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A Multiplex Gene Expression Assay for Direct Measurement of RNA Transcripts in Crude Lysates of the Nematode *Caenorhabditis elegans* Used as a Bioanalytical Tool

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Abstract: Gene expression profiling in *Caenorhabditis elegans* has been demonstrated to be a potential bioanalytical tool to detect the toxic potency of environmental contaminants. The RNA transcripts of genes responding to toxic exposure can be used as biomarkers for detecting these toxins. For routine application in environmental quality monitoring, an easy-to-use multiplex assay is required to reliably quantify expression levels of these biomarkers. In the present study, a bead-based assay was developed to fingerprint gene expression in *C. elegans* by quantitating messenger RNAs (mRNAs) of multiple target genes directly from crude nematode lysates, circumventing RNA extraction and purification steps. The assay uses signal amplification rather than target amplification for direct measurement of toxin-induced RNA transcripts. Using a 50-gene panel, the expression changes of four candidate reference genes and 46 target mRNAs for various contaminants and wastewaters were successfully measured, and the expression profiles indicated the type of toxin present. Moreover, the multiplex assay response was in line with previous results obtained with more time-consuming reverse-transcription quantitative polymerase chain reaction and microarray analyses. In addition, the transcriptomic profiles of nematodes exposed to wastewater samples and extracts prepared from tissues of swimming crabs were evaluated. The profiles indicated the presence of organic pollutants. The present study illustrates the successful development of a multiplex fluorescent bead–based approach using nematode *C. elegans* crude lysates for gene expression profiling of target RNAs. This method can be used to routinely fingerprint the presence of toxic contaminants in environmental samples and to identify the most biologically active fraction of the contaminant mixture in a toxicity identification and evaluation approach. *Environ Toxicol Chem* 2023;42:130–142. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: *Caenorhabditis elegans*; nematode bioassay; transcriptomics; biomarkers; multiplex assay; water quality

INTRODUCTION

Gene expression profiling in the soil-dwelling nematode *Caenorhabditis elegans* is a valuable tool to detect toxic contaminants in the environment (Karengera et al., 2021). The genome of this invertebrate has been completely sequenced, and many of its genes and signaling pathways are functionally characterized and conserved in higher organisms (Gartner et al., 2000; Hillier et al., 2005; Kaletta & Hengartner, 2006). This makes *C. elegans* a suitable model organism for toxicological assessments because comparable responses between the nematode and higher organisms are to be expected (Baugh et al., 2005; Hillier et al., 2005). It was recently shown that both specific and general toxic effects of chemical toxicants can be detected by transcriptional analysis of exposed *C. elegans* (Karengera et al., 2021; Karengera, Sterken, et al., 2022). 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 gene expression

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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, for example, aryl hydrocarbon receptor activity or estrogenic activity, or nonspecific indicators of general toxic effects, for example, cell viability or stress (Escher et al., 2021; Wernersson et al., 2015). Hence, a battery of bioassays is often required for testing various types of pollutants present in samples (Jia et al., 2015). In contrast, exposure of a certain cell (e.g., cell line), tissue, or organism to a chemical of interest followed by gene expression analysis is a general and flexible approach that may provide insights into the type of toxic mechanism(s) involved (Moffat et al., 2015; Neumann & Galvez, 2002; Nuwaysir et al., 1999; Poynton et al., 2008). Different toxicants might up- or down-regulate 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 by Neumann and Galvez (2002). Such molecular markers can be applied, for instance, to monitor the quality of water sources or other matrices. For routine application, an easy-to-use multiplex assay should be developed to reliably quantify simultaneously the expression levels of a panel of established transcript biomarkers; that is, the selected target transcripts should be incorporated in one assay enabling the detection and identification of different contaminants in complex sample types based on the diagnostic expression profiles they induce.

Gene expression platforms such as microarrays (Nuwaysir et al., 1999), RNA sequencing (RNA-seq; Wang et al., 2009), and reverse-transcription quantitative polymerase chain reaction (RT-qPCR; Wong & Medrano, 2005) 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 (Bourdon-Lacombe et al., 2015; Rao et al., 2019). In contrast, an RT-qPCR assay can only analyze a small number of genes; but it is a more sensitive, accurate, and robust method and is widely used for validation of transcriptomic data (Morey et al., 2006; Wang et al., 2006). To quantify gene transcripts, all of these technologies utilize complementary DNA (cDNA) synthesized from the RNA template, which involves a reverse transcription 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 reverse transcription reaction (choice of reverse-transcriptase, primer design, enzyme, or assay volume, among others; Cocquet et al., 2006; Sieber et al., 2010). Moreover, highly skilled laboratory workers are required to perform such experimental procedures. Therefore, there is a need for an alternative method to translate transcriptome-scale research into a simple and cost-effective assay that can be used for decision-making in water quality management.

Fluorescent bead–based analysis using branched DNA (bDNA) technology is an emerging technique for gene expression profiling (Collins et al., 1997; Scerri et al., 2019; Tsongalis, 2006). In contrast to the aforementioned gene expression platforms, the bDNA technique uses signal amplification rather than target amplification to measure messenger RNAs (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 such assay, incorporating bDNA technology for the direct measurement of RNA transcripts (Scerri et al., 2019).

Several studies have used bDNA technology to detect contaminants in real (environmental) samples (Baldacchino et al., 2018; Bodero et al., 2019; Flagella et al., 2006; Metzger et al., 2013; Mills & Gallagher, 2017; Papadopoulou et al., 2019), but none of them has used the nematode *C. elegans*. This nematode is, however, a very useful tool to analyze environmental samples, for example, soil or water, because of its smaller transcriptome space, higher proportion of functionally characterized genes, and ease of high-throughput culture/testing of aquatic samples. The present study aimed (1) to develop a high-throughput bDNA assay for gene expression profiling of *C. elegans* transcriptional biomarkers for several contaminants and (2) to apply the newly developed multiplex assay to a test set of representative environmental samples. Eventually, we selected 46 DEGs from previous research (Karengera, Sterken, et al., 2022; Karengera, Verburg, et al., 2022) to adapt to the bDNA assay for quantitating mRNA transcripts directly from crude nematode lysates without 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 which can bioaccumulate environmental contaminants.

MATERIALS AND METHODS

Sample preparations

Aflatoxin B1 from *Aspergillus flavus* (AFB1; ≥98% purity), benzo[*a*]pyrene (B[a]P; ≥96% purity), and Aroclor 1254 (polychlorinated biphenyl [PCB] 1254; analytical standards grade) were purchased from Sigma-Aldrich (St. Louis, MO). Solutions of these toxicants were prepared in dimethyl sulfoxide (DMSO), as described by Karengera, Sterken, et al. (2022). Water samples were obtained from a sampling campaign in the city of Sneek, in The Netherlands, as described by Verburg et al. (2019). These included wastewater samples originating from community, hospital, and nursing home wastewaters; wastewater-treatment plant (WWTP) influent; WWTP effluent; surface waters 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 the present study were WWTP influents and associated effluents (sampled at the same time) from various locations in The Netherlands and Germany (van Heijnsbergen et al., 2022). Prior to use in nematode exposure, water samples were centrifuged and filtered to remove suspended solids; they were then used without further extraction or preconcentration (Karengera, Verburg, et al., 2022).

Organic pollutants from swimming crab tissues were prepared as follows: Crabs collected in Hangzhou Bay in China were separated into the raw edible lipid part (fat) and raw meat, followed by sample homogenization in 1 ml of Milli-Q water using an ultrasound homogenizer (Scientz; DY89). Ten grams of each sample were dried overnight at 35 °C and mixed with 1 g of $NaSO_4$. After that, hexane/acetone (1:1) liquid–liquid extraction methods were applied, as described by Liu et al. (2009). The extracted samples were desulfurated by adding tetrabutyl ammonium sulfite (US Environmental Protection Agency method 3660), and further cleanup was performed using a multilayer acid-base silica column, as described by Murk et al. (1996). High-performance liquid chromatography (Agilent 1200 series) with an autosampler was applied to analyze levels of the 15 polyromantic hydrocarbons (Supporting Information, Table S5). An excitation wavelength of 290 nm and an emission wavelength of 430 nm were employed using a fluorescence detector (Waters 470 Scanning). For separation, a C18 column at 30 °C was used. Peaks were identified by comparing the retention time with that of a standard using the method described by Tfouni et al. (2007). In a final step, the extracts were dissolved in DMSO and stored at −20 °C until use.

Nematode culture and exposure

Synchronized L4-stage larvae of *C. elegans* wild-type Bristol N2 strain were cultured as described by Karengera, Sterken, et al. (2022). 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, nematodes were exposed to 30 µM of each toxicant (with the final DMSO concentration of 0.5%). 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 min at 1000 rpm at 20 °C (Avanti J-15 Centrifuge; Beckman Coulter). Then, the nematode pellets were transferred into 2-ml microtubes (Eppendorf® Safe-Lock tubes; Biopur®) and flash-frozen in liquid nitrogen for 1 min 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). The experimental setup for nematode exposure to the studied contaminants is added as Supporting Information, Figure S1A–D.

Nematode lysis

Nematode 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, nematode 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, followed by bead-beating homogenization (6500 rpm, three cycles of 20 s each with an intercycle pause of 30 s) using a Precellys® Evolution homogenizer. Samples were then incubated at 65 °C for 30 min. During this incubation, the samples were vortexed at maximum speed for 1 min every 10 min. After this step, the samples were centrifuged at 16 000 *g* for 15 min (at room temperature) to pellet any remaining cellular debris, followed by the transfer of supernatants to new test tubes and storage at −80 °C until multiplex assay analysis.

Multiplex assay design

Target mRNA markers were selected among the DEGs found in previous transcriptomics studies with microarrays (Karengera, Sterken, et al., 2022; Karengera, Verburg, et al., 2022) in which expression of 46 targets was already confirmed/validated using RT-qPCR. Multiplex panels containing target-specific probe sets and magnetic capture beads (premixed and ready to use) were purchased from Thermo Fisher Scientific. Two multiplex panels were designed: one with 14 target mRNAs (14-plex assay; see pilot study in Supporting Information) 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 test the bead-based multiplex assay with crude nematode lysates. 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 nematode homogenization protocol. Subsequently, the approach was applied using a 50-gene panel including 46 target mRNAs previously found to respond to various contaminants and four 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 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 microarray and RT-qPCR studies. Because these assessments and checks were successful, the 50-plex was used to fingerprint WWTP influents originating from various locations and organic pollutants in extracts from swimming crab tissues.

Multiplex assay procedure

Target mRNA transcripts were quantified in nematode lysates using a QGP gene expression assay (Thermo Fisher) performed 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

TABLE 1: Target messenger RNAs in *Caenorhabditis elegans* analyzed using the bead-based 50-plex gene expression assay

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

Probe sets were designed to specifically hybridize to each of the 50 targets. *vmo-1* = vitelline membrane outer layer 1 homolog; *acdh-1* = acyl-coenzyme A dehydrogenase; *cyp* = cytochrome P450; *fat-5* = fatty acid desaturase; *lipl-3* = lipase like; *clec* = C-type lectin; *dhs-23* = dehydrogenases, short chain; *gst-20* = glutathione S-transferase; *mdh-1* = malate dehydrogenase; *cdr-1* = cadmium responsive; *par-5* = abnormal embryonic partitioning of cytoplasm; *rpl-6* = ribosomal protein, large subunit; *asp-13* = aspartyl protease; *col-160* = collagen; *lips-6* = lipase related; *tag-297* = temporarily assigned gene name; *chil-28* = pseudogene (chitinase like); *ugt* = uridine diphosphate glucuronosyltransferase; *tbg-1* = tubulin gamma chain; *cpt-3* = carnitine palmitoyl transferase; *wrt-4* = warthog; *cest-29* = pseudogene (carboxyl esterase domain containing); *spl-2* = sphingosine phosphate lyase.

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 a 40-µl sample, that is 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 quantified in triplicate by adding 40 µl of working homogenizing solution (instead of nematode lysate) to the working bead mix. Next, the hybridization plate was pressure-sealed, placed in the shaking incubator, and incubated for 20 h at 54 ± 1 °C at 600 rpm. After incubation, the protocol (MAN0017862) was resumed. Plates were read using the MAGPIX system equipped with Luminex xPONENT software (Ver 4.2.1705.0).

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 FI (MFI) values were considered for further analysis. Data processing was carried out using a QGP Data Analysis Software (Ver 1.1), which is freely available online (ThermoFisher). In this software, the data quality control parameters were set as follows: 10 MFI for maximum background, 20% for technical precision (coefficient of variation), 30,000 MFI for saturation, and 35 as the required minimum number of beads (the MAGPIX instrument automatically counts the beads contained in each well). The limit of detection was determined by multiplying three times (set as the default in the software) the background signal's standard deviation plus the mean background signal. To determine gene expression level, the average signal 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 the average net MFI. Next, for each sample, each test gene signal (average net MFI) was divided by the reference gene signal (average 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 other three reference candidates were unsatisfactory because they showed too much variation. 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 $p < 0.05$. Statistical significance was tested using a two-tailed Student *t* test in Excel ($p < 0.05$).

RESULTS

Multiplex assay technical evaluation

A first check was carried out on the adequacy of bead-beating homogenization of nematodes. The bead-beating homogenization was efficient for nematode lysis because 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 then conducted to formulate the design and protocols of the bead-based multiplex assay with *C. elegans*. The measured MFI values for the different mRNAs (14-plex) in the nematode lysates were proportional to the dilution used (see Figure 1 in the pilot study; $R^2 > 0.9$). Hence, we were confident in proceeding 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 four 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. In all dilutions of surface water and community wastewater samples MFI signals resulted in accurate data; that is, target signals were generally within the suggested range of 70–130 of the percentage recovery values (Supporting Information, Table S1B). Exceptions were seen for warthog (*wrt-4*) in the 1:25 diluted sample (<6 MFI) and saturation for ribosomal protein, large subunit (*rpl-6*); collagen (*col-160*); and C24B9.3 in the 1:5 dilution (>30,000 MFI; Supporting Information, Table S1A,B). All bead counts extended the minimum of 35, except for undiluted community wastewater samples (Supporting Information, Table S1C).

To select reference genes for normalization, the expression stability of four candidate genes, abnormal embryonic partitioning of cytoplasm (*par-5*), *tbg-1*, *rpl-6*, and malate dehydrogenase (*mdh-1*), was evaluated. The transcripts of *par-5* and *tbg-1* were the most stable (variation <20%) and were further used for normalization of data (Supporting Information, Table S1D). 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, that is, investigating 46 target genes and two reference 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, this used in the full-scale experiment.

Relatively low bead counts were observed in some samples with undiluted lysate input. These samples were presumed to be too thick and viscous, which could have led to bead loss during washing steps. Furthermore, there was an increase as well as a decrease in mRNA levels which fell beyond the assay detection range. For instance, 303 MFI was measured for carboxyl esterase domain containing (*cest-33*) in nematodes treated with 0.5% DMSO (control for pure compound exposure samples). Following exposure to 30 μM of AFB1, the transcripts of this gene increased dramatically, leading to the assay saturation with 44,151 MFI. 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 from being transferred properly to the sample probe tube. Using undiluted tissue lysates sometimes resulted in signal saturation (>30,000 MFI). Therefore, the nematode lysates should adequately be diluted to avoid such issues. Diluting lysates could also reduce bead loss during the washing steps and facilitate the sorting and reading of magnetic beads by the Luminex xMAP reader. The results of the 50-plex were compared with 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 (Supporting Information, Table S2A,B). Although all 50 mRNAs were successfully measured in all samples, three mRNA targets were excluded from the analysis (glutathione *S*-transferase *gst-20*, *rpl-6*, and *col-160*) because their MFI signals were saturated (>30 000) at the dilution used (Supporting Information, Table S2B). Unexpectedly, signal saturation was also observed for the reference gene *par-5* in many samples, whereas all MFI signals measured for the 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 mRNA 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 (Supporting Information, Table S2C). These samples were excluded from further analysis.

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 a microarray study in which expression of some genes was already confirmed by RT-qPCR analysis (Karengera, Sterken, et al., 2022; Karengera, Verburg, et al., 2022). From the

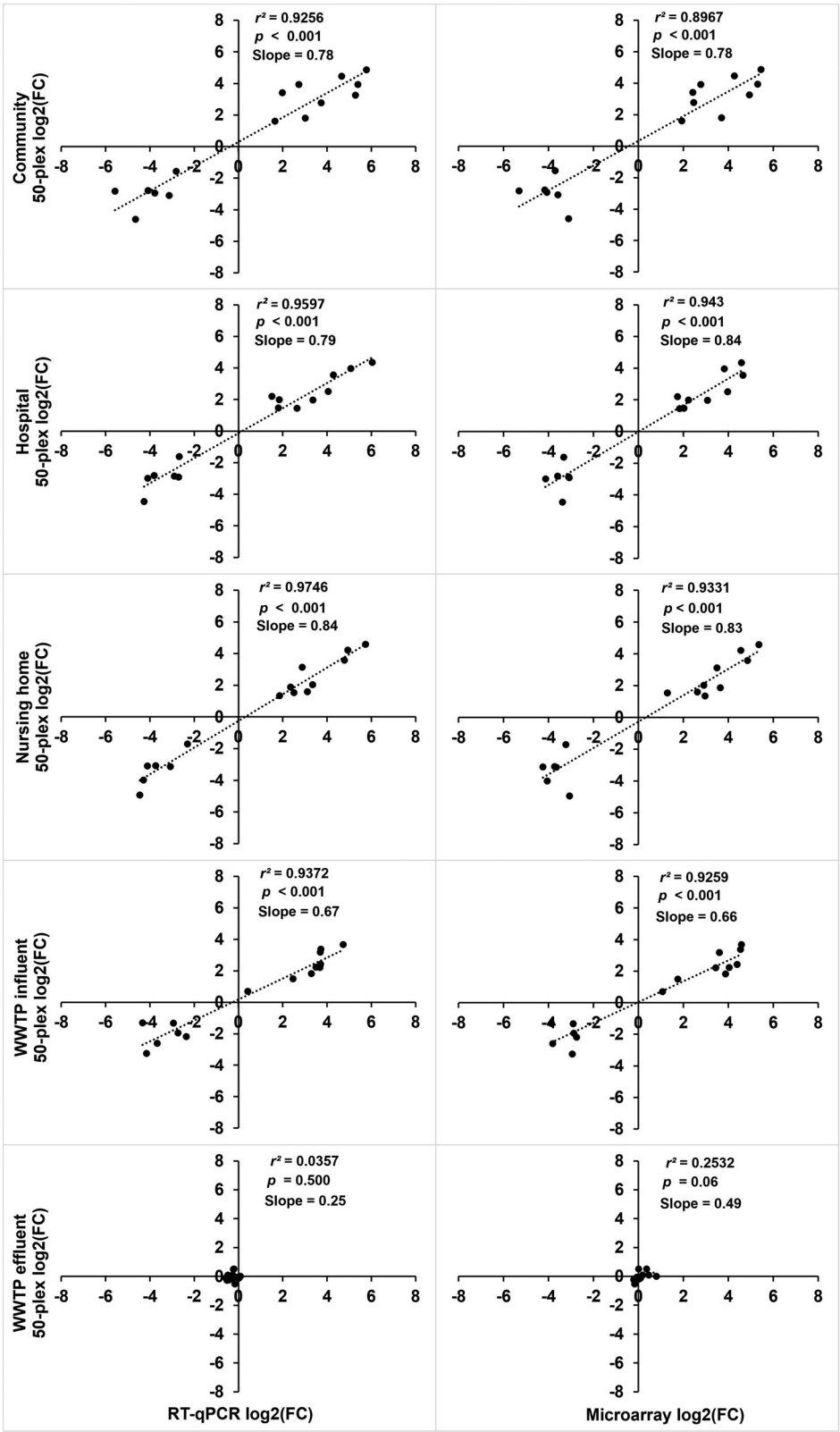

FIGURE 1: Comparison between messenger RNA (mRNA) expression measurements by the bead-based 50-plex assay, reverse-transcription quantitative polymerase chain reaction (RT-qPCR), and microarray assays for a selection of 15 target genes. The regression plots for log2-transformed mRNA expression data in the nematodes treated with (waste)water are shown. The analysis involved 15 target mRNAs responding to the (waste)water originating from the community, a hospital, a nursing home, and 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 $p < 0.05$. FC = fold change; WWTP = wastewater-treatment plant.

46 selected target mRNAs, 12 mRNAs responded to AFB1, B(a)P, or PCB1254, and 38 mRNAs responded to (waste)water samples. Among these targets, nine of the 12 and 15 of the 38 transcripts were previously validated/confirmed by RT-qPCR analysis.

For all treatment types, a significant correlation ($R^2 > 0.8$, $p < 0.01$) was observed between the fold changes of expression measured by the 50-plex assay and RT-qPCR analysis (Figures 1 and 2). Similarly, a comparison between the 50-plex assay and microarray analysis also showed a significant correlation ($R^2 > 0.8$, $p < 0.01$; Figures 1 and 2). Overall, this showed that the newly developed 50-plex for analysis of mRNA transcripts in *C. elegans* results in the same outcomes as microarray and RT-qPCR analyses; but it is much more easier, cheaper, and faster. A summary comparing the approximate time and cost of 50-plex and

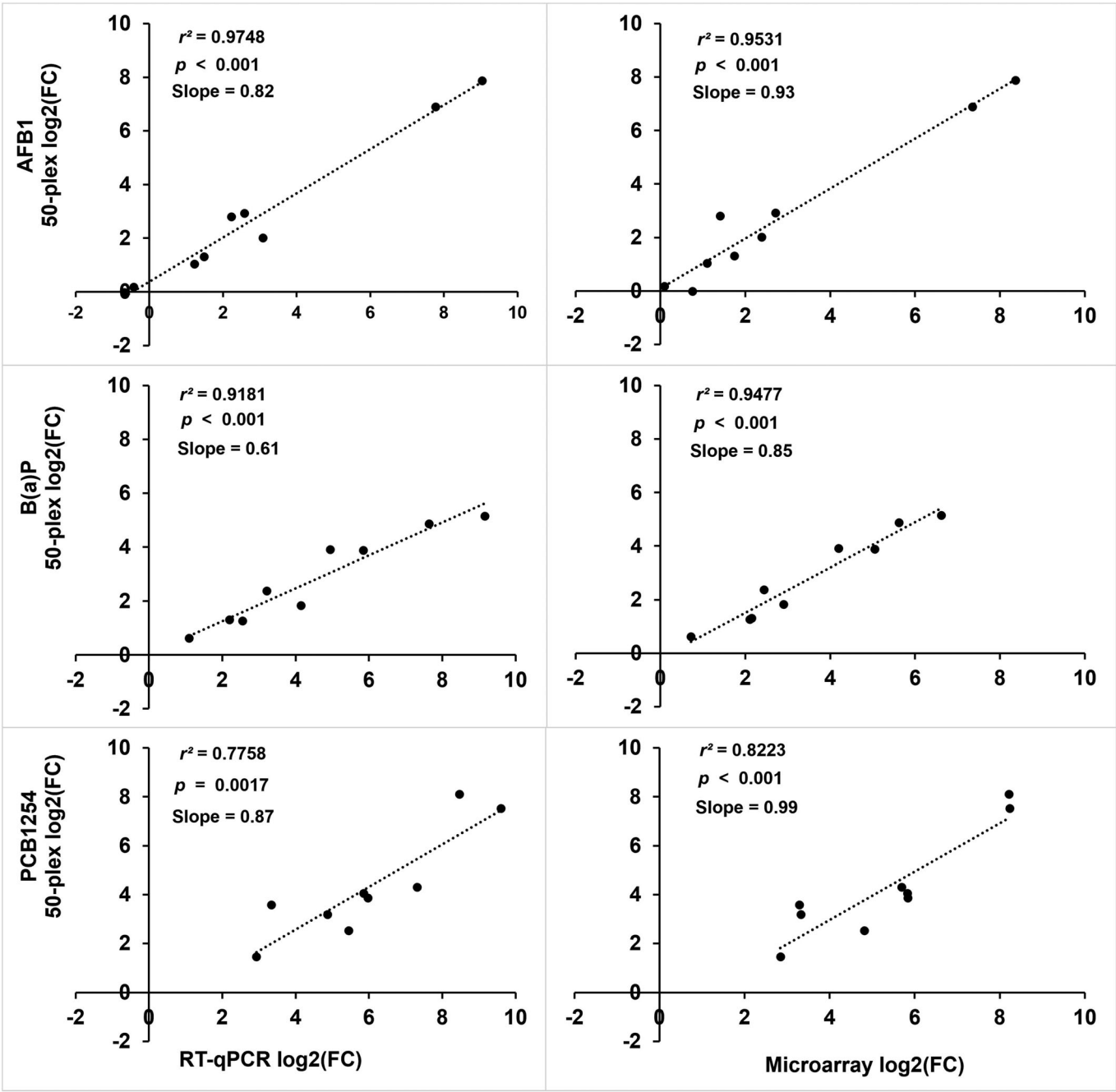

FIGURE 2: Comparison between messenger RNA (mRNA) expression measurements by the bead-based 50-plex assay, reverse-transcription quantitative polymerase chain reaction (RT-qPCR), and microarray assays for a selection of nine target genes. The regression plots for log2-transformed mRNA expression data in nematodes treated with indirect-acting toxicants are shown. The analysis involved nine target mRNAs of the genes responding to aflatoxin B1, benzo[*a*]pyrene, or Aroclor 1254. 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 $p < 0.05$. AFB1 = aflatoxin B1; FC = fold change; B(a)P = benzo[*a*]pyrene; PCB1254 = Aroclor 1254 (polychlorinated biphenyl 1254).

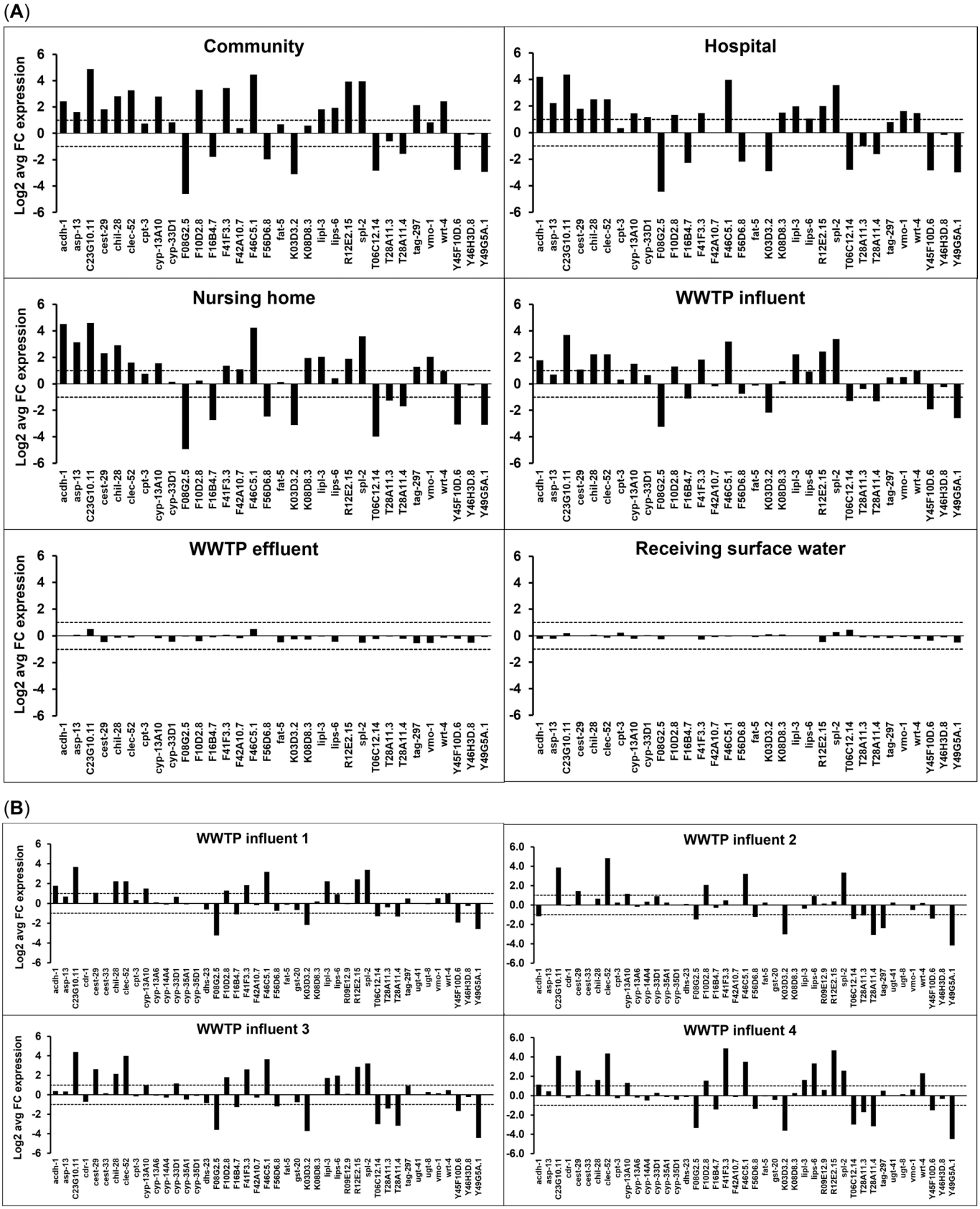

FIGURE 3: (Continued)

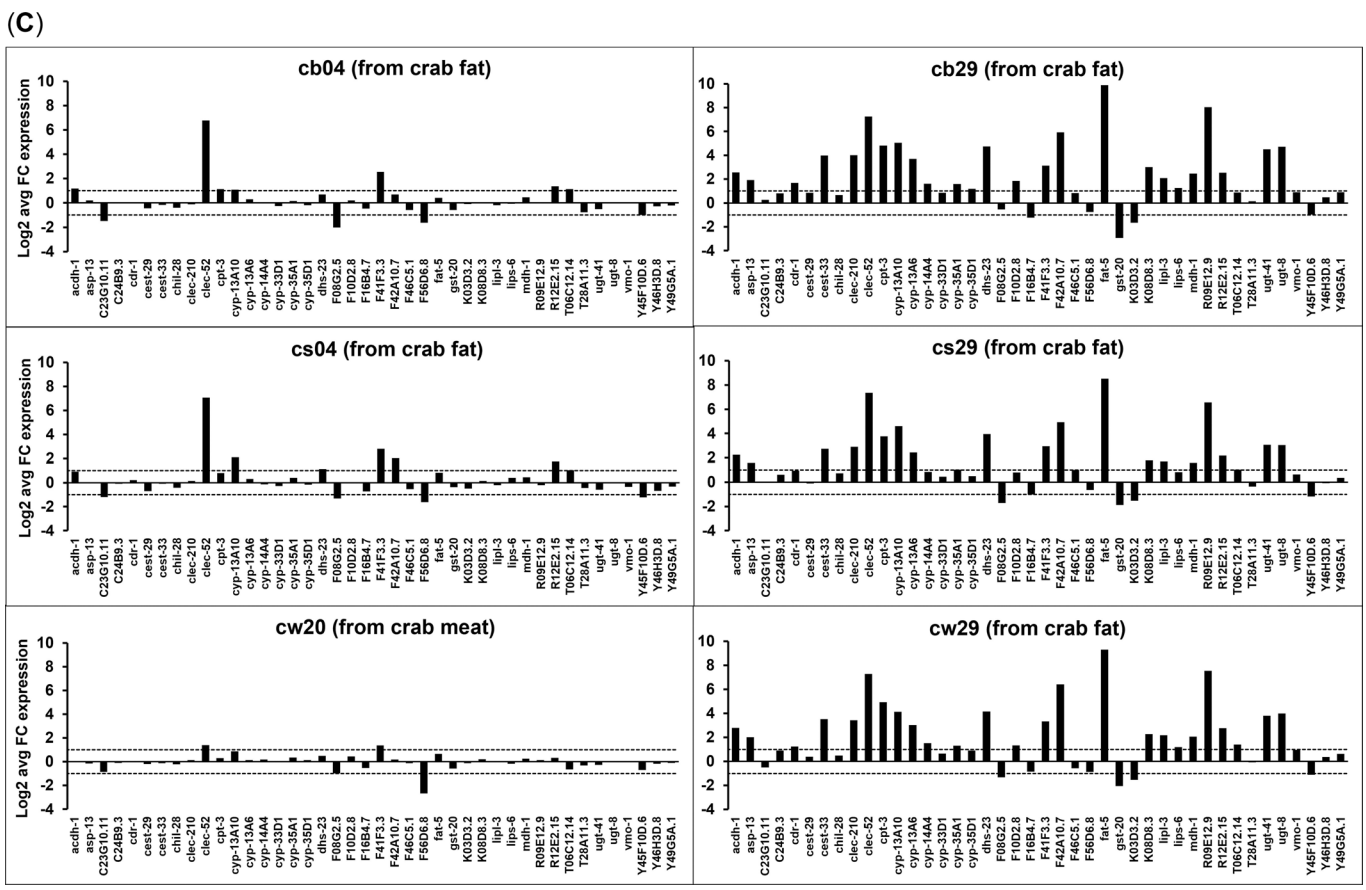

FIGURE 3: Transcriptional fingerprint of exposed *Caenorhabditis elegans* analyzed using a bead-based 50-plex gene expression assay. The transcriptional profiles in nematodes exposed to (**A**) untreated and treated wastewaters, (**B**) wastewater inflows to wastewater-treatment plants (WWTPs) from various locations, and (**C**) organic pollutant extracts from swimming crabs collected in Hangzhou Bay in China. An exposure sample from a nonreceiving surface water was used as control in (**A**), WWTP effluents related to each of the influent samples tested were used as control in (**B**), and an exposure sample with 0.5% dimethyl sulfoxide was used as control in (**C**). The fold change of gene expression level was calculated as the relative messenger RNA amount of a target gene in a test sample and a control sample, normalized to the housekeeping gene *tbg-1* (tubulin gamma chain). 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. FC = fold change; *acdh-1* = acyl-coenzyme A dehydrogenase; *asp-13* = aspartyl protease; *cdr-1* = cadmium responsive; *cest* = carboxyl esterase domain containing; *chil-28* = pseudogene (chitinase like); *clec* = C-type lectin; *cpt-3* = carnitine palmitoyl transferase; *cyp* = cytochrome P450; *dhs-23* = dehydrogenases, short chain; *fat-5* = fatty acid desaturase; *gst-20* = glutathione *S*-transferase; *lipl-3* = lipase like; *lips-6* = lipase related; *mdh-1* = malate dehydrogenase; *spl-2* = sphingosine phosphate lyase; *tag-297* = temporarily assigned gene name; *ugt* = uridine diphosphate glucuronosyltransferase; *vmo-1* = vitelline membrane outer layer 1 homolog; *wrt-4* = warthog.

RT-qPCR for carrying out this gene expression assay is provided in Supporting Information, Table S4.

50-plex fingerprinting of polluted field samples

Compared with the nematodes exposed to surface water (control), gene expression patterns induced by wastewater samples revealed a clear difference. As depicted in Figure 3A, untreated wastewaters significantly triggered differential expression of target genes, whereas 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 up-regulated C23G10.11, F46C5.1, and sphingosine phosphate lyase 2 (*spl-2*; >3 log2-fold change) in all samples. The transcripts of F08G2.5, K03D3.2, and Y49G5A.1 were among the most down-regulated ones.

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

TABLE 2: Differential messenger RNA expression fold changes in the nematode exposed to the organic pollutants extracted from swimming crab tissues

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

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. Bold values are the expression fold changes which are statistically significant ($p < 0.05$).

acdh-1 = acyl-coenzyme A dehydrogenase; *asp-13* = aspartyl protease; *cdr-1* = cadmium responsive; *cest* = carboxyl esterase domain containing; *chil-28* = pseudogene (chitinase like); *clec* = C-type lectin; *cpt-3* = carnitine palmitoyl transferase; *cyp* = cytochrome P450; *dhs-23* = dehydrogenases, short chain; *fat-5* = fatty acid desaturase; *lipl-3* = lipase like; *lips-6* = lipase related; *mdh-1* = malate dehydrogenase; *ugt* = uridine diphosphate glucuronosyltransferase; *vmo-1* = vitelline membrane outer layer 1 homolog.

and cw29 samples showed comparable responses; and the same was the case for cb04 and cs04 samples (Figure 3C). These samples were the extracts obtained from specifically the edible lipid part (fat) of crabs. Transcriptional effects by sample cw20, which 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 down-regulated 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 for cw20 samples. Of these transcripts, *clec-52*, fatty acid desaturase 5 (*fat-5*), and R09E12.9 were the most up-regulated (>6 log2-fold change) by cb29, cs29, and cw29. The transcript of *clec-52* was also the most up-regulated for cs04 (7.1 log2-fold), cb04 (6.8 log2-fold), and cw20 (1.4 log2-fold). Chemical compositions of these samples (i.e., extracts from swimming crab tissues) are provided as Supporting Information, Table S5, and correlations between observed patterns and chemical composition are discussed.

DISCUSSION

In the present study, a multiplex fluorescent bead–based assay for the nematode *C. elegans* was successfully developed using a QGP assay, to analyze the expression of target mRNA transcripts in nematodes exposed to model environmental contaminants and to polluted and control water samples. Marker genes were selected for AFB1, B(a)P, and PCB1254 and to differentiate polluted wastewater from treated wastewater or surface water. These targets represent important pollutants that are routinely monitored by management agencies. The 50-plex assay yielded distinct expression fingerprints, indicating the possible presence of pollutants in samples. Generally, the assay produced low background signals and a good correlation with microarray and RT-qPCR analyses. This assay is suited to quantify expression levels of target mRNAs directly in diluted crude lysates of nematodes (e.g., worms directly exposed to unconcentrated water samples).

In this assay, it was found that lysate dilution can be challenging because the incorrect sample input may result in an increase or decrease in mRNA levels that fall beyond the assay detection range. For instance, signal saturation was observed for some genes like *par-5*, *rpl-6*, and *col-160* encoding, respectively, 14-3-3 protein (Morton et al., 2002), 60 S ribosomal (Hansen et al., 2007), and collagen proteins (Johnstone, 1994), which are known to be among the most abundant proteins in nematode. Such transcripts cannot be reliably quantified in this multiplex assay unless a lysate is correctly diluted. Other challenges may arise from the "hook effect" phenomenon, which is common in fluorescent dye–based assays when the concentration of a target analyte is too high (do Carmo Dias Gontijo et al., 2016; Genshaft et al., 2016; Ross et al., 2020). This can be addressed by designing a multiplex panel set that combines only the mRNA transcripts that express within a comparable range in response to a particular stimulus/exposure, as previously discussed (Metzger et al., 2013). Also, it is recommended that a relatively high and a relatively low dilution of a lysate sample be tested to prevent underestimation or missing the signal.

The profiling outcomes showed that wastewater samples were contaminated with pollutants that regulated the expression of several of the selected target genes, whereas relatively clean samples including treated WWTP effluent and the receiving surface water did not affect 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 analyzed. Interestingly, *spl-2*, *clec-52*, C23G10.11, and F46C5.1, previously found to be the most up-regulated genes in wastewaters (Karengera, Verburg, et al., 2022), were also among the top induced transcripts in all WWTP influents analyzed by the 50-plex assay. The nematode *spl-2* and *clec-52*, respectively, encode the sphingosine phosphate lyase and C-type lectin (CLEC) proteins, which are involved in the nematode defense response to bacterial infection (Huang et al., 2020; Irazoqui et al., 2010). This suggests that the tested wastewater samples also were contaminated with pathogens, which is in line with early microarray findings (Karengera, Verburg, et al., 2022). 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 with other studies. The extracts were, however, assessed using a battery of other bioassays, and various toxic effects were confirmed in these samples. The most potent samples in our study (cb29, cs29, and sw29) also had the highest toxic potency in the other bioassays (Supporting Information, Table S5). Because the nematode genes responding to B(a)P and PCB were already evaluated previously (Karengera, Sterken, et al., 2022), 11 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, bioassays revealed the up-regulation of dehydrogenases, short chain (*dhs-23*); cytochrome P450 13A6 (*cyp-13A6*); *cyp-13A10*; uridine diphosphate glucuronosyltransferase (*ugt-8*); *ugt-41*; *spl-2*; *cest-33*; *clec-210*; and R09E12.9, which are all xenobiotic response genes (Karengera, Sterken, et al., 2022; Stasiuk et al., 2019). Interestingly, the transcripts that were originally included in the assay as markers of wastewater pollutants (Karengera, Verburg, et al., 2022), such as *fat-5*, *clec-52*, acyl-coenzyme A dehydrogenase (*acdh-1*), lipase like (*lipl-3*), aspartyl protease (*asp-13*), carnitine palmitoyl transferase (*cpt-3*), F42A10.7, F41F3.3, K08D8.3, and R12E2.15, were also considerably up-regulated in the extracts from crab tissues. The proteins encoded by these genes play various functions in nematode such as the fatty acid metabolic process by *fat-5* (Brock et al., 2006), *lipl-3* (O'Rourke & Ruvkun, 2013), and *cpt-3* (Van Gilst et al., 2005) or the dietary response by *acdh-1* (MacNeil et al., 2013) and *asp-13* (Qi et al., 2017). 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.

CONCLUSIONS

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 the present 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 as well as prototypical compounds (including their mixtures) to develop and validate more transcriptional biomarkers for monitoring of bioactive contaminants. Importantly, the gene expression signatures (fingerprints) for various classes of pollutants could help to establish a "reference gene expression database" or a "gene expression signature library" that may be utilized in multiplex assays for biomarker detection to classify unknown contaminants in samples and to infer their mechanisms of action. 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.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5505.

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Author Contributions Statement—**Antoine Karengera**: Conceptualization; Investigation; Data curation; Statistical analysis; Methodology; Visualization; Writing—original draft; Writing—review & editing. **Cong Bao**: Writing—review & editing. **Toine F. H. Bovee**: Conceptualization; Writing—review & editing. **Inez J. T. Dinkla**: Supervision; Conceptualization; Writing—review & editing. **Albertinka J. Murk**: Supervision; Conceptualization; Writing—review & editing.

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