SENSING HYDROPHILIC CONTAMINANTS:

transcriptional response of *Caenorhabditis elegans* as biosensor for water quality

Antoine Karengera

Propositions

1. The power of *C. elegans* to withstand the exposure to raw wastewaters is a major step forward towards testing the effects of hydrophilic compounds that are hard to extract or concentrate in water sample.
   (this thesis)

2. The application of transcription-based bioanalysis in the nematode *C. elegans* will create a bridge linking the *in vitro* and *in vivo* tools for water quality assessment.
   (this thesis)

3. The best way to make the general public understand and trust scientific achievements is to support scientists in their communication efforts rather than giving this task to communication specialists.

4. RNA technology has significantly improved human health.

5. A leadership's insensitive attitude towards the needs of compatriots is the most important setback for a democratization process.

6. A major breakthrough in poverty and corruption eradication in sub-Saharan African countries will only be achieved by abandoning kinship-related systems.

7. Children who grow up in poverty can never become economically successful when trapped in the mentality of their families fate.

Propositions belonging to the thesis, entitled

"Sensing hydrophilic contaminants: transcriptional response of *Caenorhabditis elegans* as biosensor for water quality"

Antoine Karengera

Wageningen, 7 September 2022

Sensing hydrophilic contaminants: transcriptional response of *Caenorhabditis elegans* as biosensor for water quality

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Sensing hydrophilic contaminants: transcriptional response of *Caenorhabditis elegans* as biosensor for water quality

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Thesis

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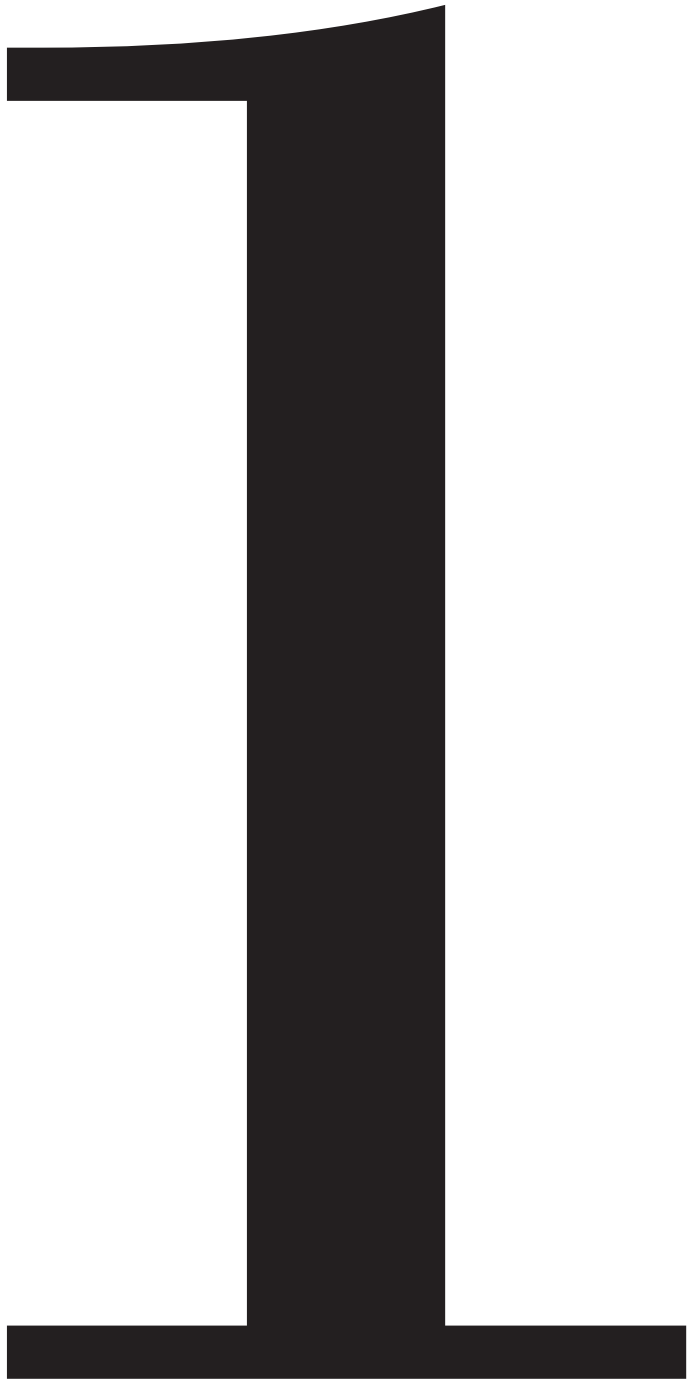

Chapter 1

General introduction

1. Water quality challenges: is your water safe?

Growing chemical and pharmaceutical use and unregulated disposal of chemicals as well as untreated or poorly treated waste waters pose a worldwide risk to sources for drinking water production and environmental water quality (Fig. 1). A large category of chemical contaminants, including many hydrophilic compounds, are frequently found in aquatic environments, especially agrochemicals [1-3] pharmaceuticals [4, 5], personal care products, industrial by-products, metabolites and many others [6-8]. Plant protection products and herbicides can enter surface waters via discharged waters from greenhouse horticultural practices [9] or as runoff from treated parcels or municipalities. Also, old chemical waste dumps present a serious environmental risk by releasing pollutants, especially the more water soluble ones, directly to ground- or surface water [10, 11]. Many compounds that are released into waste water treatment plants may survive the water treatment, whether or not as metabolites or by-products, and end up in environmental water matrices as micro-pollutants [12, 13]. So, even treated effluents may contain a wide range of natural and synthetic chemicals [14].

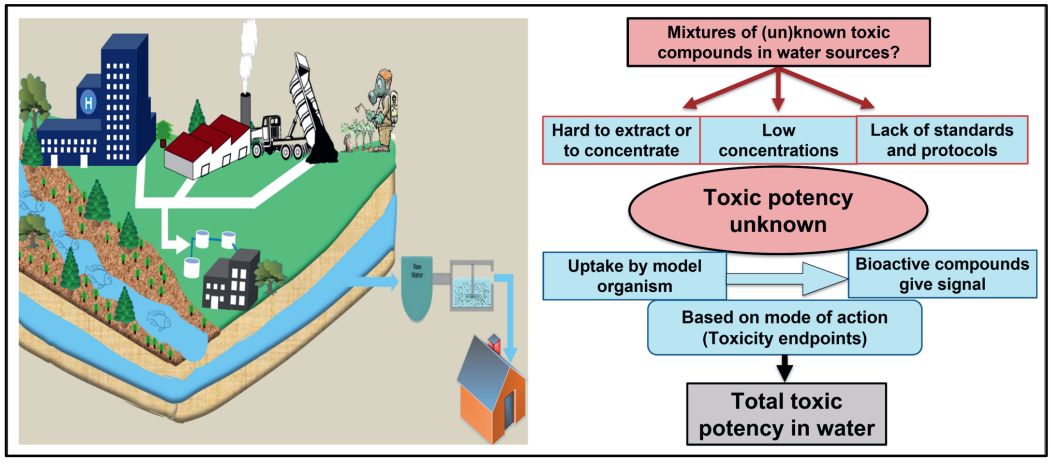

Figure 1. Possible sources of hydrophilic contaminants, their pathways to surface water and groundwater, and a summary of key water quality monitoring challenges associated with these pollutants.

Current methods for chemical monitoring of water quality mainly focus on lipophilic substances that are easy to extract and concentrate. Furthermore, the existing regulatory methods for drinking water are mostly directed towards the measurement of already known problematic or regulated chemicals [15]. This may lead to an underestimation or incorrect interpretation of human and environmental risks. The knowledge gaps on hydrophilic

substances are extensive, such as the lack of sensitive analytical chemical techniques for their concentration and identification, lack of certified reference materials, lack of data related to their fate, toxicity, and their behavior in environment, and lack of knowledge of their mixture effects [16, 17]. Contaminants which are not analyzed in the standard monitoring program could end up, for example, in drinking water without this being known since especially polar pollutants may not be removed by regular drinking water treatment [6] nor be stopped with carbon filters [18].

In addition, known substances for which chemical analytical methods do exist could exist at very low concentrations below detection limits [8]. Several other pollutants are present at detectable low concentrations that are generally considered to pose no acute risk [19-21]. However, the existing risk assessment procedures tend to overlook potential mixture effects [6, 20, 22] or long term exposure effects. Despite the low concentrations of these water pollutants there are concerns about their potential toxic effects [12, 23, 24]. The development of new chemical analysis tests for individual chemical agents including hydrophilic ones takes many years and requires huge resources, and then still the total toxic potency of the mixtures will not be known. Therefore, an efficient and cheap method is highly needed to assess the toxic potencies of hydrophilic chemical mixtures in water resources and distinguish between samples that pose reasons for concern and samples that do not. The method aimed in this thesis could be used to guide the chemical analysis to identify yet unknown toxic substances and their sources in groundwater or surface waters, before they are used for potable water production. It can also be used for monitoring the removal efficiency of (micro)pollutants during wastewater treatment and for assessing the quality of the resulting effluent and receiving waters.

2. Added value of a Bioassay: testing of the presence and toxicity of pollutants

Classic techniques used for water quality assessment and characterization typically rely on identifying and quantifying individual chemicals [25]. This approach can be helpful for evaluation of the risk posed by known chemicals, but may underestimate the risk posed by mixtures of known and unknown contaminants. Unlike chemical analysis, bioassay analyses can be used to directly characterize potential cumulative effects of bioactive substances on living cells, tissues or organisms without necessarily needing to know all the components of the samples [25, 26]. Bioassays can also be used to prioritize water sources based on ecotoxicological risks and prevent unnecessary costly chemical analysis at sites that pose low (eco)toxicological risks [27]. Typically, a bioassay measures biological effects of substances rather than identifying the substances. To identify the substance(s) responsible for a particular effect detected with a bioassay, an Effect-Directed Analysis (EDA) can be performed [28-30]. This is a powerful tool combining bioassay analyses with chromatographic separation and chemical analysis to identify drivers of bioassay activity.

Several studies involving the EDA approach investigating toxicity of water samples are reported [28, 31-34].

Bioassays can be used in vitro, by monitoring responses (parts of) cells in culture [35] or in vivo, utilizing a whole living system [36]. Most of the existing in vitro and in vivo bioassays are either very specific indicators, based on one or a few biological responses (e.g., estrogenic activity [37], aryl hydrocarbon receptor activity [38], thyroid hormone disrupting activity [39], oxidative stress response [40], and others) or are non-specific indicators of general toxic effects (e.g., mortality, fertility, reproduction, and others) [25, 36]. Hence, a battery of bioassays is often required for testing various types of bioactive pollutants present in water samples as demonstrated in [41-43]. Both in vivo and in vitro bioassays can be combined in a battery of tests to assess ecotoxicological risk in water [27, 44].

Escher et al. (2014) provided insights into in vitro bioanalytical tools used for water quality assessment [25]. These assays were performed in a controlled environment (e.g., a test tube or microtiter plate) and they were carried out using isolated tissue, enzymes, or cell culture. Various in vitro bioassays for water quality monitoring are compiled in the European technical report on aquatic effect-based tools under the Water Framework Directive [36]. In that report, some advantages are credited to the in vitro assays like the possibilities to analyze many different matrixes (e.g., concentrated extracts of surface water, sediment or pore water samples, biological tissues, passive samplers and effluents). This report also acknowledges in vitro techniques to be highly sensitive and require relatively small amounts of samples and short exposure time compared to the time required for an in vivo assay. Moreover, in vitro assays provide the opportunity to conduct high throughput toxicity screening and automated applications [39, 45]. Nevertheless, the in vitro assays are usually applicable for extracts or concentrated samples, and this may be irrelevant for hydrophilic compounds. Also, the in vitro systems studies are highly simplified when compared to the complexity of whole organisms and their applications overlook potential interactions between different structures of an organism (e.g., receptors, cells and organs) [36].

Various tests involving whole organism bioassays for the assessment of water quality monitoring are available including standardized testing methods according to OECD (Organization for Economic Co-operation and Development) or ISO (International Organization for Standardization). The Daphnia test method is designed to assess acute toxicity in *Daphnia magna* to evaluate the effects of chemicals towards daphnids or invertebrates [46]. The fish embryo acute toxicity (FET) test is another assay designed to determine the acute or lethal effects of chemicals on embryonic stages of fish and as a model for vertebrates without formally conducting animal experiments [47]. Other zebrafish bioassays similar to FET have been reviewed recently by Di Paolo and co-workers (2015) [48]. Their review paper extensively discusses the potential contribution of zebrafish-based assays for EDA approaches. Freshwater algal growth inhibition test is another OECD

method utilized to determine the effects of a chemical on the growth of freshwater microalgae and/or cyanobacteria [49]. Nematodes are another category of test organisms used as bioindicators for ecotoxicity testing [50]. These include a standardized method (ISO 10872) for determining the toxicity of environmental samples on growth, fertility and reproduction of *Caenorhabditis elegans* [51]. The advantage of using such in vivo tests is that also hydrophilic toxicants can be tested by direct exposure of the animals to the water samples where in vitro assays usually need extracts.

3. Toxicogenomics-based bioanalysis approaches

Typical toxicological tests assessing the adverse effects of chemicals on living organisms have traditionally focused on cytological, physiological, metabolic, and morphological endpoints. There is however a need for novel testing approaches that can help to overcome some of the existing limitations of traditional toxicity tests. Collins and colleagues (2008) advocated a shift in toxicology from observational science at the level of disease-specific animal models in vivo to predictive science focusing on broad inclusion of target-specific and mechanism-based biological observations [52]. Also, the OECD has launched a program on the development of Adverse Outcome Pathways (AOPs), which is considered as a central element of a toxicological knowledge framework to support chemical risk assessment based on mechanistic reasoning [53]. The new science of toxicogenomics combines RNA transcript (transcriptomics), protein (proteomics) and metabolite (metabolomics) profiling with conventional toxicology to investigate the interaction between genes and environmental stress fit this new approach [54, 55].

Toxicogenomic data available in literature is mainly derived from transcriptomics analyses. The organisms' genes may be "turned on" (activated) or "turned off" (deactivated) in response to external stimuli (e.g., pathological conditions or exposure to environmental agents) leading to adverse effects in their metabolism. For each gene included in the assay, the gene expression level is quantified experimentally after exposure and in a control condition in order to determine the differential expression of genes. A gene transcript is considered to be differentially expressed if the difference between the two experimental conditions (mostly reported as expression fold change) is statistically significant [56]. Several technologies exist to determine the gene expression changes of a biological system after exposures to substances. These include microarrays [57], RNA sequencing (RNA-Seq) [58], or reverse transcription quantitative polymerase chain reaction (RT-qPCR) analyses methods [59]. Microarrays and RNA-Seq techniques allow a genome-wide analysis of gene transcription levels enabling the simultaneous identification of a large number of genes [60, 61]. By contrast, RT-qPCR assays can only analyze a finite number of genes, but it is the most sensitive out of the three and is widely used for validation of transcriptomic data [62, 63].

Transcriptome analysis is considered as a new way of understanding biological systems and their response to toxic insult. Suter et al. (2004) explains how gene expression profiling can be used to assess the toxic potential of a compound and to understand its mechanisms of toxic action [64]. The approach consists of the identification of toxicity-related gene expression signature (fingerprint) of the suspect toxic compound and compare it to the reference transcriptomic profiles of compounds whose toxicological profile is well-established. This approach is comparable to another toxicogenomic method known as "signature matching" as explained in [56, 57], where compound-induced gene expression patterns (referred to as signatures) are evaluated against a pre-existing compound signature library in order to make predictions about their potential toxicity. Assuming that compounds inducing similar gene expression signatures will have similar effects in a biological system, transcriptomic read-outs could provide more accurate quantification of the whole mixture toxicity and provides insights into the hazards posed by chemicals in comparison to the traditional approaches like structure-activity relationships (SAR) which rely mainly on structural similarities to infer mechanisms of toxicity of suspected toxicants [65]. Nevertheless, considering the amount of chemicals in use, more transcriptomic studies are still needed to develop a reference gene expression database. Such a database can be established by following a three-part strategic approach as proposed in [55]. This strategy includes (1) the selection of chemical agents that induce specific types of toxicity, (2) the selection of several structurally and functionally diverse chemicals that produce comparable patterns of toxicity, and (3) using nontoxic isomers of toxicants to analyze effects in target and nontarget tissues.

The data derived from toxicogenomic studies can be used to assess potential hazards posed by chemical substances. There are many areas of toxicology to which transcriptomics approaches can be applied such as the prediction of the toxicological modes of action of compounds (mechanistic toxicogenomics), toxicity screening of chemical agents (predictive toxicogenomics), environmental monitoring or risk assessment [57, 66-68]. Gene expression profiling can also help to identify genes that may be candidate biomarkers for specific toxic effects [55]. Transcriptomic effect-based monitoring has been previously proposed for assessment of relative contributions of point sources to pollution and the efficacy of pollution remediation [69]. Schriks and co-workers (2014) demonstrated how transcriptomics approaches can be used to assess water quality including the concept of No Observable Transcriptional Effect Level (NOTEL) (i.e., the concentration level of a chemical below which no significant changes in gene expression occur) that might be used to investigate the impact of environmental contaminants as well as mixtures [70]. Schriks's report and other literature [56, 71], nevertheless, also admit the existence of some challenges in the application of transcriptomics technology such as limited sequence data for the organism of interest or the occurrence of confounding factors that can be difficult to be differentiated from the toxicant-induced gene expression. Likewise, Escher et al. (2021) point out the potentials of omics technologies in water quality bioanalysis, but also

acknowledge that some harmonization and methodological refinements are still necessary before adopting this approach as a reliable tool for water quality assessment [25].

4. *C. elegans* as a model organism for toxicity testing

Introduced in 1960s and 1970s as model organism for biological research [72], the nematode *Caenorhabditis elegans* has attracted increasing attention in biomedical and environmental toxicology [73]. Several studies have been carried out previously to predict the toxicity of chemical substances in *C. elegans* as a test organism [74, 75]. This soil-dwelling nematode provides particular experimental advantages such as small size, ease to handle, bacterivorous, short life cycle, and relatively cheap to maintain in an ordinary laboratory setting [76, 77]. Its culture can be conducted on solid support or in liquid, in Petri dishes, tubes, or well plates and the treatment with toxicants can be set up as an acute or chronical exposure by injection, feeding, or soaking [76]. Importantly, *C. elegans* provides the opportunity to assess transcriptional effects of toxicants using the gene expression profiling approach as described above. This is because the nematode's genome has already been fully sequenced [78] and many genes or signaling pathways are conserved in higher organisms, making it suitable as a model for risk assessments [79-81]. Additionally, there are several open access databases about the biology of *C. elegans* which provide various information related to the nematode genes and genomes, e.g. WormBase (www.wormbase.org), WormAtlas (https://wormatlas.org) among others.

Leung and co-workers (2008) describe in detail the potentials of *C. elegans* as a model organism in the fields of toxicology such as neurotoxicology, genetic toxicology, and environmental toxicology [76]. The authors also present *C. elegans* as a useful test model for high-throughput experiments including genome-wide screening for molecular targets of toxicity and rapid toxicity assessment for new chemicals. The aforementioned transcriptomics approaches could be considered as part of the genome-wide screens and can be a useful tool for assessing the toxicants (and mixtures) with a poorly understood mode of action.

Like any other organism, the nematodes exposure to xenobiotics can trigger adverse effects at a molecular level. This happens due to the interactions that can take place between xenobiotics and biological targets resulting in molecular initiating events (e.g., gene activation) within the framework of adverse outcome pathways (AOP) [82]. *C. elegans* is equipped with various biological macromolecules whose functions can be influenced by chemical exposure. Take, for instance, nuclear receptors (NRs) which are capable to directly bind to DNA and regulate the expression of adjacent genes [83]. There are 284 NRs receptors in *C. elegans* and many of them are involved in several vital functions of the nematode including those comparable to the NRs of vertebrates [84, 85].

Figure 2. Image of *Caenorhabditis elegans* taken with a binocular microscope. This image shows eggs, larvae and adult worms crawling on Nematode Growth Medium (NGM) in Petri dish (Photo by A. Karengera).

Fig. 3 depicts how the NR-xenobiotic interaction can influence biological functions via gene expression regulation. Another example are cell cycle checkpoint proteins which play an essential role in the maintenance of genomic stability following DNA damage. In response to DNA injuries, the activation of checkpoints can result in cell cycle arrest which allows time for DNA repair [86]. The failure to restore the DNA integrity can lead to problems such as mutations, apoptosis, among many others [87, 88]. Such molecular processes can be evaluated by using transcriptomics approaches.

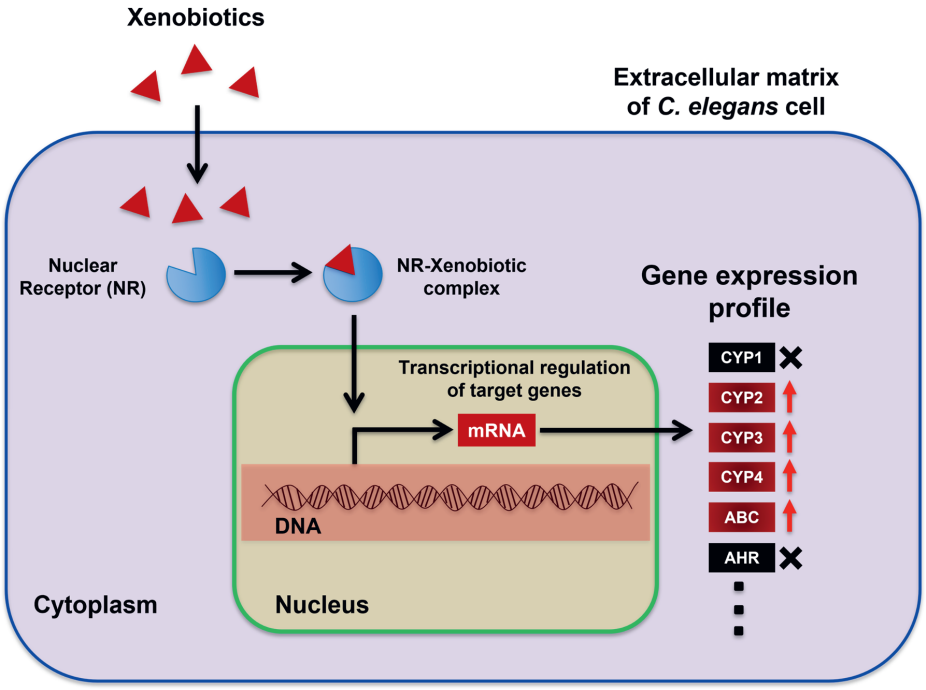

Figure 3. Activation of a nuclear receptor (NR) in living cell. After entering the cell, the xenobiotic interacts with the NR. The formed NR-xenobiotic complex is then translocated into nuclei where it binds response elements of DNA and regulate the transcriptional levels of target genes. Red arrow pointing up (↑) indicates the upregulation of the nematode genes involved in a particular biological process by xenobiotic exposure whereas a cross (X) indicates that the process is not affected by xenobiotics.

5. Hydrophilic compounds still understudied

Current standards for water quality assessment focus predominantly on problematic lipophilic substances from the past which account for a small number of compounds compared to thousands of old and new industrial chemicals present in the environment. If one considers the mixture complexity of substances present in water matrices, chemical assessment is expected to reveal just a tip of the iceberg of contamination. Moreover, there is a big concern about the presence of yet unknown hydrophilic compounds that could present environmental or human health risk. Metabolization of compounds by phase 1 and phase 2 metabolism mostly render them more water soluble to enhance excretion via e.g. urine and bile [89]. Metabolism can also occasionally generate toxic metabolites via a process known as bioactivation [90, 91], especially for the substances often categorized as

"indirect-acting" in reference to the chemical agents with little or no toxicological activity, that become toxic upon metabolic activation [92, 93]. Therefore in sewage many pharmaceutical are expected to be present as relatively hydrophilic metabolites. For several practical reasons, including problems on isolating and concentrating hydrophilic compounds from water samples, current methods cannot identify or quantify all these compounds and therefore not guarantee the water quality. And again, if they could be analyzed the actual toxicological risk that these compounds or mixture thereof pose would still be unknown. In vivo bioassay methods could allow for the detection of the toxic potency of mixtures of known and unknown hydrophilic chemicals in water samples. By exposing sentinel species to the water samples the molecular response to potentially present hydrophilic toxic compounds provides a functional endpoint. Using small organisms makes the test faster as the effective body burden will be reached much faster in small than in large organisms and exposure volumes can be really small. Nematodes offer the advantage that they are invertebrates widely used as an alternative for laboratory use of animals [94]. This approach could provide an easily applicable and cost-effective method for assessing the toxicological safety of water sources.

6. Aim of research

This thesis concerns the development of an invertebrate bioassay that can simultaneously identify and quantify the toxic potency of multiple compounds in a water sample without the need for extraction. The aim for this thesis was to develop a bioassay based on the transcriptomic response of the nematode *C. elegans* to toxic compounds. This aim was translated into the following research objectives:

1. To evaluate the transcriptional responsiveness of *C. elegans* to the toxic effects of direct-acting model compounds by genome-wide gene expression profiling.

2. To evaluate the transcriptional inducibility of *C. elegans* biotransformation enzymes in response to indirect-acting model compounds.

3. To evaluate the applicability of the *C. elegans* bioassay for fingerprinting the toxic potency of hydrophilic contaminants present in (waste)waters.

4. To develop a fast and easy-to-use multiplex gene expression assay based on characteristic gene markers associated with the toxicants as found in the *C. elegans* model.

7. Outline of the thesis

Four research questions are derived from the above objectives:

A. *Can a practical nematode bioassay method be developed for (hydrophilic) compounds?*

B. *To what extent is the nematode transcriptionally responsive to model contaminants?*

C. *What are the most relevant mechanisms represented among the differentially expressed genes (DEGs) in nematode in response to exposure to contaminants?*

D. *Can a dedicated multiplex gene expression assay be developed for fast and easy quantification of toxic potencies of (hydrophilic) contaminants in (water) samples?*

The question 1 – 3 are addressed in **Chapter 2 – 5**. **Chapter 2** and **Chapter 3** mainly focus on the experiments designed to fill information gaps in literature about the operating protocols for the testing of model toxicants in *C. elegans*. In **Chapter 2**, an experimental set up was designed for faster handling and easier nematode exposure in liquid medium. Non-lethal concentrations were first determined for three direct-acting genotoxic model compounds (MMS, ENU, and HCHO) to establish the appropriate dose for gene expression profiling assays in *C. elegans*. This experiment also helped identifying the potential sources of experimental and biological variation for this kind of studies. After successfully testing direct-acting toxicants, the next step was to investigate the transcriptional effects of compounds that require metabolic conversion to become active toxicants (referred to as indirect-acting chemical agents). *C. elegans* is reported to be deficient in cytochrome CYP1-like P450 metabolism and that its aryl hydrocarbon receptor (AhR) homolog encoded by ahr-1 purportedly does not interact with dioxins or any other known xenobiotic ligand. Therefore, gene expression profiling was carried out in the nematodes exposed to four prototypical toxicants whose biological effects are known to be mediated by CYP1 (for bioactivation) or AhR pathways (**Chapter 3**). The studied compounds include aflatoxin B1 (AFB1), benzo[a]pyrene (B(a)P), PCB mixture Aroclor 1254 (PCB1254) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) as representative compounds in the toxic classes of mycotoxins, polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), and dioxins. Following successful transcription-based nematode assay development with pure compounds, the new bioassay was applied to environmental polluted samples (**Chapter 4**). In this chapter, the toxic potencies of hydrophilic contaminants were determined in wastewaters before and after treatment by a wastewater treatment plant (WWTP) and effluent receiving surface waters.

To make it faster and easier to answer question 4, a fluorescent bead-based multiplex assay was developed (**Chapter 5**). The method relies on branched DNA (bDNA) technology allowing a direct measurement of mRNA transcripts of 50 target genes in the tissues lysates of nematodes, so without the need for further sample preparation. The newly developed

assay was validated and applied to detect the transcriptional response of *C. elegans* to (waste)waters and mixtures of organic pollutants in extracts from swimming crab tissues.

In **Chapter 6**, the results of this research are discussed in the broader context of fingerprinting toxic potencies of hydrophilic bioactive contaminants in (waste)water. This chapter also discusses how the effect-based bioanalysis approach developed in this research can be translated to a practical method for water quality monitoring. Also, future research directions and recommendations are proposed.

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Chapter 2a

Early life developmental effects induced by dioxin and PCBs in novel bioassays with *C. elegans*

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Abstract

In this study, we assessed the effects of dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), two mixtures of Polychlorinated biphenyl's (PCBs, Clophen A50 and Aroclor 1254), marine sediments and swimming crab tissue extracts, on the early life stage (ELS) of the nematode *Caenorhabditis elegans* (*C. elegans*). In the initial design, gravid *C. elegans* females were first exposed to the test chemicals mixed with agar for 72 hours. The nematodes were transferred to agar culture plates to lay eggs. The development of the hatched larva was then observed. In an improved, more practical design, the nematode eggs were isolated from gravid females and directly exposed to the test compounds in solution. In both methods, the development of nematode larvae from developmental stage L1 to L4 was observed after 72 hours of development. Field samples (marine sediment and swimming crab tissues) were tested using both nematode bioassays and an *in vitro* reporter gene assay (DR-CALUX) to quantify the dioxin equivalent potency (TEQ). Exposure to dioxin, PCBs, and field sample extracts delayed the early life development of *C. elegans*. In the maternal exposure assay, exposure to 10 pM Clophen A50 and 10 pM TCDD for 72 hours significantly inhibited or delayed larva maturation from the L3 to L4 stage with respectively 60% and 50%. In the direct egg exposure design, exposure to 5 µM Aroclor 1254, or 10 nM and 10 µM TCDD significantly delayed larva maturation from the L3 to L4 stage, but to a lesser extent (respectively 20%, 20% and 40%) and at a higher concentration. In general, both approaches demonstrated a considerable reduction in L3 to L4 development after exposure to dioxins or PCBs. All the field samples contained dioxin-like potencies (TEQ: 0.67 - 4.91 ng/kg equal to 0.2 – 1.47 pM TCDD), which reduced the L3-L4 larva development by 40% - 60%. In this study, we used two newly developed *in vivo* bioassays to quantify the effects of persistent organic pollutants on the ELS of the nematode *Caenorhabditis elegans*. The indirect exposure is more realistic and more sensitive, but the egg-exposure method facilitates rapid *in vivo* testing of many samples.

1. Introduction

Polychlorinated biphenyl (PCBs) and dioxins are organic pollutants that persist in the environment. Dioxins are a family of complex chemicals with similar molecular structures, and they include polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) [1]. The toxicity of dioxins is mainly mediated via the aryl hydrocarbon receptor (AhR) that requires the planar configuration [2]. The PCBs are divided into two congeners groups based on their molecular structures: “dioxin-like” PCBs (DL-PCBs) and “non-dioxin-like” PCBs (NDL-PCBs). NDL-PCBs comprise the larger proportion of PCBs in the environment [3]. In the present study, we used two comparable technical mixtures of PCBs: Aroclor 1254 and Clophen A50. Both of them mainly consist of NDL-PCBs, implying that their mechanism action is mostly not mediated via the AhR. Dioxins and PCBs belong to the persistent organic pollutants (POPs). They are recalcitrant lipophilic chemicals that accumulate in sediments and bioaccumulate in the food chain [4, 5]. POPs can be transferred vertically from the mother to their offspring [6] in egg-laying species and mammals (prenatally and via lactation) and in addition teratogenic effects are possible. Dioxins and PCBs induce numerous adverse effects, including disrupting growth and early life development, inducing hepatotoxicity, and interfering with reproduction and early development [7-9]. The effects are most severe in developing organisms (humans and wildlife) and may last the entire lifetime of an organism [10-12]. Therefore, it is important to assess the toxic potency of such POPs that are ubiquitously present in sediments or wildlife. We used *in vitro* and *in vivo* bioassays to assess the toxic potency of dioxins and PCBs in benthic aquatic and marine ecosystems because chemical methods cannot assess the combined toxic effects of such mixtures.

The DR-CALUX *in vitro* bioassay is an efficient method for quantifying the potency of dioxins and dioxin-like compounds (DLCs) in sediments and assessing their interaction with AhRs [13]. The relative toxicity in a sample is expressed as the toxic equivalency (TEQ), relative to the 2,3,7,8-tetrachorodibenzo-p-dioxin (TCDD) level, the most potent congener. TEQ is calculated based on an elaborate chemical analysis of all AhR antagonists present in a sample by multiplying each concentration by their toxic equivalency factor (TEF) [14]. The DR-CALUX directly quantifies the TEQ, and the outcome often is expressed as bioanalytical equivalents (BEQ) [15]. Early developmental defects occur after non-lethal exposure of developing amphibian and fish embryos to NDL-PCBs and DL-compounds. The developmental effects of these compounds on invertebrates are less studied. Also, there is no rapid assay or method for analyzing the adverse developmental effects of NDL-PCBs and DL-PCBs together with DLCs on a small scale. Ideally, such assay is performed with small invertebrates with a short life-cycle which can also represent effects in vertebrates.

In the present study, we explored the suitability of *C. elegans*, a free-living nematode and a model organism for *in vivo* assays for PCBs’ toxicity assays. The nematode was selected

because it has a short lifecycle, many progenies, and the ease of cultivation in a laboratory setting. In addition, because *C. elegans* are self-fertilizing hermaphrodites, each progeny represents a genetic clone. Although *C. elegans* is a relatively simple organism, it shares most of its developmental molecular signals with higher organisms like humans. *C. elegans* is a validated model organism for developmental studies [16]. For instance, *C. elegans* has also been used for toxicity studies in assessing the adverse effects of heavy metals [17]. The nematode genome has been fully sequenced, facilitating gene expression studies. Recent studies have shown that the effects of genotoxic compounds are concentration-dependent effects [18, 19]. For example, benzo(a) pyrene (B (a) P) and Aroclor 1254, PCB mixtures, induce the expression of toxicity-related genes in *C. elegans* genes [19].

In the present study, we describe two straightforward and inexpensive methods for analyzing the effects of direct and indirect exposure to dioxins and PCBs on the early development of *C. elegans* larvae. Gravid nematodes were either fed on the compounds, or their eggs were isolated first and directly exposed to the chemicals. The maternal exposure method mimics the natural exposure to these compounds. The exposure of gravid *C. elegans* method was developed nine years ago and has been in application since then but has never been published. The test was repeated with standard compounds to compare the effect with the egg-exposure method. The direct egg-exposure method is fast and easy, and suitable for testing many samples. In general, we analyzed the effects of dioxin (TCDD), a mixture of two PCBs (Aroclor 1254 and Clophen A50), and field samples contaminated with these compounds (sediments and edible crab) on the early life of *C. elegans* development. We found that the compounds and extracts disrupted the early life development of *C. elegans* in a consistent and dose-dependent manner.

2. Material and methods

2.1. Chemicals

All chemical reagents used in this study were of analytical grade (purity > 99%). Nitric acid (65%) and hydrogen peroxide (70%) were purchased from Changmu company LTD (Hangzhou, China). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Aroclor 1254 (PCB1254) (analytical standards grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Clophen A50 was purchased from Promochem (Wesel, Germany), and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) was purchased from Schmidt B.V. (Amsterdam, The Netherlands).

2.2. Nematode strain and cultivation

C. elegans wild-type strain (*Bristol N2*) nematodes were obtained from the Nematology group at Wageningen University and were maintained on Nematode Growth Medium (NGM) agar plates at 16 °C. The media was changed every month with fresh NGM agar seeded with *E-coli* bacteria. The *E coli*-NGM was prepared as previously described [18, 20].

2.3. Experimental set-up

The effect of dioxin and PCBs on the early developmental life of *C. elegans* was assessed using two newly developed methods: 1) indirect exposure through the mother and without further exposure of the laid eggs and 2) direct exposure to the isolated eggs. Both exposure methods were performed at 20 ◦C.

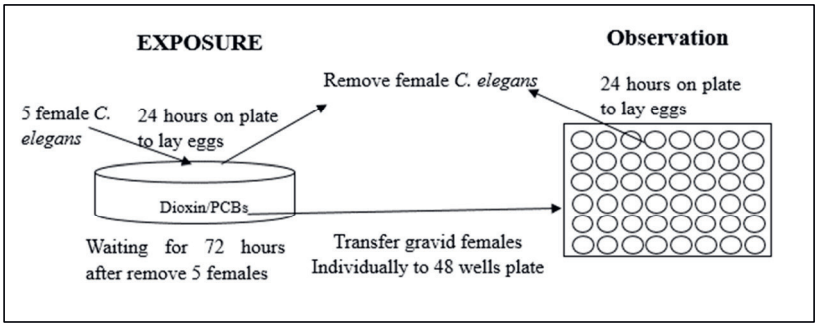


Figure 1. Schematic overview of the maternal-exposure bioassay method.

For the maternal exposure method (Figure 1), dioxin, PCBs, or field sample extracts were dissolved in DMSO before dilution in pre-heated NGM. Briefly, five gravid *C. elegans* females from a clean culture plate were transferred onto NGM agar supplemented with the test compounds. After 24 hours, the gravid *C. elegans* were removed, only leaving behind the eggs. After 72 hours, newly developed and exposed gravid females were transferred to 48-well tissue culture plates (one nematode per well). Each well was filled with 130 µl of K-medium. The exposed gravid *C. elegans* were removed from the K-medium after laying eggs. The number of offspring produced in 48-well tissue culture plates was counted, and for all larvae the L1 - L4 developmental stage was determined 72 hours later.

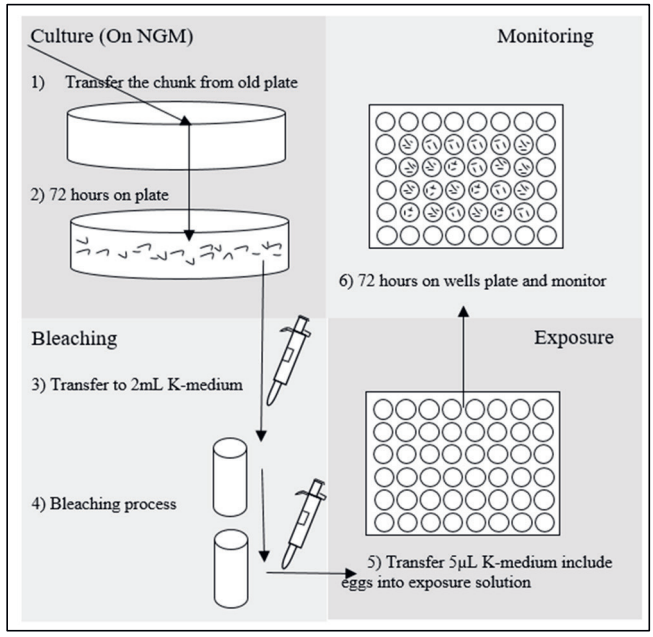


Figure 2. Schematic overview of the direct egg exposure bioassay method.

For the direct egg-exposure method (Figure 2), adult nematodes were transferred from agar-culture to liquid culture medium and, after 72 hours, concentrated in a 2 ml tube through centrifugation at 13,000 rpm. Sodium hypochlorite (1 ml; 5 %) was then added to the pellet for bleaching treatment [18] to eliminate nematodes in all developmental stages except the eggs. The pellets only containing eggs were dissolved in 0.5 ml K-medium and a mixture of deionized distilled H_2O and salts (2 mM NaCl and 13 mM KCl). Thereafter, 5 µL of eggs in K-medium was diluted with fresh 2 ml K-medium and aliquoted in 48-well tissue culture plates, ensuring that each well contained at least ten eggs. The number of eggs was

confirmed using a microscope. Dioxin, PCBs, or field sample extracts were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in all the plates was 0.5%, a concentration at which no significant effects on *C. elegans* were observed [21]. The eggs and subsequent nematode reproductive stages in the well-plates were monitored every 24 hours for 72 hours using a stereo microscope as previously described based on their size and activities [22].

2.4. Field samples

Apolar, non-organic extracts from eleven sediment samples from various places in the Netherlands were prepared, and their effect on the performance of offspring from exposed nematodes was tested using the indirect exposure nematode assay. The concentrations of polar in the agar plates were equal to that in the wet sediment samples to mimic natural exposure. Field samples (marine sediment and swimming crab samples) with dioxin and PCBs residues were collected in 1996 from the Netherlands (sediment, 2009 [23]) and China (Hangzhou Bay area sediment and crabs 2019 [24]). For the extraction of field samples, 10 g of each sample was dried overnight at 35°C and mixed with 1g of NaSO4. A hexane/acetone (1:1) liquid-liquid extraction method was used [25, 26]. The samples were de-sulphurated by adding tetrabutylammonium sulfite [27]. The samples were further cleaned using a multilayer acid-base silica column as previously described and dissolved in DMSO [28].

2.5. DR-CALUX bioassay

The (concentration) extracts dissolved in DMSO were analyzed using the DR-CALUX bioassay as previously described [29]. Briefly, 40 µL of the sample in DMSO was dissolved in 2 mL of the cell culture incubation medium. After 24 h, the medium was aspirated, and the cells were washed and lysed before measuring the luciferase content using a Luminometer. The residue contents were expressed as bioanalytical equivalents (BEQ) [15], calculated using the TCDD calibration curve (curve fitting of the Sigmoid dose-response variable slope) using GraphPad Prism software v 5.0.

2.6. Statistical analysis

Data were analyzed using the GraphPad PRISM 5.0 software. Comparisons between groups were performed using the unpaired t-test at a two-tailed $P < 0.05$.

3. Results

Supplementary figure C1 shows the absolute number and the relative distribution of larvae over the developmental stages per exposure and time point. There was no significant difference in the absolute number of nematode offsprings between the exposed and control groups at any time. Also, there were no visible malformations or behavioral changes under

either exposure method. However, exposure to dioxins and mixture of PCBs delayed larvae development, shown by few larvae that had reached the L4 stage within 72 hours.

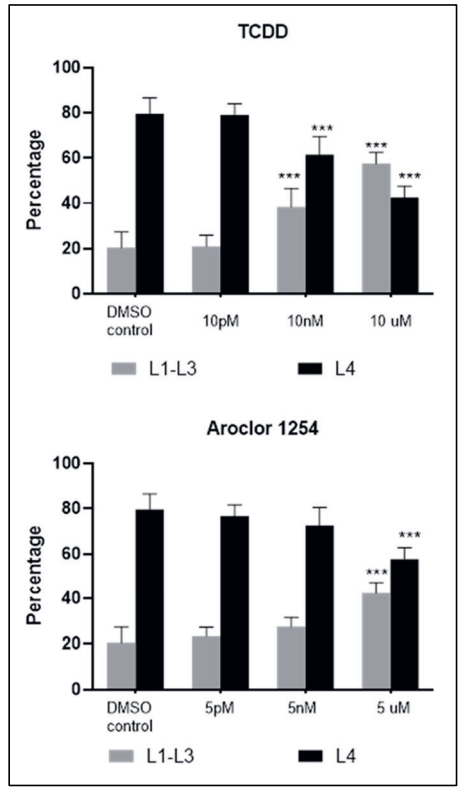


Figure 3. Proportional distribution in developmental stages of *C. elegans* larvae from eggs directly exposed to (a) the dioxin, 2, 3,7,8-tetrachloordibenzo-p-dioxine (TCDD), and (b) the technical PCB mixture Aroclor 1254. The larvae at stage 4 (L4) or stage 1-3 (L1 – L3) were determined after 72 hours of exposure. At least ten nematode eggs were exposed each time. *** represents $P < 0.0001$.

The effects of exposure to the PCB mixture Clophen A50 and TCDD in the maternal exposure method on larval development were recorded every 24 hours. As this was very time-consuming and did not yield extra relevant information, it was decided for further experiments to only observe after 72 hours. The number of larvae at a specific developmental stage (L1 - L4) at 72 hours after removing the gravid nematode from the observation wells is shown in Figure 3. Exposure to Clophen A50 and TCDD exposed groups delayed *C. elegans* development in a dose-dependent manner, particularly from the L3 to L4. The lowest Clophen A50 or TCDD concentrations of 10 pM induced significant ELS effects. Compared to the control groups, 10 pM Clophen A50 and TCDD decreased the

number of L4-stage larvae by 60%, whereas a 10 µM TCDD concentration caused an 80% decrease in L4-stage larvae development. The larval development not only stalled at stage L3, but the larvae in this larval stage died in a dose-dependent manner (Figure C2). This occurred for TCDD and Clophen A50 groups.

The developmental effects of the technical PCB mixture Aroclor 1254 and TCDD were tested in the egg exposure method and the developmental progress assessed after 72 hours. In the control group, 80% of the larvae had reached stage L4 after 72 hours, which is in accordance with other studies [30]. Compared to the control group, the percentage of nematodes in L4 was significantly lower after exposure to 5 µM Aroclor 1254 (20% less), 10 nM TCDD (20% less), and 10 µM TCDD (40% less) (Figure 4). In addition, the nematodes in stage L3 in the 5 µM Aroclor, 10 nM TCDD, and 10 µM TCDD group died (Figure C3).

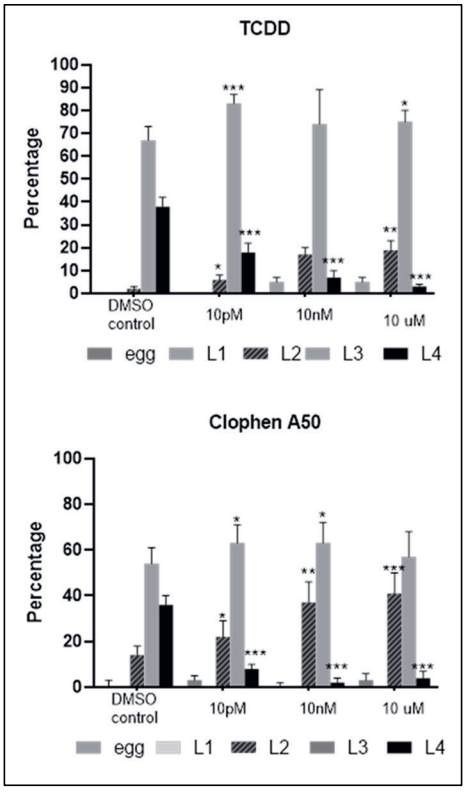
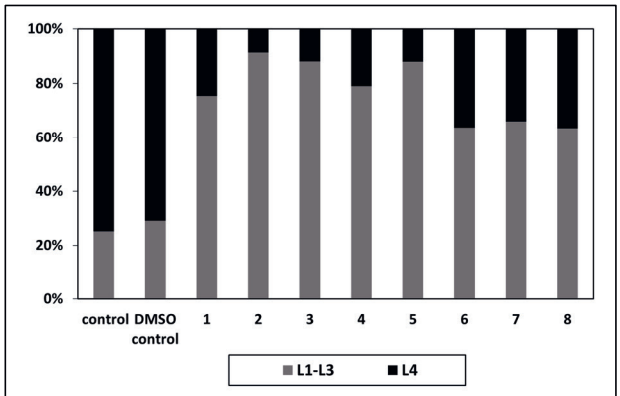


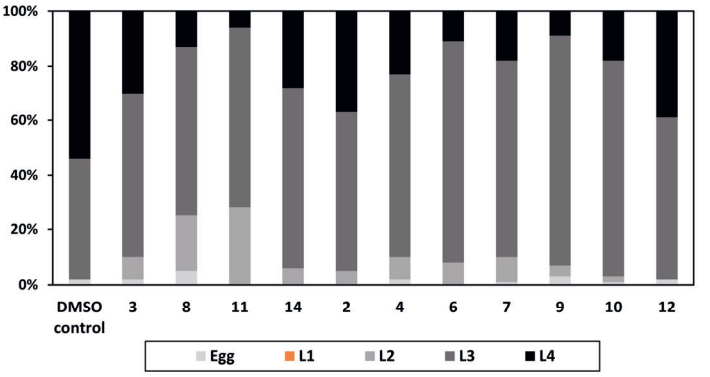
Figure 4. Proportional distribution in developmental stages of *C. elegans* larvae hatched from eggs laid by gravid females exposed to (a) dioxin, 2, 3,7,8-tetrachloordibenzo-p-dioxine (TCDD), and (b) a mixture of PCB (Clophen A50). The developmental stage of the larvae was recorded over 72 hours after egg-laying. At least ten nematodes were assessed for a given POP. * represents $P < 0.05$; ** represents $P < 0.001$; *** represents $P < 0.0001$.

In the egg-exposure method, swimming crabs (fats or meat) and marine sediment extracts significantly reduced the L3 to L4 development by 40-60% (Figure 5) for the TEQ 0.67 - 4.91 ng/kg (equal to 0.2 – 1.47 pM TCDD). The maternal exposure method findings for the Dutch sediment extracts are shown in Figure 6. The reduction in L4 larvae was between 20% and 80% compared with the L1-L3 larvae exposed to 0.3 - 15.6 ng/kg TEQ, equal to 0.09 – 4.66 pM TCDD. All the TCDD-equivalent toxic potencies (expressed as BEQ or TEQ) of the samples were analyzed using the DR-CALUX bioassay. The results are shown in the tables under figures 5 and 6.



Code	types	BEQ ng/kg	Decreased L4
1	Crab Fat	4.91	***
2	Crab Fat	4.09	***
3	Crab Fat	4.91	***
4	Crab Fat	4.58	***
5	Crab Meat	0.14	***
6	Crab Meat	0.70	**
7	Sediment	0.80	**
8	Sediment	0.67	**

Figure 5. The effects of crab extracts on the ELS development of *C. elegans.* The experiment was performed by exposing *C. elegans* eggs to the extracted residues. Sample 1–4 were extracted from the fatty parts, and samples 5–6 were extracted from the meat of swimming crabs. Samples 7–8 were extracted from marine sediments. All the samples from the Hangzhou bay region in China. BEQs of the sediments and crab samples were determined using an *in vitro* reporter gene assay (DR-CALUX bioassay). * represents $P < 0.05$; ** represents $P < 0.001$; *** represents $P < 0.0001$.



Code	locations	types	TEQ ng/kg	Decreased L4
2	Nieuwe waterweg beneluxtunnel	Sediment	16	n.s.
3	Nieuwe waterweg splistingsdam	Sediment	78	**
4	Loswal Noord Vak 53	Sediment	10	**
6	Noordwijk 2km offshore	Sediment	3	***
7	Ijmuiden outer harbour	Sediment	23	***
8	Ijmuiden inner harbour	Sediment	156	***
9	NZK10 Buitenhuizen	Sediment	87	***
10	NZK18 Amerikahaven	Sediment	12	***
11	NZK29 Oragnjesluis	Sediment	31	***
12	IJssdmmer Enkhuizen	Sediment	36	n.s.
14	Oosterschelde Oesterput	Sediment	29	***

Figure 6. The effects of sediment extracts on the early-stage development of *C. elegans.* The gravid *C. elegans* was fed on food supplanted with sediment extracts. The effect of the compound on the ELS development of the nematode was analyzed after 72 hours of gravid *C. elegans* exposure to the compound. The compounds were extracted from 11 marine sediments obtained from the Netherlands [23]. TEQs of the sediment extracts were determined using *in vitro* reporter gene assay (DR-CALUX bioassay). * represents P < 0.05; ** represents P < 0.001; *** represents P < 0.0001.

4. Discussion and conclusions

The two newly developed exposure methods revealed that TCDD and PCB mixtures significantly disrupt the early life development of *C. elegans*, also when tested at environmentally relevant contamination levels. The crab samples represented the typical marine contamination with dioxin-like compounds. Our bioassays showed that the dioxin-like compounds averaged 3.73 TEQ ng/kg in crab tissues and 4.87 TEQ ng/kg in sediment samples. This is in the same range as the 3.8 TEQ ng/kg that Knutzen and colleagues (2003) found for total dioxin-like compounds in liver and hepatopancreas of crabs samples [31]. In a related study, Manning et al. (2017) reported 4.9 – 22 TEQ ng/kg in marine crab samples

in Australia [32]. Our results demonstrate the relevant sensitivity of our bioassays in detecting realistic levels of dioxin-like compounds in the marine ecosystems.

The effect of the compounds on the larvae was stronger for the maternal exposed eggs than via direct egg exposure. This could be due to a higher exposure of the females through the skin and in the food. Also, the nematode eggshell may be less permeable to the chemicals [33] and may lack specific pathways targeted by dioxin and PCBs. In addition, accumulated POPs in the lipids are deposited in the yolk and from the developmental start directly available for the developing larvae as soon as it uses the lipids. In addition, it cannot be excluded that the female nematode has experienced sublethal effects from the exposure, possibly resulting in lower quality eggs. Further it has been shown in fish that due to bioamplification, the internal PCB levels in developing larvae are the highest just before the larvae start free feeding [11].

Epidemiological studies have shown that dioxin compounds exert their toxic effects via activation of the Aryl hydrocarbon Receptor (AhR) pathway. Most sensitive are teratogenic effects and this includes early life developmental delay [18, 34]. However, in most cases, invertebrate AhR homologs do not bind dioxins and related chemicals [35]. Also dioxin-like compounds do not activate the AhR of *C. elegans* [19, 36]. Alternatively, the ELS effects of dioxin may be related to narcosis (general toxicity), a characteristic of most organic xenobiotics, which induces non-specific disruption of the integrity and functioning of cell membranes [19, 37]. Clophen A50 delays the early life development of amphibians (*Xenopus laevis* and *Rana temporaria*) by prolonging the metamorphosis which is suggested to be mediated by thyroid hormone disruption [38]. In *C. elegans*, PCBs and dioxins inhibit the vitellogenin metabolism. Nematodes produce sterols in their intestines early in life, which are transported by vitellogenins [39]. Vitellogenin is transferred into the egg by the female, which may explain why the maternal exposure route is more sensitive. PCBs and dioxins have been shown to inhibit vitellogenin metabolism also in other aquatic vertebrates like zebrafish [40], or the fish Abramis brama and Cyprinus carpio [41]. Developmental effects of PCBs in nematodes are comparable to those seen in *ahr-1* mutant strains and include regulating fatty acid synthesis and homeostasis [42]. Aroclor 1254 dysregulates the expression of lipid metabolism genes in *C. elegans* [19]. Aroclor 1254 also downregulates the *C. elegans* development, lipogenesis, or membrane fluidity like *daf-22* [43], *emb-8* [44], *fasn-1* [45], or *pcyt*-1 [46] (which were upregulated), and *lips-15* [47] genes. Therefore, although the effects of dioxin or NDL-PCBs on fatty acid and vitellogenin metabolism is yet unclear, it is advisable to study this in detail further.

In the present study, the maternal exposure suggests that Clophen A50 could be passed to the offspring although indirect or epigenetic effects cannot be excluded either. In a side-experiment, exposure has been extended over multiple generations by having the F1 gravid females lay their eggs on new exposure plates and repeating the observations. This did not

increase the sensitivity of the assay compared to only exposing the first generation (data not shown). Interestingly, the longer exposed animals developed morphological effects. In the F3-generation, the unexposed nematodes were easy to pick up when transferring them using pig hair. Notably, the exposed animals appeared “older” than the control animals, in which their skin was softer and wrinkled and they did not show the same elasticity. Shortly after transfer to microscope slides that were covered with thin glass, the exposed animals burst, whereas those in the control group sustained the light pressure without any problem. This suggest cholesterol, fatty acids, or lipids deficiency, which is essential for maintaining the membrane rigidity [48, 49].

The more realistic maternal exposure route increased sensitivity to 10 pM TCDD and 10 pM – 10 nM mixture of PCBs. However, the maternal exposure method currently is more time-consuming, whereas the egg exposure method is more rapid. A faster bioassay procedure can provide the scanning tool to identify dioxin and non-dioxin-like compounds in toxicity identification evaluation study. The maternal exposure method could be used when a more detailed study is desired.

In conclusion, this study revealed that environmental (10 pM – 10 nM) or seafood residue levels (BEQ 0.7 - 4.91 ng/kg, equal to 0.2 – 1.47 pM) of dioxin and NDL-PCBs compounds disrupt the early life of *C. elegans*. The egg-exposure method showed that exposure to 10 nM and 10 µM of dioxin and NDL-PCBs significantly slowed down and inhibited L3 to L4 *C. elegans* development at 72 hours. In the maternal exposure method exposure to as low as 10 pM of dioxin and NDL-PCBs disrupted the early development of the nematodes. The maternal exposure method mimics the natural exposure and the full life cycle effects of the compounds. The egg-exposure method is more suitable as a rapid bioassay to test many samples. Both bioassays have their own added value for assessing marine, environmental, and seafood contamination with POPs. Further studies are advised to explore the mechanism underlying the effects of dioxin and NDL-PCBs on the ELS of *C. elegans,* especially at the L3 to the L4 stage and on the vitellogenin and lipid physiology.

Supporting information of chapter 2a

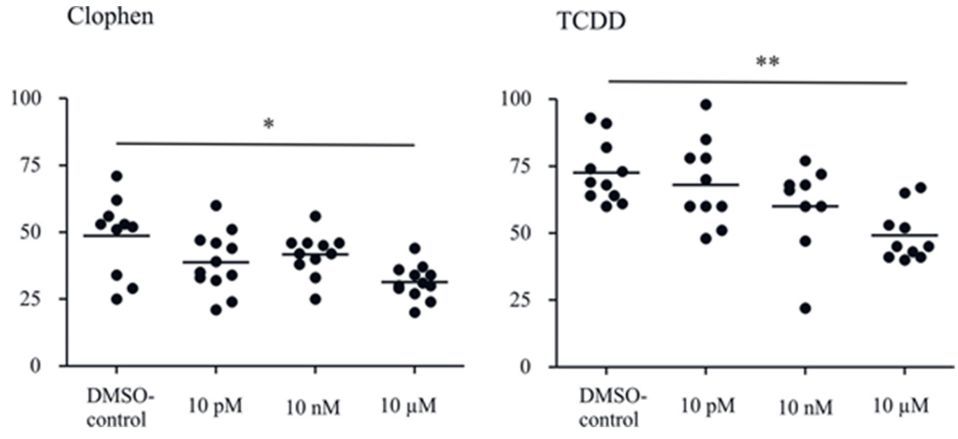


Figure C1. Number of *C. elegans* offspring exposed via maternal exposure to TCDD or Clophen A50, offspring counted after 72 hours of development . *represents P < 0.01; ** represents P < 0.001.

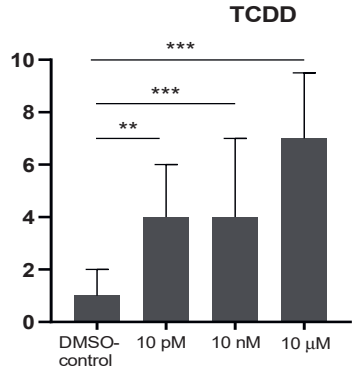


Figure C2 the average number of dead *C. elegans* offspring after exposure to TCDD at 72 hours by maternal exposure method. The assessment was performed after 72 hours after exposure of the mother nematodes. ** represents P < 0.001; *** represents P < 0.0001.

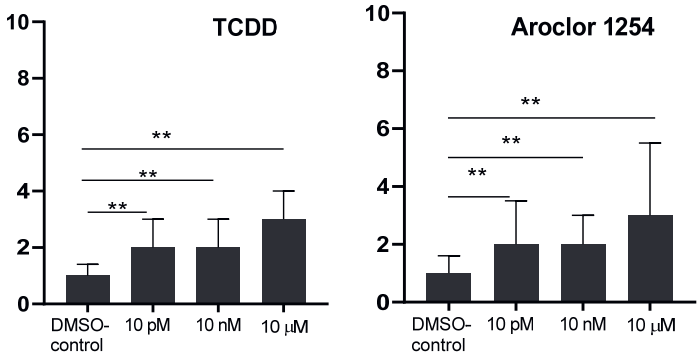


Figure C3 the average number of dead first-generation C. elegans offspring after exposure to TCDD at 72 hours by egg exposure method. The assessment was performed after 72 hours after exposure of the mother nematodes. ** represents P < 0.001.

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Chapter 2b

Development of a transcription-based bioanalytical tool to quantify the toxic potencies of hydrophilic compounds in water using the nematode *Caenorhabditis elegans*

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Abstract

Low concentrations of environmental contaminants can be difficult to detect with current analytical tools, yet they may pose a risk to human and environmental health. The development of bioanalytical tools can help to quantify toxic potencies of biologically active compounds even of hydrophilic contaminants that are hard to extract from water samples. In this study, we exposed the model organism *Caenorhabditis elegans* synchronized in larval stage L4 to hydrophilic compounds via the water phase and analyzed the effect on gene transcription abundance. The nematodes were exposed to three direct-acting genotoxicants (1 mM and 5 mM): N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS). Genome-wide gene expression analysis using microarrays revealed significantly altered transcription levels of 495 genes for HCHO, 285 genes for ENU, and 569 genes for MMS in a concentration-dependent manner. A relatively high number of differentially expressed genes was downregulated, suggesting a general stress in nematodes treated with toxicants. Gene ontology and Kyoto encyclopedia of genes and genomes analysis demonstrated that the upregulated genes were primarily associated with metabolism, xenobiotic detoxification, proteotoxic stress, and innate immune response. Interestingly, genes downregulated by MMS were linked to the inhibition of neurotransmission, and this is in accordance with the observed decreased locomotion in MMS-exposed nematodes. Unexpectedly, the expression level of DNA damage response genes such as cell-cycle checkpoints or DNA-repair proteins were not altered. Overall, the current study shows that gene expression profiling of nematodes can be used to identify the potential mechanisms underlying the toxicity of chemical compounds. *C. elegans* is a promising test organism to further develop into a bioanalytical tool for quantification of the toxic potency of a wide array of hydrophilic contaminants.

1. Introduction

Chemical substances in the environment may pose a risk to human and environmental health. The contamination by pollutants with potential genotoxic and mutagenic effects has been previously documented in water sources [1]. Compounds may be present as parent molecules as well as their metabolites for which no analytical techniques exist yet or for which concentration is usually too low to detect chemically [2]. Hydrophilic pollutants are even more difficult to analyze in water because methods for extraction hardly exist [3, 4]. This poses a problem, as testing the quality of drinking water sources typically involves the presence of hydrophilic compounds, of which especially genotoxic and endocrine disrupting compounds are of concern [2, 5]. The development of chemical analytical tests for known individual agents will take many years and requires huge resources, still leaving questions on the total toxic potency of mixtures of these compounds such as mutagenicity or genotoxicity. In addition, biotransformation products may occur that we are not even aware of.

Living organisms, however, respond to bioactive compounds that they are exposed to. Biologically active contaminants undetectable by chemical analyses can still leave their signature in those organisms [6]. This signature can be an alteration of gene expression patterns reflecting the mode of toxic action of the causative agent. In addition, transcriptional effects of chemical toxicants are not only mechanism-specific but could also be used to assess the toxic potency of complete mixtures [7, 8].

Several developmental and toxicological studies have been conducted with the free-living soil nematode *Caenorhabditis elegans* as a model organism [9, 10]. It provides particular experimental advantages such as small size, ease to handle, short life cycle, being invertebrate and relatively cheap to maintain in an ordinary laboratory setting. Most importantly, its genome has been completely sequenced and many genes or signaling pathways, particularly the ones involved in DNA damage response (DDR), are well conserved between *C. elegans* and higher organisms, hence comparable responses between the nematode and higher organisms are to be expected [11, 12].

Several genes encoding DNA damage checkpoint proteins have been identified in *C. elegans* and are essential in sensing and responding to aberrations in their genetic material [13]. The activation of checkpoints in response to DNA injuries typically stalls cell cycle progression to allow time for repair. If checkpoints fail to restore the DNA integrity, mutations can take place, and as response cell apoptosis occurs to prevent further problems [14]. Other cellular responses that can be expected in response to genotoxic stress, include transcription regulating genes related to DNA repair, biotransformation enzymes, innate immune response and other mechanisms [15]. Previous studies in *C. elegans* have primarily concentrated on ionizing radiation [16, 17], where pro-apoptotic genes such as *egl-1* and *ced-13* were transcriptionally induced in response to DNA damage. Although, several

transcriptomics studies investigating transcriptional effects of other chemical agents have been carried out in the nematode [18, 19], to our knowledge, this is the first genome-wide transcriptome study in *C. elegans* treated with genotoxic chemicals.

The afore described information has motivated us to use the transcriptional response of *C. elegans* for developing a small-scale *in vivo* bioassay as a biodetection and early warning system for the presence of genotoxic compounds. To develop such a bioanalytical tool, we chose N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS) as model compounds for determining genotoxic effects. These toxicants are known to directly react with nucleophilic sites (-NH, -OH and -SH) of macromolecules such as nucleic acids (i.e., DNA and RNA), enzymes, structural proteins, and other biological molecules [20]. It has been conclusively shown that ENU and MMS, which are monofunctional alkylating compounds, induce DNA injuries by reacting preferentially with ring nitrogen (N) and extra cyclic oxygen (O) atoms of nucleotides [21]. Exposure to HCHO induces formation of crosslinks of DNA and proteins through electrophilic attacks ultimately leading to the impairment of normal cellular functions [22].

The aim of this study was to develop an *in vivo* bioassay based on the genome-wide transcriptional response of *C. elegans* to direct-acting genotoxic compounds. Such an assay could simultaneously identify and quantify the toxic potency of single toxicants or mixtures. Gene expression profiling can provide insights in the type of toxic mechanisms involved and can be translated towards the nature of the toxicants present in a sample. Microarray analyses showed several genes in *C. elegans* whose expression level was differentially affected after 4 hours exposure to the model genotoxicants. Surprisingly, no change was found in expression of most DDR genes, including the ones encoding for checkpoints and DNA repair proteins. The bioassay was validated by gene expression analyses using quantitative reverse transcription polymerase chain reaction (RT-qPCR) of selected gene targets from the microarray data.

2. Material and methods

2.1. Handling of nematode cultures

C. elegans wild-type strain (Bristol N2) nematodes were maintained on Nematode Growth Medium (NGM) agar plate at 16 °C [23]. Subsequently, nematode stocks were renewed every month using fresh NGM agar seeded with *E-coli* bacteria as source of food [24]. The experiments were conducted by using a nematode population of *C. elegans* N2 larvae (L4), grown synchronously at 20 °C for 48 hours (starting from synchronized eggs) on a freshly prepared NGM agar plate seeded with *E. coli* strain OP50 to feed the nematodes. Synchronization was carried out by bleaching gravid nematodes with 5% sodium hypochlorite solution [25].

2.2. Chemical exposure

Exposure media. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were used without further purification. The stock solution of formaldehyde (HCHO) was prepared using the method of Moerman and Baillie [26]. 61 mg of paraformaldehyde (Sigma-Aldrich P6148-500G) was warmed in 25 mL of 65°C Milli-Q water and 0.1 M NaOH was added to clear the solution. The solution was diluted to 100 mL by adding M9 buffer, giving 20 mM solution adjusted to pH 7.04 with 0.1 M HCl. This solution was aliquoted in 1 mL, frozen, and stored at -20 °C. Methyl methanesulfonate (MMS, ≥ 99% purity) and N-ethyl-N-nitrosourea (ENU, 57% purity) were always freshly dissolved in M9 buffer to obtain 20 mM stock solutions which were further diluted to make the required concentrations. To obtain the required exposure concentration, stock solutions were further diluted in M9 buffer that was prepared according to Sulston et al. [27].

Exposure samples. A 4-hour exposure to the abovementioned toxicants were started in the synchronized L4 juvenile population. For microarray experiments, approximately 900 nematodes were exposed to two concentration (1 mM and 5 mM) and a control of M9 buffer in 1.5 mL Safe-Lock micro test tubes at 20°C. Special care was taken to avoid temperature and developmental stage effects as these had shown to be relevant in the pilot experiment **(Suppl. Pilot Study)**. After exposure, the nematodes were immediately pelleted by spinning the tubes in microcentrifuge for 20 seconds, 18,400 x g at room temperature, followed by removal of the supernatants. Subsequently, pellets were kept in the same exposure test tubes and flash-frozen in liquid nitrogen for 1 minute before storing them at -80 °C until extraction of RNA. Three independent biological replicates were used per treatment in microarray experiments. For toxicity tests, duplicate samples were analyzed per treatment as described below.

2.3. Determination of non-lethal concentration

Non-lethal concentrations were determined for MMS, ENU, and HCHO to select the appropriate dose for microarray experiments. *C. elegans* L4-stage juveniles were exposed to three concentrations (1 mM, 5 mM, &10 mM), and a control of M9 buffer. For each exposure sample, 25 to 30 nematodes per independent test were divided in 4 wells of a 96-well tissue culture plate and treated in 135 µL of the exposure medium and control. After 4 hours exposure, the nematodes were inspected under a stereo microscope to determine morbidity and mortality. The nematodes which failed to move in response to gentle probing by a platinum-wire based worm picker [23] were counted as dead. Two independent biological replicates were used per treatment.

2.4. Microarray gene expression analysis

RNA isolation. RNA was isolated following standard protocols as previously described [28]. In short, a Maxwell® 16 AS2000 instrument with a Maxwell® 16 LEV simplyRNA Tissue Kit (both Promega Corporation, Madison, WI, USA) was used following the manufacturer's protocol only modified at the lysis step. Each sample was treated with homogenization buffer (200 µl) and lysis buffer (200 µl), additionally, 10 µl of 20 mg/ml stock solution of proteinase K was added. Thereafter, the samples were incubated at 65°C for 10 minutes to digest and remove proteins while shaking at 1,000 rpm in a Thermomixer (Eppendorf, Hamburg, Germany). Subsequently, samples were cooled on ice and loaded into the cartridges provided by the manufacturer, thereby resuming the standard protocol.

Microarray preparation, hybridization, and scanning. Gene expression was measured using the Agilent *C. elegans* (V2) Gene Expression Microarray 4x44K slides following a procedure described before [28]. 'Two-Color Microarray-Based Gene Expression Analysis; Low Input Quick Amp Labeling'—protocol, version 6.0 from Agilent (Agilent Technologies, Santa Clara, CA, USA) was followed by cDNA synthesis, labelling with cy3 and cy5 dyes, and subsequent hybridization. Scanning was done using an Agilent High Resolution C Scanner with the settings as recommended by the protocol. For retrieval of the intensities the accompanying software was used (Agilent Feature Extract, v. 10.7.1.1). The array probe annotation was updated by blasting the probes against WS258 with blast (version 2.6.0, windows x64), using nblast with parameters: word size 7, reward 1, pentaly -3, and evalue 1. Probes with multiple hits were flagged and ignored in the analysis of affected genes. This microarray system holds 43,803 *C. elegans* probes. Detection is possible over a 5-log expression scale. In total, we could detect expression of 18,447 genes, representing > 90 % of the nematode genome.

Normalization and pre-processing. Normalization of the data was done using the Limma package in "R" (version 3.4.2, x64) in RStudio (version 1.1.383). Arrays were normalized without background correction, normalization within arrays was done using the Loess method and between arrays using the quantile method [29, 30]. The obtained values were $\log_2$ transformed and used for subsequent analysis. Initial analysis revealed the presence of batch- and dye-linked effects. Therefore, a batch correction was performed by fitting the gene expression to the linear model **(Suppl. Eq. S1)**. After batch correction, a $\log_2$ ratio with the mean was calculated **(Suppl. Eq. S2)**. For further analysis, the expression values of three biological replicates were averaged. The raw data of this experiment were submitted to ArrayExpress (E-MTAB-10265), accessible at https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10264/.

Statistical data analysis. We did not employ intensity thresholds: we considered all data going into the analysis. Importantly, all models were using data taken from the level of the measurement (spots). Results were post-hoc translated to genes. To detect if the arrays

were technically correct, we conducted a correlation analysis on the log_2 ratio with the mean values using the *cor* function in "R". To identify the factors that can underlie variation in gene-expression, principal component analysis (PCA) was calculated on the log_2 ratio with the mean values using the *prcomp* function in "R". To evaluate whether nematodes gene expression responded in a concentration-related manner a *"concentration-dependent linear model"* incorporating the compound as well as the exposure concentration (0, 1, and 5 mM) was applied to ENU, HCHO, and MMS separately **(Suppl. Eq. S3)**. The resulting p-values from linear model were corrected for multiple testing using the *p.adjust* function with the Benjamini and Hochberg method [31]. To assess the differentially expressed genes (DEG) per exposure condition, we took a high significance level of $-log_{10}(p) > 4$ (i.e., $p < 0.0001$; FDR < 0.05). Custom written scripts for the microarray analysis are available at https://git.wur.nl/published_papers/karengera_2021_bioanalytical_tool_genotox.

Gene ontology (GO) and pathway enrichment analysis. All DEG lists generated by microarray experiments were uploaded to the "*Database for Annotation, Visualization and Integrated Discovery"* (DAVID) v6.8 [32] for KEGG pathway and for GO analyses in three categories, including biological processes (BP), molecular functions (MF), and cellular components (CC). For the enrichment analysis, settings were limited to Gene Ontology (GOTERM_BP_ALL, GOTERM_MF_ ALL, and GOTERM_CC_ ALL). A threshold False Discovery Rate (FDR) ≤ 0.05 was considered as strongly enriched in the annotation categories. The resulting GO terms were further used as input in the online software ReViGo [33] to summarize and remove the redundant terms. All default parameters were kept unchanged during the analysis.

2.5. Validation of 12 gene targets from microarray data by RT-qPCR

cDNA synthesis. RT-qPCR analyses were conducted on samples from control and nematodes exposed to 5 mM of toxicants. The RNA samples used in these experiments were from the same batches as used in the microarrays. The complementary DNA (cDNA) was synthesized from RNA template via reverse transcription (RT). The Invitrogen™ SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme was used following the manufacturer's protocol. In short, 500 ng of total RNA was used as starting material in a 20 μL RT reaction. Each RT reaction involved two-minute digestion of genomic DNA (gDNA) using EZ DNase enzyme provided in the kit, followed by RT reaction in a T100™ Thermal Cycler. The annealing of primers was performed by incubation of samples at 25 °C for 10 minutes, reverse transcription at 50 °C for 10 minutes and inactivation of transcriptase enzyme at 85 °C for 5 minutes. The resulting cDNA was stored at -80 °C until further analysis.

PCR primer design and PCR reaction. Gene-specific PCR primers (115-200 bp) were designed by using three online database tools including Primer-BLAST (National Center for Biotechnology Information), Primer3 Input v. 0.4.0, and OligoAnalyzer v3.1 (Integrated DNA

Technologies, Inc.). The specificity of primer pairs was initially checked in Primer-BLAST and confirmed by melting curve analysis. A temperature gradient qPCR (56 °C to 62 °C) was run to determine the optimal annealing temperature of each primer set. Prior to use in RT-qPCR, the cDNA stock obtained from a 500 ng RNA template was diluted 1:5 to match a 100-ng template as recommended by Invitrogen's user guide (Pub. No. MAN0015862 Rev. B.0. SuperScript™ IV VILO™ Master Mix). The 20 μL qPCR reaction mixtures were made of 6.8 μL PCR-grade (RNAse-free) water, 10 μL iQ™ SYBR® Green Supermix, 0.6 μL of specific forward and reverse primers (10 μM concentrated) and 2 μL of cDNA (5 ng/μL). Three independent biological replicate samples were analyzed per treatment and three technical replicates withing each sample were used. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s. Melting curve analysis was performed from 62 °C to 95 °C with an increment of 0.5 °C to confirm the amplification.

Validation of housekeeping genes for normalizing RNA expression. Eight candidate housekeeping genes were selected from our experiment and from the published studies [34]. The preliminary expression stability of these genes was further assessed in our microarray data. The expression levels of candidate housekeeping genes were measured by RT-qPCR method followed by ranking them according to expression stability, meaning that their levels were not influenced by the exposure. The selection of the most stable genes and the choice of the optimal number of housekeeping genes were computed by using geNorm algorithm according to Vandesompele et al. [35] in "R" program v 3.5.2.

3. Results

3.1. Concentration-response relationship for lethal toxicity to *C. elegans*

To determine the non-toxic concentration range for studying the transcriptional effects of different genotoxic compounds, we first performed a concentration-response test (**Fig. 1**). The survival in all ENU exposed nematodes was not significantly different from control, and in the MMS-exposed nematodes only the 10 mM exposure resulted in significant (26%) reduced survival. Exposure to 5 and 10 mM HCHO resulted in significant dose-related mortality of respectively 39 and 70%.

The toxicity of the compounds was also qualitatively analyzed by comparing the swimming motions of the nematodes in the exposure media versus untreated worms. The worms exposed to 1 mM ENU and 1 mM MMS were actively swimming with a typical oscillatory movement [36] similar to untreated nematodes. In contrast, most of the worms in 5 mM and 10 mM (ENU and MMS) or in 1 mM and 5 mM (HCHO) were motionless but slowly moved their bodies upon gentle touches with worm-picker probes. In 10 mM HCHO, most nematodes were lying with their bodies stretched motionless and barely moved upon

probing. Based on the toxicity tests, we selected 5 mM as the maximum exposure concentration for microarray experiments.

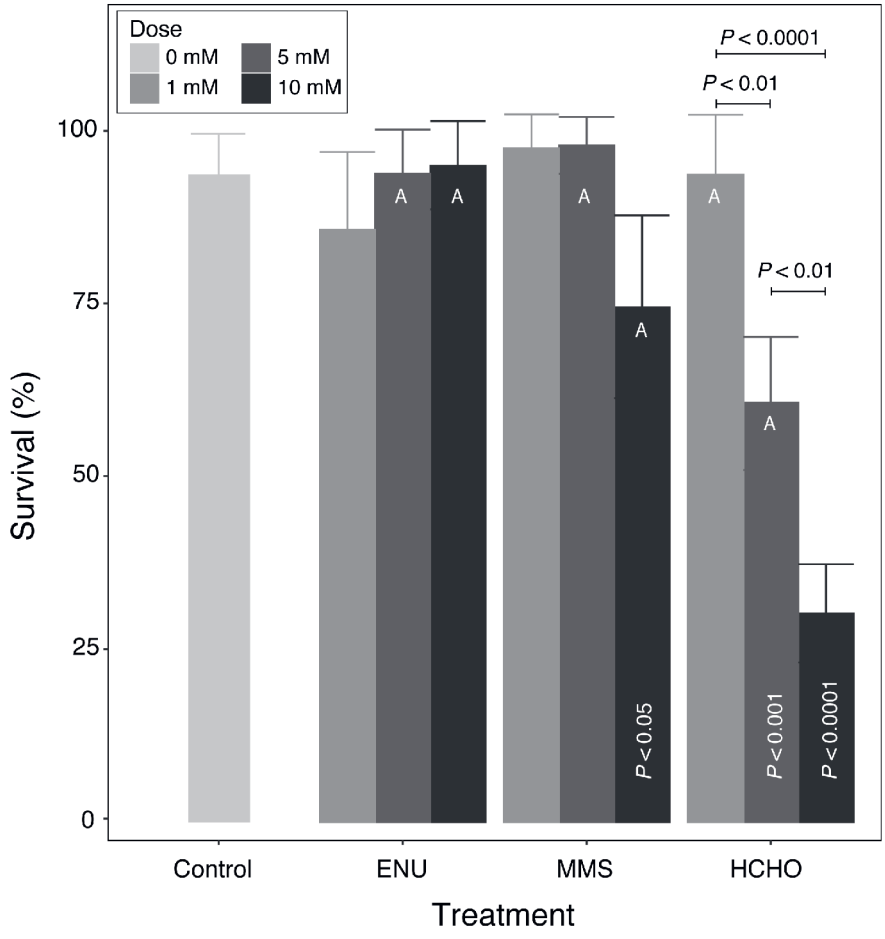

Figure 1. Concentration-response relationship for lethal toxicity. Survival of *C. elegans* L4 larval stage after four hours exposure to 1mM, 5mM, or 10mM of N-ethyl-N-nitrosourea (ENU), methyl methanesulfonate (MMS), and formaldehyde (HCHO). Plotted values are the means ± standard deviation (SD) for two independent biological replicates (n = 2) with about 25 to 30 nematodes divided in 4 wells per test. P-values (logistic regression) displayed vertically in chart bars indicate the significance of treatment compared to the untreated (control) samples while the horizontal Bonferroni-corrected P-values (logistic regression) (above the bars) show the significance of difference between concentrations within treatment. In groups indicated with **A** the nematodes displayed abnormal (reduced) swimming behavior.

3.2. Transcriptional response to genotoxic compounds

Pilot study **(Suppl. Pilot Study)** revealed the need to take extra caution on the experimental set-up to reduce potential batch development variation and temperature fluctuation effects. To determine the number of DEGs we used a concentration-dependent linear model. In general, there was a relatively high proportion of downregulated transcripts**,** where genes whose transcription was repressed counted 59 % (for ENU), 46% (for HCHO), and 49% (for MMS) of the total DEGs in each treatment **(Table 1)**. Relatively little overlap was found between gene transcripts affected by different treatments **(Fig. 2)**. Only 7 genes (T20D4.12, C17C3.3, T07G12.5, K12C11.7, *ins-20*, C28G1.2, and D1086.2) overlapped between all three treatments and were all downregulated. These genes encode proteins involved in acyl-coA metabolic process (C17C3.3), transmembrane transport (T07G12.5), copper ion transmembrane transport (K12C11.7), and hormone activity (*ins-20*), whereas the function of T20D4.12, C28G1.2, and D1086.2 are not yet known.

Table 1. The number of differentially expressed genes (DEGs) in *C. elegans* L4 following four-hour exposure to 1 mM and 5 mM N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS). Data were analyzed by a concentration-dependent linear model ($-\log_{10}(p) > 4$; FDR < 0.05).

	Upregulation	Downregulation	Total
ENU	117	168	285
HCHO	267	228	495
MMS	292	277	569

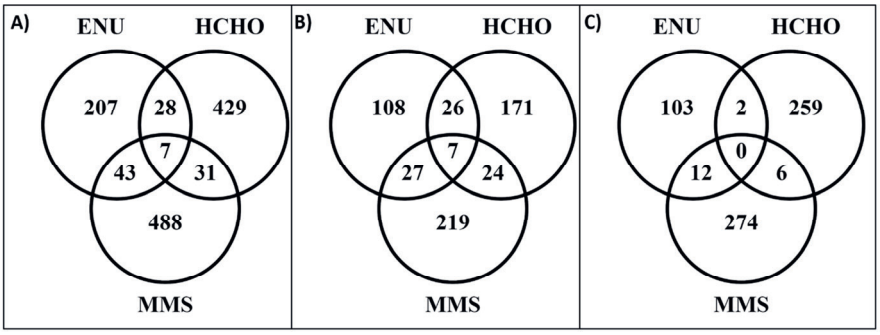

Figure 2. Overlapping of differentially expressed genes (DEGs). Included are genes that responded to the treatments with N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS) in a concentration-dependent manner, as analysed by a linear model incorporating compound as well as exposure concentration. (**A**) the overlap between all differentially expressed genes, (**B**) the overlap between down-regulated genes, and (**C**) the overlap between up-regulated genes.

To check whether these results were robust, a correlation analysis between the pilot study (2-hour exposure) and the main microarray experiment (4-hour exposure time) was conducted. We found a strong positive correlation for the array spots with the significance -log10(p) > 4 **(Fig. 3)**, meaning that despite differences in the exposure duration, similar transcriptional response trends were measured.

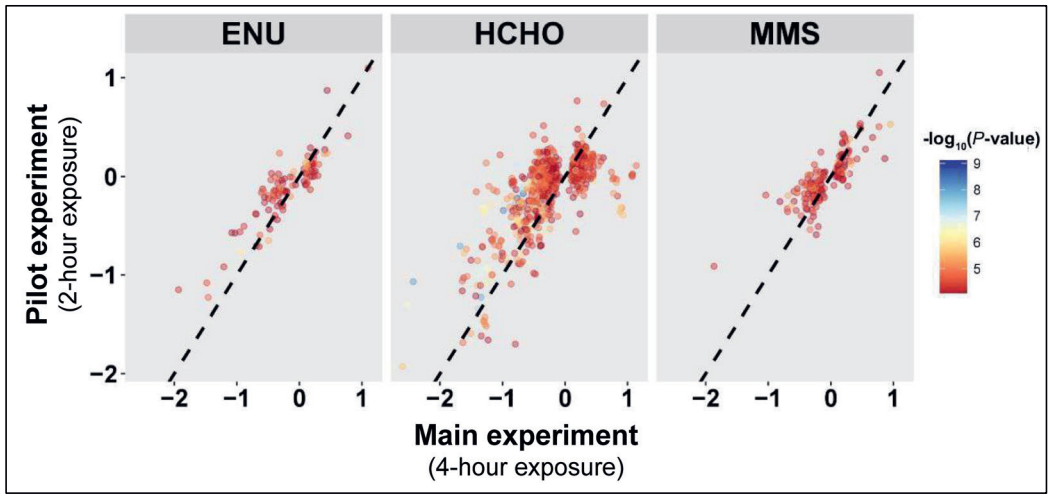

Figure 3. Comparison of two independent microarray experiments. The outcome of the linear model of main experiment was compared to pilot **(Supp. Pilot Study)**. Genes with a significant change in the expression above $-\log_{10}(p) = 4$ (displayed in the plot as color-coded dots based on their significance) showed a strong correlation (R = 0.89, R = 0.71, and R = 0.80, for N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS), respectively; $p < 1*10^{-38}$). These results show that despite the two-fold difference in the exposure duration, transcriptional effects of the toxic compounds are robust and replicable.

3.3. Functional analysis of differentially expressed genes (DEGs)

In general, a significant upregulation of genes related to metabolism and xenobiotics detoxification was found**.** For instance, concentration-dependent linear model analysis revealed that 57 out of 117 DEGs represented nearly half (49%) of the upregulated transcripts by ENU were enriched in metabolic processes, as annotated in the DAVID software **(Fig. 4A and Suppl. Table S2).** The affected genes include transcription factors belonging to nuclear hormone receptor family (*nhr-106, nhr-201, nhr-203, nhr-202*, and *nhr-237*) regulating gene expression, cytochrome P450 enzymes of phase 1 metabolism *(cyp-13A1, cyp-13A10, cyp-13A8, cyp-13A9, cyp-14A2*, and *cyp-33C8*), and phase 2 detoxification enzymes like glutathione-S-transferase enzymes *(gst-5, gst-6, gst-7, gst-8, gst-12, gst-13, gst-14, gst-31, gst-33,* and *gst-37*) or UDP-glucuronosyltransferase (*ugt-16, ugt-25, ugt-33,* and *ugt-44*). Metabolic processes were also induced by HCHO and MMS treatment (**Suppl. Table S2**), including the expression of *ugt-21, ugt-47, ugt-63, ugt-65*, and *cyp-29A2* genes by HCHO or *gst-30* by MMS. The results of GO analysis also revealed an upregulation of genes related to immune system process in the nematodes treated with ENU and MMS **(Fig. 4A and Table 2)**. KEGG pathway enrichment analysis demonstrated that both HCHO and MMS induced the upregulation of genes involved in protein processing in

endoplasmic reticulum (cel04141) **(Suppl. Table S1)**. In addition, GO analysis in biological process (BP) category found that the induced transcripts by HCHO were related to protein folding (GO:0006457), while the upregulated genes by MMS were associated with response to topologically incorrect protein (GO:0035966), response to endoplasmic reticulum stress (GO:0034976), and endoplasmic reticulum unfolded protein response (GO:0030968) **(Suppl. Table S2)**.

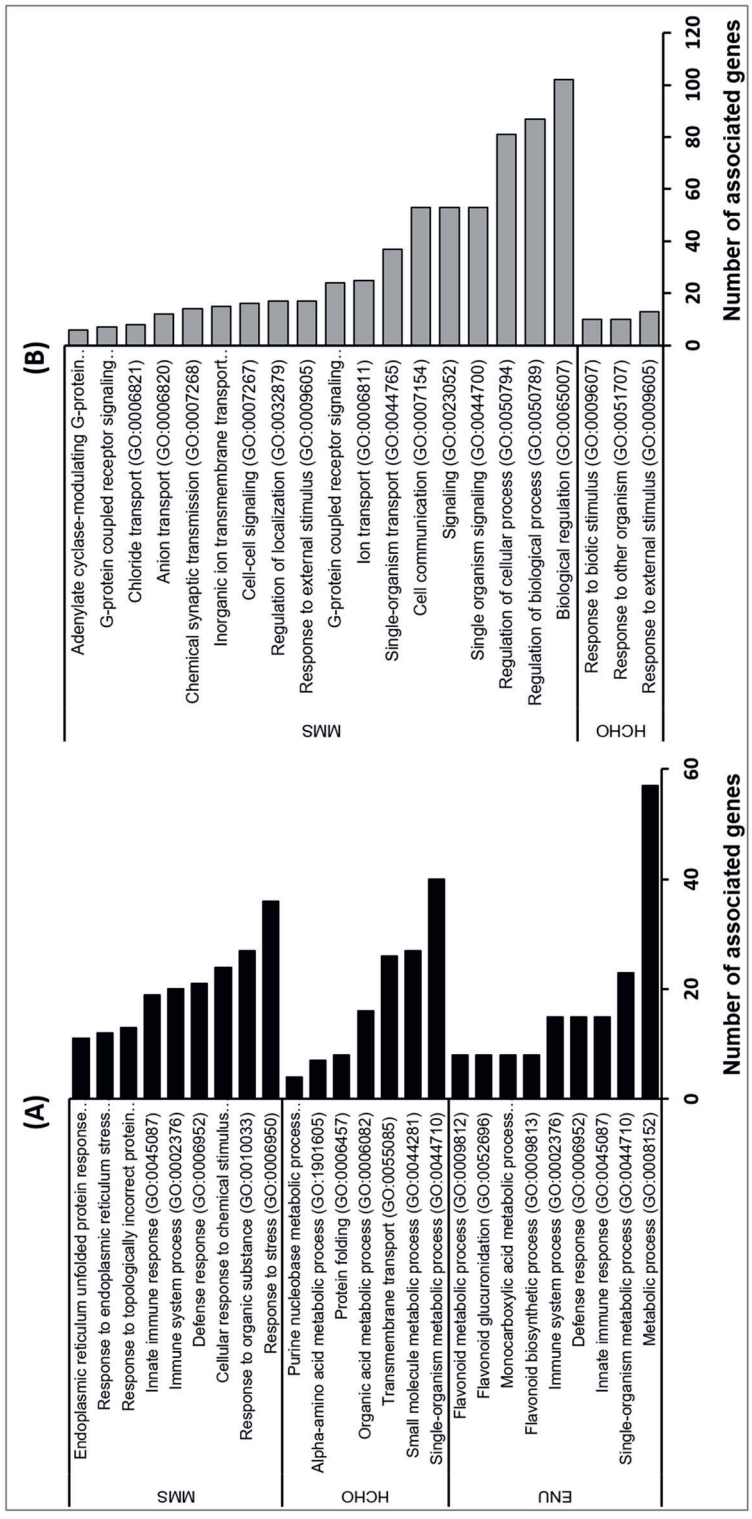

Figure 4. Gene ontology enrichment analysis of differentially expressed genes in *C. elegans* L4. Plotted are gene ontologies in biological process category associated with upregulated genes (A) and with downregulated gene transcripts (B) following 4 hours exposure to 1mM and 5mM N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS). The X-axis denotes number of genes significantly enriched in a gene ontology (GO) term (FDR < 0.05).

A significant downregulation of genes involved in nerve impulse transmission was found in the nematodes treated with MMS **(Fig. 4B and Table 2).** These include genes like *mgl-1, dop-1, gab-1, gar-3, gar-1*, and *ser-4*, which were enriched in neuroactive ligand-receptor interaction pathway (cel04080). Treatment with HCHO reduced the expression levels of genes encoding proteins involved in the nematode defence responses during pathogen attacks from fungi or bacteria (e.g., *cnc-2, cnc-5, cnc-11, atg-16.2, lgg-1, nlp-29, sta-2, elt-7*, and *vhp-1*) **(Fig. 4B and Table 2)**. Additional results of KEGG pathway and GO enrichment analysis are provided as supplementary information, including pathways **(Suppl. Table S1)**, biological process (BP) terms **(Suppl. Table S2)**, molecular function (MF) terms **(Suppl. Table S3)**, and cellular component (CC) terms **(Suppl. Table S4).**

The individual annotation of all DEGs in DAVID software resulted in a list of genes that can be linked to the modes of action expected from the tested genotoxic model compounds such as transcriptional regulation, cell cycle regulation, proteotoxic stress, apoptosis, and other mechanisms. Nevertheless, KEGG pathway and GO analysis did not reveal any of the well-characterized DDR genes encoding for cell cycle checkpoints and DNA repair proteins to be transcriptionally affected by any of the studied toxicants.

Table 2. Potential biological functions of genes down- or up-regulated in *C. elegans* L4 following 4 hours exposure to 1mM and 5mM N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS). Differentially expressed genes (DEGs) are shown with their corresponding expression fold change (FC) values and the statistical significance expressed as –log10(p).

Treatment	Gene	Protein	FC in 1mM	FC in 5mM	-log$_{10}$(p)
Defense response					
ENU	*atg-16.2*	Autophagic-related protein 16.2	-1.05	-1.24	5.57
HCHO	*atg-16.2*	Autophagic-related protein 16.2	-1.06	-1.40	4.07
HCHO	*cnc-11*	Caenacin (Caenorhabditis bacteriocin)	-1.74	-3.01	4.53
HCHO	*cnc-2*	Caenacin (Caenorhabditis bacteriocin)	-3.52	-19.70	6.06
HCHO	*cnc-5*	Caenacin (Caenorhabditis bacteriocin)	-1.29	-2.00	4.38
HCHO	*elt-7*	Transcription factor ELT-7	-1.10	-1.39	5.57
HCHO	*lgg-1*	Protein LGG-1	-1.05	-1.72	4.84
HCHO	*nlp-29*	QWGYGGY-amide	-2.84	-8.96	4.00
HCHO	*sta-2*	Signal transducer and activator of transcription b	-1.24	-2.28	4.38
HCHO	*vhp-1*	Tyrosine-protein phosphatase VHP-1	-1.03	-2.08	5.46
Signal transmission					
HCHO	*dop-1*	Dopamine receptor	-1.18	-1.45	4.09
MMS	*dop-1*	Dopamine receptor	-1.16	-1.53	6.11
MMS	*gab-1*	Gamma-aminobutyric acid receptor subunit beta	-1.05	-1.23	5.32
MMS	*gar-1*	Probable muscarinic acetylcholine receptor GAR-1	-1.03	-1.26	4.24

MMS	*gar-3*	Muscarinic acetylcholine receptor GAR-3	-1.15	-1.46	4.55
MMS	*mgl-1*	Metabotropic gLutamate receptor family	-1.07	-1.27	4.57
MMS	*ser-4*	SERotonin/octopamine receptor family	-1.13	-1.33	5.02
Innate immune response					
ENU	*cdr-4*	Cadmium responsive	1.43	3.48	5.47
MMS	*cdr-4*	Cadmium responsive	1.48	3.45	5.51
MMS	*fbxa-105*	F-box A protein	1.29	1.98	4.46
ENU	*fbxa-105*	F-box A protein	1.26	1.78	4.08
MMS	*gst-13*	Glutathione S-Transferase	1.33	2.25	6.33
ENU	*gst-13*	Glutathione S-Transferase	1.13	1.52	4.59
ENU	K08D8.4	hypothetical protein	1.24	2.51	5.07
MMS	K08D8.4	hypothetical protein	1.31	2.64	5.31
MMS	*lec-11*	Galectin	1.32	1.87	4.69
ENU	*lec-11*	Galectin	1.05	1.60	4.54
ENU	*ugt-44*	UDP-Glucuronosyltransferase	1.21	2.51	4.53
MMS	*ugt-44*	UDP-GlucuronosylTransferase	1.28	1.96	4.34
Oxidative stress response					
ENU	*gst-12*	Glutathione S-Transferase	1.57	3.55	5.04
MMS	*gst-12*	Glutathione S-Transferase	1.50	2.53	4.61
MMS	*gst-13*	Glutathione S-Transferase	1.33	2.25	6.33
ENU	*gst-13*	Glutathione S-Transferase	1.13	1.52	4.59
ENU	*gst-14*	Glutathione S-Transferase	1.45	2.80	4.74
ENU	*gst-31*	Glutathione S-Transferase	2.30	5.83	4.24
ENU	*gst-33*	Glutathione S-Transferase	1.49	3.68	6.68
ENU	*gst-37*	Glutathione S-Transferase	1.11	1.95	4.33
ENU	*gst-5*	Glutathione S-Transferase	1.56	4.43	7.39
ENU	*gst-6*	Probable glutathione S-transferase 6	1.20	1.60	4.83
ENU	*gst-7*	Probable glutathione S-transferase 7	1.14	1.78	4.59
ENU	*gst-8*	Probable glutathione S-transferase 8	1.18	1.66	5.01
Proteotoxic stress response					
MMS	*cul-6*	Cullin-6	1.14	2.14	4.45
MMS	*fbxa-158*	F-box A protein	1.43	3.36	7.48
MMS	*fbxa-75*	F-box A protein	1.64	6.81	6.58
MMS	*pals-22*	Protein containing ALS2cr12 (ALS2CR12) signature	1.15	1.46	5.37
MMS	*skr-3*	SKp1 Related (ubiquitin ligase complex component)	1.17	1.80	5.30
MMS	*skr-4*	SKp1 Related (ubiquitin ligase complex component)	1.10	1.58	5.87
Unfolded protein stress					
MMS	*arf-1.1*	ADP-ribosylation factor 1-like 1	1.17	2.18	5.21
MMS	C04F12.1	hypothetical protein	1.49	2.69	4.83
MMS	*ckb-2*	Choline kinase B2	2.30	6.74	6.08
MMS	*ckb-4*	Choline Kinase B	1.15	1.80	9.06
MMS	*cup-2*	Derlin-1	1.17	1.67	6.13

MMS	*dnj-7*	DNaJ domain (prokaryotic heat shock protein)	1.15	1.47	4.84
HCHO	*dnj-7*	DNaJ domain (prokaryotic heat shock protein)	1.10	1.36	4.70
MMS	*hsp-4*	Heat Shock Protein	1.34	2.53	6.69
MMS	*nsf-1*	Vesicle-fusing ATPase	1.05	1.35	5.41
HCHO	*pdi-2*	Protein disulfide isomerase	1.18	1.51	4.60
MMS	*pdi-2*	Protein disulfide isomerase	1.12	1.33	4.51
MMS	*rer-1*	Protein RER1 homolog	1.17	1.41	4.54
MMS	*sel-1*	Suppressor/Enhancer of LIN-12	1.03	1.27	5.34
MMS	Y54G2A.18	hypothetical protein	1.17	1.87	6.16

3.4. Validation of microarray data

To confirm the robustness of the results of microarray experiments, 12 gene targets selected from the main experiment were independently tested by using RT-qPCR experiment. RT-qPCR data were normalized by using four housekeeping genes including *csq-1, mdh-1, and pmp-3* selected from literature [34] and *ver-3* (from our study). The expression stability of these housekeeping genes in the exposed and unexposed nematodes was confirmed in our experimental conditions. RT-qPCR results showed similar expression trends to microarray outcomes **(Suppl. Table S5)**.

4. Discussion

The nematode *C. elegans* has become an invaluable model organism in high-throughput screening assays to predict the toxicity of chemical substances [18, 19]. Despite this, very little was found in the literature on the whole-genome transcriptional effects of genotoxic compounds. In this work, we have successfully developed a bioassay based on exposure of L4 larval stage *C. elegans* as a test organism and using ENU, HCHO, and MMS as model genotoxicants. As shown by microarray results, we have established the optimal experimental conditions for generating optimal gene expression profiles of *C. elegans* in response to chemical exposure in liquid medium. Most importantly we showed that transcriptional effects were chemically specific and concentration dependent. In this assay we have also identified and validated 4 stable housekeeping genes that can be used to normalize gene expression quantitation in nematode by using RT-qPCR assay.

To our knowledge, no research has been carried out yet on genome-wide transcriptional responses of *C. elegans* to HCHO, ENU, and MMS or any comparable compounds that are known to directly induce DNA damage stress. Consequently, there was insufficient information in literature about the operating protocols including the exposure concentration for the testing of these substances in the nematodes. Therefore, we tested first the lethal toxicity of each compounds followed by the selection of the highest sublethal concentration to nematodes to maximize the occurrence of biological disturbances transcriptionally

detectable. In addition, from our pilot microarray study we learned that subtle differences in the experimental temperature and the developmental synchronization of nematode culture could be major confounding variables that can influence gene expression profiling results.

The exposure concentrations used in this study caused a significant proportion of differentially expressed genes (DEGs) to be downregulated. For HCHO-treated nematodes, transcriptional downregulation was in line with the toxicity results, where the nematodes showed the highest mortality rate and impaired mobility, suggesting a predominance of general toxicity. A similar observation was previously reported in *Daphnia magna*, where high concentrations of copper, cadmium and zinc mostly trigged the transcriptional responses of general stress-related processes [37]. Some genes among DEGs repressed by HCHO treatment have been previously characterized to have antibacterial and antifungal activity in *C. elegans* such as *nlp-29* gene [38]. This suggests that nematodes affected by HCHO may be more vulnerable to infections.

The compounds tested in this study are known to react directly with biological molecules especially DNA and proteins via alkylation [20]. Consequently, upon exposure the nematodes were expected to initiate repair mechanisms in response to various molecular damages. In accordance with the proteotoxic behaviors of HCHO and MMS, there was indeed transcriptional evidence suggestive of protein damage stress, such as the upregulation of genes involved in the response to endoplasmic reticulum stress, unfolded proteins, or topologically incorrect protein. These results match those observed in other studies, in which several genes inducible by protein damage in *C. elegans* were identified for unfolded protein stress (*ckb-2, ckb-4*, C04F12.1, *pdi-2, dnj-7, cup-2, hsp-4, sel-1, arf-1.1, rer-1, nsf-1*, and Y54G2A.18) [39] and for proteotoxic stress response (*fbxa-75, fbxa-158, pals-22, skr-3, skr-4* and *cul-6*) [40].

While we anticipated that MMS and ENU would induce comparable transcriptional effects due to their close mode of toxicity as alkylating agents, ENU treatment did not appear to induce a significant expression among genes involved in proteotoxic stress response. Instead, expression of genes related to innate immune response, especially *cdr-4, fbxa-105, gst-13, lec-11, ugt-44*, and K08D8.4 was increased by both ENU and MMS treatments. In addition, the results of this study correlated with the mode of toxicity of ENU known to induce oxidative stress, as revealed by the transcriptional upregulation of peroxisome pathway (cel04146) and notably glutathione S-transferase (GST) gene family, such as *gst-5, gst-6, gst-7, gst-8, gst-12, gst-13, gst-14, gst-31, gst-33*, and *gst-37*, which have been previously linked to the oxidative stress resistance in *C. elegans* in response to exposure with chemicals like paraquat and juglone [41].

Our finding showed that MMS induced transcriptional repression of genes involved in nerve impulse transmission along a neuron, including *mgl-1, dop-1, gab-1, gar-3*, *gar-1*, and *ser-4*. These results correlated with our toxicity tests where the nematodes treated with MMS

showed signs of the reduced motility. This is in the line with earlier literature that found an important relationship between the affected genes and locomotion in nematodes, such as dopaminergic receptor *dop-1* [42], serotonin receptor *ser-4* [43], or muscarinic receptor *gar-3* [44]. One may speculate the inactivation of neurotransmitters or receptor proteins by MMS through alkylation reaction resulted in the impairment of motor activity.

Having discussed how the exposure conditions investigated in our study induced the general stress in the nematodes, this raises the question of whether specific toxicity mechanisms may be eclipsed by the general ones as speculated by Gou *et al.* [7]. Our study suggests that the nematode might attempt to shut down parts of its gene-expression machinery to alleviate the general toxicity. In doing so, many biological processes may be affected, including processes related to the specific mode of action of the compound. Indeed, this could explain why we did not find significant changes in the transcription of genes that play a critical role in the maintenance of DNA integrity in *C. elegans* such as DNA-damage checkpoints or DNA repair proteins. Similar findings were reported upon exposure to X-ray radiation as this did not affect mRNA expression levels of DNA repair genes [16], leading to the hypothesis that DDR genes in *C. elegans* might be instead regulated through posttranscriptional modifications of checkpoint proteins. Alternatively, the increase in metabolism and detoxification processes in the nematodes may have substantially reduced the efficacy of the tested toxicants, thus protecting the nematodes from genotoxic effects.

Despite the lack of expression change in DDR genes in our study, there was neither enough evidence to be able to conclude that DNA damage stress did not take place in the nematodes upon exposure to these compounds. Such DNA damage was demonstrated in a recent study showing that MMS-exposure of *C. elegans* generated a high number of mutations via base methylations [45], and chemical mutagenesis in the nematode with ENU and HCHO is also well known [46]. Moreover, our findings showed a significant change in the expression levels of genes like *gei-17* and *cul-6*, which have been reported to be involved in DNA-damage response in *C. elegans* [47]. They were categorised differently in our study based on alternative functional descriptions and therefore not identified by either KEGG pathway or GO analyses. In addition, several genes involved in apoptosis or transcriptional regulation were expressed in this study, and data from literature has linked these processes to genotoxic response [17]. Similarly, the induction of innate immune response found in our assay is reported in literature to be also triggered upon DNA damage stress [15].

Another aim of this study was to identify candidate marker genes that can be used to detect target toxicants. Previous studies have shown the potential of gene expression analysis to specifically detect contaminants in environmental samples [8]. Based on our experimental work, several genes were found as potential candidate biomarkers for the detection of the tested compounds. In brief, each candidate gene expression biomarker complies with three criteria as proposed by Gou et al. [7], including: a) the genes with chemical-specific

response, b) the genes whose expression was concentration-dependent, and c) the genes which are linked to a specific mode of action related to the toxicant.

Overall, our study implies that both general as well as specific toxicity mechanisms of the tested compounds were operational in the nematodes and can be detected transcriptionally. Hence, this study will serve as a base for developing transcriptional biomarkers for detecting a wide array of bioactive contaminants, including hydrophilic ones that are hard to detect chemically. To determine reliable biomarkers, more studies like this should be carried out on several model compounds, mixtures and toxicants that require metabolic bioactivation. Our study successfully demonstrated the capability of this nematode to respond by a specific mode of action making it suitable for detection of specific compounds. It also showed that very high exposure concentrations most likely induce general stress that can mask specific effects. Further studies should focus on lower exposure concentrations to enable quantification of key transcriptional events specific to the mode of activity of a target compound. The applicability of this bioassay can be further improved by conducting transcriptomic concentration-response analysis of model compounds to define the concentration range detectable by this method and relate this to concentrations expected in field situations. The bioassay is expected to be not only mechanism-specific but also to indicate the exposure to compounds at concentrations far below those inducing physiological responses and before chronic effects become detectable. Thereby it could complement single-compound bioassays like CALUX [48] or ToxTracker assay [49].

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Supporting information of chapter 2b

S1. Pilot microarray analysis

1. Introduction

Gene expression in *Caenorhabditis elegans* was measured by microarrays. The experiment consisted of exposure samples using three direct-acting genotoxic model compounds including N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO) and methyl methanesulfonate (MMS) dissolved in M9 buffer. Pilot study was the first pass carried out with two main goals: (i) to identify the sources of experimental and biological variation, and (ii) to get preliminary results on differentially expressed genes. This study revealed that subtle differences in the experimental temperature and the developmental synchronization of nematode culture could be major confounding variables that can influence gene expression profiling results. The outcomes from this experiment were used to reduce batch variation in the main microarray experiment as described in manuscript.

2. Material and Methods

Chemical exposure. The nematode treatment was carried out as described in the manuscript with minor adjustments. Briefly, the pilot experiment was conducted by using synchronized culture of *Caenorhabditis elegans* (L4 larval stage, 48 hours old grown at 20^{o}C) obtained via eggs that were prepared by treating gravid nematodes with 5% sodium hypochlorite solution (Bleaching) [50]. The nematodes were exposed to 1 mM and 5 mM N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS) dissolved in M9 buffer for 2 hours at 20 °C. The experiment was run in two biological replicates, and approximately 1200 animals were treated per each sample. At the end of exposure, the nematodes were immediately pelleted by spinning once in centrifuge (18,400 x g at room temperature, followed by removal of the supernatants) to collect the pellets, which were then flash frozen in liquid nitrogen for 1 minute before storage at -80 °C until RNA extraction.

Microarray preparation, hybridization, and scanning. Total RNA was isolated following standard protocols, previously described in [28]. The gene-expression was measured using the Agilent *C. elegans* (V2) Gene Expression Microarray 4x44K slides. For cDNA synthesis, labelling with cy3 and cy5 dyes, and subsequent hybridization, the 'Two-Color Microarray-Based Gene Expression Analysis; Low Input Quick Amp Labeling'—protocol, version 6.0 from Agilent (Agilent Technologies, Santa Clara, CA, USA) was followed. Scanning was done using an Agilent High Resolution C Scanner with the settings as recommended by the protocol. For extraction of the intensities the accompanying software was used (Agilent Feature Extract, v. 10.7.1.1). The array probe annotation was updated by blasting the probes

against WS258 with blast (version 2.6.0, windows x64), using nblast with parameters: word size 7, reward 1, pentaly -3, and evalue 1. Probes with multiple hits were flagged and ignored in the analysis of affected genes.

Normalization and pre-processing of microarray raw data. Normalization of the data was carried out using the Limma package in "R" (version 3.4.2, x64) in RStudio (version 1.1.383). Arrays were normalized without background correction, normalization within arrays was done using the Loess method and between arrays using the quantile method [29, 30]. The obtained values were $\log_2$ transformed and were used for subsequent analysis. Furthermore, a $\log_2$ ratio with the mean (R) was calculated, where each spot was expressed as a fraction of the intensity of the same spot over all samples:

$$\boldsymbol{R_{i,j} = log2(\frac{y_{i,j}}{\overline{y}_i})}$$

where y is the intensity of spot i (1, 2, 3, ..., 45220) of sample j (1, 2, 3, ..., 12).

Data that was analysed by linear model was first batch corrected:

$$\boldsymbol{B_{i,j} = y_{i,j} - (\overline{y}_{batch,i} - \overline{y}_{total,i})}$$

Where B is the batch corrected value, and difference between the batch average and the total average for a spot i (1, 2, 3, ..., 45220) is subtracted from the normalized intensity. The raw data of the pilot study experiment were submitted to ArrayExpress (E-MTAB-10264).

Statistical data analysis. To detect if the arrays were technically correct, Pearson correlations were calculated in "R" using the *cor* function (version 3.4.2, x64), where normalized intensities of each sample were compared to all other samples. To identify which factors underlying variation in gene expression, principal component analysis (PCA) was calculated on the $\log_2$ ratio with the mean values using the *prcomp* function in "R". To identify gene expression changes linked to the chemical treatment of nematodes, the expression was investigated using a linear model:

$$\boldsymbol{B_{i,j} \sim T_j + e_j}$$

where T is the treatment of sample j and e is the unexplained variation (error). The three treatments (ENU, HCHO, and MMS) were pairwise compared to the control treatment. The resulting p-values were corrected for multiple testing using the *p.adjust* function with the Benjamini & Hochberg method [31]. To assess the differentially expressed genes (DEG) per exposure condition, we took a high significant level of $-\log_{10}$(p-value) > 4 (i.e., $p < 0.0001$; FDR < 0.05). Further, we particularly assessed the expression of four reference genes encoding for heat shock proteins (*hsp-16.2* and *hsp-16.41*) and for vitellogenin proteins (*vit-*

1 or *vit-2*), which are indicative of heat-stress [28] and the age of the nematode population [51], respectively.

3. Results and discussion

In this experiment all samples correlated strongly, indicating that the arrays were technically sound. The analysis also revealed a batch effect. Gene expression analysis showed some fluctuation in the expression levels of *hsp-16.2* and *hsp-16.41* genes in both exposed and unexposed nematode population. Differential expression of these genes has been previously linked to the transcriptional response to heat stress in *C. elegans* [28]. In addition, a slight developmental difference between two independent nematode culture batches was observed, as suggested by the expression levels of *vit-1* and *vit-2* genes that are indicative of the age of the nematode population [51]. This illustrated the need to take extra caution on the experimental set-up to reduce potential batch development variation and temperature fluctuation effects.

We identified 12 genes whose expression levels were significantly differentially affected by HCHO treatment, including three upregulated (*gst-23*, T07D3.4, and B0024.15) and 9 downregulated (*asic-2*, *cnc-1*, *gap-2*, *grsp-2*, C25E10.5, F40H7.12, T24E12.5, W09G12.7, and Y69H2.3) gene transcripts. No significant differential gene expression was found in the nematodes treated with ENU and MMS. From these data it was hypothesized that the exposure time of 2 hours was too short, hence explaining the small numbers of differentially expressed genes (DEG).

Overall, pilot study revealed that slight differences in the experimental temperature and the developmental synchronization of nematode culture could be major confounding variables that can influence transcriptional effects. From this study we learned two major lessons: (1) It is paramount to synchronize the nematode population to the required developmental stage. The accuracy of synchronization could be confirmed by observing the nematodes under a stereo microscope. (2) To reduce potential temperature effects, it is important to carefully maintain a constant temperature for the period of exposure time, keeping the nematodes inside an incubator as much as possible. A temperature data logger can be used to monitor the temperature in the incubator. These adjustments were applied in the full-scale experiment (as described in the manuscript) and significantly reduced batch variation in gene expression.

S2. The analysis of main microarray experiment

1. Batch effect correction

A batch correction was performed by fitting the gene expression to the linear model:

Equation S1. $$I_{i,j} = B_j + D_j + T_j + e_{i,j}$$

where I is the log_2-transformed intensity of spot *i* (1, 2, ..., 45220) of sample *j* (1, 2, ..., 24). This was fitted over biological replicate B (1-3), each containing all treatments, dye D (either cy3 or cy5) and treatment T (control, MMS, ENU, or HCHO) and an error term e. Batch correction was performed by subtracting the batch and dye effects per spot per sample.

2. Calculating average expression values

After batch correction, the log_2 ratio with the mean (R) was calculated, where each spot was expressed as a fraction of the intensity of the same spot over all samples:

Equation S2. $$R_{i,j} = log2(\frac{y_{i,j}}{\bar{y}_i})$$

where y is the untransformed intensity of spot *i* (1, 2, ..., 45220) of sample *j* (1, 2, ..., 24). This resulted in log2 transformed and further referred to as the log2 ratio with the mean.

3. Linear model analysis

To evaluate whether transcriptional effects were concentration-dependent, we used a linear model analysis incorporating the compound as well as the exposure concentration. This model was applied to ENU, HCHO, and MMS separately, by

Equation S3. $$I_{B,i,j} \sim C_j + e_j$$

where I_B is the batch-corrected intensity of spot *i* of sample *j*, C is the concentration (0, 1, or 5 mM), and e is the unexplained variance (error).

As for the compound-dependent model analysis above, The resulting p-values were corrected for multiple testing using the *p.adjust* function in R program with the Benjamini and Hochberg method [31]. We set a significance threshold of –log10(p) > 4 (FDR = 0.011, FDR = 0.0059, and FDR = 0.0053 for ENU, HCHO, and MMS, respectively).

Suppl. Table S1. KEGG pathway enrichment for up- and downregulated genes analyzed in DAVID software (False Discovery Rate, FDR ≤ 5%).

Treatment	GO term	Category	#genes	%	FDR (%)
Upregulation					
HCHO	cel04141	Protein processing in endoplasmic reticulum	9	3.4	0.2
MMS	cel04141	Protein processing in endoplasmic reticulum	10	3.5	0.0
MMS	cel00480	Glutathione metabolism	4	1.4	4.6
ENU	cel00980	Metabolism of xenobiotics by cytochrome P450	7	6.0	0.0
ENU	cel00982	Drug metabolism - cytochrome P450	7	6.0	0.0
ENU	cel00480	Glutathione metabolism	5	4.3	0.0
Downregulation					
MMS	cel04080	Neuroactive ligand-receptor interaction	6	2.2	0.0
ENU	cel04146	Peroxisome	5	3.0	0.0

Suppl. Table S2. Gene Ontology (GO) enrichment analysis in biological processes (BP) category for up- and downregulated genes analyzed in DAVID software (False Discovery Rate, FDR ≤ 5%).

Treatment	GO term	Category	#genes	%	FDR (%)
Upregulation					
HCHO	GO:0044710	single-organism metabolic process	40	15.2	3.7
HCHO	GO:0044281	small molecule metabolic process	27	10.2	0.0
HCHO	GO:0055085	transmembrane transport	26	9.8	1.9
HCHO	GO:0006082	organic acid metabolic process	16	6.1	1.5
HCHO	GO:0006457	protein folding	8	3.0	1.3
HCHO	GO:1901605	alpha-amino acid metabolic process	7	2.7	4.5
HCHO	GO:0046394	carboxylic acid biosynthetic process	7	2.7	5.0
HCHO	GO:0006144	purine nucleobase metabolic process	4	1.5	1.4
MMS	GO:0006950	response to stress	36	12.6	0.0
MMS	GO:0010033	response to organic substance	27	9.4	0.0
MMS	GO:0071310	cellular response to organic substance	24	8.4	0.0
MMS	GO:0070887	cellular response to chemical stimulus	24	8.4	0.0
MMS	GO:0006952	defense response	21	7.3	0.0
MMS	GO:0002376	immune system process	20	7.0	0.0
MMS	GO:0045087	innate immune response	19	6.6	0.0
MMS	GO:0006955	immune response	19	6.6	0.0
MMS	GO:0035966	response to topologically incorrect protein	13	4.5	0.0
MMS	GO:0071383	cellular response to steroid hormone stimulus	13	4.5	0.7
MMS	GO:0048545	response to steroid hormone	13	4.5	0.7
MMS	GO:0043401	steroid hormone mediated signaling pathway	13	4.5	0.7
MMS	GO:0071396	cellular response to lipid	13	4.5	0.7
MMS	GO:0033993	response to lipid	13	4.5	0.8
MMS	GO:0009755	hormone-mediated signaling pathway	13	4.5	0.8
MMS	GO:0071407	cellular response to organic cyclic compound	13	4.5	1.4
MMS	GO:0009725	response to hormone	13	4.5	1.7
MMS	GO:0032870	cellular response to hormone stimulus	13	4.5	1.7
MMS	GO:0014070	response to organic cyclic compound	13	4.5	2.3
MMS	GO:0071495	cellular response to endogenous stimulus	13	4.5	4.6
MMS	GO:0034976	response to endoplasmic reticulum stress	12	4.2	0.0
MMS	GO:0030968	endoplasmic reticulum unfolded protein response	11	3.8	0.0
MMS	GO:0034620	cellular response to unfolded protein	11	3.8	0.0
MMS	GO:0006986	response to unfolded protein	11	3.8	0.0
MMS	GO:0035967	cellular response to topologically incorrect protein	11	3.8	0.0
ENU	GO:0008152	metabolic process	57	49.1	4.5
ENU	GO:0044710	single-organism metabolic process	23	19.8	4.0
ENU	GO:0045087	innate immune response	15	12.9	0.0
ENU	GO:0006955	immune response	15	12.9	0.0
ENU	GO:0002376	immune system process	15	12.9	0.0
ENU	GO:0006952	defense response	15	12.9	0.0
ENU	GO:0006063	uronic acid metabolic process	8	6.9	0.0
ENU	GO:0052696	flavonoid glucuronidation	8	6.9	0.0
ENU	GO:0009813	flavonoid biosynthetic process	8	6.9	0.0
ENU	GO:0019585	glucuronate metabolic process	8	6.9	0.0
ENU	GO:0052695	cellular glucuronidation	8	6.9	0.0
ENU	GO:0009812	flavonoid metabolic process	8	6.9	0.0
ENU	GO:0032787	monocarboxylic acid metabolic process	8	6.9	1.2
Downregulation					
HCHO	GO:0009605	response to external stimulus	13	5.7	2.8
HCHO	GO:0043207	response to external biotic stimulus	10	4.4	0.0
HCHO	GO:0051707	response to other organism	10	4.4	0.0
HCHO	GO:0009607	response to biotic stimulus	10	4.4	0.0
HCHO	GO:0098542	defense response to other organism	9	4.0	0.1
HCHO	GO:0009617	response to bacterium	7	3.1	1.7
HCHO	GO:0050832	defense response to fungus	5	2.2	0.3
HCHO	GO:0009620	response to fungus	5	2.2	0.3
MMS	GO:0065007	biological regulation	102	37.0	0.3
MMS	GO:0050789	regulation of biological process	87	31.5	1.8
MMS	GO:0050794	regulation of cellular process	81	29.3	0.2
MMS	GO:0044700	single organism signaling	53	19.2	0.8
MMS	GO:0023052	signaling	53	19.2	0.9
MMS	GO:0007154	cell communication	53	19.2	1.2
MMS	GO:0007165	signal transduction	48	17.4	4.9
MMS	GO:0044765	single-organism transport	37	13.4	3.8
MMS	GO:0006811	ion transport	25	9.1	0.6
MMS	GO:0007186	G-protein coupled receptor signaling pathway	24	8.7	2.7
MMS	GO:0034220	ion transmembrane transport	18	6.5	3.8
MMS	GO:0032879	regulation of localization	17	6.2	0.2
MMS	GO:0009605	response to external stimulus	17	6.2	3.6
MMS	GO:0007267	cell-cell signaling	16	5.8	0.0
MMS	GO:0098660	inorganic ion transmembrane transport	15	5.4	1.9
MMS	GO:0098916	anterograde trans-synaptic signaling	14	5.1	0.0
MMS	GO:0099537	trans-synaptic signaling	14	5.1	0.0
MMS	GO:0007268	chemical synaptic transmission	14	5.1	0.0
MMS	GO:0099536	synaptic signaling	14	5.1	0.0
MMS	GO:0006820	anion transport	12	4.3	1.5
MMS	GO:0006821	chloride transport	8	2.9	0.6
MMS	GO:0015698	inorganic anion transport	8	2.9	1.2
MMS	GO:0007187	G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	7	2.5	0.1
MMS	GO:1902476	chloride transmembrane transport	7	2.5	2.4
MMS	GO:0098661	inorganic anion transmembrane transport	7	2.5	4.1
MMS	GO:0007188	adenylate cyclase-modulating G-protein coupled receptor signaling pathway	6	2.2	0.7
MMS	GO:0007193	adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway	4	1.4	0.7

Suppl. Table S3. Gene Ontology (GO) enrichment analysis in molecular function (MF) category for up- and downregulated genes analyzed in DAVID software (False Discovery Rate, FDR ≤ 5%).

Treatment	GO term	Category	#genes	%	FDR (%)
Upregulation					
HCHO	GO:0003824	catalytic activity	84	31.8	2.1
HCHO	GO:0016757	transferase activity, transferring glycosyl groups	15	5.7	0.1
ENU	GO:0003824	catalytic activity	48	41.4	0.1
ENU	GO:0016740	transferase activity	26	22.4	0.1
ENU	GO:0016491	oxidoreductase activity	12	10.3	3.0
ENU	GO:0015020	glucuronosyltransferase activity	8	6.9	0.0
ENU	GO:0008194	UDP-glycosyltransferase activity	8	6.9	0.1
ENU	GO:0020037	heme binding	8	6.9	0.1
ENU	GO:0046906	tetrapyrrole binding	8	6.9	0.2
ENU	GO:0016758	transferase activity, transferring hexosyl groups	8	6.9	1.7
ENU	GO:0016757	transferase activity, transferring glycosyl groups	8	6.9	4.0
ENU	GO:0004497	monooxygenase activity	7	6.0	0.1
ENU	GO:0005506	iron ion binding	7	6.0	0.3
ENU	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	7	6.0	0.4
ENU	GO:0004364	glutathione transferase activity	5	4.3	0.0
ENU	GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	5	4.3	0.6
Downregulation					
HCHO	GO:0004197	cysteine-type endopeptidase activity	5	2.2	4.1
MMS	GO:0060089	molecular transducer activity	34	12.3	0.4
MMS	GO:0004872	receptor activity	34	12.3	0.4
MMS	GO:0099600	transmembrane receptor activity	33	12.0	0.3
MMS	GO:0004871	signal transducer activity	33	12.0	1.2
MMS	GO:0038023	signaling receptor activity	29	10.5	3.8
MMS	GO:0004888	transmembrane signaling receptor activity	28	10.1	3.5
MMS	GO:0004930	G-protein coupled receptor activity	24	8.7	1.8
MMS	GO:0015075	ion transmembrane transporter activity	21	7.6	1.8
MMS	GO:0022838	substrate-specific channel activity	15	5.4	2.4
MMS	GO:0005216	ion channel activity	14	5.1	4.8
MMS	GO:0022836	gated channel activity	11	4.0	1.9
MMS	GO:0008509	anion transmembrane transporter activity	10	3.6	0.3
MMS	GO:0015108	chloride transmembrane transporter activity	8	2.9	0.9
MMS	GO:0015103	inorganic anion transmembrane transporter activity	8	2.9	2.0
MMS	GO:0005254	chloride channel activity	7	2.5	3.3
MMS	GO:0005253	anion channel activity	7	2.5	3.9
MMS	GO:0008227	G-protein coupled amine receptor activity	4	1.4	1.8

Suppl. Table S4. Gene Ontology (GO) enrichment analysis in cellular component (CC) category for up- and downregulated genes analyzed in DAVID software (False Discovery Rate, FDR ≤ 5%).

Treatment	GO term	Category	#genes	%	FDR (%)
Upregulation					
HCHO	GO:0005783	endoplasmic reticulum	15	5.7	1.5
HCHO	GO:0033178	proton-transporting two-sector ATPase complex, catalytic domain	4	1.5	2.3
MMS	GO:0005622	intracellular	77	26.9	2.8
MMS	GO:0012505	endomembrane system	19	6.6	2.1
MMS	GO:0005783	endoplasmic reticulum	17	5.9	0.0
MMS	GO:0044432	endoplasmic reticulum part	14	4.9	0.0
MMS	GO:0005789	endoplasmic reticulum membrane	10	3.5	0.4
MMS	GO:0042175	nuclear outer membrane-endoplasmic reticulum membrane network	10	3.5	0.5
MMS	GO:0005788	endoplasmic reticulum lumen	4	1.4	0.8
Downregulation					
HCHO	GO:0005576	extracellular region	19	8.4	0.0
HCHO	GO:0005615	extracellular space	13	5.7	0.0
HCHO	GO:0044421	extracellular region part	13	5.7	0.0
MMS	GO:0071944	cell periphery	45	16.3	0.0
MMS	GO:0005886	plasma membrane	43	15.6	0.0
MMS	GO:0044459	plasma membrane part	28	10.1	2.1
MMS	GO:0097458	neuron part	18	6.5	0.0
MMS	GO:0043005	neuron projection	13	4.7	0.6
MMS	GO:0045202	synapse	12	4.3	0.5
MMS	GO:0030424	axon	9	3.3	2.7

Table S5. Validation of microarray gene expression data by RT-qPCR analysis. Included are gene expression data of 12 gene targets that were selected from our main microarray experiment based on their annotation and their potential association to the mechanisms of toxicity of the tested compounds. The table contains normalized relative expression fold changes of target genes in the treated nematodes compared to the untreated. Positive fold change values (upregulation) and negative fold change values (downregulation) at a significance level of $p \leq 0.001$. RT-qPCR were run in three independent biological replicates and three technical replicate per each test using the same RNA templates from 5mM-treated microarray samples. Asterisks (*) denote the gene expression fold changes measured by microarray and were also found significant in RT-qPCR with $p \leq 0.0001$

	ENU				HCHO				MMS			
Gene	**Array**	**P-value**	**RT-qPCR**	**P-value**	**Array**	**P-value**	**RT-qPCR**	**P-value**	**Array**	**P-value**	**RT-qPCR**	**P-value**
tor-2	1.4*	< 0.0001	1.7	< 0.0001	1.7*	< 0.0001	2.7	< 0.0001	1.3	NS	1.6	< 0.0001
rpt-1	1.1	NS	1.2	NS	1.1*	< 0.0001	1.2	NS	1.2*	< 0.0001	1.2	NS
cec-8	-1.1	NS	-1.1	NS	1.1	NS	-1	NS	1.2*	< 0.0001	1.1	NS
ttr-52	1.2*	< 0.0001	1.6	< 0.0001	1.1	NS	1.5	0.00066	1.2	NS	2.1	< 0.0001
C25F9.2	-1.9*	< 0.0001	-2.2	< 0.0001	-2.9*	< 0.0001	-3.8	< 0.0001	-1.9*	< 0.0001	-1.8	< 0.0001
tth-1	-1.2	NS	-1.2	NS	-2*	< 0.0001	-2.8	< 0.0001	-1.4*	< 0.0001	-1.3	0.00082
F40F9.10	-1.1	NS	1.1	NS	-1.4*	< 0.0001	-1.3	0.00034	-1.2*	< 0.0001	-1.1	NS
psa-3	-1.2	NS	-1.5	< 0.0001	-1.4*	< 0.0001	-1.2	NS	-1.6*	< 0.0001	-2	< 0.0001
hil-1	-2	0.0006	-2.8	< 0.0001	-2.5*	< 0.0001	-2.7	< 0.0001	-2	0.00078	-2.7	< 0.0001
hda-11	-1.9*	< 0.0001	1.2	NS	-1.6	0.0001	1.1	NS	-1.2	NS	1.4	0.00062
ceh-63	-1.3	NS	-1.5	0.00039	-1.6	0.00017	-2.1	< 0.0001	-1.4*	< 0.0001	-1.3	NS
ced-1	-1.3	< 0.0001	-1.2	NS	-1.4*	< 0.0001	-1.5	< 0.0001	-1.2	0.00044	-1.4	< 0.0001

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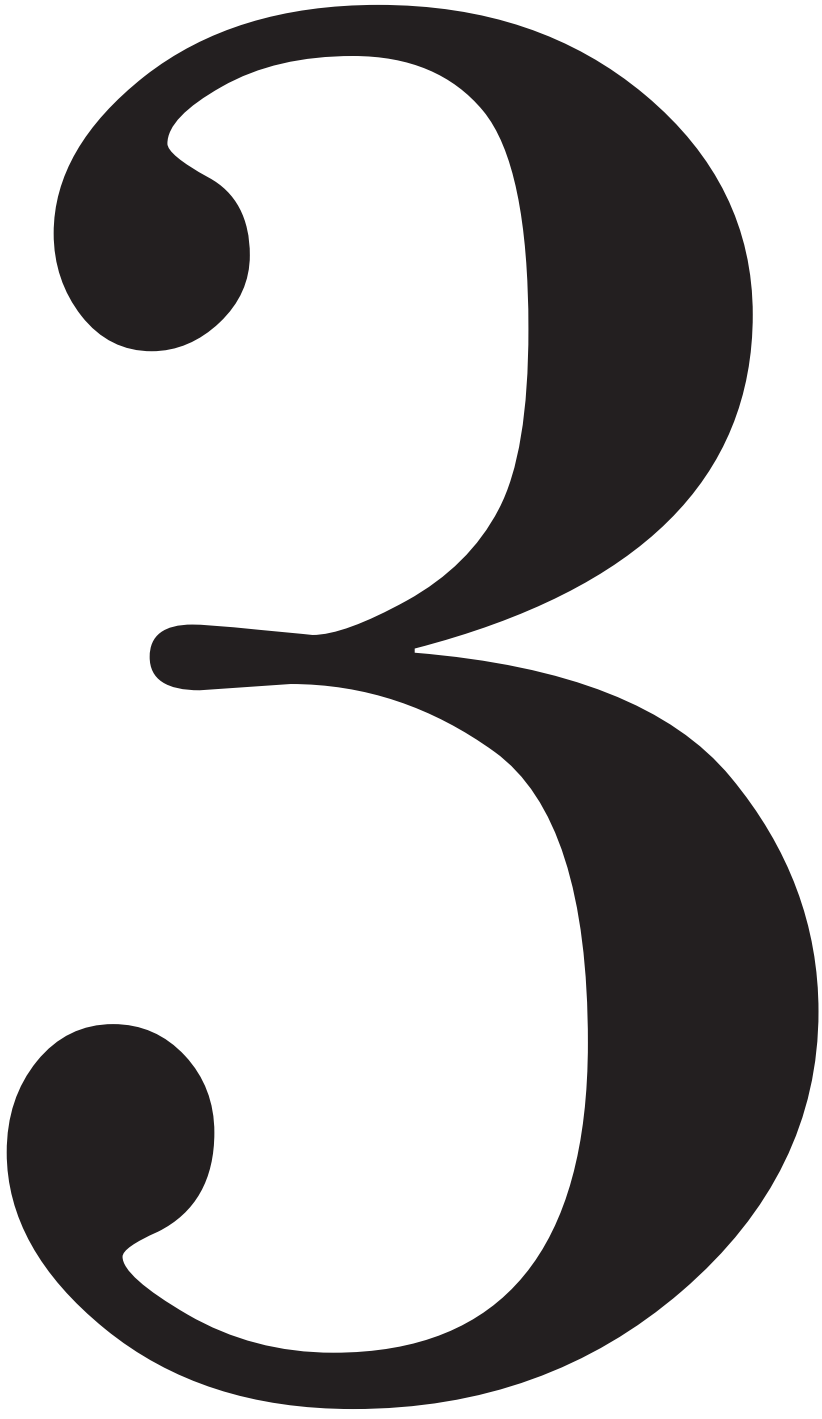

Chapter 3

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

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Abstract

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

1. Introduction

Biotransformation changes the chemical structure of xenobiotic compounds to reduce their toxicity and allow easier excretion of these compounds. Through this process, lipophilic chemicals are generally converted into more hydrophilic molecules by a series of chemical reactions. While xenobiotic biotransformation facilitates detoxification of compounds, it can occasionally generate toxic metabolites via a process known as bioactivation [1, 2]. Several substances are categorized as “indirect-acting” in reference to the chemical agents with little or no toxicological activity, that become toxic upon metabolic activation [3]. Biotransformation machinery involves several protein components like the phase I monooxygenases (also referred to as cytochrome P450s), phase II conjugation enzymes and phase III xenobiotic transport proteins [4]. The expression of genes encoding these enzymes can be transcriptionally affected by exogenous conditions including the presence of a single compound or a mixture of xenobiotics [5-7].

A recent study showed how the nematode *C. elegans* responds to direct-acting genotoxic model compounds [8]. In that study, the transcription of DNA damage repair and cell cycle checkpoints genes were not differentially affected by the selected toxicants, but several genes encoding biotransformation proteins were upregulated. Therefore, here we were interested in investigating genome-wide gene expression profiles of *C. elegans* exposed to the chemical agents that require metabolic conversion to become active toxicants.

C. elegans provides a suitable experimental model to study the effects of bioactive substances as it shares many gene functions with mammalians including those involved in xenobiotics biotransformation. For instance, the orthologs for many key mammalian redox systems have been reported in *C. elegans* including glutathione (GSH) and related systems, which are critical for detoxification of both xenobiotic and endogenous compounds in mammals [9, 10]. Furthermore, over 80 CYP genes encoding cytochrome P450 enzymes have been identified in the *C. elegans* genome [11, 12]. Based on predicted amino acid sequences, the majority of *C. elegans* CYP genes were found to be closely related to the mammalian CYP2, CYP3, and CYP4 gene families [13]. Interestingly, CYP1-like metabolism, which is indispensable for metabolizing numerous indirect-acting xenobiotics like polycyclic aromatic hydrocarbons (PAH) [14], is reported not to be present in *C. elegans* [15]. Furthermore, the mammalian aryl hydrocarbon receptor (AhR) [16, 17] which plays a central role in the toxicity of many chemical agents like dioxins and dioxin-like compounds has a homologue (AHR-1) encoded by *ahr-1* gene in *C. elegans*. The nematode protein AHR-1 regulates several physiological processes such as neuronal development [18], locomotion, egg laying, defecation behaviors, fatty acid synthesis, and others [19]. Unlike its counterpart in mammalians (AhR), the nematode AHR-1 was demonstrated not to bind to its common activators such as TCDD or β-naphthoflavone [20]. This could mean that the nematode is not sensitive to transcriptional effects of dioxins.

In this paper, we therefore investigated to which extent C*. elegans* is responsive to indirect-acting model compounds and to dioxin. We analyzed genome-wide gene expression effects of three toxicants whose mode of action is dependent on cytochrome P450-mediated metabolic activation. We selected aflatoxin B1 (AFB1), benzo[a]pyrene (B(a)P), PCB mixture Aroclor 1254 (PCB1254) as representative compounds in the toxic classes of highly genotoxic mycotoxins, polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyl (PCB). As dioxin representative we selected 2,3,7,8-tetrachlorodibenzodioxin (TCDD). All four compounds are classified as aryl hydrocarbons containing one or more aromatic rings made of delocalized π electrons which are susceptible to oxidative reactions such as epoxidation, hydroxylation and others mostly mediated by cytochrome P450 enzymes. In humans, AFB1 is mainly metabolized in the liver by CYP1A2 and CYP3A4 isoenzymes to its metabolites including the genotoxic aflatoxin B1 exo-8,9-epoxide [21]. B(a)P requires cytochrome P450 enzymes to form metabolites including the genotoxic B[a]P-7,8-diol-9,10-epoxide. In mice, the activation of B(a)P is mediated by hepatic CYP1 enzymes only, especially CYP1A1 and CYP1B1 [15, 22]. PCB1254, a mixture of several polychlorinated biphenyls, is metabolized by humans or rodents: CYP2B, CYP2C, and CYP3A enzymes into 2,3,3',4',5-pentachloro-4-biphenylol as the major metabolite [23, 24]. Like many dioxins and dioxin-like toxicants, the activity of TCDD is guided by the activation of the aryl hydrocarbon receptor (AhR) pathway [5] and via the AhR TCDD also activates CYPs belonging to the CYP1 family such as CYP1A1 [25]. Mammalian exposure to these four toxicants is linked to various effects such as immunotoxicity, oxidative stress, endocrine disruption, carcinogenicity, growth impairment, reproductive and developmental toxicity, and others [5, 15, 21, 23].

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

2. Material & methods

2.1. *C. elegans* culture

The culture of wild-type N2 (Bristol) strain of *C. elegans* was prepared as described in [8]. Synchronized populations of nematodes were obtained using a modified version of the bleaching technique [26]. Briefly, the first larval stage (L1) growth-arrested via starvation

were obtained by hatching eggs in M9 buffer [27] overnight at 20 °C with gentle agitation. The fourth larval stage (L4) nematodes were obtained after 31 ± 0.5 hours of development starting from L1 fed with *E-coli* OP50 at 20 °C.

2.2. Chemicals

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

2.3. Chemical exposure

Non-lethal concentrations used in our experiments were selected according to the study of Leung and colleagues investigating AFB1 and B(a)P metabolic activation in *C. elegans* [15]. Briefly, we first assessed whether the compound is soluble in the exposure medium. We then tested different concentration of each compound to examine which non-lethal to the nematodes. The absence of mortality among the nematodes (after the exposure period) was confirmed by visual observation through a stereomicroscope. Twenty-four-hour exposure was carried out in Falcon™ 15-mL conical tubes at 20 °C. Each sample was made of 2885 µL M9 buffer and 15 µL stock solution compound (with a final DMSO concentration of 0.5%). The solvent (0.5% DMSO) has been previously reported not to influence *C. elegans* gene expression [12] or its growth and reproduction at 24-hour exposure [15]. For single-compound exposure, the nematodes were treated in quadruplicate with 30 µM for AFB1, B(a)P, and PCB1254). As TCDD is a very potent toxic compound with lowest effect levels in the pM range (see e.g. Murk et al 1996 [28]). The relative toxic potency compared to PCB mixtures including PCB1254 (comparable to PCB A50) is 10^5 -10^6 higher [28], therefore we decided to use a higher non-lethal exposure concentration based on a previous study with *C. elegans* (Bao et al. in preparation). In that study exposure to 10 nM already resulted in significantly delayed larval developments and 10 uM still was non-lethal but the larval development was halted. Therefore we chose to expose to 10 µM TCDD. Concentration-response experiments were run in triplicate with concentration ranging from 0.01 µM to 100 µM AFB1, 0.01 µM to 40 µM B(a)P or 0.1 µM to 100 µM PCB1254. Exposure with mixtures was performed in duplicate by combining toxicants (AFB1, B(a)P, or PCB1254) at the concentration of 0.1 µM, 1 µM, or 10 µM per each compound in the mixture. Approximately 10,000 nematodes were used for each sample, and there was no feeding during the exposure period. We chose to use starved L4 larvae to minimize any developmental effects. Our preliminary experiments (data not shown), resulted in better transcriptional responses

in starved nematodes compared to the fed ones. Also, by not feeding the nematodes we expect less influence on the bioavailability or other kinetics of the toxicant as reported elsewhere [29]. After exposure, the nematodes were immediately pelleted by spinning the exposure tubes in a centrifuge for 1 minute, 400 x g (Beckman Coulter's Avanti J-15 centrifuge) at room temperature, followed by removal of the supernatants. Subsequently, pellets were transferred into 2 mL Safe-Lock micro test tubes and flash-frozen in liquid nitrogen for 1 minute before storing them at -80 °C until RNA extraction.

2.4. Microarray experiments

RNA template used in microarrays was isolated according to [8]. The mRNA expression profiles were measured using Agilent *C. elegans* (V2) Gene Expression Microarray 4×44K slides. Microarray preparation, hybridization and scanning, and normalization and pre-processing of raw data were performed as described previously in [8]. The primary data were submitted to ArrayExpress (E-MTAB-11143). KEGG pathways, Gene Ontology (GO) and functional domains involving differentially expressed genes (DEGs) were analysed by DAVID software v6.8 [30]. A threshold False Discovery Rate (FDR) ≤ 0.05 was considered as significantly enriched in the functional annotation categories.

2.5. RT-qPCR assays

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

The synthesis of cDNA from RNA template, via reverse transcription (RT), was carried out using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme and following the manufacturer's guide with minor modifications as described in [8]. PCR primer design and PCR reactions were also performed as described in [8]. Primer sequences used for RT-PCR analysis are provided as supplementary information **(Suppl. Table S1)**. Raw data were analyzed in Bio-Rad CFX Manager™ Software v3.0 and normalized to *C. elegans* tubulin gamma chain (*tbg-1*) and 14-3-3-like protein (*par-5*) as housekeeping genes. The stability of expression levels of these genes was confirmed in our experimental conditions using GeNorm approach described in [31].

2.6. Data analysis and statistics

Microarray data was statistically analyzed as described [8]. Linear model analysis was used to assess differentially expressed genes (DEG) per exposure condition whereby a threshold of p-value < 0.0001 was considered as statistically significant. Custom written scripts for the microarray analysis are available at Nematology_published_papers / Karengera_2021_Indirect_acting_xenobiotics · GitLab (wur.nl). RT-qPCR data obtained from concentration-response curves were used to calculate the "Lowest Observed Transcriptional Effect Level" (LOTEL) per gene target tested. LOTEL was considered as the lowest tested concentration that gave a statistically significant expression change for that gene transcript (p-value < 0.05). RT-qPCR data obtained with mixtures were analyzed by assuming additivity, so that the combined transcriptional effect on a particular gene equals to the sum of individual effects expected from each compound in the mixture. Experimentally obtained gene expression results (referred to as "actual effect") were then compared with its counterpart transcription level theoretically calculated by adding up the expected effect from individual compounds (referred to as "predicted effect") in that mixture. Pearson correlations were calculated between actual and predicted expressions for each compound mixture. Correlations were considered significant at p-value < 0.05. We then analysed the difference between predicted and measured values to determine additive or inhibitory effects on gene expressions. To validate microarray results, correlation between array and RT-qPCR data (presented as $\log_2$ fold changes) was determined per treatment condition using "*cor* function" in excel for computing the Pearson correlation coefficient "*R*".

3. Results

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

Since non-lethal concentrations were chosen, the nematodes treated with toxicants did not show lethality at all tested concentrations (i.e., microarray or RT-qPCR data), as confirmed by visual observation through a stereomicroscope. We analyzed global gene transcription profiles of *C. elegans* exposed to 30 µM of AFB1, B(a)P, PCB1254, and 10 µM TCDD. Compared to the untreated nematodes, 182, 86, and 321 genes were significantly up- or downregulated in the nematodes treated with AFB1, B(a)P, and PCB1254, respectively. Of

these genes, those with upregulated transcripts were remarkably predominant, as they accounted for around 87% of the total DEGs for AFB1 (159 genes) or PCB1254 (279 genes). For B(a)P treatment, all 86 DEGs were upregulated. Overlap among treatments by AFB1, B(a)P, and PCB1254 was found for 20 genes only, thus regulation of most genes was treatment-specific, especially for AFB1 and PCB1254 **(Fig. 1A)**. Interestingly, 10 µM TCDD had 1 DEG, only F59B1.8 was 2.5 fold upregulated, and this gene expressed also in the nematodes treated with 30 µM AFB1 (1.3-fold upregulation) or 30 µM PCB1254 (2.5-fold upregulation). F59B1.8 is thought to be an innate immune regulator.

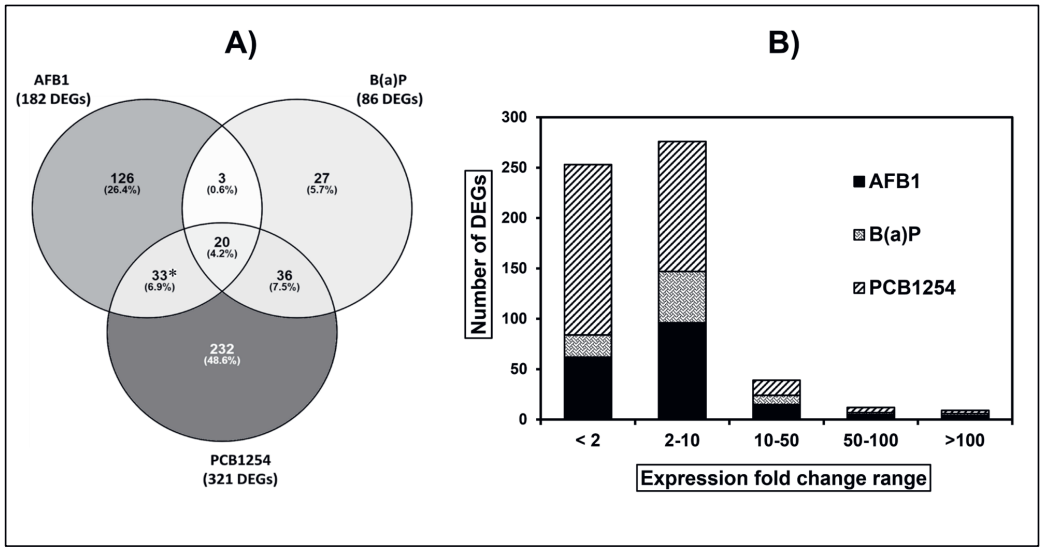

Figure 1. Overlapping of differentially expressed genes (DEGs). The Venn diagram (A) shows the number of significantly regulated genes by 30 µM of AFB1, B(a)P, and PCB1254, and their overlaps. Asterix (*) in figure (A) symbolizes the only one gene (F59B1.8) affected by 10 µM TCDD and whose expression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

Gene transcripts with more than 10-fold change in expression (microarray data) represented about 13%, 15%, and 7% of the total genes regulated by 30 µM of AFB, B(a)P, and PCB1254, respectively **(Fig. 1B)**. The expression levels of some of these genes were dramatically increased by more than 100-fold upregulation by AFB1 (*cyp-14A4, cdr-1,* F13H6.3*,* and B0205.14), B(a)P (*cyp-35C1* and *cyp-35D1*), or PCB1254 (*dhs-23*, R09E12.9,

and F25D1.5). Most genes were mainly regulated in the range of 2- to 10-fold changes including 53%, 59%, or 40% of all DEG in AFB1, B(a)P and PCB1254, respectively. Genes regulated less than 2-fold change were found for 34%, 26%, and 53% of the DEGs in AFB1, B(a)P and PCB1254, respectively.

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

Functional analysis revealed that the main part of upregulated genes (microarray data) was involved in metabolism and detoxification mechanisms of the nematode. The top affected mechanisms for AFB1, B(a)P and PCB1254 were found in the molecular function category as catalytic and oxidoreductase activities as assessed by Gene Ontology (GO) analysis **(Fig. 2 and Suppl. Table S3 – S5)**. Further analysis in biological process (BP) category showed that metabolic process counted alone about 51%, 60%, and 40% of all upregulated genes by AFB1, B(a)P, and PCB1254, respectively **(Fig. 2 and Suppl. Table S3 – S5)**. These included genes encoding nuclear hormone receptors (NHRs), phase I metabolic enzymes (cytochrome P450s), and phase II conjugation enzymes such as glutathione-S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) **(Table 1)**.

Among cytochrome P450 genes, *cyp-14A4* and *cyp-35D1* were ranked in the top induced DEGs and were specifically upregulated by AFB1 (888 fold) and B(a)P (113 fold), respectively. Likewise, the transcription of *cyp-35A3* and *cyp-13A6* were the highest upregulated cytochromes by PCB1254 (71 and 57 expression fold, respectively), but their expression was not compound specific as they were also affected by AFB1 (*cyp-13A6)* and B(a)P (*cyp-35A3*). Furthermore, the induction of ATP-binding cassette (ABC) genes (*mrp-3, pgp-1, pgp-8, and pgp-9*), which are essential in xenobiotic detoxification, was found in the nematodes treated with AFB1.

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

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

the downregulated transcripts, gene set enrichment analysis did not find any significantly affected cellular mechanism

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

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

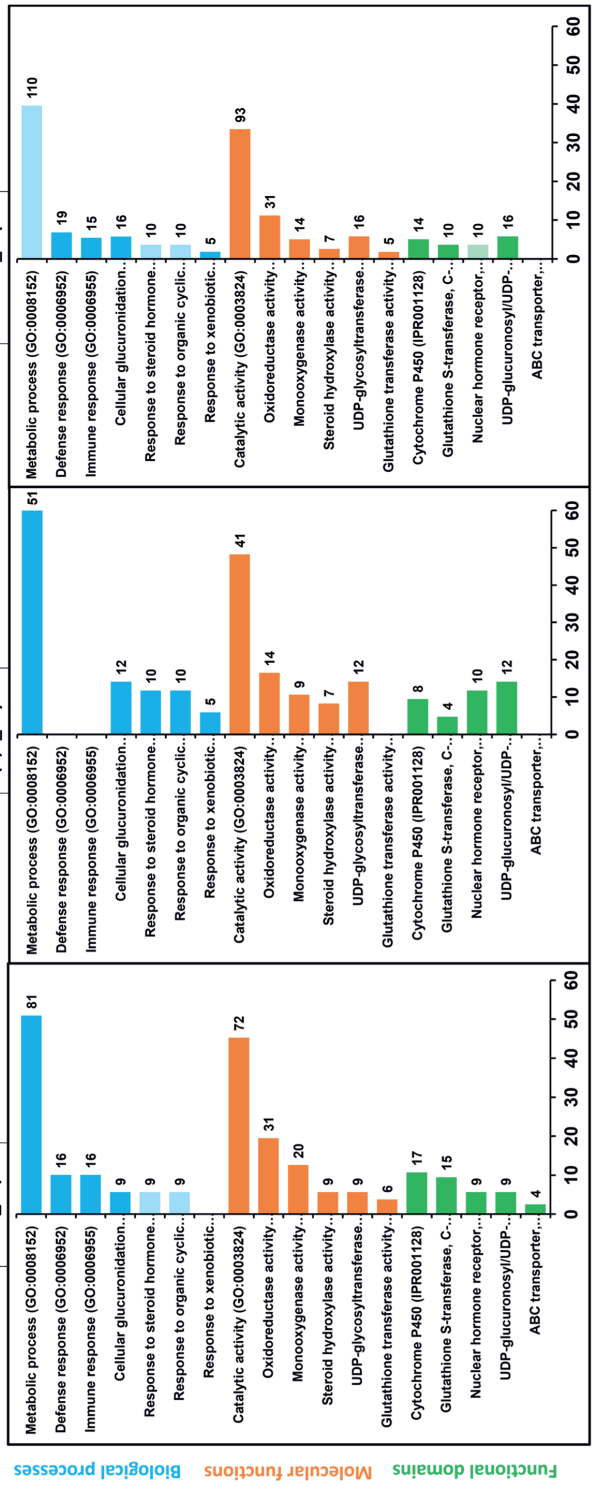

Figure 2. Gene Ontology (GO) and domain enrichment analysis terms. Plotted are gene ontologies (in biological process and molecular function categories) and functional domains associated with upregulated genes following 24 hours exposure to 30µM AFB1, B(a)P, and PCB1254. The X-axis denotes percent of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

3.3. Validation of microarray data by RT-qPCR.

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

3.4. Concentration-dependent differential gene expression

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

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

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

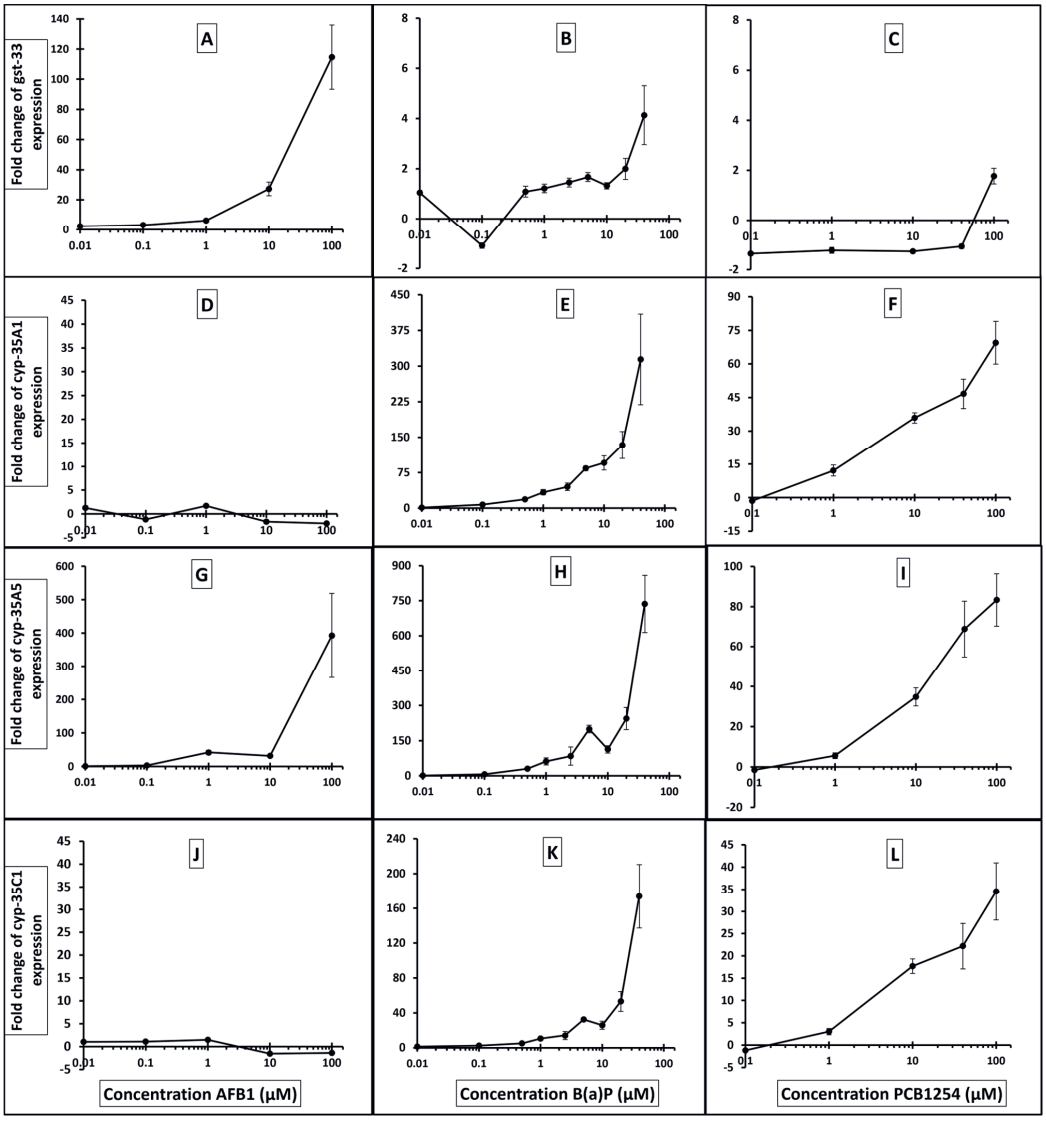

Figure 3. Concentration-response curves of differential gene expression in *C. elegans*. L4 juveniles were treated with toxicants ranging from 0.01µM to 100µM AFB1, from 0.01µM to 40µM B(a)P or from 0.1µM to 100µM PCB1254 for 24 hours. Concentration-dependent relative mRNA expression changes of gst-33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

3.5. Transcriptional effects of compounds in mixtures

We measured also joint transcriptional effects of AFB1, B(a)P, and PCB1254 tested in mixtures at the concentration of 0.1 µM, 1 µM, or 10 µM for each component. The RT-qPCR assay was used to assess the mRNA expression levels of *gst-33, cyp-14A3, cyp-35A1, cyp-35A3, cyp-35A5*, and *cyp-35C1*. Overall, significant positive correlations were found between actual and predicted expressions for compound mixtures, especially at 1 µM and 10 µM, as shown by positive correlation coefficients **(Fig. 4)**.

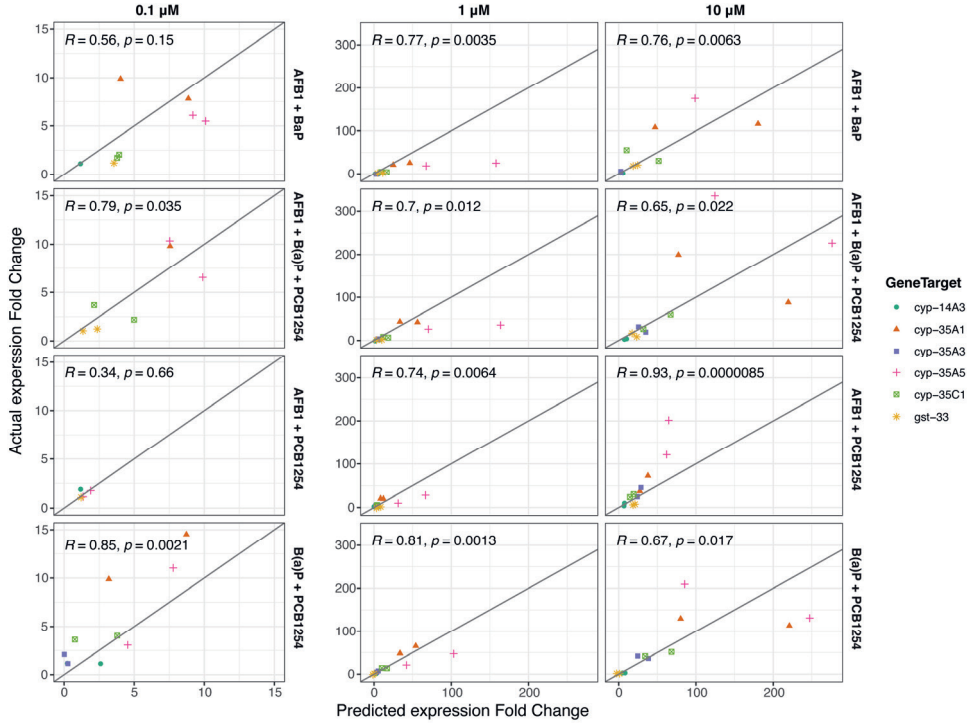

Figure 4. Comparison between actual and predicted joint transcriptional effects of AFB1, B(a)P, and PCB1254 in mixtures. 24-hour exposure was started in *C. elegans* L4 larvae with combined toxicants (AFB1, B(a)P, or PCB1254) at the concentration of 0.1µM, 1µM, or 10µM per each mixture component. The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

Nevertheless, some mixtures triggered either increase or reduction in the actual expression levels of the target genes compared to the predicted effects assuming additivity **(Suppl. Fig. S2)**, especially in the mixture containing AFB1. For instance, *gst-33* in the nematodes treated with 10-µM based mixtures was predicted to be upregulated by 22 fold (for AFB1 + B(a)P), 28 fold (for AFB1 + PCB1254), or 20 fold (for AFB1 + B(a)P + PCB1254). Instead, the actual *gst-33* expression was 20-fold, 7-fold, and 13-fold upregulation in respective aforementioned mixtures. Noteworthy, B(a)P and PCB1254 (individually or in mixture) did not have significant effect on *gst-33* expression. Based on single-compound exposure, only AFB1 induced *gst-33* expression with 29-fold upregulation in the 10 µM concentration.

4. Discussion

In this study, we determined transcriptional effects of indirect-acting model toxicants and TCDD in *C. elegans*. Several differentially expressed genes, especially those encoding biotransformation enzymes, were detected by microarrays for 30 µM AFB1, B(a)P, and PCB1254. For these three compounds, we also identified many genes whose expression is regulated by nuclear hormone receptor (NHR) transcription factors. Consistent with literature [20], our findings using microarray showed that, even at the very high exposure concentration 10 µM used, *C. elegans* is insensitive to the transcriptional effects of TCDD whose mode of action is AhR-dependent.

Compounds tested in this study are known to be metabolically activated by mammalian cytochrome P450 (CYP) enzymes. AFB1 is mainly metabolized by human CYP1A2 and CYP3A4 [21], B(a)P by CYP1A1 and CYP1B1 in mice [22], and PCB1254 by CYB2B, 2C, and 3A subfamilies in humans or rodents [23, 24]. TCDD is metabolized in rats as well as in humans by CYP1A1, but it is very persistent [25, 32]. The genes encoding phase I enzymes in *C. elegans* have been found to be closely related to the mammalian CYP2, 3, and 4 families [13], whereas CYP1-like metabolism is absent in the nematode [15]. In agreement with this literature, our findings revealed 17 CYP genes regulated by AFB1 that are related to the mammalian CYP2 (*C. elegans cyp-14A, 33C, 33E, 34A, 35A* subfamilies) and CYP3 (*C. elegans cyp-13A* subfamily and *25A2* gene) **(Suppl. Table S2)**. Among the eight CYP genes affected by B(a)P, seven are *C. elegans CYP35* family members *(cyp-35A, 35B, 35C, 35D* subfamilies) and *cyp-29A3*, which are related to the mammalian CYP2 and CYP4, respectively **(Suppl. Table S2)**. For the nematodes treated with PCB1254, we found 17 CYP genes related to the mammalian CYP2 (*C. elegans* cyp-*14A, 33B, 33C, 34A, 35A, 35C* subfamilies), and CYP3 (*C. elegans cyp-13A* subfamily) **(Suppl. Table S2)**.

Some human orthologues to *C. elegans* CYP genes, including those found in our study, have been previously reported [33]. The human *CYP4V2*, whose transcript is inducible by B(a)P in HepG2 human hepatocytes [34], is an orthologue to the nematode *cyp-29A3* that in our study was upregulated (~5-fold increase) by only B(a)P. Our data also revealed that the transcripts of both *cyp-35A3* and *cyp-35A4* were increased by B(a)P and PCB1254.

Previously, regulation of these genes was found to be restricted to the typical inducers of mammalian CYP1A such as β-naphthoflavone, PCB52, lansoprazole, and fluoranthene [12]. In human cell lines, cytochromes of CYP1A subfamily (*CYP1A1* and *CYP1A2*) are strongly inducible by B(a)P or PCB1254 [34, 35]. We also found that *C. elegans cyp-35D1*, previously reported to not be regulated by the inducers of mammalian CYP1 (like B(a)P or others) [12], was unexpectedly strongly upregulated in B(a)P exposure (~113-fold). Other nematode CYP35 regulated in our study by B(a)P or PCB1254 (e.g., *cyp-35A1, 35A5, 35B1,* and *35C1*) are reported to be orthologues to human *CYP2C18, CYP2D7,* and *CYP2E1* [33]. These human P450 proteins are not transcriptionally induced by B(a)P [34] or PCB1254 [24]. Further, our study also identified the regulation of the nematode CYP13A subfamily (*cyp-13A1, A7, A8,* and *A10*) by AFB1 or PCB1254. These genes are reported to be the orthologues to the mammalian *CYP3A4* and *CYP3A5* [33]. In comparison with the literature, *CYP3A4 can* indeed be upregulated by AFB1 in HepG2 cell line [36], while *CYP3A5* is upregulated by PCB1254 in Caco-2 cells but not in HepG2 cell line [35]. Overall, our study showed that *C. elegans* biotransformation of xenobiotics is indeed transcriptionally inducible by the studied compounds (except TCDD) via phase I metabolism comparable to mammalians.

We also found many differential expressed genes linked to the phase II metabolism of xenobiotics. These included genes encoding glutathione-S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs), which are involved in *C. elegans* resistance against oxidative stress [9, 37]. Genes encoding P-glycoproteins (*pgp-8 and pgp-9*), multidrug resistance protein (*pgp-1*), and one hypothetical protein (*mrp-3)* were upregulated in the nematodes treated with AFB1. These four genes encode ATP-binding cassette (ABC) transporters that are involved in xenobiotic detoxification by facilitating the transport of toxicants across cell membranes [38, 39] resulting in excretion. *C. elegans pgp-1* is homolog to the mammalian drug transporters such as *MDR1* and *MDR3* in humans or *Mdr1a* and *Mdr1b* in rodents [40]. Rat *Mdr1b* is transcriptionally inducible by genotoxic carcinogens including AFB1 [41]. In *C. elegans*, *pgp-1* is involved in the detoxification of heavy metals like cadmium (Cd) and arsenic (As) [42]. Compared to the Cd-regulated genes in *C. elegans* [43], many transcripts were similarly expressed in our study, including 40 and 27 genes in the nematodes treated with AFB1 and PCB1254, respectively. For AFB1-treated nematodes, the overlaps with Cd-induced genes included all four ABC transporter genes mentioned above together with the top three most expressed genes (*cyp-14A4, cdr-1,* and *cest-33*), eight cytochrome P450 genes, and five UGT genes. This suggests similar mechanisms of *C. elegans* detoxifying Cd and AFB1. The well-known cadmium-responsive gene *cdr-1* [44] was also regulated by PCB1254 and B(a)P.

NHRs are ligand-activated transcription factors that regulate several vital functions in *C. elegans* [45]. There are 284 NHRs in *C. elegans* but only few of them has been well characterized [46]. In this study, we found differential expression of many genes which are

regulated by nuclear hormone receptor (NHR) transcription factors NHR-8, NHR-86, and NHR-114. Receptor NHR-8, a homolog of mammalian liver X and vitamin D receptors, regulates *C. elegans* development, reproduction, and aging by controlling cholesterol and bile acid homeostasis [47]. NHR-114 is required for nematode fertility and germline stem cell maintenance [48], whereas NHR-86 regulates anti-pathogen responses [46]. These results suggest that the tested compounds can provoke the same responses maybe by acting as ligands to the above receptors.

The aryl hydrocarbon receptor (AhR) is another ligand-activated transcription factor which mediates biological and toxicological activity of many chemicals in mammalians including dioxins and related compounds [16, 17]. The AhR homolog (AHR-1) in *C. elegans* is encoded by the *ahr-1* gene, but the spectrum of its ligands (if there are any) is allegedly different from that of the mammalian AhR [20]. Indeed, to our knowledge, no exogenous ligand has ever been shown to directly bind and induce *C. elegans* AHR-1. AHR-1 possibly is sensitive to endogenous ligands [49, 50], and has been shown to regulate in *C. elegans* important physiological processes such as neuronal development [18], locomotion, egg laying, defecation behaviors, and fatty acid synthesis [19]. Our findings showed that only one gene (F59B1.8), involved in the nematode innate immune response [51], was regulated by TCDD. This seems to be in line with the literature that *C. elegans* AHR-1 does not bind TCDD [20], hence is transcriptionally insensitive. Nevertheless, a previous study showed that TCDD does delay the early larval development in *C. elegans* as shown by significant developmental delays for L3 larvae to reach L4 stage of larval growth, even in larvae that only were maternally exposed to levels as low as 10 nM of TCDD [Bao et al. in preparation]. These effects could be explained by baseline toxicity (known as narcosis), a characteristic of many organic xenobiotics, which typically induces non-specific disruption of the integrity and functioning of cell membranes [52].

Our study also identified gene transcripts that can be linked to the toxicological effects of AFB1, B(a)P, and PCB1254. Among the affected genes, we found those regulated by transcription factors ELT-2, MDT-15, SKN-1, or DAF-16 in *C. elegans*. ELT-2 is presumably homolog to human GATA6 and regulates genes involved in the nematodes innate immune responses [51]. MDT-15 dependent genes are linked to *C. elegans* oxidative stress resistance and cyto-protection [53]. SKN-1 is ortholog of mammalian Nrf proteins [54] and is a major regulator of the genes involved in oxidative stress response and longevity of *C. elegans* [55]. A FOXO-family transcription factor (DAF-16) and its downstream genes are linked to *C. elegans* aging and stress responses via insulin/insulin-like growth factor 1 (IGF-I) signaling [56, 57]. Furthermore, we found overlap between our data and the transcriptional profiles of other compounds in literature, like cadmium [43] and deoxynivalenol [58] known to be toxic to the *C. elegans* reproduction, development, and lifespan. Overall these findings suggest that the adverse effects expected from the tested toxicants were also represented by transcriptional profiles found in this study. Nonetheless, despite DNA-damaging

properties (especially AFB1 and B(a)P), no differential expression was found among the genes encoding DNA repair and cell cycle checkpoint proteins, which was consistent with the findings with direct-acting genotoxic model compounds [8].

In this study, we also analyzed concentration-dependent transcriptional effects of the toxicants and determined the Lowest Observed Transcriptional Effect Levels (LOTEL). This is a toxicological dose descriptor comparable to the Lowest Observed Adverse Effect Level (LOAEL) commonly used to relate the toxic effects of a chemical substance and the dose at which it takes place. Toxicogenomic studies in literature have previously advocated using threshold doses like LOTEL to evaluate toxicological profiles of chemicals [59, 60]. From concentration-response curves obtained in our study, the lowest concentration inducing transcriptional effects (LOTEL) among the tested gene targets were 0.01 µM for AFB1, 0.1 µM for B(a)P, and 1 µM for PCB1254. In comparison with literature, these LOTEL values were about 541-fold for AFB1, 2-fold for B(a)P, or 48-fold for PCB1254 smaller than the median effective concentrations (EC_{50}) for toxic effects on *C. elegans* reproduction. For 72-hour exposure, EC_{50} that caused reproductive toxicity is equivalent to 5.41 µM for AFB1 [61], 0.23 µM for B(a)P [62], and 47.82 µM for PCB52 [11]. These results suggest that transcriptional effects in *C. elegans* are occurring at a concentration much lower than developmental effects, as is also the case in vertebrates like zebrafish embryotoxicity test (ZET) [63].

Furthermore, we assessed the joint transcriptional effects of the toxicants in mixtures by comparing the actual and predicted changes in gene expression. The findings suggest possible interactions between compounds in mixtures, as shown by increase or reduction in the actual measured expression levels compared to the predicted expression of the target genes. The observed potential interactions were more apparent for the mixtures containing AFB1. According to microarray results from this study, AFB1 regulated *cyp-35A5* only among the *C. elegans* CYP-35 family members known to be strongly inducible by many xenobiotics [12]. Despite this, AFB1 combined with either B(a)P or PCB1254 in mixtures seemed to influence the joint effects by either increasing or reducing the transcription levels of other CYP-35 genes (i.e., *cyp-35A1, cyp-35A3,* and *cyp-35C1)* tested in this study. Further research is needed to elaborate the possible mechanisms underlying such interactions between toxicants.

Overall, we identified transcriptional responses of *C. elegans* to toxic substances requiring metabolic bioactivation. Several genes involved in xenobiotic biotransformation were regulated by AFB1, B(a)P, and PCB1254, suggesting that these compounds are metabolized in the nematode via phase I and II, or detoxified via transmembrane export as found for AFB1. These findings extend the knowledge on transcriptional inducibility of the nematode biotransformation enzymes in response to indirect-acting compounds. Moreover, this research adds important details about *C. elegans* gene expression profiles in response

to prototypes of mycotoxins, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls contaminants. Importantly, this study revealed differential gene expressions which can be associated with toxicological activities of the tested compounds. We also found many candidate gene transcripts that can be used as transcriptional biomarkers for detecting the presence of these compounds. Whereas the mammalian aryl hydrocarbon receptor (AhR) mediates CYP1A1 induction and toxicological effects of dioxins and a multitude of dioxin-like compounds through ligand interaction, *C. elegans* did not respond to 10 μM TCDD in our study while already exposure to 10 nM was enough to induce developmental effects (Bao et al. in preparation). It is interesting to further assess the effects of TCDD at a broader range of concentrations as well as other dioxin-like compounds.

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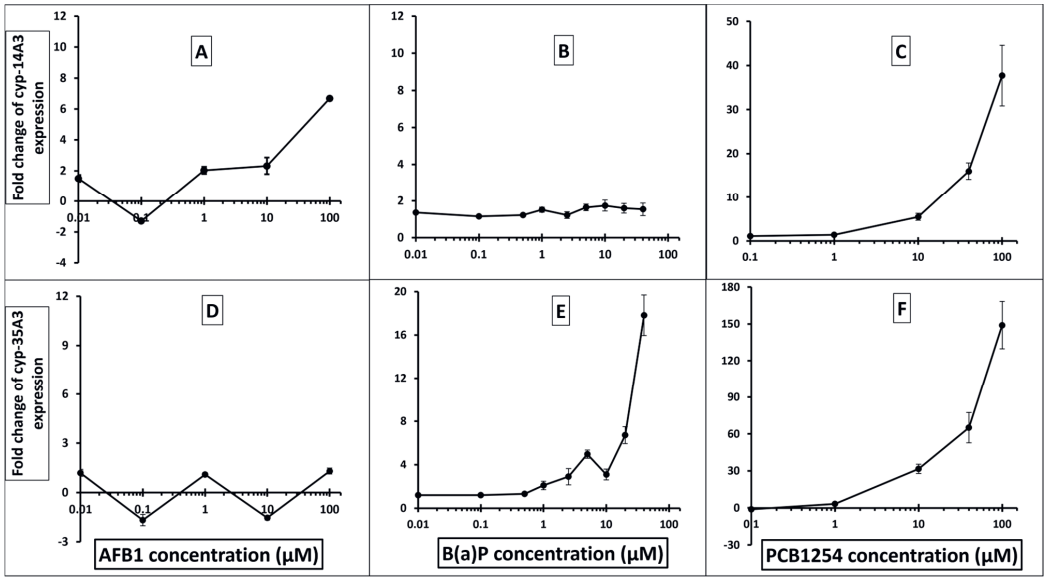
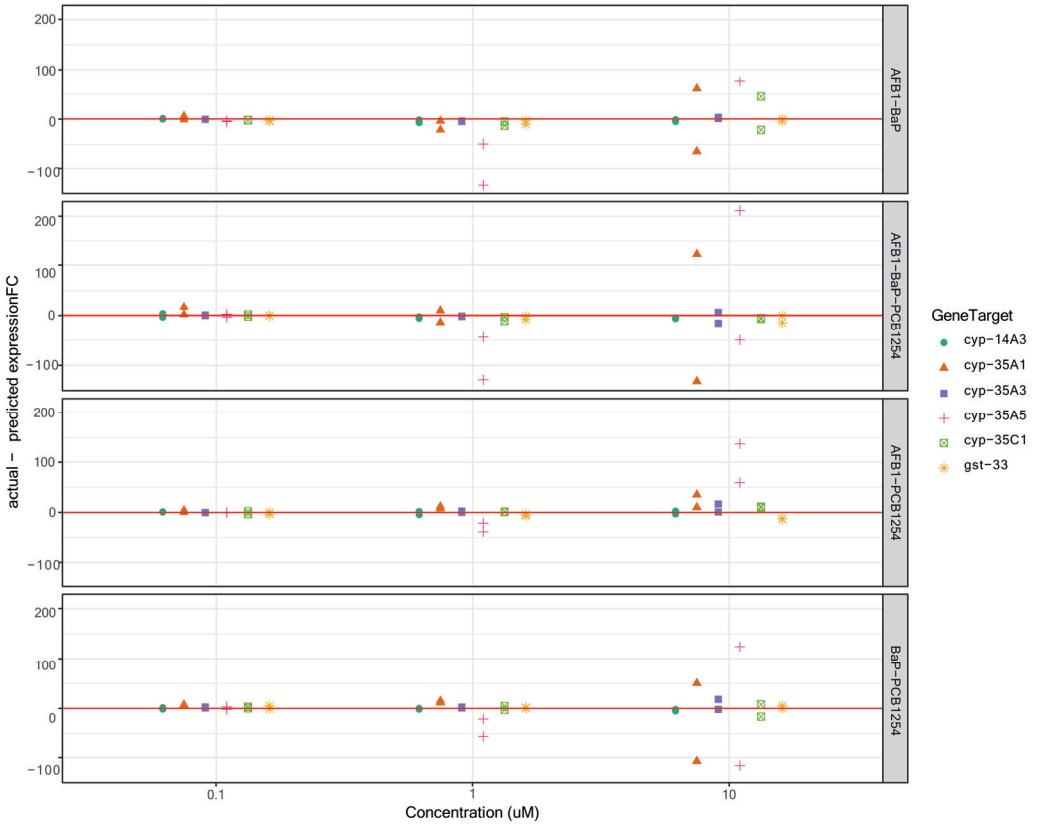
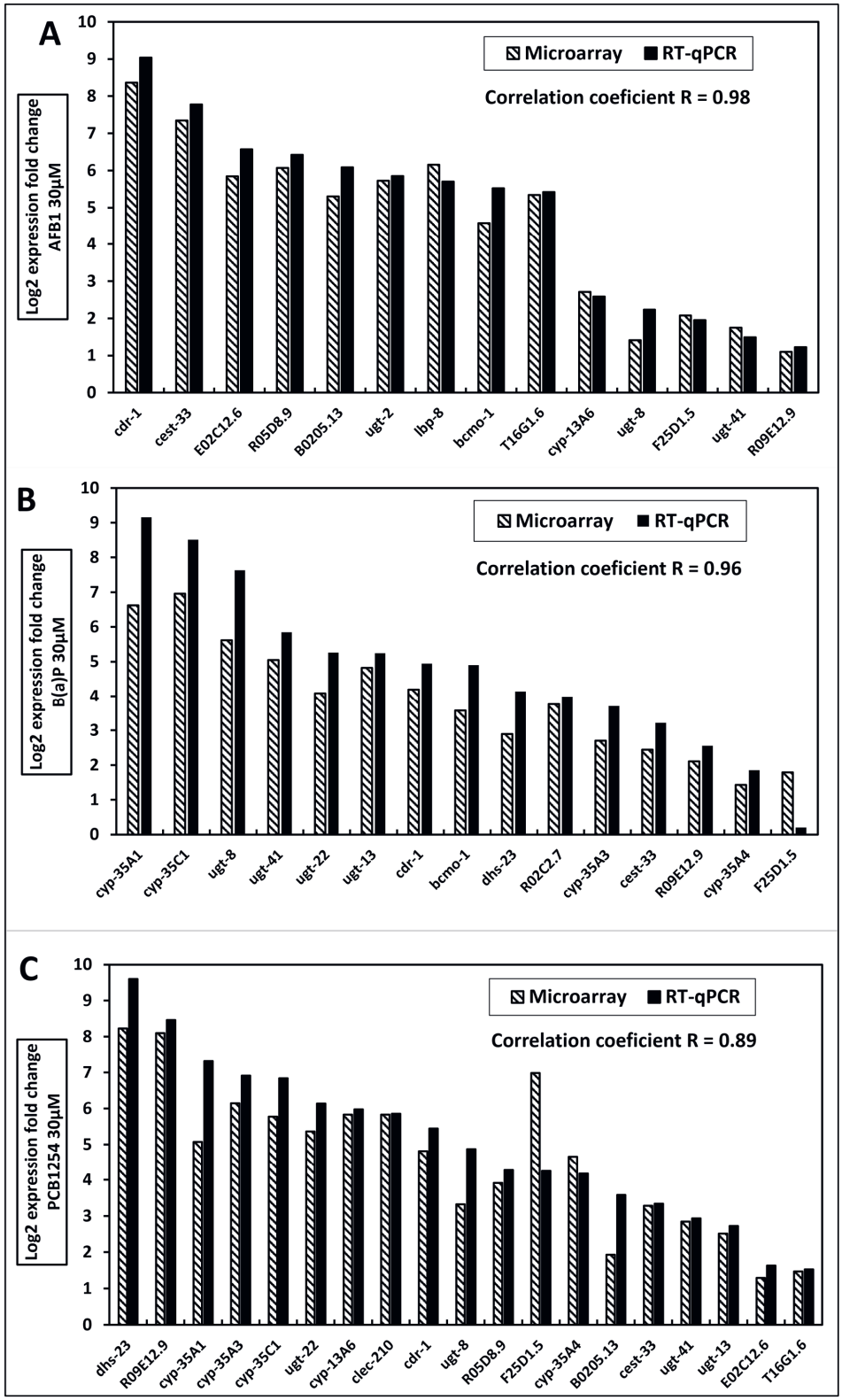
Supporting information of chapter 3



Suppl. Figure S1. Concentration-response curves of differential gene expression in *C. elegans*. L4 juveniles were treated with toxicants ranging from 0.01µM to 100µM AFB1, from 0.01µM to 40µM B(a)P or from 0.1µM to 100µM PCB1254 for 24 hours. Concentration-dependent relative mRNA expression changes of *cyp-14A3* (A, B, & C) and *cyp-35A3* (D, E, & F) genes were determined by reverse transcription polymerase chain reaction (RT-qPCR) and normalized to *C. elegans* tubulin gamma chain (*tbg-1*) and 14-3-3-like protein (*par-5*) genes. Data represent the mean fold changes ± standard error of the mean (SEM) in three independent biological replicates (n).



Suppl. Figure S2. Comparison between actual and predicted joint transcriptional effects of mixtures of AFB1, B(a)P, and PCB1254. A 24-hour exposure was started in *C. elegans* L4 larvae with combined toxicants (AFB1, B(a)P, or PCB1254) at the concentration of 0.1μM, 1μM, or 10μM per each component. The mRNA expression changes of cyp-14A3, cyp-35A1, cyp-35A3, cyp-35C1, and gst-33 were determined by reverse transcription polymerase chain reaction (RT-qPCR) and normalized to *C. elegans* tubulin gamma chain (*tbg-1*) and 14-3-3-like protein (*par-5*) genes. Data represent the differences between actual and predicted expression fold change (not log-transformed values). Two independent biological replicates were carried out.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RT-qPCR
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ugt-8
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Microarray
RT-qPCR
Correlation coeficient R = 0.96
cyp-35A1
cyp-35C1
ugt-8
ugt-41
ugt-22
ugt-13
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bcmo-1
dhs-23
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cyp-35A3
cest-33
R09E12.9
cyp-35A4
F25D1.5
C
Log2 expression fold change PCB1254 30µM
Microarray
RT-qPCR
Correlation coeficient R = 0.89
dhs-23
R09E12.9
cyp-35A1
cyp-35A3
cyp-35C1
ugt-22
cyp-13A6
clec-210
cdr-1
ugt-8
R05D8.9
F25D1.5
cyp-35A4
B0205.13
cest-33
ugt-41
ugt-13
E02C12.6
T16G1.6

Suppl. Figure S3. Validation of gene expression microarray analysis by reverse transcription polymerase chain reaction (RT-qPCR). The mRNA expression changes of the top ranked regulated genes were determined by RT-qPCR and normalized to *C. elegans* tubulin gamma chain (*tbg-1*) and 14-3-3-like protein (*par-5*) genes. Three independent biological replicates were run using the RNA template from 30µM-treated microarray samples. For these genes, log2 expression fold changes measured by microarray were compared to the log2 expression fold changes measured by RT-qPCR. The correlation coefficient R was calculated for each treatment, resulting in R = 0.98, R = 0.96, and 0.89 for AFB1, B(a)P, and PCB1254, respectively.

Suppl. Table S1. List of primer sequences used for RT-PCR analysis.

Gene symbol	NCBI_accession No	Forward primer (5'->3')	Reverse primer (5'->3')
B0205.13	NM_001047165.5	tggtgttggctattggattgg	aaaatggaccagcaccttctt
bcmo-1	NM_064328.3	gtcccagaacccaaagagtg	gagctgagtagaacatctttcca
cdr-1	NM_074585.9	tttaaaattccgtcgcttccaac	tcaaaaggggcatgagaagga
cest-33	NM_072220.5	ccgttcaaggctgctgttc	ttctcccatatctcggcgg
clec-210	NM_071454.6	cctgtgccgattgatgctc	agctttgaggtcagtgttgc
cyp-13A6	NM_063712.4	ttaggaacgacagcaaacacg	tgctccaaatgctcacaagc
cyp-35A1	NM_001356694	tgtccacgcttgatctgttc	ccaaaacacccatttcttgtct
cyp-35A3	NM_071720	gctgcgtgtttaagttggct	gttgccgtatttctttctgagc
cyp-35A4	NM_071723	cgaaaacccgctgaagtttg	caagctcggtggttgatagt
cyp-35A5	NM_071691	gaggcattgaaaagatatgacga	tttcttgaccagtcagccac
cyp-35C1	NM_171550	ttgcttcccaattgagtgct	tccaatgattaaatacagctcggc
dhs-23	NM_074419.5	gttgttgccgatgtttgcga	agcaccagcattattgaccaagat
E02C12.6	NM_073025.3	caggaacgtcgtgaaagagg	gtcttgaagctcttggaacgta
F25D1.5	NM_073303.5	gtgccaatttagctgacgga	cagagtgtgcttgtgggc
lbp-8	NM_074043.5	gatggtgacacttggcatttca	aaccaaagtgttgtaggatcgc
R02C2.7	NM_001028893.3	gtcctatactgctggagatgc	aaaacgaggccacagtatcc
R05D8.9	NM_071352.2	tcaattcaccaggtgtgcag	aaacttcatcatctcctcaaacg
R09E12.9	NM_001038410.4	gaagcaaagccatcaccgc	tgtcgccttccttcttccag
T16G1.6	NM_073833.6	gcatccagttctaaaggcagt	tcgtcgttgaacatcgtacc
ugt-13	NM_071916.7	cagcctgttttgagatcgct	tgagcgagatagagccctg
ugt-2	NM_073271.3	atttttgctcgctttacctcg	ggctctccaattgcatgtga
ugt-22	NM_070232.4	tccctgctccaacatttgac	cattttgcttaaaatgctctggc
ugt-41	NM_072417.5	tttccaagagcgactgacaa	cgatggtattctgactcgacc
ugt-8	NM_071914.5	ctgctacccaacttctcaagg	tccgagactagatggctgtc
par-5	NM_069834.6	atacgatgatatggctgccg	ggtattccttggcgagttgt
tbg-1	NM_066730.9	attctcttgtcgcccgaatc	acaacaggagagatagcgga

Suppl. Table S2. A selection of pathways conserved between *C. elegans* and mammalians which were differentially expressed in response to 30 µM of AFB1, B(a)P, or PCB1254.

Pathway	**Regulated mRNA expression in *C. elegans***			**Mammalian homolog or ortholog**	**Physiological functions in *C. elegans***	**Reference**
	30 µM AFB1	**30 µM B(a)P**	**30 µM PCB1254**			
P450-mediated metabolism	-	-	-	CYP1 family	Phase I metabolism	[13], [33] [12], [15]
	cyp-14A4, cyp-14A1, cyp-33C1, cyp-33C2, cyp-33C5, cyp-33C4, cyp-33C7, cyp-33E2, cyp-34A9, cyp-34A5, cyp-35A5	*cyp-35A1, cyp-35A3, cyp-35A4, cyp-35A5, cyp-35B1, cyp-35C1, cyp-35D1*	*cyp-14A2, cyp-14A3, cyp-34A9, cyp-33B1, cyp-33C1, cyp-35A1, cyp-35A3, cyp-35A4, cyp-35A5, cyp-35C1,*	CYP2 family		
	cyp-13A6, cyp-13A7, cyp-13A10, cyp-13A3, cyp-13A1, cyp-25A2	-	*cyp-13A6, cyp-34A10, cyp-13A9, cyp-13A8, cyp-13A10, cyp-13A7, cyp-13A1,*	CYP3 family		
	-	*cyp-29A3*	-	CYP4 family		
	pgp-1	-	-	*MDR1, MDR3*	Phase III metabolism	[42]
Nuclear Receptor	-	*nhr-8**	-	*LXR*	Development, reproduction, aging	[47]
	-	-	-	*AHR*	Neuronal development, locomotion, egg laying, defecation behaviors, fatty acid synthesis	[19] [20]

(-) No gene involved in the corresponding pathway was found in the nematode exposed to the toxicant

(*) The transcript of *nhr-8* was not differentially induced by AFB1 or PCB1254, but several downstream genes of the nematode NHR-8 were regulated in the nematodes treated with AFB1 or PCB1254. This was also observed for *nhr-86* and *nhr-114* (not included in the table as they were not differentially regulated in exposure (i.e., AFB1, B(a)P, or PCB1254) but the transcription levels of many of their downstream genes were differentially affected.

Suppl. Table S3. KEGG pathway enrichment for upregulated genes analyzed in DAVID software (False Discovery Rate, FDR ≤ 0.05).

	# genes	%	FDR
30uM AFB1 treatment			
cel00980:Metabolism of xenobiotics by cytochrome P450	9	5.7	2.51E-10
cel00982:Drug metabolism - cytochrome P450	8	5.0	2.09E-08
cel00480:Glutathione metabolism	7	4.4	1.25E-06
cel00983:Drug metabolism - other enzymes	4	2.5	4.18E-03
KEGG pathways under 30uM B(a)P treatment			
cel00980:Metabolism of xenobiotics by cytochrome P450	3	3.5	2.51E-02
cel00982:Drug metabolism - cytochrome P450	3	3.5	2.51E-02
KEGG pathways under 30uM PCB1254 treatment			
cel00980:Metabolism of xenobiotics by cytochrome P450	7	2.5	6.49E-06
cel00982:Drug metabolism - cytochrome P450	6	2.2	1.88E-04

Suppl. Table S4. Gene Ontology (GO) enrichment analysis in biological processes (BP) category for upregulated genes analyzed in DAVID software (False Discovery Rate, FDR ≤ 0.05).

	# genes	%	FDR
30uM AFB1 treatment			
GO:0055114 oxidation-reduction process	31	19.5	2.52E-11
GO:0044710 single-organism metabolic process	45	28.3	2.46E-08
GO:0009813 flavonoid biosynthetic process	9	5.7	3.36E-05
GO:0052695 cellular glucuronidation	9	5.7	3.36E-05
GO:0019585 glucuronate metabolic process	9	5.7	3.36E-05
GO:0052696 flavonoid glucuronidation	9	5.7	3.36E-05
GO:0006063 uronic acid metabolic process	9	5.7	3.36E-05
GO:0009812 flavonoid metabolic process	9	5.7	3.36E-05
GO:0045087 innate immune response	16	10.1	4.29E-05
GO:0006955 immune response	16	10.1	5.25E-05
GO:0002376 immune system process	16	10.1	5.34E-05
GO:0008152 metabolic process	81	50.9	1.09E-03
GO:0032787 monocarboxylic acid metabolic process	11	6.9	1.99E-03
GO:0006952 defense response	16	10.1	2.60E-03
30uM B(a)P treatment			
GO:0052696 flavonoid glucuronidation	12	14.1	1.10E-11
GO:0006063 uronic acid metabolic process	12	14.1	1.10E-11
GO:0009812 flavonoid metabolic process	12	14.1	1.10E-11
GO:0009813 flavonoid biosynthetic process	12	14.1	1.10E-11
GO:0052695 cellular glucuronidation	12	14.1	1.10E-11
GO:0019585 glucuronate metabolic process	12	14.1	1.10E-11
GO:0044710 single-organism metabolic process	31	36.5	5.59E-08
GO:0032787 monocarboxylic acid metabolic process	13	15.3	9.91E-08
GO:0009410 response to xenobiotic stimulus	5	5.9	2.95E-06
GO:0019752 carboxylic acid metabolic process	13	15.3	3.30E-05
GO:0043436 oxoacid metabolic process	13	15.3	3.30E-05
GO:0044281 small molecule metabolic process	17	20.0	5.89E-05
GO:0006082 organic acid metabolic process	13	15.3	6.36E-05
GO:0042221 response to chemical	23	27.1	3.76E-04
GO:0055114 oxidation-reduction process	14	16.5	6.05E-04
GO:0048545 response to steroid hormone	10	11.8	6.05E-04
GO:0071396 cellular response to lipid	10	11.8	6.05E-04
GO:0043401 steroid hormone mediated signaling pathway	10	11.8	6.05E-04
GO:0071383 cellular response to steroid hormone stimulus	10	11.8	6.05E-04
GO:0033993 response to lipid	10	11.8	6.08E-04
GO:0009755 hormone-mediated signaling pathway	10	11.8	6.12E-04
GO:0008152 metabolic process	51	60.0	7.58E-04
GO:0071407 cellular response to organic cyclic compound	10	11.8	9.23E-04
GO:0032870 cellular response to hormone stimulus	10	11.8	1.01E-03
GO:0009725 response to hormone	10	11.8	1.01E-03
GO:0014070 response to organic cyclic compound	10	11.8	1.27E-03
GO:0070887 cellular response to chemical stimulus	12	14.1	1.55E-03
GO:0071495 cellular response to endogenous stimulus	10	11.8	2.22E-03
GO:0010033 response to organic substance	12	14.1	3.04E-03
GO:0009719 response to endogenous stimulus	10	11.8	3.14E-03
GO:0071310 cellular response to organic substance	11	12.9	3.84E-03
GO:0006790 sulfur compound metabolic process	6	7.1	5.41E-03
GO:1901576 organic substance biosynthetic process	25	29.4	7.88E-03
GO:0009058 biosynthetic process	25	29.4	1.13E-02
30uM PCB1254 treatment			
GO:0019585 glucuronate metabolic process	16	5.8	1.37E-11
GO:0052696 flavonoid glucuronidation	16	5.8	1.37E-11
GO:0052695 cellular glucuronidation	16	5.8	1.37E-11
GO:0006063 uronic acid metabolic process	16	5.8	1.37E-11
GO:0009812 flavonoid metabolic process	16	5.8	1.37E-11
GO:0009813 flavonoid biosynthetic process	16	5.8	1.37E-11
GO:0044710 single-organism metabolic process	65	23.4	1.37E-11
GO:0055114 oxidation-reduction process	31	11.2	4.90E-07
GO:0032787 monocarboxylic acid metabolic process	17	6.1	1.08E-05
GO:0009410 response to xenobiotic stimulus	5	1.8	2.86E-04
GO:0006082 organic acid metabolic process	19	6.8	2.41E-03
GO:0019752 carboxylic acid metabolic process	18	6.5	2.63E-03
GO:0043436 oxoacid metabolic process	18	6.5	2.63E-03
GO:0035966 response to topologically incorrect protein	9	3.2	9.76E-03
GO:0006950 response to stress	33	11.9	1.53E-02
GO:0006952 defense response	19	6.8	1.56E-02
GO:0045087 innate immune response	15	5.4	2.08E-02
GO:0035967 cellular response to topologically incorrect protein	8	2.9	2.23E-02
GO:0006955 immune response	15	5.4	2.28E-02
GO:0010033 response to organic substance	20	7.2	2.28E-02
GO:0002376 immune system process	15	5.4	2.34E-02
GO:0071310 cellular response to organic substance	18	6.5	2.85E-02

Suppl. Table S5. Gene Ontology (GO) enrichment analysis in functional domains category for upregulated genes analyzed in DAVID software (False Discovery Rate, FDR ≤ 0.05)

	# genes	%	FDR
30uM AFB1 treatment			
IPR017972: Cytochrome P450, conserved site	17	10.7	1.63E-16
IPR002401: Cytochrome P450, E-class, group I	17	10.7	1.63E-16
IPR001128: Cytochrome P450	17	10.7	2.18E-16
IPR010987: Glutathione S-transferase, C-terminal-like	15	9.4	4.60E-14
IPR015897: CHK kinase-like	11	6.9	4.77E-13
IPR012877: Uncharacterised kinase D1044.1	11	6.9	1.38E-12
IPR012336: Thioredoxin-like fold	16	10.1	2.59E-10
IPR004046: Glutathione S-transferase, C-terminal	11	6.9	4.62E-10
IPR004045: Glutathione S-transferase, N-terminal	11	6.9	6.12E-10
IPR002213: UDP-glucuronosyl/UDP-glucosyltransferase	9	5.7	4.56E-06
IPR020904: Short-chain dehydrogenase/reductase, conserved site	7	4.4	1.41E-05
IPR002347: Glucose/ribitol dehydrogenase	8	5.0	8.02E-05
IPR005442: Glutathione S-transferase, omega-class	3	1.9	7.61E-03
IPR016040: NAD(P)-binding domain	8	5.0	8.51E-03
IPR006582: MD domain	3	1.9	9.84E-03
IPR000536: Nuclear hormone receptor, ligand-binding, core	9	5.7	3.13E-02
IPR011009: Protein kinase-like domain	12	7.5	3.13E-02
IPR011527: ABC transporter, transmembrane domain, type 1	4	2.5	3.34E-02
IPR002018: Carboxylesterase, type B	4	2.5	4.48E-02
30uM B(a)P treatment			
IPR002213: UDP-glucuronosyl/UDP-glucosyltransferase	12	14.1	4.91E-12
IPR017972: Cytochrome P450, conserved site	8	9.4	1.54E-06
IPR002401: Cytochrome P450, E-class, group I	8	9.4	1.54E-06
IPR001128: Cytochrome P450	8	9.4	1.54E-06
IPR001628: Zinc finger, nuclear hormone receptor-type	10	11.8	8.65E-05
IPR013088: Zinc finger, NHR/GATA-type	10	11.8	9.33E-05
IPR000536: Nuclear hormone receptor, ligand-binding, core	10	11.8	9.33E-05
IPR010987: Glutathione S-transferase, C-terminal-like	4	4.7	4.46E-02
30uM PCB1254 treatment			
IPR002213: UDP-glucuronosyl/UDP-glucosyltransferase	16	5.8	1.35E-11
IPR017972: Cytochrome P450, conserved site	14	5.0	1.09E-09
IPR002401: Cytochrome P450, E-class, group I	14	5.0	1.09E-09
IPR001128: Cytochrome P450	14	5.0	1.40E-09
IPR010987: Glutathione S-transferase, C-terminal-like	10	3.6	1.91E-05
IPR002347: Glucose/ribitol dehydrogenase	10	3.6	5.05E-05
IPR012336: Thioredoxin-like fold	13	4.7	5.95E-05
IPR004046: Glutathione S-transferase, C-terminal	8	2.9	1.79E-04
IPR004045: Glutathione S-transferase, N-terminal	8	2.9	2.06E-04
IPR016040: NAD(P)-binding domain	12	4.3	5.20E-04
IPR020904: Short-chain dehydrogenase/reductase, conserved site	6	2.2	3.80E-03

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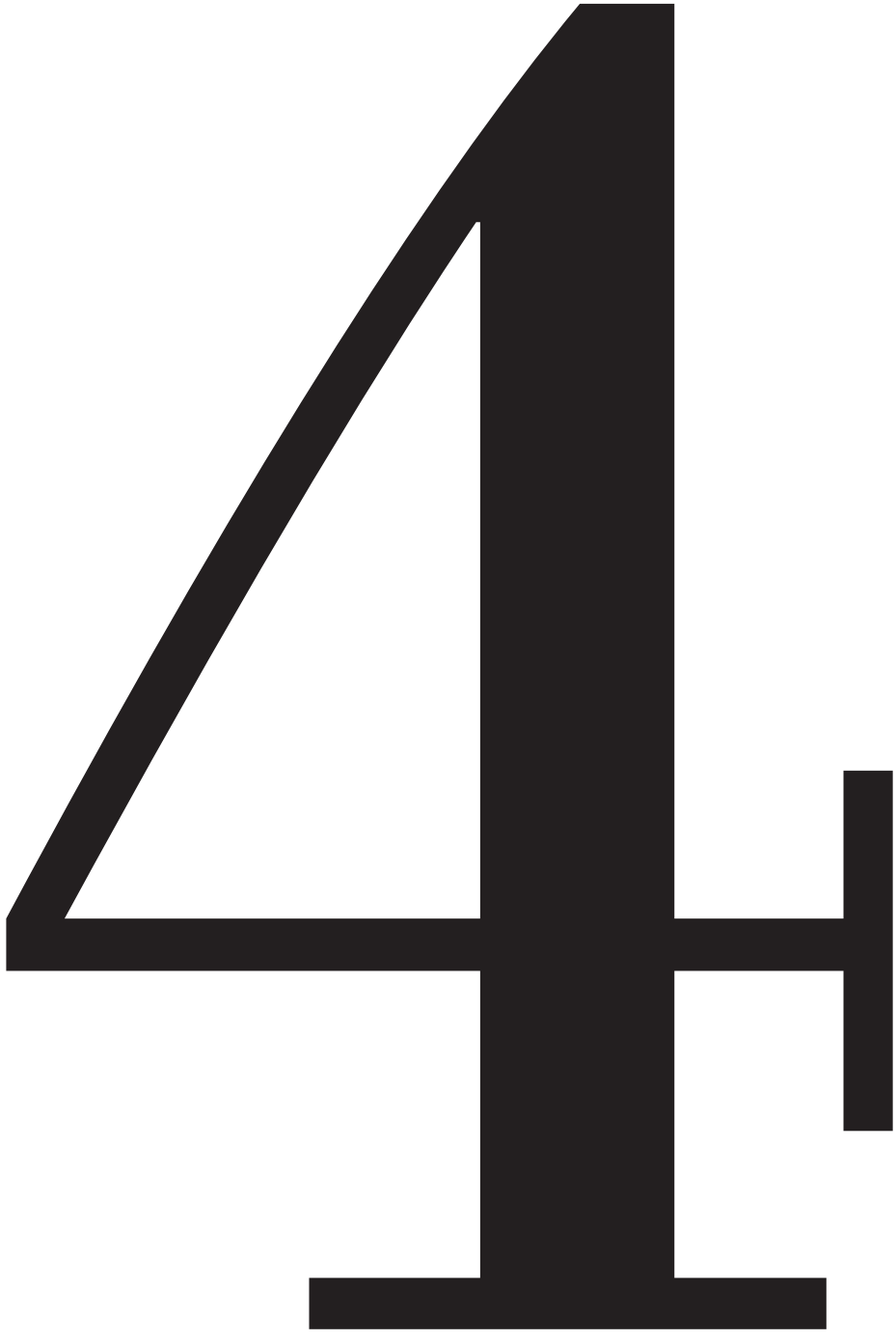

Chapter 4

Fingerprinting toxic potencies of hydrophilic contaminants in wastewater using gene expression profiling in *C. elegans* as a bioanalytical tool

Submitted for publication:
Antoine Karengera, Ilse Verburg, Mark G. Sterken, Joost A. G. Riksen, Albertinka J. Murk, Inez J. T. Dinkla. Fingerprinting toxic potencies of hydrophilic contaminants in wastewater using gene expression profiling in *C. elegans* as a bioanalytical tool.

Abstract

With chemical analysis it is impossible to qualify and quantify the toxic potency of especially hydrophilic bioactive contaminants. In this study, we applied the nematode *C. elegans* as a model organism for detecting the toxic potency of hydrophilic pollutants in wastewater samples. Gene expression in the nematode was used as bioanalytical tool to reveal the presence, type and potency of molecular pathways induced by 24-hour exposure to wastewater from a hospital (H), nursing home (N), community (C), and influent (I) and treated effluent (E) from a local wastewater treatment plant (WWTP). Exposure to influent water significantly altered expression of 464 genes, while only two genes were differentially expressed in nematodes treated with effluent. This indicates a significant decrease in bioactive pollutant-load after wastewater treatment. Surface water receiving the effluent did not induce any genes in exposed nematodes. A subset of 209 genes was differentially expressed in all untreated wastewaters, including cytochromes P450 and C-type lectins related to the nematode's xenobiotic metabolism and immune response, respectively. Different subsets of genes responded to particular waste streams making them candidates to fingerprint specific wastewater sources. This study shows that gene expression profiling in *C. elegans* can be used for mechanism-based identification of hydrophilic bioactive compounds and fingerprinting of specific wastewaters. More comprehensive than with chemical analysis, it can demonstrate the effective overall removal of bioactive compounds through wastewater treatment. This bioanalytical tool can also be applied in the process of identification of the bioactive compounds via a process of Toxicity Identification Evaluation (TIE).

1. Introduction

A multitude of chemical substances used for anthropogenic activities often end up in municipal wastewater [1, 2]. Both raw and treated effluents may contain a wide range of natural and synthetic chemicals [3]. These substances are usually present as complex mixtures whose composition is difficult to analyze by current chemical methods, among others, because they occur at levels below the limit of detection or no standards are available yet [4]. Substances like hydrophilic compounds are even more challenging for chemical analysis as they are hard to extract or concentrate [5]. Most of these pollutants, including their metabolites and reaction products, remain unknown and yet they may add to the total toxicological risk posed by the mixture [6].

Municipal wastewaters in the Netherlands are treated in wastewater treatment plants (WWTPs), which are generally designed to remove a range of contaminants like suspended solids, phosphorus, nitrogen, biodegradable organic matter, and others [7]. Unfortunately, conventional WWTPs do not completely remove all micropollutants in wastewater [5], and many chemicals originating from treated effluents can be found in receiving water bodies like groundwater or surface waters [8, 9]. Unfortunately, the available analytical methods cannot provide information about the potential toxic effects of these compounds and mixtures thereof [10]. Therefore, concerns remain, especially for hydrophilic compounds that may pose environmental health risks or contaminate drinking water sources [11].

Bioanalytical tools, also referred to as bioassays, can quantify the toxic potency of bioactive pollutants in water samples based on their combined effects [12, 13]. Bioassays can be in vitro, monitoring responses of cells in culture [14] or *in vivo*, utilizing a whole living system [15]. Most of the existing *in vitro* and in vivo bioassays are either very specific to one or few biological responses (e.g., endocrine-disrupting activity, aryl hydrocarbon receptor activity, oxidative stress response, and others) or are non-specific indicators of general toxic effects (e.g., mortality, fertility, reproduction, and others) [12, 15]. Hence, a battery of bioassays is often required for testing various types of bioactive pollutants present in water samples as demonstrated in [16].

The small nematode *Caenorhabditis elegans* has attracted attention as a model in toxicity testing. This nematode has shown its potential use as toxicological tool for water quality monitoring as shown in [17], where toxicity from pollution in rivers was assessed by measuring effects on *C. elegans* growth. Strengths and limitations for *C. elegans* used in predictive toxicology have been reviewed in [18], where good *C. elegans* culture practice (GCeCP) were proposed for reliable and repeatable data. We recently developed a gene expression-based toxicity bioassay using *C. elegans* as a test organism [19] and showed that the nematodes transcriptomic response can be used to detect the toxic potency of xenobiotics. Toxicity testing by gene expression profiling can provide insights in the type of bioactivity mechanism that is influenced and can be translated towards the nature of the risk

the substances present [20, 21]. Also, tests with single contaminants demonstrated that the magnitude of differential gene expression change that were observed can be related to the toxic potency (concentration) that the nematode is exposed to.

In the present study, we aim to evaluate the applicability of the *C. elegans* bioassay for qualification and quantification of the toxic potency of hydrophilic contaminants present in wastewater. More specifically the differential gene expression as biomarker for the toxic potency posed by contaminants in wastewater from specific sources was investigated. The samples analyzed in this study were: wastewater from hospital, nursing home, community, and WWTP influent and effluent. In addition surface water receiving treated effluent was analyzed. Prior to use in nematode exposure, all (waste)water sample were centrifuged and filtrated to remove suspended solids. This implies that, the water-soluble pollutants were the major composition of contaminants left in samples after filtration whereas the hydrophobic fraction was very low. Genome-wide gene expression profiling of *C. elegans* exposed to the filtrated wastewater samples revealed the potential of this transcription-based bioassay as a bioanalytical tool for monitoring the toxic potency of also hydrophilic compounds in wastewater without the need for extraction.

2. Material and methods

2.1. Wastewater sampling

Wastewater samples were obtained from the sampling campaign as described in [22]. Briefly, samples were collected from the city of Sneek, in the Netherlands. Wastewater samples from a community of 80 households (C), hospital (H, 300 beds), and nursing home (N, 220 beds) were taken from the receiving wells of which neither received other wastewaters nor rainwater. These wastewater streams each contributed less than 1% of the water inflow into a local municipal wastewater treatment plant (WWTP). The main WWTP influent (> 97%) originated from other sources including industrial water, households, stormwater runoff, and seepage from ground and surface waters. The WWTP influent (I) and effluent (E) samples were collected from this WWTP. The WWTP effluent is discharged into an adjacent canal, from which surface water samples were collected upstream (SW1) and downstream (SW2) of the effluent discharge point. In addition, a surface water sample (SW3) was collected from a non-receiving surface water located in a nature reserve, hardly affected by anthropogenic activities. Each sample of 2 liters was taken in high density polyethylene (HDPE) bottles (VWR, Amsterdam, The Netherlands) using an autosampler (except surface waters where grab samples were taken 1 meter from the shore at ~0.2 meter of depth). Time-proportional sampling (24-h samples) was used for C, H, and N, whereas time-proportional sampling (24-h samples) was used for I and E. All samples were transported in cooling boxes and subsequently stored at -20°C until use.

2.2. Exposure media

Prior to the use for exposure the suspended solid material was removed from water samples by centrifugation and filtration. Therefore, the water-soluble pollutants were the major composition of contaminants left in samples after filtration whereas the hydrophobic fraction should be expected to be very low. Each sample was aliquoted by transferring 10 mL to Falcon™ 15-mL conical centrifuge tubes followed by centrifugation at 3,750 rpm for 20 minutes (Avanti J-15 Centrifuge, Beckman Coulter). Next the supernatants were further filtrated using Syringe filters Millex® Hydrophilic PTFE (0.45 µm pore size). For all filtrates, pH values in a range of 8.5 - 9.8 were measured prior to the use for the nematodes exposure. *C. elegans* has been shown previously to be tolerant to such test conditions [23], thus no pH adjustment was made.

2.3. Nematode culture and exposure

Synchronized L4 stage larvae of *C. elegans* wild-type Bristol N2 strain were cultured and exposed in three biological replicates for 24 hours as described in [24]. Prior to microarray experiments we first made sure that the nematodes were alive after the exposure period exposure. The absence of mortality among the nematodes was confirmed by visual observation through a stereomicroscope. For each water sample, approximately 10,000 nematodes were used without feeding during the exposure period. After exposure, the nematode exposure tubes were spun for 1 minute at 1,000 rpm, 20 °C using a centrifuge (Avanti J-15 Centrifuge, Beckman Coulter). Subsequently, the nematode pellets were transferred into 2-mL microtubes (Eppendorf® Safe-Lock tubes, Biopur®) and flash-frozen in liquid nitrogen for 1 minute before storing them at -80 °C until the extraction of RNA.

2.4. RNA extraction

TRIzol® Reagent with the PureLink® RNA Mini Kit was used to extract total RNA as described in [24]. Briefly, TRIzol® Reagent was used to prepare nematode lysates from which crude RNA extracts were obtained using chloroform (Molecular Biology Reagent, Thermo Fisher GmbH). The RNA was subsequently isolated from the crude extracts following the manufacturer's protocol (Thermo Fisher MAN0000406) including column-based RNA isolation through binding, washing, and elution steps. A NanoDrop spectrophotometer was used to measure RNA quantity and quality (**Suppl. Table S1**), with an A260/A280 ratio of 1.8 to 2.0 as requirement for further use.

2.5. Microarray experiments

Microarray analysis was conducted as described before [19] including array preparation, hybridization, scanning, raw data normalization and pre-processing. Differential gene expression linked to the treatment was investigated by using a linear model, fitted per exposure (i.e., C, N, H, I, and E). The data obtained from SW1, SW2, and SW3 were not significantly different and were therefore used as control. The raw data of this experiment

are provided via ArrayExpress (E-MTAB-11260). To identify biological pathways and gene ontologies of differentially expressed genes (DEGs), we analysed KEGG pathways, Gene Ontology (GO) and functional domains by using DAVID software v6.8 [25]. A threshold False Discovery Rate (FDR) ≤ 0.05 was considered as significantly enriched in the annotation categories.

2.6. RT-qPCR assays

Gene expression of fifteen target genes selected from microarray data, was tested by using RT-qPCR. The cDNA was synthesized from RNA templates via reverse transcription (RT) by using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme as described in [24]. Two biological replicates were run using the same extracted RNA as used in the microarrays. Due to insufficient RNA material, the third biological replicate sample was run on microarray only and not confirmed by RT-qPCR. PCR primer design and PCR analysis were performed as described in [19]. Primer sequences used for RT-PCR analysis are provided as supplementary information (**Suppl. Table S2**). Raw data were analyzed in Bio-Rad CFX Manager™ Software v3.0, and normalized to *C. elegans* tubulin gamma chain (*tbg-1*) and 14-3-3-like protein (*par-5*) as housekeeping genes.

2.7. Data analysis and statistics

Microarray data were statistically analyzed as described by Karengera and colleagues [19]. Briefly, linear model analysis was used to assess differentially expressed genes (DEGs) per exposure condition whereby a threshold of p-value < 0.0001 was considered as statistically significant. Custom written scripts for the microarray analysis are provided at https://git.wur.nl/published_papers/karengera_2021_wastewater_fingerprinting. To analyse the variation in gene expression, principal component analysis (PCA) was applied on the $\log_2$ ratio with the mean expression values using the *prcomp* function in “R” (version 3.5.3, x64) in RStudio (version 1.1.463).

3. Results

3.1. Transcriptome response to wastewaters and treated effluent

The exposed and unexposed nematodes did not show lethality for all tested water samples, as confirmed by visual observation through a stereomicroscope. Whole-transcriptome analysis using microarrays revealed a clear difference between the gene expression patterns induced by wastewater samples before and after wastewater treatment **(Fig. 1).**

Based on the differences in expression profiles, two clusters can be distinguished, one comprising of surface water and E samples and another one comprising of untreated wastewater samples C, H, N, and I **(Fig. 2)**. The difference between the untreated wastewaters and treated effluent or surface water became also clear in principal component analysis (PCA) **(Fig. 3)**.

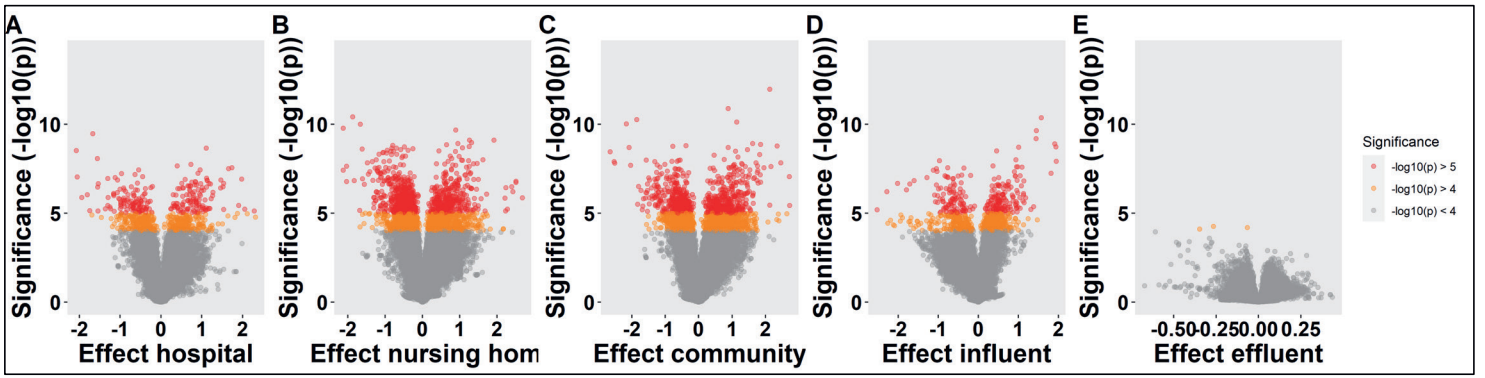

Figure 1. Volcano plots showing the distribution of gene expression changes and p-values. Each dot represents a spot on the microarray, as analysed by three linear models. On the x-axis the effect is given (a negative sign indicates lower expression over increasing concentrations, a positive sign higher expression over increasing concentrations), on the y-axis the $-\log_{10}$(p-value) obtained from the linear model. These effect plots show an obvious distinction between wastewater samples before and after treatment in a WWTP. Colours provide a visual guide for the thresholds of $-\log_{10}(p) > 4$ and $-\log_{10}(p) > 5$. (A) hospital samples, (B) nursing home samples, (C) community samples, (D) WWTP influent samples, (E) WWTP effluent samples.

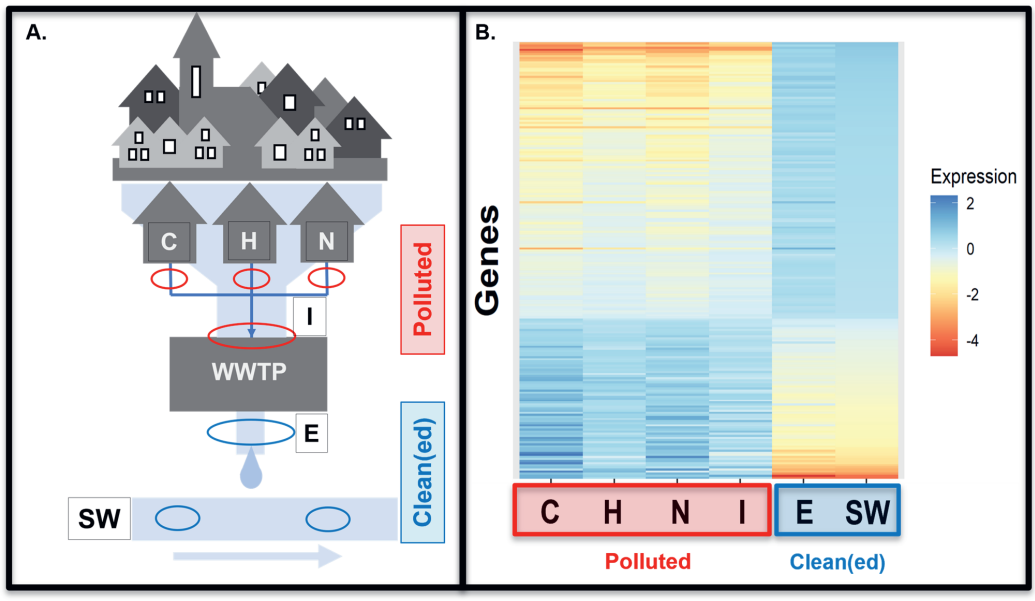

Figure 2. Comparison of gene expression profiles in nematodes treated with (waste)water samples. Sampling points are shown in (A), including wastewater Community (C), Hospital (H), Nursing home wastewater (N), WWTP influent (I), WWTP effluent (E) and surface water (SW) receiving the treated effluent. (B) is a heatmap showing the up- (red-orange) and down-regulation (blue) of C. elegans genes after exposure to different (waste)water samples. There is a clear difference between gene expression patterns before and after wastewater treatment.

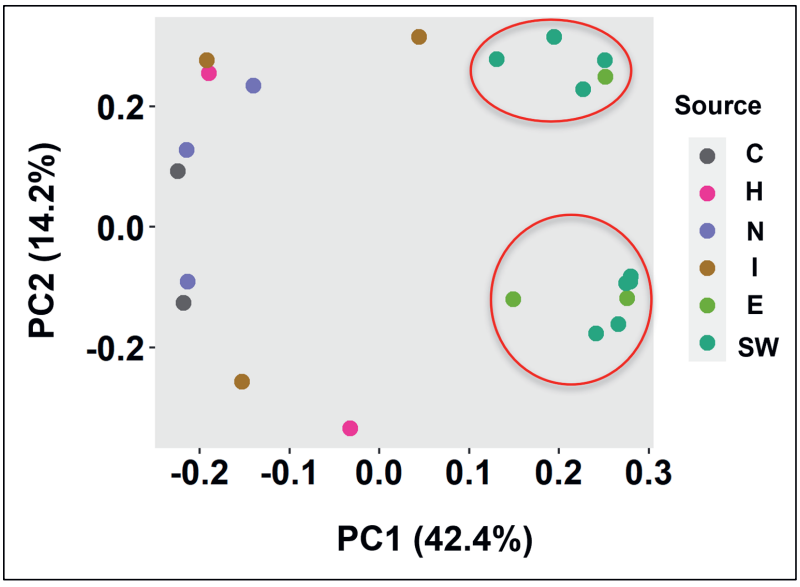

Figure 3. Principal component analysis (PCA) for variation in gene expression. The first two principal components PC1 and PC2 combined captured 56.6% of the variance and mainly separate the surface water and effluent samples from the other samples.

All four wastewater types shared 209 genes that were differentially expressed **(Fig. 4)**, representing 16%, 15%, 51%, and 45% of the total DEGs affected by sample C, N, H, and I, respectively. These genes included those encoding C-type lectin (CLEC) proteins, cytochrome P450 (CYP) and other enzymes involved in xenobiotic biotransformation. In addition, several other overlaps were found between wastewater samples **(Fig. 4)**. C23G10.11 and B0222.4 (known as *spl-2*) genes were found to be the most upregulated transcripts for all wastewater samples. Expression of sphingosine phosphate lyase encoded by *spl-2* is involved in defense response to gram-positive bacterium. The function of protein encoded by C23G10.11 is not yet known.

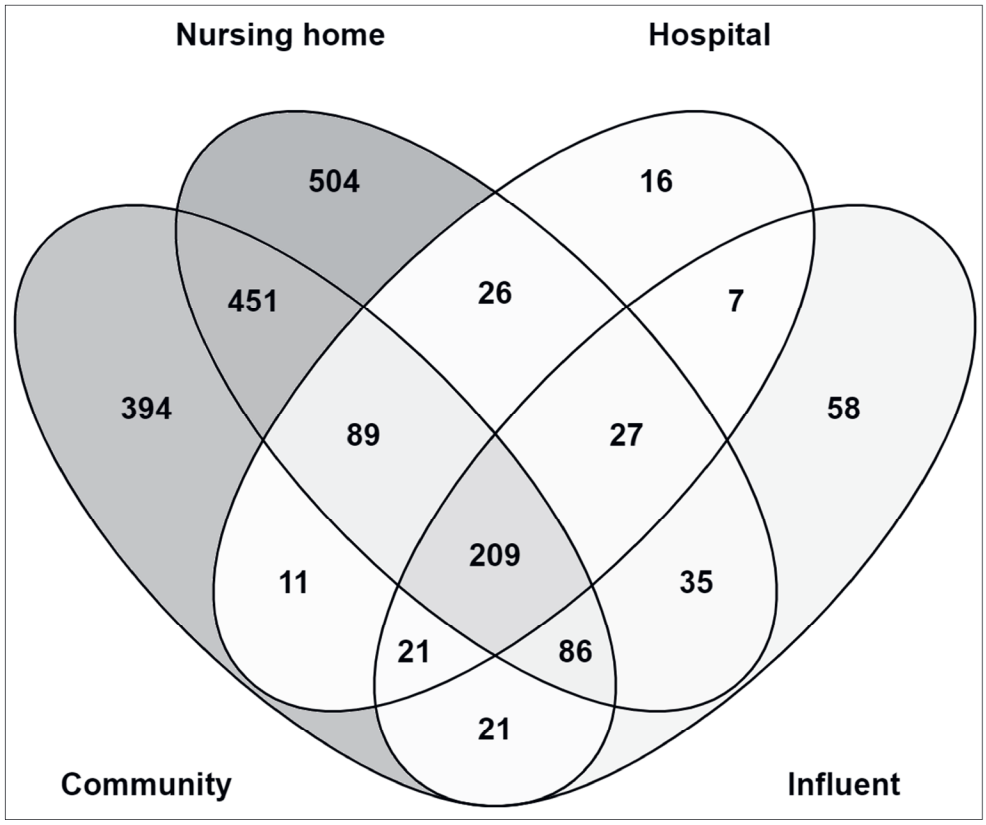

Figure 4. Differences and similarities of genes expression profiles in nematodes after exposure to different wastewater samples. The Venn diagram shows that from the 1,756 DEGs (up- or downregulated) in one or more of the polluted samples (i.e., hospital, nursing home, community, and influent), the majority (69%) of these genes were specific to community and / or nursing home wastewaters. The overlap of 209 DEGs (approx. 11%) were found in all polluted samples.

Wastewater samples from C and N induced the greatest number of DEGs **(Fig. 4)**, 1282 and 1427, respectively ($-\log_{10}(p) > 4.0$; false discovery rate, FDR < 0.01). In contrast, differential expression in sample H and I was much lower with 464 and 406 genes, respectively. Only two genes (*ncx-4* and F22B8.7) were differentially expressed in the nematodes treated with sample E and were both upregulated (1.1-fold for *ncx-4* and 1.5-fold for F22B8.7). Of these two genes, differential upregulation of F22B8.7 (1.4-fold) was also found in the sample I. Of the genes whose transcription levels (absolute-value expression) were changed more than five-fold **(Fig. 5)**, most were found in nematodes exposed to C (166 DEGs) and N (101 DEGs) wastewaters, representing 13% and 7% of total DEGs of each sample, respectively. For sample H and I, 33 and 23 DEGs representing 8% and 7%

of total DEGs of each sample were changed over five-fold. The two most upregulated genes for all wastewater were C23G10.11 (> 40-fold for sample C and N or > 20-fold for sample H and I) and B0222.4 (39-fold for C, 25-fold for H, 29-fold for N, and 23-fold for I). The decrease in expression level of T06C12.14 (40-fold for C and 15-fold for I) and Y49G5A.1 (19-fold for I and 17-fold for H) represented the most downregulated transcripts.

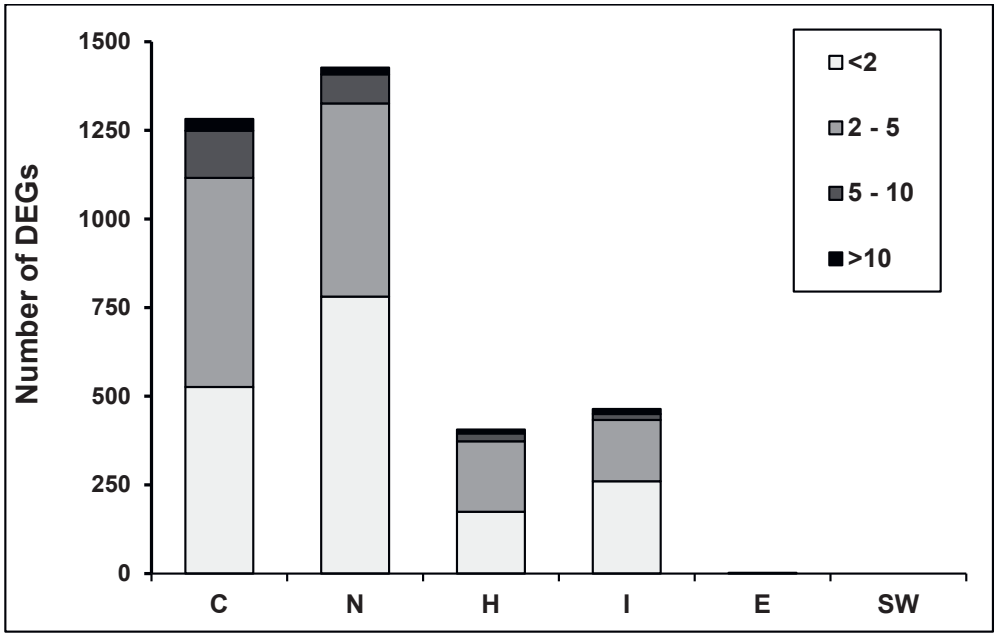

Figure 5. Expression fold change range of differentially expressed genes (DEGs) in the nematodes treated with wastewater samples. Bar charts displays the number of DEGs in each fold change range (i.e., < 2 fold, 2 – 5 fold, 5 – 10 fold, and > 10 fold) of the transcription levels induced in the nematodes treated with the samples originating from community (C), nursing home (N), hospital (H), WWTP influent, WWTP effluent (E), or surface water (SW).

3.2. Functional analysis of differentially expressed genes (DEGs)

Gene Ontology (GO) and domain enrichment analysis of DEG lists were carried out in DAVID software to identify the types of biological mechanisms underlying the nematode responses triggered by exposure to wastewater samples **(Fig. 6 and Suppl. Table S3)**. We identified a total of 36 genes encoding nuclear hormone receptors (NHRs) whose expression levels were affected by exposure. Of these genes, 10 transcripts (including *nhr-23* gene which is a critical regulator of the nematode growth and molting) were upregulated while the other 26 genes were downregulated. Many upregulated genes were related to the nematode

metabolic processes, especially those involved in the biotransformation (both phase I and phase II) of a wide range of substrates such as lipids, carbohydrates, and proteins. These biotransformation genes included those encoding cytochrome P450 (CYP), glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGT), NADPH-cytochrome P450 reductase homolog (*emb-8*), and a number of genes annotated as FAD/NADP coenzymes. Cytochrome genes *cyp-25A1, cyp-25A2, cyp-29A2, cyp-33B1, cyp-35B1,* and *cyp-37A1* were upregulated in all wastewater samples. Transcriptional repression was found for pathways involved in the metabolism of purine and pyrimidine nucleotides, and was identified in nematodes exposed to sample C and H. We also found DEGs involved in a peroxisomal pathway, including the transcripts of *acox-3*, *prx-3*, *prx-5*, *gstk-1*, *daf-22*, *ctl-2*, *ech-4*, *fard-1*, *acs-13*, C24A3.4, T20B3.1, and ZK550.6 genes upregulated by sample C and *prx-3*, C24A3.4, *daao-1*, *prx-14*, *ctl-2*, *ech-4*, *sod-1*, and *acs-13* upregulated by sample N. Genes annotated for oxidative stress response were found upregulated, including *pdi-2* and F09F3.*5* (in sample C), *pept-1* (in N), R08F11.7 (in C and N), and *col-61* (in C, H, and N samples).

Also genes involved in the *C. elegans* molting cycle processes were upregulated in C and N samples. These included the DEGs encoding collagen and cuticulin-based cuticle in the nematode. We also identified upregulation of many genes modulating growth processes in the nematodes treated with sample C. The *daf*-36 gene encoding a Rieske-like oxygenase, which is a component of *C. elegans* endocrine system, was upregulated in sample C, H, and N exposure, but not in sample I. The individual annotation (in DAVID software) of all DEGs which responded to the wastewater samples revealed several transcripts that can be linked to reproductive physiological processes in *C. elegans*. Nevertheless, reproduction related processes (GO:0000003) were not found among the significantly regulated processes as obtained by GO enrichment analysis. We also found in total 40 DEGs encoding C-type lectin (CLEC) proteins, which are related to the immune response in nematodes. Of these, 11 genes were differentially expressed in all wastewater samples including both upregulation (*clec-39, clec-52, clec-55, clec-57, clec-221,* and *clec-227)* and downregulation (*clec-45, clec-53, clec-62, clec-63, clec-147,* and *col-137)*.

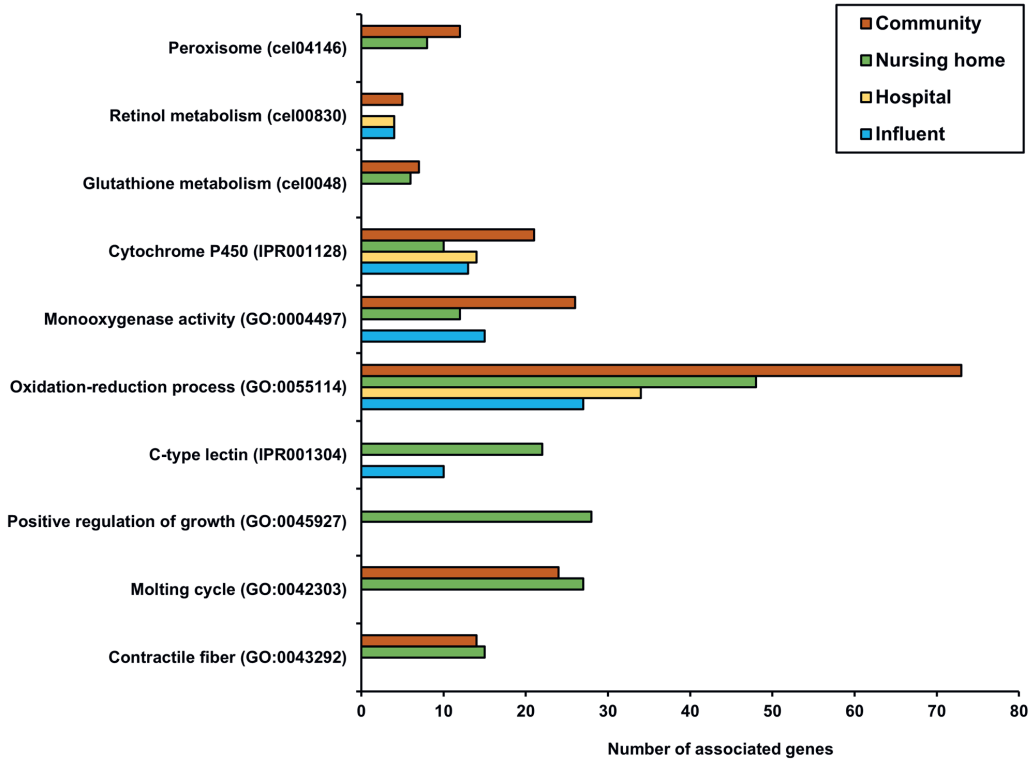

Figure 6. Some significantly upregulated genes for which enriched terms could be obtained (False Discovery Rate (FDR) < 0.05). Full results of functional enrichment analysis are provided in Suppl. Table S3.A – S3.D of supplementary information.

3.3. Validation of microarray data by RT-qPCR

To validate the microarray results we conducted RT-qPCR for 15 target genes that were among the top most affected transcripts, among those regulated in all wastewater samples, or those specifically responding to one or two wastewater samples. Overall, RT-qPCR results correlated to the microarray results **(Fig. 7)**.

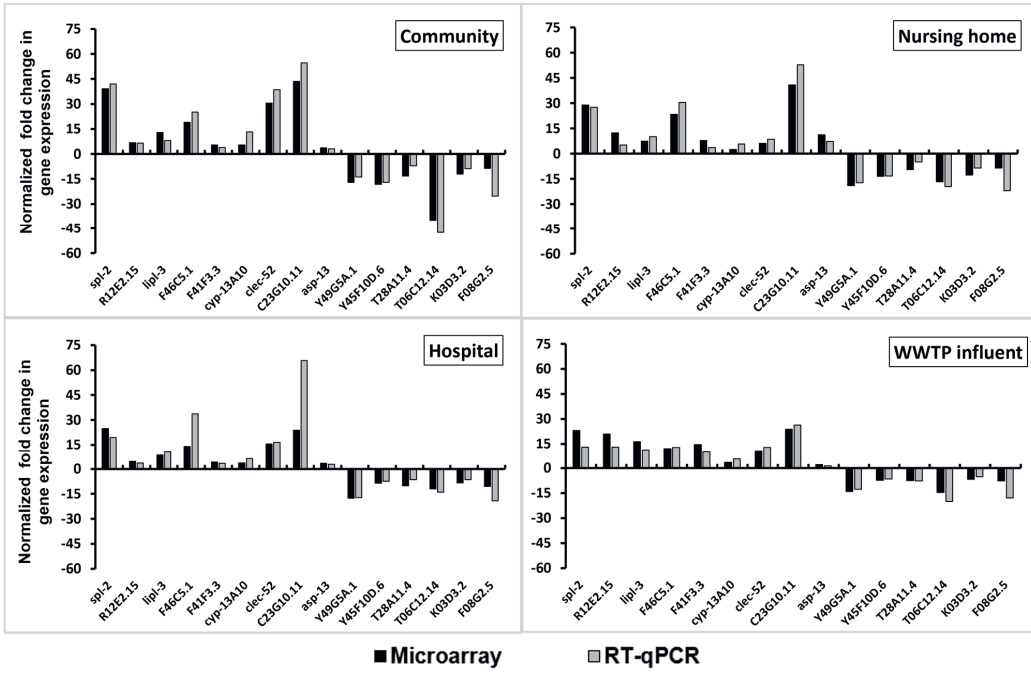

Figure 7. Validation of gene expression microarray results by reverse transcription polymerase chain reaction (RT-qPCR) for 15 target genes in two independent biological replicates using the RNA template from microarray samples. Negative values indicate downregulation and positive values upregulation of the target genes relative to two housekeeping genes (*tbg-1* and *par-5*) used to normalize the expression fold changes.

4. Discussion

In this study, we successfully applied a nematode-based assay using gene expression profiling in *Caenorhabditis elegans* to fingerprint wastewaters before and after treatment by a wastewater treatment plant (WWTP) and effluent receiving surface waters. Several genes were differentially regulated following the exposure to wastewater samples, and this effect was absent in nematodes exposed to treated effluent as well as in effluent receiving surface water. The nematodes were exposed without extraction or preconcentration of water samples, except the removal of suspended solid materials by centrifugation. This means that bioanalysis with the water-exposed nematodes will especially indicate the total toxic potencies of hydrophilic compounds that may be present in the tested samples, even at concentrations that could not yet be detected with chemical analysis.

Untreated and treated wastewater can typically contain a wide range of natural and synthetic chemical contaminants and reaction products and metabolites thereof [1-3]. The composition and type of contaminants present in each water source can vary depending on several factors [26]. The most challenging substances to detect and quantify are hydrophilic compounds which are hardly known and difficult to detect with existing chemical analytical techniques [4]. The exposure of nematodes to water samples containing hydrophilic compounds which are invisible by chemical analyses are expected to leave their signature in this invertebrate detectable by transcriptome analysis. In this study, gene expression profiling using microarray provides information about the total combined toxic potency specified per mechanism of action without the need to know the nature of the causative agents.

Although 209 genes were differentially regulated (77 upregulated DEGs and 132 downregulated DEGs) in all four types of wastewaters, these sample types also had specific DEGs that could be characteristic for the source. These included 31%, 4%, 35%, and 13% of the total DEGs specifically regulated in response to the sample C, H, N, and I exposure, respectively. There were also several DEGs regulated in the nematodes treated with the sample C, H, and N, but were not found in the sample I exposure. Compared with the total amount of DEGs found with each wastewater source, these genes comprised 74% for C, 35% for H, and 83% for N affected DEGs (including the overlaps). The expression of these genes may be linked to substances that were diluted by the additional water from other sources (which accounted 97% of the total influent) such as stormwater runoff, seepage water, and water from other community households. It is also possible that the substances in wastewater sources were degraded or have reacted before reaching the influent. More detailed study, including more sampling (time) points and combining this with a tiered approach for screening and assessment of the contaminant mixtures can reveal the most important bioactive compounds, their sources, and their fate. This is comparable to the approach of effect-directed analysis (EDA) utilizing the process similar to the toxicity identification evaluation (TIE) to identify unknown contributors to the mixture effects in water samples as described previously [12].

Only two genes were regulated in the nematodes treated with effluent, suggesting an efficient removal of pollutants by the WWTP, and none after emission of the effluent into the surface water. This means that the nematode assay could be developed into a bioanalytical tool for determining whether the toxic potency is below a threshold of ‘no indications for concern’. The small size of the nematodes and sensitivity of molecular endpoints potentially make the assay sensitive for ultra-low concentrations of contaminants. The aim, however, does not necessarily have to be to make the assay as sensitive as possible, but sensitive enough to be able to determine whether the possibly remaining contaminants do not pose a risk.

Another advantage of this small scale, bioanalytical in-vivo tool is that the DEGs provide mechanism-based information on the combined toxic potency of the contaminants present, including the unknown hydrophilic compounds. In this study, genes related to metabolic processes were affected most. These included several genes involved in the metabolic pathways such as the *emb-8* gene encoding *C. elegans* NADPH-cytochrome P450 reductase homolog (EMB-8) which governs the nematode CYP-mediated metabolism [27, 28]. There was also significant expression among the genes involved in the peroxisomal pathway, which is essential in the antioxidant defence system. Of these genes, *ctl-2* [29], *sod-1* [30], and *gsto-1* [31] are known for their central role in the detoxification of reactive oxygen species (ROS). Other genes annotated for oxidative stress response were upregulated , including *col-61, pdi-2, pept-1*, R08F11.7, and F09F3.5 transcripts. These observations do not imply a toxic risk *per se*, as explained in [32], but the involved genes do indicate exposure to compounds that trigger the organism's defense mechanism.

Wastewaters have been shown to contain endocrine disrupting compounds [33] which are highly heterogeneous in source and nature [34]. Nematodes have been shown to be sensitive for the effects and mechanisms of endocrine disrupting compounds as has been reviewed in [35]. The authors demonstrated evidence that many processes like molting or growth, regulated via hormonal pathways are also operational in *C. elegans*. In our study, the differential gene expression profile of these pathways induced by wastewater, mostly in those originating from community and nursing home, indeed suggest the suitability of *C. elegans* to indicate endocrine active compounds. The DEGs included those required for molting, growth, and reproduction processes in the nematode, and especially well-known regulators of *C. elegans* development like *nhr-23* [36], *unc-52* [37], and *daf-36* [38], together with many of their downstream genes. This finding suggest the presence of endocrine disrupting substances in the tested wastewater samples and the absence thereof in the effluent and surface water samples. The application of bioassays in high-resolution effect-directed analysis has been recently demonstrated for the identification of endocrine disrupting and mutagenic compounds in wastewater treatment plant effluents and the river Meuse [39].

Our study also identified differential expression of many genes contributing to the nematode innate immune system, especially those encoding C-type lectin (CLEC) proteins. This could be related to exposure of the nematodes to microorganisms from the wastewaters including pathogens that may trigger an immune response in the nematodes as previously reported [40]. Proteins encoded by the DEGs that we found in the wastewaters are associated with the innate immune mechanisms of invertebrates [41]. The genes *clec-52, clec-70, clec-61, tag-38, acdh-1, F55G11.7, myo-2,* Y51H4A.5, and *unc-52*, also found in the outcome of our study, were linked to the *C. elegans* infection by the bacteria *P. aeruginosa* and *S. aureus* [40]. Among the 300 CLEC genes estimated to be present in the *C. elegans* genome [42], our study showed that 40 CLEC genes responded to the wastewater exposure but not to

effluent or surface water exposure. Noteworthy, *spl-2* that was among the top upregulated transcripts by all wastewaters is also involved in the nematode defense response to a gram-positive bacterium [40]. Further transcriptomic profiling of CLEC genes in *C. elegans* exposed to various pathogen types can provide gene markers that may specifically detect those pathogens in water sources.

5. Conclusion

Overall, this study showed that gene expression profiling in *C. elegans* is a potential powerful tool for monitoring water-soluble pollutants in wastewaters. This bioanalytical assay especially is suitable for monitoring of the mechanism-specific toxic potency from hydrophilic pollutants since the nematodes can be directly exposed to even severely polluted wastewater samples without the need to pretreat or to dilute the samples. The results from this study showed a strong difference between polluted water and clean(ed) water samples in terms of gene expression profiles and intensity. Hence, our method can be used for monitoring the removal efficiency of (micro)pollutants during wastewater treatment and assessing the quality of the resulting effluent and receiving waters. In a tiered approach, this bioanalytical tool could help identify the most important bioactive compounds, their sources, and their fate. Also, the mechanistic profile of specific compounds of interest could be studied to possibly be able to identify, for instance, the presence of (recreational) drugs in wastewater. In addition, transcriptional profiles could be used to identify the presence of wastewater input or specific wastewater sources. It also is important to study the lowest induction level below which there is no indication for toxicological concern from hydrophilic compounds, compounds that are not yet easily detected, quantified, and assessed based on chemical analysis.

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Supporting information of chapter 4

Suppl. Table S1. The quality of template RNA used in microarrays as measured by NanoDrop spectrophotometer.

Batch No	Sample ID	Samples name	ng/µl	A260	A280	260/280	260/230
Batch 1	SW1	Surface water 1	161.7	4.0	2.0	2.1	2.1
	SW2	Surface water 2	202.6	5.1	2.4	2.1	1.9
	SW3	Surface water 3	197.8	4.9	2.4	2.1	1.4
	H	Hospital	205.2	5.1	2.4	2.1	1.9
	N	Nursing homes	178.9	4.5	2.1	2.1	1.4
	C	Community	150.0	3.7	1.8	2.1	1.2
	I	WWTP influent	172.2	4.3	2.1	2.1	1.8
	E	WWTP effluent	268.4	6.7	3.2	2.1	1.9
Batch 2	SW1	Surface water 1	191.6	4.8	2.3	2.1	2.5
	SW2	Surface water 2	310.4	7.8	3.7	2.1	1.7
	SW3	Surface water 3	233.6	5.8	2.8	2.1	2.3
	H	Hospital	253.4	6.3	3.0	2.1	1.9
	N	Nursing homes	289.8	7.2	3.5	2.1	2.4
	C	Community	159.2	4.0	1.9	2.1	2.1
	I	WWTP influent	269.8	6.7	3.2	2.1	2.4
	E	WWTP effluent	268.4	6.7	3.2	2.1	2.3
Batch 3	SW1	Surface water 1	116.5	2.9	1.5	2.0	0.9
	SW2	Surface water 2	135.7	3.4	1.5	2.2	1.7
	SW3	Surface water 3	138.6	3.5	1.6	2.1	1.8
	H	Hospital	138.9	3.5	1.6	2.1	2.0
	N	Nursing homes	114.0	2.9	1.3	2.1	1.6
	C	Community	71.7	1.8	0.9	2.0	1.3
	I	WWTP influent	107.4	2.7	1.3	2.1	1.7
	E	WWTP effluent	154.9	3.9	1.8	2.1	1.9

Suppl. Table S2. Primer sequences used for RT-PCR analysis to validate microarray data

Gene	Acc._No	Forward primer (5'->3')	Reverse primer (5'->3')
asp-13	NM_072831	ctatttggagtaccgcgacc	cgttgtgcaattagttcctgg
C23G10.11	NM_065953	aaaagcgtcaggaaaccact	tgttcagaaaaagtgttgctcg
clec-52	NM_068970	tgtttcgatcactgctcctc	caatgtggcacagatggact
cyp-13A10	NM_063684	ctcctagcaacacatccagaag	cgagagttcgccattgttcc
F08G2.5	NM_064500	ccaattccagtgactcctcg	aagtttctctcggcgagttc
F41F3.3	NM_071850	aaaactcatcatcgccttcgc	atggaagggaaagtggtggg
F46C5.1	NM_063478	tcttttggttgtcatcgttgc	ttgcttggactcattctgct
K03D3.2	NM_070543	cgtgggttgatggaaaggga	ggacaaccgaagtttgctgt
lipl-3	NM_070832	aatgtgctcttcgctttgtg	cccgtcatctgtagtcactg
R12E2.15	NM_001382870	ggtaacgcttgcatcggag	gtatgctcctccttgtggtg
spl-2	NM_072971	ggagattcggagacatgtgg	ggaaacattcggcagctttg
T06C12.14	NM_074575	ccccaggaagagatcagcag	gtgattgggtaatggtggcg
T28A11.4	NM_071502	tatttgcggtgtctgaggct	aagtctgttctccagcggc
Y45F10D.6	NM_070261	ccctcatcacgttgtgctct	ccgtttttcttgcagacttttgg
Y49G5A.1	NM_072012	cccattggcttttgagtacagt	ttttggcccacattcttcatcg

Suppl. Table S3.A. Significantly enriched terms obtained from the DEGs (False Discovery Rate, FDR < 0.05) in the nematodes treated with community wastewater.

	# genes	%	FDR
Upregulation			
GO:0006082~organic acid metabolic process	32	5.3	7.22E-04
GO:0006629~lipid metabolic process	32	5.3	7.22E-04
GO:0006631~fatty acid metabolic process	18	3.0	8.40E-06
GO:0006633~fatty acid biosynthetic process	10	1.7	7.67E-04
GO:0006636~unsaturated fatty acid biosynthetic process	6	1.0	6.87E-04
GO:0006637~acyl-CoA metabolic process	7	1.2	1.08E-02
GO:0008152~metabolic process	231	38.5	4.18E-03
GO:0008610~lipid biosynthetic process	14	2.3	4.17E-02
GO:0016053~organic acid biosynthetic process	12	2.0	3.25E-02
GO:0018208~peptidyl-proline modification	8	1.3	1.07E-02
GO:0019752~carboxylic acid metabolic process	31	5.2	6.58E-04
GO:0032507~maintenance of protein location in cell	6	1.0	2.22E-02
GO:0032787~monocarboxylic acid metabolic process	26	4.3	8.29E-06
GO:0033559~unsaturated fatty acid metabolic process	6	1.0	6.87E-04
GO:0035383~thioester metabolic process	7	1.2	1.08E-02
GO:0042303~molting cycle	24	4.0	2.22E-02
GO:0043436~oxoacid metabolic process	31	5.2	6.58E-04
GO:0044255~cellular lipid metabolic process	22	3.7	1.30E-02
GO:0044281~small molecule metabolic process	45	7.5	2.85E-03
GO:0044710~single-organism metabolic process	115	19.2	1.78E-13
GO:0045185~maintenance of protein location	7	1.2	6.51E-03
GO:0046394~carboxylic acid biosynthetic process	12	2.0	1.73E-02
GO:0051235~maintenance of location	43	7.2	8.05E-03
GO:0051651~maintenance of location in cell	6	1.0	3.25E-02
GO:0055114~oxidation-reduction process	73	12.2	3.40E-20
GO:0072330~monocarboxylic acid biosynthetic process	10	1.7	1.06E-03
GO:0005576~extracellular region	30	5.0	1.88E-02
GO:0005737~cytoplasm	115	19.2	3.69E-02
GO:0005778~peroxisomal membrane	5	0.8	8.79E-03
GO:0005789~endoplasmic reticulum membrane	18	3.0	1.18E-03
GO:0012505~endomembrane system	44	7.3	3.52E-04
GO:0016020~membrane	216	36.0	3.69E-02
GO:0016021~integral component of membrane	196	32.7	4.25E-02
GO:0030055~cell-substrate junction	5	0.8	2.16E-02
GO:0031224~intrinsic component of membrane	196	32.7	4.42E-02
GO:0031903~microbody membrane	5	0.8	8.79E-03
GO:0042175~nuclear outer membrane-endoplasmic reticulum membrane network	18	3.0	1.35E-03
GO:0043292~contractile fiber	14	2.3	7.98E-03
GO:0044425~membrane part	209	34.8	2.16E-02
GO:0044438~microbody part	5	0.8	1.88E-02
GO:0044439~peroxisomal part	5	0.8	1.88E-02
GO:0044444~cytoplasmic part	93	15.5	1.12E-03
GO:0044449~contractile fiber part	14	2.3	3.65E-03
GO:0055120~striated muscle dense body	10	1.7	1.88E-02
GO:0003824~catalytic activity	205	34.2	2.96E-09
GO:0004497~monooxygenase activity	26	4.3	4.01E-13
GO:0004768~stearoyl-CoA 9-desaturase activity	4	0.7	2.88E-02
GO:0005506~iron ion binding	28	4.7	6.29E-13
GO:0008395~steroid hydroxylase activity	8	1.3	1.56E-02
GO:0016215~acyl-CoA desaturase activity	4	0.7	2.88E-02
GO:0016491~oxidoreductase activity	70	11.7	1.86E-18
GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	36	6.0	2.81E-19
GO:0016709~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen	5	0.8	3.78E-02
GO:0016717~oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	4	0.7	2.88E-02
GO:0016746~transferase activity, transferring acyl groups	19	3.2	2.57E-02
GO:0016853~isomerase activity	15	2.5	5.19E-04
GO:0020037~heme binding	24	4.0	2.42E-07
GO:0046906~tetrapyrrole binding	24	4.0	2.69E-07
GO:0048037~cofactor binding	17	2.8	4.07E-02
IPR001128:Cytochrome P450	21	3.5	4.83E-11
IPR002347:Glucose/ribitol dehydrogenase	13	2.2	9.59E-04
IPR002401:Cytochrome P450, E-class, group I	21	3.5	4.16E-11
IPR011038:Calycin-like	7	1.2	1.16E-02
IPR016040:NAD(P)-binding domain	27	4.5	5.50E-09
IPR017972:Cytochrome P450, conserved site	20	3.3	9.44E-11
IPR020904:Short-chain dehydrogenase/reductase, conserved site	9	1.5	2.58E-03
cel00071:Fatty acid degradation	8	1.3	2.90E-03
cel00480:Glutathione metabolism	7	1.2	5.43E-03
cel00520:Amino sugar and nucleotide sugar metabolism	7	1.2	2.90E-03
cel00830:Retinol metabolism	5	0.8	8.27E-03
cel00980:Metabolism of xenobiotics by cytochrome P450	10	1.7	4.44E-06
cel00982:Drug metabolism - cytochrome P450	11	1.8	1.97E-06
cel01040:Biosynthesis of unsaturated fatty acids	4	0.7	3.15E-02
cel01100:Metabolic pathways	35	5.8	2.90E-03
cel01130:Biosynthesis of antibiotics	15	2.5	2.90E-03
cel01212:Fatty acid metabolism	7	1.2	9.43E-03
cel04141:Protein processing in endoplasmic reticulum	12	2.0	5.31E-03
cel04146:Peroxisome	12	2.0	2.22E-05
Downregulation			
IPR016638:Uncharacterised protein family UPF0376	10	1.497005988	0.001568144
IPR002542:Domain of unknown function DUF19	12	1.796407186	0.017176012
cel00230:Purine metabolism	11	1.646706587	2.73E-03
cel01100:Metabolic pathways	27	4.041916168	1.81E-02

Suppl. Table S3.B. Significantly enriched terms obtained from the DEGs (False Discovery Rate, FDR < 0.05) in the nematodes treated with community wastewater.

	# genes	%	FDR
Upregulation			
GO:0006082~organic acid metabolic process	28	4.8	1.14E-02
GO:0006631~fatty acid metabolic process	13	2.2	7.08E-03
GO:0006636~unsaturated fatty acid biosynthetic process	6	1.0	1.98E-03
GO:0010927~cellular component assembly involved in morphogenesis	18	3.1	3.62E-02
GO:0018996~molting cycle, collagen and cuticulin-based cuticle	26	4.4	3.58E-03
GO:0019752~carboxylic acid metabolic process	28	4.8	3.90E-03
GO:0030029~actin filament-based process	20	3.4	2.07E-02
GO:0030036~actin cytoskeleton organization	20	3.4	1.33E-02
GO:0030239~myofibril assembly	18	3.1	3.58E-03
GO:0031032~actomyosin structure organization	18	3.1	3.58E-03
GO:0031033~myosin filament organization	17	2.9	3.58E-03
GO:0031034~myosin filament assembly	17	2.9	3.58E-03
GO:0033559~unsaturated fatty acid metabolic process	6	1.0	1.98E-03
GO:0035264~multicellular organism growth	29	4.9	3.58E-03
GO:0040007~growth	37	6.3	3.44E-02
GO:0040008~regulation of growth	32	5.4	3.58E-03
GO:0040014~regulation of multicellular organism growth	29	4.9	3.86E-03
GO:0040018~positive regulation of multicellular organism growth	26	4.4	1.16E-02
GO:0042303~molting cycle	27	4.6	3.58E-03
GO:0042692~muscle cell differentiation	18	3.1	7.18E-03
GO:0043436~oxoacid metabolic process	28	4.8	3.90E-03
GO:0043623~cellular protein complex assembly	21	3.6	3.55E-02
GO:0044710~single-organism metabolic process	82	13.9	3.86E-03
GO:0045927~positive regulation of growth	28	4.8	7.18E-03
GO:0048589~developmental growth	34	5.8	3.58E-03
GO:0048638~regulation of developmental growth	32	5.4	3.58E-03
GO:0048639~positive regulation of developmental growth	27	4.6	7.30E-03
GO:0051146~striated muscle cell differentiation	18	3.1	3.58E-03
GO:0055001~muscle cell development	18	3.1	3.58E-03
GO:0055002~striated muscle cell development	18	3.1	3.58E-03
GO:0055114~oxidation-reduction process	48	8.2	1.28E-05
GO:0061061~muscle structure development	19	3.2	8.56E-03
GO:0071688~striated muscle myosin thick filament assembly	17	2.9	3.58E-03
GO:0005777~peroxisome	8	1.4	2.68E-02
GO:0030016~myofibril	10	1.7	4.01E-02
GO:0030055~cell-substrate junction	7	1.2	2.71E-03
GO:0030056~hemidesmosome	6	1.0	5.72E-03
GO:0042579~microbody	8	1.4	2.68E-02
GO:0043292~contractile fiber	15	2.6	1.12E-02
GO:0044449~contractile fiber part	13	2.2	3.02E-02
GO:0003824~catalytic activity	175	29.8	6.04E-03
GO:0004497~monooxygenase activity	12	2.0	3.78E-02
GO:0004768~stearoyl-CoA 9-desaturase activity	4	0.7	3.78E-02
GO:0005506~iron ion binding	14	2.4	2.56E-02
GO:0016215~acyl-CoA desaturase activity	4	0.7	3.78E-02
GO:0016491~oxidoreductase activity	44	7.5	8.28E-05
GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	19	3.2	8.28E-05
GO:0016717~oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	4	0.7	3.78E-02
GO:0030246~carbohydrate binding	23	3.9	3.78E-02
GO:0048037~cofactor binding	18	3.1	2.94E-02
IPR001128:Cytochrome P450	10	1.7	4.53E-02
IPR001304:C-type lectin	22	3.7	2.54E-03
IPR002035:von Willebrand factor, type A	15	2.6	3.08E-06
IPR002401:Cytochrome P450, E-class, group I	10	1.7	3.83E-02
IPR011038:Calycin-like	7	1.2	1.52E-02
IPR013098:Immunoglobulin I-set	8	1.4	3.51E-02
IPR016186:C-type lectin-like	22	3.7	7.15E-03
IPR016187:C-type lectin fold	23	3.9	7.15E-03
cel00480:Glutathione metabolism	6	1.0	3.61E-02
cel00982:Drug metabolism - cytochrome P450	6	1.0	3.61E-02
cel01100:Metabolic pathways	29	4.9	3.61E-02
cel01130:Biosynthesis of antibiotics	13	2.2	3.25E-02
cel04146:Peroxisome	8	1.4	3.25E-02

Suppl. Table S3.C. Significantly enriched terms obtained from the DEGs (False Discovery Rate, FDR < 0.05) in the nematodes treated with hospital wastewater

	# genes	%	FDR
Upregulation			
GO:0044710~single-organism metabolic process	53	26.1	1.07E-10
GO:0055114~oxidation-reduction process	34	16.7	4.94E-12
IPR001128:Cytochrome P450	14	6.9	2.64E-10
IPR002401:Cytochrome P450, E-class, group I	14	6.9	2.64E-10
IPR016040:NAD(P)-binding domain	9	4.4	4.56E-02
IPR017972:Cytochrome P450, conserved site	13	6.4	1.50E-09
cel00830:Retinol metabolism	4	2.0	1.32E-02
cel00980:Metabolism of xenobiotics by cytochrome P450	5	2.5	7.54E-03
cel00982:Drug metabolism - cytochrome P450	6	3.0	2.44E-03
cel01130:Biosynthesis of antibiotics	10	4.9	3.10E-03
Downregulation			
IPR019425:7TM GPCR, serpentine receptor class t (Srt)	6	3.0	3.59E-02
IPR003582:Metridin-like ShK toxin	7	3.5	3.97E-02
cel00230:Purine metabolism	5	2.5	9.17E-04
cel00240:Pyrimidine metabolism	4	2.0	2.83E-03
cel03020:RNA polymerase	3	1.5	6.03E-03
cel01100:Metabolic pathways	7	3.5	6.32E-03

Suppl. Table S3.D. Significantly enriched terms obtained from the DEGs (False Discovery Rate, FDR < 0.05) in the nematodes treated with WWTP influent wastewater

Upregulation	# genes	%	FDR
GO:0006636~unsaturated fatty acid biosynthetic process	4	2.0	1.32E-02
GO:0033559~unsaturated fatty acid metabolic process	4	2.0	1.32E-02
GO:0044710~single-organism metabolic process	38	19.3	1.06E-04
GO:0055114~oxidation-reduction process	27	13.7	3.29E-08
GO:0003824~catalytic activity	64	32.5	1.01E-02
GO:0004497~monooxygenase activity	15	7.6	2.47E-10
GO:0004768~stearoyl-CoA 9-desaturase activity	3	1.5	3.33E-02
GO:0005506~iron ion binding	14	7.1	3.63E-08
GO:0016215~acyl-CoA desaturase activity	3	1.5	3.33E-02
GO:0016491~oxidoreductase activity	26	13.2	6.21E-08
GO:0016705~oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	20	10.2	2.33E-14
GO:0016717~oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	3	1.5	3.33E-02
GO:0020037~heme binding	13	6.6	3.90E-06
GO:0046906~tetrapyrrole binding	13	6.6	3.90E-06
GO:0046914~transition metal ion binding	22	11.2	3.16E-02
IPR001128:Cytochrome P450	13	6.6	1.12E-09
IPR001304:C-type lectin	10	5.1	1.81E-02
IPR002401:Cytochrome P450, E-class, group I	13	6.6	1.12E-09
IPR005804:Fatty acid desaturase, type 1	3	1.5	4.31E-02
IPR016186:C-type lectin-like	10	5.1	3.29E-02
IPR016187:C-type lectin fold	10	5.1	4.31E-02
IPR017972:Cytochrome P450, conserved site	12	6.1	6.91E-09
IPR020846:Major facilitator superfamily domain	10	5.1	1.80E-02
cel00830:Retinol metabolism	4	2.0	2.58E-03
cel00980:Metabolism of xenobiotics by cytochrome P450	5	2.5	9.57E-04
cel00982:Drug metabolism - cytochrome P450	6	3.0	1.12E-04

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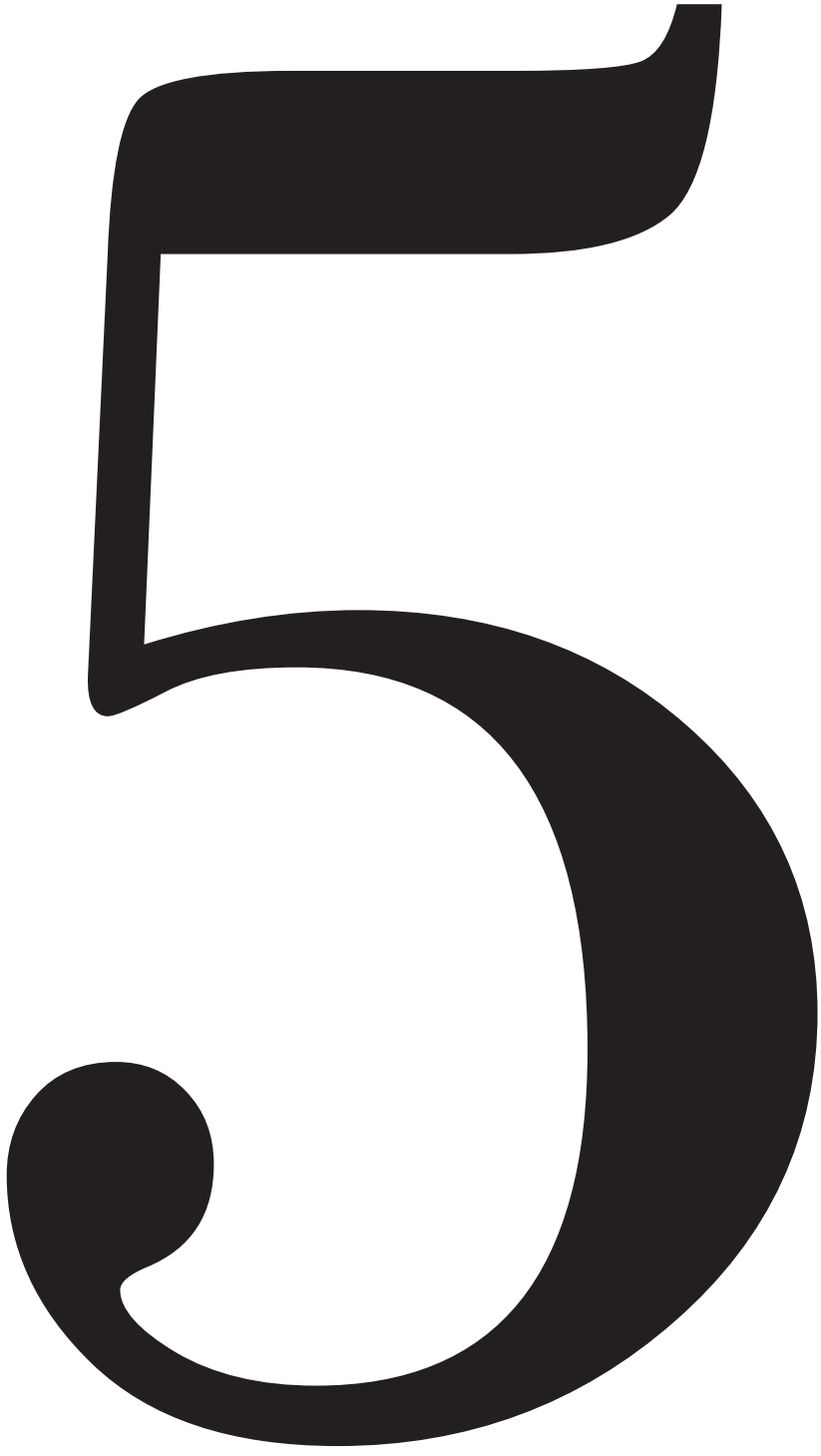


Chapter 5

A multiplex gene expression assay for direct measurement of RNA transcripts in crude lysates of the nematode *Caenorhabditis elegans* used as bioanalytical tool.

Submitted for publication:
Antoine Karengera, Cong Bao, Toine F.H. Bovee, Inez J. T. Dinkla, Albertinka J. Murk. A multiplex gene expression assay for direct measurement of RNA transcripts in crude lysates of the nematode *Caenorhabditis elegans* used as bioanalytical tool.

Abstract

Gene expression profiling in the nematode *Caenorhabditis elegans* has been demonstrated as a potential bioanalytical tool to detect the toxic potency of contaminants. RNA transcripts of genes responding to toxic exposure can be used as biomarkers for detecting these toxins. For regular application in environmental quality monitoring, an easy-to-use multiplex assay is required to routinely and reliably quantify expression levels of the biomarkers associated with the nematode response to pollutant exposure. In the current study, a bead-based assay to fingerprint gene expression in *C. elegans* by quantitating mRNAs of multiple target genes directly from crude nematode lysates without the need of RNA extraction and purification was developed. The assay uses signal amplification rather than target amplification for direct measurement of the toxin-induced RNA transcripts. Using a 50-gene panel, the expression changes of 46 target mRNAs for various contaminants and wastewaters were successfully measured and the obtained expression profiles indicated the type of toxin present in the sample. Moreover, the multiplex assay response was in line with previous results obtained with more time-consuming RT-qPCR assays and microarray analyses. In addition, the transcriptomic profiles of nematodes exposed to wastewater samples and extracts prepared from tissues of swimming crabs were evaluated, and the obtained profiles indicated the presence of organic pollutants which was confirmed by high-performance liquid chromatography (HPLC). This study illustrates the successful development of a multiplex fluorescent bead-based approach using nematode *C. elegans* crude lysates for gene expression profiling of target RNA transcripts. This method can be used to routinely fingerprint the presence of toxic contaminants in environmental samples and can be used to identify most biologically active fraction of the contaminant mixture in a toxicity identification and evaluation approach.

1. Introduction

Gene expression profiling in the soil-dwelling nematode *C. elegans* is a valuable tool to detect toxic contaminants in the environment [1]. The genome of this invertebrate has been completely sequenced and many of its genes and signaling pathways are conserved in higher organisms [2-4]. This makes *C. elegans* a suitable model organism for toxicological assessments as comparable responses between the nematode and higher organisms are to be expected [4, 5]. It was recently shown that both specific and general toxic effects of chemical toxicants can be detected by transcriptional analysis of exposed *C. elegans* [1, 6]. In response to the toxicants tested, several differentially expressed genes (DEGs) that are involved in well-defined biological functions of the nematode were found. This makes the gene expression profiling of *C. elegans* a suitable tool for the effect-based monitoring of bioactive pollutants.

Most bioassays are either very specific for a certain group of bioactive compounds, e.g. aryl hydrocarbon receptor activity or estrogenic activity, or are non-specific indicators of general toxic effects, e.g. cell viability or stress [7, 8]. Hence, a battery of bioassays is often required for testing various types of pollutants present in samples [9]. Exposure of a certain cell (e.g., cell line), tissue or organism to a chemical of interest followed by gene expression analysis may provide insights in the type of toxic mechanism(s) involved [10-13]. Different toxicants might up- or downregulate specific genes or result in specific gene expression profiles that can also be used for their detection. Hence, gene expression profiling can provide an opportunity to develop transcriptional biomarkers for assessing the toxic potencies of contaminants as described in [12]. Such molecular markers can be applied, for instance, to monitor the quality of water sources or other matrices. For regular application, an easy-to-use multiplex assay should be developed to reliably quantify simultaneously the expression levels of several biomarkers, i.e. the selected target transcripts should be incorporated in one assay enabling to detect and identify different contaminants in complex sample types.

Gene expression platforms such as microarrays [11], RNA sequencing (RNA-Seq) [14], or reverse transcription quantitative polymerase chain reaction (RT-qPCR) [15] are commonly used in toxicogenomic studies. Microarrays and RNA-Seq techniques allow a genome-wide analysis of gene transcription levels enabling the identification of a larger number of DEGs of toxicological relevance [16, 17]. In contrast, a RT-qPCR assay can only analyze a finite number of genes, but it is a more sensitive, accurate, and robust method, and is widely used for validation of transcriptomic data [18, 19]. To quantify gene transcripts, all these technologies utilize complementary DNA (cDNA) synthesized from the RNA template which involves a reverse transcription (RT) reaction. The main challenges for the success of gene expression profiling experiments using microarrays, sequencing, or RT-qPCR are the extraction of the RNA (e.g., RNA degradation, low yield, low purity, or DNA contamination) and the RT reaction (choice of reverse transcriptase, primer design, enzyme, or assay

volume among others) [20, 21]. Moreover, highly skilled laboratory workers are required to perform such experimental procedures. Therefore, there is a need of an alternative method to overcome the difficulties of classic transcriptomic technologies.

Fluorescent bead-based analysis using branched DNA (bDNA) technology is an emerging technique for gene expression profiling [22-24]. In contrast to the aforementioned gene expression platforms, the bDNA technique uses signal amplification rather than target amplification to measure mRNAs, and it does not rely on RNA extraction, cDNA synthesis, and PCR amplification. The Invitrogen™ QuantiGene™ Plex Assay (QGP, Thermo Fisher Scientific) is one of such assays incorporating bDNA technology for the direct measurement of RNA transcripts [22, 25].

There are several studies using bDNA technology to detect contaminants in real (environmental) samples [26-31], but none of them is using the nematode *C. elegans*. This nematode is however a very useful tool to analyze environmental samples, e.g. soil or water, as its whole genome is characterized. The present study aims (1) to develop a high throughput bDNA assay using Luminex magnetic beads for gene expression profiling in *C. elegans* to select and subsequently detect transcriptional biomarkers for several contaminants and (2) to apply the newly developed multiplex for fingerprinting environmental samples. Eventually, 46 DEGs were selected from our previous transcriptomic studies and successfully used to develop a nematode-based multiplex bDNA assay for quantitating mRNA transcripts directly from crude nematode lysates without the need of RNA extraction, purification or amplification. Subsequently, this assay was successfully validated and applied to detect the transcriptional response of *C. elegans* to (waste)waters and mixtures of organic pollutants in extracts from swimming crab tissues.

2. Material and Methods

2.1. Sample preparations

Aflatoxin B1 from *Aspergillus flavus* (AFB1, ≥ 98% purity), Benzo[a]pyrene (B(a)P, ≥ 96 % purity), Aroclor 1254 (PCB1254, analytical standards grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions of these toxicants were prepared in DMSO as described previously [6]. Water samples were obtained from a sampling campaign in the city of Sneek, in the Netherlands as described in [32]. These included wastewater samples originating from community, hospital, nursing home, wastewater treatment plant (WWTP) influent, WWTP effluent, surface water that receive the treated effluent, and a non-receiving surface water. All samples were transported in cooling boxes and stored at -20 °C until use. Other wastewaters tested in this study were WWTP influents and associated effluents (sampled at the same time) from various locations in the Netherlands and Germany [van Heijnsbergen et al., under review]. Prior to use in nematode exposure, water samples were processed as described previously [Karengera et al., under review]. In short, all

(waste)water samples were centrifuged and filtrated to remove suspended solids, and were used without further extraction or preconcentration.

Regarding swimming crab tissues (collected in Hangzhou bay in China), organic pollutants were extracted as described by [Bao et al. in preparation]. In short, crabs were separated into the raw edible lipid part (fat) and raw meat followed by sample homogenization in 1mL Milli-Q water using ultrasound homogenizer (Scientz, DY89). 10 grams of each sample were dried overnight at 35 °C and mixed with 1 g NaSO4. After that hexane/acetone (1:1) liquid-liquid extraction methods were applied as described in [33]. The extracted samples were de-sulphurated by adding tetrabutyl ammonium sulfite (US-EPA method No.3660) and further clean-up was performed using a multilayer acid-base silica column as described in [34]. In a final step, the extracts were dissolved in DMSO and stored at -20 °C until use.

2.2. Nematode culture and exposure

Synchronized L4 stage larvae of *C. elegans* wild-type Bristol N2 strain were cultured as described in [6]. Twenty-four-hour exposure was carried out in duplicate in Falcon™ 15 mL conical tubes at 20 °C. For each sample, approximately 10,000 nematodes were exposed in 3 mL of medium without feeding during the exposure period to minimize any potential developmental differences in the exposure patterns. For AFB1, B(a)P, and PCB1254, the nematodes were exposed to 30 µM of each toxicant (with the final DMSO concentration of 0.5%). The extract stock solutions with organic pollutants from swimming crab tissues were first diluted 10 times in DMSO before dosing (with a final DMSO concentration of 0.5%). After the exposure period, the exposure tubes were centrifuged for 1 minute at 1,000 rpm at 20 °C (Avanti J-15 Centrifuge, Beckman Coulter). Hereafter, the nematode pellets were transferred into 2 mL microtubes (Eppendorf® Safe-Lock tubes, Biopur®) and flash-frozen in liquid nitrogen for 1 minute before storing them at -80 °C until later use. Two independent biological replicate samples were analyzed per treatment (except for WWTP influents and effluents originating from other locations where only one replicate was tested).

2.3. Nematode lysis

Nematodes lysates were prepared using the QGP sample processing kit for fresh or frozen tissues (QS0106) following the manufacturer's protocol (Invitrogen's MAN0017268) with modifications. Briefly, the nematodes lysates were prepared by adding 400 µL of working homogenization solution, consisting of a combination of 4 µL proteinase K and 400 µL homogenate solution (Thermo Fisher), to the frozen pellets of the nematode samples and each mixed well by pipetting up and down several times until fully resuspended. The samples were then transferred to the tubes containing beads and proceeded with beat beating homogenization (6500 rpm, 3 cycles of 20 seconds each with an inter-cycle pause of 30 seconds) using a Precellys® Evolution homogenizer. Samples were then incubated at 65 °C for 30 minutes. During this incubation, the samples were vortexed at maximum speed

for 1 minute every 10 minutes. After this step, the samples were centrifuged at 16,000 × g for 15 minutes (at room temperature) to pellet any remaining cellular debris followed by the transfer of supernatants to new test tubes and store at -80 °C until multiplex assay analysis.

2.4. Multiplex assay design

Target mRNA markers were selected among the DEGs found in our previous transcriptomics studies with microarrays [6] [Karengera et al. under review] in which expression of 46 targets was already confirmed/validated using RT-qPCR. In collaboration with Thermo Fisher Scientific, multiplex panels containing target-specific probe sets and magnetic capture beads were manufactured and supplied (premixed and ready to use). Two multiplex panels were designed: one with 14 target mRNAs (14-plex assay, **see pilot study in Suppl. material**) and another with 50 target mRNAs (50-plex assay, **Table 1**). The 14-gene panel, designed with target mRNAs of genes responding to AFB1, B(a)P, and PCB1254, was used in a pilot experiment to formulate the design and protocols of the bead-based multiplex assay with crude nematode lysates without the need of RNA extraction and purification. A pure RNA extract of one of the samples was included in the pilot study as a positive control to determine the effectiveness of the nematodes homogenization protocol. Subsequently, the approach was applied using a 50-gene panel including 46 target mRNAs previously found to respond to various contaminants and 4 genes selected as references. Before running the full-scale experiment, the performance of the 50-plex was assessed by testing 1:1 (undiluted), 1:5, and 1:25 diluted lysates from nematodes exposed to surface water (negative control) or community wastewater (positive control). Dilutions of nematode lysates were prepared by using homogenization solution (prepared as mentioned above). After assessing its performance, the 50-plex was checked by comparing the outcome to the data from our early microarrays and RT-qPCR studies. As these assessments and checks were successful, the 50-plex was used to fingerprint WWTP influents originating from various locations and of organic pollutants in extracts from swimming crab tissues.

Table 1. Target mRNAs in *C. elegans* analysed using bead-based 50-Plex gene expression assay. Probe sets were designed to specifically hybridize to each of the 50 targets.

Target Symbol	Accession Number	Sequence Length	Probe set region
cest-33	NM_072220	1773	73-553
F10D2.8	NM_001322565	1485	6-313
F56D6.8	NM_001028064	329	2-259
vmo-1	NM_075562	688	55-507
F16B4.7	NM_071046	330	2-230
acdh-1	NM_001383261	3009	794-1285
R12E2.15	NM_001382870	268	8-246
cyp-14A4	NM_077806	1554	102-575
R09E12.9	NM_001038410	479	68-347
T28A11.3	NM_071503	683	1-585

fat-5	NM_075081	1104	427-865
lipl-3	NM_070832	1437	228-685
cyp-13A6	NM_063712	1759	344-814
Y45F10D.6	NM_070261	664	91-590
T28A11.4	NM_071502	349	4-246
C23G10.11	NM_065953	362	36-323
clec-210	NM_071454	1182	597-1025
dhs-23	NM_074419	1080	518-983
Y46H3D.8	NM_071061	744	23-563
gst-20	NM_064457	817	109-767
mdh-1	NM_072255	1149	20-449
cyp-35A1	NM_001356694	1546	37-521
T06C12.14	NM_074575	777	27-499
cdr-1	NM_074585	948	409-874
par-5	NM_069834	1106	422-814
clec-52	NM_068970	1027	62-557
cyp-35D1	NM_074643	1576	44-586
K03D3.2	NM_070543	513	31-374
rpl-6	NM_066183	769	2-472
F42A10.7	NM_065940	674	2-491
K08D8.3	NM_070059	1916	584-1059
F41F3.3	NM_071850	471	76-384
asp-13	NM_072831	1348	489-987
F46C5.1	NM_063478	764	117-658
col-160	NM_001380796	1115	9-429
lips-6	NM_069875	1214	408-899
Y49G5A.1	NM_072012	626	27-524
cyp-13A10	NM_063684	1803	543-1165
F08G2.5	NM_064500	537	28-486
tag-297	NM_064638	1940	353-821
chil-28	NC_003280	3404	101-740
ugt-41	NM_072417	1793	234-767
tbg-1	NM_066730	1480	2-479
cyp-33D1	NM_074675	1516	116-815
cpt-3	NM_065097	2217	242-815
wrt-4	NM_078192	1891	7-419
C24B9.3	NM_001028500	1420	374-827
cest_29	NC_003283	1982	97-772
spl-2	NM_072971	1772	177-679
ugt-8	NM_071914	1758	267-827

2.5. Multiplex assay procedure

Target mRNA transcripts were quantified in nematode lysates using a QGP gene expression assay (Thermo Fisher) performed as per the manufacturer's protocol (MAN0017862). All QGP reagents were purchased from Thermo Fisher. Briefly, an appropriate volume of working bead mix was prepared by combining nuclease-free water (18.5 µL), lysis mixture (33.3 µL), blocking reagent (2 µL), proteinase K (0.2 µL), capture beads (1 µL), and probe set (5 µL). This 60 µL working bead mix was pipetted into each well of the 96-well hybridization plate and subsequently 40 µL sample, i.e. nematode lysate, was added. Each sample was tested in duplicate (two technical replicates). The Assay background (i.e., the signal generated by assay in the absence of RNA) was performed in triplicate by adding 40 µL of working homogenizing solution (instead of nematode lysate) to the working bead mix. Next, the hybridization plate was sealed tight (using the pressure seal) and placed in the shaking incubator and incubated for 20 hours at 54 °C ± 1 °C at 600 rpm. After incubation, the protocol (MAN0017862) was resumed. Plates were read using the MAGPIX™ system equipped with Luminex xPONENT software (version 4.2.1705.0).

2.6. Data analysis and statistics

After reading the plate, the raw data obtained during the readout (for each gene target per well) were displayed as fluorescence intensity (FI) and only the median values (MFI) were considered for further analysis. Data processing was carried out by using a QGP Data Analysis Software (version 1.1) which is freely available online [25]. In this software, the data quality control parameters were set as follows: 10 MFI for maximum background, 20% for technical precision (% CV), 30000 MFI for saturation, and 35 as required minimum number of beads (the MAGPIX instrument automatically counts the number of beads contained in each well). The limit of detection (LOD) was determined by multiplying 3 (set as default in the software) the background signal's standard deviation plus the mean background signal. To determine gene expression, the average signal (Avg MFI) of technical replicates was first calculated for each target mRNA (including reference genes). Next, the average background signals (i.e., measured in the absence of nematode lysate) were subtracted for each gene (i.e., both targets and references), which resulted in average net MFI (Avg Net MFI). Next, for each sample, each test gene signal (Avg Net MFI) was divided by the reference gene signal (Avg Net MFI). These steps correct for deviations due to sample preparation, sample input and deviations between wells and experiments. Differential mRNA expression fold change was calculated (in each biological replicate) by dividing the normalized value for the treated samples by the normalized value from the untreated sample. The *C. elegans* tubulin gamma chain (*tbg-1*) was used as a reference gene to normalize the data (the expression stability of *tbg*-1 was first confirmed in the exposure conditions tested). The fold changes obtained in two biological replicates were averaged in excel and used for further analysis. Correlation between 50-plex and RT-qPCR data or between 50-plex and microarrays (presented as log2 average relative gene expression fold changes) was determined. Slopes

and regression coefficients were generated in excel and correlations were considered significant at p-value < 0.05.

3. Results

3.1. Multiplex assay technical evaluation

A first check was carried out on bead-beating homogenization of nematodes. The bead-beating homogenization was efficient for nematode lysis as all nematodes (~10 000 worms per sample) were entirely dissolved according to the visual observation of the lysates through a binocular microscope. A preliminary study using a 14-gene panel was conducted to formulate the design and protocols of the bead-based multiplex assay with *C. elegans*. The measured MFI (median fluorescence intensity) values for the different mRNAs (14-plex) in the nematode lysates were proportional to the dilution used (**Suppl. Figure S1**, $R^2 > 0.9$). Hence, we were confident to proceed to the full-scale test with the 50-plex panel set.

The signal linearity of the 50-plex panel set, containing 46 target mRNAs and 4 candidate genes as reference to normalize gene expression data, was assessed in 1:1 (undiluted), 1:5 and 1:25 diluted lysates of the water exposure and control samples to optimize the assay. MFI signals in all dilutions of surface water and community wastewater samples resulted in accurate data, i.e., target signals were generally within the suggested range between 70 – 130 of the % recovery values **(Suppl. Table S1.B)**. Except from *wrt-4* in 1:25 diluted sample (< 6 MFI) and saturation for *rpl-6*, *col-160,* and C24B9.3 in 1:5 dilution (> 30 000 MFI) **(Suppl. Table S1.A and B)**. All bead-counts extended the minimum of 35, except for undiluted community wastewater samples **(Suppl. Table S1.C)**.

In order to select reference genes for normalization, the expression stability of four candidate genes, *par-5, tbg-1, rpl-6,* and *mdh-1*, was evaluated. The transcripts of *par-5* and *tbg-1* genes were the most stable (variation was < 20%) and were further used for normalization of data **(Suppl. Table S1.D)**. The expression of *mdh-1* varied most, on average approximately 35%**,** and the MFI signal for *rpl*-6 was already saturated in 1:5 samples, and thus not suited for real practice. Therefore, *mdh-1* and *rpl*-6 were excluded as reference genes. Overall, the “50-plex panel set” was found to perform well, i.e. investigating 46 target genes and 2 refence genes (*par-5* and *tbg-1*). Based on the linearity of the probe set signals, the optimal dilution for real samples was considered to be 1:4 and therefore used in the full-scale experiment.

Overall, sample dilution was the major variable determining the success of this multiplex assay. The undiluted tissue lysates could trigger clogging of the sample probe-needle utilized in the MAGPIX instrument which would prevent the beads to be transferred properly to sample probe tube. Using undiluted tissue lysates can also cause signal saturation (> 30 000 MFI). Therefore, the nematode lysates should adequately be diluted to avoid such

issues. Diluting lysates could also assist in preventing beads loss during the washing steps, and facilitate the sorting and reading of magnetic beads by the Luminex xMAP reader.

3.2. Sample analysis with the 50-plex panel set

The results of the 50-plex were compared to previous data obtained from gene expression studies using microarrays and RT-qPCR analysis of *C. elegans* exposed to pure compounds (AFB1, B(a)P, or PCB1254) and (waste)water samples. Thereafter, the 50-plex assay was used to fingerprint different wastewater sources (community, hospital, and nursing home), WWTP influents originating from various locations, and mixtures of organic pollutants in extracts from swimming crab tissues.

No background signals (< 10 MFI) were observed for the above mentioned samples **(Suppl. Table S2.A)**. Although all 50 mRNAs were successfully measured in all samples, three mRNA targets were excluded from the analysis (*gst-20*, *rpl-6*, and *col-160*) as their MFI signals were saturated (> 30,000) at the dilution used **(Suppl. Table S2.B)**. Unexpectedly, signal saturation was also observed for reference gene *par-5* in many samples, while all MFI signals measured for reference gene *tbg-1* were well within the detection range of the assay (> 10 MFI or < 30,000 MFI). Therefore, *tbg-1* ultimately was the only reference gene used for normalization of mRNAs data measured by the 50-plex assay. For the whole 96-well plate, the bead counts were all above the minimum required number (> 35 beads per target per well) except for *wrt-4* and *F46C5.1* in a few samples **(Suppl. Table S2.C)**. These samples were excluded from further analysis.

3.3. Correlation between 50-plex and microarray analysis and 50-plex and RT-qPCR analysis

We set out to validate our multiplex approach by comparing gene expression levels as measured by the 50-plex assay with those determined in established microarray and RT-qPCR analysis. These included the DEGs observed after exposure to 30 µM AFB1, B(a)P, or PCB1254 and to water samples as described previously in our microarray study in which expression of some genes were already confirmed by RT-qPCR analysis [6] [Karengera et al., under review]. From the 46 selected target mRNAs, 12 mRNAs respond to AFB1, B(a)P, or PCB1254 while 38 mRNAs respond to (waste)water samples. Among these targets, 9 of the 12 and 15 of the 38 transcripts were previously validated/confirmed by RT-qPCR analysis.

A significant correlation (R^2 > 0.8, p-value < 0.01) was observed between the fold changes of expressions measured by the 50-plex assay and RT-qPCR analysis **(Fig. 1)**. Similarly, a comparison between the 50-plex assay and microarray analysis also showed a significant correlation (R^2 > 0.8, p-value < 0.01) **(Fig. 2)**. Overall, this show that the newly developed 50-plex for analysis of mRNA transcripts in *C. elegans* results in the same outcomes as microarray and RT-qPCR analysis, but it is much more easier and faster.

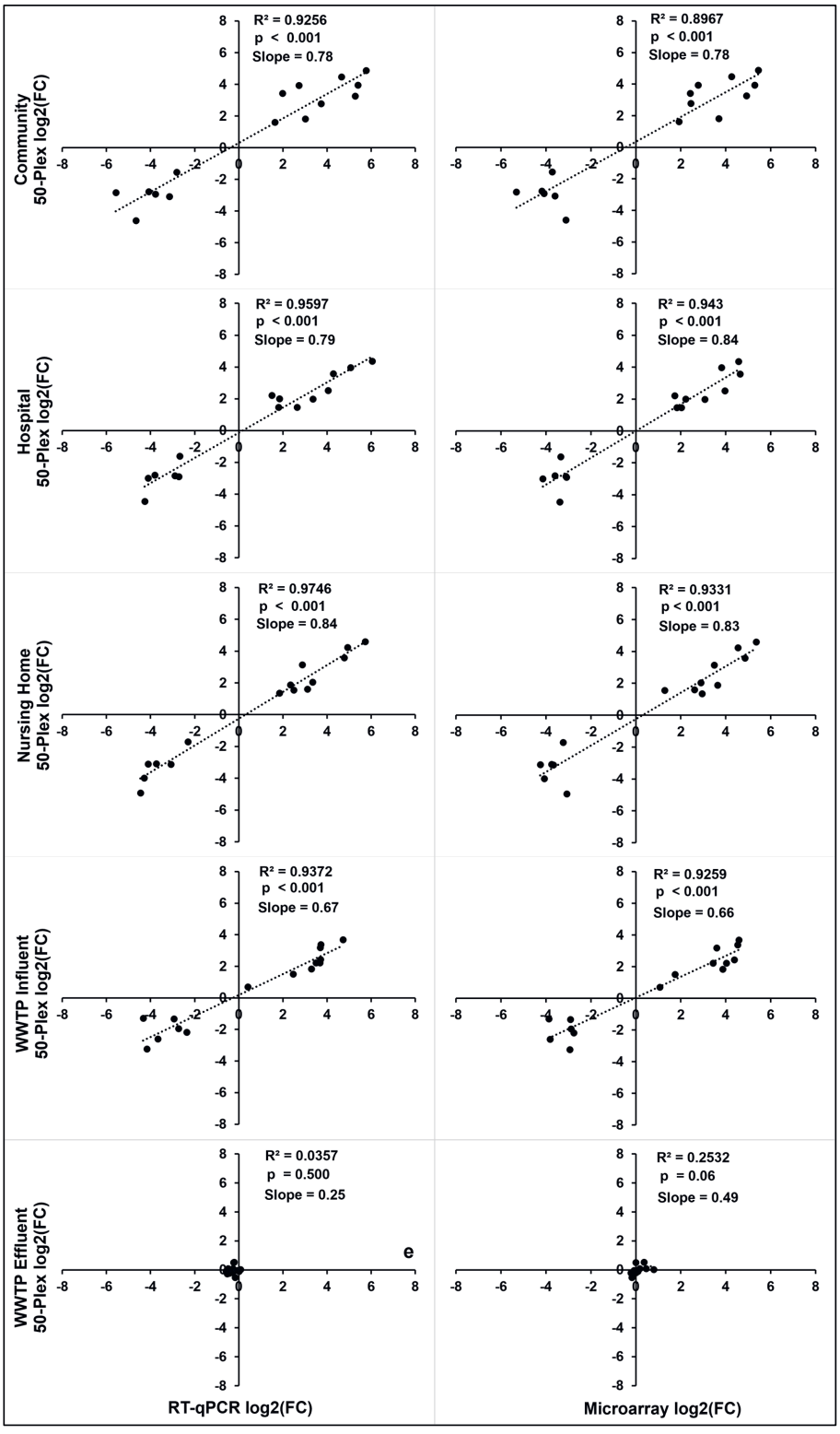

R² = 0.9256
p < 0.001
Slope = 0.78
R² = 0.8967
p < 0.001
Slope = 0.78
Community 50-Plex log2(FC)
R² = 0.9597
p < 0.001
Slope = 0.79
R² = 0.943
p < 0.001
Slope = 0.84
Hospital 50-Plex log2(FC)
R² = 0.9746
p < 0.001
Slope = 0.84
R² = 0.9331
p < 0.001
Slope = 0.83
Nursing Home 50-Plex log2(FC)
R² = 0.9372
p < 0.001
Slope = 0.67
R² = 0.9259
p < 0.001
Slope = 0.66
WWTP Influent 50-Plex log2(FC)
R² = 0.0357
p = 0.500
Slope = 0.25
R² = 0.2532
p = 0.06
Slope = 0.49
WWTP Effluent 50-Plex log2(FC)
e
RT-qPCR log2(FC)
Microarray log2(FC)

Figure 1. Comparison between mRNA expression measurements by the bead-based 50-Plex assay, RT-qPCR and microarray assays for a selection of 15 target genes. The regression plots for log2-transformed mRNA expression data in the nematodes treated with (waste)water are shown. The analysis involved 15 target mRNAs responding to the (waste)water originating from community, hospital, nursing home, wastewater treatment influent or effluent. Pearson correlation coefficients were calculated between the 50-Plex assay and RT-qPCR (right column) or between the 50-Plex assay and microarray assays (left column). Correlations were considered significant at $p < 0.05$.

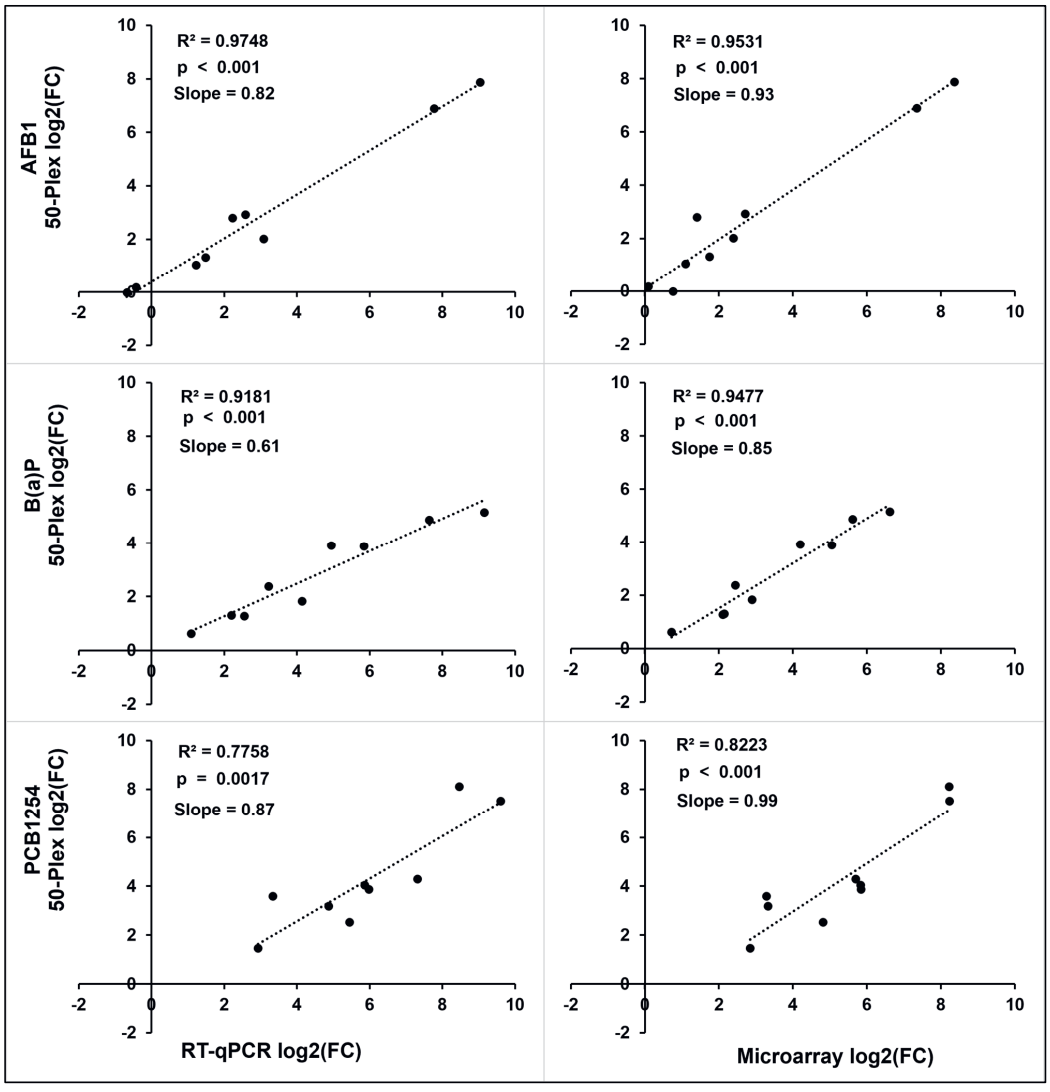


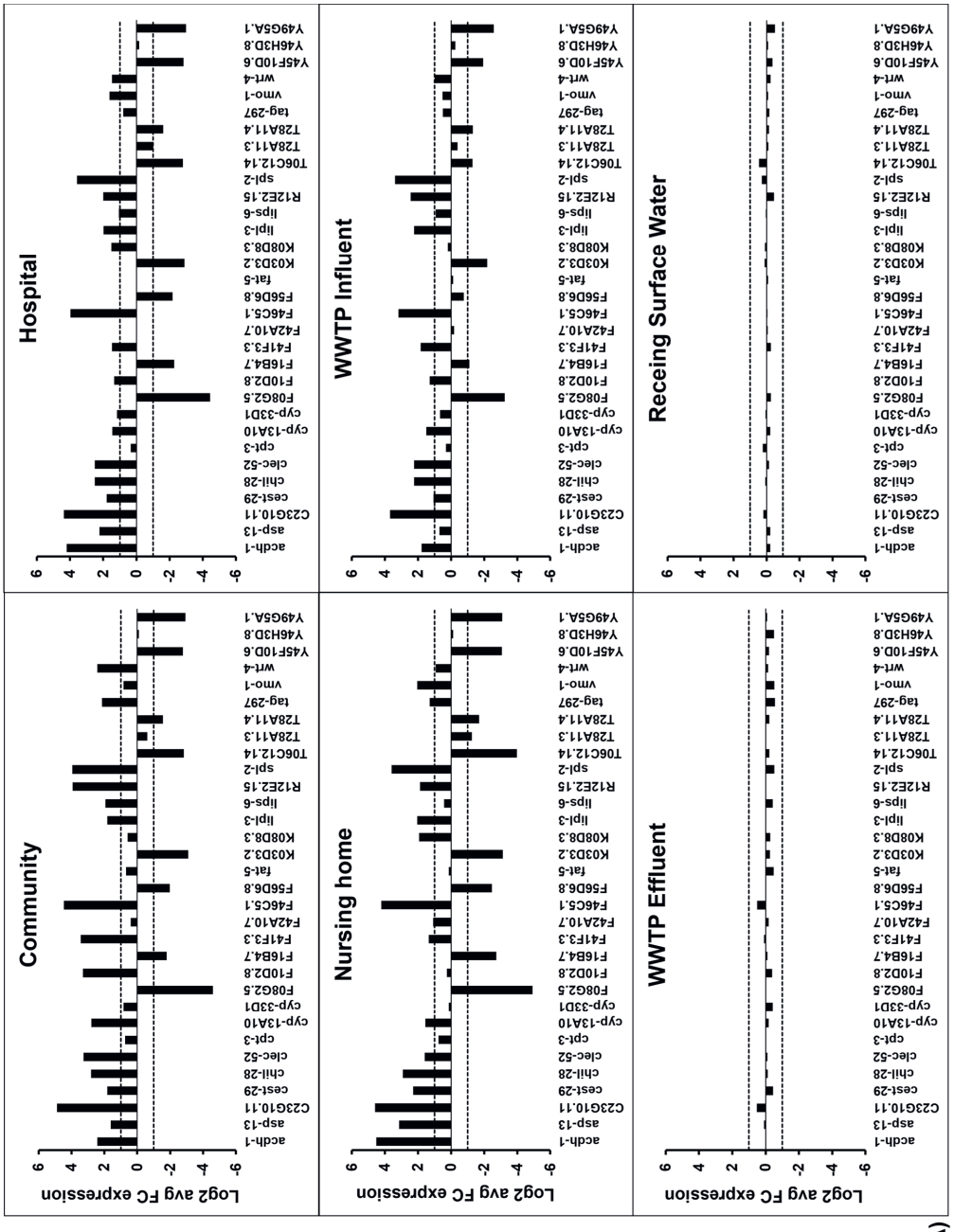
Figure 2. Comparison between mRNA expression measurements by the bead-based 50-Plex assay, RT-qPCR and microarray assays for a selection of 9 target genes. The regression plots for log2-transformed mRNA expression data in the nematodes treated with indirect-acting toxicants are shown. The analysis involved 9 target mRNAs of the genes responding to aflatoxin B1 (AFB1), benzo(a)pyrene (B(a)P), or Aroclor 1254 (PCB1254). Pearson correlation coefficients were calculated between 50-Plex assay and RT-qPCR (right column) or between 50-Plex assay and microarray assays (left column). Correlations were considered significant at $p < 0.05$.

3.4. 50-plex fingerprinting of polluted field samples

Compared to the nematodes exposed to surface water (control), gene expression patterns induced by wastewater samples revealed a clear difference. As depicted in **Fig. 3A**, untreated wastewaters significantly triggered differential expression of target genes while exposure of the nematodes to treated WWTP effluent and the receiving surface water did not result in significant transcriptional response. Among the top affected transcripts, exposure to untreated wastewater especially upregulated C23G10.11, F46C5.1 and *spl-2* (> 3 log2-fold change) in all samples. The transcripts of F08G2.5, K03D3.2 and Y49G5A.1 were among the most downregulated ones.

Gene expression profiling in the nematodes exposed to untreated WWTP influents from different locations showed comparable expression patterns (i.e. affected similar mRNAs) **(Fig. 3B)**. These target transcripts were previously selected as marker genes for wastewater, and thus confirm the consistency of the profile obtained with polluted wastewater samples. The mRNA of C23G10.11, *clec-52,* F46C5.1 and *spl-2* were among the most upregulated transcripts for all WWTP influents and their expression levels were increased above 3 log2-fold change. The transcripts of F08G2.5, K03D3.2, T28A11.4 and Y49G5A were among the most downregulated for influent wastewaters (> 3 log2-fold change).

Gene expression profiling in the nematodes exposed to organic extracts from tissues of swimming crabs from cb29, cs29, and cw29 samples showed comparable responses and the same was the case for cb04 and cs04 samples **(Fig. 3C).** These samples were the extracts obtained from specifically the edible lipid part (fat) of crabs. Transcriptional effects by sample cw20, that originated from raw crab meat, were limited and resembled the gene expression patterns obtained by cb04 and cs04. The expression levels of many mRNAs were differentially up- or downregulated above a threshold of 1 log2-fold change (**Table 2**). The affected transcripts totaled 29 for cw29, 28 for cb29, 27 for cs29, 11 for cs04, 10 for cb04, and 3 cw20 sample. Of these transcripts, *clec-52*, *fat-5* and R09E12.9 were the most upregulated (> 6 log2-fold change) by cb29, cs29 and cw29. The transcript of *clec-52* was also the most upregulated for cs04 (7.1 log2-fold), cb04 (6.8 log2-fold), and cw20 (1.4 log2-fold). Chemical compositions of these samples (i.e., the extracts from swimming crab tissues) are provided as supplementary information (**Suppl. Table S4**) and correlations between observed patterns and chemical composition are discussed.


Hospital
WWTP Influent
Receing Surface Water
Community
Nursing home
WWTP Effluent
Log2 avg FC expression
6
4
2
0
-2
-4
-6
acdh-1
asp-13
C23G10.11
cest-29
chil-28
clec-52
cpt-3
cyp-13A10
cyp-33D1
F08G2.5
F10D2.8
F16B4.7
F41F3.3
F42A10.7
F46C5.1
F56D6.8
fat-5
K03D3.2
K08D8.3
lpl-3
lips-6
R12E2.15
spl-2
T06C12.14
T28A11.3
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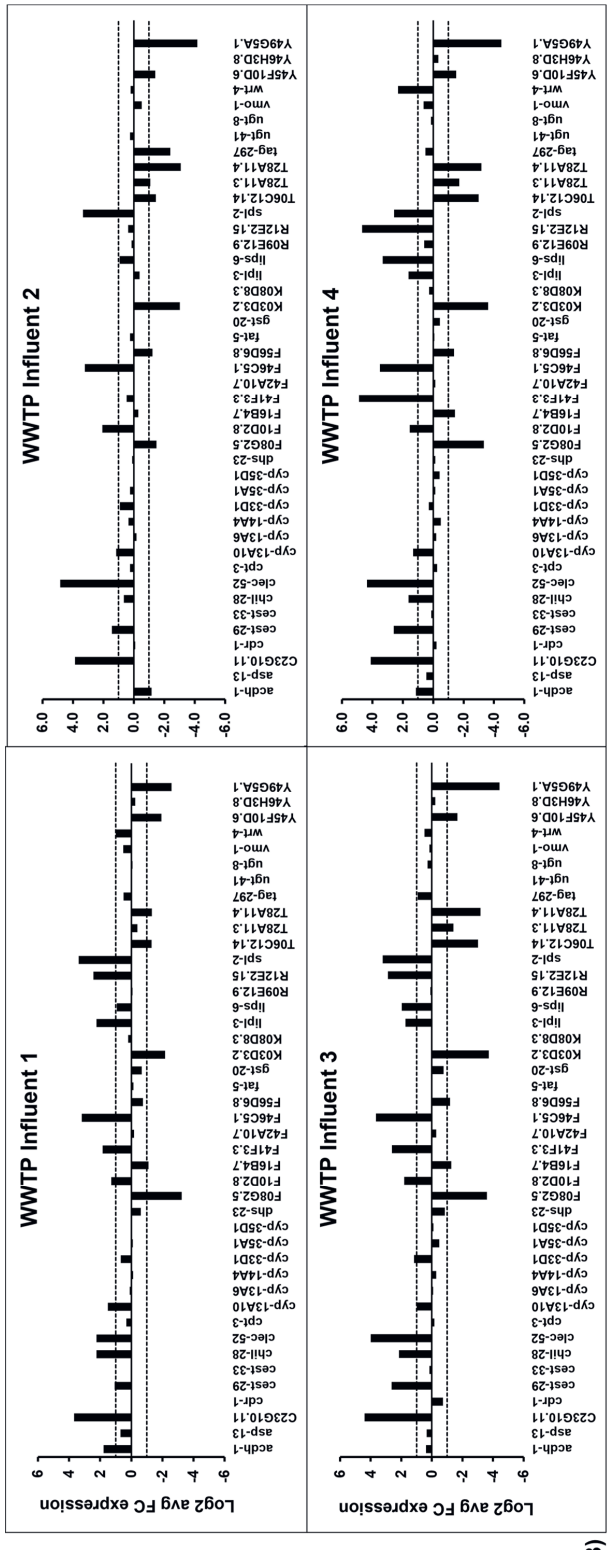
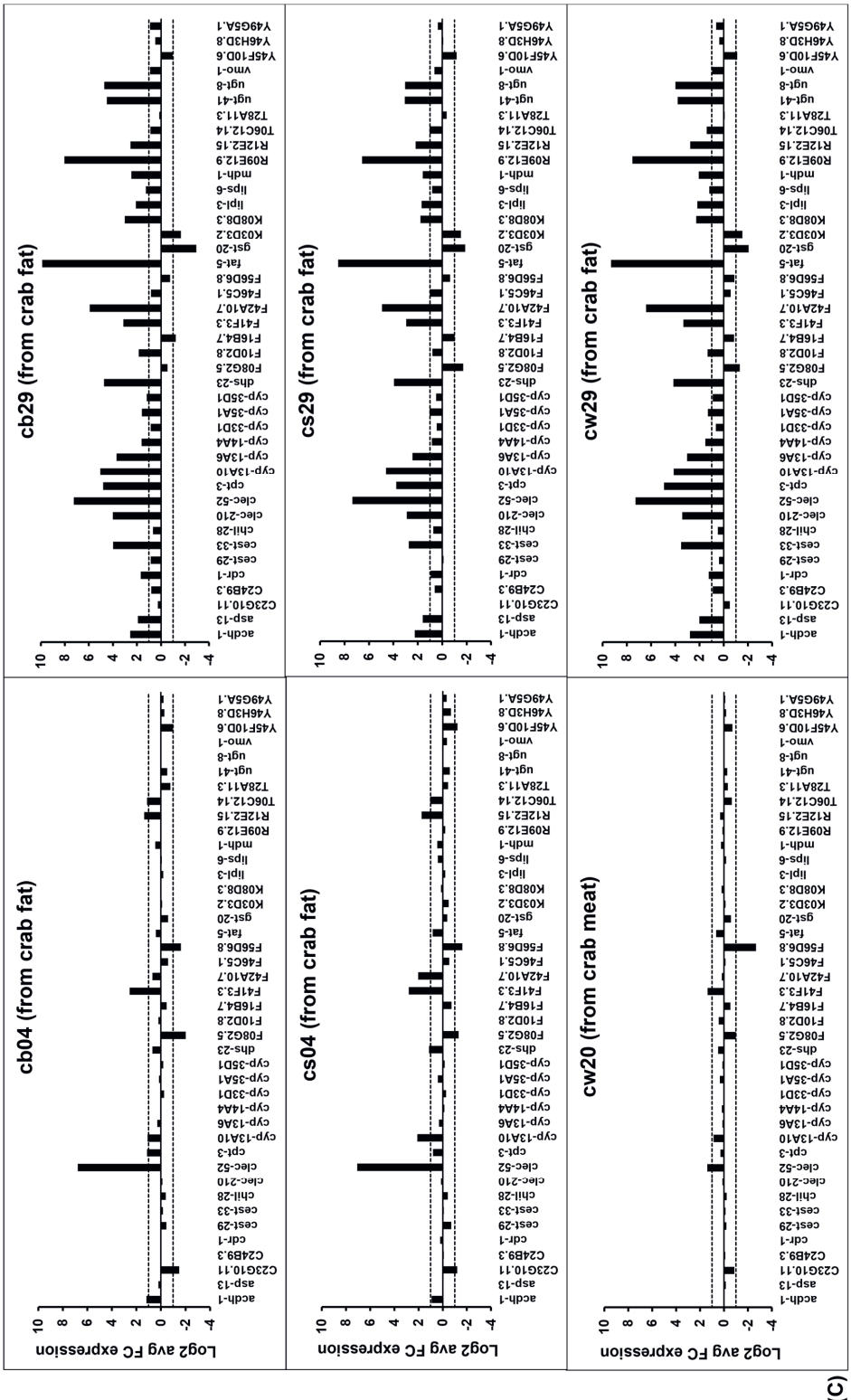
tag-297
vmo-1
wrt-4
Y45F10D.6
Y46H3D.8
Y49G5A.1

(A)


WWTP Influent 1
WWTP Influent 2
WWTP Influent 3
WWTP Influent 4
Log2 avg FC expression
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
acdh-1
asp-13
C23G10.11
cdr-1
cest-29
cest-33
chil-28
clec-52
cpt-3
cyp-13A10
cyp-13A6
cyp-14A4
cyp-33D1
cyp-35A1
cyp-35D1
dhs-23
F08G2.5
F10D2.8
F16B4.7
F41F3.3
F42A10.7
F46C5.1
F56D6.8
fat-5
gst-20
K03D3.2
K08D8.3
lipl-3
lips-6
R09E12.9
R12E2.15
spl-2
T06C12.14
T28A11.3
T28A11.4
tag-297
ugt-41
ugt-8
vmo-1
wrt-4
Y45F10D.6
Y46H3D.8
Y49G5A.1

(B)


cb04 (from crab fat)
cs04 (from crab fat)
cw20 (from crab meat)
cb29 (from crab fat)
cs29 (from crab fat)
cw29 (from crab fat)
Log2 avg FC expression
10
8
6
4
2
0
-2
-4
acdh-1
asp-13
C23G10.11
C24B9.3
cdr-1
cest-29
cest-33
chil-28
clec-210
clec-52
cpt-3
cyp-13A10
cyp-13A6
cyp-14A4
cyp-33D1
cyp-35A1
cyp-35D1
dhs-23
F08G2.5
F10D2.8
F16B4.7
F41F3.3
F42A10.7
F46C5.1
F56D6.8
fat-5
gst-20
K03D3.2
K08D8.3
lipl-3
lips-6
mdh-1
R09E12.9
R12E2.15
T06C12.14
T28A11.3
ugt-41
ugt-8
vmo-1
Y45F10D.6
Y46H3D.8
Y49G5A.1

(C)

Figure 3. Transcriptional fingerprint of exposed *C. elegans* analysed using a bead-based 50-Plex gene expression assay. The transcriptional profiles in nematodes exposed to (A) untreated and treated wastewaters; (B) wastewater inflows to WWTPs from various locations; (C) organic pollutant extracts from swimming crabs collected in Hangzhou bay in China. An exposure sample from a non-receiving surface water was used as control in (A), WWTP Effluents related to each of the influent samples tested was used as control in (B), and an exposure sample with 0.5% DMSO was used as control in (C). The fold change of gene expression level was calculated as the relative mRNA amount of a target gene in a test sample and a control sample, normalized to the housekeeping gene *tbg-1*. Positive values represent up-regulation, negative values represent down-regulation. Gene expression levels between 1 and -1 (log2 average fold change) shown in figure by plotted lines were considered as noise.

Table 2. Differential mRNA expression fold changes in the nematode exposed to the organic pollutants extracted from swimming crab tissues. The table displays log2-transformed mean expression measurements obtained from two independent biological replicate samples using the newly developed bead-based 50-plex assay. Negative value (-) indicates down regulation of the gene.

Targets	cb04	cb29	cs04	cs29	cw20	cw29
acdh-1	1.2	2.6	0.9	2.2	0.0	2.8
asp-13	0.2	1.9	0.0	1.6	-0.2	2.0
C23G10.11	-1.5	0.3	-1.2	-0.1	-0.9	-0.5
C24B9.3	0.0	0.8	-0.1	0.6	-0.1	0.9
cdr-1	-0.1	1.7	0.2	0.9	0.1	1.2
cest-29	-0.4	0.9	-0.7	-0.1	-0.2	0.4
cest-33	-0.2	4.0	-0.1	2.7	-0.1	3.5
chil-28	-0.4	0.7	-0.4	0.7	-0.2	0.5
clec-210	-0.1	4.0	0.1	2.9	0.1	3.4
clec-52	6.8	7.2	7.1	7.3	1.4	7.3
cpt-3	1.1	4.8	0.8	3.8	0.3	4.9
cyp-13A10	1.1	5.0	2.1	4.6	0.9	4.1
cyp-13A6	0.3	3.7	0.3	2.4	0.1	3.0
cyp-14A4	0.1	1.6	-0.1	0.8	0.2	1.5
cyp-33D1	-0.3	0.9	-0.3	0.4	0.0	0.7
cyp-35A1	0.2	1.6	0.4	1.0	0.3	1.3
cyp-35D1	-0.2	1.2	-0.2	0.5	0.1	0.9
dhs-23	0.7	4.7	1.1	4.0	0.5	4.2
F08G2.5	-2.0	-0.5	-1.3	-1.7	-1.0	-1.3
F10D2.8	0.2	1.9	-0.1	0.8	0.4	1.3
F16B4.7	-0.5	-1.2	-0.7	-1.0	-0.5	-0.9
F41F3.3	2.5	3.1	2.8	2.9	1.4	3.3
F42A10.7	0.7	5.9	2.0	4.9	0.2	6.4
F46C5.1	-0.6	0.8	-0.6	1.0	-0.1	-0.6
F56D6.8	-1.6	-0.8	-1.6	-0.7	-2.7	-0.9
fat-5	0.4	9.9	0.8	8.5	0.6	9.3
K03D3.2	-0.1	-1.7	-0.5	-1.5	-0.1	-1.5
K08D8.3	0.0	3.0	0.1	1.8	0.2	2.3
lipl-3	-0.2	2.1	-0.2	1.7	-0.1	2.2
lips-6	-0.1	1.3	0.4	0.8	-0.2	1.2
mdh-1	0.5	2.5	0.5	1.6	0.2	2.1
R09E12.9	0.1	8.0	-0.2	6.6	0.1	7.6
R12E2.15	1.4	2.5	1.7	2.2	0.3	2.8
T06C12.14	1.1	0.9	1.0	1.0	-0.7	1.4
T28A11.3	-0.8	0.2	-0.4	-0.4	-0.3	-0.1
ugt-41	-0.5	4.5	-0.6	3.1	-0.3	3.8
ugt-8	0.1	4.7	-0.1	3.0	0.0	4.0
vmo-1	0.0	0.9	-0.3	0.6	0.0	1.0
Y45F10D.6	-1.0	-1.0	-1.2	-1.2	-0.7	-1.1
Y46H3D.8	-0.3	0.5	-0.7	-0.1	-0.2	0.4
Y49G5A.1	-0.2	0.9	-0.3	0.4	-0.1	0.6

4. Discussion

In this study, a multiplex fluorescent bead-based assay for the nematode *C. elegans* was successfully developed using a QuantiGene™ Plex Assay, i.e. to analyze the expression of target mRNA transcripts in nematodes exposed to model contaminants and to polluted and relatively clean water samples. Marker genes were selected for AFB1, B(a)P, and PCB1254 and those responding to polluted wastewater samples but not to clean field water samples (treated wastewater or surface water). The 50-plex assay was successfully applied to fingerprint the possible presence of pollutants in samples. Generally, the assay showed a good performance as shown by low background signals and a good correlation with microarray and RT-qPCR analysis. This assay is suited to quantify expression levels of target mRNAs directly in crude lysates of nematodes (e.g., the worms directly exposed to unconcentrated water samples).

The profiling outcomes showed that wastewater samples were contaminated with pollutants that regulated the expression of several of the selected target genes, while relatively clean samples including treated WWTP effluent and the receiving surface water not or hardly affected the expression of these genes. Further analysis of WWTP influent samples from four different locations (including three new samples not tested previously with microarray or RT-qPCR) showed comparable profiles of transcriptional effects, confirming the validity of the mRNA markers analysed. Interestingly, *spl-2*, *clec-52*, C23G10.11 and F46C5.1 previously found to be the most upregulated genes in wastewaters [Karengera et al., under review], were also among the top induced transcripts in all WWTP influents analysed by 50-plex assay. The nematode *spl-2* and *clec-52* respectively encode sphingosine phosphate lyase and C-type lectin (CLEC) proteins which are involved in nematode defense response to bacterial infection [35, 36]. This suggests that the tested wastewater samples also were contaminated with pathogens, which is in line with early microarray findings [Karengera et al., under review]. The function of proteins encoded by C23G10.11 and F46C5.1 is not yet known.

Gene expression profiling of pollutants in extracts from swimming crab tissues using the newly developed 50-Plex assay identified many responsive genes among the mRNA markers tested. The assay showed that the edible fat parts were polluted with contaminants that affected many of the target genes tested whereas the relatively clean crab meat did not show significant effects on these genes. Transcriptional effects of such extracts in *C. elegans* were not previously determined so our results cannot be compared to other studies. The extracts were however assessed using a battery of other bioassays and various toxic effects were confirmed in these samples [Bao et al, in preparation]. The most potent samples in our study (cb29, cs29 and sw29) also had the highest toxic potency in the other bioassays (**Suppl. Table S3**). Since the nematode genes responding to B(a)P and PCB were already evaluated previously [6], eleven mRNAs of those genes were included in the present 50-

plex assay as biomarkers for fingerprinting crab food contaminated with mixtures of organic pollutants. The extracts from the lipid part (but not meat) of swimming crabs induced similar effects as B(a)P and PCB as pure compounds, indicating that the swimming crabs were indeed polluted with toxic substances belonging to these classes of compounds. Among the transcripts predicted to be affected by these samples, our bioassay revealed the upregulation of *dhs-23*, *cyp-13A6*, *cyp-13A10*, *ugt-8*, *ugt-41*, *spl-2*, *cest-33*, *clec-210* and R09E12.9 which are all xenobiotic response genes [6, 37]. Interestingly, the transcripts that were originally included in the assay as markers of wastewater pollutants [Karengera et al., under review], such as *fat-5*, *clec-52*, *acdh-1*, *lipl-3*, *asp-13*, *cpt-3*, F42A10.7, F41F3.3, K08D8.3 and R12E2.15, were also considerably upregulated in the extracts from crab tissues. The proteins encoded by these genes play various functions in nematode such as nematode fatty acid metabolic process by *fat-5* [38]*, lipl-3* [39]*,* and *cpt-3* [40] or nematode dietary response by *acdh-1* [41] and *asp-13* [42]. Further transcriptomic analysis of the nematode genome-wide response to these samples could show more insights on cellular mechanisms affected by these pollutants or whether the responses were related to crab fat components that were not removed from the extracts.

In summary, we have successfully developed a multiplex assay for gene expression profiling in *C. elegans* without the need of RNA extraction and purification. A panel of mRNA transcripts was effectively assembled as biomarkers and incorporated in a multiplex gene expression assay for detecting bioactive contaminants in polluted samples. The results from this study showed that the newly developed multiplex approach offers many advantages in comparison with the time-consuming RT-qPCR test, especially the direct measurement of target mRNAs in crude nematode lysates. The use of direct exposure of nematodes to even severely polluted water samples like wastewater without the need to pretreat or to dilute the samples in combination with a fast analysis of the genomic responses makes this a potentially interesting bioassay for environmental quality monitoring. We also successfully applied our bioassay to fingerprint the nematode transcriptional responses to mixtures of organic pollutants in the extracts from swimming crab tissues. Our study demonstrated that RNA transcripts of the nematode genes responding to pollutants in water or in crab tissues can be used as pollution indicators. It would therefore be interesting to test more polluted environmental samples to develop and validate more transcriptional biomarkers for monitoring of bioactive contaminants. Further, given that our multiplex gene expression assay uses magnetic beads, the applicability of this bioassay can be further improved by automating the procedure, especially washing steps, which would make high throughput screening easier.

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Supporting information of chapter 5

S1. Pilot study with 14-Plex assay

1. Introduction

A pilot experiment was carried out with the goal to develop a novel multiplex gene expression assay method that can measure mRNA transcripts directly from crude lysates of nematodes. The target genes analyzed in this assay were selected from our previous transcriptomics studies conducted with microarrays and RT-qPCR. These genes were shown before to be regulated in the nematodes in response to exposure to various contaminants. In this pilot study we formulated the design and protocols of the fluorescent bead-based multiplex assay with the nematode *C. elegans* as model organism. The outcome of this analysis was subsequently used in the full-scale assay as described in the manuscript.

2. Methods

The pilot study was conducted using a probe set designed with different 14 target mRNAs (i.e., 14-Plex assay) (**Table 1**). The multiplex panel containing target-specific probe sets and magnetic capture beads were manufactured and supplied (premixed and ready to use) by Thermo Fisher. The experiment involved the nematodes (10,000 per sample) treated with 0.5% DMSO (control) and 30µM of AFB1, B(a)P, or PCB1254. Nematode lysis was conducted by using bead beating procedure on a Precellys® Evolution homogenizer. For each lysate sample, four dilutions (1:1, 1:4, 1:16, and 1:64) were prepared and used for further analysis. A sample containing pure RNA (500 ng) isolated from the nematodes exposed to AFB (30µM) was included in the analysis and was tested in four dilutions (1:1, 1:5, 1:25, and 1:125). The sample responses were read with the MAGPIX™ system equipped with Luminex xPONENT software (4.2.1705.0). Derived raw data were presented as median fluorescence intensity (MFI) for one biological replicate analyzed in two technical replicates. Detailed information on the experimental procedures is provided in manuscript.

Table 1. Target mRNAs in C. elegans analysed using bead-based 14-Plex gene expression assay. Probe sets were designed to specifically hybridize to each of the 14 targets.

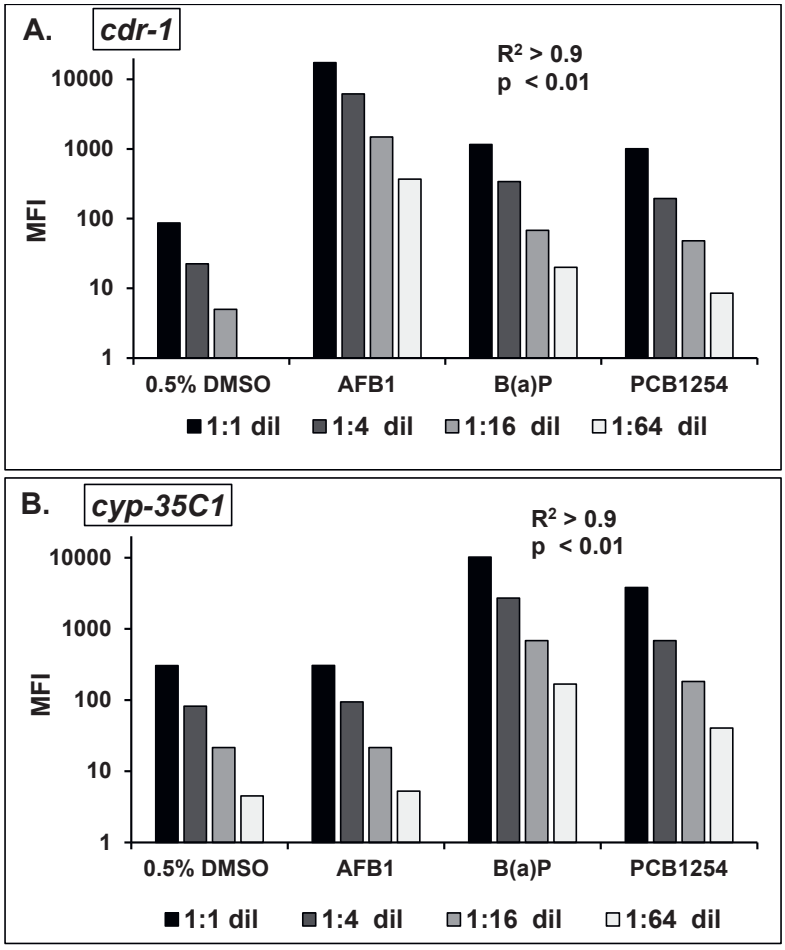
Target Symbol	Accession Number	Sequence Length	Probe set region
cest-33	NM_072220	1773	72-553
cyp-35A5	NM_071691	1741	457-881
cyp-35C1	NM_171550	1543	233-690
F25D1.5	NM_073303	1091	403-873
cyp-14A4	NM_077806	1554	102-575
F38C2.1	NM_001307174	357	25-338
cyp-13A6	NM_063712	1759	344-814
clec-210	NM_071454	1182	597-1025
cyp-35A1	NM_001356694	1546	37-521
cdr-1	NM_074585	948	409-874
par-5	NM_069834	1106	422-814
cyp-35D1	NM_074643	1576	44-586
cdr-2	NM_073713	837	23-449
B0205.14	NM_001047166	364	68-330

3. Results and discussion

Nematode samples were successfully lysed by the bead beating homogenization setup utilized in this procedure. This was confirmed by a visual observation of the lysates though a binocular microscope showing that all the nematodes were entirely dissolved. The signals (MFI) measured in the tested samples were generally within the suggested range between 70 – 130 of the % recovery values (**Suppl. Table S3.B**). The precision as coefficients of variation (%CV) of technical replicates was overall found to be below the limit of 20%. The samples with a %CV above 20% were mostly the highly diluted ones with responses close to the background (**Suppl. Table S3.C**). Saturation (the upper threshold for the intensity of the signal) occurred for few target mRNAs, such as *cest-33* (Esterase CM06B1) or *par-5* (14-3-3-like protein) genes in the undiluted (1:1) samples.

For 1:16 and 1:64 dilution samples (especially for the untreated nematodes), the measured signals (MFI) were close to the maximum background signals or even falling below. Maximum background parameter is the upper limit for which a value is considered to be within the background (noise). In this pilot assay, any signal below 10 MFI was considered as a background. Decrease in signals (MFI) was especially observed for the transcripts of the target genes that are known to express less in the absence of exposure (i.e., in control nematodes). For instance, signals measured for the target transcripts of *cdr-1* (cadmium responsive gene) and *cyp-35C1* (cytochrome P450 gene) were relatively very low in the nematodes exposed to solvent control (0.5% DMSO) especially the 1:64 dilution. The expression level of these genes was considerable in the exposed animals and could even

be quantified in the most diluted samples (**Figure 1)**. Additional data from pilot study are provided as supplementary information. Overall, bead-beating homogenization procedure was found to be efficient for nematode lysis. Hence, we considered the protocols suitable to proceed to the full-scale experiment.



Suppl. Figure S1. 14-Plex assay measurements. Bar charts correspond to the average net median fluorescence (MFI) values (displayed at log10-scale) for 2 target mRNAs of genes cdr-1 (Fig. 1A) and cyp-35C1(Fig. 1B) The measurements were run on a dilution series (1:1, 1:4, 1:16, and 1:64) of the nematode homogenates. Nematodes were treated with 0.5% DMSO (as control) or 30 µM of AFB1, BaP, or PCB1254. Significant positive correlation ($R^2 > 0.9$, $p < 0.01$) was found between the measured MFI signals and dilutions series.

Suppl. Table S1.A. Background median fluorescence intensities (MFI) signals. Assay signals below the Limit of Detection (LOD) were not used to draw quantitative conclusions about gene expression. LOD is a theoretical limit calculated based on a multiple of background standard deviations above the average background signal.

	cest-33	F10D2.8	F56D6.8	vmo-1	F16B4.7	acdh-1	R12E2.15	cyp-14A4	R09E12.9	T28A11.3	fat-5	lipl-3	cyp-13A6	Y45F10D.6	T28A11.4	C23G10.11	clec-210	dhs-23	Y46H3D.8	gst-20	mdh-1	cyp-35A1	T06C12.14	cdr-1	par-5
Background	2	2	3	3	2	2	2	3	3	2	2	3	3.5	2	3	2	2	3	3	3	4	5	3	2	3
Background	3	2	3	2	2	2	2	2	3	2	2	3	4	2	4	3	2	3	3	3	3	6	3	2	3
Measurment limits																									
Avg	2.5	2.0	3.0	2.5	2.0	2.0	2.0	2.5	3.0	2.0	2.0	3.0	3.8	2.0	3.5	2.5	2.0	3.0	3.0	3.0	3.5	5.5	3.0	2.0	3.0
StDev	0.7	0.0	0.0	0.7	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.4	0.0	0.7	0.7	0.0	0.0	0.0	0.0	0.7	0.7	0.0	0.0	0.0
LOD	4.6	2.0	3.0	4.6	2.0	2.0	2.0	4.6	3.0	2.0	2.0	3.0	4.8	2.0	5.6	4.6	2.0	3.0	3.0	3.0	5.6	7.6	3.0	2.0	3.0

Suppl. Table S1.A. (Continued)

	clec-52	cyp-35D1	K03D3.2	rpl-6	F42A10.7	K08D8.3	F41F3.3	asp-13	F46C5.1	col-160	lips-6	Y49G5A.1	cyp-13A10	F08G2.5	tag-297	chil-28	ugt-41	tbg-1	cyp-33D1	cpt-3	wrt-4	C24B9.3	cest-29	spl-2	ugt-8
Background	3	3	4	4	3	3	3	4	3	5	5	3	3	3	3	4	4	5	4	3	5	5	5	5	6
Background	3	3	4	4	3	3	3	3	3	6	4	3	3	3	3	4	4	5	3	3	4	5	4	5	6
Measurment limits																									
Avg	3.0	3.0	4.0	4.0	3.0	3.0	3.0	3.5	3.0	5.5	4.5	3.0	3.0	3.0	3.0	4.0	4.0	5.0	3.5	3.0	4.5	5.0	4.5	5.0	6.0
StDev	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.7	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.7	0.0	0.7	0.0	0.0
LOD	3.0	3.0	4.0	4.0	3.0	3.0	3.0	5.6	3.0	7.6	6.6	3.0	3.0	3.0	3.0	4.0	4.0	5.0	5.6	3.0	6.6	5.0	6.6	5.0	6.0

Suppl. Table S1.B. Average net median fluorescence intensities (Av Net MFI) and Dilution Linearity. Linearity calculates the % recovery for a dilution series ("% recovery = observed value/expected value × 100%). The data whose dilution recovery values falling between 70–130 of the % recovery values were considered to be within the linear range of the assay (green-highlighted cells indicates those outside the linear range, whereas red-highlighted cells contain MFI outside the detection range of the assay, i.e. > 10 MFI or < 30,000 MFI).

Expsoure with surface water (SW)																								
Sample	**cest-33**	**F10D2.8**	**F56D6.8**	**vmo-1**	**F16B4.7**	**acdh-1**	**R12E2.15**	**cyp-14A4**	**R09E12.9**	**T28A11.3**	**fat-5**	**lipl-3**	**cyp-13A6**	**Y45F10D.(**	**T28A11.4**	**C23G10.11**	**clec-210**	**dhs-23**	**Y46H3D.8**	**gst-20**	**mdh-1**	**cyp-35A1**	**T06C12.14**	**cdr-1**	**par-5**
SW 1:1	1164.5	165	23313	296.5	17529	6337	416	328	380	2785	238	551	601.25	1761.5	717.5	666	268	524	309	27333	23038.5	393.5	27545	619	26968
SW 1:5	222.5	30.5	6900.5	62.5	3580	1080	107	71.5	86	556	72	132.5	124.25	334.5	149.5	136.5	65	97.5	76.5	21427.5	5663.5	77.5	20434	123	27252
SW 1:25	24.5	7	1003	6.5	639	154	18	8.5	11	89.5	9	19	16.25	56.5	20.5	14.5	6	11	9.5	2609	651	11.5	1596	17	3880
Dilution Lineality																									
R-squared	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.62	1.00	1.00	0.66	1.00	0.38
SW 1:5	96	92	148	105	102	85	129	109	113	100	151	120	103	95	104	102	121	93	124	392	123	98	371	99	505
SW 1:25	55	115	73	52	89	71	84	59	64	80	63	72	65	84	69	53	46	56	62	61	57	74	39	69	71
Expsoure with community wastewater																									
Sample	**cest-33**	**F10D2.8**	**F56D6.8**	**vmo-1**	**F16B4.7**	**acdh-1**	**R12E2.15**	**cyp-14A4**	**R09E12.9**	**T28A11.3**	**fat-5**	**lipl-3**	**cyp-13A6**	**Y45F10D.(**	**T28A11.4**	**C23G10.11**	**clec-210**	**dhs-23**	**Y46H3D.8**	**gst-20**	**mdh-1**	**cyp-35A1**	**T06C12.14**	**cdr-1**	**par-5**
Community 1:1	1828.5	1832	10136	602	6170	26647	6058	360.5	419	1762	544	2271	986.75	246	302.5	15540	685	563	301	19368	14475	399.5	10078	595.5	28244
Community 1:5	284.5	415	1822	123.5	1085	7619.5	1439	81.5	99	390.5	114	424	172.25	74	60.5	3282.5	157	91	88	8921	8327.5	76	2464	122	24614
Community 1:25	52.5	51.5	314.5	19.5	184	1095	279	11.5	16	70	17.5	111.5	36.25	14	7.5	592	25	13	16	1208	1288.5	15	367	22	4431
Dilution Lineality																									
R-squared	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	0.99	0.92	0.84	1.00	1.00	1.00	0.54
Community 1:5	78	113	90	103	88	143	119	113	118	111	105	93	87	150	100	106	115	81	146	230	288	95	122	102	436
Community 1:25	92	62	86	79	85	72	97	71	81	90	77	131	105	95	62	90	80	71	91	68	77	99	74	90	90

Suppl. Table S1.B. (Continued)

Expsoure with surface water (SW)																								
Sample	**clec-52**	**cyp-35D1**	**K03D3.2**	**rpl-6**	**F42A10.7**	**K08D8.3**	**F41F3.3**	**asp-13**	**F46C5.1**	**col-160**	**lips-6**	**Y49G5A.1**	**cyp-13A10**	**F08G2.5**	**tag-297**	**chil-28**	**ugt-41**	**tbg-1**	**cyp-33D1**	**cpt-3**	**wrt-4**	**C24B9.3**	**cest-29**	**spl-2**	**ugt-8**
SW 1:1	822	343	17704	23324	448	597	2242	1769.5	1905	21016.5	205.5	1187.5	728	17440	309	657	314	6511	397.5	126	63.5	27248	211.5	281	256.5
SW 1:5	179	68.5	3811.5	39135	90.5	137	453	343	350	38417.5	49	206.5	131.5	3337	73	142	56	1168	76.5	44.5	16	18162	49.5	69.5	54
SW 1:25	23	10	581	24470.5	11	16	98	50.5	65	18954.5	5.5	29	18.5	574	12	17	9.5	168	9	7	4.5	1906.5	7.5	8	8
Dilution Lineality																									
R-squared	1.00	1.00	1.00	0.18	1.00	1.00	1.00	1.00	1.00	0.07	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.98	1.00	0.75	1.00	1.00	1.00
SW 1:5	109	100	108	839	101	115	101	97	92	914	119	87	90	96	118	108	89	90	96	177	126	333	117	124	105
SW 1:25	64	73	76	313	61	58	108	74	93	247	56	70	70	86	82	60	85	72	59	79	141	52	76	58	74
Expsoure with community wastewater																									
Sample	**clec-52**	**cyp-35D1**	**K03D3.2**	**rpl-6**	**F42A10.7**	**K08D8.3**	**F41F3.3**	**asp-13**	**F46C5.1**	**col-160**	**lips-6**	**Y49G5A.1**	**cyp-13A10**	**F08G2.5**	**tag-297**	**chil-28**	**ugt-41**	**tbg-1**	**cyp-33D1**	**cpt-3**	**wrt-4**	**C24B9.3**	**cest-29**	**spl-2**	**ugt-8**
Community 1:1	10814	331	2908.5	7875.5	705	924	12974.5	7453	26753	20722.5	1397.5	207	7257	1340.5	1133	5531	369	6842	895.5	207.5	756.5	17995	1235	5441.5	367
Community 1:5	2210	95	677	42151	143.5	190	3481	1297	9678	17470.5	226.5	38	1246	231.5	255	1095	72	1219.5	154.5	52.5	148.5	34013	192.5	1060	65.5
Community 1:25	411	17	140	29731	23	35	601	245.5	1191	2517	38.5	9	201.5	50	52	210.5	11	221	23.5	8.5	25	7062	34.5	199	10
Dilution Lineality																									
R-squared	1.00	0.99	1.00	0.75	1.00	1.00	1.00	1.00	0.97	0.56	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00
Community 1:5	102	144	116	2676	102	103	134	87	181	422	81	92	86	86	113	99	98	89	86	127	98	945	78	97	89
Community 1:25	93	89	103	353	80	92	86	95	62	72	85	118	81	108	102	96	76	91	76	81	84	104	90	94	76

Suppl. Table S1.C. Bead counts (the minimum number of beads that should be counted for each target in each well was set at 35 beads/well (highlighted cells indicate the bead counts below a limit of 35 beads per target per well)

Sample	cest-33	F10D2.8	F56D6.8	vmo-1	F16B4.7	acdh-1	R12E2.15	cyp-14A4	R09E12.9	T28A11.3	fat-5	lipl-3	cyp-13A6	Y45F10D.6	T28A11.4	C23G10.11	clec-210	dhs-23	Y46H3D.8	gst-20	mdh-1	cyp-35A1	T06C12.14	cdr-1	par-5
SW 1:1	84	84	111	121	103	110	91	150	101	123	124	121	115	130	122	116	107	101	107	154	95	119	121	144	99
SW 1:5	101	72	108	117	113	103	108	120	113	102	125	118	109	144	105	120	139	112	122	152	133	111	121	145	116
SW 1:25	88	85	99	111	92	107	82	112	94	108	117	105	109	110	117	109	90	99	132	132	128	95	95	123	112
Community 1:1	13	5	31	10	30	30	41	45	21	29	39	11	10	23	21	22	25	21	13	17	8	14	8	16	39
Community 1:5	96	74	107	98	96	96	101	104	95	116	108	103	81	110	101	121	102	99	117	123	107	106	111	119	96
Community 1:25	103	84	94	114	93	125	110	142	127	123	138	114	109	117	124	122	113	119	124	158	125	106	103	149	109

Suppl. Table S1.B. (Continued)

Sample	clec-52	cyp-35D1	K03D3.2	rpl-6	F42A10.7	K08D8.3	F41F3.3	asp-13	F46C5.1	col-160	lips-6	Y49G5A.1	cyp-13A10	F08G2.5	tag-297	chil-28	ugt-41	tbg-1	cyp-33D1	cpt-3	wrt-4	C24B9.3	cest-29	spl-2	ugt-8	Total Events
SW 1:1	125	93	179	134	102	131	111	135	66	137	123	126	71	131	97	106	131	198	113	89	49	111	113	103	110	5737
SW 1:5	136	112	156	157	124	118	125	116	53	152	122	124	54	157	92	131	177	165	135	106	50	131	115	92	109	5938
SW 1:25	126	119	129	162	106	94	104	130	55	123	121	109	78	100	94	100	138	158	116	90	49	98	125	95	118	5388
Community 1:1	9	11	18	6	19	43	22	24	27	43	13	29	27	36	35	15	34	40	38	24	7	20	18	22	17	1139
Community 1:5	127	106	139	144	92	109	113	106	59	94	112	91	52	110	91	101	109	134	123	94	57	88	95	87	96	5116
Community 1:25	147	152	160	180	99	139	115	155	61	136	121	112	60	136	125	110	149	159	139	114	46	117	128	131	132	6068

Suppl. Table S1.D. Expression stability of candidate reference genes. The gene whose expression level varied > 20% or whose MFI signal saturates was considered not stable and not suitable reference gene (green-highlighted cells indicates stable gene expression, whereas red-highlighted cells show the saturation of MFI signals measured)

	mdh-1	**par-5**	**rpl-6**	**tbg-1**
SW 1:1	23038.5	26968	23324	6511
Community 1:1	14475	28244	7875.5	6842
%CV	32.28	3.27	70.03	3.51
	mdh-1	**par-5**	**rpl-6**	**tbg-1**
SW 1:5	5663.5	27252	39135	1168
Community 1:5	8327.5	24614	42151	1219.5
%CV	26.93	7.19	5.25	3.05
	mdh-1	**par-5**	**rpl-6**	**tbg-1**
SW 1:25	651	3880	24470.5	168
Community 1:25	1288.5	4431	29731	221
%CV	46.48	9.38	13.73	19.27

Suppl. Table S2.A Background MFI signals

Sample	cest-33	F10D2.8	F56D6.8	vmo-1	F16B4.7	acdh-1	R12E2.15	cyp-14A4	R09E12.9	T28A11.3	fat-5	lipl-3	cyp-13A6	Y45F10D.6	T28A11.4	C23G10.11	clec-210	dhs-23	Y46H3D.8	gst-20	mdh-1	cyp-35A1	T06C12.14	cdr-1	par-5
Background	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	4.0	3.0	3.0	3.0	5.0	3.0	4.0	3.0	3.0	3.0	4.0	4.0	4.0	8.0	3.0	3.0	3.0
Background	3.5	3.0	3.0	3.0	3.0	3.0	3.0	3.0	4.0	3.0	3.0	3.0	6.0	3.0	4.0	3.0	3.0	4.0	4.0	4.0	4.0	8.0	4.0	3.0	3.0
Background	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.5	3.0	3.0	3.0	5.0	3.0	4.0	3.0	3.0	3.0	3.0	3.0	4.0	8.0	3.0	3.0	3.0
Measurment limits																									
Avg	3.2	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.8	3.0	3.0	3.0	5.3	3.0	4.0	3.0	3.0	3.3	3.7	3.7	4.0	8.0	3.3	3.0	3.0
StDev	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.6	0.6	0.6	0.0	0.0	0.6	0.0	0.0
LOD	4.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	4.7	3.0	3.0	3.0	7.1	3.0	4.0	3.0	3.0	5.1	5.4	5.4	4.0	8.0	5.1	3.0	3.0

Suppl. Table S2.A. (Continued)

Sample	clec-52	cyp-35D1	K03D3.2	rpl-6	F42A10.7	K08D8.3	F41F3.3	asp-13	F46C5.1	col-160	lips-6	Y49G5A.1	cyp-13A10	F08G2.5	tag-297	chil-28	ugt-41	tbg-1	cyp-33D1	cpt-3	wrt-4	C24B9.3	cest-29	spl-2	ugt-8
Background	3.0	4.0	4.0	4.0	4.0	3.0	3.0	4.0	4.0	9.5	5.0	3.0	4.0	3.0	3.0	4.0	5.0	6.0	4.0	4.0	5.0	6.0	5.0	6.0	7.0
Background	3.0	4.0	4.0	5.0	4.0	4.0	4.0	4.0	4.0	8.0	5.5	4.0	3.0	4.0	4.0	5.0	5.0	6.0	4.0	4.0	6.0	7.0	5.0	6.0	7.0
Background	3.0	4.0	5.0	5.0	4.0	3.0	4.0	4.0	3.0	8.0	5.0	4.0	3.0	3.0	4.0	5.0	5.0	6.0	4.0	4.0	6.0	7.0	5.0	6.0	7.0
Measurment limits																									
Avg	3.0	4.0	4.3	4.7	4.0	3.3	3.7	4.0	3.7	8.5	5.2	3.7	3.3	3.3	3.7	4.7	5.0	6.0	4.0	4.0	5.7	6.7	5.0	6.0	7.0
StDev	0.0	0.0	0.6	0.6	0.0	0.6	0.6	0.0	0.6	0.9	0.3	0.6	0.6	0.6	0.6	0.6	0.0	0.0	0.0	0.0	0.6	0.6	0.0	0.0	0.0
LOD	3.0	4.0	6.1	6.4	4.0	5.1	5.4	4.0	5.4	11.1	6.0	5.4	5.1	5.1	5.4	6.4	5.0	6.0	4.0	4.0	7.4	8.4	5.0	6.0	7.0

Suppl. Table S2.B. Average net median fluorescence intensities (Av. Net. MFI).Highlighted cells indicate the saturation of MFI signals measured)

Sample	cest-33	F10D2.8	PS6D6.8	vmo-1	F16B4.7	acdh-1	R12E2.15	cyp-14A4	R09E12.9	T28A11.3	fat-5	lipl-3	cyp-13A6	Y45F10D.6	T28A11.4	C23G10.11	clec-210	dhs-23	Y46H3D.8	gst-20	mdh-1	cyp-35A1	T06C12.14	cdr-1	par-5
SW1 (batch1)	182	32	8652	62	3612	1071	87	76	71	609	64	151	107	324	150	124	72	93	71	30827	6561	78	27438	127	35062
SW2_(batch1)	347	47	11616	97	5888	1764	172	101	145	996	107	201	167	612	239	239	88	138	108	29562	8858	99	27276	197	34588
SW3 (batch1)	342	43	12501	94	6660	1891	167	94	151	985	96	203	181	647	269	175	92	184	122	31584	9678	99	26762	160	33663
H_(batch1)	457	147	3453	366	1538	37131	598	108	151	556	88	1016	201	95	89	4478	86	121	118	19087	12046	97	5708	152	34646
N_(batch1)	403	64	2983	464	1001	43474	815	97	119	417	83	1009	214	84	81	5340	77	114	118	11026	12394	94	2358	190	30315
C_(batch1)	405	509	4287	187	1700	10374	2403	89	124	614	118	720	222	82	65	6062	201	107	107	12582	11429	93	3849	153	27623
I_(batch1)	219	1137	7348	120	1791	5737	614	68	98	560	66	747	142	147	77	1564	54	67	78	17501	8027	65	7837	148	25648
E_(batch1)	265	41	15458	66	5367	1821	210	72	121	926	59	161	136	565	234	223	53	139	87	31368	8158	62	23431	163	30804
SW1 (batch1)	224	38	11079	66	4792	1135	110	74	95	631	67	143	133	406	171	146	77	119	98	31031	7576	81	28477	130	33381
SW2_(batch1)	324	58	12473	100	5986	1747	182	117	130	1018	92	207	175	554	232	274	99	134	112	31519	10278	118	28607	201	35035
SW3 (batch1)	382	60	13652	106	7204	1872	170	103	144	1092	109	189	203	636	266	193	88	169	117	33304	10281	100	30036	195	34941
H_(batch1)	450	195	4236	407	1581	39750	780	111	146	552	114	1204	227	106	92	5294	103	160	117	20061	13382	122	6363	176	36553
N_(batch1)	418	82	3074	540	1085	43808	871	127	135	468	123	1169	265	97	93	5935	100	137	150	12174	14244	111	2719	209	33038
C_(batch1)	519	657	3754	205	1719	11702	2451	121	161	684	157	752	260	109	90	6723	230	162	131	14248	12904	106	4458	206	29897
I_(batch1)	320	157	7667	138	2210	6842	722	88	121	667	83	1058	181	147	93	1762	89	94	97	20161	9974	92	9916	208	30872
E_(batch1)	293	31	13862	56	4854	1592	180	58	110	801	65	181	111	505	169	203	48	125	76	28653	7669	60	21336	164	28084
SW1 (batch2)	194	44	7281	91	3895	751	57	105	87	767	80	134	126	460	232	264	100	114	106	30464	5233	97	27056	122	29391
SW2_(batch2)	328	56	7888	125	5287	1167	71	120	118	1173	122	242	192	809	330	493	119	166	172	32118	8562	126	29002	177	30684
SW3_(batch2)	329	55	9567	111	4390	1042	123	111	114	1031	87	186	172	864	295	310	91	157	167	32389	7254	113	25402	186	29102
H_(batch2)	420	172	2475	663	1351	27918	789	174	172	747	164	776	270	172	144	7415	142	187	201	16081	9736	171	3982	182	30188
N_(batch2)	390	88	1818	504	1013	35424	480	143	127	539	164	787	230	120	126	7720	120	128	159	8862	10889	129	1444	178	28708
C_(batch2)	592	623	2607	256	2235	7286	3005	167	169	1030	274	884	352	174	173	9580	294	221	185	14525	14244	158	4803	173	30842
I_(batch2)	371	115	6218	187	3446	3686	1042	126	146	1096	126	931	255	284	147	5887	106	152	160	22171	11361	122	14089	182	30996
E_(batch2)	351	41	9123	108	5979	1415	122	94	131	1301	104	221	172	850	325	607	83	136	138	30847	9071	87	25205	175	29275
SW1 (batch2)	169	45	6686	75	4102	633	67	84	76	791	71	128	96	537	205	289	68	99	99	31570	5694	84	25666	127	28038
SW2_(batch2)	357	72	7884	113	5355	1122	70	135	129	1186	121	226	188	764	377	479	115	172	183	32929	8303	138	30484	201	30132
SW3_(batch2)	270	58	9755	117	4855	1053	142	115	116	1013	120	203	175	920	308	276	100	148	152	32597	7158	111	26182	172	29633
H_(batch2)	418	173	2593	433	1467	28108	856	159	154	743	156	889	258	182	160	7620	154	171	209	16341	10231	170	3681	196	30984
N_(batch2)	414	76	1983	603	1017	34508	493	156	148	682	195	820	241	138	133	7983	112	186	187	8943	10822	144	1601	172	28580
C_(batch2)	549	659	2544	247	2179	6656	3033	173	170	952	273	883	340	179	166	9117	287	168	201	13664	13887	155	4922	211	30997
I_(batch2)	460	135	6229	206	3716	4114	1106	140	157	1102	138	1031	265	301	187	5726	136	156	177	24926	12611	153	16042	208	31457
E_(batch2)	321	62	8592	90	5954	1234	111	101	140	1282	91	243	160	875	330	551	77	151	113	34329	8844	88	27100	181	33011
0.5%DMSO (batch1)	230	27	766	29	2993	7462	75	33	44	380	24	147	70	529	21	101	21	61	35	48432	10860	36	1183	47	35367
AFB1(30uM)_(batch1)	44282	45	197	67	2456	11702	100	31770	161	480	66	274	770	871	48	69	57	480	53	45189	13008	63	660	24387	38852
BaP(30uM)_(batch1)	2141	45	393	53	2831	10460	117	61	168	450	62	197	168	609	45	102	94	377	58	46178	14315	2680	905	1616	38647
PCB1254(30uM) (batch1)	4028	47	220	47	1893	8548	96	43	16777	464	34	166	1523	558	29	83	561	15833	46	45413	12456	1219	509	482	37003
0.5%DMSO_(batch2)	353	50	1145	62	3811	3976	77	69	94	563	64	381	96	852	58	177	59	104	77	47057	12295	71	1381	90	39330
AFB1(30uM)_(batch2)	44288	59	266	66	2005	5056	96	27828	194	586	75	341	810	1095	46	96	69	345	100	45311	13092	76	427	15817	35705
BaP(30uM)_(batch2)	2279	44	951	61	4131	3785	150	66	265	630	71	288	195	579	55	243	190	416	68	44980	12366	2446	1414	1231	35937
PCB1254(30uM)_(batch2)	4787	51	492	58	2088	2959	101	55	28441	633	54	262	1398	578	43	139	1065	19739	71	44196	11246	1097	721	440	34720
0.5%DMSO (batch1)	248	38	872	40	3084	6807	111	39	54	347	32	161	73	575	25	96	36	75	44	48445	13130	42	1535	58	35215
AFB1(30uM)_(batch1)	44634	68	190	65	2309	11578	120	31911	150	531	57	257	808	920	34	62	48	434	78	46082	14033	61	682	23149	36756
BaP(30uM)_(batch1)	1872	38	368	50	2637	10544	105	46	173	436	37	185	162	623	31	123	75	359	45	44775	13574	2503	798	1567	36603
PCB1254(30uM) (batch1)	3917	49	203	51	2017	8873	111	36	17267	505	38	173	1415	606	31	102	622	16172	52	44658	12734	1269	534	487	34533
0.5%DMSO_(batch2)	380	38	1037	48	3215	3450	93	61	80	543	64	326	90	871	49	170	48	82	62	45358	11886	67	1052	90	32222
AFB1(30uM)_(batch2)	43480	57	244	62	1815	4745	71	26039	172	623	58	279	738	945	37	94	47	351	77	45154	11815	57	353	14255	33772
BaP(30uM)_(batch2)	2163	40	969	47	4045	3777	135	64	276	572	65	267	161	534	51	242	191	399	73	41666	11398	2405	1365	1179	32772
PCB1254(30uM)_(batch2)	4471	51	504	60	2217	3143	83	51	26577	568	68	195	1408	541	47	164	958	18925	72	42440	11899	1267	766	492	32541
0.5%DMSO (SedBatch1)	172	27	248	41	1004	4420	68	24	31	97	21	139	35	719	14	95	21	50	49	42658	6026	19	316	33	24489
ch04-2ah_(batch1)	213	43	131	79	1321	9979	154	27	52	83	36	159	58	485	19	37	22	152	79	43481	9119	23	945	53	25545
cw29--2ah_(batch1)	3773	123	209	160	795	19862	841	97	14044	154	30145	854	500	527	54	109	448	1893	136	14946	17718	81	1031	157	21629
ch29--2ah_(batch1)	4973	208	235	162	601	21218	763	109	19489	208	37055	873	863	580	57	202	669	2957	156	8773	44600	92	687	225	23904
cs04--2ah_(batch1)	204	36	106	52	816	6289	186	15	35	80	38	161	36	426	17	52	19	185	42	45956	7216	19	809	60	23169
cs29--2ah_(batch1)	2112	89	244	136	628	14546	473	57	6858	106	17141	595	316	491	44	136	312	1640	106	17433	24688	46	802	126	18345
cw20--2a (batch1)	238	49	46	75	1163	5544	149	31	56	108	47	238	49	654	23	86	26	117	77	46808	8449	24	280	52	26817
0.5%DMSO_(SedBatch1)	149	29	292	52	1164	4956	69	18	36	109	29	160	29	705	26	98	21	54	60	47150	6048	23	218	41	26507
ch04-2ah_(batch1)	236	55	121	82	1252	10550	183	34	63	70	52	175	64	614	24	51	27	149	82	42048	9506	35	1017	55	26455
cw29--2ah_(batch1)	4139	141	218	147	778	18887	837	107	14115	137	29044	824	543	548	49	107	480	1998	134	15198	25666	76	1089	159	21683
cb29--2ah_(batch1)	5133	196	229	149	543	19584	656	96	16894	175	32642	758	754	589	49	181	627	2736	138	8108	38979	84	690	209	21234
cs04--2ah_(batch1)	195	31	106	52	828	6333	169	16	40	97	36	170	50	385	18	59	21	163	53	45411	7536	26	788	46	23479
cs29--2ah_(batch1)	2051	73	219	108	736	13811	405	57	6487	108	16719	586	313	479	27	143	291	1585	80	16778	25008	60	751	119	18446
cw20--2a_(batch1)	244	66	72	87	1236	5678	145	30	66	123	54	225	49	716	30	92	33	138	105	46280	7688	35	270	65	25353
0.5%DMSO (SedBatch2)	141	27	185	56	744	841	42	13	30	88	10	98	23	566	21	51	14	46	117	28603	1855	12	156	28	9085
ch04-2ah_(batch2)	188	49	118	82	767	5252	338	32	51	115	45	231	56	510	37	45	28	102	121	39845	6475	28	448	45	20984
cw29--2ah_(batch2)	715	65	204	172	851	20055	315	66	1451	139	8469	1126	150	304	49	61	91	470	144	14559	13888	46	758	74	17098
cb29--2ah_(batch1)	1810	70	243	151	738	15932	307	86	5827	148	25673	1069	328	470	47	93	214	938	155	8069	23929	71	615	110	16156
cs04--2ah_(batch2)	226	43	117	62	764	4853	435	45	49	141	72	170	71	397	32	34	40	119	105	38258	7024	45	444	59	20383
cs29--2ah_(batch2)	702	63	275	145	886	14842	404	54	978	173	5236	862	156	453	42	92	80	469	117	16994	12197	53	631	75	17005
cw20--2a (batch2)	199	63	58	71	801	1914	67	41	57	153	51	162	55	622	39	39	38	95	118	40476	5855	41	153	58	22584
0.5%DMSO_(SedBatch2)	155	28	213	72	709	732	45	20	38	92	20	123	29	593	20	57	14	63	148	32770	1822	11	171	31	11119
ch04-2ah_(batch2)	214	55	116	71	738	4974	298	34	47	115	35	186	46	447	31	39	28	90	109	36930	6225	30	510	50	20317
cw29--2ah_(batch2)	680	61	178	148	832	18598	311	56	1269	149	8505	1131	142	374	50	56	85	432	138	14030	13645	49	692	81	16232
cb29--2ah_(batch2)	1633	69	193	123	662	14880	284	69	4679	130	23503	975	268	433	45	91	189	763	153	7150	22582	65	570	90	14675
cs04--2ah_(batch2)	187	45	108	66	699	3815	357	35	46	107	64	137	61	343	35	38	39	121	115	14516	5519	37	375	57	17107
cs29--2ah_(batch2)	592	63	262	115	746	13589	366	48	817	133	4577	811	127	387	45	83	83	370	131	15694	10851	60	636	65	15708
cw20--2a (batch2)	199	61	61	73	694	1698	71	36	48	118	46	151	53	505	33	41	29	95	104	33161	4640	30	153	48	17410
0.17%MeOH	251	35	1090	55	2354	1368	34	28	40	186	34	97	44	669	31	161	24	60	93	26913	3254	23	1081	44	17915
I_W22	309	134	2039	58	5440	1989	227	88	155	546	64	104	177	174	73	4339	57	81	63	28309	12287	68	8858	145	37968
E_W02	295	35	5305	89	7163	4808	199	65	145	1278	69	138	188	494	767	286	50	79	70	29701	10593	58	23979	154	34792
I_W29	412	198	2166	119	2896	7687	2016	69	179	414	88	590	210	196	69	8349	61	77	70	19074	13129	63	4033	135	33230
E_W09	307	44	4390	92	5993	4663	224	77	144	912	71	131	159	529	491	323	62	128	71	25846	9616	71	25997	171	31407
I_W31	350	133	1354	140	2055	7737	4388	62	187	287	62	419	169	123	63	4289	56	81	61	16955	9003	65	2546	137	30716
E_W11	339	49	3792	95	5828	3949	169	84	119	1038	69	129	183	413	557	228	54	97	88	25451	9499	66	19644	189	32676
ERYT	317	51	808	79	2227	2216	52	38	55	166	72	169	51	573	36	125	31	85	137	25064	7304	33	509	53	19294
AZI	188	30	529	74	1926	1343	56	24	42	150	27	114	38	493	25	104	17	72	100	27014	2980	20	500	28	17280
I_W22	216	112	1701	49	4497	1626	189	53	109	495	64	89	129	147	66	3060	35	69	63	25332	10619	51	7038	121	35122
E_W02	250	25	3598	68	5257	3517	130	48	98	990	40	117	164	372	433	232	31	64	57	24493	8692	44	20487	137	31415
I_W29	312	134	1863	106	2554	5731	1560	58	141	373	67	439	177	179	59	6542	49	75	58	16801	11056	51	3356	103	32121
E_W09	229	34	3167	73	4735	3786	175	50	102	814	55	125	176	444	459	255	42	96	53	23934	8531	60	22809	151	30665
I_W31	247	102	1139	114	1743	7123	3851	43	146	274	49	296	114	107	44	2901	35	64	54	15221	7675	49	1787	120	28963
E_W11	306	47	3867	100	6249	4025	209	91	144	1143	71	144	200	365	576	261	67	92	84	26632	9761	83	20990	166	33743
CLAR	271	50	601	74	2308	1700	62	35	65	214	30	166	43	711	41	123	34	74	133	28418	4588	28	462	50	23640
SMO	187	34	736	65	2288	1256	42	24	37	147	23	140	47	635	30	120	15	66	106	26360	3326	20	884	34	17903

Suppl. Table S2.B. (Continued)

Sample	clec-52	cyp-35D1	K03D3.2	rpl-6	F42A10.7	K08D8.3	F41F3.3	asp-13	F46C5.1	col-160	lips-6	Y49G5A.1	cyp-13A10	F08G2.5	tag-297	chil-28	ugt-41	tbg-1	cyp-33D1	cpt-3	wrt-4	C24B9.3	cest-29	spl-2	ugt-8
SW1_(batch1)	158	76	5218	40650	103	143	515	286	253	35586	59	238	93	4077	66	156	77	1296	80	36	17	24280	47	67	56
SW2_(batch1)	277	105	6694	44049	151	175	711	571	521	38084	70	377	176	5913	94	199	81	1926	131	54	35	24370	69	84	83
SW3_(batch1)	297	94	9982	42963	149	173	787	544	517	39253	62	619	215	8490	98	209	79	1973	116	40	29	23731	71	62	74
H_(batch1)	2560	111	1612	40451	174	636	1886	4331	10704	34284	181	54	720	548	162	1527	95	2423	331	61	130	43056	312	1296	84
N_(batch1)	1260	96	1380	44929	307	837	2268	6619	13396	24036	117	53	636	310	178	2111	80	2060	153	78	82	47787	433	1272	85
C_(batch1)	3612	99	1052	40628	179	263	7128	2064	14894	27551	323	48	1690	341	324	1528	101	1691	215	58	191	43938	232	1224	70
I_(batch1)	1607	68	1638	47681	94	168	1528	878	3788	39798	106	63	503	674	107	892	71	1471	163	46	53	34710	132	772	56
E_(batch1)	249	70	7921	46748	118	139	1012	657	686	46098	49	485	159	7805	65	181	57	1740	92	46	27	22630	43	51	61
SW1_(batch2)	194	85	7604	44149	128	134	507	384	426	42442	68	265	150	4414	70	148	68	1510	86	40	23	25182	54	79	65
SW2_(batch1)	278	116	7163	43429	140	192	931	652	646	36028	69	430	140	6330	100	238	89	1713	132	66	47	24976	93	85	87
SW3_(batch1)	306	105	10773	43081	165	194	843	613	588	40126	82	585	241	9504	91	213	74	2098	131	47	35	25823	66	85	72
H_(batch1)	2875	114	1704	42691	168	681	2143	4712	12302	36830	167	75	739	567	184	1717	104	2451	367	76	116	45102	295	1360	95
N_(batch1)	1380	145	1356	44117	325	855	2688	7629	14439	24489	123	67	769	353	204	2024	106	2268	177	92	84	46690	462	1262	91
C_(batch1)	3924	105	1244	46932	221	288	7690	2370	16015	27528	285	74	1821	395	387	2003	108	2064	235	90	214	42405	315	1614	93
I_(batch1)	1748	87	1999	48304	134	182	1950	1030	3976	43990	111	88	575	745	130	967	82	1826	198	49	49	38484	122	836	60
E_(batch1)	216	66	7340	45406	107	127	874	544	634	44455	52	470	174	7052	58	177	69	1746	80	37	33	21369	43	43	41
SW1_(batch2)	325	103	7568	45661	110	141	304	474	960	44441	61	287	139	5356	123	190	104	1251	117	45	20	17664	63	70	90
SW2_(batch2)	493	135	8194	45133	176	199	360	759	1651	43464	99	401	198	5578	170	287	116	1734	165	60	34	19492	90	104	126
SW3_(batch2)	410	114	7325	45668	157	175	452	550	960	43958	89	437	166	6423	187	222	106	1427	134	45	40	15883	80	78	112
H_(batch2)	2501	175	1419	44304	240	646	2158	2303	17865	23757	251	96	707	365	568	1591	144	2006	400	80	131	44828	386	989	164
N_(batch2)	1243	147	1141	45137	472	798	1505	4910	17940	12921	137	78	667	270	772	1843	120	1991	166	105	75	45600	453	824	163
C_(batch2)	3924	167	1413	45591	281	365	8590	1924	20998	20662	427	88	1512	381	1459	1741	141	2259	339	111	251	39196	368	1283	239
I_(batch2)	1519	146	2248	46536	176	241	2812	1023	12102	38704	236	104	600	902	350	1186	108	1968	256	83	98	38590	192	877	132
E_(batch2)	599	90	8263	44534	174	183	531	760	1724	42433	79	543	201	8581	160	256	84	1864	127	57	43	18444	86	69	104
SW1_(batch2)	346	106	7980	46942	86	136	255	354	561	46870	46	252	121	4631	103	188	69	1004	115	40	33	16742	58	60	76
SW2_(batch2)	559	164	8056	43354	160	227	350	664	1571	43108	88	444	207	6364	165	275	131	1815	156	68	38	20166	87	93	131
SW3_(batch2)	493	118	8391	45232	151	169	514	684	941	44239	93	435	191	6769	208	235	109	1494	139	57	46	17384	90	90	109
H_(batch2)	2237	179	1448	45476	228	617	2384	2302	18140	24051	281	95	656	350	471	1445	154	1942	396	87	145	45457	352	982	155
N_(batch2)	1196	172	1139	44951	530	792	1491	5338	18047	13063	141	89	789	294	859	1874	150	2037	194	114	92	44855	419	994	154
C_(batch2)	3915	178	1343	45925	299	365	8565	1783	19675	20100	419	112	1435	388	1433	1743	138	2183	318	119	261	37034	336	1239	227
I_(batch2)	1563	159	2568	45862	186	282	3112	1018	12699	41952	250	106	746	1151	339	1305	128	2011	254	76	136	39743	251	869	144
E_(batch2)	513	106	8238	47013	195	176	449	716	1863	46046	75	534	220	8180	143	232	89	1907	108	57	36	19083	73	71	110
0.5%DMSO_(batch1)	386	35	2648	47171	75	81	295	1201	45	46076	21	41	103	11719	23	59	40	1759	50	19	14	23574	18	41	25
AFB1(30uM)_(batch1)	752	69	4061	42819	103	163	396	3905	85	42163	51	54	755	12357	49	94	110	2477	81	31	31	26248	39	77	178
BaP(30uM)_(batch1)	702	1535	3295	43303	112	135	413	1736	79	43602	41	47	385	6002	52	92	589	3196	61	35	36	24634	35	93	1097
PCB1254(30uM)_(batch1)	942	42	2695	43342	84	110	478	1494	72	42891	40	41	1428	5964	44	77	106	2685	56	29	26	23038	27	86	273
0.5%DMSO_(batch2)	799	70	2945	45176	170	146	359	2011	144	43872	43	61	202	2829	72	118	67	2051	80	36	29	21603	34	72	84
AFB1(30uM)_(batch2)	919	63	3128	43342	135	131	277	3346	149	42379	53	54	639	3598	68	85	207	2441	99	27	31	20866	50	86	460
BaP(30uM)_(batch2)	1098	2148	2574	46493	150	102	572	1537	148	45697	52	68	281	2490	72	93	1462	2464	90	37	33	18931	48	81	2697
PCB1254(30uM)_(batch2)	1256	58	2544	45028	113	104	284	1288	80	42892	40	62	1091	1679	63	89	192	2259	74	38	38	18568	41	108	1015
0.5%DMSO_(batch1)	441	30	2885	47021	76	97	253	1345	45	45519	23	49	184	11893	36	88	31	1950	42	22	17	24524	22	44	25
AFB1(30uM)_(batch1)	768	53	3858	43633	103	143	376	4167	77	43442	43	40	781	12163	49	90	106	2773	79	35	35	24853	41	87	173
BaP(30uM)_(batch1)	658	1386	3386	44555	101	120	352	1502	61	42749	32	55	273	6098	35	84	550	3006	64	26	16	23059	31	76	1075
PCB1254(30uM)_(batch1)	952	37	2696	45014	94	121	481	1559	56	42806	35	34	1387	6041	45	86	114	2528	65	36	21	22944	24	79	362
0.5%DMSO_(batch2)	798	50	2492	45767	162	108	342	1914	144	44518	47	43	189	2470	66	92	54	2125	65	28	22	20119	44	66	65
AFB1(30uM)_(batch2)	868	62	2776	43161	114	119	251	3159	104	42565	45	42	608	2934	75	94	169	2246	85	28	37	18725	42	66	362
BaP(30uM)_(batch2)	885	2135	2548	45986	131	103	558	1554	140	42584	53	64	276	2274	52	73	1257	2232	85	34	34	17680	36	103	2741
PCB1254(30uM)_(batch2)	1219	58	2480	43692	139	95	278	1322	91	40650	48	49	1016	1679	67	77	230	2069	71	30	31	17573	48	98	1020
0.5%DMSO_(SedBatch1)	154	23	1062	45575	103	60	183	844	341	40291	21	19	93	2635	19	46	35	1138	28	15	17	7838	39	29	30
cb04-2ah_(batch1)	23709	18	1262	46689	266	89	937	1277	151	39471	37	31	225	790	44	57	32	1238	37	50	33	9356	53	256	70
cw29--2ah_(batch1)	31815	64	399	45832	10342	456	3157	3881	340	38632	104	56	2496	1596	467	131	1152	1244	85	785	41	17857	104	1043	1210
ch29--2ah_(batch1)	33340	89	379	45650	7338	787	2577	4424	954	43755	126	67	4239	2188	544	148	1813	1308	95	783	82	19013	170	1398	2064
cs04--2ah_(batch1)	24318	16	796	48182	478	61	949	856	271	39434	45	20	355	1123	27	53	26	1020	30	25	42	7272	31	202	45
cs29--2ah_(batch1)	32890	41	368	44427	4282	270	1901	3171	610	30655	76	45	3042	1124	454	150	657	1216	72	335	60	14316	78	666	629
cw20--2a_(batch1)	510	29	1397	49111	155	84	766	974	466	41526	42	32	225	1975	28	79	48	1269	48	24	42	8894	60	83	64
0.5%DMSO_(SedBatch1)	117	29	1104	48719	113	57	180	796	452	41819	31	27	97	2326	31	66	37	1039	32	16	30	7151	53	51	41
cb04-2ah_(batch1)	24980	27	1421	45495	293	80	1113	1357	154	37351	40	30	231	892	37	71	48	1252	44	52	59	9500	60	285	67
cw29--2ah_(batch1)	23586	78	429	43224	9764	466	2954	3627	322	36406	103	57	2625	1655	493	108	1048	1294	85	848	92	17000	124	1005	1300
ch29--2ah_(batch1)	29502	76	386	43466	6911	831	2338	4032	987	38090	97	76	4449	2157	579	147	1641	1250	107	669	70	17247	163	1326	2082
cs04--2ah_(batch1)	25279	15	902	48224	460	63	870	971	350	39147	38	22	457	952	33	58	34	1131	36	31	47	7597	49	240	51
cs29--2ah_(batch1)	33901	51	423	43933	4631	305	1845	3064	633	30142	72	41	2817	1096	434	150	582	1205	71	359	66	14702	75	703	598
cw20--2a_(batch1)	594	37	1356	44423	164	93	774	982	503	34128	39	33	230	2043	32	78	52	1344	59	30	69	8822	87	84	70
0.5%DMSO_(SedBatch2)	123	18	577	42553	61	29	78	439	44	24023	37	23	27	759	12	58	47	401	46	11	66	4206	97	78	85
cb04-2ah_(batch2)	18043	42	1190	43867	164	77	1354	1167	130	34958	62	32	153	425	69	79	60	1094	61	40	99	9818	100	182	97
cw29--2ah_(batch2)	31614	59	522	44440	13852	256	1178	5237	72	24408	110	59	787	474	624	143	205	1115	87	422	105	18546	121	555	249
ch29--2ah_(batch2)	31622	90	497	44522	10137	370	1341	4315	192	23985	117	63	2070	1285	649	138	574	1120	98	402	112	14996	143	743	566
cs04--2ah_(batch2)	24372	45	871	44485	576	92	1868	996	72	22815	89	40	356	763	86	74	59	1128	63	42	88	9563	73	389	103
cs29--2ah_(batch2)	31968	59	586	41064	4301	228	1444	3666	398	20146	106	56	1769	439	758	164	167	1279	80	217	104	16056	86	444	171
cw20--2a_(batch2)	508	40	1068	48063	142	81	376	903	99	35430	45	31	126	651	30	70	60	1163	56	23	61	9084	79	174	97
0.5%DMSO_(SedBatch2)	114	16	586	46457	60	40	86	524	52	29259	44	25	32	876	11	63	55	504	53	11	76	4281	97	72	92
cb04-2ah_(batch2)	17431	40	1073	42708	130	59	1356	956	105	32303	59	35	154	372	61	77	48	1131	57	41	81	9420	94	153	81
cw29--2ah_(batch2)	30404	57	493	44628	12881	227	1044	4424	75	23886	103	55	855	438	537	136	195	1064	85	359	102	18798	102	482	212
ch29--2ah_(batch2)	29255	62	433	43720	8987	336	1108	3232	149	22097	101	63	1714	1199	614	135	539	1072	91	445	96	14301	122	734	540
cs04--2ah_(batch2)	20664	44	767	40537	454	82	1420	861	53	27189	95	54	212	646	65	63	54	925	57	41	74	7321	80	278	110
cs29--2ah_(batch2)	33411	52	551	41976	3448	213	1230	3177	282	17347	100	57	1436	449	668	127	156	1139	75	189	101	13710	106	400	163
cw20--2a_(batch2)	492	47	985	39560	143	80	244	840	82	25037	43	43	97	602	24	79	56	894	60	26	61	7597	101	139	98
0.17%MeOH	176	29	1051	39863	81	59	52	481	181	15339	35	39	71	2490	20	62	47	779	49	16	58	5856	81	64	59
I_W22	10984	69	2461	44759	59	113	772	1265	9900	44078	78	47	318	3572	98	449	73	2693	179	34	27	28718	92	801	75
E_W02	425	82	20099	46463	74	114	587	1316	1073	41044	40	889	172	9858	609	319	63	2626	95	34	35	21622	41	76	78
I_W29	6768	77	1611	40922	92	154	8086	1570	19061	36255	231	49	518	930	1339	1373	86	2756	252	40	74	27425	380	730	115
E_W09	364	77	17665	42519	100	137	1157	951	1344	34285	54	812	195	8930	586	264	65	2167	85	38	41	17914	45	70	89
I_W31	9313	63	1224	40648	79	145	15429	1271	9634	33843	574	28	447	967	595	784	65	1997	121	31	195	25785	296	693	74
E_W11	430	70	15828	42119	85	124	591	1049	956	39486	60	871	187	10300	451	269	68	2206	96	44	42	19707	52	125	69
ERVT	160	39	911	45362	98	131	69	731	116	19440	39	41	83	2086	25	66	70	895	65	20	79	6397	115	67	91
AZI	113	33	880	43612	58	60	71	488	101	13232	34	36	69	1908	17	60	51	803	56	15	55	5355	81	54	78
I_W22	9439	64	1973	46397	67	93	524	943	8053	44981	71	44	363	2559	96	353	49	1961	127	45	31	25375	112	575	58
E_W02	304	55	16486	46530	59	92	375	997	893	45019	40	788	139	7759	433	200	42	2123	72	34	16	17996	36	63	62
I_W29	6074	65	1391	42264	84	124	6894	1151	16662	40855	213	40	370	711	1142	1113	55	2279	205	38	60	26264	264	627	118
E_W09	299	48	15005	47605	75	98	864	816	939	45746	40	759	174	7445	494	198	55	1967	83	33	38	17443	41	51	69
I_W31	7444	36	1094	45748	64	130	14184	1094	9156	42370	492	34	277	792	524	659	45	1730	100	32	137	26975	240	557	64
E_W11	545	86	18020	44223	101	146	597	1010	1004	36020	66	802	158	10720	479	287	62	2211	120	45	37	21055	53	122	79
CLAR	151	37	1244	50178	87	84	110	685	131	19173	53	40	78	2448	28	96	64	976	66	22	76	6772	126	67	85
SMO	171	25	949	44945	71	71	67	533	191	16051	32	35	49	1632	18	63	43	781	41	13	60	5667	83	36	77

Suppl. Table S2.C. Bead counts (highlighted cells indicate the bead counts which are below a limit of 35 beads per target per well)

Location (96-well plate)	Sample	cest-33	F10D2.8	F56D6.8	vmo-1	F16B4.7	acdh-1	R12E2.15	cyp-14A4	R09E12.9	T28A11.3	fat-5	lipl-3	cyp-13A6	Y45F10D.6	T28A11.4	C23G10.11	clec-210	dhs-23	Y46H3D.8	gst-20	mdh-1	cyp-35A1	T06C12.14	cdr-1	par-5
1(1,A1)	SW1_(batch1)	110	88	104	128	107	127	91	123	121	119	113	111	94	124	85	111	117	96	125	166	119	111	109	125	110
2(1,A2)	SW1_(batch1)	99	70	75	103	79	105	80	79	104	91	127	89	71	98	103	118	92	105	101	112	89	91	96	122	91
3(1,A3)	SW1_(batch2)	98	90	87	110	92	90	100	118	117	103	101	117	92	125	88	99	117	82	98	128	89	92	100	111	104
4(1,A4)	SW1_(batch2)	74	79	85	94	102	113	102	104	115	109	109	87	84	109	82	98	99	102	110	109	105	101	106	97	93
5(1,A5)	0.5%DMSO_(batch1)	96	83	82	104	82	105	104	107	105	85	100	101	77	108	84	100	90	95	110	112	104	102	89	108	81
6(1,A6)	0.5%DMSO_(batch1)	72	67	68	81	86	89	69	77	63	80	74	77	76	60	76	108	79	63	95	89	83	80	76	76	76
7(1,A7)	0.5%DMSO_(SedBatch1)	65	69	87	89	82	95	87	89	88	77	84	89	66	87	83	89	79	81	94	101	64	70	91	92	86
8(1,A8)	0.5%DMSO_(SedBatch1)	83	60	85	83	79	100	87	107	114	101	100	103	78	105	92	117	87	79	108	112	81	101	86	103	85
9(1,A9)	0.5%DMSO_(SedBatch2)	85	79	86	104	87	98	106	98	98	89	97	86	87	102	92	114	87	79	113	115	87	95	93	97	96
10(1,A10)	0.5%DMSO_(SedBatch2)	71	81	84	106	88	103	92	95	88	104	94	99	66	85	82	88	91	80	89	93	71	89	79	99	81
11(1,A11)	I_W22	64	62	85	80	90	88	76	77	74	79	85	85	77	85	72	98	80	90	77	111	82	75	90	89	84
12(1,A12)	I_W22	82	60	74	85	87	95	77	122	79	93	80	78	71	81	56	121	92	73	105	100	77	85	91	84	84
13(1,B1)	SW2_(batch1)	88	72	90	103	80	115	100	96	96	111	100	88	96	105	72	84	90	79	101	107	93	80	86	107	81
14(1,B2)	SW2_(batch1)	95	67	79	90	80	86	77	94	89	92	101	96	88	91	70	107	94	63	98	111	92	78	88	96	87
15(1,B3)	SW2_(batch2)	81	72	83	97	92	113	86	115	116	98	104	100	64	101	92	110	112	78	93	116	112	88	91	101	105
16(1,B4)	SW2_(batch2)	95	93	109	96	100	111	89	95	106	102	100	104	86	118	91	123	98	93	107	138	112	103	99	130	102
17(1,B5)	AFB1(30uM)_(batch1)	67	62	75	98	70	98	76	114	100	91	99	94	80	99	80	82	92	84	89	98	84	76	94	126	84
18(1,B6)	AFB1(30uM)_(batch1)	63	63	75	73	82	84	63	92	79	97	89	78	52	92	70	93	72	79	74	88	86	78	66	86	67
19(1,B7)	cb04-2ah_(batch1)	67	61	80	92	76	75	71	68	64	83	102	68	62	94	82	96	85	81	81	103	89	74	78	93	76
20(1,B8)	cb04-2ah_(batch1)	93	74	76	111	79	103	90	121	93	83	104	119	104	105	82	104	98	92	106	115	92	91	91	115	92
21(1,B9)	cb04-2ah_(batch2)	91	76	89	96	93	130	107	107	123	96	110	111	99	90	115	98	108	98	97	100	136	91	91	99	93	106
22(1,B10)	cb04-2ah_(batch2)	79	73	88	111	86	121	93	73	107	82	87	106	68	101	82	116	91	79	99	117	113	81	105	93	87
23(1,B11)	E_W02	86	55	83	70	66	94	66	88	88	71	86	87	60	94	76	81	71	78	78	79	79	87	88	96	71
24(1,B12)	E_W02	76	64	73	62	66	88	63	86	89	84	83	73	73	72	61	84	81	56	74	103	58	80	85	104	87
25(1,C1)	SW3_(batch1)	94	58	86	79	80	89	77	103	95	101	94	91	64	112	94	100	95	71	82	85	86	85	79	73	78
26(1,C2)	SW3_(batch1)	84	62	93	88	90	113	101	111	96	84	91	85	87	108	83	103	114	78	83	119	118	79	76	103	94
27(1,C3)	SW3_(batch2)	84	89	107	112	87	105	95	97	96	93	114	109	107	98	99	99	91	90	104	124	120	88	89	129	117
28(1,C4)	SW3_(batch2)	76	81	99	99	88	97	107	108	81	94	100	115	95	95	82	117	104	86	131	124	97	96	93	117	114
29(1,C5)	BaP(30uM)_(batch1)	93	67	89	93	85	94	82	111	102	99	89	102	78	117	91	110	110	102	99	113	118	123	123	96	110	85
30(1,C6)	BaP(30uM)_(batch1)	63	81	69	84	81	82	71	99	75	73	96	96	99	90	66	80	65	68	79	98	82	85	79	96	78
31(1,C7)	cw29--2ah_(batch1)	71	65	77	89	69	72	87	83	84	79	96	80	55	69	68	85	85	66	68	104	73	76	68	92	66
32(1,C8)	cw29--2ah_(batch1)	100	80	76	99	71	99	90	88	99	91	120	108	88	87	80	76	102	91	97	113	91	88	72	92	92
33(1,C9)	cw29--2ah_(batch2)	98	89	70	105	90	108	95	92	98	111	136	124	93	102	78	115	99	94	108	126	104	89	92	100	130
34(1,C10)	cw29--2ah_(batch2)	105	85	99	96	96	102	97	91	89	98	134	109	90	114	101	103	99	84	100	163	125	76	108	117	87
35(1,C11)	I_W29	95	75	80	114	79	114	100	96	92	102	96	95	91	88	100	118	94	95	97	131	106	80	97	83	95
36(1,C12)	I_W29	71	78	88	92	94	87	106	94	96	94	90	81	92	105	87	103	84	80	79	110	100	82	94	115	101
37(1,D1)	H_(batch1)	67	77	93	92	84	95	104	117	97	95	96	116	79	118	95	118	116	85	91	137	91	82	93	96	105
38(1,D2)	H_(batch1)	84	59	97	117	72	101	91	96	105	97	94	99	59	78	98	102	111	75	79	123	89	86	87	110	97
39(1,D3)	H_(batch2)	94	77	121	101	104	107	101	108	103	98	106	108	104	99	99	136	103	91	106	125	97	93	106	122	97
40(1,D4)	H_(batch2)	101	79	114	114	84	103	115	89	114	119	123	113	120	107	92	89	96	112	105	137	123	86	104	129	94
41(1,D5)	PCB11254(30uM)_(batch1)	85	78	96	91	100	107	91	107	104	103	88	106	81	111	93	121	114	113	99	113	103	102	112	125	101
42(1,D6)	PCB11254(30uM)_(batch1)	76	49	69	86	66	90	93	96	92	91	99	99	86	102	92	85	83	88	87	94	84	81	81	114	69
43(1,D7)	cb29--2ah_(batch1)	75	64	71	85	62	81	80	74	82	71	92	89	60	94	79	82	82	77	69	85	69	79	81	89	85
44(1,D8)	cb29--2ah_(batch1)	82	80	68	93	74	112	86	93	109	99	104	94	85	91	85	84	103	103	92	107	87	84	80	94	99
45(1,D9)	cb29--2ah_(batch2)	96	79	93	108	80	118	95	99	98	102	96	99	94	111	78	98	93	97	94	125	93	83	80	95	102
46(1,D10)	cb29--2ah_(batch2)	91	77	83	105	106	114	90	106	96	87	109	102	87	115	84	98	87	89	100	133	97	83	93	90	99
47(1,D11)	E_W09	79	68	88	99	91	109	97	103	93	84	94	111	84	104	106	87	103	82	123	142	84	81	84	106	102
48(1,D12)	E_W09	75	69	91	99	94	101	91	92	88	85	109	95	88	96	83	87	85	62	82	136	94	84	80	101	98
49(1,E1)	N_(batch1)	80	81	81	91	87	87	88	79	92	104	94	93	78	98	76	94	95	73	95	124	96	83	90	104	87
50(1,E2)	N_(batch1)	92	89	97	128	83	98	91	96	107	100	99	121	103	98	103	135	97	96	90	137	87	89	85	108	105
51(1,E3)	N_(batch2)	99	90	98	114	85	127	107	127	106	108	118	116	81	112	106	109	97	87	117	139	111	101	103	112	109
52(1,E4)	N_(batch2)	81	78	86	94	87	96	85	90	87	82	95	98	98	92	71	108	91	76	107	120	112	80	93	78	78
53(1,E5)	0.5%DMSO_(batch2)	104	80	108	97	89	101	112	106	120	124	90	104	78	105	105	93	124	96	117	141	126	96	83	119	127
54(1,E6)	0.5%DMSO_(batch2)	81	62	94	93	97	80	70	88	83	81	100	87	78	87	96	103	111	78	95	93	89	82	80	100	88
55(1,E7)	co04--2ah_(batch1)	76	53	63	83	72	99	62	102	83	94	101	81	79	97	82	97	81	79	99	120	93	89	80	95	84
56(1,E8)	co04--2ah_(batch1)	89	84	90	97	84	122	85	97	102	98	102	102	83	102	92	91	85	100	109	127	92	80	85	119	80
57(1,E9)	co04--2ah_(batch2)	92	80	109	87	68	112	99	116	118	106	104	110	73	112	87	104	91	88	95	125	89	90	85	110	104
58(1,E10)	co04--2ah_(batch2)	83	81	92	109	77	99	82	112	92	91	102	78	78	102	85	111	110	67	104	117	106	75	92	99	100
59(1,E11)	I_W31	87	82	102	99	80	95	95	93	96	114	94	116	78	95	94	118	105	89	115	131	99	77	115	105	90
60(1,E12)	I_W31	88	90	99	90	92	97	90	106	97	98	101	110	102	99	93	130	106	78	94	114	120	100	117	97	103
61(1,F1)	C_(batch1)	92	82	89	92	95	100	94	117	109	105	101	110	86	97	101	130	97	81	85	138	105	79	101	101	103
62(1,F2)	C_(batch1)	76	72	74	89	72	112	98	89	85	78	82	93	74	92	90	97	87	67	108	109	96	81	107	105	112
63(1,F3)	C_(batch2)	103	83	109	108	85	116	101	108	96	111	107	115	110	80	98	128	91	76	89	129	103	81	101	123	119
64(1,F4)	C_(batch2)	80	83	82	99	60	94	100	86	93	80	112	111	90	99	84	112	92	67	110	122	99	68	97	93	93
65(1,F5)	AFB1(30uM)_(batch2)	90	90	71	92	82	86	70	95	78	116	102	92	90	95	82	115	83	96	85	105	107	82	98	98	84
66(1,F6)	AFB1(30uM)_(batch2)	70	64	74	108	89	90	73	90	101	92	99	104	85	105	65	96	83	78	95	105	83	77	86	108	82
67(1,F7)	cs29--2ah_(batch1)	76	72	78	90	71	88	89	79	109	80	125	93	74	95	84	89	75	66	71	124	79	74	74	91	87
68(1,F8)	cs29--2ah_(batch1)	100	80	88	104	85	111	94	82	115	102	109	96	79	106	94	91	100	100	90	136	96	107	90	89	85
69(1,F9)	cs29--2ah_(batch2)	69	72	91	92	70	96	95	107	109	94	110	115	80	99	81	91	86	86	90	121	112	86	85	85	93
70(1,F10)	cs29--2ah_(batch2)	95	79	93	119	99	119	81	111	108	93	104	125	104	127	97	103	101	101	104	134	95	80	101	112	81
71(1,F11)	E_W11	74	59	72	90	70	95	80	81	66	89	93	94	79	87	87	94	102	75	80	108	97	54	80	121	88
72(1,F12)	E_W11	77	66	78	85	71	97	82	88	80	86	94	76	72	77	93	89	72	72	82	96	98	68	85	109	97
73(1,G1)	I_(batch1)	76	85	107	95	90	100	81	105	99	96	111	116	88	105	107	104	86	81	97	120	115	94	114	105	103
74(1,G2)	I_(batch1)	70	65	100	80	70	96	78	99	88	90	80	91	69	95	93	80	91	74	90	120	86	89	90	84	91
75(1,G3)	I_(batch2)	102	76	102	103	87	112	88	106	95	105	86	98	88	96	78	98	88	87	111	150	121	89	109	112	126
76(1,G4)	I_(batch2)	84	64	97	117	89	117	92	86	91	108	83	91	81	105	77	111	98	87	98	135	94	95	94	137	96
77(1,G5)	BaP(30uM)_(batch2)	106	68	106	105	98	129	81	94	89	101	119	105	91	111	90	111	90	81	88	134	105	102	114	125	116
78(1,G6)	BaP(30uM)_(batch2)	102	70	95	99	91	94	82	104	91	105	106	99	81	104	98	102	122	84	87	124	98	110	89	111	96
79(1,G7)	cw20--2a_(batch1)	89	71	99	87	71	89	68	103	97	106	94	93	82	123	89	88	85	79	98	118	100	82	97	110	91
80(1,G8)	cw20--2a_(batch1)	80	91	82	102	93	113	96	98	107	96	133	102	83	115	85	99	78	65	105	120	100	95	106	106	92
81(1,G9)	cw20--2a_(batch2)	91	74	101	113	99	112	95	107	131	85	111	111	97	82	85	114	104	88	117	140	99	85	104	115	101
82(1,G10)	cw20--2a_(batch2)	90	68	92	111	98	86	87	92	113	95	96	84	69	117	106	93	77	81	101	106	100	101	92	98	90
83(1,G11)	ERYT	65	70	83	102	83	109	96	103	105	102	114	102	91	122	85	98	105	81	111	122	100	96	92	104	107
84(1,G12)	CLAR	89	73	91	113	83	122	87	90	98	83	81	81	66	102	84	85	100	93	86	133	87	94	82	111	99
85(1,H1)	E_(batch1)	95	77	88	98	65	86	87	93	104	88	100	105	85	106	70	100	87	93	95	130	110	77	88	107	98
86(1,H2)	E_(batch1)	79	72	87	84	62	97	94	108	99	86	112	110	85	71	83	96	73	77	68	118	94	66	88	101	85
87(1,H3)	E_(batch2)	65	63	76	86	87	95	96	71	95	91	63	92	107	77	85	93	88	81	79	86	106	72	77	81	86
88(1,H4)	E_(batch2)	56	60	69	93	87	72	63	81	80	79	66	93	97	108	70	80	79	80	71	98	83	73	71	84	88
89(1,H5)	PCB11254(30uM)_(batch2)	90	61	76	72	81	110	73	80	78	95	96	75	80	84	69	108	101	82	74	95	74	74	76	87	74
90(1,H6)	PCB11254(30uM)_(batch2)	103	68	97	86	78	90	100	92	101	90	90	100	87	94	97	99	93	74	93	119	82	73	89	93	84
91(1,H7)	Background0	69	63	88	99	67	88	80	105	85	93	91	82	85	102	86	92	76	64	90	108	78	70	83	93	80
92(1,H8)	Background0	98	74	97	117	100	102	102	98	133	94	110	119	87	136	106	119	113	114	117	144	90	114	104	128	107
93(1,H9)	Background0	92	86	79	105	97	112	99	115	112	108	99	103	86	121	83	108	121	110	118	121	84	100	88	110	70
94(1,H10)	0.175%EtOH	91	77	90	79	89	89	71	110	89	84	97	79	88	113	102	105	103	80	104	115	93	79	87	98	90
95(1,H11)	AZI	48	46	67	71	60	51	58	78	70	61	69	67	56	82	65	59	53	39	64	69	67	57	55	79	70
96(1,H12)	SMO	78	71	84	101	71	89	79	93	126	117	88	106	97	92	87	111	87	92	106	148	82	102	93	118	87

Suppl. Table S2.C. (Continued)

Location (96-well plate)	Sample	clec-52	cyp-35D1	K03D3.2	npl-6	F42A10.7	K08D8.3	F41H3.3	asp-13	F46C5.1	col-160	lips-6	Y49G5A.1	cyp-13A10	F08G2.5	tag-297	chil-28	sqt-41	tbg-1	cyp-33D1	cpt-3	wrt-4	C24B9.3	cest-29	spl-2	sqt-8	Total Events
1(1,A1)	SW1_(batch1)	133	98	139	143	118	111	102	113	59	123	149	111	69	141	95	109	117	135	114	108	61	111	114	90	94	5591
2(1,A2)	SW1_(batch1)	115	106	132	125	89	101	83	90	42	106	71	104	66	92	85	93	87	122	108	89	46	95	97	84	64	4682
3(1,A3)	SW1_(batch2)	127	93	141	152	102	112	104	128	56	126	90	108	64	123	100	95	100	131	113	93	52	99	102	91	79	5129
4(1,A4)	SW1_(batch2)	119	85	135	109	81	108	107	118	50	99	114	93	59	114	95	85	117	128	92	93	38	88	95	81	75	4846
5(1,A5)	0.5%DMSO_(batch1)	103	73	114	95	84	99	94	115	52	61	110	66	51	105	73	107	99	114	96	94	45	75	102	82	81	4604
6(1,A6)	0.5%DMSO_(batch1)	79	79	97	82	74	70	75	76	38	68	87	78	52	94	73	84	103	91	93	69	31	64	84	62	79	3822
7(1,A7)	0.5%DMSO_(SedBatch1)	96	77	97	92	80	90	92	86	41	81	89	79	50	76	59	75	100	114	93	97	29	74	69	63	76	4059
8(1,A8)	0.5%DMSO_(SedBatch1)	92	96	125	119	77	99	85	100	56	103	103	88	67	90	78	97	105	120	95	82	39	78	95	74	71	4570
9(1,A9)	0.5%DMSO_(SedBatch2)	115	92	115	124	93	88	91	100	42	100	111	84	50	121	83	94	102	118	104	88	40	88	84	103	95	4695
10(1,A10)	0.5%DMSO_(SedBatch2)	96	105	100	119	99	98	76	90	51	89	103	75	50	108	81	86	103	101	115	87	40	93	92	79	80	4414
11(1,A11)	I_W22	79	87	115	96	70	79	85	83	42	88	91	70	57	104	76	77	91	109	84	72	46	76	64	82	67	4045
12(1,A12)	I_W22	90	88	97	117	80	85	103	99	47	91	104	86	55	123	76	97	104	107	99	80	32	97	84	68	80	4321
13(1,B1)	SW2_(batch1)	79	97	123	126	87	74	101	89	50	111	92	80	59	110	88	81	104	136	108	96	37	73	87	65	97	4570
14(1,B2)	SW2_(batch1)	86	92	131	132	70	87	79	102	48	96	88	82	45	84	92	80	93	124	90	88	36	86	80	84	89	4373
15(1,B3)	SW2_(batch2)	113	98	127	98	84	108	111	117	56	119	108	103	61	109	94	86	104	132	100	103	38	85	120	87	91	4872
16(1,B4)	SW2_(batch2)	99	93	156	143	99	111	91	100	60	106	117	96	60	120	93	105	105	131	96	97	55	98	125	100	84	5140
17(1,B5)	AFB1(30nM)_(batch1)	110	81	122	136	79	88	96	107	47	115	84	91	41	102	76	87	100	118	102	78	37	79	79	58	81	4406
18(1,B6)	AFB1(30nM)_(batch1)	81	70	96	115	78	72	77	92	36	98	93	78	37	97	73	83	77	101	76	61	40	80	60	52	60	3824
19(1,B7)	cb04-2ah_(batch1)	100	95	104	96	78	76	74	95	54	101	75	91	46	87	83	73	92	101	96	81	26	79	73	90	82	4049
20(1,B8)	cb04-2ah_(batch1)	118	94	120	130	91	104	95	110	60	108	93	110	62	93	74	74	103	103	122	111	87	42	91	101	78	4829
21(1,B9)	cb04-2ah_(batch2)	112	97	124	121	95	110	101	140	64	98	112	103	68	118	93	99	129	121	89	104	43	110	110	105	82	5045
22(1,B10)	cb04-2ah_(batch2)	103	83	118	106	94	95	97	102	40	89	79	105	67	112	74	79	121	108	114	63	41	90	81	86	76	4561
23(1,B11)	E_W02	86	83	109	91	74	101	64	75	40	76	95	95	58	74	62	81	73	91	91	67	35	77	78	54	58	3866
24(1,B12)	E_W02	82	66	92	76	77	77	57	89	41	60	83	64	50	71	70	71	85	98	82	73	34	83	65	59	66	3696
25(1,C1)	SW3_(batch1)	83	92	127	118	86	94	80	108	50	108	95	97	52	89	69	88	113	122	105	89	41	76	87	66	79	4365
26(1,C2)	SW3_(batch1)	94	76	133	115	85	112	98	107	49	96	95	92	56	94	91	97	119	122	98	67	37	77	83	83	97	4616
27(1,C3)	SW3_(batch2)	116	103	127	138	78	123	87	123	53	93	104	104	63	111	98	120	125	129	105	88	52	106	99	97	82	5067
28(1,C4)	SW3_(batch2)	119	119	123	137	108	105	103	111	59	117	83	94	50	130	100	92	116	123	110	96	44	104	92	84	99	5014
29(1,C5)	BaP(30uM)_(batch1)	101	105	140	122	101	94	87	102	38	110	127	112	64	108	83	96	132	121	100	91	48	76	99	83	95	4917
30(1,C6)	BaP(30uM)_(batch1)	85	84	116	92	95	86	86	94	34	81	82	91	40	101	82	75	95	106	89	90	47	81	75	72	86	4100
31(1,C7)	cw29--2ah_(batch1)	94	66	92	84	93	88	81	97	39	80	87	71	45	87	58	68	82	76	85	71	28	63	72	70	76	3780
32(1,C8)	cw29--2ah_(batch1)	114	86	107	127	96	101	105	105	51	108	104	93	58	101	63	71	112	102	69	86	40	94	91	75	92	4531
33(1,C9)	cw29--2ah_(batch2)	104	110	112	130	122	113	119	140	60	129	116	86	55	108	95	77	103	122	89	120	50	105	88	88	75	5062
34(1,C10)	cw29--2ah_(batch2)	104	102	134	120	110	110	124	127	47	118	112	87	69	114	87	87	94	105	120	106	107	77	99	97	77	5064
35(1,C11)	I_W29	119	87	138	117	100	96	112	108	67	109	101	88	48	109	91	104	101	123	109	114	35	98	90	73	87	4837
36(1,C12)	I_W29	103	89	123	103	93	94	79	124	47	108	123	105	55	104	83	88	121	109	109	86	44	82	89	88	78	4630
37(1,D1)	H_(batch1)	131	88	152	111	95	105	94	98	66	94	114	93	58	112	96	110	118	139	113	85	42	89	98	98	85	4918
38(1,D2)	H_(batch1)	100	85	108	120	96	99	101	101	49	114	98	80	59	105	81	86	107	143	105	99	48	86	99	79	74	4628
39(1,D3)	H_(batch2)	129	111	148	145	114	98	107	133	51	109	111	105	69	123	96	105	124	151	108	113	35	81	104	89	86	5251
40(1,D4)	H_(batch2)	131	110	119	146	99	114	118	133	54	112	116	102	52	97	89	100	108	121	111	88	55	116	108	87	91	5239
41(1,D5)	PCB1254(30nM)_(batch1)	114	84	127	126	99	104	89	117	41	105	93	87	72	121	108	85	119	123	128	106	44	99	85	59	95	4974
42(1,D6)	PCB1254(30nM)_(batch1)	84	65	129	123	87	94	77	98	46	93	106	89	51	106	79	78	89	107	93	84	40	80	84	82	77	4293
43(1,D7)	cb29--2ah_(batch1)	109	88	104	88	76	87	85	92	45	97	88	93	55	89	72	79	96	83	87	93	36	66	85	83	85	4018
44(1,D8)	cb29--2ah_(batch1)	116	90	121	131	83	101	93	119	50	107	110	94	62	108	86	87	129	133	98	90	37	111	98	96	88	4726
45(1,D9)	cb29--2ah_(batch2)	111	75	97	134	110	86	95	102	41	122	96	88	69	112	86	85	109	131	101	83	38	100	91	63	99	4728
46(1,D10)	cb29--2ah_(batch2)	129	105	122	124	116	90	100	105	37	103	113	97	60	121	104	91	111	104	104	93	35	92	91	90	85	4843
47(1,D11)	E_W09	89	84	119	134	93	103	95	105	54	116	97	100	51	125	92	98	129	113	100	96	45	107	101	92	96	4838
48(1,D12)	E_W09	101	85	137	95	90	83	73	83	52	102	104	86	46	103	87	78	97	101	84	113	35	84	101	80	87	4452
49(1,E1)	N_(batch1)	102	74	105	104	98	98	86	99	49	93	82	99	69	88	85	98	103	116	91	86	45	72	82	76	80	4430
50(1,E2)	N_(batch1)	103	95	144	132	115	126	105	123	52	139	108	97	62	110	115	136	98	132	107	85	47	90	116	101	79	5151
51(1,E3)	N_(batch2)	133	86	165	144	95	96	112	123	55	121	117	108	68	115	98	124	115	155	105	108	41	115	111	109	93	5391
52(1,E4)	N_(batch2)	90	90	126	116	74	100	99	116	52	97	111	95	62	95	95	94	97	123	104	90	25	61	81	75	74	4505
53(1,E5)	0.5%DMSO_(batch2)	146	100	120	116	107	91	97	139	60	107	126	97	62	112	95	123	116	145	102	112	56	99	103	104	104	5284
54(1,E6)	0.5%DMSO_(batch2)	104	72	135	111	83	103	81	94	43	105	91	95	40	89	76	92	97	107	85	74	42	78	84	76	76	4329
55(1,E7)	cs04--2ah_(batch1)	105	100	126	93	98	92	97	102	46	103	103	96	54	98	84	88	105	125	102	92	35	83	90	85	93	4439
56(1,E8)	cs04--2ah_(batch1)	118	91	128	122	102	100	88	97	60	108	101	97	68	101	93	117	105	124	112	84	45	85	109	81	80	4813
57(1,E9)	cs04--2ah_(batch2)	141	113	132	129	101	107	102	112	51	94	111	104	68	119	94	106	103	137	103	107	55	92	99	101	111	5046
58(1,E10)	cs04--2ah_(batch2)	98	99	105	128	78	102	76	116	44	110	107	83	48	108	80	75	101	109	90	85	37	82	84	81	82	4552
59(1,E11)	I_W31	130	92	128	130	100	95	107	107	50	105	124	87	67	123	82	92	134	132	124	86	49	99	108	92	82	4989
60(1,E12)	I_W31	93	90	122	144	115	95	114	122	64	98	109	91	69	122	93	105	111	127	113	109	38	100	90	79	84	5008
61(1,F1)	C_(batch1)	107	94	127	134	117	95	100	128	59	112	102	99	71	106	78	95	96	144	108	90	40	102	106	103	82	4985
62(1,F2)	C_(batch1)	101	71	107	136	93	90	99	102	56	114	102	73	69	81	77	103	92	114	111	93	45	112	88	98	84	4556
63(1,F3)	C_(batch2)	127	89	125	152	118	107	125	124	66	107	112	107	71	121	110	117	96	119	95	95	52	105	96	107	88	5201
64(1,F4)	C_(batch2)	107	90	103	142	101	93	99	102	54	120	96	101	68	106	86	109	94	107	103	94	45	101	95	77	113	4712
65(1,F5)	AFB1(30nM)_(batch2)	111	98	116	86	96	116	77	102	47	90	102	112	58	97	77	82	116	126	82	87	44	87	78	81	82	4534
66(1,F6)	AFB1(30nM)_(batch2)	83	78	137	105	94	98	73	110	47	103	113	97	70	109	86	93	115	101	100	100	34	88	88	100	79	4488
67(1,F7)	cs29--2ah_(batch1)	120	76	101	102	88	73	91	114	50	107	85	86	59	85	69	76	87	98	99	84	36	89	78	65	83	4234
68(1,F8)	cs29--2ah_(batch1)	109	85	109	137	96	97	95	118	64	93	97	79	67	90	85	88	122	101	110	90	44	88	88	93	102	79	4768
69(1,F9)	cs29--2ah_(batch2)	109	89	99	142	98	80	99	82	34	122	101	99	55	87	83	87	76	112	100	100	45	107	93	89	86	4589
70(1,F10)	cs29--2ah_(batch2)	140	86	146	138	112	121	109	98	58	113	112	115	75	110	94	93	97	145	117	102	40	100	91	94	107	5179
71(1,F11)	E_W11	86	84	134	128	84	103	68	100	39	99	99	88	60	90	89	83	106	100	98	76	41	86	83	79	37	4255
72(1,F12)	E_W11	77	83	107	114	77	79	78	80	42	115	90	104	49	98	85	75	92	102	88	70	38	76	79	54	58	4100
73(1,G1)	I_(batch1)	97	86	134	127	114	123	93	97	47	107	101	102	53	98	98	92	116	120	116	99	48	105	90	86	84	4913
74(1,G2)	I_(batch1)	96	78	108	109	103	90	74	96	56	79	98	77	50	92	79	78	89	108	84	104	45	75	86	100	87	4300
75(1,G3)	I_(batch2)	120	81	150	107	111	112	106	113	68	112	101	98	64	105	82	116	121	125	114	98	48	98	99	90	76	5028
76(1,G4)	I_(batch2)	90	113	115	140	79	111	101	107	51	108	90	89	63	111	68	98	105	115	84	89	36	91	98	114	88	4781
77(1,G5)	BaP(30uM)_(batch2)	109	131	143	130	120	101	106	127	51	118	109	105	57	119	95	105	137	165	121	112	48	104	99	82	105	5258
78(1,G6)	BaP(30uM)_(batch2)	106	116	122	128	87	97	103	121	41	116	91	96	66	107	94	98	104	122	122	88	47	76	98	81	96	4867
79(1,G7)	cw20--2a_(batch1)	121	93	150	137	75	104	104	120	60	114	91	85	49	109	84	89	104	148	109	101	32	80	86	83	90	4727
80(1,G8)	cw20--2a_(batch1)	112	96	124	114	93	109	92	133	45	101	105	105	52	132	101	95	120	148	120	114	35	101	118	90	95	4992
81(1,G9)	cw20--2a_(batch2)	114	94	126	150	106	110	103	117	59	101	100	88	55	114	89	98	114	125	125	104	42	95	93	82	93	5058
82(1,G10)	cw20--2a_(batch2)	113	79	116	115	89	94	79	110	49	89	108	102	56	105	78	91	98	126	83	84	50	95	89	83	75	4619
83(1,G11)	ERYT	112	99	119	107	105	88	91	118	46	98	105	108	70	119	95	101	102	123	98	116	35	90	98	79	94	4864
84(1,G12)	CLAR	102	98	109	104	83	96	73	95	47	94	92	85	63	79	79	93	97	141	93	103	39	86	86	95	88	4533
85(1,H1)	E_(batch1)	96	106	126	140	90	95	94	99	51	98	96	83	47	113	88	78	114	108	86	70	40	113	89	104	82	4638
86(1,H2)	E_(batch1)	100	79	115	109	75	79	100	81	41	109	87	87	53	113	76	92	90	100	104	86	41	79	79	77	69	4308
87(1,H3)	E_(batch2)	91	86	121	103	95	97	62	94	59	80	84	96	40	92	75	91	93	130	98	83	44	87	78	73	70	4215
88(1,H4)	E_(batch2)	74	78	98	115	59	96	67	82	50	74	80	74	36	95	61	68	80	116	100	65	34	91	79	64	58	3865
89(1,H5)	PCB1254(30nM)_(batch2)	91	85	105	116	88	81	86	84	41	74	92	71	49	101	76	94	109	106	105	86	23	78	65	67	81	4119
90(1,H6)	PCB1254(30nM)_(batch2)	116	98	115	104	89	105	78	96	41	92	100	98	61	111	85	80	95	129	112	85	42	81	87	68	71	4511
91(1,H7)	Background0	127	86	130	107	67	99	70	77	48	82	106	79	68	100	87	93	93	106	84	94	37	78	118	84	73	4290
92(1,H8)	Background0	135	120	140	136	117	143	111	119	65	86	116	103	64	109	93	126	148	129	119	118	52	102	121	118	104	5517
93(1,H9)	Background0	101	98	146	114	111	110	97	117	51	87	103	94	71	105	101	106	108	129	110	88	64	86	97	102	84	5007
94(1,H10)	0.17%MeOH	105	99	136	132	90	95	96	100	43	91	108	104	51	99	66	85	117	127	97	99	54	90	90	82	81	4639
95(1,H11)	AZI	86	57	65	97	63	72	65	68	27	90	71	74	38	65	53	50	72	85	66	64	30	64	78	58	55	3174
96(1,H12)	SMO	98	85	129	118	97	95	91	113	62	115	112	94	56	136	107	100	136	127	117	116	42	93	109	98	98	4949

Suppl. Table S3. Polyaromatic hydrocarbons (PAHs) pollutants measured in the extracts from swimming crab tissues. The most (geno) toxic PAHs are highligted, including Benz-a-anthracene, Benzo[e]pyrene, and Dibenzo [a, h] fluoranthene

Conc ug/kg)	Tissue type	Acenaphthylene	Phenanthrene	Anthracene	PYRENE	Benz-a-anthracene	Benzo-b-fluoranthene	Benzo-k-fluoranthene	Benzo[a]pyrene	Naphthalene	Acenaphthene	fluorene	Fluoranthene	Dibenzo [a, h] fluoranthene	Benzo [g, h, i] pyrene	Indene [1,2,3-cd] pyrene	SUM (all)	3 Most (geno)toxic PAHs (SUM)
cb04	Fat	0.00	0.00	0.00	241.61	0.00	21.58	0.00	28.25	360.64	801.94	1250.35	560.36	241.55	0.00	712.13	**4218.41**	269.80
cb29	Fat	142.44	19.61	5.81	34.79	84.08	90.90	0.00	203.98	107.64	66.04	216.23	0.00	124.55	552.94	443.74	**2092.75**	412.61
cs04	Fat	0.00	0.00	0.00	0.00	2.45	0.00	0.00	0.00	1076.23	181.33	503.33	910.78	259.38	2100.28	373.38	**5407.13**	261.83
cs29	Fat	6.54	0.00	0.05	0.00	0.00	0.00	0.00	209.76	467.61	0.00	0.00	0.00	418.59	0.00	0.00	**1102.55**	628.35
cw20	Meat	42.41	0.00	50.10	0.00	0.00	1387.75	134.84	0.00	0.00	0.00	0.00	224.22	56.10	0.00	0.00	**1895.42**	56.10
cw29	Fat	24.67	0.00	93.34	0.00	310.15	0.00	561.30	0.00	193.67	0.00	0.00	47.05	471.71	0.00	0.00	**1701.89**	781.86

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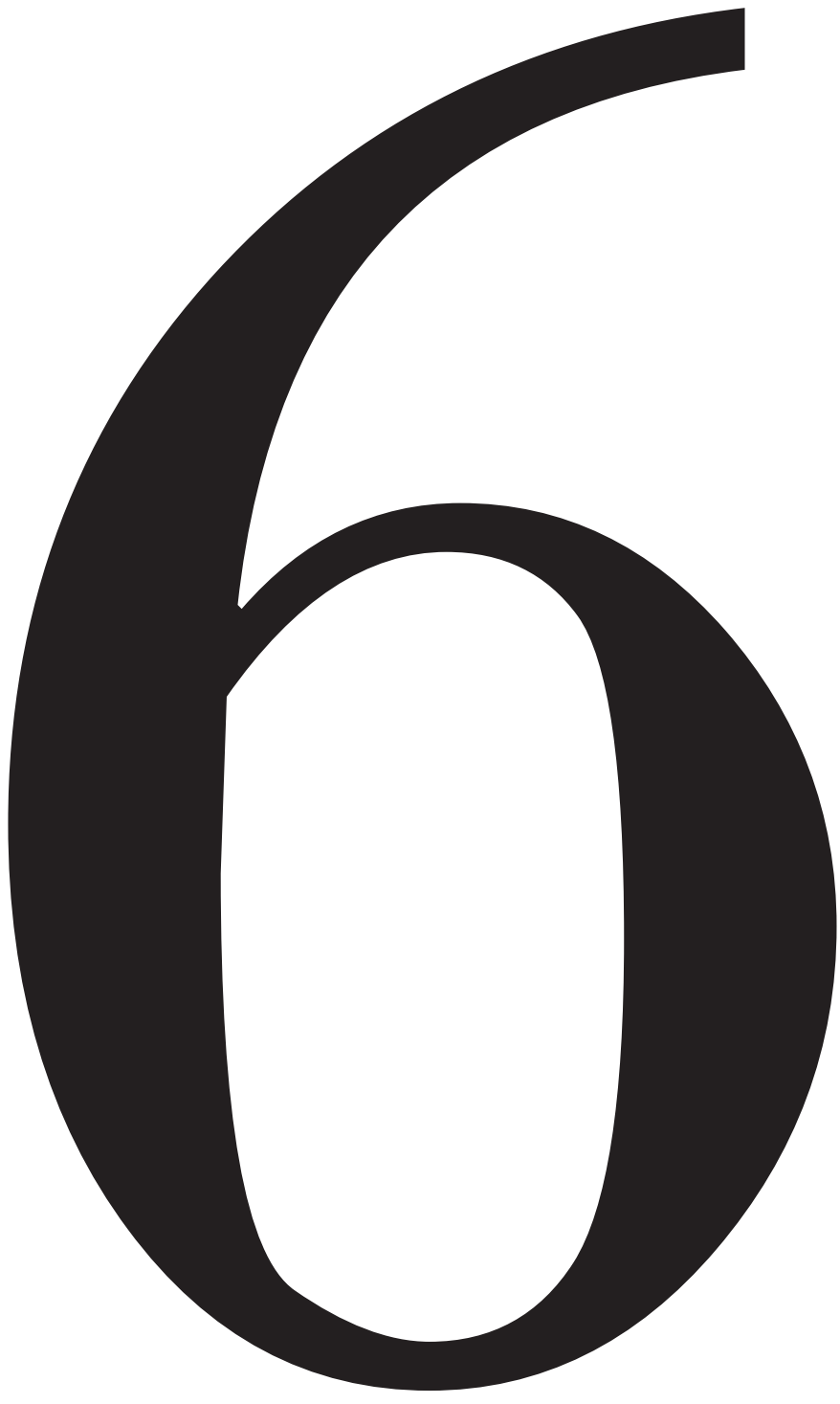


Chapter 6

General discussion and outlook

1. Introduction

The research presented in this thesis describes the development, validation, and application of a comprehensive effect-based bioanalytical tool to detect the presence and toxic potency of (un)known (hydrophilic) contaminants in water sources. With current analytical chemical approaches only few of the many chemical contaminants (mostly hydrophilic ones) can be effectively measured, and yet the risk that the analyzed compounds and their mixtures may pose to human and environmental health remains unknown. The newly developed bioassay is based on transcriptomics technologies for gene expression profiling in the nematode *C. elegans* as test organism (**Fig. 1**). Gene transcripts were altered in response to toxic model agents and untreated and treated waste water samples and samples from receiving surface water. Several gene transcripts responding to various adverse effects of contaminants were found and were successfully used as biomarkers to fingerprint toxic potencies in the various (water) samples. These marker genes were also employed to develop a dedicated multiplex gene expression assay that enables the fast and easy measurement of mRNA transcripts directly from crude lysates of nematodes. The newly developed bioassay is suitable for further development towards a routine tool for toxicological risk assessment to distinguish between polluted and good quality water samples, and to fingerprint the presence of toxic (hydrophilic) bioactive contaminants.

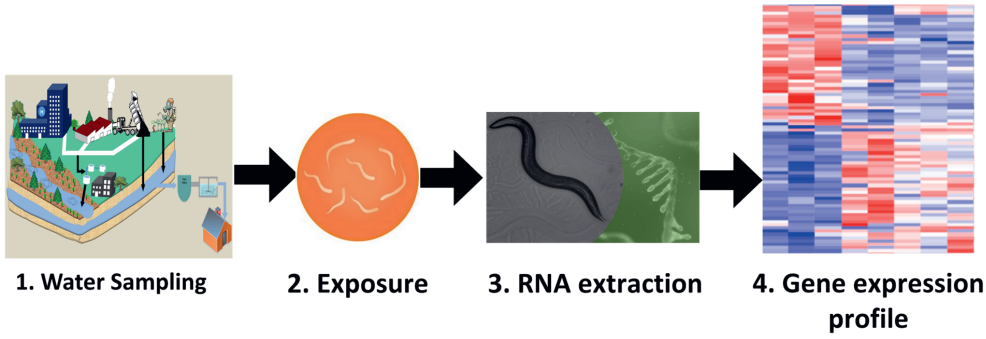
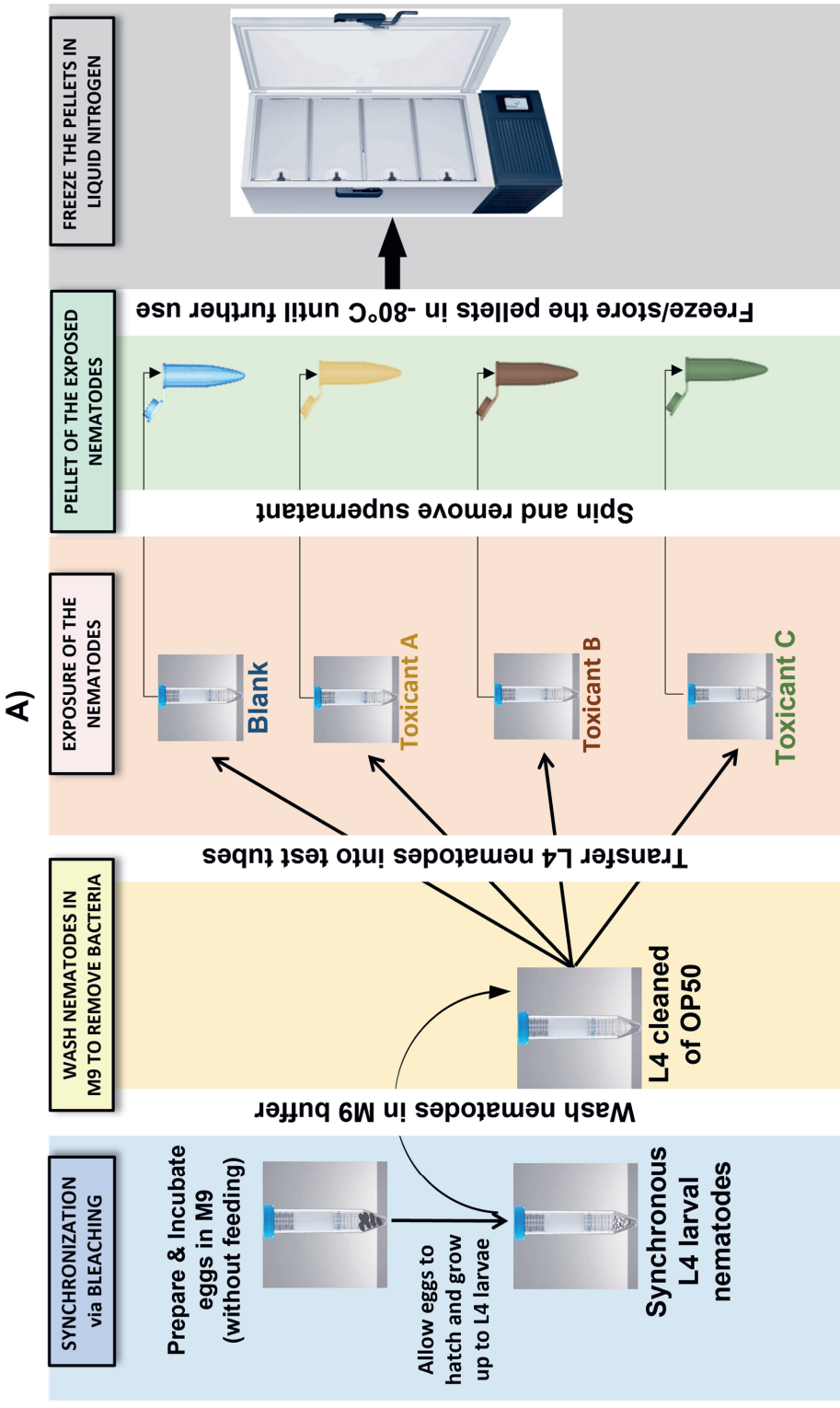


Figure 1. A summary of the procedure of transcription-based bioassay using *C. elegans* as a model organism to detect toxic potentials of (hydrophilic) contaminants.

In this chapter, the findings of research described in this thesis are discussed in relation to the four research questions posed in **Chapter 1**.

A. Can a practical nematode bioassay method be developed for (hydrophilic) compounds?

During the research (as presented in **Chapters 2 – 5**) it became clear that a successful bioassay method using the nematode *C. elegans* as model organism could indeed be developed. **Fig. 2** depicts a summary flowchart that was followed to optimize the handling and exposure of nematodes **(A),** and to determine the optimal exposure dosage **(B)**. Our research corroborates reports in literature that the assays using *C. elegans* provide outstanding advantages as it allows the combination of aspects of both in vivo and in vitro methods, especially due to its simplicity and manipulability [1, 2]. The assay can be versatile and cheap: the nematode culture, maintenance or exposure can be carried out using standard relatively inexpensive laboratory apparatus such as micro test tubes, Falcon™ 15-mL conical tubes, multiwell plates, or petri dishes. The nematodes can be cultured and tested in liquid or solid media [3]. For liquid exposure, the test material may be dissolved in media like M9 buffer [4] or K-medium [5] with or without feeding. On solid media, the nematode can be maintained and exposed on nematode growth medium (NGM) agar [6] with feed for a longer period.


A)
SYNCHRONIZATION via BLEACHING
Prepare & Incubate eggs in M9 (without feeding)
Allow eggs to hatch and grow up to L4 larvae
Synchronous L4 larval nematodes
WASH NEMATODES IN M9 TO REMOVE BACTERIA
Wash nematodes in M9 buffer
L4 cleaned of OP50
Transfer L4 nematodes into test tubes
EXPOSURE OF THE NEMATODES
Blank
Toxicant A
Toxicant B
Toxicant C
PELLET OF THE EXPOSED NEMATODES
Spin and remove supernatant
Freeze/store the pellets in -80°C until further use
FREEZE THE PELLETS IN LIQUID NITROGEN

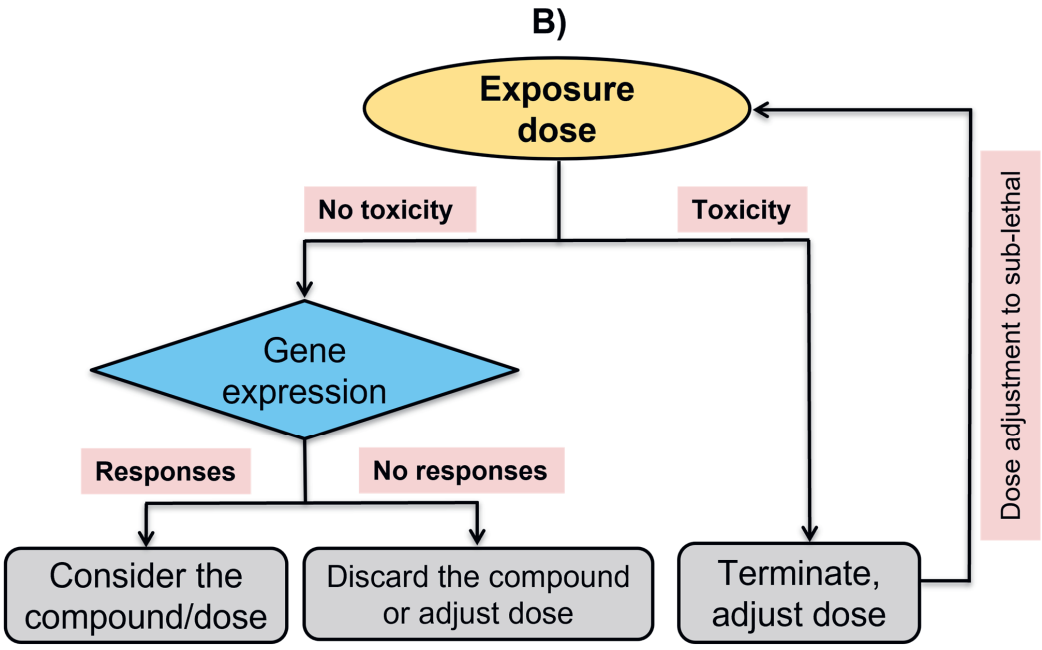


Figure 2. Experimental setup for nematode handling and exposure to contaminants (A), and the identification of optimal dosage regimes for gene expression analysis (B).

Furthermore, the nematode provides an opportunity to carry out a direct exposure to raw environmental samples (even the severely polluted ones like wastewater samples without the need to pretreat or to dilute) (**Chapter 4**). This exposure procedure can facilitate the detection of unknown hydrophilic compounds which cannot (or hardly) be extracted and concentrated in a quantitative way and that would otherwise get lost. This bioassay is scalable from hundreds to ten thousands of nematodes according to the analysis needs. While our protocols rely on manual estimation of the amount of nematodes, specialist worm counting machinery is available for the automated counting of large numbers accurately [7, 8].

It is important to control some potential confounding variables such as experimental temperature or developmental synchronization of nematode cultures as subtle differences can influence gene expression profiling results. Following the study in **Chapter 2**, the synchronized larval stage L4 was found to be the most suitable stage facilitating a successful exposure procedure. These L4 were treated without feeding to minimize potential developmental differences in the exposure patterns as some less hydrophilic compounds will at least partially bind to the bacterial feed. According to the literature, feeding the

nematodes during exposure could influence the bioavailability or other kinetics of the toxicants [9-11]. We previously carried out preliminary experiments to compare the transcriptional responsiveness of the fed nematodes versus unfed, and it appeared that the nematodes exposed with feeding tend to respond transcriptionally less than the starved ones. Nevertheless, starvation in late L4 larval stage can induce some stresses including the inhibition of egg-laying resulting in a so-called “bagging” where the embryos hatch within the body of the parent worms [12, 13]. This should not be a concern for our protocols as we exposed early L4 nematodes for 24 hours, particularly in **Chapter 3 – 5**, hence not reaching the gravid stage that occurs within 24 to 48 hours at 20 °C [14]. No concern about nematode death due to starvation periods of 24 hours or less was reported before [13], which is in accordance with our observations that no control or exposed nematodes died when starved for 24 hours (**Chapter 3 – 5)**.

This research revealed that the transcription levels of a significant proportion of genes were downregulated in response to direct-acting DNA-damaging agents (**Chapter 2**). The shutting down (downregulation) of the nematode gene-expression machinery can be presumed to be the nematode defensive mechanisms to alleviate the general stress toxicity. This could be related to the high exposure concentration, although at sublethal levels. A similar observation was previously reported in *Daphnia magna* [15], where high-concentration exposure with metals mostly trigged the downregulation of several gene families at higher concentrations. Overall, the assay procedure was successfully developed for *C. elegans* and was applied to assess biological effects of various contaminants.

B. To what extent is the nematode transcriptionally responsive to model contaminants?

Both general stress as well as specific mechanisms of toxicity of the tested contaminants are operational in the nematodes and can be detected transcriptionally, as was discussed in detail in **Chapter 2 – 4**. The nematodes responded to prototypical chemical agents for various mechanisms of toxicity including both direct-acting toxicants (as discussed in **Chapter 2**) as well as indirect-acting compounds (**Chapter 3**). In both exposure conditions, the mechanisms related to the known modes of toxicity of the studied compounds were represented among the differentially expressed genes (DEGs). The gene expression profiles of different toxic compounds showed limited overlap, especially for the alkylating agents (direct acting compounds) whose toxicities rely on the direct reaction with biological molecules such as DNA and proteins [16-18]. This implies that the transcriptional regulation of gene expression in nematodes was largely compound-specific.

In real life the exposure to contaminants barely involves single contaminants. The nematode *C. elegans* has been previously advocated as a promising alternative model for mixture toxicity testing [1]. In our research, transcriptional profiling in the nematodes treated with mixtures of xenobiotics indeed suggested some additivity between compounds (**Chapter 3**).

This chapter also discusses the concentration-dependent transcriptional effects of the tested toxicants. The analysis of concentration-response curves of the tested gene targets revealed that a toxicological dose descriptor, the “Lowest Observed Transcriptomic Effect Levels (LOTEL)” is applicable to the gene expression assay. This concept is comparable to No Observable Transcriptional Effect Level (NOTEL) (i.e., the concentration level of a chemical below which no significant changes in gene expression occur) previously promoted as a dose descriptor that can be helpful to assess the impacts of environmental contaminants as well as mixtures [15, 19, 20]. This implies that our transcription-based nematode bioassay can be applied in a similar approach. For decision makers it is important to know above which threshold there could be reason for concern/further action. For this the transcriptional effects in nematodes exposed to the water should be related to physiological and even (eco)toxicological effects in nematodes and other organisms. The final step would be to determine the maximum transcriptional effect levels in the nematodes below which adverse (eco)toxicological effects are unlikely to occur.

Most xenobiotics undergo biotransformation to render them harmless and enhance excretion, and thus this may alter their biological effects [21-23]. The biotransformation machinery includes proteins like phase I monooxygenases (also referred to as cytochrome P450s), phase II conjugation enzymes, and phase III xenobiotic transport proteins [24]. The activity of these functions can also be influenced by exogenous conditions like chemical exposure [25-27]. The genes encoding phase I enzymes in *C. elegans* are closely related to the mammalian CYP2, 3, and 4 cytochrome families [28], while CYP1-like metabolism is absent [29]. This research showed several genes encoding the nematode metabolic enzymes whose transcripts were differentially regulated in response to xenobiotics (**Chapter 2, 3, and 4**). In agreement with literature [29], this research provides evidence supporting the lack of a cytochrome CYP1-like P450 metabolism in *C. elegans* (**Chapter 3**). The CYP1 proteins are essential for metabolizing and bioactivating numerous indirect-acting xenobiotics like polycyclic aromatic hydrocarbons (PAHs) [30]. The outcome of the study presented in **Chapter 3** also reveals that the nematode exposure to benzo(a)pyrene (a PAH toxicant metabolized exclusively by CYP1 enzymes in vertebrate [29, 31]) induces the *C. elegans* gene transcripts related to vertebrate CYP2 and CYP4 and these different enzymes will almost certainly result in the formation of different metabolites than is the case with mammals. The CYP1 proteins in vertebrates are upregulated via the Aryl hydrocarbon receptor (AhR). This receptor plays a central role in modes of action of dioxins and dioxin-like compounds [32, 33]. Therefore our finding that the nematode was insensitive to the transcriptional effects of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (**Chapter 3**) is consistent with the absence of not inducing CYP1. This nematode insensitivity towards TCDD transcriptional effects also is consistent with literature that AhR homolog (AHR-1) encoded by the *ahr-1* gene in *C. elegans* purportedly does not interact with dioxins or any other known xenobiotic ligand [34]. Nevertheless, in **Chapter 2a** TCDD exposure does delay the early larval development in *C. elegans*, even in larvae that only were maternally exposed to this

toxicant. This suggests that the developmental effects occur via another mechanism that is not mediated by the AhR.

Interestingly, genotoxic-stress-response genes (such as cell-cycle checkpoints or DNA-repair proteins) in *C. elegans* genes are not transcriptionally regulated by either direct-acting (**Chapter 2**) or indirect-acting genotoxic agents (**Chapter 3**). For benzo(a)pyrene exposure, genotoxicity was not expected in *C. elegans* since its metabolism does not produce genotoxic metabolites [29]. However, direct-acting toxicants like N-ethyl-N-nitrosourea (ENU), formaldehyde (HCHO), and methyl methanesulfonate (MMS) analyzed in **Chapter 2b** have been reported before to produce DNA injuries in nematodes [35, 36]. We cannot yet explain the apparent lack of transcriptional responses of the *C. elegans* DNA-damage response (DDR) genes and also did not directly study the occurrence of DNA injuries. Further research will have to unveil whether other genes may need to be included in our transcription-based nematode bioassay to be able to assess transcriptional impacts of genotoxic stresses.

Overall, this research shows that the nematode is transcriptionally responsive to contaminants and this is very well reproducible in controlled exposure. Of course, a further transcriptomic profiling is needed to study e.g. what other mechanisms could be affected by TCDD exposure. Also, for the most relevant compounds and mixtures at broader ranges of concentrations and combinations should be tested to further characterize the *C. elegans* sensitivity towards this and other classes of contaminants.

C. What are the most relevant mechanisms represented among the differentially expressed genes (DEGs) in nematode in response to exposure to contaminants?

Gene expression profiling can assist to gain insights into the interaction between biological systems and their responses to toxic insults [37-42]. In **Chapter 1**, the transcriptomics-based bioanalysis approach is discussed as a promising monitoring tool for assessing the toxic potential of pollutants and to understand the modes of action involved. Our research revealed various biological mechanisms that are involved in the nematode response to contaminants exposure. The observed mechanisms responding to the tested pollutants could be grouped in two categories, (1) the mechanisms associated with the nematode adaptive response to the exposure and (2) the mechanisms linked to the toxicological effects of the involved contaminants.

In relation to adaptive response mechanisms, the studies in **Chapter 2 – 4** show differential expression of several genes which are involved in xenobiotics biotransformation and detoxification pathways in *C. elegans*. These include genes encoding proteins like nuclear hormone receptors (NHRs), cytochrome P450s (but not CYP1), glutathione-S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs) as well as xenobiotic efflux transporters. Transcriptional responses were also observed among the genes connected to protective

functions in the nematode such as antioxidant defence, antimicrobial activity, or immune response. Many of these genes shares similarities with their counterparts (also referred to as orthologues) in vertebrate species including their biological functions such as metabolic pathways [43-46] or protective roles [47-49]. The activation of adaptive stress response pathways may not be a direct indicator of toxic events per se, as explained in [50], but the involved genes do indicate the presence of the stressors that trigger the nematode's defense mechanisms. Thus, the expression of these genes can be used, for instance, as a proxy to detect the presence of the triggers for these responses.

Toxicological effects that are known to be linked to the tested contaminants were represented among regulated genes. For exposure with alkylating agents, known to be extremely reactive towards biological molecules such as nucleic acids or cellular proteins [16], we show in **chapter 2** that these compounds triggered transcriptional regulation among the nematode genes involved in proteotoxic and neurotoxic stress responses. Gene transcripts related to the nematode physiology such as fertility, reproduction, development, lifespan, aging, or cytoprotective processes were found in transcriptional profiles of indirect-acting xenobiotic toxicants whose toxic effects rely on metabolic activation [23] (**Chapter 3**). In response to wastewater exposure, several genes involved in the nematode physiological processes such as molting, growth, reproduction, or development processes were differently transcriptionally regulated. These effects possibly were triggered by the presence of endocrine-disrupting pollutants which are prevalent in wastewater [51] (**Chapter 4**). Overall, several relevant and well known biological mechanisms were represented among the gene expression profiles triggered in the nematode by various contaminants. This confirms that the transcription-based bioanalysis approach can simultaneously detect the toxic potential present for multiple mechanisms in a single test.

D. Can a dedicated multiplex gene expression assay be developed for fast and easy quantification of toxic potencies of (hydrophilic) contaminants in (water) samples?

The results of studies presented in **Chapter 2 – 4** prove that various toxic mechanisms of contaminants are operational in nematode and that they are detectable by using gene expression profiling approach. The next challenge of our research was to develop a dedicated multiplex method to reliably quantify the expression levels of target gene transcripts (also referred to as transcriptional biomarkers). The method was successfully developed by involving the mRNA transcripts of 46 target genes (as biomarkers) that were selected from the DEGs responding to indirect-acting toxicants (**Chapter 3**) or wastewater (**Chapter 4**) as summarized in **Fig. 3**. The newly developed method was validated by fingerprinting transcriptional effects of pure compounds, mixtures of organic pollutants (extracted from the tissues of contaminated crabs) or environmental polluted water samples (**Chapter 5**).

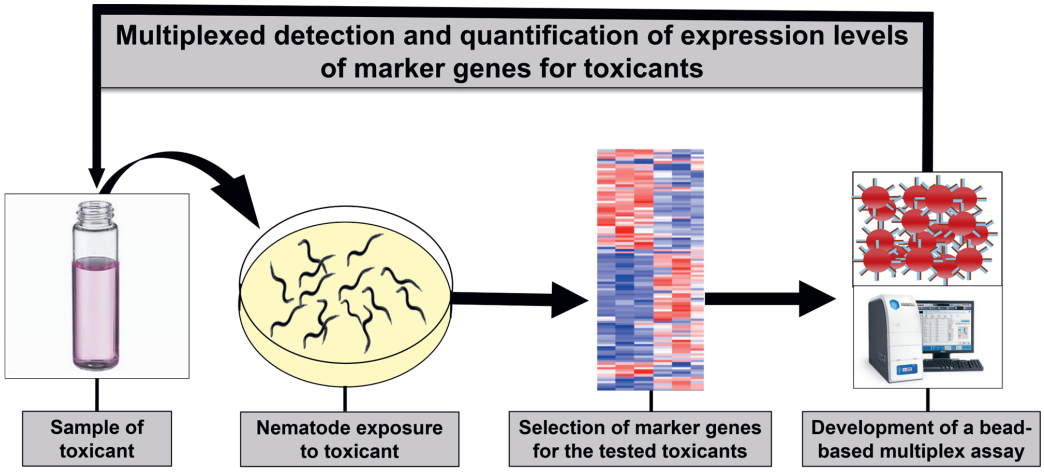


Figure 3. Schematic representation of the major steps followed to develop a multiplex gene expression profiling assay in using *C. elegans* as a model organism.

The novel multiplex gene expression assay developed uses Luminex® xMAP technology (x = analyte, MAP = Multi-Analyte Profiling) [52] (**Chapter 5**). This technology provides many advantages compared to the classical transcriptomics analysis in terms of sample preparation (e.g., sample volume and other redundant consumables). The MAGPIX® system utilized for developing our assay allows a multiplexed detection and quantification of up to 50 target mRNA transcripts in a single reaction volume and can read a 96-well plate in 60 minutes. This method relies on branched DNA (bDNA) technology for analyzing gene expression levels [53-55]. In contrast to the other transcriptomics technologies that require RNA extraction, cDNA synthesis, or PCR amplification [39, 56, 57], the multiplex assay developed in this research allows a direct detection of mRNA transcripts in tissues lysates of nematodes because the bDNA technology used by this assay employs linear signal amplification rather than exponential amplification of RNA targets [53-55]. It can therefore help to circumvent several challenges linked to RNA extraction (e.g., RNA degradation, low yield, low purity, or DNA contamination) or the synthesis of complementary DNA (cDNA) via reverse transcription reaction [58, 59]. And of course it is much faster and easier to perform. Overall, the new assay provides an opportunity to develop a dedicated bioanalytical tool to reliably assess the toxic potencies of (hydrophilic) contaminants by fingerprinting the expression levels of relevant gene markers. Application of this approach to real life polluted water and animal samples shows that it can indeed successfully indicate the absence of toxic potency (**Chapter 5**).

2. Main conclusions

1. This research reveals the potential of gene expression profiling in nematode as an effect-based bioanalytical tool for fingerprinting toxic potencies of bioactive contaminants.

2. It can be regarded as a potential powerful bioanalytical tool in comparison to classical assays which are usually too general or too specific.

3. General toxicity as well as specific toxicity mechanisms induced by exposure to chemical agents are operational in the nematodes and can be detectable via gene expression profiling.

4. The nematode biotransformation enzymes are transcriptional inducible by xenobiotic exposure. Nevertheless, this research provides evidence supporting the absence of the CYP1-like metabolism as previously reported in literature, illustrating that not all induction routes found in vertebrates are also present in the nematode.

5. Despite its involvement in the regulation of *C. elegans* important physiological processes (e.g., neuronal development, locomotion, egg laying, defecation behaviors, or fatty acid synthesis), transcriptional profiles identified in our research confirm previous reports suggesting that the nematode AhR homolog (AHR-1) does not interact with TCDD, hence the organism seems transcriptionally insensitive to this compound while still developmental effects were observed.

6. The transcription-based nematode bioassay can be used to characterize adverse outcome mechanisms for the (unknown) hydrophilic compounds, compounds that we cannot, or hardly, extract and concentrated in a quantitative way.

7. This bioassay can be used for monitoring the removal efficiency of (micro)pollutants during wastewater treatment and assessing the quality of the resulting effluent and receiving waters.

8. The newly developed multiplex approach overcomes the typical challenges (such issues include, for instance, RNA quality, transcript degradation, enzymatic manipulation, and others) connected to classical technologies that rely on complementary DNA (cDNA) synthesis for measuring mRNA transcripts.

3. Perspectives for translating the nematode bioassay to a practical method for water quality monitoring

The outcomes from this research open doors to the prospect of developing a nematode bioassay that can assist with environmental monitoring. In line with transcriptomics approaches described in **Chapter 1**, our assay can help to characterize the mechanisms of the toxic effects of the unknown hydrophilic compounds, chemicals that cannot, or hardly, be extracted and concentrated in a quantitative way. Transcription-based responses of a nematode can be translated into a dedicated effect-based bioanalytical tool that can be used to reliably detect the toxic potential of various water pollutants. For regular application, an easy-to-use dedicated bioassay should be developed using transcriptional biomarkers for assessment of toxic potencies of pollutants as summarized in **Fig. 4**. Such an assay requires the careful selection of relevant genes whose transcripts can be utilized as biomarkers for target contaminants. Therefore, the marker genes already identified in this research are a promising starting point.

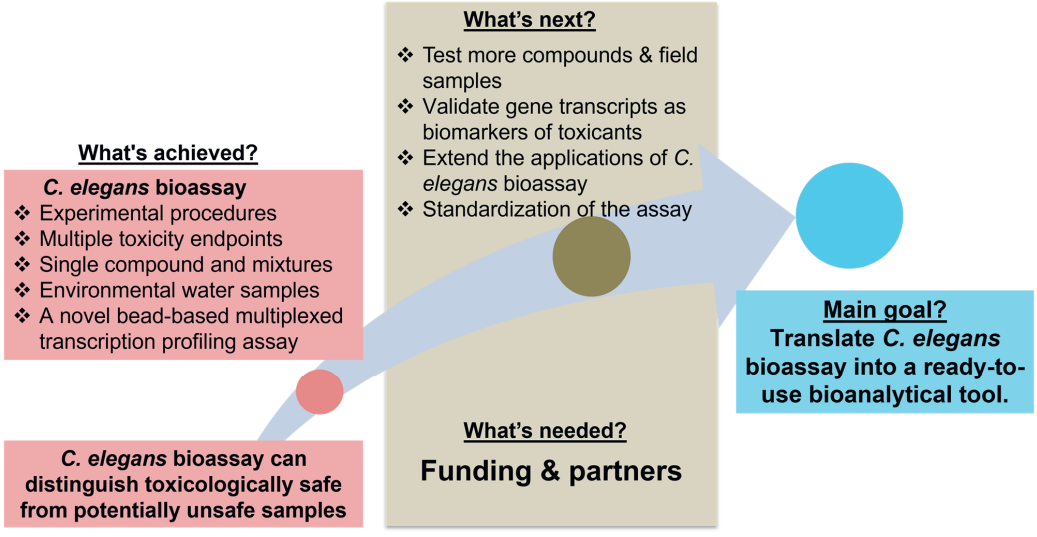


Figure 4. Current status of the newly developed *C. elegans* bioassay and perspectives to translate it into a bioanalytical tool for water quality monitoring.

A reliable transcriptional biomarker should fulfill three main criteria as proposed by Gou et al. [19]: a) it should have an expression response that is specific to a group of chemicals (assuming that the toxicants inducing similar gene expression signatures will have similar effects in nematodes as has been shown for other organisms), b) it should have an expression response that is concentration-dependent, and c) it should have an expression

response that is linked to a specific mode of action of toxic agents. Toxicity-related gene expression signatures (fingerprints) are needed for several prototypical compounds (including their mixtures) for various classes of toxicity. This could help to establish a kind of “reference gene expression database” or “gene expression signature library” that may be utilized to infer mechanisms of action of unknown contaminants. A similar approach has been previously recommended by various authors [37, 39, 60]. According to the strategic approach proposed by Paules (2003) [42], the reference gene expression database for the nematode *C. elegans* may mainly include a) the gene expression data from various chemical agents that induce specific types of toxicity and b) the gene expression data from structurally and functionally diverse chemicals that produce comparable pattern of toxicity. Considering the amount of chemicals in use, our research acknowledges that more transcriptomic studies are still needed to develop a reliable reference gene expression database. In relation to the water quality monitoring tool, as a starting point, the library of gene markers should be developed and validated first with the model chemical agents selected among priority substances under the EU Water Framework Directive. Overall, the above-described approach could help to make nematode bioassay a practical tool for effect-based water quality monitoring.

4. Potential application areas and advantages of transcription-based nematode bioassay compared to the existing bioassay approaches

Existing in vitro or in vivo bioassays are either very specific to one or few biological responses or are non-specific indicators of general toxic effects [50, 61]. In contrast, the transcription-based bioassay developed in this research can simultaneously reveal the type of toxic mechanism as well as the response magnitude related to the nature of the toxicants present in the test samples. Therefore, our novel bioassay provides a new tool to the toolkit of water quality managers, as it is capable of detecting not merely the presence, but rather the toxic potencies related to the presence of pollutants in water. Its application can provide data that are more specific and informative than standard bioassays such as Daphnia [62], fish [63], or mussel [64, 65] based assays. Furthermore, our assay is more versatile than very dedicated single-compound bioassays like CALUX systems [66]. The transcription-based nematode bioassay approach can be applied as:

a) An early warning system to indicate the safety, or risk levels created by pollutants in environmental samples. This can help management distinguish between samples with and without priority for further actions because of indications for toxicological concerns.

b) A monitoring tool for the water quality by distinguishing toxicologically safe from unsafe waters. For this the transcriptional effects in nematodes exposed to the water must be related to physiological and even (eco)toxicological effects occurring in organisms of interest. The maximum transcriptional effect levels in the

nematodes should be known below which adverse (eco)toxicological effects are unlikely to occur (at the short or long term).

c) A monitoring tool to assess the effectiveness of wastewater treatment technologies for evaluating the removal of bioactive (micro)pollutants (especially the unknown ones) prior to the disposal or reuse.

The application of bioassays in general and the assay described in this thesis in particular does require water quality managers to approach pollutants in water from a toxicological and/or environmental risk perspective rather than from a traditional chemical analytical approach in which type and concentrations of a limited number of known compounds are monitored rather than their combined toxic potency. Both the diversity, hydrophilicity and low concentration aspects of emerging pollutants will demand more effect-based than compound-based approaches in the future.

5. Recommendations for further research

This thesis describes the feasibility of exposure and responsiveness of the nematode *C. elegans* L4 stage to the model toxicants (**Chapter 2 and 3**) or wastewaters before and after treatment in a wastewater treatment plant (WWTP) (**Chapter 4**). The outcomes from these studies revealed that gene expression profiling can provide insights in the type of toxic mechanisms involved and can be translated towards the nature of the pollutants present in the test samples. Furthermore, the magnitude of transcriptional response of a target gene can be related to the exposure concentration (**Chapter 3**). A dedicated bioassay was successful developed and validated based on multiplexed detection and quantification of expression levels of marker genes for toxicants exposure (**Chapter 5**). Nevertheless, there is still a need to test more contaminants and combinations thereof in order to select and validate gene transcripts which can be used as reliable transcriptional biomarkers for toxicological assessment. The following recommendations are proposed for future research to translate this assay into a ready-to-use bioanalytical tool as an early warning system that can indicate the safety or detect the presence of hazardous pollutants:

1. Additional transcriptional profiling assays for several model toxicants in order to build a library of candidate gene markers that can serve as reference signatures to measure the toxic potency of the (un)known pollutants in samples.

2. Further studies analyzing transcriptomic concentration-responses of nematode to various toxic model compounds to define the concentration range detectable by transcription-based nematode bioassay and relate this to concentrations expected in field situations.

3. Dose-response studies to be performed with known mixtures of (hydrophilic) model compounds in different combinations and relative concentrations. Also for detection

of biotoxins related to toxic algal blooms the current fast, small scale, invertebrate, mechanisms-specific bioassay could be very valuable.

4. Further studies to assess a broader range of transcriptional effects of TCDD, especially in life stages where TCDD effects on development have repeatedly been shown. It is advisable to test a broader range of concentrations as general toxic mechanisms may mask more subtle chronic effects, as well as other dioxin-like compounds and PCBs.

5. A toxicity identification evaluation (TIE) approach with stepwise fractionation of wastewater samples to identify (hydrophilic) compounds responsible for the toxic effects observed in our research.

6. It would be interesting to explore the option for producing transcriptional reporters for the top expressed genes obtained in our research to possibly design transgenic *C. elegans* strains for endpoints that are most relevant in toxicity assessment of bioactive contaminants in wastewater and raw drinking water. This would enable even faster detection and quantification of specific toxic potencies of hydrophilic compounds, something which is not possible with in vitro reporter gene assays or with in vivo reporter gene assays with more vulnerable species.

7. Since *C. elegans* can only be applied for direct monitoring of toxic potencies in fresh water, it is advisable to develop a version of the current transcriptional effect bioassay with marine nematodes for brackish and marine water as well. This especially would be interesting for marine biotoxins that still are not easy to monitor.

8. The newly developed multiplex gene expression assay utilizing magnetic beads is very suitable for (semi)automatization of the procedure, especially washing steps, which would make high throughput screening easier. This development is recommended for increasing the practical applicability.

9. Collaboration with water quality managers is very important to optimize the applicability of the *C. elegans* bioassay for the toxicological risks in their water systems that they are mostly concerned about. In this respect it also is important they will be able to translate the test outcomes into clear conclusions of safe, doubtful or not good water qualities.

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Summary

Only few of the many chemical pollutants in water can be effectively measured by current analytical techniques. Especially hydrophilic compounds are hard to extract and unknown compounds (e.g., metabolites and reaction products) are hard to identify. Also, chemical techniques cannot provide information about the potential toxic effects of these compounds and mixtures thereof. Bioassays can quantify the toxic potency of bioactive pollutants, but most of the existing bioassays are either very specific for one or a few compounds or are non-specific indicators for general toxic effects. In this research, an invertebrate assay was successfully developed based on the genetic response of the nematode *Caenorhabditis elegans* to toxic compounds. The newly developed bioanalytical approach can simultaneously detect multiple mechanisms of toxicity in single test.

In **Chapter 1**, the relevance of our research is explained by introducing water quality monitoring challenges especially the presence of hydrophilic chemicals. This chapter also describes how transcriptomics-based bioanalysis approaches can be applied using the invertebrate *Caenorhabditis elegans* to detect the toxic potency of water contaminants.

In **Chapter 2**, the nematode responsiveness is evaluated. Exposure to the technical PCB mixtures Clophen A50 and the model dioxin TCDD (2,3,7,8-tetrachlorodibenzodioxin) was found to delay the early larval development in *C. elegans*. Significant developmental delays for L3 larvae to reach L4 stage of larval growth were observed, even in larvae that were only maternally exposed to levels as low as 10 nM. Further experiments in this chapter show that the nematode exposure to model compounds for direct genotoxicity resulted in transcriptional regulation of the genes that are linked to the mode of action of the tested toxicants (HCHO, ENU, and MMS). However, no differential expression was found among the genes involved in DNA damage response of nematode after exposure to the tested toxicants.

In **Chapter 3**, the inducibility of biotransformation enzymes in *C. elegans* is analyzed by testing four indirect-acting model xenobiotic compounds. The nematode P450 orthologs to vertebrate CYP2, CYP3, and CYP4 cytochrome metabolism pathways were shown to be activated by AFB1, B(a)P, and PCB1254 exposure. The lack of CYP1-like metabolism in the nematode previously reported in literature was also supported by gene expression profiling results in this research. The results discussed in this chapter also support the hypothesis in literature that *C. elegans* aryl hydrocarbon receptor (AhR) homolog encoded by *ahr-1* does not play its role of modulating gene expression as is the case in vertebrates.

In **Chapter 4**, the newly developed transcription-based bioanalysis approach is applied to environmentally polluted samples. The gene expression levels in the nematode exposed to wastewater inflow received by WWTP were significantly altered for several gene transcripts, which was not the case with nematodes exposed to the treated effluent or receiving surface water. The removal of toxic potencies implies a significant decrease in bioactive pollutant-load by the wastewater treatment. This chapter describes the development and proof of

principle of a novel method that can quantify the presence and fate of toxic potencies of unknown hydrophilic, including emerging contaminants and the effect of environmental technological processes to remove these mostly unknown contaminants.

In **Chapter 5**, a design of a novel bead-based multiplexed transcription profiling in *C. elegans* is described. Gene makers selected from the earlier studies as presented in chapters 3 & 4 were used to develop and validate a dedicated multiplex gene expression assay for fingerprinting the toxic potencies in polluted samples. The advantages of the novel method approach are also discussed in this chapter, especially the possibility to measure mRNA levels of multiple target genes directly from crude nematode lysates without the need of RNA extraction or cDNA synthesis.

In **Chapter 6**, the results of this research are discussed in broader context of fingerprinting toxic potencies of (hydrophilic) bioactive contaminants in (water) samples. Importantly, this chapter discusses how the effect-based bioanalysis approach developed in this research can be further developed into practical methods for water quality monitoring. Also, future research directions and recommendations are proposed.

Author's publications

A. Karengera, M.G. Sterken, J.E. Kammenga, J.A.G. Riksen, I.J.T. Dinkla, A.J. Murk, Differential expression of genes in *C. elegans* reveals transcriptional responses to indirect-acting xenobiotic compounds and insensitivity to 2,3,7,8-tetrachlorodibenzodioxin, Ecotoxicology and Environmental Safety 233 (2022) 113344. https://doi.org/10.1016/j.ecoenv.2022.113344

A. Karengera, C. Bao, J.A.G. Riksen, H.P.J. van Veelen, M.G. Sterken, J.E. Kammenga, A.J. Murk, I.J.T. Dinkla, Development of a transcription-based bioanalytical tool to quantify the toxic potencies of hydrophilic compounds in water using the nematode Caenorhabditis elegans, Ecotoxicology and Environmental Safety 227 (2021) 112923. https://doi.org/10.1016/j.ecoenv.2021.112923.

A. Karengera, I. Verburg, M.G. Sterken, J.A. G. Riksen, A.J. Murk, Inez J.T. Dinkla. Fingerprinting toxic potencies of hydrophilic contaminants in wastewater using gene expression profiling in *C. elegans* as a bioanalytical tool. (Under review). Journal: Archives of Environmental Contamination and Toxicology.

A. Karengera, C. Bao, T.F.H. Bovee, I.J.T. Dinkla, A.J. Murk. A multiplex gene expression assay for direct measurement of RNA transcripts in crude lysates of the nematode *Caenorhabditis elegans* used as bioanalytical tool. (Accepted). Journal: Environmental Toxicology and Chemistry.

C. Bao, **A. Karengera**, I.J.T. Dinkla, J. Kammenga, W. Wieland, A.J. Murk. Early life developmental effects induced by dioxin and PCBs in novel bioassays with *C. elegans*. (In submission).

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“If you can dream it, you can do it. Your limits are all within yourself” — *Brian Tracy*

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About the Author

Antoine Karengera was born on 20th of June 1984 in Northern province of Rwanda. In 2008 he started to study Pharmacy at the National University of Rwanda, where he obtained his bachelor's degree in 2012. Antoine is currently a Registered Pharmacist by Rwanda National Pharmacy Council. In 2013 he was awarded the Erasmus Mundus scholarship to pursue a master's degree in Chemical Innovation and Regulation. This programme allowed him to study at the University of Algarve (Portugal) and University of Barcelona (Spain). In September 2015, Antoine obtained his Master's degree with a thesis focused on *Evaluation of the cytotoxicity of transition metal complexes: DNA cleavage by copper complexes in cells*. In August 2016, Antoine moved to Leeuwarden (Netherlands) to start a new journey working as a PhD candidate at Wetsus in the theme of Genomics Based Water Quality Monitoring in collaboration with Marine Animal Ecology (MAE) Group of Wageningen University. His PhD research focused on *development of an invertebrate bioassay for detection of toxic potency of hydrophilic contaminants in water*. During his PhD research, Antoine also followed and completed the Dutch national programme for vocational postgraduate training in toxicology for registration as European Recognized Toxicologist (ERT). He is currently registered under status of a Toxicologist-in-Training by the Netherlands Society of Toxicology (NVT). In 2021, before finishing his PhD, he started to work as a toxicologist for Toxicology & Environmental Research and Consulting (TERC) at Dow Chemical Company. In June 2022, Antoine, his wife and their two children became Dutch citizens through naturalization.

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The SENSE Research School declares that **Antoine Karengera** has successfully fulfilled all requirements of the educational PhD programme of SENSE with a work load of 48.9 EC, including the following activities:

SENSE PhD Courses

- Research in context activity: 'Supervising students and teach them how to perform research using the Nematodes, and transferring knowledge on (eco)toxicogenomics to fellow researchers, and on gene expression analysis tools' (2018-2020)

Other PhD and Advanced MSc Courses

- Risk assessment, Wageningen University (2017)
- Laboratory Animal Science, Utrecht University (2017)
- Ecotoxicology, Vrije Universiteit Amsterdam/Wageningen University (2017)
- Toxicogenomics, Maastricht University (2017)
- Occupational toxicology, Radboud University (2018)
- Cell Toxicology, Leiden University (2018)
- Epidemiology, Utrecht University (2018)
- Mutagenesis and Carcinogenesis, Leiden University (2019)
- Organ Toxicology, Radboud University (2020)
- Starting course, and Presentation skills, WETSUS (2016)
- Communication Styles, WETSUS (2016)
- Image Processing for Scientists: Adobe Illustrator, WETSUS(2016)
- Illustrations for Scientific Publications, WETSUS (2017)
- How to supervise BSc/MSc students WETSUS(2017)
- Design of Experiments WETSUS (2017)
- Talent, WETSUS(2018)
- Scientific Writing, WETSUS (2019)

Management and Didactic Skills Training

- Co-organise Wetsus Challenge Day recruitment (2017 and 2018)
- Supervising one BSc student with thesis (2019), and supervising one MSc student and two students with internship (2019)

Oral Presentations

- *Nematode Bioassay for Water Quality Monitoring. Bioassays: added value for water quality monitoring* (webinar), 10th December 2020, Leeuwarden, The Netherlands

SENSE coordinator PhD education

Dr. ir. Peter Vermeulen

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