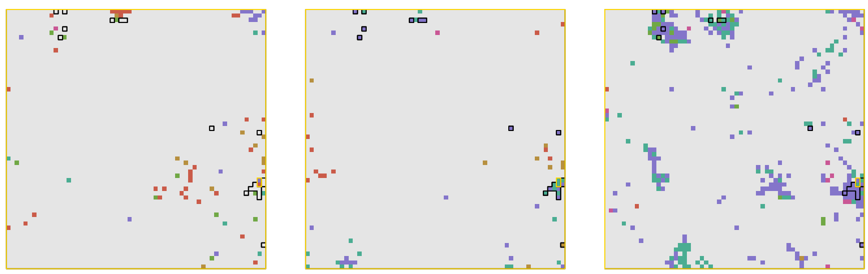
Here, we evaluated the top ten affected toxnodes of our model substances. We focused on toxnodes identified with the help of the Hill-Gauss model (for top ten toxnodes identified with the Gauss-Gauss model, compare Tables B.3-B.5 in Appendix B). Additionally, we analyzed the commonly affected toxnodes by diclofenac and naproxen as potential COX-inhibition toxnodes. For the toxnodes discussed here, we evaluated if the clustering of genes matches with our knowledge about biological functions of the respective transcripts.

DIURON Of the three model compounds, diuron exhibits the most distinct early regulation. The most prominent toxnodes (Table 3.3) contain genes for phase I (cyp1a, cyp1b1, cyp1c1, cyp1c2) and phase II (sult6b1) biotransformation enzymes. Toxnode #1117 contains genes



(a) Diuron (b) Diclofenac (c) Naproxen

Figure 3.16: Predicted sensitivity dynamics of significantly affected toxnodes. Dynamics are predicted from parameter estimates of Hill-Gauss model. Each line represents one toxnode. Colours indicate first time point of significant regulation: 3 hpe (); 6 hpe (); 12 hpe (); 24 hpe (); 48 hpe (); 72 hpe (); Horizontal dashed line represents lethal sensitivity at 72 hpe



(a) Diuron (b) Diclofenac (c) Naproxen

Figure 3.17: Onset of toxnode regulation induced by model compounds projected on ZETAC. Significantly affected toxnodes are colored according to their first time point of regulation: 3 hpe (); 6 hpe (); 12 hpe (); 24 hpe (); 48 hpe (); 72 hpe (). Yellow framed toxnodes are general toxnodes significantly affected by all model substances. Black framed toxnodes are affected by diclofenac and naproxen but not by diuron.

Table 3.3: Top ten toxnodes regulated by Diuron. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval. Positive CI-differences indicate upregulation, negative indicate downregulation of respective toxnodes.

toxnode #	Membergenes	ratio Lethal	ratio Sublethal	$maxS50$	$hillslope$	μ	$sigma$	err	sum(CI-difference)
3567	cyp1a	15.8	15.8	0.204 0.333	0.8 1.1	8.6 27.3	1.22 11.66	0.29 0.47	73.4
1303	cyp1b1	10.1	10.1	0.118 0.265	0.8 1.4	1.5 3.8	1.41 2.18	0.44 0.67	21
3568	cyp1c2, cyp1c1, ENSDARG00000097714	9.4	9.4	0.124 0.218	0.9 1.4	19 22.3	0.56 0.75	0.61 0.77	11.7
3507	sult6b1	7.9	7.9	0.094 0.213	0.6 1.2	3.8 11.7	0.78 1.62	0.23 0.35	7.8
1061	prf1.5, grk1b	23.3	23.3	0.014 4.333	0.1 1.2	1.6 4.1	0.14 0.64	0.73 1	3.6
727	cxcr3.3	0.4	0.4	0.003 0.028	1 17.4	3.4 73.7	0.4 21.85	0.6 0.9	3.2
1002	grapb	4	4	0.038 0.242	0.7 10.1	4.6 7.8	0.14 0.51	0.57 0.86	1.4
3184	zgc:110373	36.1	36.1	0.091 2.889	0.2 3	67.5 74.9	0.13 0.2	0.71 1.1	-1.4
1117	saga, arr3a	0.5	0.5	0.003 0.036	0.5 18	4.3 73.9	0.25 21.64	0.93 1.25	1.4
3539	amy2a, zgc:92137, ela3l	2.9	2.9	0.041 0.06	1.8 5	65.1 74.8	0.14 0.27	0.46 0.59	-1.4

Table 3.4: Top ten toxnodes regulated by Diclofenac. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval. Positive CI-differences indicate upregulation, negative indicate downregulation of respective toxnodes.

toxnode #	Membergenes	ratio Lethal	ratio Sublethal	*maxS50*	*hillslope*	μ	*sigma*	*err*	sum(CI-difference)
1260	cyp2k18	2	1.9	0.204 0.282	2.2 9.2	44.8 54.4	0.24 0.47	0.82 1.24	12.1
1259	si:zfos-411a11.2	1.9	1.8	0.186 0.281	0.7 2.5	45.8 54.6	0.19 0.39	0.22 0.34	8.6
1079	lepa, pth1a	1.9	1.7	0.192 0.28	1.8 6.9	45 55.2	0.22 0.52	0.69 0.92	5.5
1968	si:dkey-1c7.3	1.9	1.7	0.197 0.277	3.8 35.1	38.1 50.7	0.17 0.52	0.51 0.75	-3.9
3446	ugt1a4, ugt1a5, ugt1a6, ugt1a7, ugt1a2, ugt1a1, ugt1ab	2.1	1.9	0.215 0.282	1.6 4.8	44.6 54.1	0.23 0.41	0.18 0.27	3.7
1137	fosab	1.6	1.5	0.164 0.268	1.6 14.7	44.6 57.4	0.21 0.71	0.75 1.13	3
1440	crygmxl2	1.2	1.1	0.099 0.252	0.5 4	5.1 7.4	0.14 0.35	0.43 0.64	-2.9
1500	crygm3, crygm2d12, crygm1, crygm2d13, crygm2d7, crygm2d3	2	1.8	0.197 0.281	0.6 1.9	5.5 6.9	0.14 0.24	0.42 0.49	-2.6
1199	nfe2l2b	1.1	1	0.097 0.252	0.1 3.8	9.2 74.1	0.42 20.69	0.46 0.7	2.5
1557	si:ch211-251b21.1	2	1.9	0.197 0.281	0.9 27.7	5.2 7.2	0.15 0.5	0.28 0.42	-1.8

for two members of the arrestin family. This protein family is involved in dampening the signal cascade of G-protein coupled receptors. One of these proteins, Saga, is involved in desensitization of the photo-activated transduction cascade. The up-regulation goes in line with an observed weakening of locomotor response after diuron exposure (Leuthold, in preparation). Among the top ten toxnodes the $minEC_{50}$ for toxnode regulation is predicted to be approximately one order of magnitude lower than the $minEC_{50}$ for lethality (no sublethal effects were observed for diuron). However, there are also toxnodes (e.g., #3539) for which the EC_{50}s are only separated by a factor of two, or toxnodes (e.g., #1117 or #727) for which the predicted $minEC_{50}$ for toxnode regulation is even higher than the apical EC_{50}.

DICLOFENAC Similarly as for diuron, the most prominent toxnodes affected by diclofenac also contain phase I (cyp2k18, si:zfos-411a11.2) and phase II (ugt1) biotransformation enzymes, albeit different ones than induced by diuron (Table 3.4). Additionally, two toxnodes of down-

Table 3.5: Top ten toxnodes regulated by Naproxen. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval. Positive CI-differences indicate upregulation, negative indicate downregulation of respective toxnodes.

toxnode #	Membergenes	ratio Lethal	ratio Sublethal	*maxS50*	*hillslope*	μ	*sigma*	*err*	sum(CI-difference)
1260	cyp2k18	5.8	2.8	0.013 0.017	1.6 2.6	62.2 74.4	0.26 0.45	0.29 0.45	34.2
3312	serpinh1b	5.4	2.7	0.011 0.017	0.9 1.5	52.1 74.6	0.67 0.96	0.24 0.36	25
1139	slc25a38a, c7b	2.7	1.3	0.007 0.013	1.8 2.8	61.8 74.8	0.15 0.34	0.33 0.46	16.1
1320	si:rp71-80o10.4	3	1.5	0.006 0.016	0.5 2	61.9 74.9	0.37 0.81	0.4 0.62	15.1
1140	si:ch1073-165f9.2, isg15	2.4	1.2	0.006 0.012	1.8 3.4	60.9 74.8	0.16 0.46	0.6 0.81	14.2
1259	si:zfos-411a11.2	5.7	2.8	0.012 0.017	0.9 1.8	65 74.8	0.21 0.37	0.25 0.39	13.5
1078	mmp13a	3.6	1.8	0.007 0.016	0.8 2.6	63.2 74.7	0.14 0.28	0.41 0.64	13.2
3477	ahsg2	3.9	1.9	0.007 0.016	1.4 5.2	67.2 74.8	0.13 0.23	0.97 1.54	- 12.5
3493	slc16a9a	4.2	2.1	0.008 0.017	0.9 2.5	63 74.8	0.15 0.32	0.38 0.6	12.2
1080	cart3, LOC101885512, ccl34a.4, bmb	3.3	1.6	0.008 0.013	0.8 1.8	69.1 74.9	0.46 0.68	0.48 0.59	11.7

regulated crystallins (#1500 and 1440) belong to the most affected toxnodes (#1500 is also down-regulated in diuron). The up-regulation of toxnode #1079 (up-regulated by naproxen as well), comprising of the genes for leptin alpha and thyroid hormone 1, indicates a disturbance of the thyroid system. The level of leptin alpha is thought to regulate the activity of the thyroid system (Flier et al., 2000), therefore the clustering of those two genes in one toxnode also match with our knowledge of underlying biological processes.

For diclofenac the ratio between apical $minEC_{50}$ and toxnode EC_{50} is not larger than two for any of the top ten toxnodes.

NAPROXEN Naproxen shows the highest number of significantly affected toxnodes and the latest effect on the ZETU, with most toxnodes not affected until 12 hpe. Its most prominent toxnode is the same as in diclofenac, containing cyp2k18 (Table 3.5).

Additionally, one toxnode includes serpinh1b, a gene for a collagen specific chaperone. Toxnode #1139 (also regulated by diclofenac) contains the gene for slc25a38a, a mitochondrial membrane carrier involved in heme synthesis and c7b, a part of the membrane attack complex. Whether the clustering of those genes indeed reflect involvement in a common biological process is not clear. However, it was shown before that formation of a membrane attack complex indicates an immunological response (Bayly-Jones et al., 2017). Toxnode #1140 contains, besides a non-annotated transcript, the gene isg15, an ubiquitin-like protein, assigned to different cellular processes, e.g., directing ligated proteins to intermediate filament. The up-regulated toxnode #1080 (also showing significant regulation in response to diuron and a clear trend of regulation in response to diclofenac) contains the genes cart3, coding for a peptide regulating energy metabolism, apoptosis facilitator Bcl-2-like protein 14 (LOC101885512), the gene for the chemokine ccl34a.4 and brambleberry. The direct functional derivation of this toxnode is again not straightforward, but the presence of Bcl-2-like protein 14 indicates involvement in apoptosis. It should be mentioned that up-regulated toxnode #1134 (ranked 62 among naproxen affected toxnodes) contains the gene for COX2, junbb, and RNaseMRP. It is not clear whether this up-regulation is a direct effect of inhibiting COX2 or if this effect is a secondary effect due to activation of an inflammatory response.

For naproxen the ratio between apical $minEC_{50}$ and toxnode EC_{50} shows a maximum of 5.8 for toxnode #1260. Although this toxnode could be suspected to represent a COX inhibitor specific effect, it shows clearly different ratios for diclofenac and naproxen. Interestingly the ratios mainly differ when comparing to lethal effects and not so much when comparing to sublethal effects.

In summary, all model substances clearly affect a limited number of toxnodes in a time- and concentration-dependent manner. For some of these toxnodes we can derive hypotheses about their involvement in the response of the embryos towards the compounds. For some toxnodes the establishment of those connections will need further investigation.

3.4.6 *Toxnodes specifically affected by COX-inhibitors*

With regard to environmental diagnostics, we would like to define a set of toxnodes, indicating a certain mode of action (MOA). Two of our model substances (i.e., diclofenac and naproxen) are known COX-inhibitors. Thus, the question we are trying to answer is, if there is a common MOA of those COX-inhibitors in zebrafish embryos, which becomes visible on transcriptome level.

A number of 19 toxnodes showed significant up- or down-regulation (in the same direction) in response to naproxen and diclofenac, but no significant regulation in response to diuron. The regulation of one of these nodes (toxnode 1019) is exemplarily shown in Figure 3.18. In Figure 3.18a) it is demonstrated that the two member transcripts of the toxnode are indeed co-expressed over different time points and concentrations, while in Figure 3.18c) it is shown how well the model fits for the Hill-Gauss model actually represent the measured data. The estimated parameters for these nodes for diclofenac and naproxen are summarized in Tables 3.6 and 3.7.

Besides the already mentioned toxnodes 1260 and 1079, another toxnode (#3445) containing genes for three phase II biotransformation enzymes, namely cbr1l (carbonyl reductase), mgst3b (Microsomal glutathione S-transferase) and ugdh (UDP-glucose 6-dehydrogenase) is regulated by both diclofenac and naproxen.

An important toxnode for COX-inhibitors might be toxnode #1019, containing the genes timp2b and clusterin. The enzymatic product of COX, prostaglandin H-2 (PGH(2)) can be converted to prostaglandin E-2 (PGE(2)) by prostaglandin E synthase. Increased abundance of COX-inhibitors has been shown to induce TIMP-1 production in human rheumatoid synovial fibroblast, while PGE(2) dampens this effect (Takahashi et al., 1997). In response to naproxen and diclofenac, we see the toxnode up-regulated This is plausible since with COX-inhibition we expect less of PGE(2)). At the same time, timp2a has been shown to play an important role in ZFE development, and both, too much and too less of the transcripts, has been demonstrated to be harmful (Zhang et al., 2003). This toxnode thus indicates a direct connection between COX-inhibition and transcriptional regulation.

Further toxnodes specifically reacting towards diclofenac and naproxen are #1198 containing the membrane transporter abcc2, which is known to transport especially phase II biotransformation products including conjugated drugs out of cells (Jedlitschky et al., 2006) or toxnode #1200 containing a range of different U5 spliceosomal RNAs.

3.4.7 *Assessing regulation of affected toxnodes in the toxicogenomic universe*

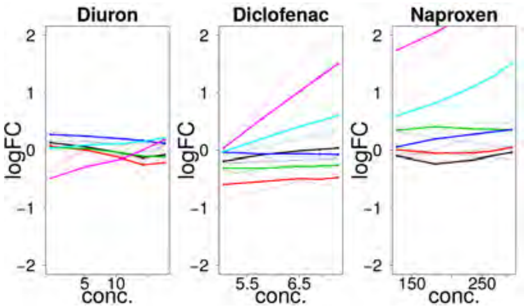
The framework of the toxicogenomic universe allows us to easily assess which of the exposure settings (i.e., compounds) used to infer the toxicogenomic universe significantly affect toxnodes of interest. This can give helpful information for the functional interpretation and compound or MOA specificity of important toxnodes (keeping in mind that most datasets integrated in the universe only contain a very limited range of exposure settings). To exemplify this, we identified substances significantly affecting the toxnodes, which we identified as COX-specific above. The results are summarized in Table 3.8. We see that most toxnodes are not specific for COX-inhibitors alone. Even toxnode #1019, for which we derived a clear hypothesis about the regulation by COX-inhibition is found to be affected by substances unsuspicious of inhibiting COX. However, the combination of nodes seems to be unique for the examined COX-inhibitors, since none of the compounds in Table 3.8 appears to affect all COX toxnodes.

Table 3.6: COX-Toxnodes regulated by Diclofenac. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval. Positive CI-differences indicate upregulation, negative indicate downregulation of respective toxnodes.

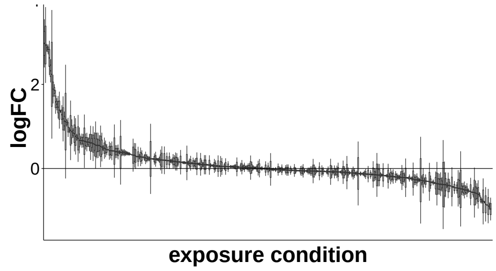
toxnode #	Membergenes	ratio Lethal	ratio Sublethal	$maxS50$	$hillslope$	μ	$sigma$	err	sum(CI-difference)
1260	cyp2k18	2	1.9	0.204 0.282	2.2 9.2	44.8 54.4	0.24 0.47	0.82 1.24	12.1
1079	lepa, pth1a	1.9	1.7	0.192 0.28	1.8 6.9	45 55.2	0.22 0.52	0.69 0.92	5.5
1137	fosab	1.6	1.5	0.164 0.268	1.6 14.7	44.6 57.4	0.21 0.71	0.75 1.13	3
3445	cbr1l, mgst3b, ugdh	0.9	0.9	0.095 0.201	0.4 1.5	57 63.3	0.14 0.37	0.2 0.26	1.7
1198	abcc2, AL929435.1 (antisense RNA)	1.3	1.2	0.11 0.257	0.3 4	45 56.2	0.14 0.63	0.39 0.53	1.5
3447	dhrs13l1, tpmt.2, aifm4	0.9	0.8	0.095 0.19	0.8 2.6	49.7 57.7	0.15 0.48	0.31 0.38	1.1
1138	LOC100000332	1	0.9	0.096 0.145	1.8 8.6	39.5 59.2	0.21 1.38	0.34 0.51	0.8
1019	timp2b, clu	1.2	1.1	0.134 0.167	4.1 10.8	61.2 74.8	0.27 0.84	0.42 0.56	0.8
1200	U5 spliceosomal RNA	1.1	1.1	0.132 0.162	5.5 10.8	61.2 74.7	0.31 0.86	0.39 0.46	0.7
3193	hbegfa	0.9	0.8	0.095 0.123	2.3 5.8	42.8 72.4	0.53 1.65	0.17 0.25	0.5
3552	g0s2, si:ch211-198c19.3	1.3	1.2	0.139 0.182	2.5 9	60.7 74.6	0.28 0.91	0.58 0.76	0.4
1076	atf3, crema, BX005417.1 (antisense RNA)	1.5	1.4	0.161 0.261	1.3 8.7	40.2 55.2	0.17 0.57	0.47 0.59	0.4
3554	GLDC	1	0.9	0.098 0.139	2 9.5	43.7 74.7	0.6 5.3	0.26 0.39	0.3
1968	si:dkey-1c7.3	1.9	1.7	0.197 0.277	3.8 35.1	38.1 50.7	0.17 0.52	0.51 0.75	-3.9
1139	slc25a38a, c7b	1	0.9	0.096 0.161	2.2 8.4	61.7 74.7	0.15 0.57	0.51 0.7	0.2
1919	apoea, si:ch73-263o4.3	1	0.9	0.095 0.256	0.3 1.8	35.5 41.8	0.14 0.36	0.22 0.29	-0.1
360	tmem121a	1	0.9	0.097 0.18	1.1 133.2	6 72.2	0.16 21.13	0.45 0.7	-0.1
3314	btg2, zgc:85866, jun, ier2, dusp1	1.1	1	0.112 0.196	2.2 7	50.6 58.3	0.2 0.71	0.31 0.37	0
3448	gstp1, gsto2, cmbl, sqrdl	0.8	0.8	0.095 0.162	1.1 2.9	60.5 74.7	0.14 0.55	0.27 0.33	0

Table 3.7: COX-Toxnodes regulated by Naproxen. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval. Positive CI-differences indicate upregulation, negative indicate downregulation of respective toxnodes.

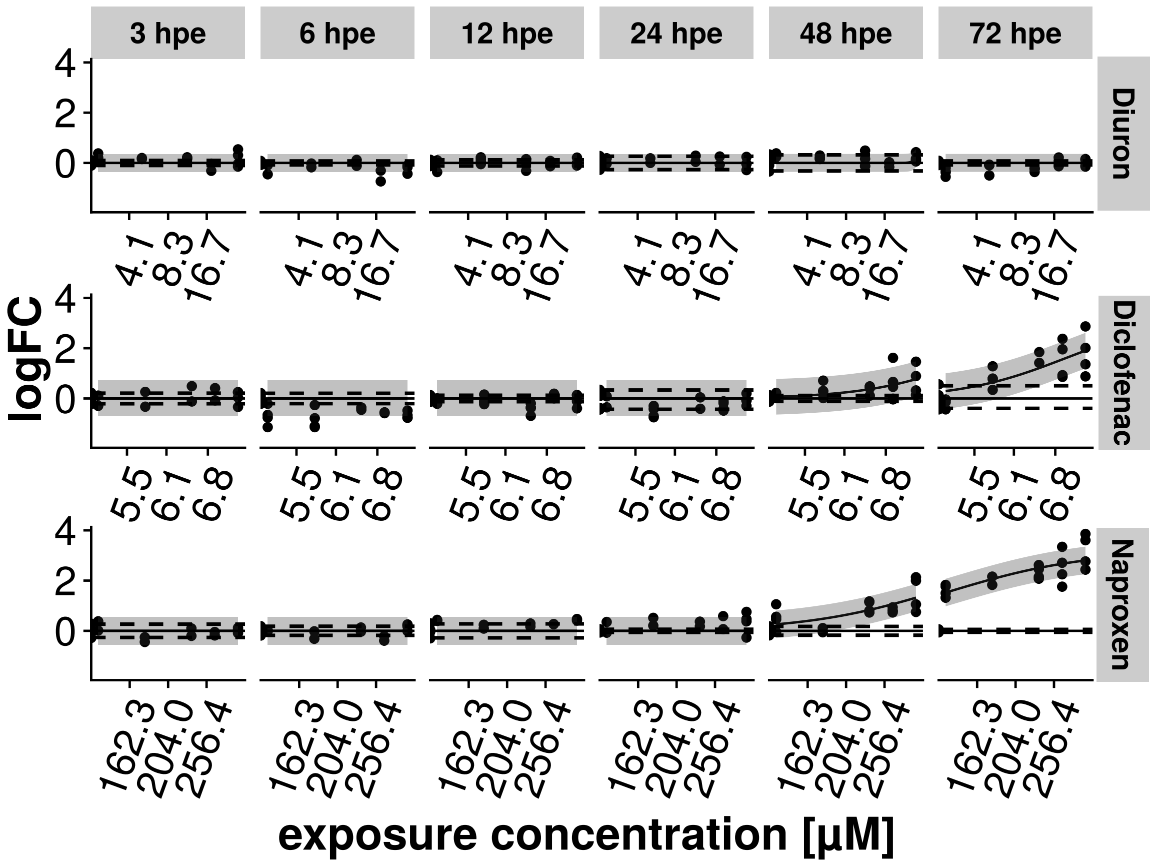
toxnode #	Membergenes	ratio Lethal	ratio Sublethal	$maxS50$	$hillslope$	μ	$sigma$	err	sum(CI-difference)
1260	cyp2k18	5.8	2.8	0.013 0.017	1.6 2.6	62.2 74.4	0.26 0.45	0.29 0.45	34.2
1079	lepa, pth1a	5.5	2.7	0.011 0.017	1.4 3	64.6 74.8	0.17 0.28	0.53 0.71	8.7
1137	fosab	2.2	1.1	0.005 0.011	1.4 5.9	61.6 74.6	0.15 0.44	0.49 0.77	2.8
3445	cbr1l, mgst3b, ugdh	4.6	2.3	0.007 0.017	0.1 0.6	55.8 74.7	0.3 0.64	0.32 0.41	3.9
1198	abcc2, AL929435.1 (antisense RNA)	3.7	1.8	0.007 0.016	0.8 2.7	61.8 74.7	0.17 0.48	0.49 0.66	3.4
3447	dhrs13l1, tpmt.2, aifm4	3.8	1.9	0.005 0.017	0.1 0.8	29.8 45.1	0.21 0.69	0.54 0.69	2.4
1138	LOC100000332	2.1	1	0.005 0.01	1 3.9	62 74.6	0.17 0.43	0.34 0.55	6.6
1019	timp2b, clu	2.7	1.3	0.006 0.012	1.7 3.3	62.2 74.8	0.16 0.34	0.33 0.44	8.7
1200	U5 spliceosomal RNA	2.5	1.2	0.006 0.011	1.7 3	60.3 73.6	0.18 0.5	0.45 0.54	5.4
3193	hbegfa	1.5	0.7	0.003 0.008	0.2 2.8	55.2 74.8	0.24 1.53	0.3 0.48	3.9
3552	g0s2, si:ch211-198c19.3	3.5	1.7	0.007 0.016	0.6 1.9	62.6 74.9	0.14 0.31	0.39 0.53	8
1076	atf3, crema, BX005417.1 (antisense RNA)	4.9	2.4	0.009 0.017	1.6 4.4	62.9 74.8	0.21 0.58	0.39 0.5	5.7
3554	GLDC	2.6	1.3	0.006 0.013	1.4 3.3	59.9 74.6	0.17 0.59	0.2 0.31	7.5
1968	si:dkey-1c7.3	0.6	0.3	0.001 0.003	0.4 2	36.1 58.7	0.14 1.17	0.37 0.59	-0.3
1139	slc25a38a, c7b	2.7	1.3	0.007 0.013	1.8 2.8	61.8 74.8	0.15 0.34	0.33 0.46	16.1
1919	apoea, si:ch73-263o4.3	4	2	0.008 0.017	1 3.3	45.2 55.7	0.17 0.39	0.3 0.41	-2.7
360	tmem121a	0.7	0.3	0.001 0.004	0.3 5.4	5.6 73.6	0.25 21.83	0.66 1.04	-0.1
3314	btg2, zgc:85866, jun, ier2, dusp1	2.2	1.1	0.005 0.008	2.7 4.5	62.9 74.8	0.17 0.33	0.3 0.36	1.4
3448	gstp1, gsto2, cmbl, sqrdl	5	2.5	0.009 0.017	0.5 1.1	57.1 74.7	0.33 0.53	0.44 0.55	6



(a) Single transcript regulation for three model substances diuron (left), diclofenac (middle) and naproxen (right)



(b) Regulation of toxnode across complete toxicogenomic universe



(c) Model fit of Hill-Gauss model

Figure 3.18: Example regulation of potential COX toxnode #1019: timp2b/clusterin. In a) the concentration-response curves for both transcripts are shown in thin lines, thick lines represent the toxnode "code" (i.e., the summary of the toxnode). Colors represent different time points. in b) the regulation of the two member transcripts across all conditions of the toxicogenomic universe are shown as boxplot. In c) the model fit of the Hill-Gauss model is illustrated. Shaded region indicates the 95% confidence interval.

Table 3.8: Substances significantly affecting COX-toxnodes (toxnodes identified to be affected by known COX-inhibitors and not affected by diuron as exemplary "non-COX-inhibitor")

Toxnode	**Substances**
1260	amiodarone, cyanopeptolin, oil emulsion
1079	–
1137	SiNP , retinoic acid, perchloroethylene, isoniazid, 1-naphthylisothiocyanate, adefovir, sertraline, beclomethasone, leflunomide
3445	oil emulsion
1198	amidarone, paraquat
3447	percholorethylene, paraquat
1138	SiNP, retinoic acid, perchloroethylene, isoniazid, flusilazole, valproic acid, G3-polyamidoamine, beclomethasone, trimethyltin chloride
1019	SiNP, isoniazid
1200	–
3193	1-naphthylisothiocyanate, trimethyltin chloride
3552	beclomethasone
1076	SiNP, trimethyltin chloride
3554	perchloroethylene
1139	flusilazole, valproic acid, leflunomide
3314	–
3348	paraquat
360	–
1919	TCDD
1968	trichloroethylene, flusilazole, G3-polyamidoamine, beclomethasone

3.5 DISCUSSION

We suggested an experimental design and analysis framework to determine informative and toxicogenomic profiles of environmental contaminants. The resulting profiles should help in establishing robust molecular fingerprints of defined modes of action (MOAs), applicable to the bioanalytical dissection of environmental extracts. The profiles, exemplarily inferred here, still need to be investigated further with regard to robustness (e.g., inter-laboratory comparisons, comparison with compounds expected to act similarly). Confidence of inferred model parameters, describing node response dynamics, varied between nodes and should be improved especially on the concentration scale by including more concentrations in the experimental design (compare Figures B.3-B.8 in Appendix B). However, for toxnodes showing distinct regulation in response to a substance, model parameters can be well defined. The quality of the Zebrafish Embryo Toxicogenomic Universe (ZETU) is dependent on the quality of the respective input datasets. If more high-quality datasets should become available in future, the robustness of the ZETU might still improve.

3.5.1 *"Self-organization" of toxicogenomic knowledge*

The application of self-organizing maps helps to integrate existing toxicogenomic knowledge in our analysis pipeline. At the same time it reduces the number of entities to be analyzed by (at least) one order of magnitude, without removing any transcripts from further analysis. This is especially important when moving to comparison of several different compounds, and filtering out non-responsive transcripts is not feasible any more. The projection of results on the map opens up the possibility of visual inspection of any transcriptome wide results (e.g., *log*FCs, Figures 3.8-3.10; or parameter estimates, Figure 3.17). Additionally, we suspect that results on toxnode level will be more robust than findings on single gene level, since "exceptional" single gene changes will be dampened by other members of the same toxnode. When applying regression models to the toxnodes, one benefits from more data to estimate parameter values (here we treated the member-transcripts of each toxnode as pseudo-replicates).

Certainly, effects on the transcriptome which are very substance specific (especially if they are more subtle) and would be detected by gene-wise analysis might get lost in the toxicogenomic universe. Thus, to reveal substance specific AOPs, our analysis strategy might not be the first choice. However, with regard to diagnosis of environmental extracts, we can anyhow only expect to be able to detect and interpret robust signals for certain effect categories. Transcripts which show a robust signal on single gene level and are not correlated to other transcripts will end up in single-member toxnodes, as it was the case for cyp2k18 or cyp1a in our example.

Acknowledging the fact that most robust transcriptome effects do not appear in much lower concentrations than apical effects (in our experiments, ratios were not much higher than 2-3 in most cases), one could argue that in a complex mixture one might only be able to detect an unspecific "general" stress response. This could actually be the case for a equitoxic mixture of many compounds with different mode of actions. However, previous findings indicate that in environmental mixtures we would expect a limited number of driver substances, being responsible for the majority of a toxicological effect (Altenburger et al., 2004). In those cases a toxicogenomic study could help in dissecting exactly those important driving MOAs.

3.5.2 *Regression models to describe toxnode behavior*

For a quantitative description and the ability of extrapolation toxnode behavior, we developed and applied two different regression models to the toxicodynamic maps of our three model substances. The Hill-Gauss model can describe the behavior of most toxnodes quite well. The Gauss-Gauss model would be preferred in many instances over the Hill-Gauss model, however, we should prefer working with the Hill-Gauss model whenever possible, since it allows applying classic toxicological assumptions. Anyway, some nodes clearly show a biphasic behavior on the concentration scale, which makes it necessary to apply the Gauss-Gauss model. Hermsen et al. (2012) speculated that such kind of responses indicate a homeostatic "collapse", when the limit of adaption has been reached for the respective pathway. To capture such responses in a more general way, one could also consider integrating a multiphase Hill-model (e.g., Di Veroli et al., 2015), which is easier to interpret but harder to infer. Therefore, more data would be needed on the concentration scale to estimate the parameters of such a model. When interpreting the estimated parameters of each node, one has to be aware of the uncertainty connected to the estimation. We showed that the width of confidence intervals can differ from node to node. Especially, the parameters describing concentration dependence are not well defined in many cases. Therefore, the experimental design should be adjusted to account for more concentrations (one can reduce the amounts of replicates of treatments in exchange). Sometimes, the control levels are not adequately captured by only two replicates. Here, it should be considered to use at least three (if not four) replicates to establish a robust control level. Not all nodes can be adequately represented by our proposed regression models. However, nodes showing a multiphasic behavior (maybe because of complex indirect regulation loops) are hard to interpret biologically, anyway. Since they are neither strongly correlated with the exposure setting nor with apical outcome, these nodes probably represent transient regulatory responses. In the setting of analyzing environmental extracts it is not clear in which state those nodes will be captured. Therefore, they may be neglected at this point.

3.5.3 *Time and concentration dependent transcriptomics*

We could clearly demonstrate here that important toxnodes can show substance specific response dynamics. This can be due to differences in toxicokinetic or toxicodynamic processes. To get to a representative, comparable and extrapolatable toxicogenomic profile of a toxicant, it is therefore essential to capture and understand as far as possible the time and concentration dependence. Otherwise, one is in high risk to derive false conclusions from the resulting profiles. Our findings show that not only concentration dependence (as it has already been shown by Smetanová et al., 2015, for example) but also time dependence is in many cases easily described. Therefore, we should move on from assuming binary gene responses. It cannot be resolved here, if the time sequence of regulated nodes, which we can observe for all substances, is the progression of a toxicodynamic process, or if it represents different effects on different developmental stages of the zebrafish embryo. Therefore, our suggested pipeline might provide extended information when applied on a model, not developing as much as the ZFE during the exposure period (or alternative *in vitro* systems).

3.5.4 *Detection of a mode of action in the ZFE transcriptome*

We strived for exemplarily establishing a toxicogenomic profile for COX-inhibition. Therefore, we selected two model compounds known as COX-inhibitors. The selected COX-inhibitors diclofenac and naproxen show many differences in their effects on the ZFE, on transcriptome as well as on apical effect level. For naproxen sublethal effects occur at clearly lower concentrations than lethal effects (Ratio $\frac{LC_{50}}{EC_{50}}$ between 2.7 (24 hpe) and 2.1 (72 hpe)), while for diclofenac this distance is smaller (Ratio $\frac{LC_{50}}{EC_{50}}$ between 2.0 (24 hpe) and 1.0 (72 hpe)). This corresponds to a much more extensive effect on the transcriptome by naproxen compared to diclofenac in our exposure setting. Additionally, the concentration ratios between lethal effect and transcript effect ($\frac{min(LC_{50})}{min(EC_{50})_{Toxnode}}$) are larger for most toxnodes affected by naproxen compared to diclofenac. Interestingly, the ratios between sublethal effects and transcript effect ($\frac{min(EC_{50})_{apical}}{min(EC_{50})_{Toxnode}}$) are similar for diclofenac and naproxen exposures. We can derive several hypotheses from this: the observed transcript regulation in diclofenac and naproxen mainly corresponds to sublethal effects; most likely COX-inhibition alone cannot explain the lethality induced by diclofenac and naproxen in the ZFE, and there might be additional toxicity mechanisms besides COX-inhibition leading to adverse effects. It is possible that naproxen has a stronger COX-inhibiting effect than diclofenac, and for diclofenac the effect by COX-inhibition may be masked by an onset of baseline toxicity in lower concentrations.

When comparing the estimated time parameters (i.e., μ and *sigma*) of the COX-toxnodes for diclofenac and naproxen it is obvious that they show different dynamics. Most toxnodes show an earlier time point of maximum sensitivity in diclofenac compared to naproxen. This goes in line with the observed dynamics of apical effects (see Figure 3.2), which increase earlier with diclofenac exposure than with naproxen exposure. This indicates a quicker uptake of diclofenac into the embryos (thus, a toxicokinetic effect).

However, we identified commonly regulated toxnodes in the transcriptome profiles, which might represent specific responses towards COX-inhibition. The most distinct regulations were observed for genes of biotransformation enzymes, such as cyp2k18 or ugt1. Additionally, the induction of lepa and pth1a lets us suspect perturbation of the thyroid pathway. By comparing with other studies, we found that most of the common COX toxnodes are also regulated by compounds which are no COX-inhibitors. This could be explained by multiple targets of the COX product PGH(2) or other products of the same pathway such as PGE(2). Therefore, some of the targets can obviously also be affected by other substances via different pathways, and a specific COX-inhibition effect can probably only be detected by considering a combination of toxnodes. Additionally, there might be also rather unspecific effects (compare Chapter 2). For example, the down-regulation of crystallins has been observed as an unspecific effect in ZFEs, possibly a result of developmental delay (Hermsen et al., 2013).

In summary, our experimental design and analysis pipeline has proven to be helpful in deriving informative and comparable toxicogenomic profiles of three model substances. It was possible to identify toxnodes, indicating the effect of COX-inhibitors on zebrafish embryos. Most likely, one isolated node will not be specific enough to define this MOA (since cyp2k18, the thyroid system or timp2b might also be triggered by different mechanisms), but combining the information of the toxnodes might allow to identify the effects of COX-inhibitors also in a mixture. An in-depth analysis of COX-inhibitor specific toxnodes and integration of prior knowledge about the member-genes of the respective nodes allows to gain knowledge about the affected processes in the zebrafish embryo when inhibiting COX. If the affected processes, indicated by regulated toxnodes, go in line with our previous knowledge about COX-inhibition in the same or in other species (i.e., in mammals), this additionally strengthens our assumption that the observed molecular responses indeed directly result from the inhibition of COX (and do not represent unspecific secondary responses). However, it is not essential to know the function of each affected toxnode, before using them as exposure indicators.

A combination of toxnodes indicating a COX-inhibition profile could be applicable for diagnostics of chemical mixtures. However, to be able to detect and interpret such profiles in a mixture, it will be essential to understand how the toxicogenomic profile combine in a mixture. This will be the topic of the following chapter.

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REFERENCES

Altenburger, R., H. Walter, and M. Grote (Dec. 2004). "What Contributes to the Combined Effect of a Complex Mixture?" In: *Environmental Science & Technology* 38.23, pp. 6353–6362. DOI: 10.1021/es049528k.

Andrews, F., B. Croke, and A. Jakeman (Oct. 2011). "An open software environment for hydrological model assessment and development." In: *Environmental Modelling & Software* 26.10, pp. 1171–1185. DOI: 10.1016/j.envsoft.2011.04.006.

Bahamonde, P. A., A. Feswick, M. A. Isaacs, K. R. Munkittrick, and C. J. Martyniuk (2016). "Defining the role of omics in assessing ecosystem health: Perspectives from the Canadian environmental monitoring program." In: *Environmental Toxicology and Chemistry* 35.1, pp. 20–35. DOI: 10.1002/etc.3218.

Ballman, K. V., D. E. Grill, A. L. Oberg, and T. M. Therneau (2004). "Faster cyclic loess: Normalizing RNA arrays via linear models." In: *Bioinformatics* 20.16, pp. 2778–2786. DOI: 10.1093/bioinformatics/bth327.

Bates, D. M. and D. G. Watts (Aug. 1988). *Nonlinear Regression Analysis and Its Applications.* Ed. by D. M. Bates and D. G. Watts. Wiley Series in Probability and Statistics. Hoboken, NJ, USA: John Wiley & Sons, Inc. DOI: 10.1002/9780470316757.

Bayly-Jones, C., D. Bubeck, and M. A. Dunstone (Aug. 2017). "The mystery behind membrane insertion: a review of the complement membrane attack complex." In: *Philosophical Transactions of the Royal Society B: Biological Sciences* 372.1726, p. 20160221. DOI: 10.1098/rstb.2016.0221.

Bolstad, B., R. Irizarry, M. Astrand, and T. Speed (Jan. 2003). “A comparison of normalization methods for high density oligonucleotide array data based on variance and bias.” In: *Bioinformatics* 19.2, pp. 185–193. DOI: 10.1093/bioinformatics/19.2.185.

Bolker, B. and R. D. C. Team (2017). *bbmle: Tools for General Maximum Likelihood Estimation.* R package version 1.0.19.

Brinke, A. and S. Buchinger (2017). “Toxicogenomics in Environmental Science.” In: *In vitro Environmental Toxicology - Concepts, Application and Assessment.* Ed. by G. Reifferscheid and S. Buchinger. Cham: Springer International Publishing, pp. 159–186. DOI: 10.1007/10_2016_15.

Busch, W., S. Schmidt, R. Kühne, T. Schulze, M. Krauss, and R. Altenburger (Aug. 2016). “Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring.” In: *Environmental Toxicology and Chemistry* 35.8, pp. 1887–1899. DOI: 10.1002/etc.3460.

Carpenter, B., A. Gelman, M. D. Hoffman, D. Lee, B. Goodrich, M. Betancourt, M. Brubaker, J. Guo, P. Li, and A. Riddell (2017). “Stan: A Probabilistic Programming Language.” In: *Journal of Statistical Software* 76.1. DOI: 10.18637/jss.v076.i01.

Dean, J. L., Q. Jay Zhao, J. C. Lambert, B. S. Hawkins, R. S. Thomas, and S. C. Wesselkamper (Jan. 2017). “Application of Gene Set Enrichment Analysis for Identification of Chemically-Induced, Biologically Relevant Transcriptomic Networks and Potential Utilization in Human Health Risk Assessment.” In: *Toxicological Sciences* 157.1, pp. 85–99. DOI: 10.1093/toxsci/kfx021.

Di Veroli, G. Y., C. Fornari, I. Goldlust, G. Mills, S. B. Koh, J. L. Bramhall, F. M. Richards, and D. I. Jodrell (2015). “An automated fitting procedure and software for dose-response curves with multiphasic features.” In: *Scientific Reports* 5.1, p. 14701. DOI: 10.1038/srep14701.

Duan, Q. Y., V. K. Gupta, and S. Sorooshian (1993). “Shuffled complex evolution approach for effective and efficient global minimization.” In: *Journal of Optimization Theory and Applications* 76.3, pp. 501–521. DOI: 10.1007/BF00939380.

Dudoit, S., Y. H. Yang, M. J. Callow, and T. P. Speed (2002). “Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments.” In: *Statistica Sinica* 12, pp. 111–139.

Edgar, R., M. Domrachev, and A. E. Lash (Jan. 2002). “Gene Expression Omnibus: NCBI gene expression and hybridization array data repository.” In: *Nucleic Acids Research* 30.1, pp. 207–10. DOI: 10.1093/nar/30.1.207.

Feswick, A., M. Isaacs, A. Biales, R. W. Flick, D. C. Bencic, R.-L. Wang, C. Vulpe, M. Brown-Augustine, A. Loguinov, F. Falciani, et al. (Oct. 2017). “How consistent are we? Interlaboratory comparison study in fathead minnows using the model estrogen 17α-ethinylestradiol to develop recommendations for environmental transcriptomics.” In: *Environmental Toxicology and Chemistry* 36.10, pp. 2614–2623. DOI: 10.1002/etc.3799.

Flier, J. S., M. Harris, and A. N. Hollenberg (2000). “Leptin, nutrition, and the thyroid: The why, the wherefore, and the wiring.” In: *Journal of Clinical Investigation* 105.7, pp. 859–861. DOI: 10.1172/JCI9725.

Goutelle, S., M. Maurin, F. Rougier, X. Barbaut, L. Bourguignon, M. Ducher, and P. Maire (2008). “The Hill equation: A review of its capabilities in pharmacological modelling.” In: *Fundamental and Clinical Pharmacology* 22.6, pp. 633–648. DOI: 10.1111/j.1472-8206.2008.00633.x.

Grubbs, F. E. (1950). “Sample criteria for testing outlying observations.” In: *The Annals of Mathematical Statistics* 21.1, pp. 27–58. DOI: 10.1214/aoms/1177729885.

Hermsen, S. a. B., T. E. Pronk, E.-J. van den Brandhof, L. T. M. van der Ven, and A. H. Piersma (2012). “Triazole-induced gene expression changes in the zebrafish embryo.” In: *Reproductive toxicology (Elmsford, N.Y.)* 34.2, pp. 216–24. DOI: 10.1016/j.reprotox.2012.05.093.

Hermsen, S. A. B., T. E. Pronk, E.-J. van den Brandhof, L. T. M. van der Ven, and A. H. Piersma (Oct. 2013). “Transcriptomic analysis in the developing zebrafish embryo after compound exposure: individual gene expression and pathway regulation.” In: *Toxicology and applied pharmacology* 272.1, pp. 161–71. DOI: 10.1016/j.taap.2013.05.037.

Hill, A. V. (1910). “The possible effects of the aggregation of the molecule of hemoglobin on its dissociation curves.” In: *Journal of Physiology* 40, pp. iv–vii. DOI: 10.1017/CBO9781107415324.004.

Jedlitschky, G., U. Hoffmann, and H. K. Kroemer (2006). “Structure and function of the MRP2 (ABCC2) protein and its role in drug disposition.” In: *Expert Opinion on Drug Metabolism & Toxicology* 2.3, pp. 351–366. DOI: 10.1517/17425255.2.3.351.

Jin, D., T. T. Ni, J. Sun, H. Wan, J. D. Amack, G. Yu, J. Fleming, C. Chiang, W. Li, A. Papierniak, et al. (Sept. 2014). “Prostaglandin signalling regulates ciliogenesis by modulating intraflagellar transport.” In: *Nature Cell Biology* 16.9, pp. 841–851. DOI: 10.1038/ncb3029.

Klüver, N., C. Vogs, R. Altenburger, B. I. Escher, and S. Scholz (2016). “Development of a general baseline toxicity QSAR model for the fish embryo acute toxicity test.” In: *Chemosphere* 164, pp. 164–173. DOI: 10.1016/j.chemosphere.2016.08.079.

Kohonen, T. (1982). “Self-organized formation of topologically correct feature maps.” In: *Biological Cybernetics* 43.1, pp. 59–69. DOI: 10.1007/BF00337288.

Leek, J. T., W. E. Johnson, H. S. Parker, A. E. Jaffe, and J. D. Storey (Mar. 2012). “The sva package for removing batch effects and other unwanted variation in high-throughput experiments.” In: *Bioinformatics* 28.6, pp. 882–883. DOI: 10.1093/bioinformatics/bts034.

Leuthold, D. (in preparation). “Optimierung und Erweiterung des akuten Fischembryotoxizitätstests für das Umweltmonitoring.” PhD thesis.

Li, Q., P. Wang, L. Chen, H. Gao, and L. Wu (2016). “Acute toxicity and histopathological effects of naproxen in zebrafish (Danio rerio) early life stages.” In: *Environmental Science and Pollution Research* 23.18, pp. 18832–18841. DOI: 10.1007/s11356-016-7092-4.

Lonappan, L., S. K. Brar, R. K. Das, M. Verma, and R. Y. Surampalli (2016). “Diclofenac and its transformation products: Environmental occurrence and toxicity - A review.” In: *Environment International* 96, pp. 127–138. DOI: 10.1016/j.envint.2016.09.014.

Nelder, J. A. and R. Mead (1965). “A Simplex Method for Function Minimization.” In: *The Computer Journal* 7.4, pp. 308–313. DOI: 10.1093/comjnl/7.4.308.

Nuwaysir, E. F., M. Bittner, J. Trent, J. C. Barrett, and C. A. Afshari (Mar. 1999). “Microarrays and toxicology: The advent of toxicogenomics.” In: *Molecular Carcinogenesis* 24.3, pp. 153–159. DOI: 10.1002/(SICI)1098-2744(199903)24:3<153::AID-MC1>3.0.CO;2-P.

Oaks, J. L., M. Gilbert, M. Z. Virani, R. T. Watson, C. U. Meteyer, B. A. Rideout, H. L. Shivaprasad, S. Ahmed, M. J. Iqbal Chaudhry, M. Arshad, et al. (2004). “Diclofenac residues as the cause of vulture population decline in Pakistan.” In: *Nature* 427.6975, pp. 630–633. DOI: 10.1038/nature02317.

OECD (July 2013). *Test No. 236: Fish Embryo Acute Toxicity (FET) Test.* OECD Guidelines for the Testing of Chemicals, Section 2 July. OECD Publishing, pp. 1–22. DOI: 10.1787/9789264203709-en.

Perkins, E. J., J. K. Chipman, S. Edwards, T. Habib, F. Falciani, R. Taylor, G. Van Aggelen, C. Vulpe, P. Antczak, and A. Loguinov (Jan. 2011). “Reverse engineering adverse outcome pathways.” In: *Environmental Toxicology and Chemistry* 30.1, pp. 22–38. DOI: 10.1002/etc.374.

Perkins, E. J., T. Habib, B. L. Escalon, J. E. Cavallin, L. Thomas, M. Weberg, M. N. Hughes, K. M. Jensen, M. D. Kahl, D. L. Villeneuve, et al. (Aug. 2017). “Prioritization of Contaminants of Emerging Concern in Wastewater Treatment Plant Discharges Using Chemical:Gene Interactions in Caged Fish.” In: *Environmental Science & Technology* 51.15, pp. 8701–8712. DOI: 10.1021/acs.est.7b01567.

R Core Team (2016). *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing. Vienna, Austria.

Ricciotti, E. and G. A. FitzGerald (May 2011). "Prostaglandins and Inflammation." In: *Arteriosclerosis, Thrombosis, and Vascular Biology* 31.5, pp. 986–1000. DOI: 10.1161/ATVBAHA.110.207449.

Ritchie, M. E., B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi, and G. K. Smyth (Apr. 2015). "limma powers differential expression analyses for RNA-sequencing and microarray studies." In: *Nucleic Acids Research* 43.7, e47–e47. DOI: 10.1093/nar/gkv007.

Scholze, M., W. Boedeker, M. Faust, T. Backhaus, R. Altenburger, and L. H. Grimme (2001). "A general best-fit method for concentration-response curves and the estimation of low-effect concentrations." In: *Environmental Toxicology and Chemistry* 20.2, pp. 448–457. DOI: 10.1897/1551-5028(2001)020<0448:AGBFMF>2.0.CO;2.

Schiller, V., A. Wichmann, R. Kriehuber, C. Schäfers, R. Fischer, and M. Fenske (Dec. 2013). "Transcriptome alterations in zebrafish embryos after exposure to environmental estrogens and anti-androgens can reveal endocrine disruption." In: *Reproductive Toxicology* 42, pp. 210–223. DOI: 10.1016/j.reprotox.2013.09.003.

Schroeder, A. L., D. Martinović-Weigelt, G. T. Ankley, K. E. Lee, N. Garcia-Reyero, E. J. Perkins, H. L. Schoenfuss, and D. L. Villeneuve (Feb. 2017). "Prior knowledge-based approach for associating contaminants with biological effects: A case study in the St. Croix River basin, MN, WI, USA." In: *Environmental Pollution* 221, pp. 427–436. DOI: 10.1016/j.envpol.2016.12.005.

Smetanová, S., J. Riedl, D. Zitzkat, R. Altenburger, and W. Busch (Sept. 2015). "High-throughput concentration-response analysis for omics datasets." In: *Environmental Toxicology and Chemistry* 34.9, pp. 2167–2180. DOI: 10.1002/etc.3025.

Snape, J. R., S. J. Maund, D. B. Pickford, and T. H. Hutchinson (Apr. 2004). "Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology." In: *Aquatic Toxicology* 67.2, pp. 143–154. DOI: 10.1016/j.aquatox.2003.11.011.

Spiess, A.-N. and N. Neumeyer (2010). "An evaluation of R2 as an inadequate measure for nonlinear models in pharmacological and biochemical research: a Monte Carlo approach." In: *BMC Pharmacology* 10.1, p. 6. DOI: 10.1186/1471-2210-10-6.

Takahashi, S., T. Inoue, M. Higaki, and Y. Mizushima (1997). "Cyclooxygenase inhibitors enhance the production of tissue inhibitor-1 of metalloproteinases (TIMP-1) and pro-matrix metalloproteinase 1 (proMMP-1) in human rheumatoid synovial fibroblasts." In: *Inflammation Research* 46.8, pp. 320–323. DOI: 10.1007/s000110050194.

Tamayo, P., D. Slonim, J. Mesirov, Q. Zhu, S. Kitareewan, E. Dmitrovsky, E. S. Lander, and T. R. Golub (1999). "Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation." In: *Proceedings of the National Academy of Sciences of the United States of America* 96.6, pp. 2907–12. DOI: 10.1073/PNAS.96.6.2907.

Thomas, R. S., B. C. Allen, A. Nong, L. Yang, E. Bermudez, H. J. Clewell, and M. E. Andersen (July 2007). "A Method to Integrate Benchmark Dose Estimates with Genomic Data to Assess the Functional Effects of Chemical Exposure." In: *Toxicological Sciences* 98.1, pp. 240–248. DOI: 10.1093/toxsci/kfm092.

Tixier, C., H. P. Singer, S. Oellers, and S. R. Müller (Mar. 2003). "Occurrence and Fate of Carbamazepine, Clofibric Acid, Diclofenac, Ibuprofen, Ketoprofen, and Naproxen in Surface Waters." In: *Environmental Science & Technology* 37.6, pp. 1061–1068. DOI: 10.1021/es025834r.

Törönen, P., M. Kolehmainen, G. Wong, and E. Castrén (May 1999). "Analysis of gene expression data using self-organizing maps." In: *FEBS Letters* 451.2, pp. 142–146. DOI: 10.1016/S0014-5793(99)00524-4.

Velki, M., C. Di Paolo, J. Nelles, T. B. Seiler, and H. Hollert (2017). "Diuron and diazinon alter the behavior of zebrafish embryos and larvae in the absence of acute toxicity." In: *Chemosphere* 180, pp. 65–76. DOI: 10.1016/j.chemosphere.2017.04.017.

Verenitch, S. S., C. J. Lowe, and A. Mazumder (2006). "Determination of acidic drugs and caffeine in municipal wastewaters and receiving waters by gas chromatography-ion trap tandem mass spectrometry." In: *Journal of Chromatography A* 1116.1-2, pp. 193–203. DOI: 10.1016/j.chroma.2006.03.005.

Wagner, J. (Aug. 1968). "Kinetics of pharmacologic response I. Proposed relationships between response and drug concentration in the intact animal and man." In: *Journal of Theoretical Biology* 20.2, pp. 173–201. DOI: 10.1016/0022-5193(68)90188-4.

Wehrens, R. and L. M. C. Buydens (2007). "Self- and super-organizing maps in R: The kohonen package." In: *Journal of Statistical Software* 21.5, pp. 1–19. DOI: 10.18637/jss.v021.i05.

Wirth, H., M. Löffler, M. Von Bergen, and H. Binder (2011). "Expression cartography of human tissues using self organizing maps." In: *BMC Bioinformatics* 12, p. 306. DOI: 10.1186/1471-2105-12-306.

Wirth, H., M. von Bergen, and H. Binder (2012). "Mining SOM expression portraits: feature selection and integrating concepts of molecular function." In: *BioData Mining* 5.1, p. 18. DOI: 10.1186/1756-0381-5-18.

Woo, J. H., Y. Shimoni, W. S. Yang, P. Subramaniam, A. Iyer, P. Nicoletti, M. Rodríguez Martínez, G. López, M. Mattioli, R. Realubit, et al. (July 2015). "Elucidating Compound Mechanism of Action by Network Perturbation Analysis." In: *Cell* 162.2, pp. 441–451. DOI: 10.1016/j.cell.2015.05.056.

Wood, S. N. (2003). "Thin plate regression splines Duchon spline." In: *Journal of the Royal Statistical Society B* 65.1, pp. 95–114. DOI: 10.1111/1467-9868.00374.

Yang, H., Y. Zhou, J. Gu, S. Xie, Y. Xu, G. Zhu, L. Wang, J. Huang, H. Ma, and J. Yao (2013). "Deep mRNA Sequencing Analysis to Capture the Transcriptome Landscape of Zebrafish Embryos and Larvae." In: *PLoS ONE* 8.5. DOI: 10.1371/journal.pone.0064058.

Yates, A., W. Akanni, M. R. Amode, D. Barrell, K. Billis, D. Carvalho-Silva, C. Cummins, P. Clapham, S. Fitzgerald, L. Gil, et al. (2016). "Ensembl 2016." In: *Nucleic Acids Research* 44.D1, pp. D710–D716. DOI: 10.1093/nar/gkv1157.

Zhang, J., S. Bai, C. Tanase, H. Nagase, and M. P. Sarras (2003). "The expression of tissue inhibitor of metalloproteinase 2 (TIMP-2) is required for normal development of zebrafish embryos." In: *Development Genes and Evolution* 213.8, pp. 382–389. DOI: 10.1007/s00427-003-0333-9.

4 TOXICOGENOMIC MIXTURE EFFECTS

4.1 INTRODUCTION

"What I cannot create, I do not understand"
— R. Feynman

Toxicogenomic methods have been shown to bear the potential for elucidating molecular effects of single substances. In the previous chapter it was exemplarily shown how comprehensive molecular toxicodynamic profiles of chemicals can be inferred from time- and concentration resolved transcriptome data. Applying an analysis pipeline integrating self-organizing maps (SOMs) and regression modeling, *toxicogenomic landscapes* (i.e, toxicogenomic profiles of compound or exposure conditions, projected on a universal coordinate system, compare Chapter 3) for three model substances could be retrieved. Subsequently, characteristic patterns for the MOA of cyclooxygenase (COX) inhibition could be derived from the landscapes. It is conceivable that in future, toxicogenomic landscapes of environmental extracts could be retrieved and scanned for such MOA specific patterns. In this way, toxicogenomic methods could serve as non-target bioanalytical tools to diagnose and categorize dominating biological effects of environmental extracts (compare Section 1.3).

4.1.1 *Application of toxicogenomics in environmental monitoring*

The potential for applying toxicogenomic methods in environmental monitoring has been discussed (e.g., Bahamonde et al., 2016) and evaluated in a few case studies: One approach is the comparison of toxicogenomic profiles induced by environmental extracts or conditions (i.e., in caged individuals) to those induced by selected reference compounds. Bluhm et al. (2014), for example, analyzed gene regulations in ZFEs caused by exposure against sediment extracts and compared these to effects of selected single compound exposures. However, gene regulation induced by the sediment extracts showed large differences compared to the single compound exposures and the authors summarized that "in addition to differences in time of exposure and concentrations applied, additive, synergistic as well as potential antagonistic effects might impede a direct comparison". They concluded that "profiles of gene expression levels of these complex mixtures will hardly correlate with profiles of single substances and therefore, contaminants can hardly be detected based on a comparison of expression level profiles". A more advanced approach was suggested by Schroeder et al. (2016), who combined the *prediction* of generic biological effects on molecular level, based on chemical analytics, with the *measurement* of biological effects, based on the exposure of cell-based bioassays to environmental extracts or conditions. Here, an expectation about regulated genes or pathways is formulated based on a list of chemicals detected at a specific site and from literature-curated chemical-gene interactions (such as the Comparitive Toxicogenomics Database (CTD), Davis et al., 2017). These expectations can subsequently be compared to measured gene regulation induced in caged individuals or individuals exposed to environmental extracts. Thereby, major biological effects and candidate substances potentially contributing to the effects can be identified. This approach was followed by Perkins et al. (2017) and Schroeder et al. (2017), for example. They could demonstrate the potentially added value for environmental monitoring by providing hypotheses about biological effects on selected organisms and linking these effects to priority substances detected at a specific site.

However, concentration- or time-dependence of toxicogenomic effects has not been considered in such studies of environmental samples since concentration dependent data of single component responses is rarely available. This also implies that combination effects of effective compounds could not be taken into account so far. However, in the environment we usually face a complex mixture of compounds. For apical endpoints it has been shown that effects of environmental mixtures are commonly induced by more than one compound (i.e., combination effects occur). Furthermore, the proportion of individual substances contributing to the total effect may differ depending on the respective effect level (Altenburger et al., 2004, submitted). The occurrence of concentration dependent effects as well as combination effects might therefore explain some of the unexpected effects by the environmental sample, reported by Bluhm et al. (2014), for example. Also in the approach by (Schroeder et al., 2016) the neglect of combination effects may lead to false conclusions.

4.1.2 *Mixture toxicology*

When following an integrated approach of chemical and biological effect analytics, as suggested by Schroeder et al. (2016), signals not expected from analytically detected compounds might hint at the presence of not detected compounds. However, those signals might also result from combination effects of multiple components (for definition of mixture terminology see terminology box 4.1). Therefore, if we want to interpret toxicogenomic landscapes of mixtures in the light of our existing knowledge about single compound landscapes, we should get an idea about how such single substance landscapes or characteristic MOA patterns (like the ones exemplarily established for COX-inhibition in Chapter 3) combine in mixtures (Altenburger et al., 2012). Otherwise it will be virtually impossible to distinguish "unexpected" signals, resulting from components not detected analytically or from unknown synergistic effects, from technically predictable combination effects.

Therefore, several questions should be raised in this context: Are the same toxnodes (in the context of the previously established ZETU, compare Chapter 3) affected by the mixture as by the single compounds or is regulation suppressed for some of the nodes? Do unexpected toxnodes get regulated by the mixture? Is it possible to recover an established MOA (such as COX-inhibition) in the toxicogenomic landscape of the mixture? Are there combination effects occurring (either qualitative combination effects, i.e., toxnodes affected in the mixture not found to be affected by one of the components, or quantitative, i.e., toxnodes

Terminology Box 4.1: Mixture terminology

SINGLE COMPOUND EFFECT Effect of the exposure to a single compound on the toxicogenomic landscape or single toxnodes.

MIXTURE EFFECT Effect of the exposure to a mixture of two or more active or inert compounds on toxicogenomic landscape or single toxnodes. May be equivalent to single compound effect if only one component of the mixture is active. With this, the term *mixture effect* is used differently here, than in some other parts of mixture toxicology literature (e.g., Kortenkamp et al. (2009) use the term mixture toxicity synonymously with combined effect).

COMBINED EFFECT Mixture effect resulting from the joined action of two or more compounds in a mixture. In the context of toxicogenomic landscapes there may be *a) qualitative combined effects*, i.e., more toxnodes are affected than would be expected by the most potent single compound exposure; or *b) quantitative combined effects*, i.e., single toxnodes are affected stronger (respectively more sensitive) than would be expected by the most potent single compound exposure.

are affected stronger than expected from the most potent component)? And in case of combination effects occurring, are they predictable?

These kind of questions have been discussed in the field of (eco)-toxicology for decades (Altenburger et al., 2013), and there are well established concepts which have been shown to reliably predict mixture effects of several contaminants (reviewed in, e.g. Cedergreen et al., 2013). The most commonly applied concepts are concentration addition (CA) and independent action (IA). While the concept of CA has been shown to be the most accurate prediction in the case of similar acting compounds (e.g., Altenburger et al., 2000; Altenburger et al., 2004; Silva et al., 2002), IA serves as the most accurate prediction of mixture toxicity in the case of dissimilar acting compounds (Backhaus et al., 2000; Faust et al., 2003) (more details on the mentioned concepts in Section 4.2).

However, in spite of many studies about toxicogenomic mixture effects, the mentioned concepts have almost exclusively been discussed regarding apical endpoints, and there is little knowledge about combination effects and the applicability of CA and IA on transcriptome level (Altenburger et al., 2012). Despite the lack of mechanistic pharmacological support, the two concepts usually (and often implicitly) applied in toxicogenomic studies are effect addition (EA) (e.g., Yang et al., 2007, assuming that effects of single components linearly add up) or the assumption of a boolean mixture (e.g., Schroeder et al., 2017, assuming those genes which have been observed to be regulated in response to any of a mixtures components will be regulated in a mixture exposure). The reasons for applying EA or boolean mixture assumptions instead of CA or IA might be primarily due to lacking concentration response information. First indications of the applicability of pharmacologically based mixture concepts (i.e., CA or IA) are given by De Coninck et al. (2014), who investigated the effects of binary mixtures on the transcriptome of *Daphnia pulex* and could identify pathways for which interactions between the different stressors could be suspected based on the IA assumption. Recently, Labib et al. (2017) analyzed the transcriptome of mice lung tissues exposed to eight different PAHs and their mixtures in three doses. For six cancer related pathways the mixture concepts of IA, CA and generalized concentration addition (GCA) were evaluated, while IA was predicting pathway perturbations most accurately. While these studies provide a good starting point, they do not explicitly evaluate combination effects and rely on very limited concentration information (one concentration in the study of De Coninck et al. (2014) and three in the study of Labib et al. (2017)).

Therefore, the goal of this study was to systematically evaluate how toxicogenomic profiles combine in a mixture as well as to evaluate the predictability of mixture and combined effects. To achieve this, the toxicogenomic effects of a mixture consisting of the three compounds diuron, diclofenac and naproxen were measured. Of those compounds the individual toxicogenomic landscapes had been thoroughly characterized before (compare Chapter 3). We evaluated, if combination effects occur on transcriptome level and if the mentioned pharmacologically based mixture concepts (CA, IA) are suited to describe the combination effects. Additionally, we used the concepts to predict a complete time and concentration resolved toxicogenomic landscape of the mixture. Last but not least, we evaluated if landscape patterns from the single substances could be recovered in the mixture landscape. The mixture predictions were framed within the ZETU, which was inferred in Chapter 3. This reduced the number of mixture predictions by one order of magnitude, gave a better data foundation for prediction and provided an opportunity for visually inspecting overall qualitative validity of the mixture predictions.

4.2 METHODS

A mixture of the three compounds diuron, diclofenac and naproxen was designed in such a way that distinct combination effects could be expected. A diagonal mixture design was chosen (Berenbaum, 1981), i.e., a mixture of constant ratio was applied in several dilutions. Experimental design and data analysis for the exposure were the same as for single substance profiling (described in Chapter 3), with exposure concentrations ranging from $LC_{0.5}$ to LC_{25} of the mixture, ZFEs being exposed from 24 hpf to 27, 30, 36, 48, 72, and 96 hpf, and changes in transcriptome measured at the end of exposure. A concentration response curve for lethality including the selected exposure concentrations is shown in Figure C.1 in Appendix C. The time and concentration resolved toxicogenomic landscape of the mixture was evaluated for combination effects. Next, several mixture concepts were evaluated for their qualitative and quantitative predictivity of the combination effects and of the whole mixture landscape. The mixture concepts evaluated were boolean mixture (BO), effect addition (EA), concentration addition (CA) and independent action (IA). Partly, the concepts had to be adapted to be applicable to toxicogenomic data, as it is described below. All data analyses were performed using the software R (version 3.3.1, R Core Team, 2016). The data discussed in this chapter have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE109498 (`https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109498`). All scripts used for analyses and figures in this chapter have been deposited in GitHub and are accessible in the GitHub repository *ZFEmixtomics* (`https://github.com/anschue/ZFEmixtomics`).

4.2.1 *Boolean mixtures*

In many studies concentration dependence of chemical effects on molecular level has not been investigated. This is the case for most toxicogenomic profiles established so far (compare Chapter 2) and is also reflected in databases like the CTD (Davis et al., 2017). In this case, assumptions about mixture effects can only be formulated in a boolean way, i.e, all transcripts affected by one of the substances in the mixture are expected to be affected by the mixture also. A boolean approach is followed by Schroeder et al. (2017), for example (compare Section 4.1.1). The concept can be summarized by Equation 4.1:

$$E_{mix} = E_i||E_{i+1}||E_{i+2}||...||E_n, \tag{4.1}$$

where E_{mix} is a boolean variable (i.e., can be either *true* or *false*) describing if a gene or toxnode is affected by the mixture exposure or not; E_n is a boolean variable describing if a gene or toxnode if affected by the nth mixture component (irrespective of exposure conditions).

4.2.2 *Effect addition in toxicogenomics*

The concept of effect addition (EA) is the implicit assumption in many toxicogenomic mixture studies, when the components in the analyzed mixture are applied in the same (single) concentrations as they were applied separately (compare Yang et al., 2007, for example). The assumption behind this concept is that the effect of a mixture at a given concentration is the sum of effects of the components at this concentration. No concentration response modeling is needed to formulate mixture hypotheses. It can be summarized by Equation 4.2:

$$E_{mix}(c_{mix}) = \sum_{i=1}^{n}(E_i(c_{mix} * p_i)), \tag{4.2}$$

where $E_{mix}(c)$ is the effect of the mixture at a given mixture concentration, $E_i(c)$ is the effect of compound i at a given concentration, p_i is the proportion of compound i in the mixture, and c_{mix} is the concentration of the mixture.

4.2.3 *Concentration addition in toxicogenomics*

In the previous chapter it was shown that the concentration dependence of toxicogenomic responses can be described by simple regression models. One of the two regression models, which were developed here, is the Hill-Gauss-model, which allows the concept of concentration addition (CA) to be easily applied to toxicogenomic effects. The effect concentrations of the mixture can be iteratively calculated for each toxnode using Equation 4.3:

$$EC_{X^{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{X^i}}\right)^{-1}, \tag{4.3}$$

where $EC_{X^{mix}}$ is the effect concentration of the mixture leading to X% effect, EC_{X^i} is the effect concentration of compound i leading to X% effect, and p_i is the proportion of compound i in the mixture. A few challenges exist, when applying the concept of CA for toxicogenomic response modeling: In contrast to lethal effects, toxicogenomic effects can be stimulatory or inhibiting compared to the reference. This means that in some cases genes or toxnodes (compare Chapter 3) can be stimulated by one compound and inhibited by another. In these cases, however, one would not expect similar action anyway and rather refer to the model of independent action (see below). Here, to be able to make a CA prediction for every toxnode anyway, the direction of regulation of the majority of compounds (i.e., two compounds) was assumed as the direction of regulation induced by the mixture. For all three substances the estimated parameters for the selected direction of regulation was taken for mixture prediction.

Another hurdle to overcome is that both mixture concepts, CA and IA, assume a common maximum effect for all mixture components. For lethality this is assumed to be 0% survivors (respectively 100% dead), but for transcript abundance the maximum is not trivial to capture or estimate. It might be gene or toxnode specific and depend on the dynamic range in abundance of the respective transcript (which we usually do not know). This problem was already tackled in Chapter 3 and the maximum measured effect across all conditions for each toxnode was considered as a proxy for the maximum effect of the respective toxnode.

CONCENTRATION ADDITION FOR BIPHASIC CONCENTRATION RESPONSE RELATIONSHIPS In Chapter 3 it was shown that the concentration dependence of some toxnodes seems to be biphasic. Here, we focus on monotone concentration response relationships. However, it was also evaluated whether the consideration of biphasic responses can explain deviations from mixture predictions with the help of an extended concentration addition concept, which was suggested by Martin-Betancor et al. (2015). Details are given in Appendix C.

4.2.4 *Independent action for toxicogenomics*

The concept of CA only helps to predict combined effects for toxnodes regulated in one direction. Similarly, the concept of independent action (IA) is only used to describe proportional effects into one direction, until now. However, since the concept is based on the notion that compounds act independent from each other, it bears the potential to be extended to cases of diverging effect directions.

We translated the concept of IA to the application on toxicogenomic responses by transforming the absolute *log*FCs into relative *log*FCs (compare Equation 4.4). From those, a combined relative *log*FC was retrieved and subsequently transformed back into an absolute *log*FC. We considered up- and down-regulation separately at first, summing up the absolute *log*FCs for up- and down-regulation in the end. This implies that the combined effect of up- and down-regulation is weighted by the respective maximum effects. In a binary mixture of two compounds which are exactly complementary (one inducing a toxnode, one inhibiting it) the net effect will be zero if the maximum effects in both directions are the same; In a binary mixture with maximum positive *log*FC of 5 and maximum negative *log*FC of -2, the combined effect of high effect concentration will be 3 (however it is possible that the *log*FC rises to 5 temporarily in an equieffective mixture ratio). The adapted IA model is represented by Equation 4.4:

$$\begin{aligned} E(c_{mix}) = &\left[1 - \prod_{i=0}^{n_{up}} \left(1 - \frac{logFC(c_{iup})}{maxlogFC_{up}}\right)\right] * maxlogFC_{up} \\ &+ \left[1 - \prod_{i=0}^{n_{down}} \left(1 - \frac{logFC(c_{idown})}{maxlogFC_{down}}\right)\right] * maxlogFC_{down}, \end{aligned} \tag{4.4}$$

where $E(c_{mix})$ is the mixture effect at a given mixture concentration, $logFC(c_i)$ is the *log*FC induced by compound i, $maxlogFC_{up}$ is the

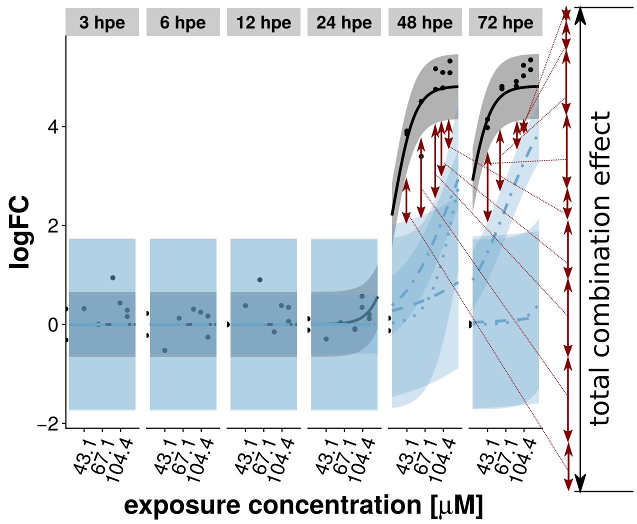


Figure 4.1: Demonstration of combination effect identification. Effect and 95% CI of single substance (■); Effect and 95% CI of mixture (■). Arrows illustrate the quantification of a quantitative combination effect.

maximum up-regulation *log*FC across all conditions and $maxlogFC_{down}$ is the maximum down-regulation *log*FC across all conditions.

4.2.5 *Confidence interval for predictions*

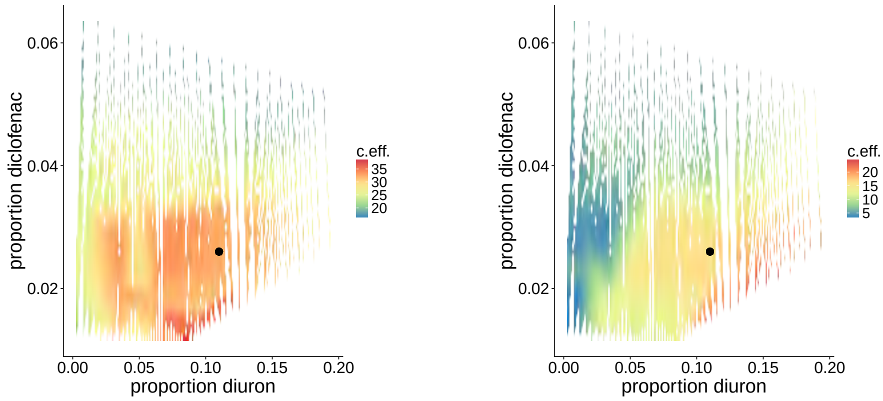
The mixture predictions for each toxnode are based on fitted regression models of different certainty. To account for prediction errors of single compound models, bootstrapping was applied to estimate a confidence interval for each mixture prediction. In short, the error distribution (compare Equation 3.5) was randomly sampled for each compound, using a substance specific distribution given by the fitted standard deviation. With this, a mixture effect was predicted and the process was repeated 100 times. Afterwards, the Hill-Gauss model described in Chapter 3 (respectively the Gauss-Gauss model) was fitted to the bootstrapped data points of predicted mixture effect. This gave a measure of uncertainty for mixture prediction but also allowed to qualitatively predict which toxnodes will be affected by the mixture by comparing the retrieved 95%CI with the respective 2.5%/97.5% quantile of controls (complementary to the approach of selecting significantly affected toxnodes for single substances).

IDENTIFICATION OF COMBINATION EFFECTS In order to determine which of the toxnodes show combination effects in response to the mixture, the 95%CI of the most effective component of each toxnode was compared with the 95%CI of the measured (and modeled) mixture effect (compare figure). The gap sizes between the CIs at each measured time point/concentration were summed up. A significant combination effect was assessed to occur, if the sum of gaps was greater than zero.

4.2.6 *Mixture design*

To experimentally test the validity of the described mixture concepts, the mixture composition for an experiment has to be carefully chosen in order to induce combination effects. If the proportion of one compound in the mixture is too high, measurable effects will only be attributable to this compound, no combination effect will be visible, and therefore the validity of the mixture concepts cannot be evaluated. This is especially important for mixtures with only few components. For toxicogenomic effects the task of optimizing the mixture proportions is even more challenging, since thousands of endpoints (genes/toxnodes) are measured at the same time, and single components show differing sensitivities for each endpoint. This means that a mixture proportion optimized to show a strong combination effect for one toxnode might result in weak or no combination effects for other toxnodes. Therefore, for our purpose of studying the predictivity of the mixture concepts, the mixture composition had to be optimized to yield a maximum average distance between predicted mixture effect and the most effective single compound (i.e., a strong combination effect) across all or a range of selected toxnodes. Additionally, the combination effect should occur at sublethal concentrations. For this reason, also the hypothetical exposure concentrations of the mixture had to be taken into account, when optimizing the mixture composition.

To find a mixture proportion accounting for all mentioned requirements, we determined the expected combination effects for a range of different mixture compositions. A grid of different mixture proportions was assembled, roughly aligned along the $LC_{0.5}$ and LC_{25} of each substance to make sure that each substance would be present in the mixture in an effective proportion. For the different mixture compositions, CA was used to predict lethality and derive a hypothetical exposure range for transcriptome measurements using the approach described in Section 3.2.2. Then, the mixture effect was predicted in this concentration range for the regulation of each toxnode, applying the concepts of CA and IA. From this, the difference of the area under the curve of CA/IA prediction and the respective most effective individual components was derived for each toxnode. The average difference for all toxnodes or for selected toxnodes then gave an indication of how distinct a combination effect would be measurable. In Figure 4.2 the expected average combination effects for all toxnodes significantly regulated in at least two of the substances for different mixture proportions, assuming CA or IA, are shown. As a compromise between inclusion of all substances



(a) CA (b) IA

Figure 4.2: Predicted combination effects on transcriptome level for different mixture compositions of diuron, diclofenac and naproxen. Proportions are shown for diuron and diclofenac, naproxen proportion is determined by the respective remaining proportion. Color code shows the difference of integral between mixture prediction and most effective single component. Black dot illustrates mixture composition chosen for experimentation.

in a comparable effective proportion and expecting a distinct combination effect, the following (molar) mixture proportions were selected for experimentation: diuron–0.110; diclofenac–0.026; naproxen–0.864.

4.3 results

We evaluated the toxicogenomic profile induced by the three-component mixture of diuron, diclofenac and naproxen. The Zebrafish Embryo Toxicogenomic trAnscriptome Coordinate system (zetac) from the previous chapter was applied to infer a toxicogenomic landscape of the mixture and get a structured overview over molecular effects of the mixture. In this process we determined whether single compound and moa-specific toxnodes (here, cox-toxnodes identified in Chapter 3) are also significantly affected in the mixture. Next, we evaluated if and for which toxnodes combination effects appeared and how well these could be predicted. Additionally to the toxnodes showing significant combination effects, we evaluated the predictivity of the mixture concepts for the whole toxicogenomic landscape. Besides a qualitative evaluation, we quantitatively compared the effect induced by the mixture with a prediction based on the single compound effects and determined some possible causes for deviations from mixture predictions.

4.3.1 *Toxnodes affected by mixture exposure (mixture effects)*

Using the same procedure as described in Chapter 3 (applying the identical ZETAC), 145 toxnodes were identified as significantly affected by the mixture (compared to 422 toxnodes affected by at least one of the components). The top ten nodes are listed in Table 4.1. They contain toxnodes identified as top-toxnodes for diclofenac and naproxen (e.g, toxnodes #1260, #1259) and toxnodes identified as top-toxnodes for diuron (e.g., toxnode #3567). Of the 145 toxnodes affected by the mixture, 19 were not identified as regulated by any of the single substance exposures earlier. However, most of those "new" toxnodes only showed a weak regulation with a summed difference between control and treatment CIs below 0.5 for 15 of those nodes. Of the 19 potential COX-toxnodes, 18 were identified as affected by the mixture (compare Table 4.2).

4.3.2 *Identification and predictability of combination effects on transcriptome level*

When evaluating the effects of a mixture, one of the major questions we should consider is if an observed mixture effect indeed resulted from the joint action of the components (i.e, is a combination effect), or if the mixture effect just resembled the effect of the most effective component. On the scale of the observed toxicogenomic landscape we can already conclude that combination effects were present since the measured landscape did not resemble the landscape expected from one compound but showed to be a combination of the components (see above), thereby exhibiting a *qualitative* combination effect (compare terminology box 4.1). Zooming in to the single toxnode scale, we may additionally ask if there were toxnodes which are affected stronger than it would have been expected by any of the single compounds alone or even stronger than the sum of effects of all single components, thereby exhibiting a *quantitative* combination effect.

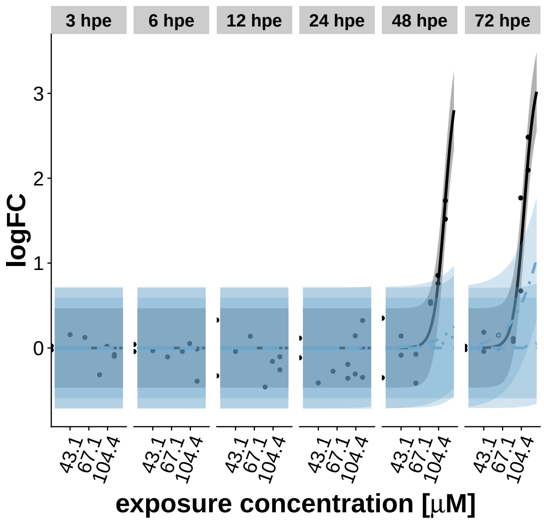
IDENTIFICATION OF QUANTITATIVE COMBINATION EFFECTS
Toxnodes which show a significant quantitative combination effect were identified by comparing the modeled CIs of the mixture effect with the CI of the strongest single compound effect (using the Hill-Gauss regression model) for each toxnode. When considering a 95% CI this resulted in nine (up-regulated) toxnodes with a quantitative combination effect (compare Figure 4.3 for single substance effects and Figure 4.5 for the respective CA, IA and EA predictions). When considering a 75% CI, for 32 toxnodes (28 up, 4 down) a quantitative combination effect was identified. This corresponds to less than 1% of all toxnodes, but to 21% of those toxnodes significantly affected by the mixture exposure. One of the nodes showing a distinct combination effect was toxnode 1138,

Table 4.1: Top ten toxnodes regulated in the ZFE after exposure to a mixture of diuron, diclofenac and naproxen. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval.

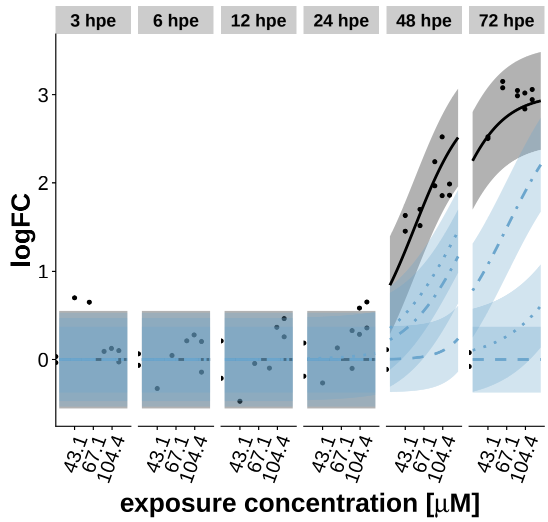
toxnode #	Membergenes	ratio Lethal	ratio Sublethal	$maxS50$	$hillslope$	μ	$sigma$	err	sum(CI-difference)
3567	cyp1a	5.5	3.7	0.025	0.6	4.8	2.1	0.53	68.4
				0.061	1.4	73.4	21.71	0.8	
1260	cyp2k18	6.4	4.3	0.032	2.4	57.6	0.24	0.31	38.3
				0.064	8.1	73.5	0.53	0.46	
1259	si:zfos-411a11.2	8.7	5.8	0.043	1.3	64.2	0.19	0.26	16.9
				0.066	2.4	74.7	0.33	0.41	
3477	ahsg2	3.1	2.1	0.016	0.8	68.7	0.13	0.52	-
				0.03	2	74.9	0.17	0.78	10.9
3312	serpinh1b	1.1	0.7	0.004	0.3	33.8	0.46	0.22	9.9
				0.01	1.2	58.4	1.1	0.34	
1140	si:ch1073-165f9.2, isg15	1.4	1	0.009	3.4	59.8	0.35	0.54	7.5
				0.011	6.1	74.7	1.03	0.72	
1139	slc25a38a, c7b	1.5	1	0.01	2.7	60.8	0.19	0.33	6.8
				0.015	4.4	74.8	0.54	0.45	
3493	slc16a9a	1.9	1.3	0.009	1.8	61.2	0.17	0.44	5.9
				0.022	5.1	74.7	1.35	0.69	
1199	nfe2l2b	4.8	3.2	0.016	1	41.9	0.29	0.29	5.1
				0.062	3.4	69.1	1.31	0.47	
3507	sult6b1	1.2	0.8	0.006	0.5	3.1	2.89	0.23	5
				0.011	1.5	73	21.97	0.36	

Table 4.2: COX-Toxnodes regulated by Mixture. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval.

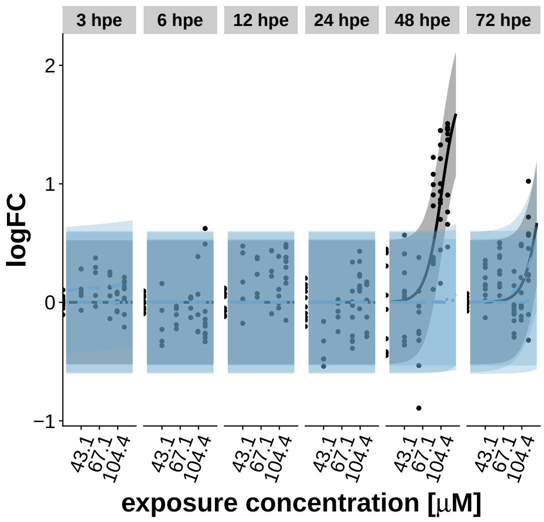
toxnode #	Membergenes	ratio Lethal	ratio Sublethal	$maxS50$	$hillslope$	μ	$sigma$	err	sum(CI-difference)
1260	cyp2k18	6.4	4.3	0.032 0.064	2.4 8.1	57.6 73.5	0.24 0.53	0.31 0.46	38.3
1079	lepa, pth1a	1.2	0.8	0.005 0.009	0.9 3.8	44.8 74	0.66 1.69	0.55 0.72	1.1
1137	fosab	1.5	1	0.009 0.014	4 11	46.8 59.8	0.23 0.96	0.43 0.66	3
3445	cbr1l, mgst3b, ugdh	4	2.7	0.015 0.061	0.1 0.7	53 66.1	0.14 0.3	0.27 0.35	3.3
1198	abcc2, AL929435.1 (antisense RNA)	2	1.3	0.011 0.024	0.8 3.3	47.8 62.7	0.17 0.61	0.5 0.67	0.6
3447	dhrs13l1, tpmt.2, aifm4	2.2	1.4	0.011 0.021	0.4 1.2	40.1 43.7	0.22 0.41	0.35 0.45	3.3
1138	LOC100000332	1.3	0.9	0.009 0.01	5.7 10.1	60 74.6	0.41 1.2	0.2 0.32	3.3
1019	timp2b, clu	1.3	0.9	0.007 0.011	3.1 9.8	60.5 74.8	0.21 1.15	0.35 0.47	1.6
1200	U5 spliceosomal RNA	1.4	0.9	0.009 0.01	9.3 31.9	61.9 74.7	0.33 0.79	0.58 0.69	0.8
3193	hbegfa	1.1	0.7	0.005 0.008	1.4 4.1	47.4 74.6	1.29 2.6	0.18 0.27	1.7
3552	g0s2, si:ch211-198c19.3	1.6	1.1	0.009 0.016	1.1 3.4	61.4 74.7	0.17 0.49	0.47 0.63	2.3
1076	atf3, crema, BX005417.1 (antisense RNA)	1.5	1	0.009 0.012	2.1 5.2	44 74.3	0.7 1.86	0.4 0.5	0.8
3554	GLDC	1.7	1.2	0.01 0.023	1.4 2.6	51.7 58.3	0.15 0.47	0.14 0.21	4
1968	si:dkey-1c7.3	0.6	0.4	0.003 0.007	0.4 38.3	1.9 72.1	0.3 21.77	0.52 0.81	-0.2
1139	slc25a38a, c7b	1.5	1	0.01 0.015	2.7 4.4	60.8 74.8	0.19 0.54	0.33 0.45	6.8
1919	apoea, si:ch73-263o4.3	3	2	0.014 0.054	0.3 1.9	41.3 46.3	0.16 0.36	0.23 0.31	-2.2
360	tmem121a	4	2.6	0.006 0.062	0.2 13.4	4.9 9.5	0.16 1.37	0.39 0.6	0
3314	btg2, zgc:85866, jun, ier2, dusp1	1.4	0.9	0.009 0.012	3.8 6.5	42.7 55.7	0.22 0.55	0.26 0.32	0.3
3448	gstp1, gsto2, cmbl, sqrdl	1.5	1	0.008 0.012	0.5 1.1	64.1 74.9	0.37 0.61	0.27 0.34	3.9



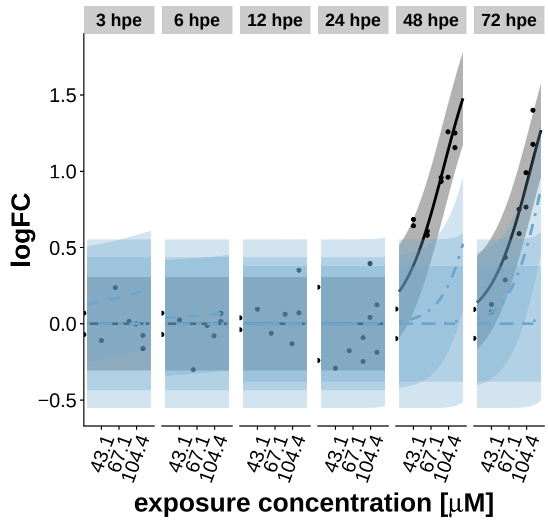
(a) Toxnode 1138



(b) Toxnode 1259



(c) Toxnode 3314



(d) Toxnode 3554

Figure 4.3: Effect of mixture and single substances (modeled) for four example toxnodes showing distinct combination effect. Effect and 95% CI of single substance (■); Effect and 95% CI of mixture (■)

for example. The node was clearly affected stronger than would be expected by any of the single substances (compare Figure 4.3a) and even stronger than would be expected when summing up the effects of the single substances (this expected effect is represented by the EA concept (yellow line) in Figure 4.5a.

QUALITATIVE PREDICTIVITY FOR THE OCCURRENCE OF QUANTITATIVE COMBINATION EFFECTS The next question arising is if the observed combination effects were also predictable. To answer this question, the mixture concepts of CA, IA and EA were applied to predict the mixture effects and compare it with the measurements.

First, we evaluated how well it was predictable from the single substance exposures *if* a significant (quantitative) combination effect will occur. The concept of CA predicted a significant combination effect for four toxnodes (using a CI of 95%), of which three indeed showed a measured combination effect. For the fourth one (toxnode #1019) the quantitative regulation was overestimated and showed a bit higher variance in the experiment than expected. Therefore, no significant combination effect was detected experimentally. The concept of IA and EA did not predict any toxnodes with combination effect for a CI of 95%. For a CI of 75% CA predicted combination effects for 21 up-regulated and 4 down-regulated toxnodes, of which 15 up-regulated toxnodes indeed showed a combination effect. The concept of IA did not predict a combination effect for a CI of 75%. The concept of EA predicted a combination effect for two up-regulated toxnodes for a CI of 75% (which both indeed showed a combination effect). For overlaps of predicted with measured combination effects also compare Venn diagrams in Figure 4.4.

QUANTITATIVE PREDICTIVITY FOR COMBINATION EFFECTS Next, we evaluated how well the observed combination effects could be predicted *quantitatively*. Therefore, we determined how accurately the sensitivity of toxnodes (i.e., the minimum EC_{50} as inverse of the parameter $maxS50$ in the hill-gauss model) could be predicted. As prediction deviation ratio (PDR) we determined the ratio between predicted and measured minimum EC_{50}. When applying the CA concept, of the nine toxnodes with strongest combination effect, for seven toxnodes (respectively 78%) the predicted $minEC_{50}$ was in the range of factor two compared to the experimentally determined $minEC_{50}$ (i.e., the PDR was in the range between 0.5-2.0. This evaluation has been applied by Belden et al. (2007) as "a benchmark for discussing the accuracy of the [mixture] models"). The remaining two toxnodes were more sensitive than predicted (also compare Section 4.3.5). Of the 32 toxnodes showing a combination effect, considering a 75% CI, the $minEC_{50}$ was predicted in the range of factor two for 24 nodes (75%). Of the remaining eight toxnodes, for six sensitivity was underestimated; for only one

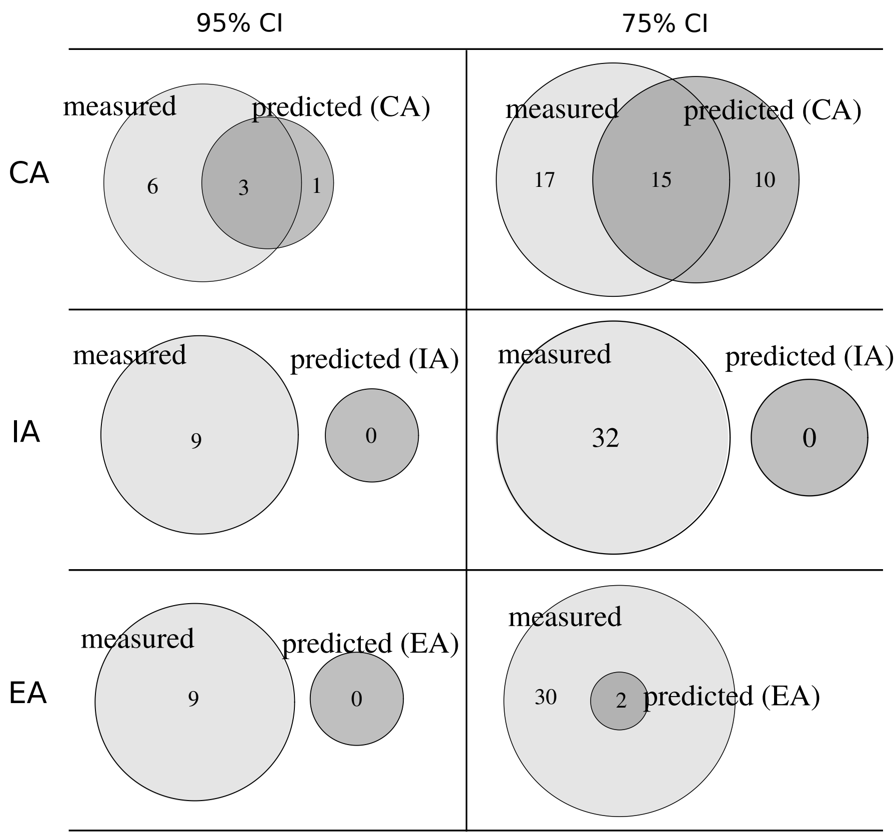


Figure 4.4: *Qualitative* predictivity for the occurrence of (quantitative) combination effects: Overlaps of predicted versus measured combination effects for different mixture concepts and confidence intervals (CIs).

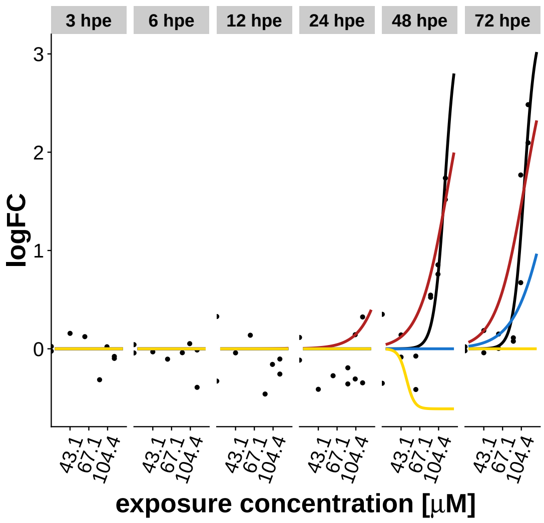
toxnode sensitivity was overestimated, and for one the wrong direction of regulation was predicted.

For the concept of IA and EA the predictions are less accurate and in many cases underestimating sensitivity. With IA only 4 (95% CI)/15 (75% CI) toxnodes are predicted correctly, with EA 2/13 (also compare Table 4.6). In conclusion, it can be shown here that distinct qualitative as well as quantitative combination effects occur on the level of transcript regulation in our case study. The toxnode sensitivities, observed here, were predicted best using the concept of CA.

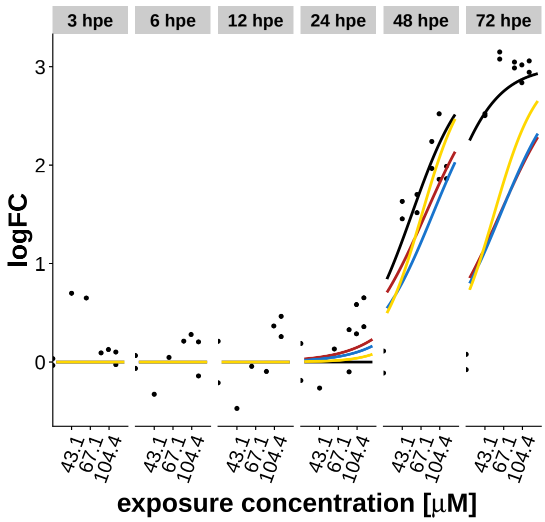
4.3.3 *Prediction of qualitative toxicogenomic mixture landscape – which toxnodes are affected?*

It could be demonstrated above that the concept of CA performed best in predicting the sensitivities of those toxnodes which exhibit a distinct combination effect. Next, we wanted to find out if (and which) mixture concepts could be used to predict the whole toxicogenomic landscape of the mixture (additionally comprising of toxnodes showing only a weak or no combination effect) from the toxicodynamic landscapes of the single compounds.

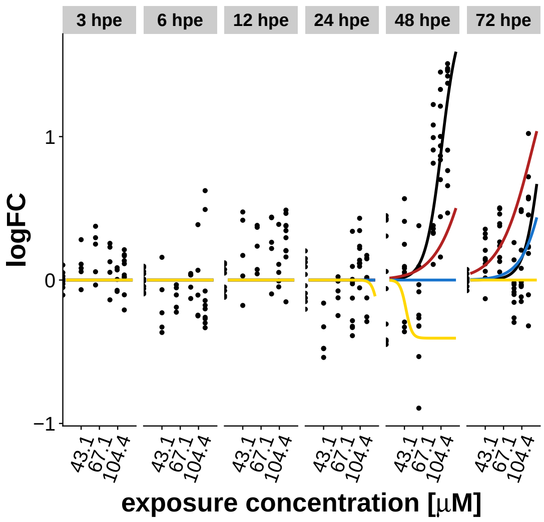
In a first step we evaluated how well it can be predicted *which* toxnodes will be significantly affected by the mixture. As described above, of the 422 toxnodes which could be expected to be affected by the mixture (based on a boolean mixture assumption, i.e., because they are affected by at least one of the components) only 126 were actually identified to be significantly affected by the mixture (probably mostly due to lower concentrations of the components in the mixture compared to the single compound exposures). Here, the first objective was to determine if the mixture concepts of CA, IA or EA can give a more precise expectation than a boolean concept, about *which* toxnodes will be affected by the mixture. For this, the confidence intervals (CIs) of the CA, IA and EA predictions were compared with the 2.5, respectively 97.5 % quantile of control measurements. Only toxnodes showing a gap between the prediction CIs and the control quantiles for one of the treatment conditions were defined to be affected in the mixture. As it is summarized in Table 4.3 for up-regulated toxnodes and Table 4.4 for down-regulated toxnodes, the number of false positive predicted toxnodes (for up-/down-regulation) decreased from 172/126 (Boolean) to 55/26 (CA), 4/2 (IA) and 2/1 (EA). While CA produced more false-positive predictions than IA and EA, there were also less false-negative results with only 25/38 false-negative predictions in comparison to 68/53 (IA) and 70/53 (EA). The number of false-positive and false-negative predictions in relation to the number of true positive and true negative predictions can be translated into scores measuring the quality of binary predictions such as sensitivity and precision (compare Table 4.5). Prediction precision ($\frac{\text{true positives}}{\text{true positives+false positives}}$) was highest



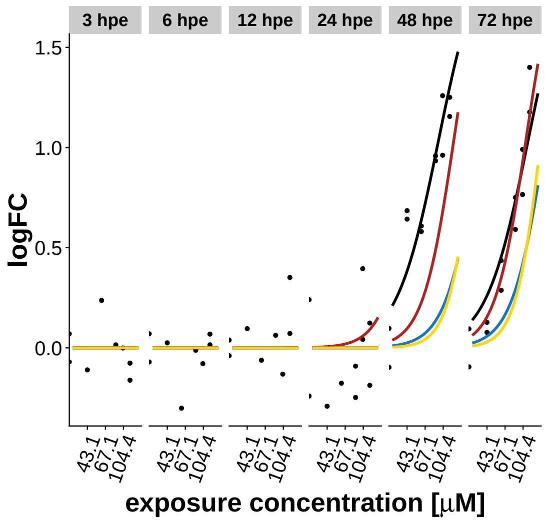
(a) Toxnode 1138



(b) Toxnode 1259



(c) Toxnode 3314



(d) Toxnode 3554

Figure 4.5: Effect of mixture and single substances (modeled) for four example toxnodes showing distinct combination effect: measured effect (●); modeled effect (▬); CA (▬); IA (▬); EA (▬).

Table 4.3: Number of predicted and measured toxnodes significantly upregulated by mixture exposure. CA:concentration-addition; IA:independent action; EA: effect addition; BO: boolean mixture

	Measured	CA	IA	EA	BO
upregulated	90	120	26	22	255
true positive	90	65	22	20	83
true negative	3510	3451	3502	3504	3334
false positive	–	55	4	2	172
false negative	–	25	68	70	7

Table 4.4: Number of predicted and measured toxnodes significantly downregulated by mixture exposure. CA:concentration-addition; IA:independent action; EA: effect addition; BO: boolean mixture

	Measured	CA	IA	EA	BO
downregulated	55	43	4	3	167
true positive	55	17	2	2	41
true negative	3545	3515	3539	3540	3415
false positive	–	26	2	1	126
false negative	–	38	53	53	14

for EA (0.91 for up-regulation/0.67 for down-regulation), followed by IA (0.85/0.5), CA (0.54/0.4) and the boolean approach (0.33/0.25). In contrast, sensitivity ($\frac{\text{true positives}}{\text{true positives+false negatives}}$) was highest for the boolean approach (0.92 for up-regulation/0.75 for down-regulation), followed by CA (0.72/0.31), IA (0.24/0.04), and EA (0.22/0.04). A conclusive measure for model accuracy is given by the F1-score, the harmonic mean of sensitivity and specificity. The F1-score was significantly higher for CA predictions (0.62/0.35) than for the boolean approach (0.48/0.37), IA (0.38/0.07), and EA (0.36/0.07). Therefore, we can conclude that for a prediction of *which* toxnodes are affected by a mixture, the concept of CA gives less sensitive but more precise estimates than the boolean approach. Overall, estimates are most accurate using the CA approach, which balances best the sensitivity and precision of predictions.

4.3.4 *Prediction of quantitative toxicogenomic mixture landscapes – how are the toxnodes affected?*

The concept of CA overall performed best in predicting *which* toxnodes are affected by the mixture exposure experiment analyzed here. In a next step the objective was to determine if it is also predictable from the single compound exposures, *how* the toxnodes are affected by the

Table 4.5: Qualitative prediction scores for mixture models (up-regulation/down-regulation). CA: concentration addition; IA: independent action; EA: effect addition; BO: boolean mixture

	CA	IA	EA	BO
sensitivity	0.72/0.31	0.24/0.04	0.22/0.04	0.92/0.75
precision	0.54/0.4	0.85/0.5	0.91/0.67	0.33/0.25
F1-Score	0.62/0.35	0.38/0.07	0.36/0.07	0.48/0.37

mixture in a time and concentration dependent manner. Therefore, CA (as the best performing concept in the previous step) was applied to predict time and concentration dependent *in silico* toxicogenomic landscapes for the mixture and compare it with the landscapes inferred from our experimental mixture exposure. The *in silico* and experimental landscapes are shown in Figure C.2 and Figure C.3, the differences between prediction and measurement in Figure C.4 (Appendix C). The most prominent structures in the landscapes were correctly predicted (especially in the later time points and higher concentrations). However, by visual inspection it already becomes clear that there were also toxnodes distributed across the map, for which regulation cannot be correctly predicted, either because there was a false-positive/false-negative qualitative prediction, or the quantitative prediction was not met by the experimental observations.

MAPPING THE PREDICTION DEVIATION RATIO To evaluate in more detail the quantitative predictive power of the mixture concepts on whole transcriptome level for our case study, again the prediction deviation ratio (PDR) between predicted and measured minimum EC_{50} was determined (compare Section 4.3.2), this time for all toxnodes. In Figure 4.6, the predicted and measured sensitivities and PDRs (using CA) for all toxnodes were projected on a map applying the ZETAC. This allows a general visual inspection of the predictivity for the whole transcriptome. Clearly, the most prominent structures in the landscapes (the three "clusters" in the top region of the map, the cluster in the middle right region and the cluster in the middle left region) showed consistency between prediction and measurement. However, several toxnodes could be identified on the map, for which the measured values did not agree with the predicted ones.

DISTRIBUTION OF PREDICTION DEVIATION RATIOS For a comprehensive evaluation of the PDRs for the different mixture concepts, Figure 4.7 shows the distribution of PDRs for different groups of selected toxnodes: toxnodes with significant combination effect, COX-toxnodes, significantly affected toxnodes, and all toxnodes. In Table 4.6 the proportions of toxnodes are summarized, for which the PDR was smaller or equal to 2. Overall, CA gave the most accurate predictions of $minEC_{50}$. As already mentioned above, the proportion of toxnodes for which predictions met the measured sensitivities was highest for toxnodes with a significant combination effect (78% using CA). Similarly, sensitivities for 74% of the proposed COX-toxnodes could be correctly predicted. For those toxnodes, identified to be significantly affected by the mixture, still 61% of the sensitivities met the predictions, while for all toxnodes only 31% of the predictions were consistent with the measurements. Interestingly, the proportion of correctly predicted sensitivities for specific diuron/naproxen or diuron/diclofenac toxnodes turned out to be lower than for COX-toxnodes (i.e., specific diclofenac/naproxen toxnodes). For IA and EA the distribution of PDRs is much wider (Figure 4.7) and the proportions of correctly predicted toxnodes lower (maximum 68% for COX-toxnodes using IA; maximum 58% for COX-toxnodes using EA). This leads to the conclusion that similarly as for qualitative predictions, the concept of CA also turned out to result in the most accurate quantitative predictions in comparison with IA and EA. The advantage of CA was most pronounced for toxnodes showing distinct combination effects.

COMBINATION OF QUALITATIVE AND QUANTITATIVE PREDICTIONS Qualitative and quantitative prediction as described above can also be joined. In this case, sensitivity of nodes was set to zero if there was no significant effect predicted or measured. Table 4.7 and Figure C.5 in Appendix C show the results for joining qualitative and quantitative prediction. When evaluating the complete map, the proportion of toxnodes with a PDR in the range of two was increased from 24-31% (only quantitative prediction) to 96% (quantitative and qualitative prediction combined). For significantly affected toxnodes, the proportion decreased slightly using CA and drastically, when using IA or EA (due to the large number of false-negative predictions using those concepts). For COX-toxnodes the proportion of toxnodes with a PDR in the range of two was increased slightly from 74% to 79 % when using CA. For IA or EA, the correctly predicted proportion decreased from 68%/58% to 37%/26%.

4.3.5 *Deviations from predictions*

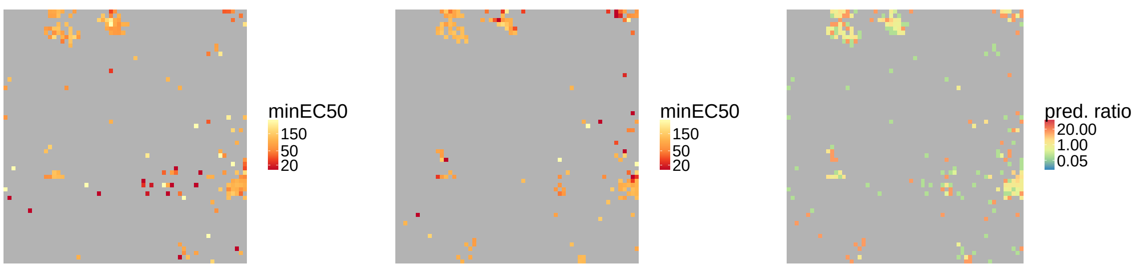
As it was shown above, the predicted sensitivity was close to the measured sensitivity for roughly 80 % of toxnodes, which showed a distinct combination effect, and for 60% of all significantly affected toxnodes, when applying CA for prediction. This means that at the same time 20% to 40% of the toxnodes remained for which the measurements deviate from the predictions. Here, a few selected examples for toxnodes with over- and underestimated sensitivity are shown. Three examples for underestimation of toxicity were toxnodes 1260 (CA-ratio: 2), 1020

Table 4.6: Proportion of toxnodes, for which measured minEC50 are within a range of 2 of prediction. CA: concentration addition; IA: independent action; EA: effect addition.

Nodes	CA	IA	EA
All toxnodes	0.31	0.30	0.24
Toxnodes affected by mixture	0.61	0.47	0.53
COX-Toxnodes	0.74	0.68	0.58
Diuon/Naproxen-Toxnodes	0.55	0.50	0.45
Diuron/Diclofenac-Toxnodes	0.25	0.50	0.50
Toxnodes with significant combination effect (95%CI)	0.78	0.44	0.22
Toxnodes with significant combination effect (75%CI)	0.75	0.47	0.41

Table 4.7: Proportion of toxnodes, for which measured minEC50 are within a range of 2 of prediction, after combination with qualitative prediction. CA: concentration addition; IA: independent action; EA: effect addition.

Nodes	CA	IA	EA
All toxnodes	0.96	0.96	0.96
Toxnodes affected by mixture	0.46	0.13	0.10
COX-Toxnodes	0.79	0.37	0.26
Diuon/Naproxen-Toxnodes	0.65	0.45	0.40
Diuron/Diclofenac-Toxnodes	0.50	0.75	1.00

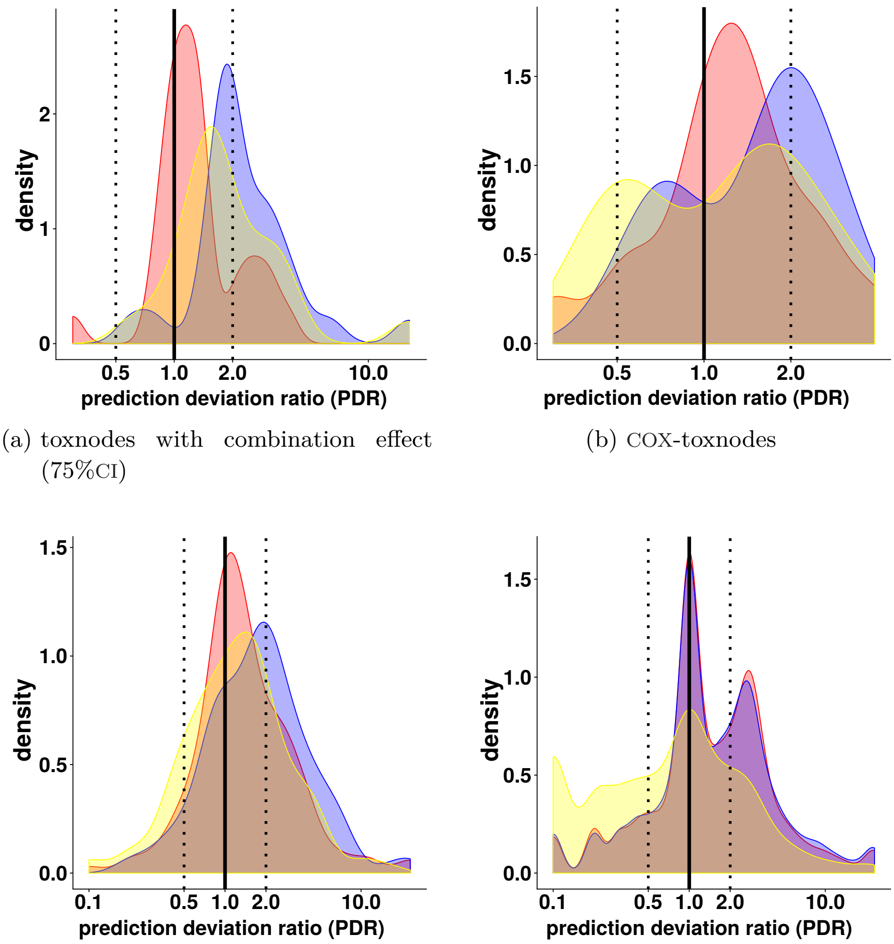


(a) predicted minEC50 (µmol) (b) measured minEC50 (µmol) (c) prediction deviation ratio

Figure 4.6: Predicted minEC50, measured minEC50, and prediction deviation ratio (PDR) for mixture. All toxnodes neither predicted nor measured to be affected are plotted in gray.

Table 4.8: Ratios of predicted and measured min $EC50$ for COX-toxnodes. CA: concentration addition; IA: independent action; EA: effect addition. The ratios representing the most accurate prediction for each toxnode are printed in bold.

Toxnode #	measured min EC_{50}	ratio CA	ratio IA	ratio EA
1260	25.9	**2**	2.7	2.1
1139	83.7	**1.3**	2	1.8
1079	120.9	0.5	**0.7**	0.5
1019	114.1	0.3	1.5	**1.4**
3552	58.8	**1.4**	2.4	1.9
3554	44	**2.5**	3	3.5
1138	106.5	**1.1**	1.9	-1.6
1200	101.9	**0.8**	1.9	1.5
1076	91.1	0.6	**0.8**	0.7
3448	93.4	**1**	0.7	0.5
1137	96.6	**1**	3.4	3
3445	33.5	2.7	**0.5**	**0.5**
1198	74.6	1.4	1.8	**1.2**
3193	130.5	**1.5**	1.8	1.6
1968	346.9	**1**	-1	-0.2
3447	66.1	3.9	**0.7**	0.4
1919	50.7	1.8	**1**	0.6
3314	101.2	**1.3**	1.9	-0.2
360	15.2	-22.9	-22.9	-6.1



(a) toxnodes with combination effect (75% CI)

(b) COX-toxnodes

(c) toxnodes significantly affected by mixture

(d) all toxnodes

Figure 4.7: Distribution of prediction deviation ratios for selected groups of toxnodes. CA (■); IA (■); EA (■). Prediction deviation ratios above 1 indicate underestimation of toxnode sensitivity by the prediction, ratios below 1 indicate overestimation.

(CA-ratio: -2.4) and 3447 (CA-ratio:3.9), shown in Figure 4.8 and Figure 4.9. While for toxnode 1260 the deviation could actually be a biological effect, detailed examination of toxnode 1020 revealed that the wrong prediction was based on different directions of regulation over time visible in the single compounds. Since the regression model was not able to capture this kind of response, the mixture prediction was based on one of the directions and could not capture the effect in the other direction, which lead to an underestimation of effect. In the case of toxnode 3447 a look at the single compound measurements revealed that regulation showed a biphasic behavior on the concentration scale. Therefore, we checked the prediction for biphasic-CA. This resulted in a much better prediction for this toxnode than monotonic CA. The predictions based on the gauss-gauss model are shown in Figure 4.12.

An example for overestimation of toxicity is shown for toxnodes 1363 (CA-ratio:0.1) in Figure 4.10. It remains unclear why the distinct regulation measured in diuron exposure did not reappear in the mixture, and a biological cause could be suspected here.

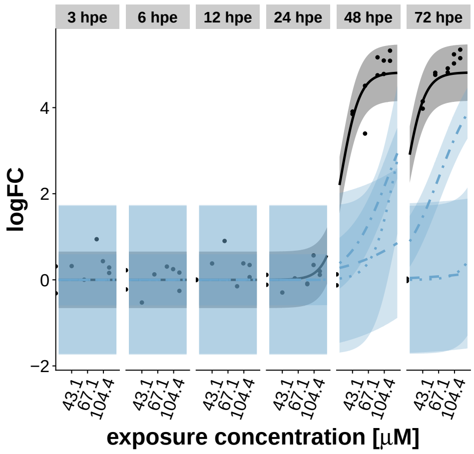
These examples show that deviations from the mixture predictions may indicate a biological (possibly synergistic/antagonistic) effect, but detailed examination is still needed to exclude the possibility of a "wrong" prediction, e.g. based on misfits in single compound models. However, predictions using the concept of CA did significantly reduce the number of "surprising" mixture effects in our case study compared to commonly applied concepts like EA or boolean mixture.

In general, accuracy of mixture prediction was better the clearer the reaction towards single substances were. This is shown in Figure 4.13: For toxnodes with a high summed CI-difference (CI-differences are summed across the three single substances) there is a higher proportion of toxnodes with a PDR below 2.

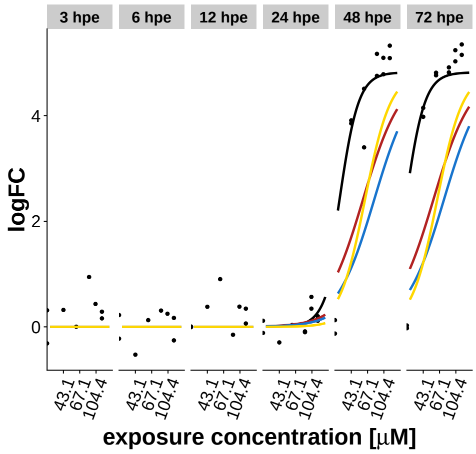
4.4 DISCUSSION

Regarding environmental monitoring, the presence of chemical mixtures in the environment has been termed "the elephant in the room" (Schroeder et al., 2016), especially in the application of high-throughput bioanalytical methods. The potential of toxicogenomic methods in environmental monitoring will be heavily dependent on our understanding about combination effects on molecular level (Altenburger et al., 2012). To begin elucidating the basic concepts with which toxicogenomic profiles combine in a mixture, we conducted a mixture exposure with a ternary mixture of the three earlier characterized compounds diuron, diclofenac and naproxen.

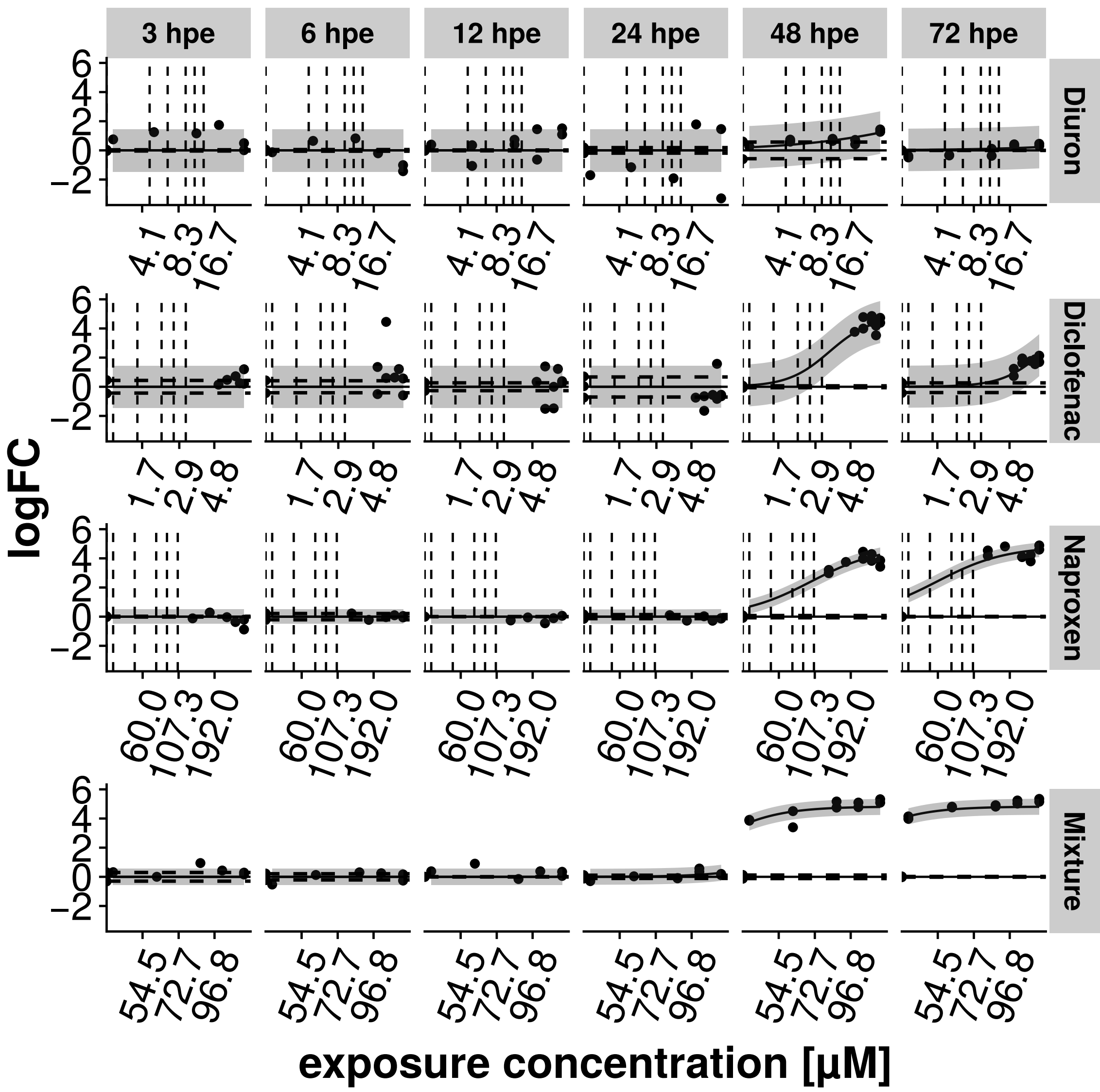
The results of the mixture exposure showed that significant and predictable combination effects could be clearly identified. Moreover, the effect profile of COX-inhibition could be recovered in the toxicogenomic landscape of the mixture. The predictability of the whole toxicogenomic



(a) Modeled single substance and mixture effects: effect and 95% CI of single substance (■); Effect and 95% CI of mixture (■)

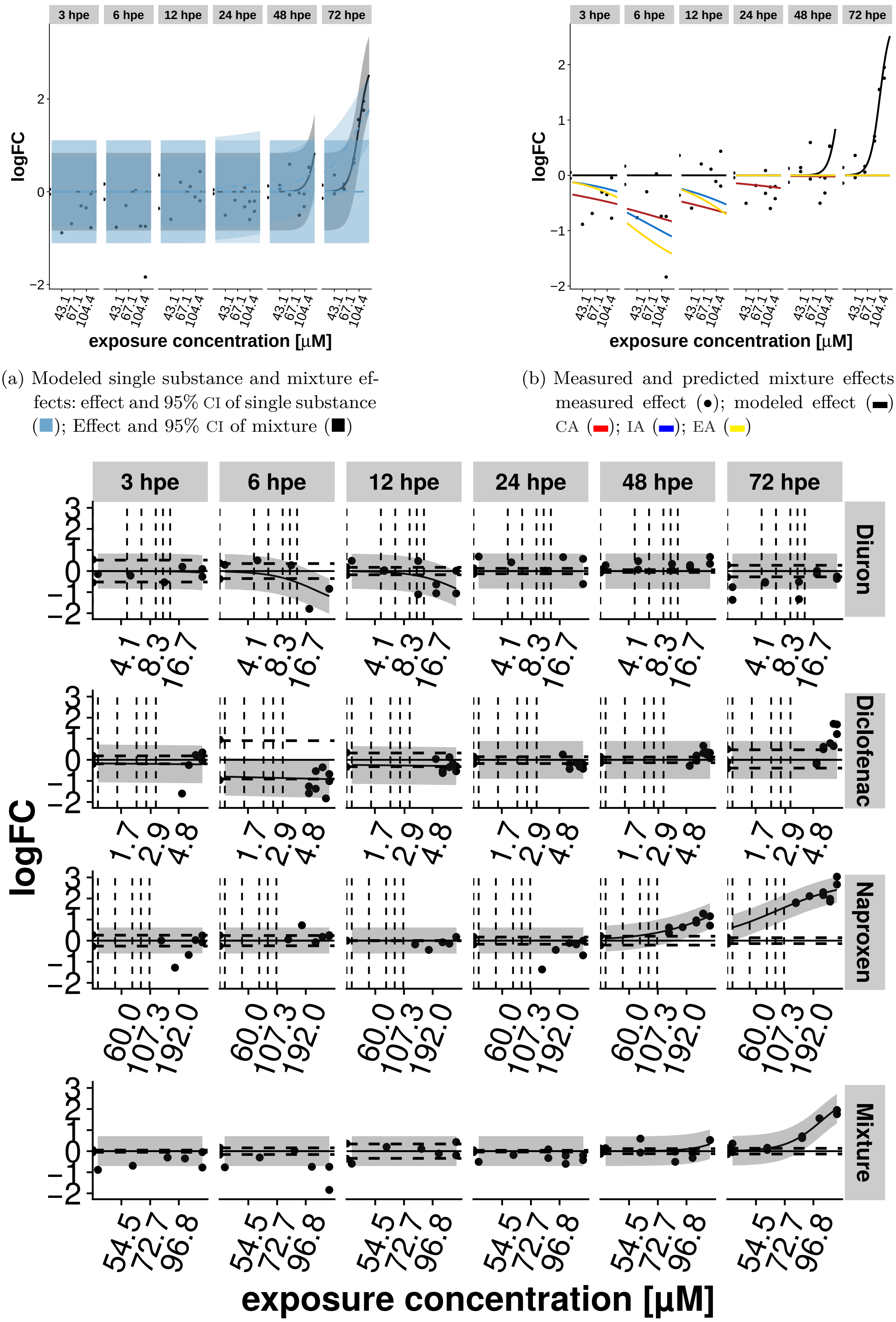


(b) Measured and predicted mixture effects: measured effect (•); modeled effect (▬); CA (▬); IA (▬); EA (▬)



(c) Single substance and mixture effects: measured effect (•); modeled effect and 95%CI (▬); dashed lines indicate concentrations of single compounds in the mixture exposure.

Figure 4.8: Predicted and measured single compound and mixture effects for toxnode 1260. The mixture effect is underestimated for this toxnode (PDR = 2.0).

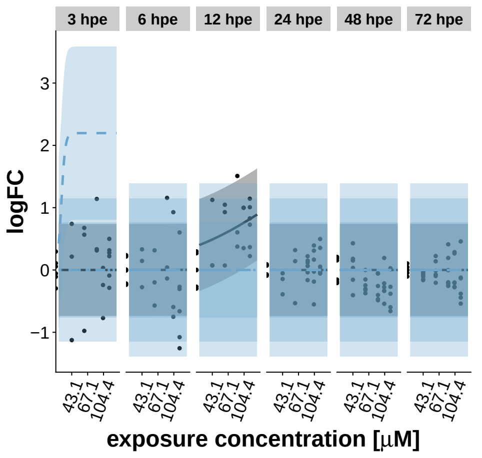


(a) Modeled single substance and mixture effects: effect and 95% CI of single substance (■); Effect and 95% CI of mixture (■)

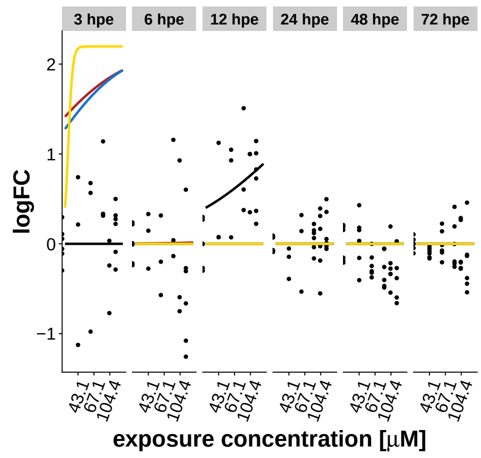
(b) Measured and predicted mixture effects: measured effect (•); modeled effect (▬); CA (▬); IA (▬); EA (▬)

(c) Single substance and mixture effects: measured effect (•); modeled effect and 95%CI (▬); dashed lines indicate concentrations of single compounds in the mixture exposure.

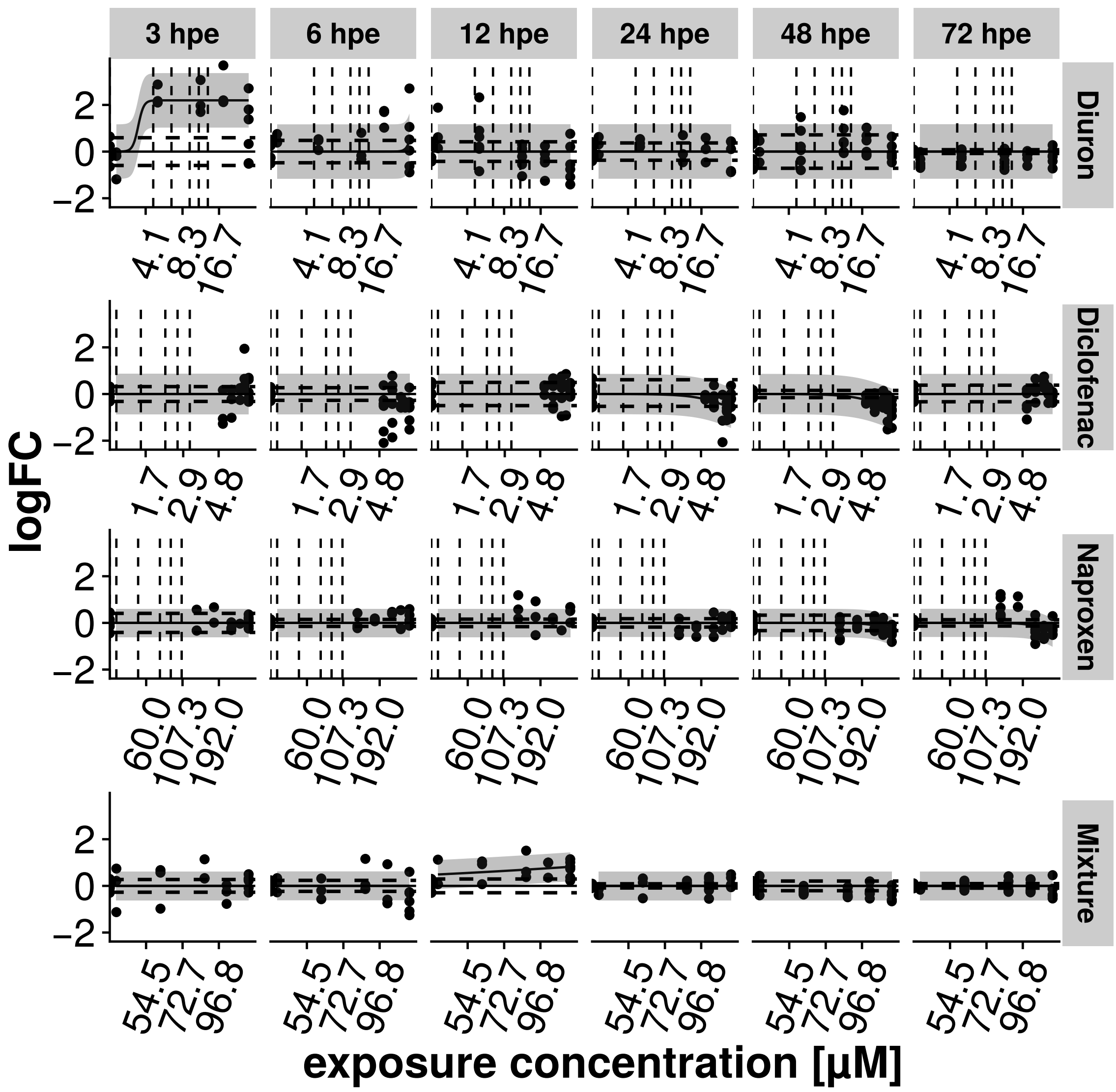
Figure 4.9: Predicted and measured single compound and mixture effects for toxnode 1020. The mixture effect is predicted in the wrong direction for this toxnode.



(a) Modeled single substance and mixture effects: effect and 95% CI of single substance (■); Effect and 95% CI of mixture (■)

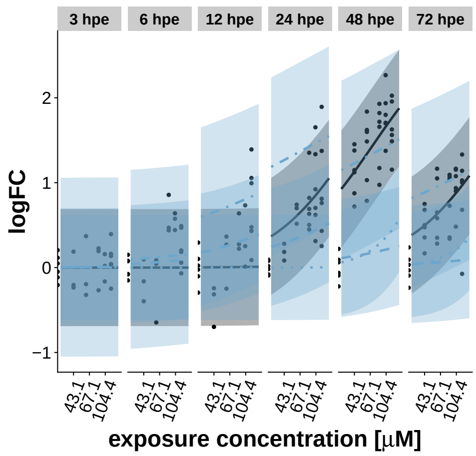


(b) Measured and predicted mixture effects: measured effect (●); modeled effect (▬); CA (▬); IA (▬); EA (▬)

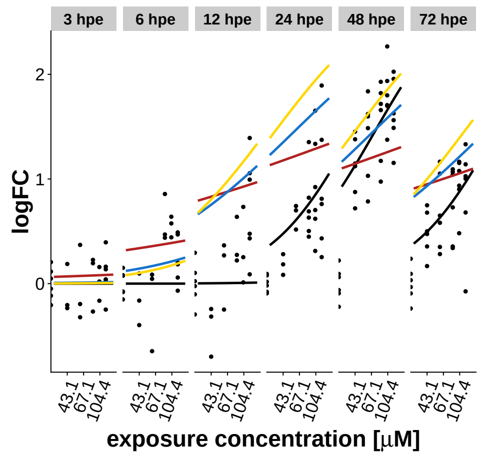


(c) Single substance and mixture effects: measured effect (●); modeled effect and 95%CI (▬); dashed lines indicate concentrations of single compounds in the mixture exposure.

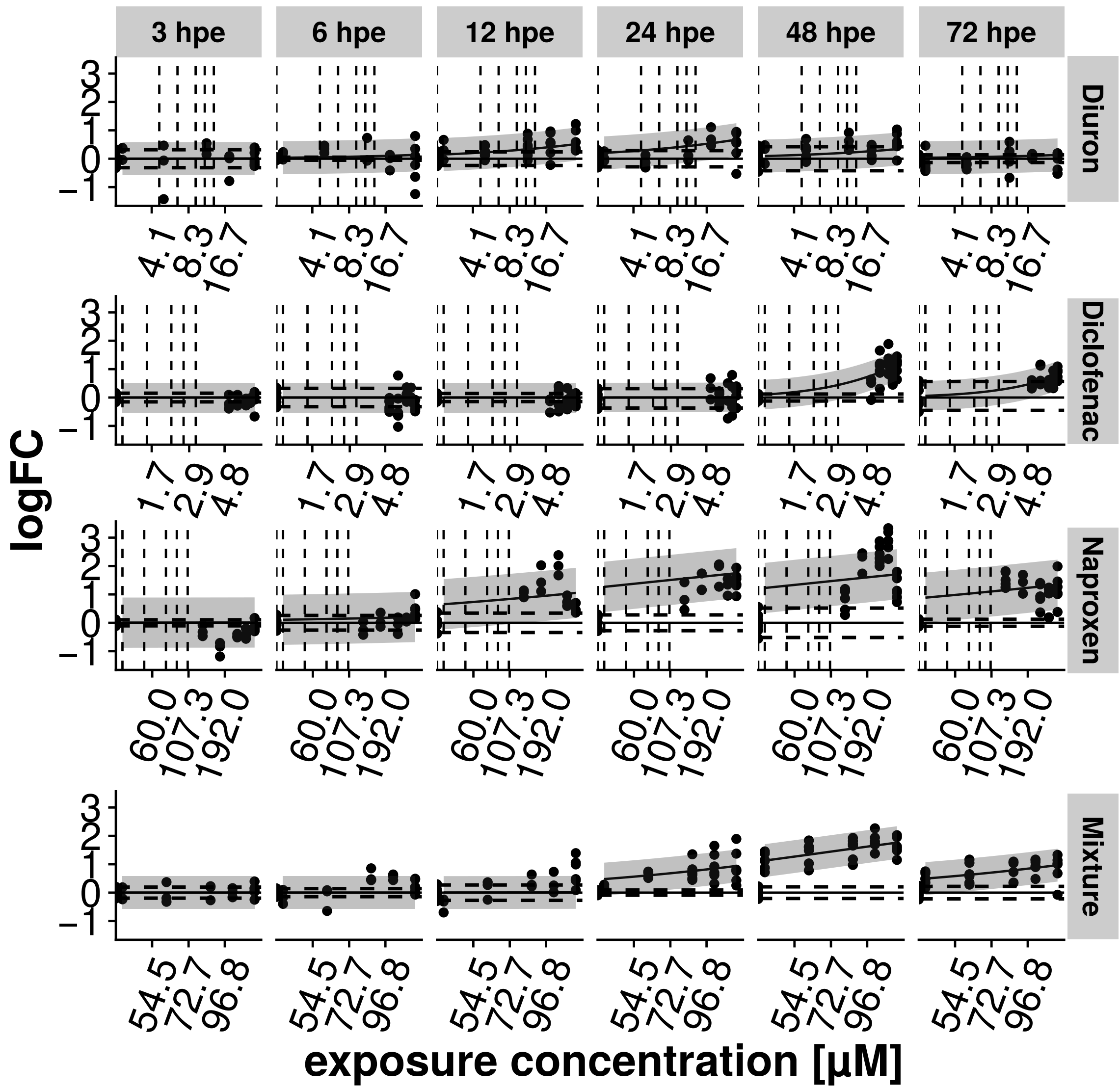
Figure 4.10: Predicted and measured single compound and mixture effects for toxnode 1363. The mixture effect is overestimated for this toxnode (PDR = 0.1).



(a) Modeled single substance and mixture effects: effect and 95% CI of single substance (■); Effect and 95% CI of mixture (■)

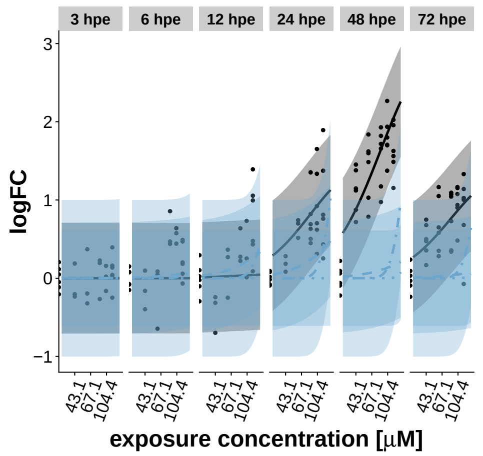


(b) Measured and predicted mixture effects based on biphasic response: measured effect (●); modeled effect (▬); CA (biphasic) (▬); IA (▬); EA (▬)

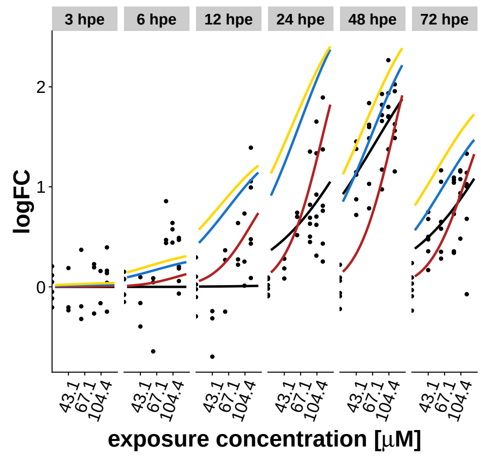


(c) Single substance and mixture effects: measured effect (●); modeled effect and 95%CI (▬); dashed lines indicate concentrations of single compounds in the mixture exposure.

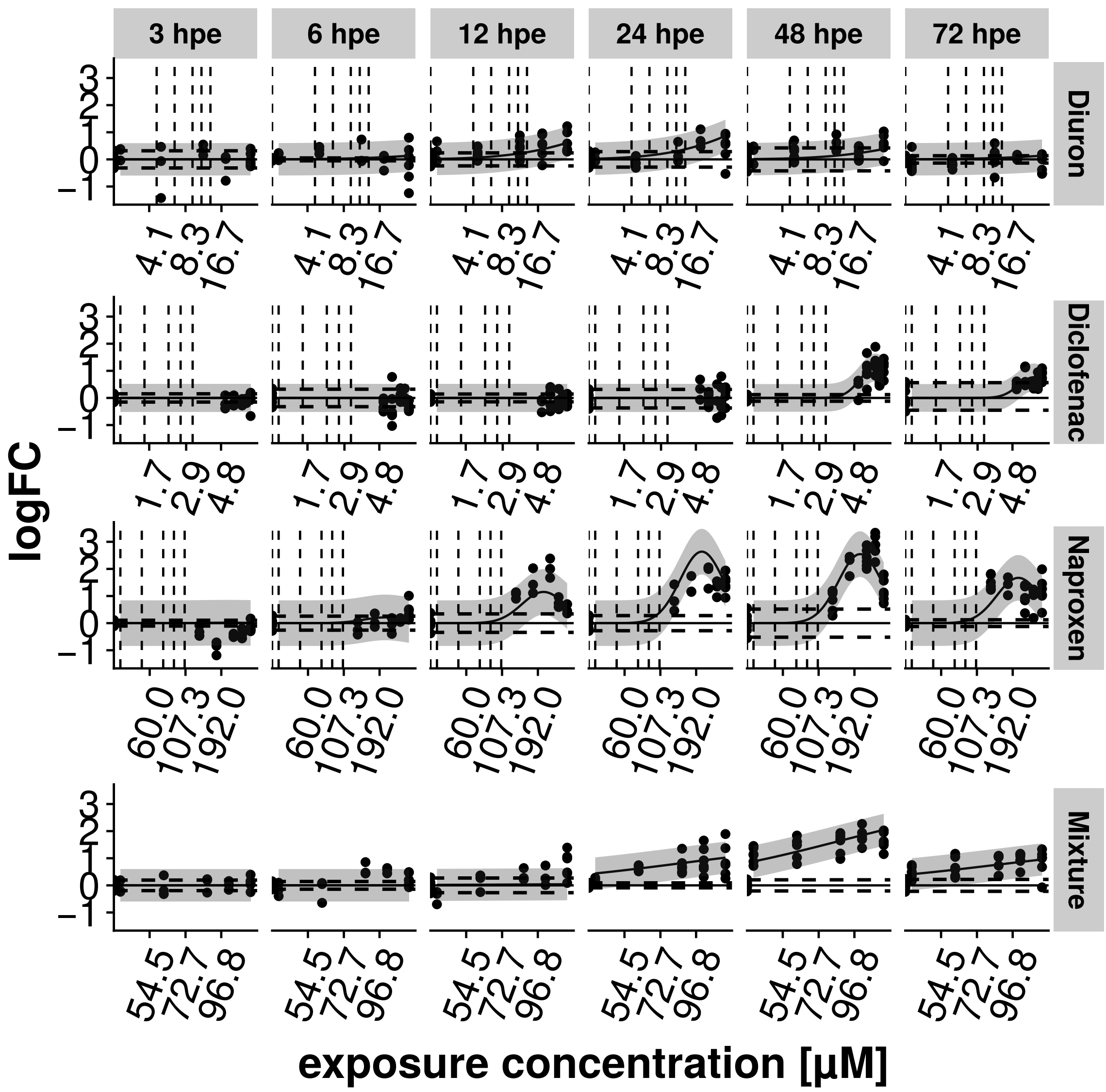
Figure 4.11: Predicted and measured single compound and mixture effects for toxnode 3447 based on monotonic response.



(a) Modeled single substance and mixture effects: effect and 95% CI of single substance (■); Effect and 95% CI of mixture (■)

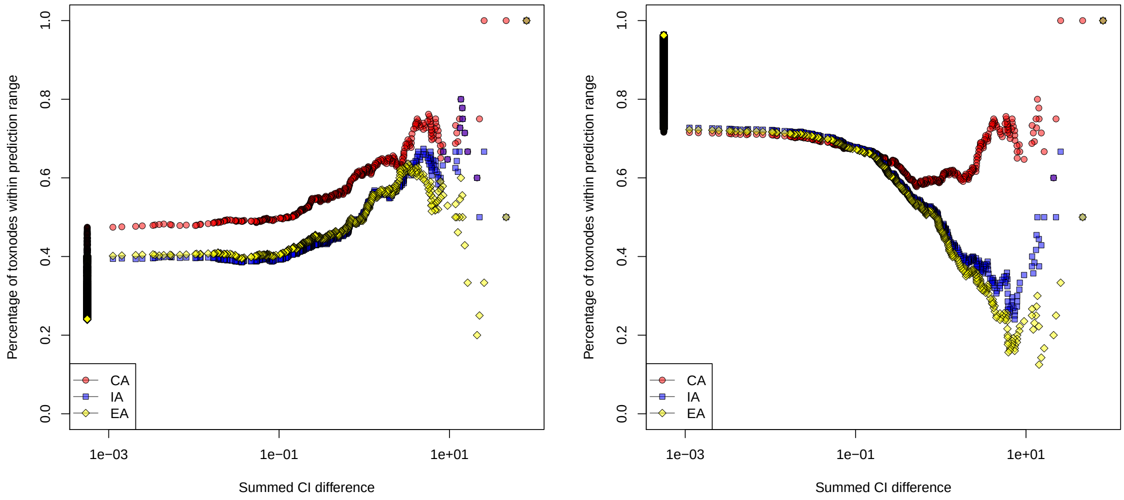


(b) Measured and predicted mixture effects based on biphasic response: measured effect (●); modeled effect (▬); CA (biphasic) (▬); IA (▬); EA (▬)



(c) Single substance and mixture effects: measured effect (●); modeled effect and 95%CI (▬); dashed lines indicate concentrations of single compounds in the mixture exposure.

Figure 4.12: Predicted and measured single compound and mixture effects for toxnode 3447 based on biphasic response.



(a) Only quantitative prediction

(b) Quantitative and qualitative prediction

Figure 4.13: Proportion of correctly predicted toxnodes over summed CI-differences. CI-difference is summed across all conditions of single substance exposures.

mixture landscape was primarily limited by uncertain descriptions of the single component responses, which will be discussed in more detail below.

4.4.1 *Experimental design, model and prediction uncertainties*

While experimental efforts for the analysis of combination effects in a mixture exposure increase with each additional mixture component (as each component has to be characterized on its own first), the quantitative evaluation of combination effects is becoming easier with more mixture components. In the case of only three components the mixture has to be carefully designed in order to be able to evaluate prediction concepts for combination effects. For a toxicogenomics experiment this is especially challenging, since optimization must be performed for a large number of endpoints (i.e., genes or toxnodes) simultaneously. Another challenge is the uncertainty of model prediction for some of the toxnodes single compound exposures, be it because of biological variation, or because the concentration range in the single compound exposure did not cover the whole concentration response range. While in a single bioassay setting one would usually prevent such cases with the help of range finding experiments, in an omics setting we cannot get around those uncertainties. Here, those challenges were tackled with a carefully selected and phenotypically anchored single component exposure as well as an optimization approach for the mixture design, maximizing the expected combination effect. Uncertainty of predictions were quantified in the modeling process, including a bootstrapping approach for mixture prediction (which also allowed for qualitative predictions about which toxnodes will be affected by the mixture exposure). A factor potentially contributing to biased predictions could also be the unknown maximum effect for each toxnode's regulation. Here, we assumed the maximum (respectively minimum) *log*FC across all single component conditions of our three model compounds to represent the maximum effect (for all three compounds). Therefore, if a compound did not affect a toxnode, effects were assumed to occur in higher concentrations than the measured ones. This implies that extrapolations to higher exposure concentrations have to be treated with care. At the same time, assigning the same maximum effect to all compounds allowed us to use CA across the whole range of effect levels. Since, we did not observe noticeable plateaus in the concentration responses well below the assumed maximum effect, we deem this approach reasonable. Otherwise, if such plateaus were observed, one should, for example, make use of an toxic unit extrapolation approach (Scholze et al., 2014). Anyway, the assumed maximum effect for each toxnode will have to be reviewed, when more single substance exposures are performed in the future.

4.4.2 *Recovery of effect profiles in toxicogenomic mixture landscapes – qualitative combination effects*

In the previous chapter a set of toxnodes were proposed as effect profile for COX-inhibition. Here, it could be shown that this effect profile could be recovered in a mixture of two compounds used as COX-inhibitors (diclofenac, naproxen) and one used as a herbicide (diuron): Of the 19 proposed COX-toxnodes, 18 were found to be regulated in the mixture. For 14 of the COX-toxnodes the predicted sensitivity was in the range of factor two from the measured one. Moreover, also toxnodes found to be affected by diuron alone, were found to be affected by the mixture. Although one compound might not be sufficient to define an effect group profile, it could still be observed how the effects of the different compounds in the mixture combined on transcriptome level. For example, the up-regulation of a combination of compound or MOA specific biotransformation enzymes (e.g., cyp1a for diuron, cyp2k18 for diclofenac and naproxen) could be observed in the mixture exposure. Approximately 15% of the toxnodes significantly affected by the mixture were not significantly affected by any of the components, however most of those only showed a weak regulation. These could be signs of interactions of the compounds, however, biological variation might also be the cause for the regulation of those toxnodes.

4.4.3 *Occurrence of quantitative combination effects*

When several components in the mixture affect the same toxnodes, this might result in a quantitative combination effect. The results of our mixture evaluations show that quantitative combination effects can be expected on transcriptome level (i.e., single toxnodes are affected significantly stronger than would be expected by exposure to any of the single components). We found that the mixture effect of approximately every fifth toxnode which was significantly affected by the mixture exposure resulted of a combined effect. While in some cases the mixture effects only differed slightly from the effects of the most effective substance, the extent of combination effect will potentially be higher in mixtures with more components (as discussed by Faust (1999), the maximum ratio between the EC_X predicted by CA and the EC_X predicted by IA corresponds to the number of mixture components). This is especially relevant for the evaluation of toxicogenomic portraits of environmental extracts, since the joint action of many substances can lead to a "something-from-nothing" effect (Silva et al., 2002), i.e., an effect of the mixture, when no effect from the single components would be expected (by the respective compound concentrations).

4.4.4 *Predictability of combination effects*

To evaluate the quantitative predictability of combination effects, a newly developed regression model for time and concentration dependent transcript regulation was applied here (compare Chapter 3) in combination with mixture concepts established for apical endpoints. With the help of these concepts it has been shown for apical as well as receptor based bioassays (Altenburger et al., submitted) that mixture effects can be predicted from known effects of the mixtures components.

In the context of toxicogenomics several studies have evaluated mixture effects (reviewed in Altenburger et al., 2012). However, only few studies explicitly relate their findings to expectations based on theoretical mixture concepts. De Coninck et al. (2014) investigated the effects of binary mixtures on the transcriptome of *Daphnia pulex* and could identify pathways for which interactions between different stressors could be suspected based on the IA assumption. Recently, Labib et al. (2017) analyzed the transcriptome of mice lung tissues exposed to eight different PAHs and their mixtures in three doses. For six cancer related pathways the mixture concepts of IA, CA and GCA were evaluated, while IA was predicting pathway perturbations most accurately. Both mentioned studies provide a good starting point for mixture evaluation, whereas they both do not explicitly evaluate combination effects of the compounds and rely on very limited concentration response information.

It was shown here that of the evaluated mixture concepts (boolean, EA, IA, CA) the concept of concentration addition (CA) gave the most accurate quantitative predictions of combination effects. More precisely, for close to 80% of the toxnodes, which showed a significant combination effect, the predicted sensitivity (using CA) was in the range of factor 2 of the measured sensitivity. Similar proportions have been found for apical endpoints before (Belden et al., 2007). This strengthens the applied regression model and also the pharmacological assumptions behind the model and the CA concept.

However, the results of this work clearly differ from the findings of Labib et al. (2017), who found CA as overestimating the toxicogenomic effects. One reason for this could be the different approach of gene aggregation, since Labib et al. (2017) aggregated genes based on pathway annotation, while commonly co-regulated genes were clustered here. Additionally, the mixture predictions by Labib et al. (2017) were based on only three concentrations, which might also include a bias in the findings.

Interestingly, we observed that sensitivities for toxnodes with combination effects were rather underestimated and almost never overestimated. It could be hypothesized that this resembles a synergistic effect for immediately affected molecular targets, which gets weaker as the signal evolves in its cascade. This would go in line with the predictions of Fitzgerald et al. (2006), who showed that feedback-loops in a signaling cascade should stabilize a non-interacting combination effect.

4.4.5 *Predictability of whole toxicogenomic mixture landscape*

It was also evaluated here how well the toxicogenomic mixture landscape can be predicted as a whole. We evaluated (qualitative) predictions of *which* toxnodes in the ZETU will be affected by the mixture, and (quantitative) predictions of *how* the toxnodes are affected.

For qualitative predictions for the whole toxicogenomic landscape, the CA concept gave the best balance between sensitivity and precision of the predictions. Interestingly, qualitative prediction was more accurate for up-regulation than for down-regulation (irrespective of the mixture concept applied). This might be an indication for different types of responses, with up-regulation being an "active" response to substance exposure, while down-regulation might primarily be a sign of "passive" secondary effects, unspecific disregulation, or developmental delay (compare Chapter 2).

Applying mixture concepts, we cannot only make qualitative statements about *which* toxnodes are affected but also quantitative predictions *how* they should be affected by a mixture. We tested the quantitative power of the concept of concentration addition by predicting a concentration and time resolved set of *in silico* toxicogenomic landscapes for the mixture. The predicted set of landscapes resembled the main structures visible in the experimental ones. To get a more detailed view on the quantitative predictivity, we determined prediction ratios for the $minEC_{50}$ for each toxnode. For up to 61% of the toxnodes affected by the mixture the predicted $minEC_{50}$ was in the range of factor 2 of the experimentally determined value. When evaluating MOA specific toxnodes (here potential COX-toxnodes) this proportion increased to 80 % (when combining quantitative with qualitative predictions).

Certainly, the predictions using the concept of CA are far from perfect, and measurements deviate in many cases from the predictions. Those deviations may have several reasons. Most importantly, prediction accuracy depends on the valid characterization of single substance effects. The more subtle an effect appears on the transcriptome, the less likely a mixture effect will be correctly predicted. However, in some cases, also more distinct effects are not captured correctly by the applied regression model. We showed that in some cases a biphasic model (such as the suggested Gauss-Gauss model) has to be applied to capture the single compound responses. A biphasic CA model, suggested by Martin-Betancor et al. (2015), could improve predictivity for selected nodes showing a biphasic concentration-response relationship. In other cases transcript regulation direction changed over time. This effect cannot be captured by the applied regression models and can also lead to false mixture predictions. Therefore, if we want to use the CA predictions as null-hypothesis to identify interaction effects between mixture components on a genome scale, we first have to further reliably separate technically from biologically caused deviations.

4.5 CONCLUSION

In summary, despite the mentioned shortcomings of the predictions, the pharmacologically based concept of concentration addition significantly improves the prediction of mixture effects on transcriptome level, compared to the more trivial assumptions usually made until now. On the other hand, our results should also challenge the assumption that mixture effects might be to complex to predict on transcriptome level (Bluhm et al., 2014). The superiority of CA for the prediction of mixture effects, which was shown here, will even be more pronounced in mixtures containing more components since combination effects are more likely to occur. This has several important implications for the interpretation of (past and future) omics mixture experiments because mixture effects which might have been interpreted as result of compound interaction so far might actually just be caused by a concentration dependent combination effect. The results are also highly relevant when applying integrated approaches of molecular effect prediction and molecular effect surveillance like it is suggested by Schroeder et al. (2016). Here, one should be aware of combination effects possibly leading to effects in the surveillance which cannot be explained by adding up effects of detected chemicals. On the other hand, effects might also "disappear", i.e., be diluted in a mixture due to their concentration dependence. Additionally, as it was already discussed in Chapter 3, toxicogenomic profiles may show time dependent changes, which also should be considered in a mixture assessment. All of this demonstrates that the inclusion of concentration and time information in toxicogenomic databases would provide major added value to the applicability of *omics* data to environmental risk assessment and monitoring since it would allow for a more accurate evaluation of mixture effects.

The three component mixture applied here is still far away from a complex mixture, typically found in the environment. Still, these findings support the notion of the potential application of toxicogenomic tools to detect dominating effect profiles in complex mixtures. This would require that effect profiles established from single component exposures can be recovered in mixtures and that there are no dominating interactions leading to unexplainable effects (Altenburger et al., submitted). Both requirements were found to be met in the described mixture case study.

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REFERENCES

Altenburger, R., T. Backhaus, W. Boedeker, M. Faust, M. Scholze, and L. H. Grimme (2000). “Predictability of the Toxicity of Multiple Chemical Mixtures To Vibrio Fischeri : Mixtures Composed of Similarly Acting Chemicals.” In: *Environmental Toxicology and Chemistry* 19.9, pp. 2341–2347. DOI: 10.1002/etc.5620190926.

Altenburger, R., H. Walter, and M. Grote (Dec. 2004). “What Contributes to the Combined Effect of a Complex Mixture?” In: *Environmental Science & Technology* 38.23, pp. 6353–6362. DOI: 10.1021/es049528k.

Altenburger, R., S. Scholz, M. Schmitt-Jansen, W. Busch, and B. I. Escher (Mar. 2012). “Mixture Toxicity Revisited from a Toxicogenomic Perspective.” In: *Environmental Science & Technology* 46.5, pp. 2508–2522. DOI: 10.1021/es2038036.

Altenburger, R., T. Backhaus, W. Boedeker, M. Faust, and M. Scholze (2013). “Simplifying complexity: Mixture toxicity assessment in the last 20 years." In: *Environmental Toxicology and Chemistry* 32.8, pp. 1685–1687. DOI: 10.1002/etc.2294.

Altenburger, R., W. Busch, B. I. Escher, G. Jakobs, M. Krauss, P. A. Neale, A. C. Almeida, H. Hollert, R. Schlichting, Y. Shao, et al. (submitted). "Mixture effect detection in complex contaminated samples – an interlaboratory comparative study using bioassays."

Backhaus, T., R. Altenburger, W. Boedeker, M. Faust, M. Scholze, and L. H. Grimme (Sept. 2000). "Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri." In: *Environmental Toxicology and Chemistry* 19.9, pp. 2348–2356. DOI: 10.1002/etc.5620190927.

Bahamonde, P. A., A. Feswick, M. A. Isaacs, K. R. Munkittrick, and C. J. Martyniuk (2016). "Defining the role of omics in assessing ecosystem health: Perspectives from the Canadian environmental monitoring program." In: *Environmental Toxicology and Chemistry* 35.1, pp. 20–35. DOI: 10.1002/etc.3218.

Belden, J. B., R. J. Gilliom, and M. J. Lydy (July 2007). "How well can we predict the toxicity of pesticide mixtures to aquatic life?" In: *Integrated Environmental Assessment and Management* 3.3, pp. 364–372. DOI: 10.1002/ieam.5630030307.

Berenbaum, M. C. (1981). "Criteria for analyzing interactions between biologically active agents." In: *Advances in Cancer Research* 35, pp. 269–335. DOI: 10.1016/S0065-230X(08)60912-4.

Bluhm, K., J. C. Otte, L. Yang, C. Zinsmeister, J. Legradi, S. Keiter, T. Kosmehl, T. Braunbeck, U. Strähle, and H. Hollert (2014). "Impacts of different exposure scenarios on transcript abundances in Danio rerio embryos when investigating the toxicological burden of riverine sediments." In: *PLoS ONE* 9.9. DOI: 10.1371/journal.pone.0106523.

Cedergreen, N., C. Svendsen, and T. Backhaus (2013). "Chemical Mixtures : Concepts for Predicting Toxicity." In: *Encyclopedia of Environmental Management*. Ed. by S. E. Jorgensen. New York: Taylor and Francis, pp. 2572–2581. DOI: 10.1081/E-EEM-120046684.

Davis, A. P., C. J. Grondin, R. J. Johnson, D. Sciaky, B. L. King, R. McMorran, J. Wiegers, T. C. Wiegers, and C. J. Mattingly (Jan. 2017). "The Comparative Toxicogenomics Database: update 2017." In: *Nucleic Acids Research* 45.D1, pp. D972–D978. DOI: 10.1093/nar/gkw838.

De Coninck, D. I. M., J. Asselman, S. Glaholt, C. R. Janssen, J. K. Colbourne, J. R. Shaw, and K. a. C. De Schamphelaere (Mar. 2014). "Genome-wide transcription profiles reveal genotype-dependent responses of biological pathways and gene-families in Daphnia exposed to single and mixed stressors." In: *Environmental science & technology* 48.6, pp. 3513–22. DOI: 10.1021/es4053363.

Edgar, R., M. Domrachev, and A. E. Lash (Jan. 2002). "Gene Expression Omnibus: NCBI gene expression and hybridization array data repository." In: *Nucleic Acids Research* 30.1, pp. 207–10. DOI: 10.1093/nar/30.1.207.

Faust, M., R. Altenburger, T. Backhaus, H. Blanck, W. Boedeker, P. Gramatica, V. Hamer, M. Scholze, M. Vighi, and L. H. Grimme (2003). "Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action." In: *Aquatic Toxicology* 63.1, pp. 43–63. DOI: 10.1016/S0166-445X(02)00133-9.

Faust, M. (1999). "Kombinationseffekte von Schadstoffen auf aquatische Organismen: Prüfung der Vorhersagbarkeit am Beispiel einzelliger Grünalgen." PhD thesis. Universität Bremen.

Fitzgerald, J. B., B. Schoeberl, U. B. Nielsen, and P. K. Sorger (2006). "Systems biology and combination therapy in the quest for clinical efficacy." In: *Nature Chemical Biology* 2.9, pp. 458–466. DOI: 10.1038/nchembio817.

Kortenkamp, A., T. Backhaus, and M. Faust (2009). *State of the Art Report on Mixture Toxicity.* Tech. rep., pp. 1–391. DOI: http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf.

Labib, S., A. Williams, B. Kuo, C. L. Yauk, P. A. White, and S. Halappanavar (July 2017). "A framework for the use of single-chemical transcriptomics data in predicting the hazards associated with complex mixtures of polycyclic aromatic hydrocarbons." In: *Archives of Toxicology* 91.7, pp. 2599–2616. DOI: 10.1007/s00204-016-1891-8.

Martin-Betancor, K., C. Ritz, F. Fernández-Piñas, F. Leganés, and I. Rodea-Palomares (2015). "Defining an additivity framework for mixture research in inducible whole-cell biosensors." In: *Scientific Reports* 5, p. 17200. DOI: 10.1038/srep17200.

Perkins, E. J., T. Habib, B. L. Escalon, J. E. Cavallin, L. Thomas, M. Weberg, M. N. Hughes, K. M. Jensen, M. D. Kahl, D. L. Villeneuve, et al. (Aug. 2017). "Prioritization of Contaminants of Emerging Concern in Wastewater Treatment Plant Discharges Using Chemical:Gene Interactions in Caged Fish." In: *Environmental Science & Technology* 51.15, pp. 8701–8712. DOI: 10.1021/acs.est.7b01567.

R Core Team (2016). *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing. Vienna, Austria.

Scholze, M., E. Silva, and A. Kortenkamp (2014). "Extending the applicability of the dose addition model to the assessment of chemical mixtures of partial agonists by using a novel toxic unit extrapolation method." In: *PLoS ONE* 9.2. DOI: 10.1371/journal.pone.0088808.

Schroeder, A. L., G. T. Ankley, A. H. Keith, and D. L.Villeneuve (2016). "Environmental Surveillance and Monitoring - The Next Frontiers for High-Throughput Toxicology." In: *Environmental Toxicology and Chemistry* 35.3, pp. 513–525. DOI: 10.1002/etc.3309.

Schroeder, A. L., D. Martinović-Weigelt, G. T. Ankley, K. E. Lee, N. Garcia-Reyero, E. J. Perkins, H. L. Schoenfuss, and D. L. Villeneuve (Feb. 2017). “Prior knowledge-based approach for associating contaminants with biological effects: A case study in the St. Croix River basin, MN, WI, USA.” In: *Environmental Pollution* 221, pp. 427–436. DOI: 10.1016/j.envpol.2016.12.005.

Silva, E., N. Rajapakse, and A. Kortenkamp (Apr. 2002). “Something from “Nothing” - Eight Weak Estrogenic Chemicals Combined at Concentrations below NOECs Produce Significant Mixture Effects.” In: *Environmental Science & Technology* 36.8, pp. 1751–1756. DOI: 10.1021/es0101227.

Yang, L., J. R. Kemadjou, C. Zinsmeister, M. Bauer, J. Legradi, F. Müller, M. Pankratz, J. Jäkel, and U. Strähle (Jan. 2007). “Transcriptional profiling reveals barcode-like toxicogenomic responses in the zebrafish embryo.” In: *Genome Biology* 8.10, R227. DOI: 10.1186/gb-2007-8-10-r227.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

A non-target bioanalytical tool for the diagnosis of chemical effects on (aquatic) organisms could provide a tremendous improvement for assessing and safeguarding environmental health. A few case studies have demonstrated how *omics* techniques can offer non-targeted biological effect information for environmental sites (Perkins et al., 2017; Schroeder et al., 2016; Schroeder et al., 2017). However, some complications still need to be overcome until an application like envisioned in Section 1.3 is feasible. This includes the development of efficient and informative experimental designs and analysis strategies, which facilitate the reproducibility and interconnection between different studies, as well as the consideration of mixture effects. This dissertation aimed at tackling these challenges arriving at the following key findings:

- In a meta-analysis of published transcriptome datasets, genes could be identified which show a common trend of transcriptional regulation in response to exposure against specific groups of chemicals, or in specific exposure settings. It was shown that experimental factors, such as embryo age or the applied effect concentration may significantly alter the type or extent of transcriptome responses. Thus, heterogeneous exposure settings probably obscure a more pronounced overlap in responses, which would have been expected for groups of commonly acting substances, for example.
- The algorithm of self-organizing maps (SOMs) offers a promising strategy for integrating various toxicogenomic datasets, thereby increasing robustness, digestibility and ability to interpret the data as well as compare the effects of different compounds or exposure settings. Here, ZFE transcriptome data from 358 different exposure conditions were used to compile a Zebrafish Embryo Toxicogenomic Universe (ZETU). This provides a universal coordinate system to project toxicogenomic compound landscapes.
- A regression model was developed, which can sufficiently describe time and concentration dependent regulation for a majority of genes, facilitating extrapolation and mixture prediction. Additionally, our results confirmed that gene regulation in response to chemical exposure is time and concentration dependent.
- Combination effects could be observed and predicted on transcriptome level. The concept of concentration addition (CA) performed best in comparison to the concepts of independent action (IA), effect addition (EA), and boolean mixture (BO) regarding qualitative and quantitative prediction of combination effects.

- The concept of CA also provided a reasonable prediction of the toxicogenomic landscape which can be expected from a mixture exposure. The prediction quality primarily depended on the quality of single compound profiles.

These findings may have several implications for further research and applications of toxicogenomics, which will be discussed in the following.

Robustness of toxicogenomic profiles could be increased by clustering similar responding transcripts and establishing time and concentration response relationships.

ROBUSTNESS AND REPRODUCIBILITY There are concerns about high variation and low reproducibility in *omics* experiments. Interlaboratory studies by Vidal-Dorsch et al. (2016) and Feswick et al. (2017), comparing transcriptome measurements, showed that there can be significant differences between the results of different laboratories, even when analyzing aliquots of the same tissue sample. Certainly, *omics* measurements reflect biological variance between individuals, and the choice of array or sequencing technology, as well as differences in laboratory practices may add additional sources of variation. However, our analyses showed an overall concordance in transcript regulation between independent experiments, which were conducted several months apart. Distinct toxnode regulations could be recovered in independent experiments. Strikingly, our extrapolation efforts with the help of regression modeling could correctly predict gene regulation for lower exposure concentrations. A potential gain of robustness can be achieved by clustering the transcripts (e. g., as performed here with self-organizing maps, in principle other methods are conceivable too and future research might come up with even better methods). Due to the clustering, outlying measurements have less weight in the analysis. It should be subject of further research, whether clustering approaches like SOMs could also lead to more robust results across different laboratories.

Another important step to increase robustness is the establishment of concentration (and time) response relationships. Thereby one adds biological significance to a mere statistical one, when assembling gene lists based on statistical tests. In this work, a regression model was suggested, which is suitable to establish exposure concentration and time dependent response relationships for single genes or gene sets (here, toxnodes). From evaluating the certainty of the fitted model parameters we can conclude that the experimental design used here should be adapted to assess more concentrations. Anyway, the experimental design and analysis pipeline, which was suggested here, demonstrates an approach to infer robust toxicogenomic profiles.

GENERALITY Closely connected to the issue of robustness is the one of generality. If we want to follow the vision of diagnosing environmental health with the help of *omics* techniques, this implies a categorization of pharmacological or toxicological effects (e.g., COX inhibitor, estrogenic, cyp P450 inducer). This categorization will only be possible if we can identify general patterns in transcriptome responses. Primarily, the answers to two questions need to be found: a) What do all toxicogenomic profiles/landscapes *within* a group of similar acting compounds have in common?; and b) what is the *difference* from compounds with other MOAs? At the same time we will have to differentiate between patterns indicating a specific molecular response and patterns which are more general or dependent on other factors, e.g. toxicokinetic properties of substances. In other words, we should identify the points at which different molecular AOPs converge.

The elucidation of commonalities in toxicogenomic profiles is a prerequisite for the application in effect categorization.

In my perception, the issue of generality was no major focus in toxicogenomic studies published so far. Here, it was a central goal to evaluate common responses in published datasets (Chapter 2) and develop an experimental design and analysis pipeline improving our ability to compare responses between compounds (Chapter 3).

If we want to use toxicogenomic patterns to categorize biological effects, a challenge lies in an incomplete reference grouping. While for compounds applied as pharmaceuticals usually the therapeutic target (in mammals) is known, there might be additional unknown targets. For other compounds and other organisms there might be no MOA known at all (such as for the effects of diuron in the ZFE). Therefore, the definition of characteristic patterns probably has to be tackled from two ends: On the one hand, we should find common characteristics for compounds with *known* common MOA; on the other hand we should use a sufficient data set of toxicogenomic profiles to group *compounds* according to their toxicogenomic effect profile in an unsupervised manner.

COMPLEXITY OF GENE REGULATION AND COMBINATION EFFECTS Given the complex interactions between elements of molecular regulatory networks, it may be suspected that regulation of genes or gene sets is hardly predictable and governed by emergent properties (especially when evaluating mixture effects). This was suspected by Bluhm et al. (2014), for example, who speculated that the toxicogenomic profile of a mixture might not resemble the profiles of its components. However, in our case study the opposite was true. The regulation of most genes (respectively toxnodes in the context of our analysis pipeline) was well predictable also in the context of a mixture exposure. Additionally, the majority of combination effects, which occurred in the mixture exposure, could be predicted by applying the concept of concentration addition (CA).

Regulation of gene transcription is complex – however, patterns can be described by simple regression modeling.

A boolean mixture concept proved to be too simplistic to describe mixture effects on transcriptome, CA describes mixture effects sufficiently in many cases.

On the other hand, some studies oversimplify expected mixture effects by assuming boolean mixture effects (Perkins et al., 2017; Schroeder et al., 2016; Schroeder et al., 2017), which turned out to give very imprecise expectations in our case study. To be able to account for combination effects in mixture predictions, toxicogenomic profiles should include concentration dependence in future studies. The encouraging findings of our case study regarding the predictability of mixture and combination effects should be deliberately extended to multi-component mixtures in future.

INTERPRETATION OF REGULATED GENES One of the greatest remaining challenge for the application of *omics* techniques in (eco)toxicological sciences is the functional interpretation of the identified (significantly) regulated genes. To functionally interpret gene expression data, Subramanian et al. (2005) established the method of gene set enrichment analysis (GSEA). Recently, Dean et al. (2017) suggested to combine GSEA with benchmark dose modeling to retrieve compound specific dose ranges leading to the activation of enriched signaling pathways. Additionally, commercial software tools like *Ingenuity Pathway Analysis* provide extensive curated knowledge bases integrated with sophisticated analysis tools (Krämer et al., 2014). However, there are also doubts about the *guilt-by-association* strategy to predict gene functions, and despite all annotation efforts it is still not trivial to derive a functional interpretation from a toxicogenomic profile. This is partly due to the multi-functionality of many genes (Gillis and Pavlidis, 2011), but also to the lack of concentration response relationships. These would support functional association of genes and allow differentiating between specific and more general toxnodes. In this work, the functional interpretation of affected gene sets was not the main focus and only exemplarily performed. However, the COX-inhibitor pattern in the toxicogenomic landscape, which was identified here, provides a good starting point for mechanistically evaluating the effect of inhibiting COX in the ZFE.

The interpretation of regulated genes remains challenging. The establishment of reference databases including concentration (and time) dependent toxicogenomic exposure and effect patterns would be helpful for the application of toxicogenomics in environmental monitoring.

With regard to the development of a non-target bioanalytical tool, I would recommend the establishment of reference profiles for a set of representative compounds, for which we have knowledge about their molecular action. Subsequently, the regulation of specific gene clusters (e.g., toxnodes) may be connected to the exposure to a specific compound group.

Additionally, toxicogenomic patterns should be connected to expected adverse outcomes. This could either be achieved by direct correlation between toxicogenomic patterns and adverse effects ("phenotypic anchoring", Paules, 2003) or by retrieving information about disturbed molecular processes from a toxicogenomic profile and subsequently predicting higher level effects with the help of adverse outcome pathways (AOPs) (compare Brockmeier et al., 2017).

To make the establishment of a reference database of toxicogenomic exposure and effect patterns economically feasible, one could consider the application of reduced arrays (see below).

In the long term, the usefulness of functional annotation databases like Gene Ontology (GO, Ashburner et al., 2000), Molecular Signatures Database (MSigDB, Liberzon et al., 2011), or Comparitive Toxicogenomics Database (CTD, Davis et al., 2017), would significantly increase with the inclusion of quantitative exposure information and concentration response relationships.

EMBRYO DEVELOPMENT While the zebrafish embryo offers several advantages as model organism, it poses the challenge that it undergoes quite drastic changes during the course of the exposure. While we could successfully describe the changes in gene regulation induced by compound exposure, we neglected (i.e., normalized) the changes of gene expression due to embryo development. To get closer to a mechanistic understanding of compound action, a future goal should be to describe regulation of each gene (or toxnode) in normal development and then model the deviations induced by chemicals.

ECONOMIC COSTS A major confounding factor for establishing extensive toxicogenomic datasets is the price of *omics* measurements. Therefore, there have been efforts to use reduced arrays implying much lower costs, which allows to establish extensive data sets (compare Subramanian et al., 2017). Recently, a reduced array for the zebrafish transcriptome was suggested (Wang et al., 2018) including representative genes for a range of biological pathways. However, this selection was not based on zebrafish experimental data, but focused on orthologues of genes known to be important in mammalian toxicology. Therefore, as an alternative approach to design a reduced zebrafish array we would suggest to select a representative gene for each toxnode of the Zebrafish Embryo Toxicogenomic Universe (ZETU).

Reduced arrays may facilitate high throughput transcriptome profiling.

First, it might be necessary though to derive whole transcriptome toxicogenomic profiles of a broader range of chemicals. The experimental design applied here could be used as a guidance for such experimentation. After updating the ZETU, representative genes for each toxnode might be chosen for a reduced array.

SOMs could guide the design of efficient reduced arrays.

BIG DATA PARADIGM In the field of high-content methodologies, where massive amounts of data are generated, there has been a paradigm shift in scientific methodology, which has been described as a shift from theoretical science ("second paradigm") and computational science ("third paradigm") to exploratory science ("fourth paradigm") (Bell et al., 2009; Kitchin, 2014). This shift has let scientists declare the "end of theory" and propose to let "the numbers speak for themselves"

(Anderson, 2008). Indeed, the approaches taken in many (eco)toxicogenomic studies seem to follow this envisioned change in scientific methods, such that explicit study hypotheses are rarely stated or falsified. Rather, the classical approach in ecotoxicogenomics is the measurement and analysis of an *omics* profile after a more or less arbitrarily chosen compound exposure. Subsequently the data are screened for somewhat interesting patterns or structures. This leads to findings *what* happens, but rarely it can be resolved *why* something happens. While in some areas data might indeed be rich enough to be used in a solely exploratory fashion, in my perception toxicogenomics is far from this point. In spite of over 2×10^6 gene expression profiles in the Gene Expression Omnibus (GEO) database, this still represents a quite strict selection of biological instances, and as Leonelli (2014) states, the selection "is not the result of scientific choices, which can therefore be taken into account when analyzing the data. Rather, it is the serendipitous result of social, political, economic and technical factors". In this work I tried to demonstrate from different ends that application of high-content data can (and should) be combined with theoretical reasoning to progress and apply our understanding of biological processes. Only careful experimental design and "ancient" mixture theory allowed to make a step forward in *omics* mixture toxicology. This supports the notion of Mazzocchi (2015), who stated that with the big data era "there is no 'end of theory' but only new opportunities".

High content data in toxicogenomics does not supersede careful experimental design based on theoretical concepts.

In summary, it can be concluded that transcriptome analyses bear the potential as a non-target bioanalytical tool. Many of the previously identified drawbacks and hurdles can be overcome by conducting more strategic experiments and establishing valid toxicogenomic concentration response profiles. My dissertation is meant to be a step into this direction. It offers a strategy how to acquire and analyze time and concentration resolved toxicogenomic data as well as a strategy to predict and validate combination effects, which may be a valuable improvement when aiming at diagnosing chemical mixtures with the help of *omics* analyses.

REFERENCES

Anderson, C. (2008). *The End of Theory: The Data Deluge Makes the Scientific Method Obsolete*. WIRED. URL: `https://www.wired.com/2008/06/pb-theory/` (visited on 01/11/2018).

Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, J. T. Eppig, et al. (2000). "Gene ontology: Tool for the unification of biology." In: *Nature Genetics* 25.1, pp. 25–29. DOI: 10.1038/75556.

Bell, G., T. Hey, and A. Szalay (Mar. 2009). "COMPUTER SCIENCE: Beyond the Data Deluge." In: *Science* 323.5919, pp. 1297–1298. DOI: 10.1126/science.1170411.

Bluhm, K., J. C. Otte, L. Yang, C. Zinsmeister, J. Legradi, S. Keiter, T. Kosmehl, T. Braunbeck, U. Strähle, and H. Hollert (2014). "Impacts of different exposure scenarios on transcript abundances in Danio rerio embryos when investigating the toxicological burden of riverine sediments." In: *PLoS ONE* 9.9. DOI: 10.1371/journal.pone.0106523.

Brockmeier, E. K., G. Hodges, T. H. Hutchinson, E. Butler, M. Hecker, K. E. Tollefsen, N. Garcia-Reyero, P. Kille, D. Becker, K. Chipman, et al. (Aug. 2017). "The Role of Omics in the Application of Adverse Outcome Pathways for Chemical Risk Assessment." In: *Toxicological Sciences* 158.2, pp. 252–262. DOI: 10.1093/toxsci/kfx097.

Davis, A. P., C. J. Grondin, R. J. Johnson, D. Sciaky, B. L. King, R. McMorran, J. Wiegers, T. C. Wiegers, and C. J. Mattingly (Jan. 2017). "The Comparative Toxicogenomics Database: update 2017." In: *Nucleic Acids Research* 45.D1, pp. D972–D978. DOI: 10.1093/nar/gkw838.

Dean, J. L., Q. Jay Zhao, J. C. Lambert, B. S. Hawkins, R. S. Thomas, and S. C. Wesselkamper (Jan. 2017). "Application of Gene Set Enrichment Analysis for Identification of Chemically-Induced, Biologically Relevant Transcriptomic Networks and Potential Utilization in Human Health Risk Assessment." In: *Toxicological Sciences* 157.1, pp. 85–99. DOI: 10.1093/toxsci/kfx021.

Feswick, A., M. Isaacs, A. Biales, R. W. Flick, D. C. Bencic, R.-L. Wang, C. Vulpe, M. Brown-Augustine, A. Loguinov, F. Falciani, et al. (Oct. 2017). "How consistent are we? Interlaboratory comparison study in fathead minnows using the model estrogen 17α-ethinylestradiol to develop recommendations for environmental transcriptomics." In: *Environmental Toxicology and Chemistry* 36.10, pp. 2614–2623. DOI: 10.1002/etc.3799.

Gillis, J. and P. Pavlidis (Feb. 2011). "The impact of multifunctional genes on guilt "by association "analysis." In: *PLoS ONE* 6.2. Ed. by J. Bader, e17258. DOI: 10.1371/journal.pone.0017258.

Kitchin, R. (July 2014). "Big Data, new epistemologies and paradigm shifts." In: *Big Data & Society* 1.1, pp. 1–12. DOI: 10.1177/2053951714528481.

Krämer, A., J. Green, J. Pollard, and S. Tugendreich (Feb. 2014). "Causal analysis approaches in Ingenuity Pathway Analysis." In: *Bioinformatics* 30.4, pp. 523–530. DOI: 10.1093/bioinformatics/btt703.

Leonelli, S. (2014). "What difference does quantity make? On the epistemology of Big Data in biology." In: *Big Data & Society* 1.1, pp. 1–11. DOI: 10.1177/2053951714534395.

Liberzon, A., A. Subramanian, R. Pinchback, H. Thorvaldsdóttir, P. Tamayo, and J. P. Mesirov (2011). "Molecular signatures database (MSigDB) 3.0." In: *Bioinformatics* 27.12, pp. 1739–1740. DOI: 10.1093/bioinformatics/btr260.

Mazzocchi, F. (2015). "Could Big Data be the end of theory in science? A few remarks on the epistemology of data-driven science." In: *EMBO reports* 16.10, pp. 1250–1255. DOI: 10.15252/embr.201541001.

Paules, R. (May 2003). "Phenotypic Anchoring: Linking Cause and Effect." In: *Environmental Health Perspectives* 111.6, a338–a339. DOI: 10.1289/ehp.111-a338.

Perkins, E. J., T. Habib, B. L. Escalon, J. E. Cavallin, L. Thomas, M. Weberg, M. N. Hughes, K. M. Jensen, M. D. Kahl, D. L. Villeneuve, et al. (Aug. 2017). "Prioritization of Contaminants of Emerging Concern in Wastewater Treatment Plant Discharges Using Chemical:Gene Interactions in Caged Fish." In: *Environmental Science & Technology* 51.15, pp. 8701–8712. DOI: 10.1021/acs.est.7b01567.

Schroeder, A. L., G. T. Ankley, A. H. Keith, and D. L.Villeneuve (2016). "Environmental Surveillance and Monitoring - The Next Frontiers for High-Throughput Toxicology." In: *Environmental Toxicology and Chemistry* 35.3, pp. 513–525. DOI: 10.1002/etc.3309.

Schroeder, A. L., D. Martinović-Weigelt, G. T. Ankley, K. E. Lee, N. Garcia-Reyero, E. J. Perkins, H. L. Schoenfuss, and D. L. Villeneuve (Feb. 2017). "Prior knowledge-based approach for associating contaminants with biological effects: A case study in the St. Croix River basin, MN, WI, USA." In: *Environmental Pollution* 221, pp. 427–436. DOI: 10.1016/j.envpol.2016.12.005.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, et al. (Oct. 2005). "Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles." In: *Proceedings of the National Academy of Sciences of the United States of America* 102.43, pp. 15545–50. DOI: 10.1073/pnas.0506580102.

Subramanian, A., R. Narayan, S. M. Corsello, D. D. Peck, T. E. Natoli, X. Lu, J. Gould, J. F. Davis, A. A. Tubelli, J. K. Asiedu, et al. (2017). "A Next Generation Connectivity Map: L1000 Platform and the First 1,000,000 Profiles." In: *Cell* 171.6, 1437–1452.e17. DOI: 10.1016/j.cell.2017.10.049.

Vidal-Dorsch, D. E., S. M. Bay, S. Moore, B. Layton, A. C. Mehinto, C. D. Vulpe, M. Brown-Augustine, A. Loguinov, H. Poynton, N. Garcia-Reyero, et al. (2016). "Ecotoxicogenomics: Microarray interlaboratory comparability." In: *Chemosphere* 144, pp. 193–200. DOI: 10.1016/j.chemosphere.2015.08.019.

Wang, P., P. Xia, J. Yang, Z. Wang, Y. Peng, W. Shi, D. L. Villeneuve, H. Yu, and X. Zhang (Jan. 2018). "A Reduced Transcriptome Approach to Assess Environmental Toxicants Using Zebrafish Embryo Test." In: *Environmental Science & Technology* 52.2, pp. 821–830. DOI: 10.1021/acs.est.7b04073.

APPENDIX

A

SUPPLEMENTAL INFORMATION CHAPTER 2

A.1 QUALITY CONTROL, NORMALIZATION

All studies included in the meta-analysis followed MIAME-Guidelines meaning that raw data and sufficient sample and array annotation was supplied by the authors for a reanalysis (Brazma et al., 2001). If available, the processed signal intensity was taken as transcription measure, for other arrays background subtraction was applied. For Affymetrix arrays the package "oligo" was used to extract transcription measures from the raw data (Carvalho and Irizarry, 2010). As quality control the inter-quartile ranges (iqr) of each array was compared within each dataset. If the iqr of a single array diverged for more than 3 standard deviations from the median iqr in one dataset, this array was not further considered in the analysis (see figure S2 for an example). If duplicate probe spots were present on the array the median signal was taken as probe value. Probes that did not show a signal above the negative control probes in most samples (0.9*(Number of Samples in Dataset/Number of Contrasts in Dataset)) were removed from analysis. If no negative control probes were present on the array, the 5% quantile of all arrays in the dataset was taken as negative control cutoff. If batch information was available for a dataset, batch correction was applied using the empirical Bayes based method "ComBat" (Johnson et al., 2007), with compound name, concentration and measurement time (hpf) given as covariates. This method has outperformed other batch correction methods in comparison studies (Chen et al., 2011).

A.2 ANNOTATION

The platform information files were downloaded from GEO or ArrayExpress in *.soft format when available, otherwise probe sequence information was directly downloaded from the manufacturer homepage. From each platform file the probe IDs and corresponding nucleotide sequences were extracted and stored in FASTA format. The probes were then mapped against the *Danio rerio* genome (DanRer10/GRCz10, September 2014) using stand-alone BLAT (Kent, 2002) with maximum Intron size set to 380000 (which is the maximum known intron size for *Danio rerio* according to Moss et al. (2011)), the minimum Identity to 95% the tileSize to 9 and stepSize to 5, the minimum Score to 19. These settings were chosen for a fine mapping, also allowing for shorter probe sequences (as it is the case for Affymetrix Arrays). Only hits uniquely mapping to a certain region of the zebrafish genome were further considered. The mapped regions were subsequently annotated with Ensembl Gene IDs. If a probe "exon" showed overlap of at least one nucleotide with an annotated exon or UTR in the Ensembl Database (Ensembl Release 80, May 2015), it was considered a valid annotation. If no annotation was found in the Ensembl Database, the RefSeq-Database was queried additionally (RefSeq annotation of GRCz10, 26.09.2014).

In parallel to genome mapping of the probes, the probes were mapped to the *Danio rerio* cDNA database (Ensembl Release 80, May 2015) using BLAT (maximum Intron size = 0, remaining parameters as in genome mapping), as in genome annotation only unique hits were considered. If there was conflicting information between genome and cDNA annotation the corresponding probes were not considered for analysis. Only probes with explicit annotation were considered in further analysis. If several probes existed for one gene, the probe with the highest IQR was taken for analysis.

For Affymetrix Arrays annotation of single probes were summarized on probeset level. If more than 50% of probes in a probeset were uniquely annotated with the same Gene ID, the respective Gene ID was considered a valid annotation for the probeset.

A.3 RETRIEVAL OF GENESETS FOR FUNCTIONAL ANNOTATION

For functional annotation a manually combined library of gene sets was created from selected databases deemed to be most relevant for zebrafish gene annotation. All genes annotated with a common annotation term were grouped together in one gene set. Usually each gene is member of several different gene sets. Only gene sets containing more than 10 and less than 400 genes were considered for analysis. Annotation of KEGG zebrafish pathways was retrieved using the R package "KEGGREST" (Tenenbaum, 2015), ZFIN annotation was directly downloaded from the ZFIN database (`www.zfin.org/downloads`). Gene Ontology and Interpro-Domain annotation was retrieved using the R package "biomaRt" (Durinck et al., 2005, 2009). Zebrafish WikiPathways were directly downloaded from the WikiPathways database (`www.wikipathways.org`).

Additionally, gene sets containing genes with common transcription factor binding sites as defined in the TRANSFAC database (version 7.4, http://www.gene-regulation.com/) were retrieved from the Molecular Signatures Database (v5.2) (Subramanian et al., 2005). For enrichment analysis of transcription factor binding sites, zebrafish genes were converted to their human orthologues using the HGNC Database (Gray et al., 2015).

A.4 SUPPLEMENTAL FIGURES

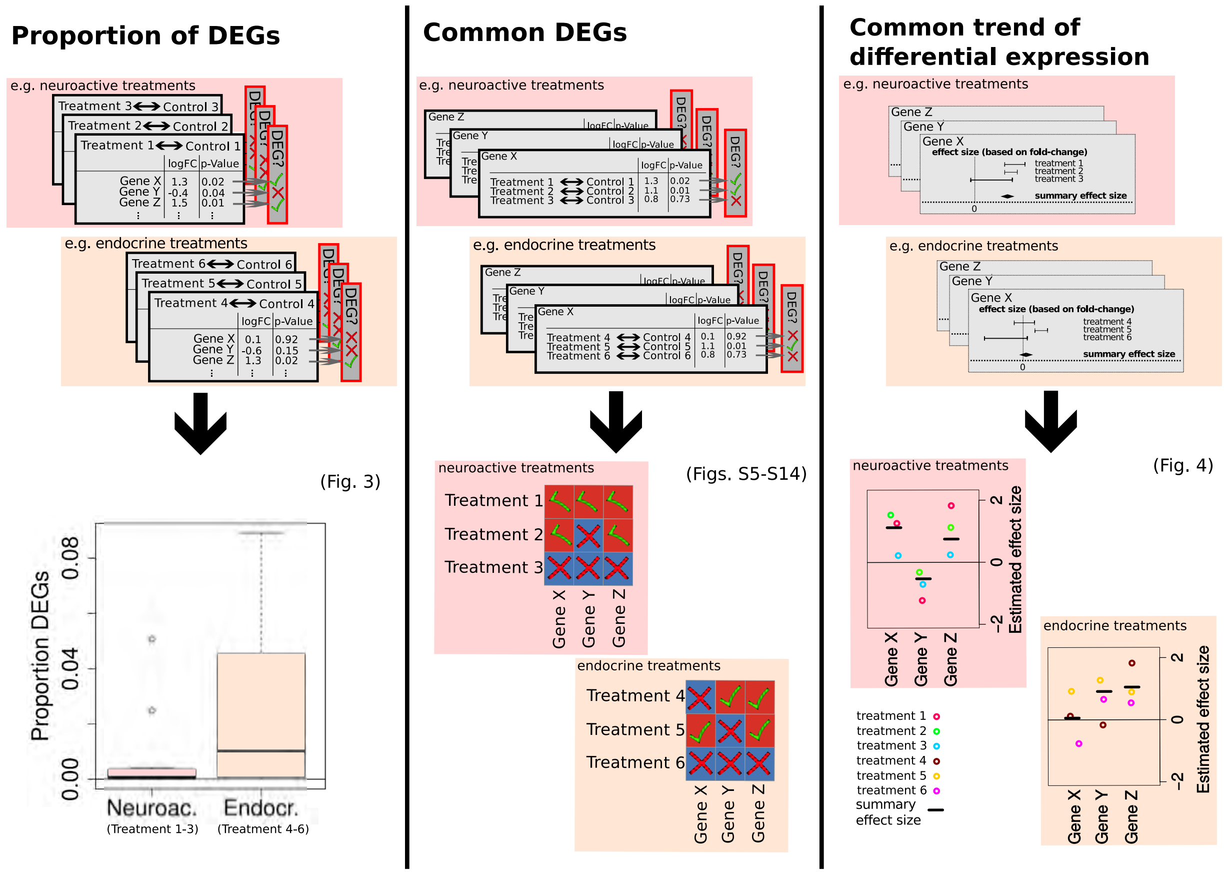


Figure A.1: Meta-analysis approach: From left to right: 1) Analysis of proportion of differentially transcribed/expressed genes (DEGs); Distributions of DEG proportions are compared across treatment groups (e.g treatments using neuroactive substances vs. treatments using endocrine acting substances); 2) Those genes are sought after which are differentially expressed in all (many) treatments of one treatment group; 3) Genes are sought after which show common trends of differential expression (determined by summary effect size)

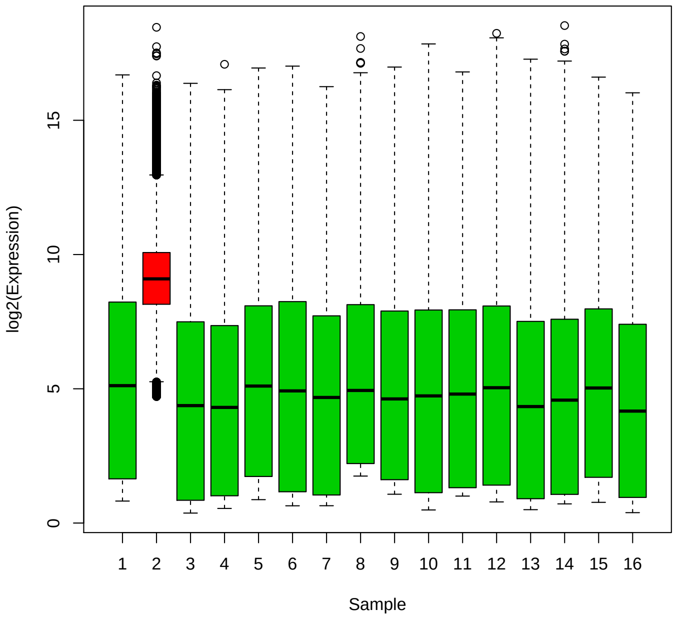
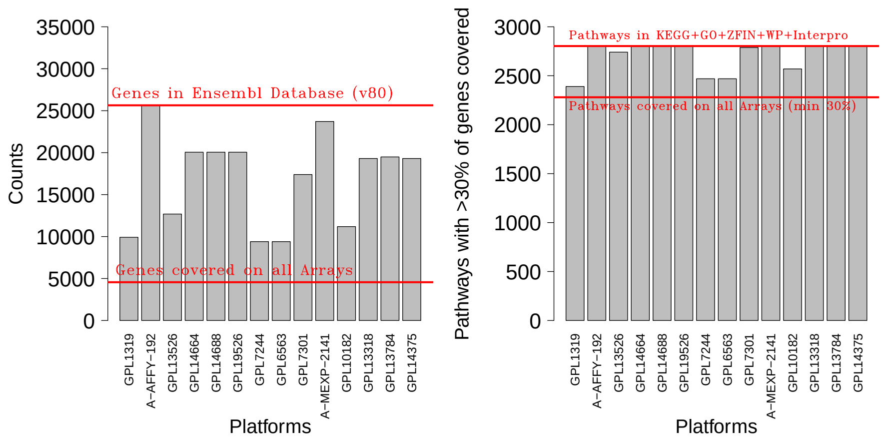
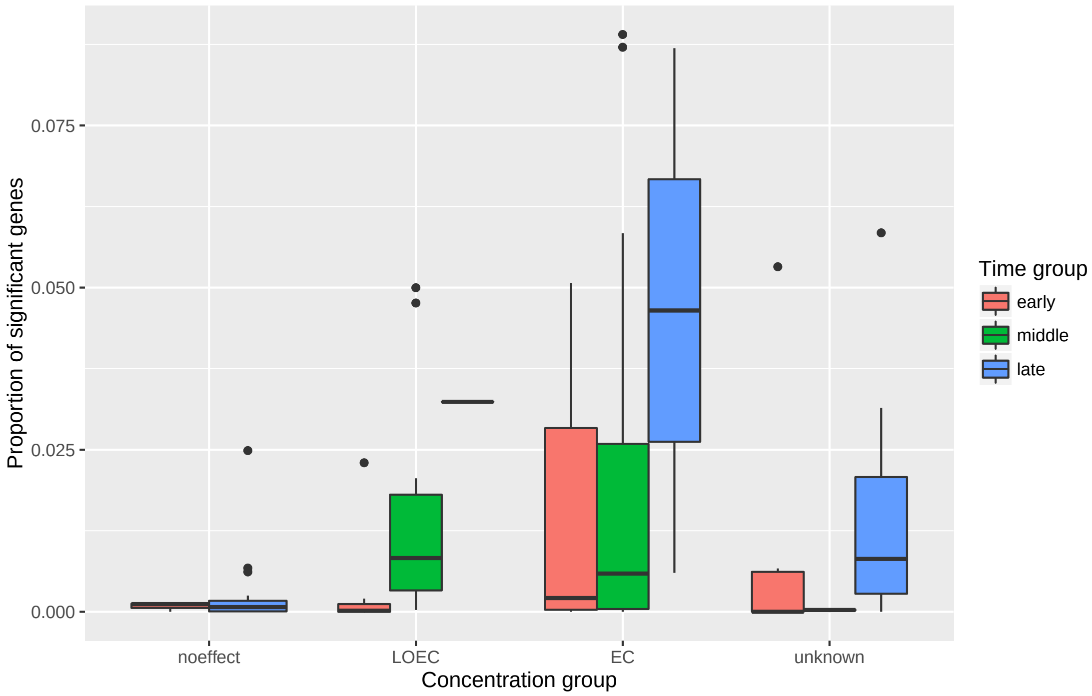


Figure A.2: Boxplot of signal intensity distribution within samples of one dataset. The interquartile range of Sample 2 (red) is more than three standard deviations smaller than the median iqr in the dataset. It is not further considered in the analyis.

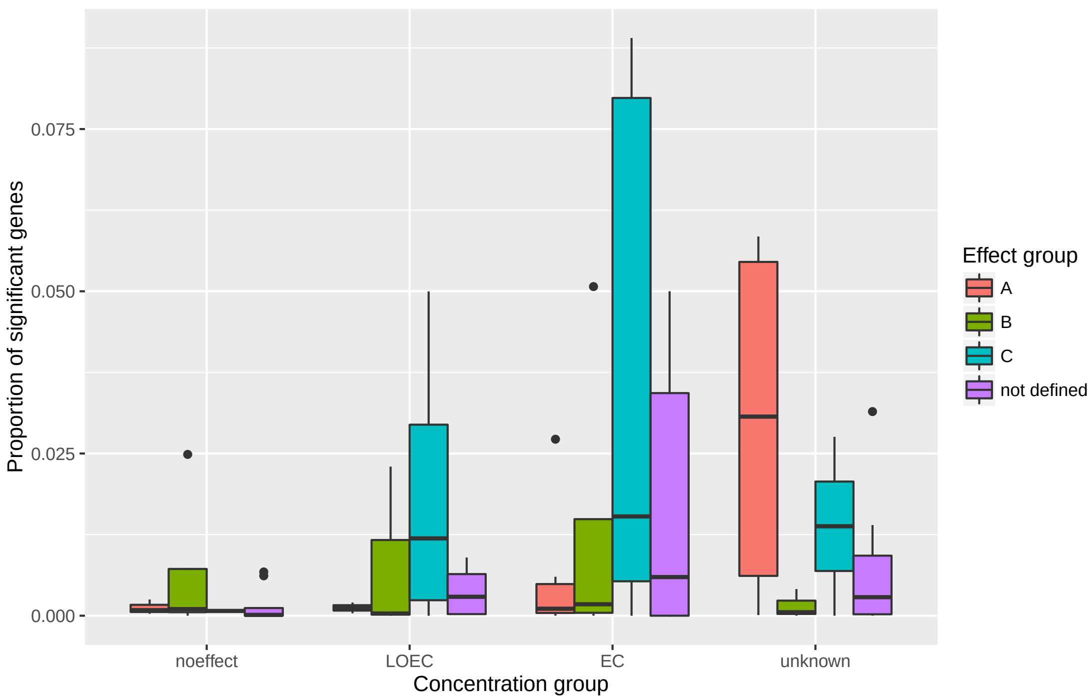


(a) genes (b) gene sets

Figure A.3: Coverage of the recent *Danio rerio* transcriptome (danRer10/Ensembl v80) by the microarray platforms used in the analyzed studies. (A) Bars represent the number of unique genes covered on the array. Red lines show the number of genes in the Ensembl Database, and the number of gene covered on all arrays. The plot shows that many platforms only reliably cover roughly 50% of all genes in the database. Consequently, no more than 12,000 genes can be compared across 50% of all contrasts. If one wants to compare across all contrasts, this is only possible for roughly 5,000 genes. (B) Coverage of database genesets wikipahtways, KEGG, GO, ZFIN and Interpro by the microarray platforms used in the analyzed studies. Bars represent the number of pathways of which a minimum of 30% of genes are covered on the respective array. Red lines show the number of pathways in all databases, and the number of pathways covered on all arrays. The plot shows that nearly all gene sets are covered by a significant amount of genes on all arrays.



(a) Concentration and Time



(b) Concentration and Mode of action

Figure A.4: Boxplot of proportions of differentially expressed genes (DEGs) among all measured genes in respective treatments sorted according to time, concentration and mode of action

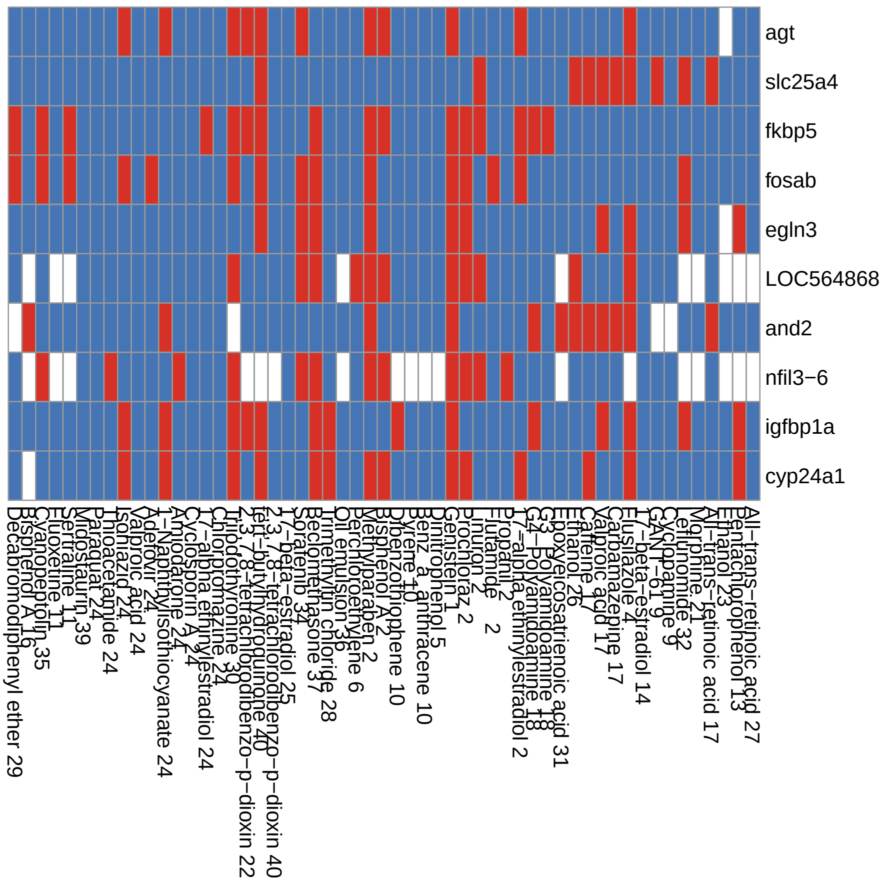


Figure A.5: Top 10 occuring differentially expressed genes (DEGs) across all treatments. Red = differentially expressed, blue = not differentially expressed, white = no information.

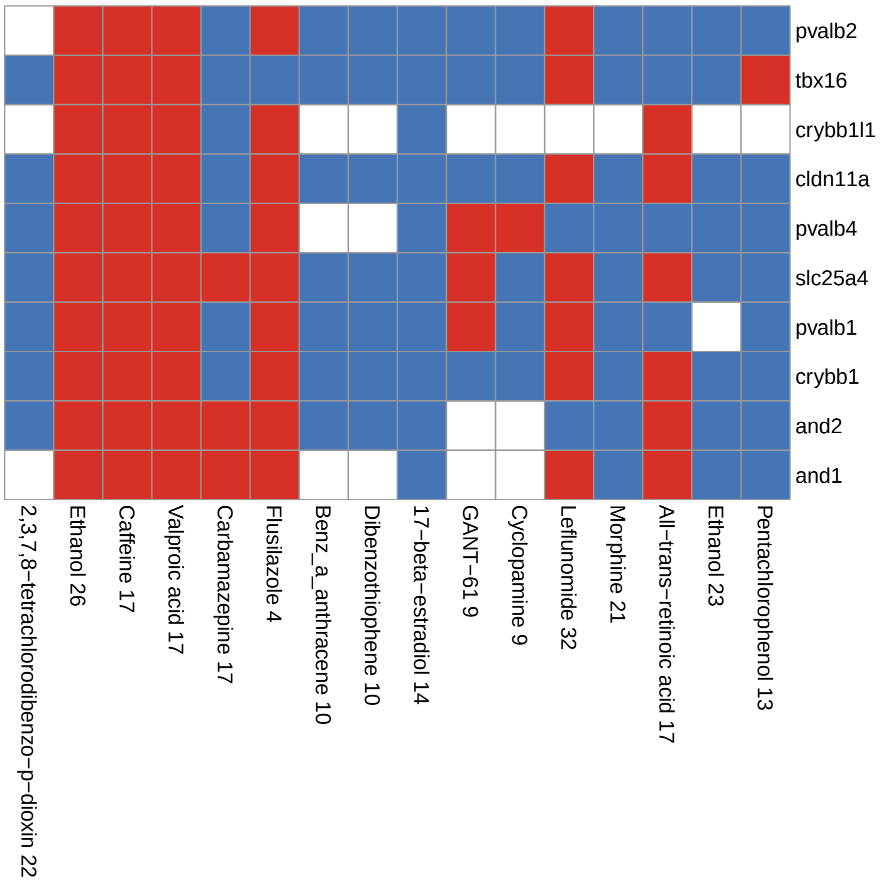


Figure A.6: Top 10 occuring differentially expressed genes (DEGs) across early treatments (exposure end before 24 hpf). Red = differentially expressed, blue = not differentially expressed, white = no information.

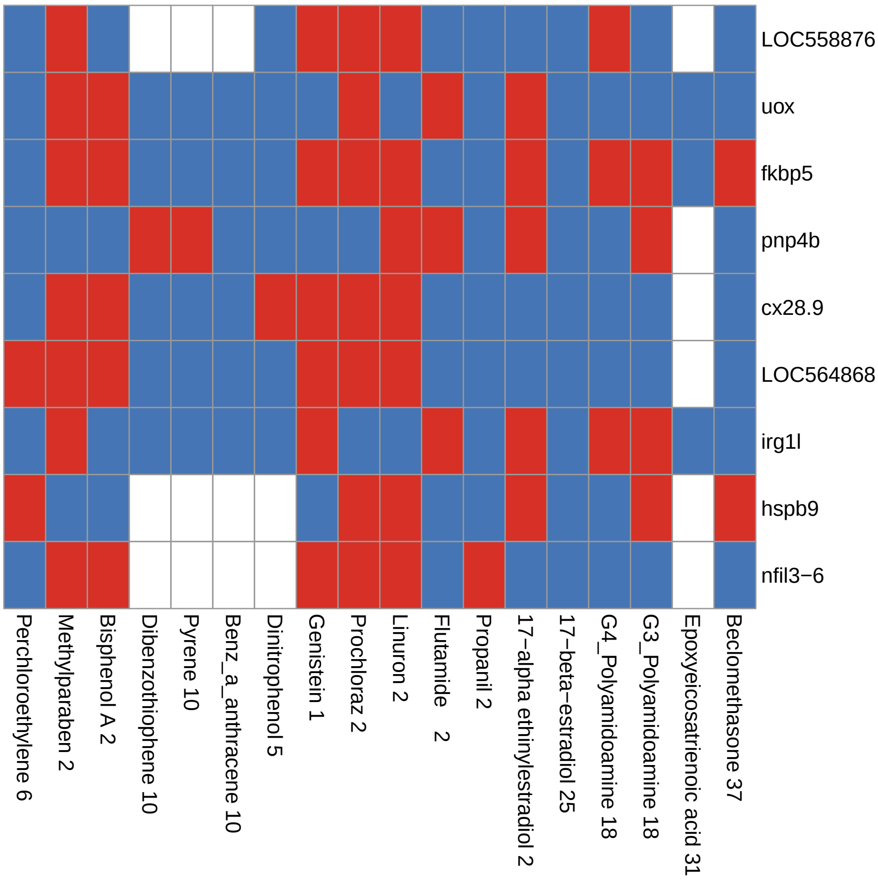


Figure A.7: Top 10 occuring differentially expressed genes (DEGs) across middle treatments (exposure end before 50 hpf). Red = differentially expressed, blue = not differentially expressed, white = no information.

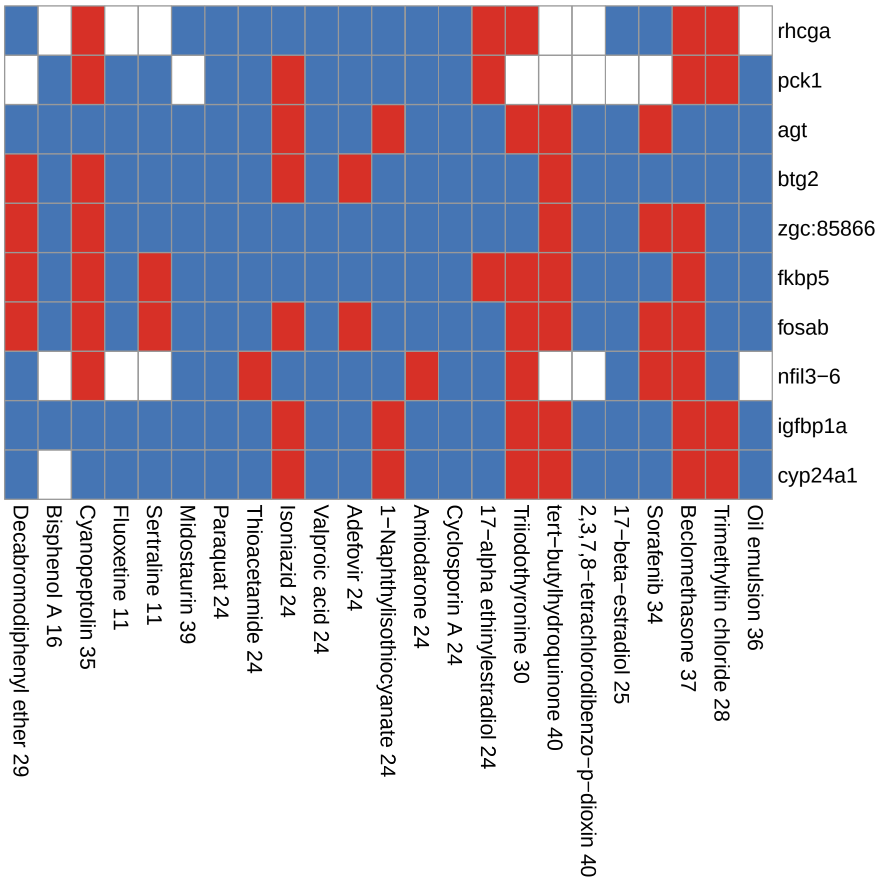


Figure A.8: Top 10 occuring differentially expressed genes (DEGs) across late treatments (exposure end after 50 hpf). Red = differentially expressed, blue = not differentially expressed, white = no information.

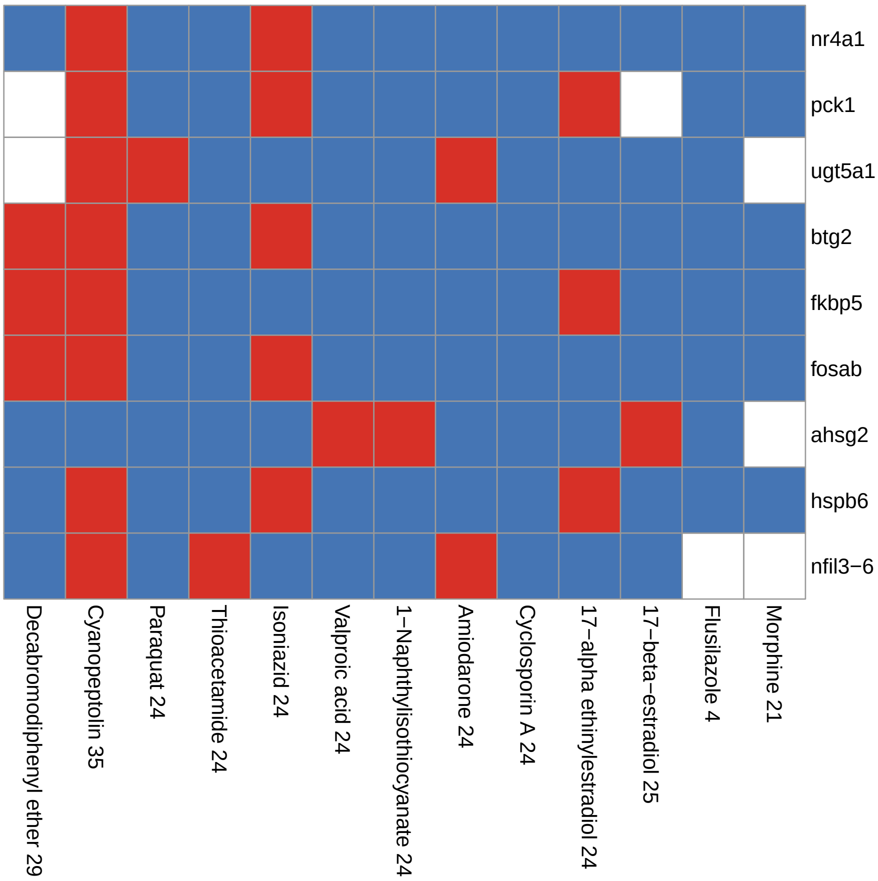


Figure A.9: Top 10 occuring differentially expressed genes (DEGs) across no-effect treatments (applied concentration had no reported phenotypic effect in the experiment). Red = differentially expressed, blue = not differentially expressed, white = no information.

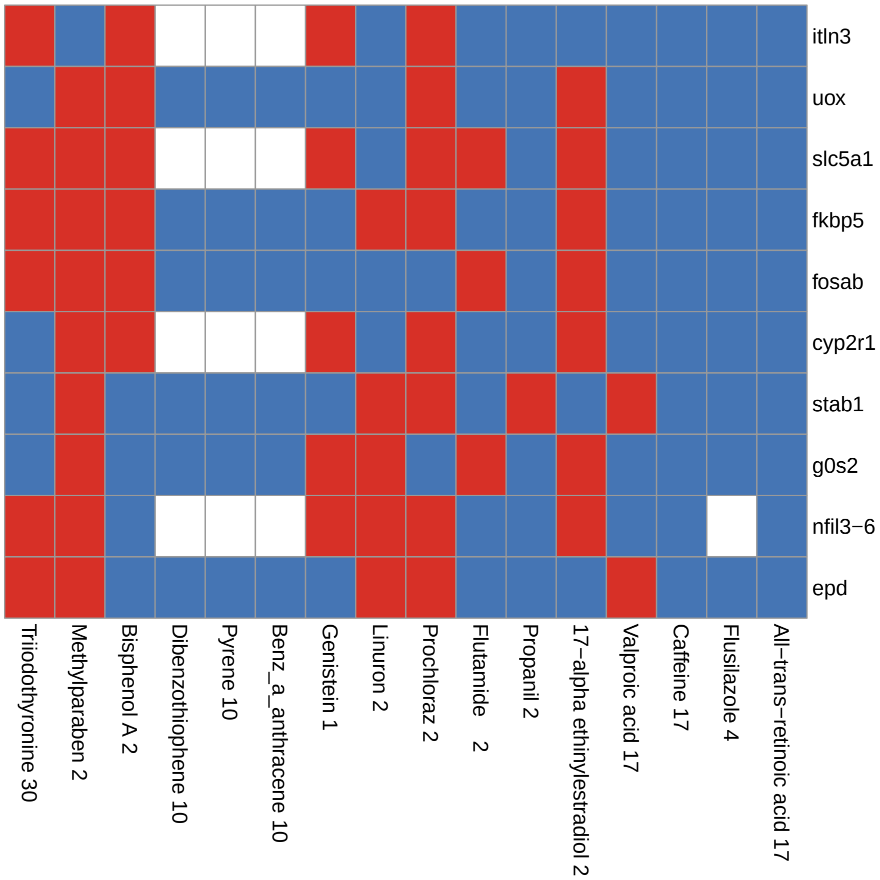


Figure A.10: Top 10 occuring differentially expressed genes (DEGs) across LOEC treatments (applied concentration was lowest observed effect concentration or some not precisely defined low effects up to EC_{10}). Red = differentially expressed, blue = not differentially expressed, white = no information.

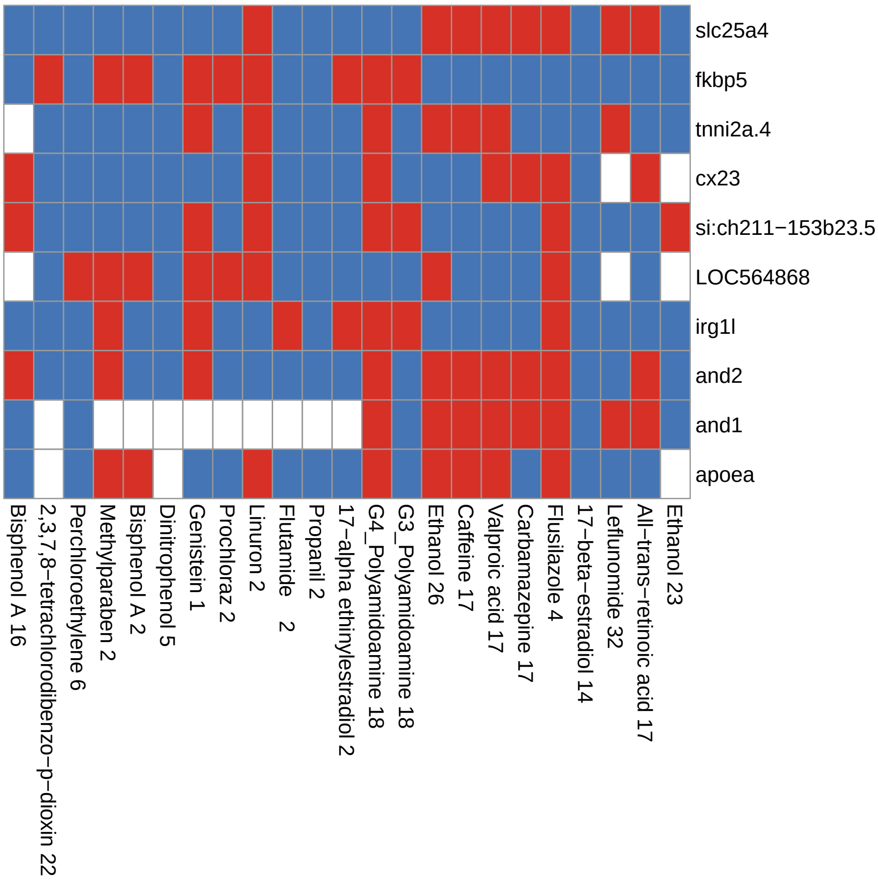


Figure A.11: Top 10 occuring differentially expressed genes (DEGs) across EC treatments (applied concentration reported to induce visible or lethal effects). Red = differentially expressed, blue = not differentially expressed, white = no information.

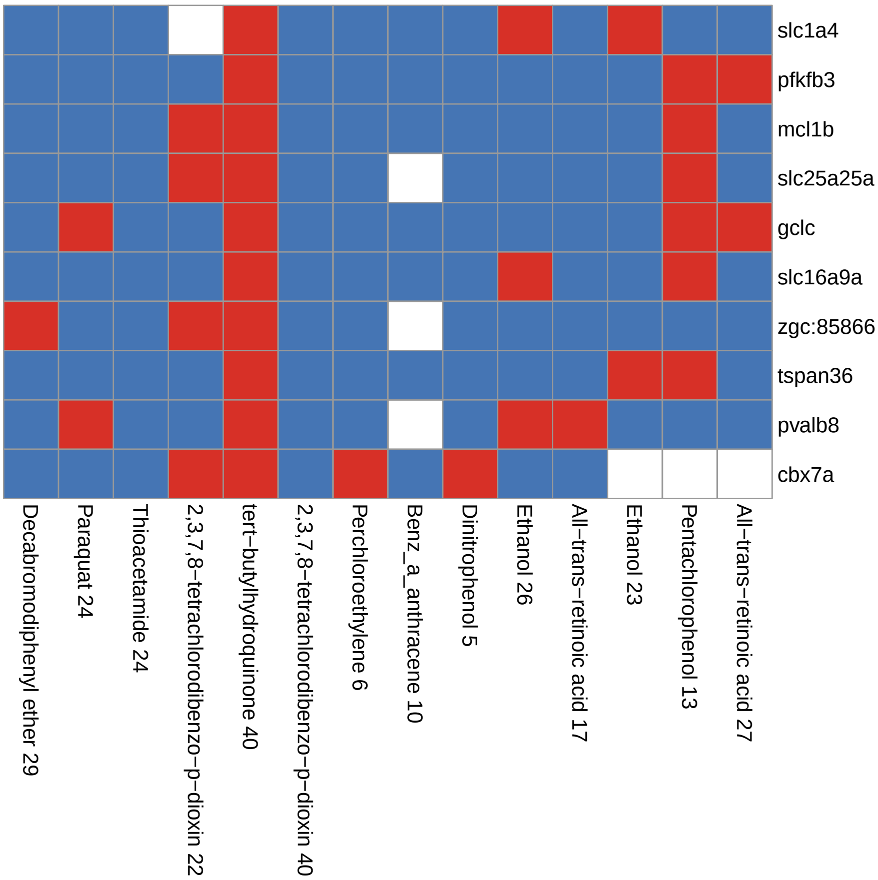


Figure A.12: ./Plots/Top 10 occuring differentially expressed genes (DEGs) across carcinogenic/teratogenic treatments. Red = differentially expressed, blue = not differentially expressed, white = no information.

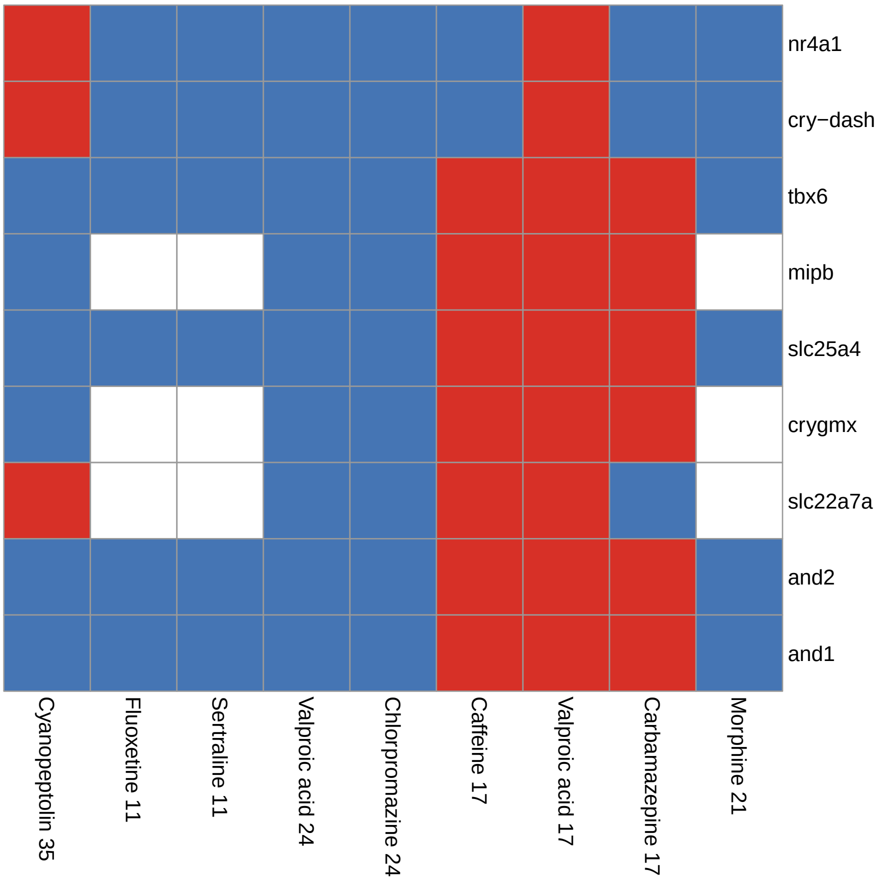


Figure A.13: Top 10 occuring differentially expressed genes (DEGs) across neuroactive treatments. Red = differentially expressed, blue = not differentially expressed, white = no information.

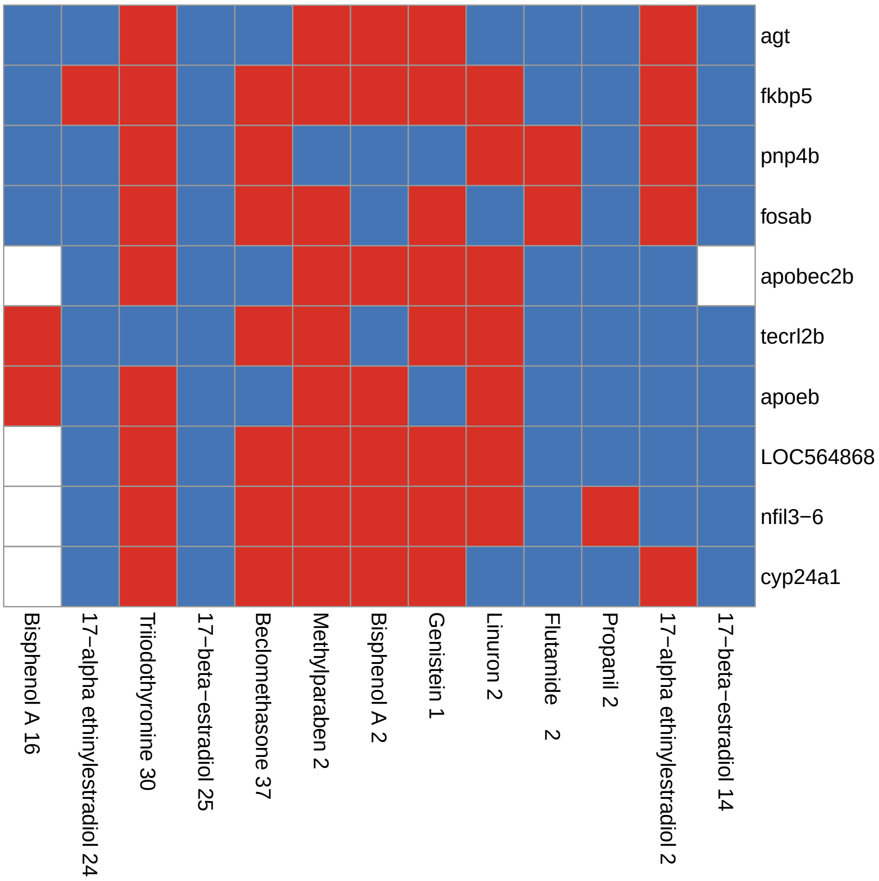
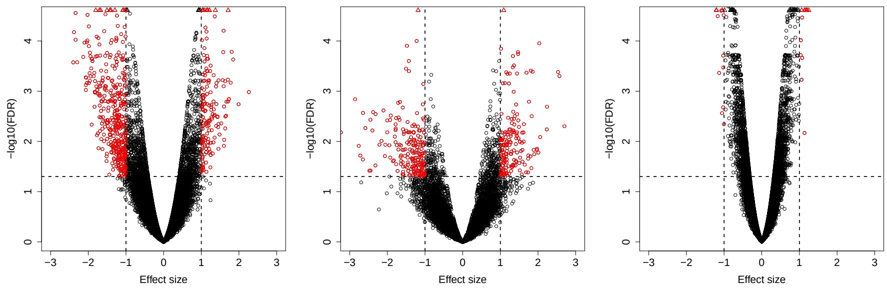
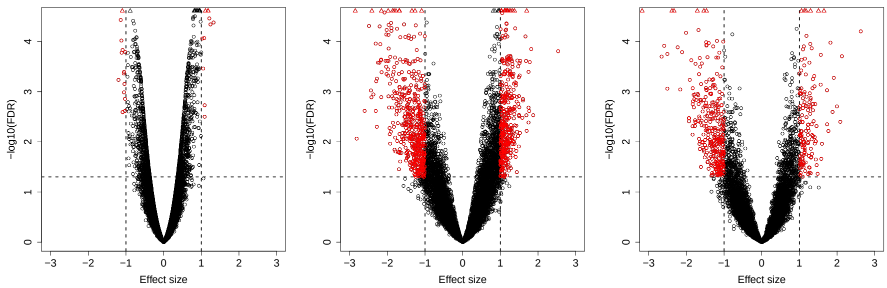


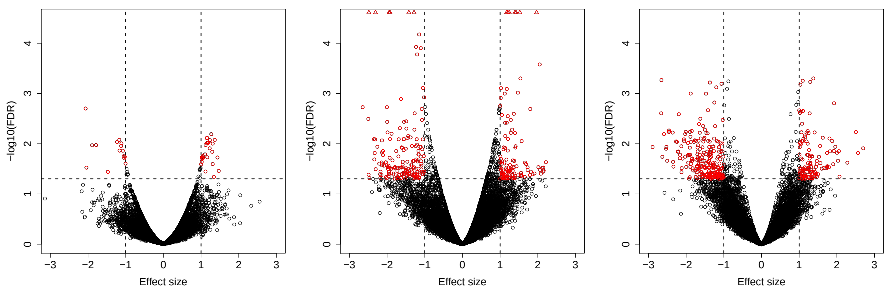
Figure A.14: Top 10 occuring differentially expressed genes (DEGs) across endocrine treatments. Red = differentially expressed, blue = not differentially expressed, white = no information.



(a) early measurements (b) middle measurements (c) late measurements



(d) no effect concentrations (e) LOEC concentrations (f) EC concentrations



(g) reactive/teratogen compounds (h) neuroactive compounds (i) endocrine compounds

Figure A.15: Volcaonplot of summary effect sizes. Each tested gene is represented by a dot, red dots represent genes with FDR smaller than 0.05 and an absolute effect size larger than 1. Dotted lines depict cutoffs.

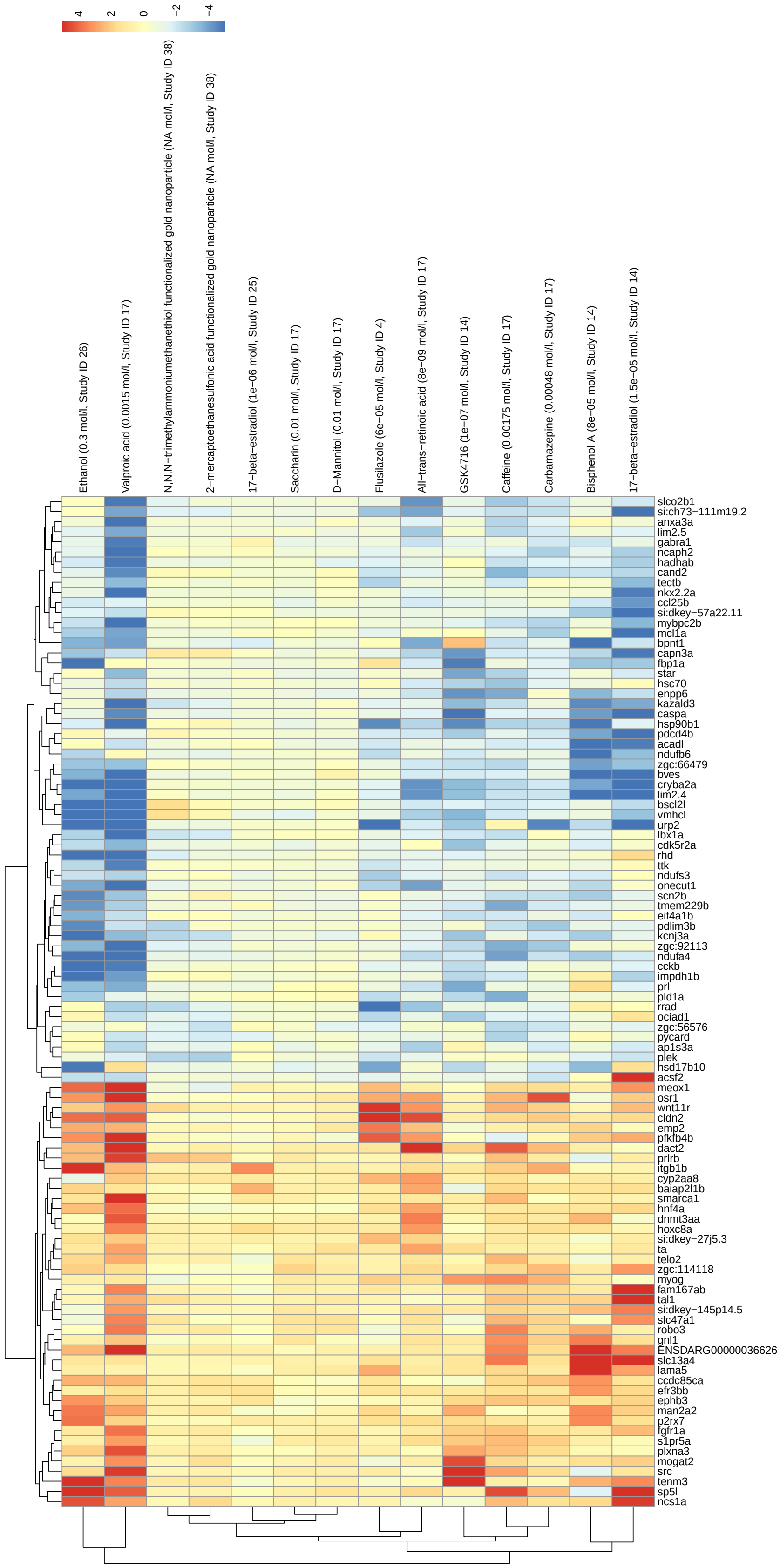


Figure A.16: Heatmap of single effect sizes of top 100 "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in early measurements (exposure end before 24 hpf). Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

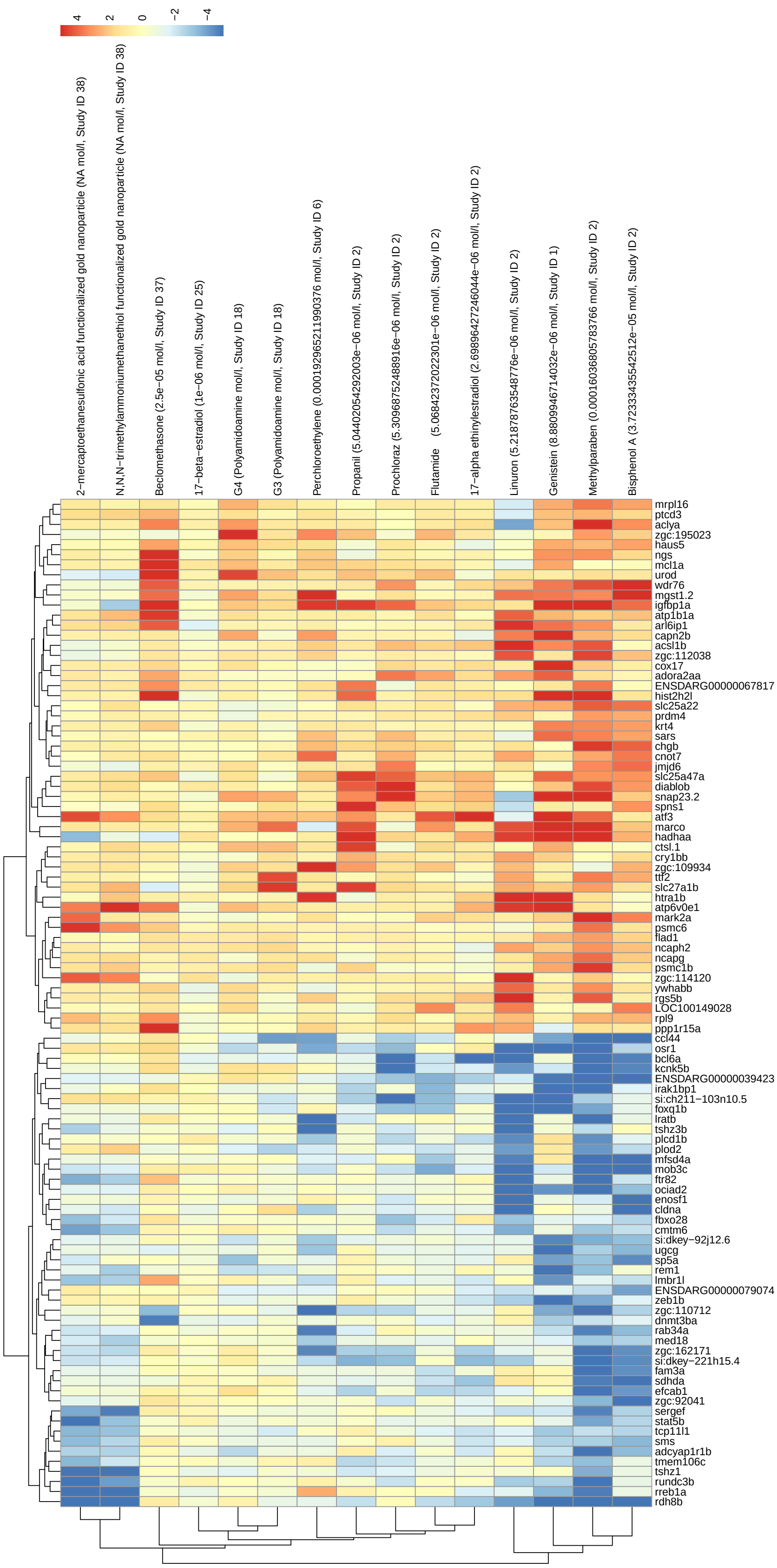


Figure A.17: Heatmap of single effect sizes of top 100 "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in middle measurements (exposure end before 50 hpf). Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

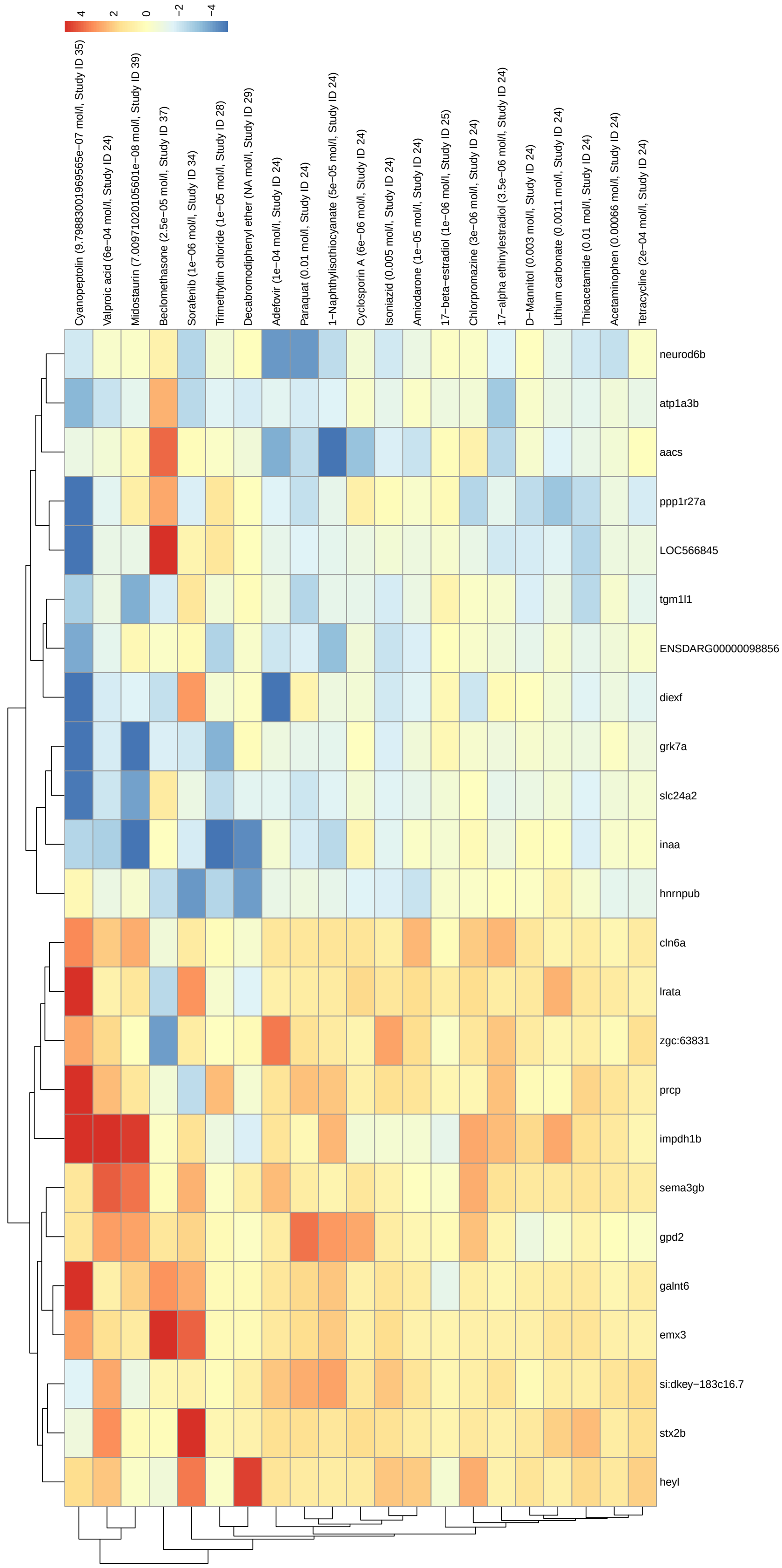


Figure A.18: Heatmap of single effect sizes of all significant "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in late measurements (exposure end after 50 hpf). Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

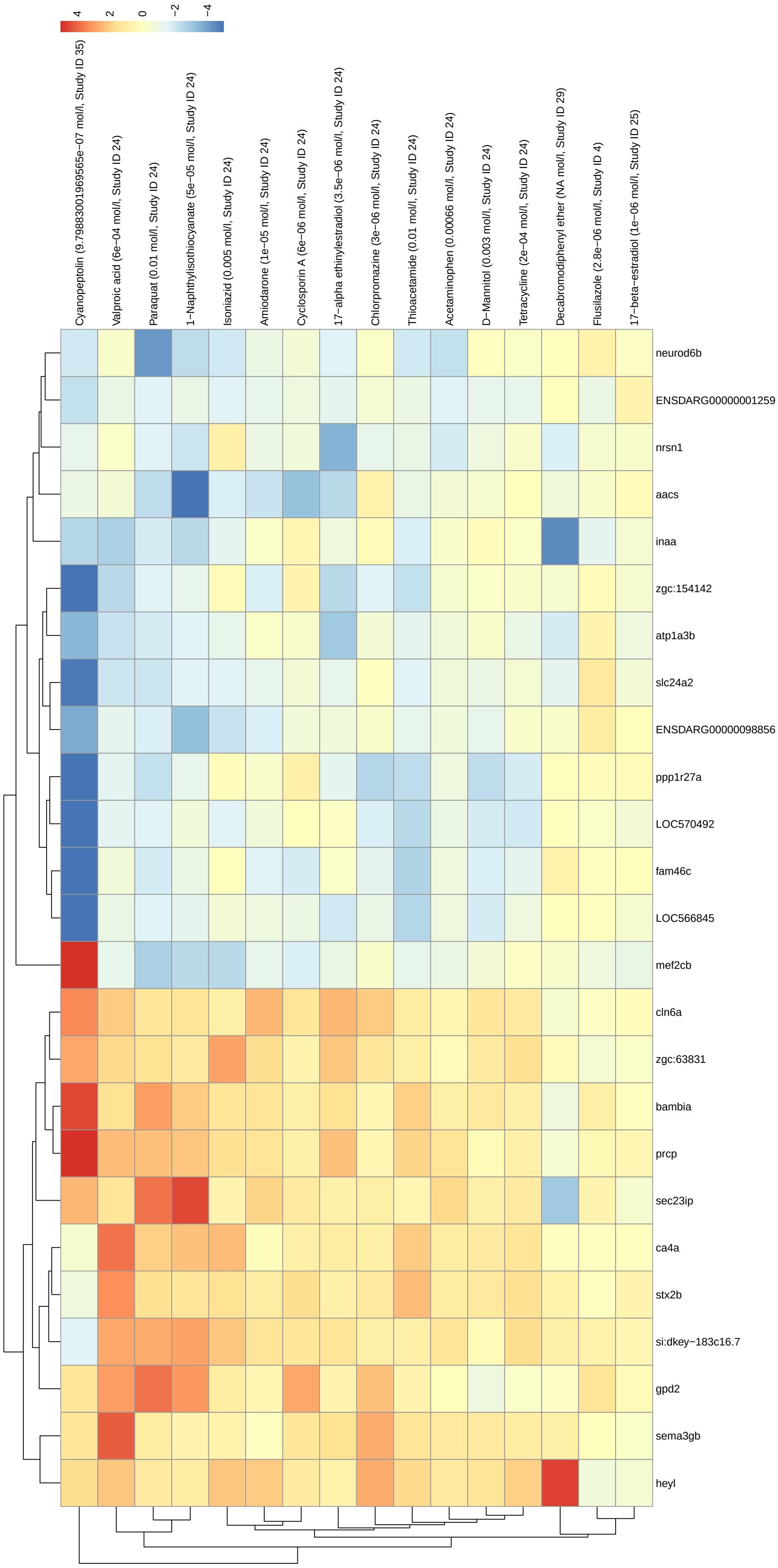


Figure A.19: Heatmap of single effect sizes of all significant "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in no-effect experiments (applied concentration had no reported phenotypic effect in the experiment). Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

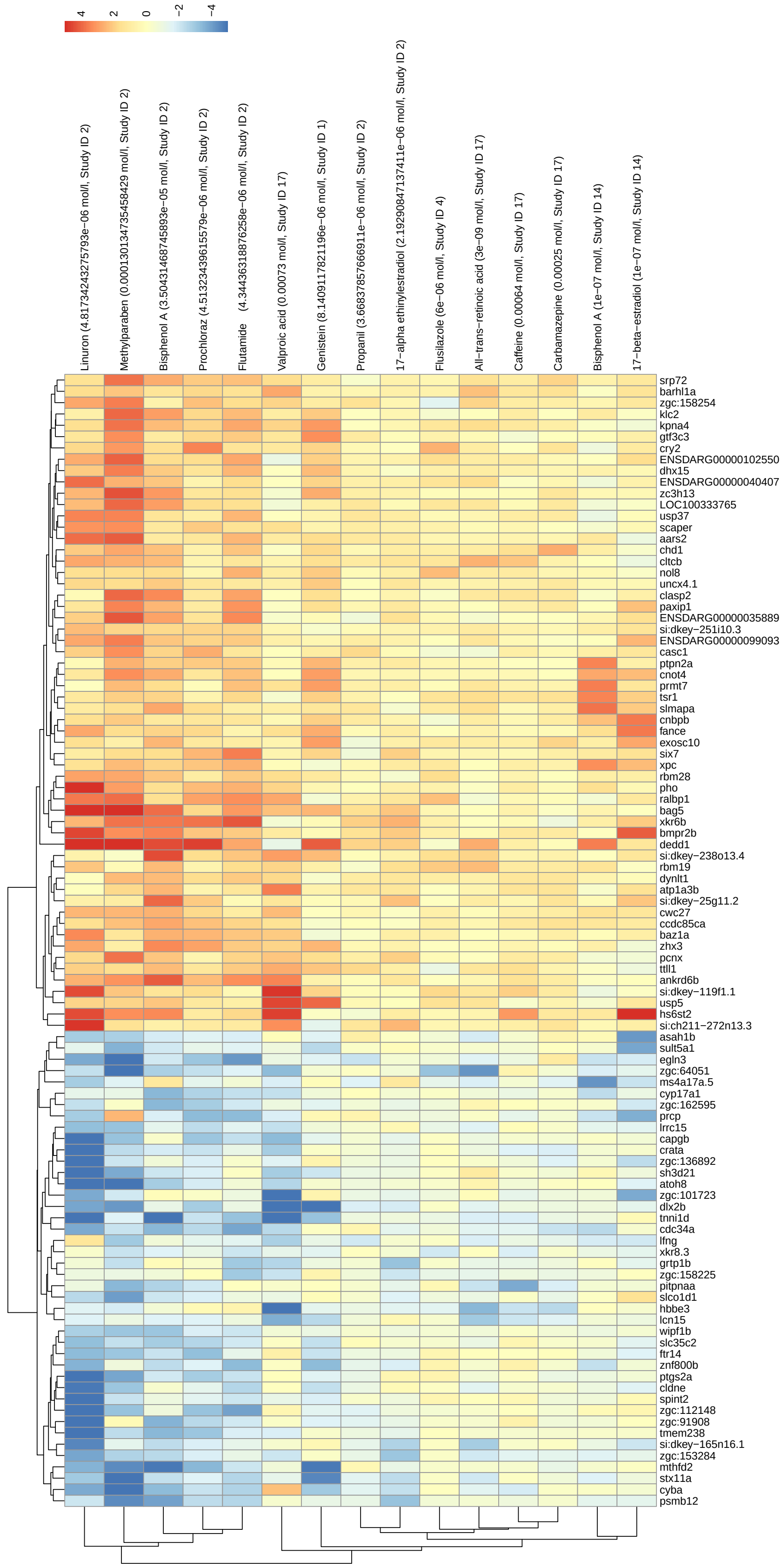


Figure A.20: Heatmap of single effect sizes of top 100 "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in LOEC experiments (applied concentration was lowest observed effect concentration or some not precisely defined low effects up to EC_{10}). Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

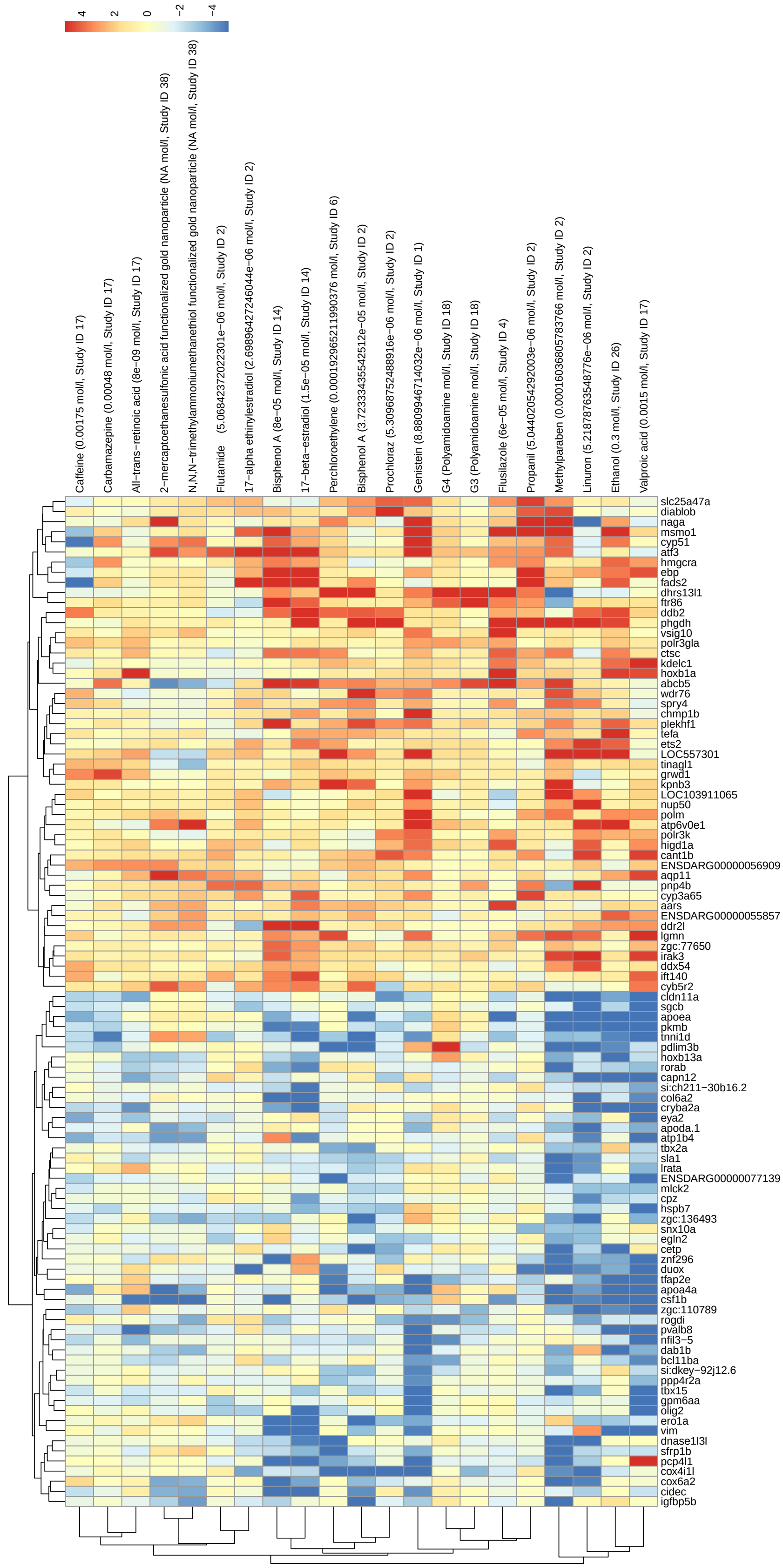


Figure A.21: Heatmap of single effect sizes of top 100 "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in EC experiments (applied concentration reported to induce visible or lethal effects). Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

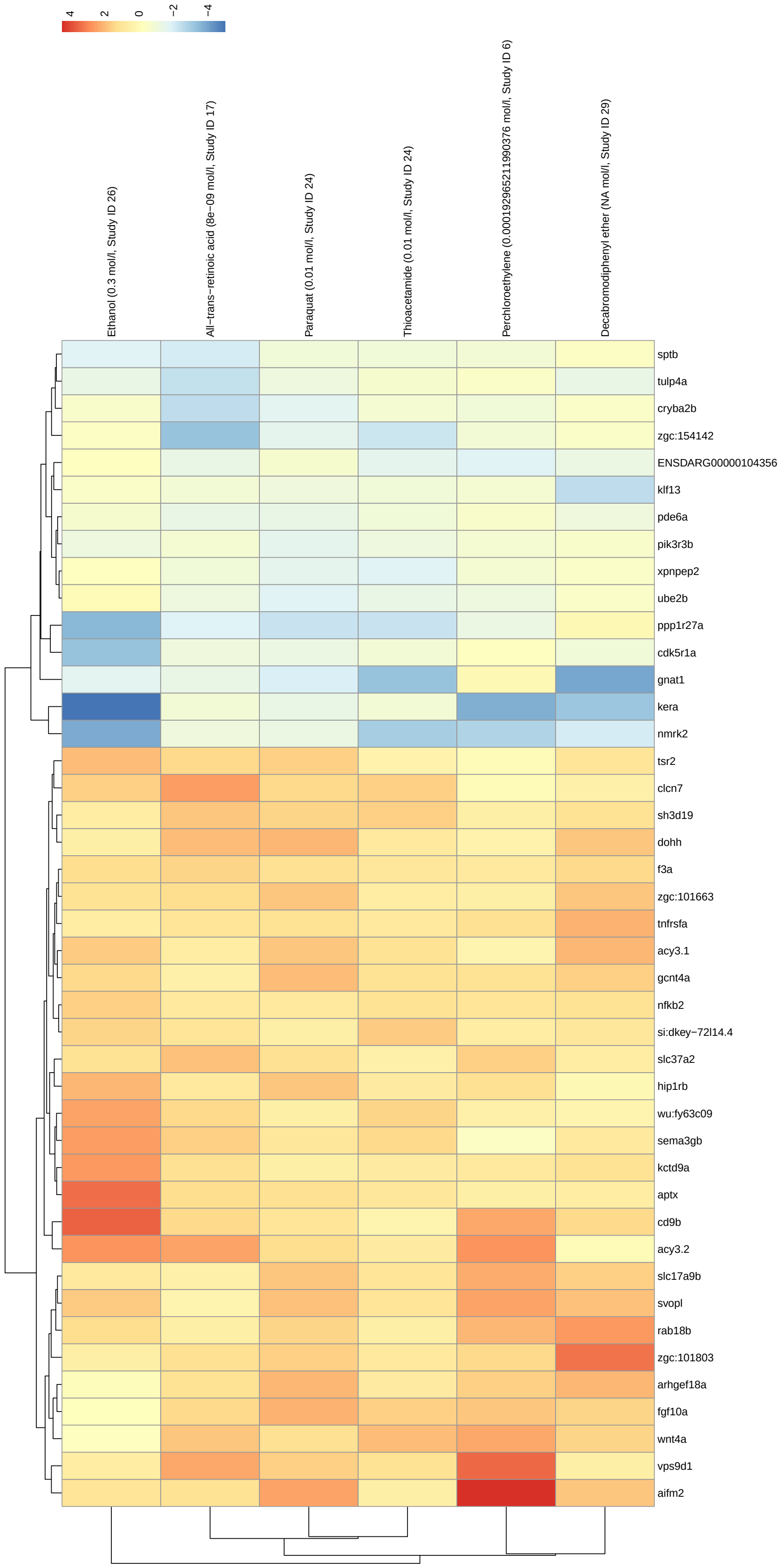


Figure A.22: Heatmap of single effect sizes of all significant "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in exposures to reactive compounds. Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

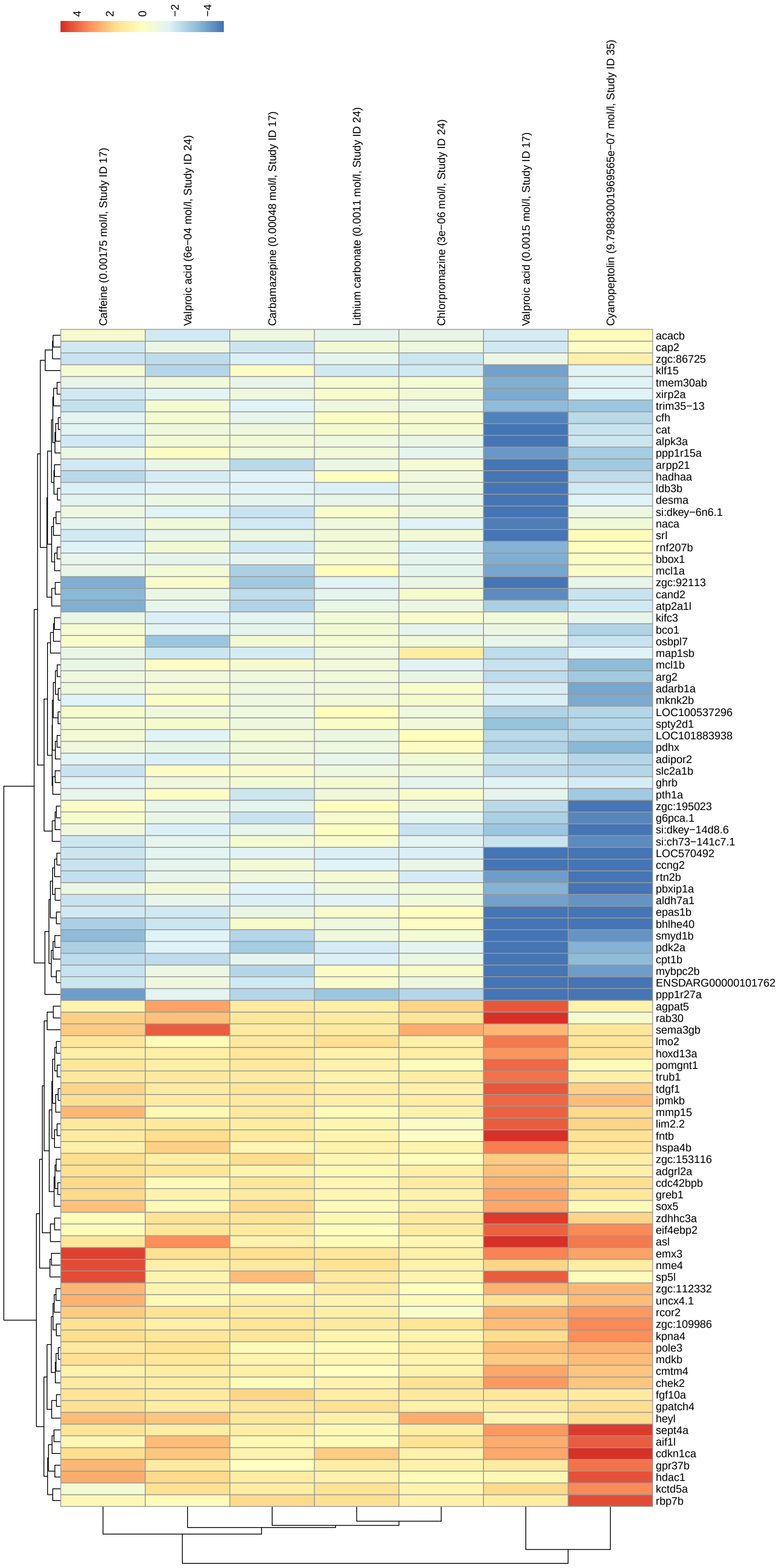


Figure A.23: Heatmap of single effect sizes of top 100 "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in exposures to neuroactive compounds. Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

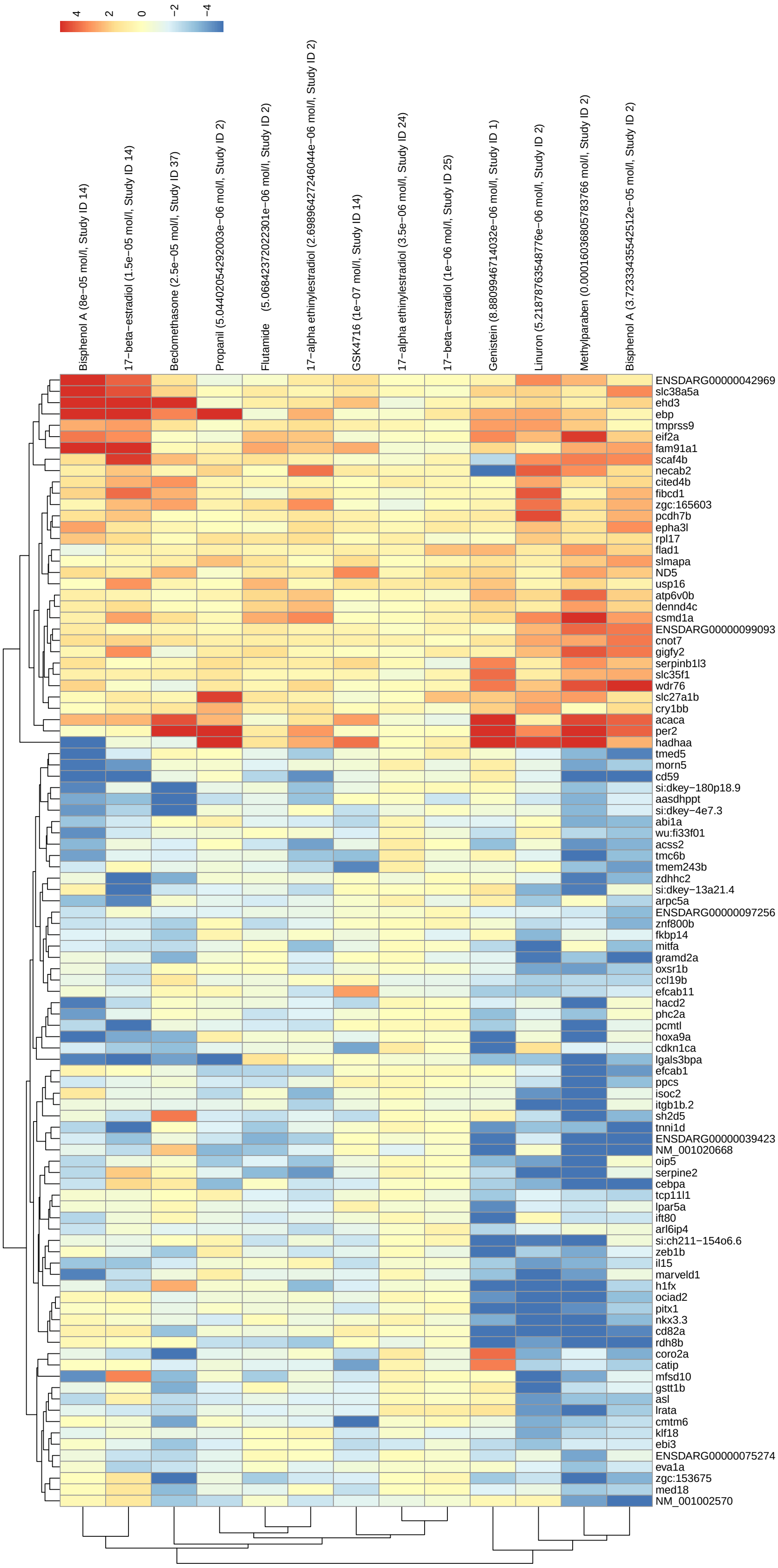


Figure A.24: Heatmap of single effect sizes of top 100 "meta-genes" (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) detected in exposures to endocrine compounds. Values above 0 = increased expression after chemical exposure, values below 0 = decreased expression after chemical exposure. Values above 5 and below -5 are clipped.

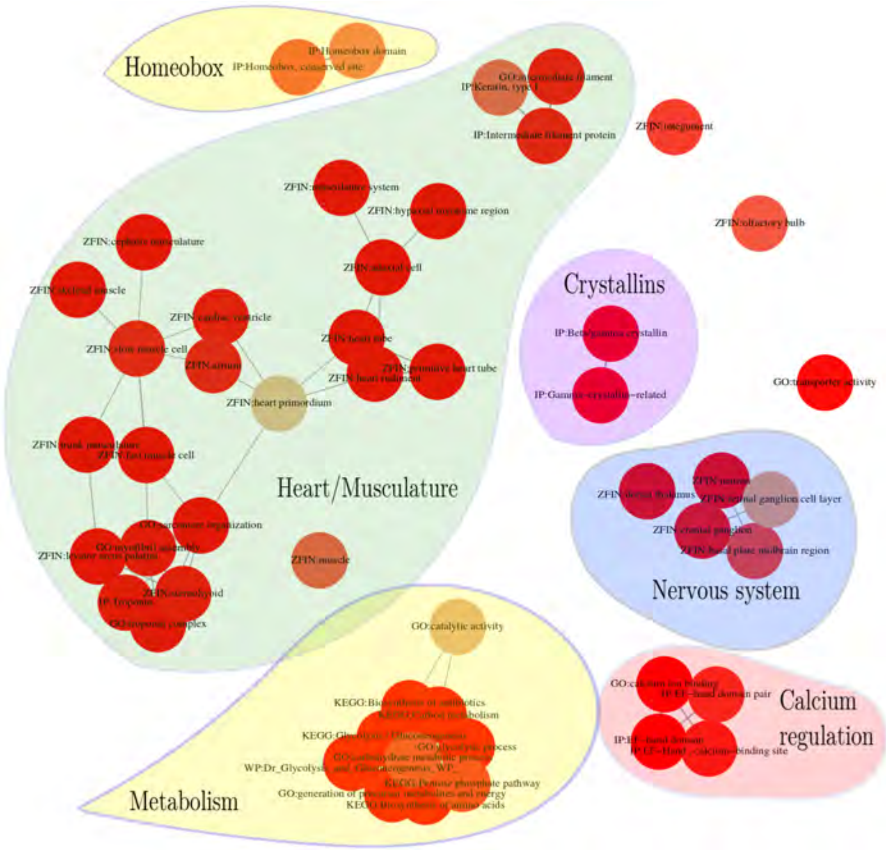


Figure A.25: Clusterplot of significantly enriched gene sets in meta-genes (= genes significantly differentially expressed in a subgroup as indicated by effect size analysis) of early experiments (measurement not later than 24 hpf). Gene sets with overlapping genes are connected Several functional/anatomical clusters were found.

REFERENCES

Brazma, A., P. Hingamp, J. Quackenbush, G. Sherlock, P. Spellman, C. Stoeckert, J. Aach, W. Ansorge, C. A. Ball, H. C. Causton, et al. (Dec. 2001). “Minimum information about a microarray experiment (MIAME)–toward standards for microarray data.” In: *Nature Genetics* 29.4, pp. 365–371. DOI: 10.1038/ng1201-365.

Carvalho, B. S. and R. A. Irizarry (Oct. 2010). “A framework for oligonucleotide microarray preprocessing.” In: *Bioinformatics* 26.19, pp. 2363–2367. DOI: 10.1093/bioinformatics/btq431.

Chen, C., K. Grennan, J. Badner, D. Zhang, E. Gershon, L. Jin, and C. Liu (Feb. 2011). “Removing Batch Effects in Analysis of Expression Microarray Data: An Evaluation of Six Batch Adjustment Methods.” In: *PLoS ONE* 6.2. Ed. by D. Kliebenstein, e17238. DOI: 10.1371/journal.pone.0017238.

Durinck, S., Y. Moreau, A. Kasprzyk, S. Davis, B. De Moor, A. Brazma, and W. Huber (2005). “BioMart and Bioconductor: A powerful link between biological databases and microarray data analysis.” In: *Bioinformatics* 21.16, pp. 3439–3440. DOI: 10.1093/bioinformatics/bti525.

Durinck, S., P. T. Spellman, E. Birney, and W. Huber (Aug. 2009). “Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt.” In: *Nature Protocols* 4.8, pp. 1184–1191. DOI: 10.1038/nprot.2009.97.

Gray, K. A., B. Yates, R. L. Seal, M. W. Wright, and E. A. Bruford (Jan. 2015). “Genenames.org: the HGNC resources in 2015.” In: *Nucleic Acids Research* 43.D1, pp. D1079–D1085. DOI: 10.1093/nar/gku1071.

Johnson, W. E., C. Li, and A. Rabinovic (Jan. 2007). “Adjusting batch effects in microarray expression data using empirical Bayes methods.” In: *Biostatistics* 8.1, pp. 118–127. DOI: 10.1093/biostatistics/kxj037.

Kent, W. J. (Apr. 2002). “BLAT—The BLAST-Like Alignment Tool.” In: *Genome Research* 12.4, pp. 656–664. DOI: 10.1101/gr.229202.

Moss, S. P., D. A. Joyce, S. Humphries, K. J. Tindall, and D. H. Lunt (2011). “Comparative analysis of teleost genome sequences reveals an ancient intron size expansion in the zebrafish lineage.” In: *Genome biology and evolution* 3, pp. 1187–96. DOI: 10.1093/gbe/evr090.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, et al. (Oct. 2005). “Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.” In: *Proceedings of the National Academy of Sciences of the United States of America* 102.43, pp. 15545–50. DOI: 10.1073/pnas.0506580102.

Tenenbaum, D. (2015). *KEGGREST: Client-side REST access to KEGG.* R package version 1.10.0.

B

SUPPLEMENTAL INFORMATION CHAPTER 3

B.1 EXPOSURE

Wild-type adult zebrafish, originally received from OBI petshop (Leipzig, Germany) were kept in 120 L fish tanks containing carbon-filtered tap water at 26(1) °C under a 12h:12h dark-light cycle. Eggs were collected approximately 1 hour after light onset and inspected using a light microscope. Fertilized eggs were incubated at 26(1) °C, while unfertilized eggs were discarded. For lethality experiments, which were conducted to find adequate exposure concentrations for transcriptome experiments, nine embryos per replicate were distributed into three 7.5 mL glas vials, each containing 6 mL exposure or control medium. Vials were sealed and incubated in a climate chamber shaking (Edmund Bühler SM-30 Control, 26(1) °C, 75 rpm, 12h:12h light:dark) till 96 hours post fertilization (hpf). At 48 hpf, 72 hpf and 96 hpf sublethal and lethal effects were recorded. For transcriptome experiments 20 embryos per replicate were transfered to two 20 mL GC-glas vials at 24 hpf. A volume of 18 mL of exposure medium was added to each vial and vials were sealed and incubated in a climate chamber (same settings as above) until sampling. Embryos were exposed to a range of 5 different concentrations of toxicant and RNA was extracted at 6 different timepoints (see figure 3.3). Exposure medium for all experiments was prepared by dissolving the substance in freshly prepared ISO-water (pH 7.4, oxygenized) (Diclofenac, Naproxen) or in 0.1 % methanol as solubilising agent (Diuron).

B.2 RNA EXTRACTION AND ISOLATION

At observation time points (specified above), two vials of embryos (20 in total) were transfered into 150 µL Eppendorf tubes. RNA was extracted by addition of 150 µL of Trizol and homogenization using a T10 basic

Ultra-Turrax (IKA, Werke GmbH & Co. KG, Germany) for 20 sec at maximum speed. RNA isolation was performed using a pipetting robot (Microlab Star, Hamilton Life Science Robotics, Germany) following the manual provided for Total RNA Extraction Kit MagMAX 96 for microarrays and conducted in a 96-well plate. Quality of isolated RNA was assesed using a Bioanalyzer (Agilent 2100 Technologies, Waldborn) and the Agilent RNA 6000 Nano Kit. RNA samples were used for further processing if RIN values derived from ribosomal RNA absorption adopted values > 7 and calculated concentrations exceeded $100\,\mathrm{ng\,\mu L^{-1}}$. RNA samples were diluted to a concentration level of $25\,\mathrm{ng\,\mu L^{-1}}$ by addition of RNAse free water.

B.3 MEASUREMENT OF TRANSCRIPT ABUNDANCE (TA)

Procedure for transcriptome measurement is described in detail in (Agilent, 2012) and will be outlined here in brief only. To measure transcript abundance RNA was first transcribed into complementary DNA. Afterwards, the cDNA was transcribed and amplified into cRNA, while incorporating fluorescently labeled cytidine nucleotides. The cRNA was fragmented and hybridized with Oaklabs ArrayXS Zebrafish microarray slides. All these steps were applied according to the manufacturer's protocol (Agilent, 2012) and using the equipment recommended in the protocol.

Microarray slides were scanned with the Agilent High-Resolution Microarray Scanner (settings: Area $61\,\mathrm{mm} \times 21.6\,\mathrm{mm}$, image resolution $3\,\mathrm{\mu m}$, intensity $100\,\%$, modus single pass). Intensity values were extracted from captured images using Agilent Feature Extraction Software (Version 11.5.1.1).

RNA samples in the Diclofenac experiment were partly processed by Oaklabs GmbH (Berlin, Germany). Potential batch effect, caused by handling the samples in two different laboratories was accounted for during data analysis.

B.4 REMOVAL OF BATCH EFFECT

Due to technical reasons, parts of the arrays of the Diclofenac exposure were hybridized and measured by Oaklabs GmbH (Hennigsdorf, Germany).

B.5 PARAMETER BOUNDARIES

The maximum slope we can unravel depends on the spacing of our concentrations. It would describe a change of effect from approximately 1% to 99% in between two measured concentrations. This implies an effect of 99% in the X_{50} multiplied by the square root of the dilution factor (DF):

$$Effect = \frac{maxFC}{1 + \exp\left(-slope * (\log c - \log X_{50})\right)} \tag{B.1}$$

$$\Leftrightarrow \quad = \frac{maxFC}{1 + \exp\left(\log\left(c^{-slope}\right) - \log\left(X_{50}^{-slope}\right)\right)} \tag{B.2}$$

$$\Leftrightarrow \quad = \frac{maxFC}{1 + \exp\left(\log\left(\frac{c^{-slope}}{X_{50}^{-slope}}\right)\right)} \tag{B.3}$$

$$\Leftrightarrow \quad = \frac{maxFC}{1 + \left(\frac{c}{X_{50}}\right)^{-slope}} \qquad \text{insert values} \tag{B.4}$$

$$0.99 * maxFC = \frac{maxFC}{1 + \left(\frac{X_{50} * \sqrt{DF}}{X_{50}}\right)^{-slope}} \tag{B.5}$$

$$\Leftrightarrow \quad \sqrt{DF}^{-slope} = \frac{1}{0.99} - 1 \tag{B.6}$$

$$\Leftrightarrow \quad slope = -\frac{\log\left(\frac{1}{0.99} - 1\right)}{\log\left(\sqrt{DF}\right)} \tag{B.7}$$

For the minimum slope the calculation is the similar. Here we expect, that a minimum slope that still can be unraveled by our measurements would describe a change of 1% effect across the measured concentration range. This implies an effect of 50.5% at a concentration of EC_{50} multiplied by the square root of the concentration range.

The lowest σ we can measure depends on the spacing of our time-points. If an effect occurs only at one time point, there should be less than 1% effect at the preceding/succeeding measured time points. In our setting the smallest factor between two time points is 2/3 (between 48 and 72h):

$$Sensitivity = maxS50 * \exp\left(-0.5 * \left(\frac{\log t - \log \mu}{\sigma}\right)^2\right) \tag{B.8}$$

$$0.01 * maxS50 = maxS50 * \exp\left(-0.5 * \left(\frac{\log 2/3}{\sigma}\right)^2\right) \tag{B.9}$$

$$\Leftrightarrow \quad \frac{\log(2/3)}{\sigma} = \sqrt{-2 * \log 0.01} \tag{B.10}$$

$$\Leftrightarrow \quad \sigma = \frac{\log(2/3)}{\sqrt{-2 * \log 0.01}} \tag{B.11}$$

This implies that the term $\exp(-0.5 * (\frac{log(48/72)}{\sigma})^2)$, which describes the time dependence of the sensitivity should be 0.01.

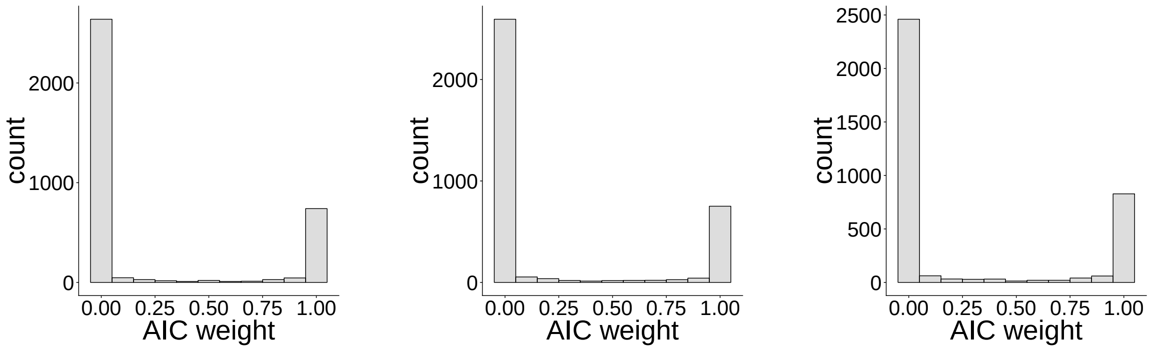
Table B.1: Parameter boundaries for Hill-Gauss-model (Equation 3.5). Concrange: Ratio between highest and lowest concentration applied for respective substance. DF: Smallest dilution factor between applied concentrations for respective substance.

parameter	**min**	**max**	**reasoning**
slope	$-\frac{\log\left(\frac{1}{0.505}-1\right)}{\log\sqrt{concrange}}$	$-\frac{\log\left(\frac{1}{0.99}-1\right)}{\log\sqrt{DF}}$	Minimum slope would imply a maximum increase in effect across the tested range of 1%. Maximum slope would imply a maximum increase in effect of 98% between two tested concentrations.
maxSen	$\frac{1}{concrange*max(\text{appl. concentration})}$	$\frac{concrange}{min(\text{appl. concentration})}$	X_{50} can be below or above tested concentrations. It is not discrimibable from the data, if it is just above or well above (resp. below)
σ	$\frac{\log 1.5}{\sqrt{-2*\log 0.01}}$	$\frac{\log 72/3}{\sqrt{-2*\log 0.99}}$	Minimum sigma would imply a 1% effect in the timepoint succeeding the maximum; Maximum sigma would imply a 99% effect at most distant time point
μ	3	72	the highest (measured) sensitivity of a gene can only be in the range of measured time points

Table B.2: Parameter boundaries for Gauss-Gauss-model (Equation 3.6). Concrange: Ratio between highest and lowest concentration applied for respective substance. DF: Smallest dilution factor between applied concentrations for respective substance.

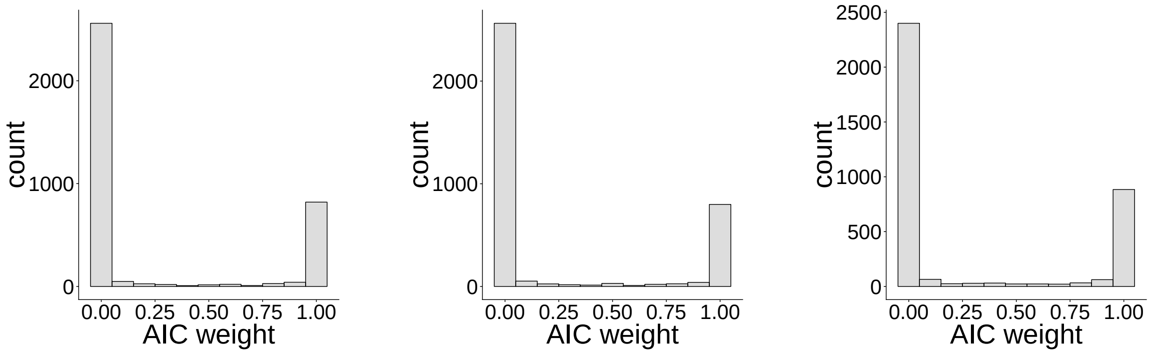
parameter	min	max	reasoning
sconc	$\frac{\log DF}{\sqrt{-2*\log 0.01}}$	$\frac{\log Concrange}{\sqrt{-2*\log 0.99}}$	Minimum slope would imply a maximum increase in effect across the tested range of 1%. Maximum slope would imply a maximum increase in effect of 98% between two tested concentrations.
mconc	$\frac{1}{concrange*max(\text{appl. concentration})}$	$\frac{concrange}{min(\text{appl. concentration})}$	X_{50} can be below or above tested concentrations. It is not discrimibable from the data, if it is just above or well above (resp. below)
σ	$\frac{\log 1.5}{\sqrt{-2*\log 0.01}}$	$\frac{\log 72/3}{\sqrt{-2*\log 0.99}}$	Minimum sigma would imply a 1% effect in the time point succeeding the maximum; Maximum sigma would imply a 99% effect at most distant time point
μ	1.5	75	the highest (measured) sensitivity of a gene can only be in the range of measured time points. The limits are set a bit wider to avoid boundary effects

B.6 SUPPLEMENTAL FIGURES



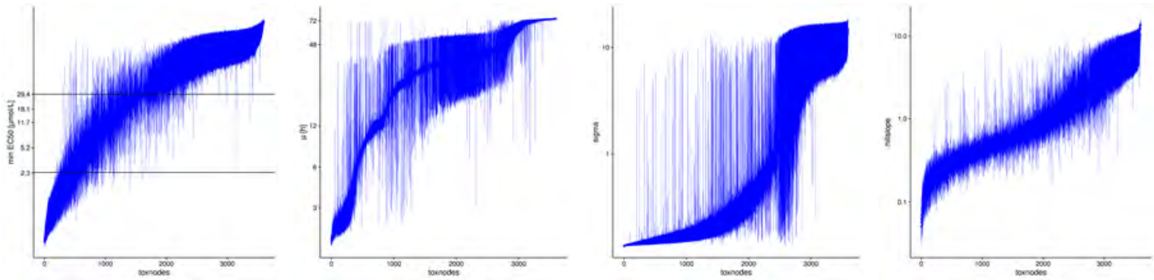
(a) Diuron (b) Diclofenac (c) Naproxen

Figure B.1: Histogram of AIC$_c$-weights compared to spline. The spline is preferred over the regression model for a majority of the toxnodes. However for roughly 25% of the nodes the regression models are preferred over the much more flexible spline.



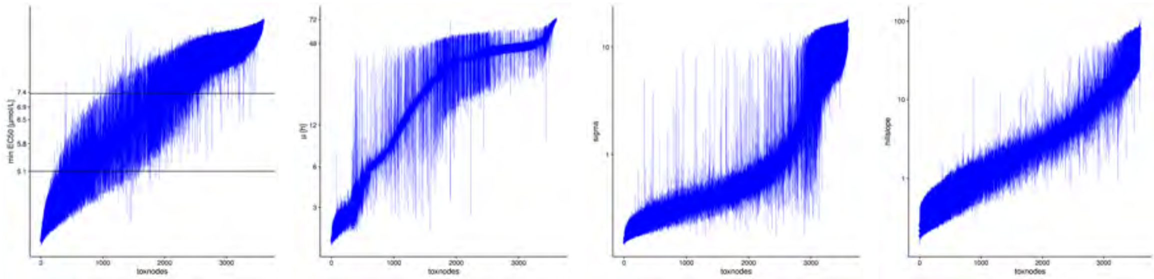
(a) Diuron (b) Diclofenac (c) Naproxen

Figure B.2: Histogram of AIC$_c$-weights compared to spline. The spline is preferred over the regression model for a majority of the toxnodes. However for roughly 25% of the nodes the regression models are preferred over the much more flexible spline.



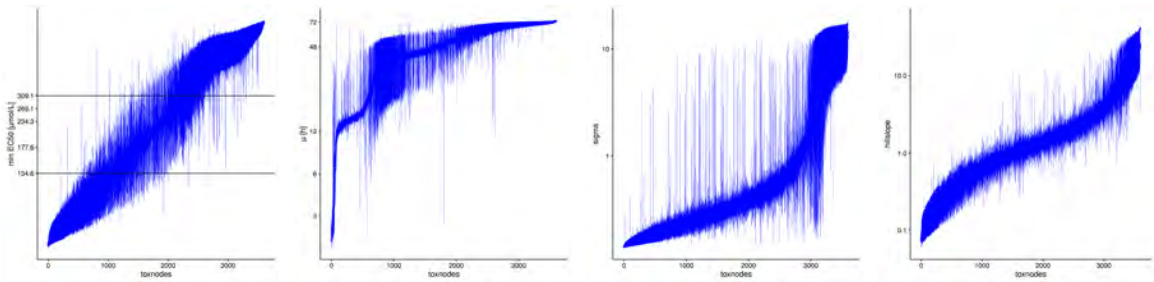
(a) minEC50 (b) μ (c) sigma (d) hillslope

Figure B.3: Uncertainty of parameter estimates (Hill-Gauss model, diuron) across all toxnodes. Each blue line represents the 50% confidence interval for one toxnode.



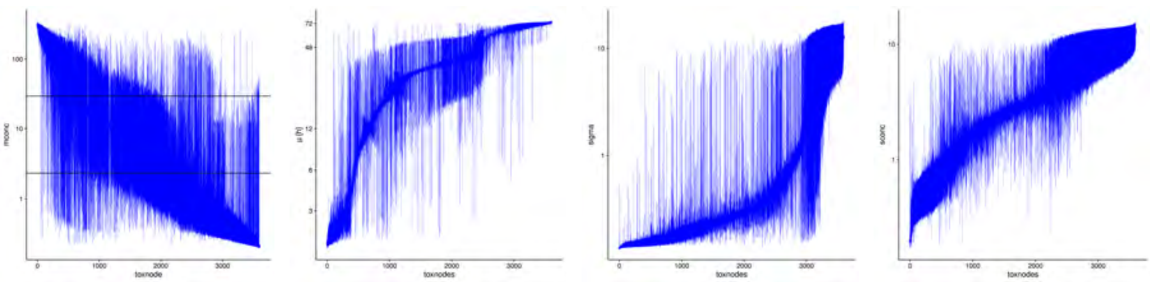
(a) minEC50 (b) μ (c) sigma (d) hillslope

Figure B.4: Uncertainty of parameter estimates (Hill-Gauss model, diclofenac) across all toxnodes. Each blue line represents the 50% confidence interval for one toxnode.



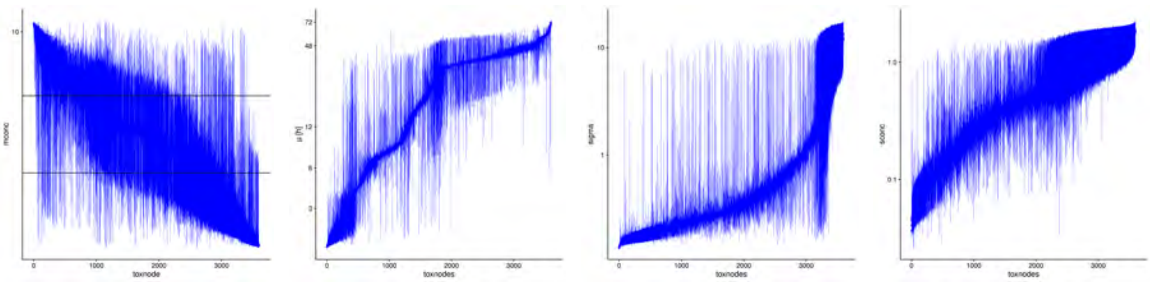
(a) minEC50 (b) μ (c) sigma (d) hillslope

Figure B.5: Uncertainty of parameter estimates (Hill-Gauss model, naproxen) across all toxnodes. Each blue line represents the 50% confidence interval for one toxnode.



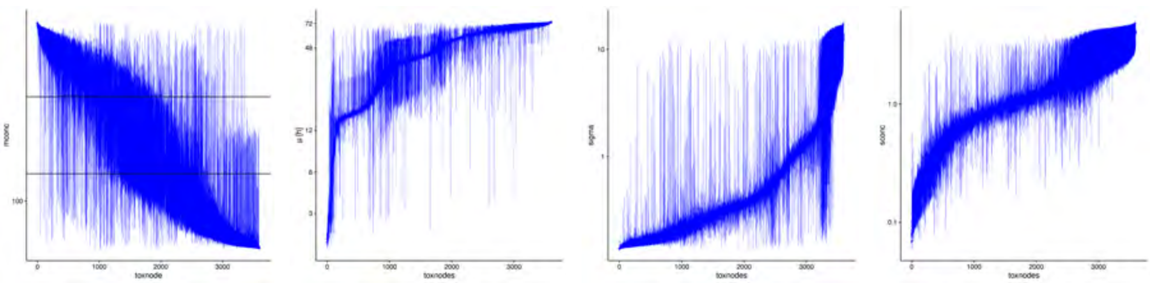
(a) mconc (b) μ (c) sigma (d) sconc

Figure B.6: Uncertainty of parameter estimates (Gauss-Gauss model, diuron) across all toxnodes. Each blue line represents the 50% confidence interval for one toxnode.



(a) mconc (b) μ (c) sigma (d) sconc

Figure B.7: Uncertainty of parameter estimates (Gauss-Gauss model, diclofenac) across all toxnodes. Each blue line represents the 50% confidence interval for one toxnode.



(a) mconc (b) μ (c) sigma (d) sconc

Figure B.8: Uncertainty of parameter estimates (Gauss-Gauss model, naproxen) across all toxnodes. Each blue line represents the 50% confidence interval for one toxnode.

B.7 SUPPLEMENTAL TABLES

Table B.3: Top ten toxnodes regulated by diuron given by Gauss-Gauss model. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval.

toxnode #	Membergenes	ratio Lethal	ratio Sublethal	*mconc*	*sconc*	μ	*sigma*	*err*	sum(CI-difference)
3567	cyp1a	15.8	15.8	45.1	2.0	12.4	2.08	0.25	76.3
				85.2	2.6	16.8	2.83	0.37	
1303	cyp1b1	10.1	10.1	22.0	1.2	1.5	1.95	0.43	21.8
				131.7	2.6	3.6	2.87	0.66	
3568	cyp1c2, cyp1c1, NA	9.4	9.4	31.3	1.3	18.7	0.83	0.59	12
				81.1	2.1	21.9	1	0.74	
3507	sult6b1	7.9	7.9	55.9	1.7	6.5	1.19	0.22	8.3
				267.4	3.1	12.2	1.96	0.34	
1362	crybb1l3, NA, NA, si:ch1073-	0.5	0.5	3.5	1	1.8	0.28	0.62	3.6
	82l19.1			27.1	11.2	3.6	0.67	0.77	
1117	saga, arr3a	0.5	0.5	0.7	0.7	1.7	0.17	0.64	3.2
				200.3	15.2	3.6	0.68	0.87	
1115	crygm5, si:dkey-57a22.15	19.2	19.2	8.3	0.7	1.9	0.28	0.76	3
				21.2	3.5	4	0.75	1.02	
1243	tecrl2b, aglb, NA	11.8	11.8	10.8	0.7	1.6	0.73	0.56	2.9
				14.7	1	3.2	1.26	0.7	
1380	srgn, hspb2, capn3a	0.4	0.4	3.8	0.9	2.1	0.14	0.57	2.3
				53.4	14.9	3.4	0.4	0.73	
651	prss59.2	0.7	0.7	0.2	1.8	18.6	0.13	0.59	-2.2
				250.2	17.2	30.5	0.22	0.88	

Table B.4: Top ten toxnodes regulated by Diclofenac given by Gauss-Gauss model. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval.

toxnode #	Membergenes	ratio Lethal	ratio Sublethal	*mconc*	*sconc*	μ	*sigma*	*err*	sum(CI-difference)
1260	cyp2k18	2	1.9	3.7 10.4	0.5 2.5	46.5 54.6	0.18 0.3	0.82 1.21	12.1
1259	si:zfos-411a11.2	1.9	1.8	3.7 10.5	0.4 2.5	55.1 56.7	0.15 0.19	0.23 0.34	8.5
1079	lepa, pth1a	1.9	1.7	3.7 10.4	0.4 2.5	49.4 55.8	0.17 0.29	0.71 0.94	4.9
1500	crygm3, crygm2d12, crygm1, crygm2d13, crygm2d7, crygm2d3	2	1.8	4.1 10.2	0.2 2.5	5.1 7.3	0.13 0.2	0.42 0.49	-4.5
3446	ugt1a4, ugt1a5, ugt1a6, ugt1a7, ugt1a2, ugt1a1, ugt1ab	2.1	1.9	3.6 10.3	0.4 2.5	47.2 55.1	0.17 0.29	0.17 0.27	3.8
1968	si:dkey-1c7.3	1.9	1.7	3.8 10.3	0.4 2.5	41.8 51	0.13 0.24	0.52 0.79	-3.6
1137	fosab	1.6	1.5	4.0 10.5	0.2 2.4	46.3 56.6	0.16 0.35	0.75 1.14	3.4
1440	crygmxl2	1.2	1.1	3.7 10.4	0.4 2.5	4.8 7.7	0.13 0.2	0.42 0.64	-3
1002	grapb	1.3	1.2	3.6 5.7	0.1 0.5	1.9 6.5	0.46 1.43	0.71 1.04	2.7
1199	nfe2l2b	1.1	1	3.8 10.3	0.3 2.5	38.9 53.1	0.19 0.43	0.43 0.65	2.4

Table B.5: Top ten toxnodes regulated by Naproxen given by Gauss-Gauss model. Ratio Lethal: $min(LC_{50})/min(EC_{50})_{Toxnode}$; Ratio Sublethal: $min(EC_{50})_{apical}/min(EC_{50})_{Toxnode}$. Parameter estimates given as ranges of 95% confidence interval.

toxnode #	Membergenes	ratio Lethal	ratio Sublethal	*mconc*	*sconc*	μ	*sigma*	*err*	sum(CI-difference)
1260	cyp2k18	5.8	2.8	89.7	1.5	60.2	0.32	0.28	33.6
				662.5	5.8	63.9	0.4	0.44	
3312	serpinh1b	5.4	2.7	475.6	1.4	49.4	0.88	0.27	23.7
				707.1	2.3	74.2	1.24	0.42	
1139	slc25a38a, c7b	2.7	1.3	378.1	0.8	65.9	0.24	0.32	16.5
				595.9	1.2	74.8	0.36	0.44	
1259	si:zfos-411a11.2	5.7	2.8	61.4	1.5	62.5	0.24	0.21	15.6
				699.7	5.7	74.7	0.48	0.32	
1320	si:rp71-80o10.4	3	1.5	466.3	1	65	0.52	0.39	15.6
				706.8	1.6	74.9	0.84	0.59	
1140	si:ch1073-165f9.2, isg15	2.4	1.2	387.9	0.7	65.6	0.3	0.59	14.5
				629.6	1.2	74.9	0.54	0.8	
1078	mmp13a	3.6	1.8	336.2	0.8	64.5	0.18	0.38	13.7
				702.7	1.8	74.9	0.32	0.6	
3493	slc16a9a	4.2	2.1	261.9	0.8	62.9	0.2	0.38	12.5
				702.5	2.7	74.9	0.43	0.6	
1080	LOC557301, LOC101885512,	3.3	1.6	528.8	1.2	70.2	0.6	0.45	12.4
	NA, bmb			707.1	1.7	75	0.77	0.56	
3477	ahsg2	3.9	1.9	64.7	0.9	67.3	0.13	0.98	-
				686.5	5.7	74.9	0.18	1.53	12.4

REFERENCES

Agilent (2012). *One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling V6.6. Protocol.*

C

SUPPLEMENTAL INFORMATION CHAPTER 4

C.1 CONCENTRATION ADDITION FOR BIPHASIC CONCENTRATION RESPONSE RELATIONSHIPS

In Chapter 3 we showed that the concentration dependence of some toxnodes are biphasic. Martin-Betancor et al. (2015) suggested an extension of the concentration addition concept for biphasic concentration response relationship, which we exemplary applied for our toxicogenomic dataset. Martin-Betancor et al. suggest to represent the effective doses by a two-dimensional vector consisting of E_p, and D_p. D_p represents the dose leading to an effect E_p, with $-100 < p < 100$, representing the fraction of a maximum effect E_{max}. For $p < 0$ the effects for the increasing part of the biphasic curve are retrieved, $p > 0$ describes the decreasing part of the curve, $p = 0$ implicates the maximum effect. Combination effects can be predicted by applying the concept of concentration addition separately to E_p (equation C.1) and D_p (equation C.2).

$$E_{(p)mix} = \left(\sum_{i=1}^{n} \frac{j_i}{E_{(p)i}} \right)^{-1} \tag{C.1}$$

$$D_{(p)mix} = \left(\sum_{i=1}^{n} \frac{j_i}{D_{(p)i}} \right)^{-1} \tag{C.2}$$

C.2 SUPPLEMENTAL FIGURES

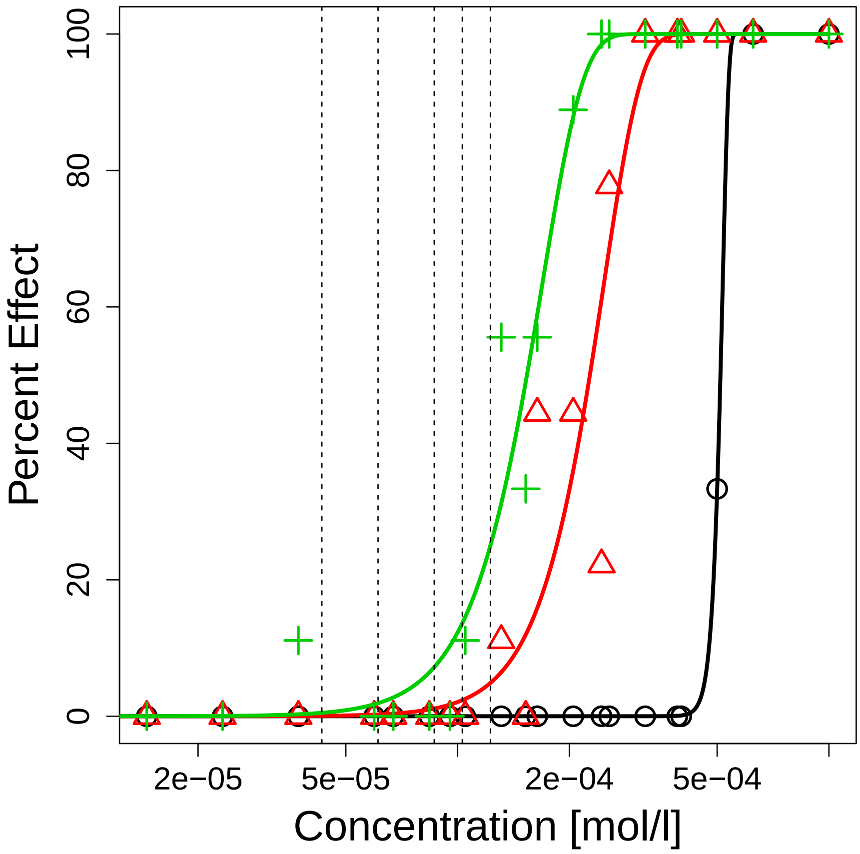


Figure C.1: Concentration response relationships for apical effects induced by mixture of diuron (11%), diclofenac (2.6%), and naproxen (86.4%). Vertical lines illustrate exposure concentrations for transcriptome experiments. Curves illustrate best fit model for lethality, colours indicate different exposure durations. Lethal effects: 24-48hpf (○); 24-72hpf (△); 24-96hpf (+).

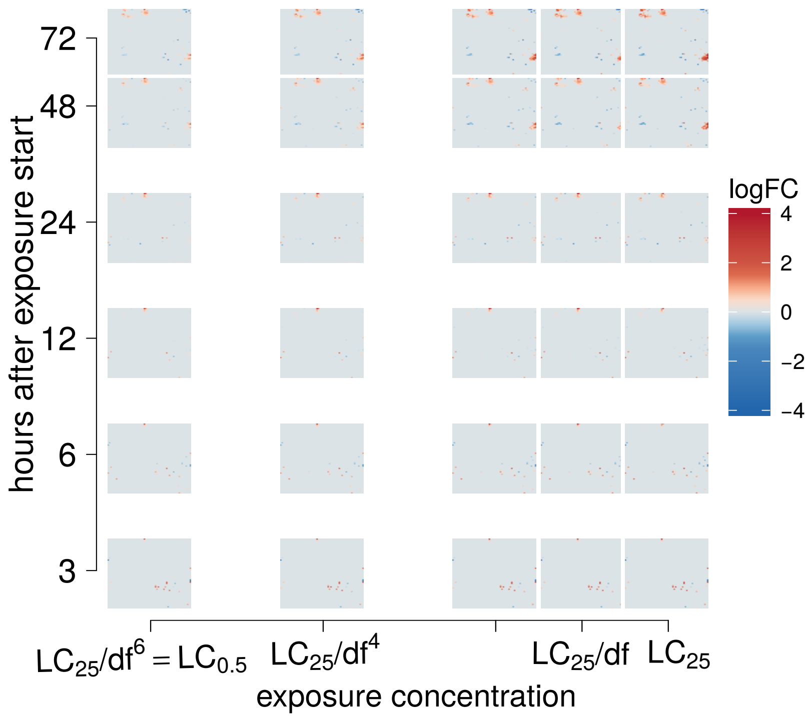


Figure C.2: *In silico* predicted toxicogenomic landscapes for mixture. All logFCs for toxnodes not predicted as significantly regulated by CA were set zero.

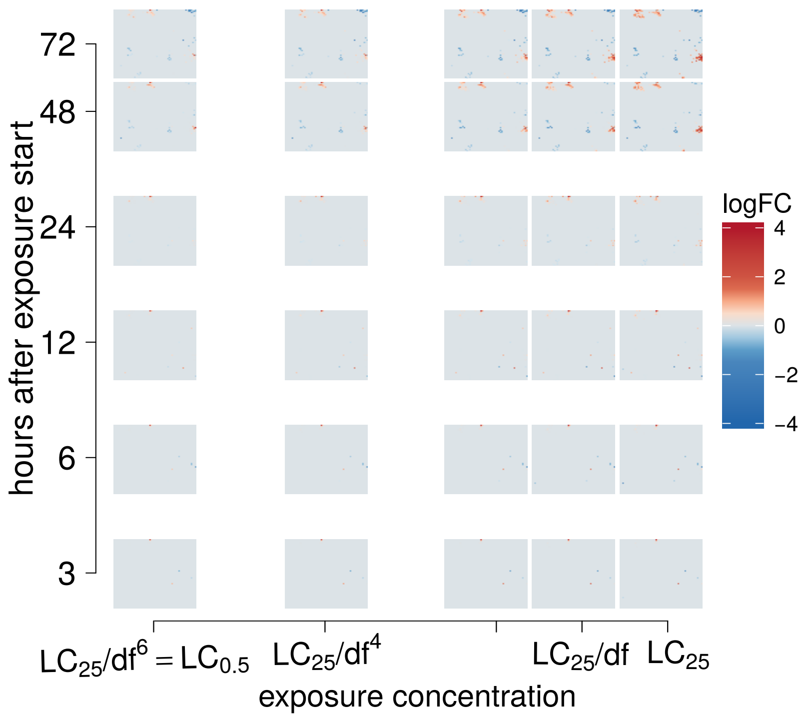


Figure C.3: Measured toxicogenomic landscapes for mixture. All toxnodes not identified as significantly affected in the mixture experiment were set to zero.

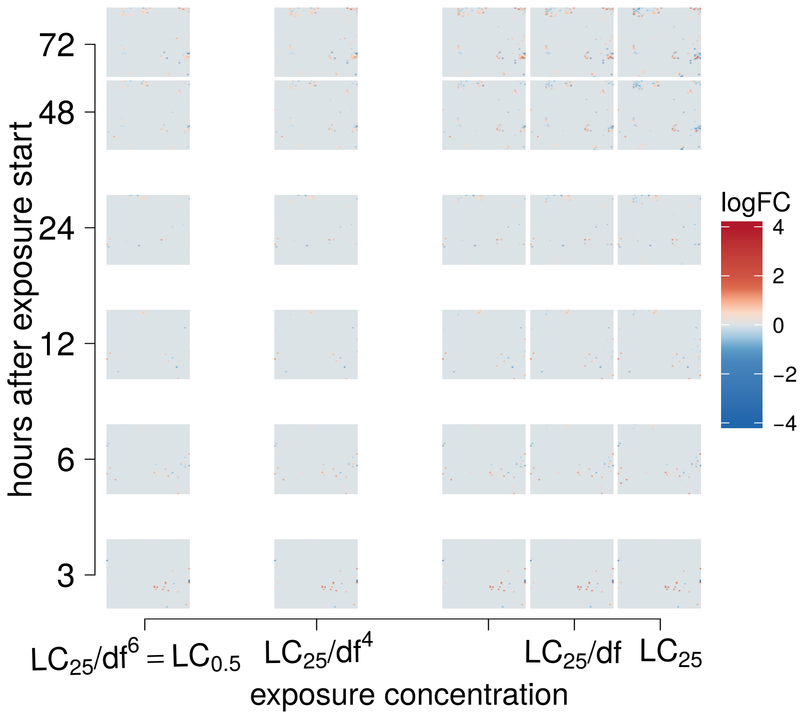
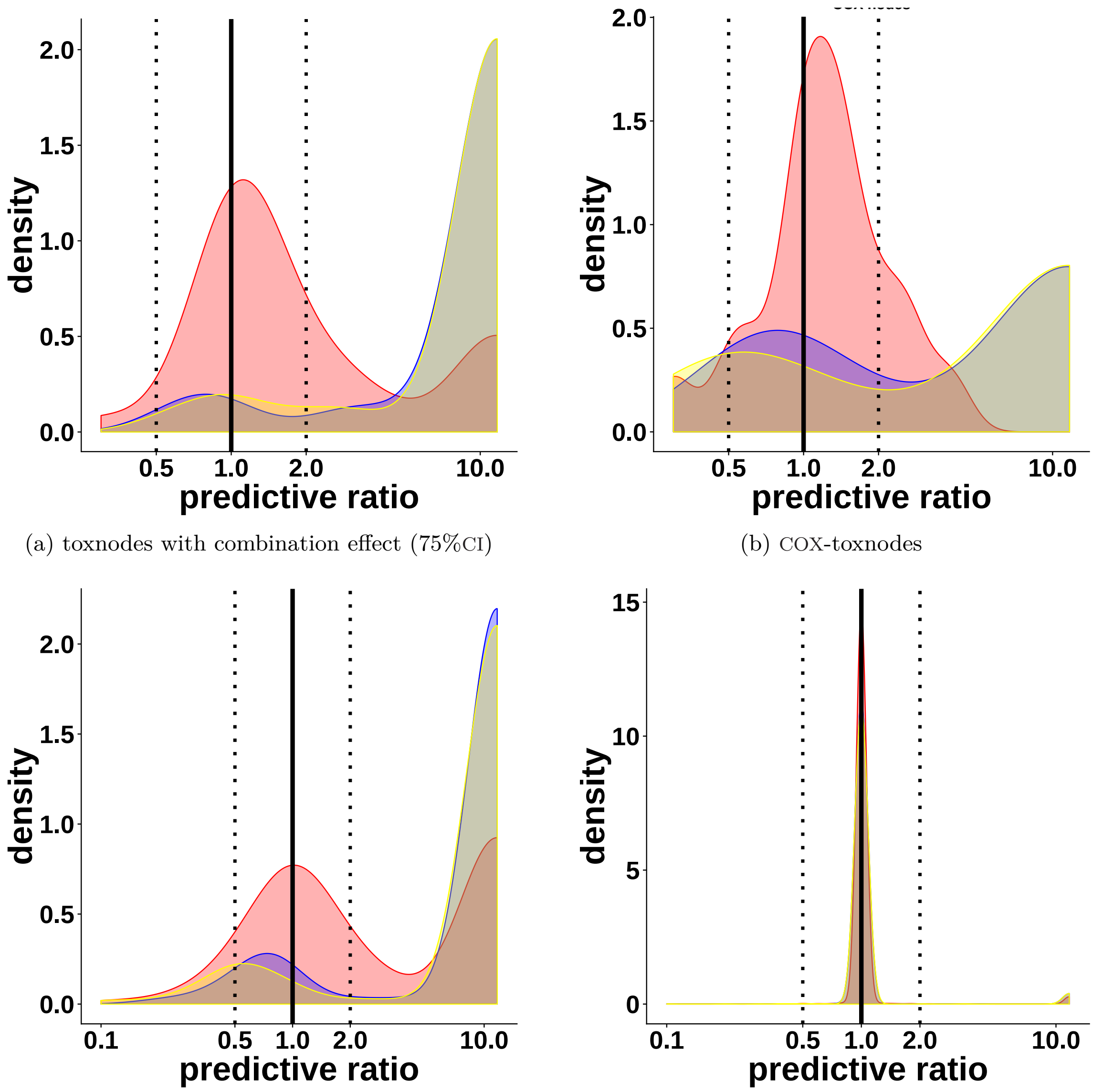


Figure C.4: Difference between predicted and measured toxicogenomic panorama for mixture



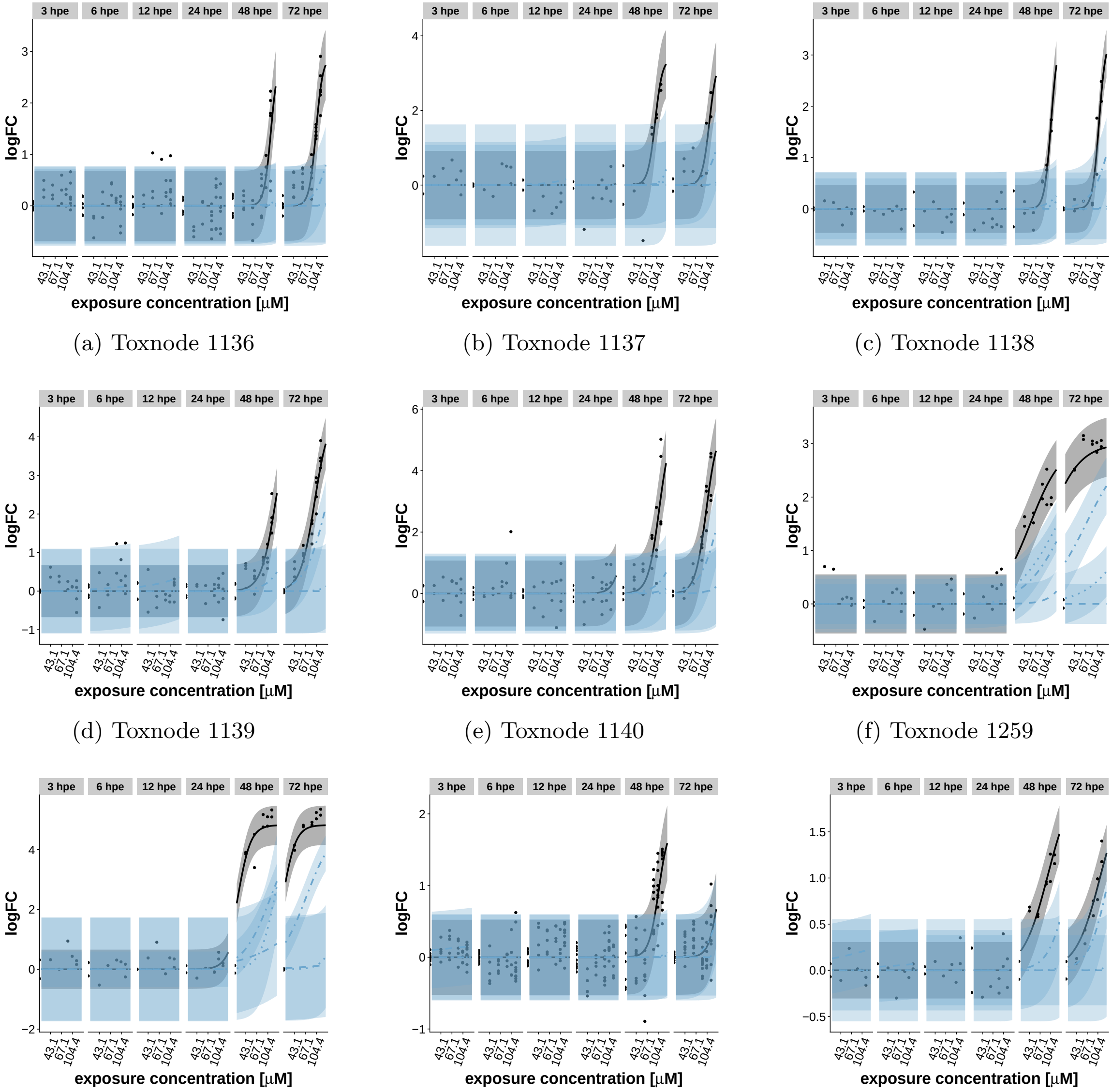
(a) toxnodes with combination effect (75%CI)

(b) COX-toxnodes

(c) toxnodes significantly affected by mixture

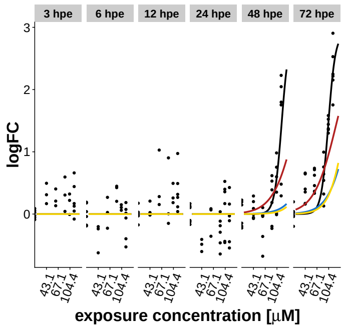
(d) all toxnodes

Figure C.5: Distribution of prediction ratios for combination of qualitative and quantitative prediction. CA (■);IA (■); EA (■). Predictive ratios above 1 indicate underestimation of toxnode sensitivity by the prediction, ratios below 1 indicate overestimation.

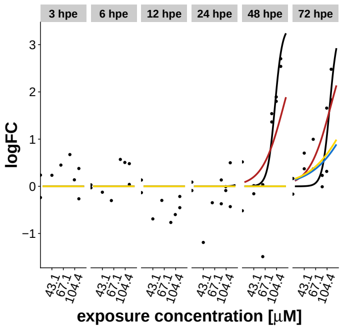


(a) Toxnode 1136
(b) Toxnode 1137
(c) Toxnode 1138
(d) Toxnode 1139
(e) Toxnode 1140
(f) Toxnode 1259
(g) Toxnode 1260
(h) Toxnode 3314
(i) Toxnode 3554

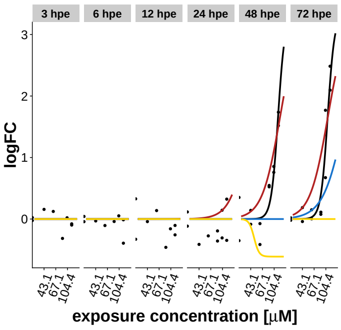
Figure C.6: Effect of mixture and single substances (modeled) for toxnodes showing distinct combination effect. Effect and 95% CI of single substance (■); Effect and 95% CI of mixture (■)



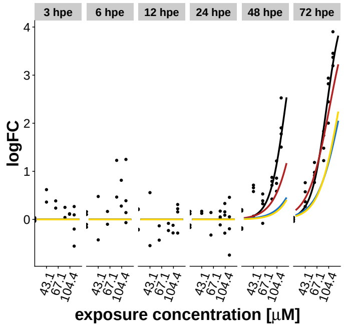
(a) Toxnode 1136



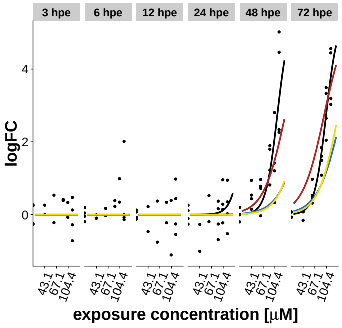
(b) Toxnode 1137



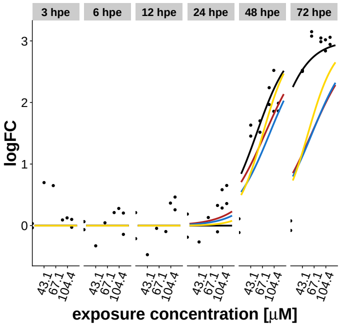
(c) Toxnode 1138



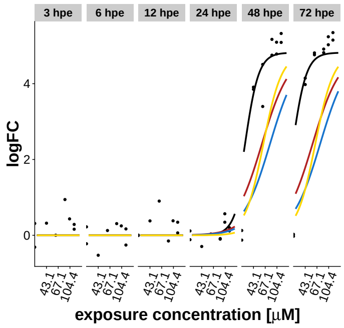
(d) Toxnode 1139



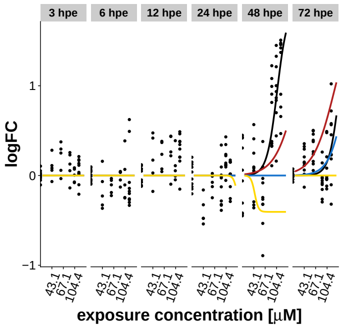
(e) Toxnode 1140



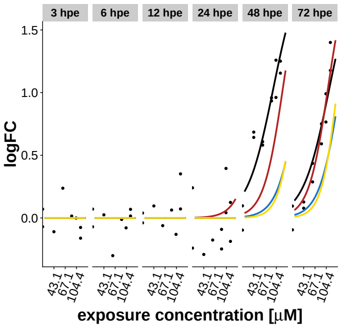
(f) Toxnode 1259



(g) Toxnode 1260



(h) Toxnode 3314



(i) Toxnode 3554

Figure C.7: Effect of mixture and single substances (modeled) for toxnodes showing distinct combination effect: measured effect (•); modeled effect (▬); CA (▬); IA (▬); EA (▬).

REFERENCES

Martin-Betancor, K., C. Ritz, F. Fernández-Piñas, F. Leganés, and I. Rodea-Palomares (2015). “Defining an additivity framework for mixture research in inducible whole-cell biosensors.” In: *Scientific Reports* 5, p. 17200. DOI: 10.1038/srep17200.

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Leipzig, 25. Februar 2019

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*__Schüttler, A.__, Reiche, K., Altenburger, R., Busch, W. (2017): The transcriptome of the zebrafish embryo after chemical exposure – a meta-analysis. *Toxicological Sciences*, 157.2, pp. 291–304, https://doi.org/10.1093/toxsci/kfx045

Fetter, E., Smetanova, S., Baldauf, Lisa; Lidzba, A., Altenburger, R., **Schüttler, A.**, Scholz, S. (2015): Identification and characterisation of androgen-responsive genes in zebrafish embryos. *Environmental Science & Technology*, 49.19, pp. 11789–11798, https://doi.org/10.1021/acs.est.5b01034.

Schüttler, A., Reiche, K., Altenburger, R., Busch, W. (2017): Zebrafish transcriptomes after chemical exposure - a quest for common grounds (Platform presentation). *SETAC Europe 27th annual meeting.* Brussels, Belgium.

Schüttler, A., Reiche, K., Altenburger, R., Busch, W. (2015): Sorting the signals - Meta-Analysis of transcriptome data of zebrafish embryos after chemical exposure (Poster). *SETAC Europe 25th annual meeting.* Barcelona, Spain.

Schüttler, A., Reiche, K., Altenburger, R., Busch, W. (2015): Transcriptome studies in ecotoxicology need time and dose resolved data (Poster). *Statistical Methods for Omics Data Integration and Analysis.* Valencia, Spain.

Schüttler, A., Reiche, K., Altenburger, R., Busch, W. (2015): Meta-analysis of transcriptome data of zebrafish embryos reveals low overlap in gene expression after chemical exposure (Poster). *ICCE 2015.* Leipzig, Germany.

*Publication is part of this PhD thesis. I performed the data analysis and wrote main parts of the manuscript.

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