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A Reduced Transcriptome Approach to Assess Environmental Toxicants Using Zebrafish Embryo Test

Pingping Wang^{†,1}, Pu Xia^{†,1}, Jianghua Yang[†], Zhihao Wang[†], Ying Peng[†], Wei Shi[†], Daniel L. Villeneuve[‡], Hongxia Yu[†], and Xiaowei Zhang^{†,*}

[†]State Key Laboratory of Pollution Control & Resource Reuse, School of the Environment, Nanjing University, Nanjing, P. R. China, 210023

[‡]United States Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, MN, USA

Abstract

Omics approaches can monitor responses of biological pathways at genome-scale, which are useful to predict potential adverse effects by environmental toxicants. However, high throughput application of transcriptomics in chemical assessment is limited due to the high cost and lack of matured dose response models. Here, a reduced zebrafish transcriptome (RZT) approach was developed to represent the whole transcriptome and to profile bioactivity of chemical and environmental mixtures in zebrafish embryo. Quantitative analysis of RZT gene expression by RNA-ampliseq technology was used to identify differentially expressed genes (DEGs) at 32 hpf following exposure to reference chemical, BPA, and four water samples ranging from wastewater to drinking water. Concentration-response modeling was used to calculate the effect concentrations (ECs) of DEGs and corresponding molecular pathways. RZT gene set of 1637 zebrafish Entrez genes was designed to cover a wide range of biological processes, and faithfully captured gene-level and pathway-level changes by toxicants compared with the whole transcriptome. The RZT-ampliseq-embryo approach was able to differentiate low dose response from a wide spectrum of biological activities for BPA. Finally, water quality was benchmarked based on the sensitivity distribution curve of biological pathways detected using RZT-ampliseq-embryo, and the most sensitive biological pathways were identified. RZT-ampliseq-embryo approach provides an efficient and cost-effective tool to derive pathway-based information of environmental toxicants.

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*Correspondence: Xiaowei Zhang, PhD, Prof, School of the Environment, Nanjing University, Nanjing, 210089, China; Tel.: 86-25-89680623, Fax: 86-25-89680623, howard50003250@yahoo.com, zhangxw@nju.edu.cn.

¹these two authors contributed equally to this paper

1. INTRODUCTION

A major challenge with regard to prioritizing environmental chemicals and/or assessing the hazard of complex mixtures is the lack of sufficient toxicological information for thousands of chemicals and endless possibility of mixtures. Toxicity pathway profiling could help to predict potential apical toxicity and prioritize and guide subsequent testing of the chemicals.

¹ Traditionally, monitoring and assessment of mixtures have relied on chemistry analyses. Although high throughput targeted and non-targeted analytical methods have been developed for detection of hundreds of chemicals present in complex environmental samples, chemical-focused analyses cannot detect contaminants with unknown structure, and cannot explain the cumulative toxicity of mixtures.^{2, 3} Effect-based approaches, such as high content screening, can provide assessments of biological activity of environmental mixture.^{2, 3} However, most current cell-based HTS assays are limited in their coverage of biological pathways, and consequently their ability to predict a wide range of potential adverse outcomes.

High throughput in vitro bioassays such as traditional ToxCast program⁴ have been extensively used to capture molecular response signatures, and have already showed some success in screening for apical toxicity effects, such as carcinogenicity, hepatotoxicity, or endocrine disruption, caused by pure chemicals⁵⁻⁹ and complex aquatic matrices^{9,10, 11}. However, in vitro bioassays are generally cell-based or cell-free systems, inherently limited by their lack of biological complexity. The toxicity induced by chemical may be associated with modulation of normal signaling and metabolic pathways in a multi-cellular system. The inherent limitations of in vitro cell systems could be overcome using a series of in vivo high throughput model (such as Daphnia, C elegans, zebrafish).¹² Zebrafish embryos in particular have received considerable attention as an efficient alternative model that can be used to monitor altered molecular response induced by environmental stimuli,¹³ and linked with apical endpoints such as developmental and neuro toxicities.^{12, 14}

Transcriptomics can provide a global observation of the transcriptional activities of molecular responses, such as activation states of signaling and metabolic pathways, in

response to individual chemicals and mixtures. Such data could be used to identify molecular initiating events, adaptive changes and points of departure (PODs) involved in toxicities or other adverse outcomes¹⁵. Furthermore, transcriptional effects can be connected to the population level through increasing levels of biological complexity¹⁶. Most of the published transcriptomics studies in ecotoxicology are restricted to investigation of few exposure concentrations in favor of testing more replicates per concentration. However, the analysis of few predefined concentrations might not allow conclusions regarding concentration-dependent dynamics of molecular reactions and might therefore lead to false conclusions.¹⁷ Improved concentration-response modeling and analysis of transcriptome data could improve studies on toxic modes of action (eg. primary, secondary molecular mechanism) and effect pathways, and enable predictions of mixture effects from single chemicals by applying models such as independent action and concentration addition^{18–20}. However, due to the relative high cost and complexity of data analysis, concentration-response modeling analysis for transcriptome approach was impractical for thousands of chemicals and environmental mixtures.

Because gene expression is coordinately regulated in all signaling and metabolic pathways, reduced transcriptome approaches are promising for profiling the molecular pathways modulated by chemicals. Certain representative genes in a network could represent all other members of that network.²¹ Current gene panels for reduced transcriptome, like L1000 landmark genes²² has been reported to represent human whole-genome expression. A recent study has reported 917 pathway reporter genes²¹ specifically targeting 154 human signaling pathways. We have recently developed an omics approach to profile the disrupted biological activity of water samples in human cells by a reduced human transcriptome (RHT) method and dose response models²³.

The objectives of this study were three folds. First, to curate a reduced gene set from zebrafish transcriptome (RZT) that can comprehensively represent biological pathways and toxicologically relevant processes, and be quantified by Ion Ampliseq Technology (RZT-ampliseq) (Figure 1). Second, to develop a chemical test protocol integrating RZT-ampliseq in zebrafish embryo (RZT-ampliseq-embryo) and dose-response modeling. Bisphenol A (BPA), an endocrine disruptor frequently detected in water samples²⁴, was selected as a reference chemical. Third, to evaluate the performance of RZT-ampliseq-embryo for use in hazard assessment of environmental mixtures. The mixture samples tested in this study were a set of water extracts which have been previously characterized by a battery of *in vitro* assays²⁵ and reduced human transcriptome (RHT) method²³.

2. MATERIALS & METHODS

2.1. Design of a gene set for reduced zebrafish transcriptome

The RZT gene set was selected to represent the key biological pathways and toxicologically relevant processes in zebrafish (*Danio rerio*) genome (Figure 1). First, a list of genes associated with key biological pathways (in Entrez ID formats) was curated from three databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG),²⁶ zebrafish orthologs of L1000 landmark genes²² and zebrafish orthologs of pathway reporter genes (Table 1).²⁷ The centrality values of genes were calculated using CentiScaPe in Cytoscape

software.²⁸ Centrality values are node parameters demonstrating the relevant position of nodes in a whole network. Higher centrality value suggests more central roles of a gene in biological pathways. Then the numbers of significantly enriched KEGG pathways and GO terms (adjusted p-value<0.05) were calculated in clusterProfiler²⁹ by walking down the list of curated genes from high to low centrality values by adding 100 genes each time.²⁹ We selected a minimum number of genes playing central roles in biological pathways, which may significantly represent the maximum number of biological pathways. Additionally, a list of toxicology-relevant genes was curated to include the following: (1) genes measured as endpoints in ToxCast¹, (2) genes corresponding to molecular initiating events (MIEs) and key events (KEs) associated with adverse outcome pathways (AOPs) described in the AOP-Wiki (https://aopwiki.org/wiki/index.php/Main_Page), (3) genes listed in graphical model of the fish hypothalamic–pituitary–gonadal (HPG) axis³⁰ and (4) a set of manually retrieved genes associated with development^{31–38} (Table 1). Finally, to avoid potential amplification bias during mRNA quantification, the combined genes were submitted for online multiplex primers design by Ion Ampliseq Designer, where the genes with high transcript abundance across zebrafish transcriptome were removed from the RZT gene set.

2.2. *In silico* validation of RZT

The numbers of KEGG pathways or GO terms covered by genes in the RZT gene set were calculated to evaluate the biological coverage of RZT. Additionally, the numbers of significantly enriched KEGG pathways and GO terms associated with the RZT gene set were compared to a randomly selected gene set (repeated N=1000) using clusterProfiler.²⁹

Furthermore, to evaluate the representation of RZT gene set on the global expression patterns and sensitivity of whole zebrafish transcriptome, five microarray datasets of transcriptomic experiments were used in *in silico* simulation analysis, which cover five distinct life stages of zebrafish (Table S1). The global expression patterns of the previous study were simulated with principal component analysis (PCA) by using genes from RZT gene set and whole genome using edgeR.³⁹ The sensitivities of zebrafish transcriptome to toxicants in two dose-dependent whole transcriptome data were evaluated by transcriptional point of departure (POD_t) calculated by ten previously reported approaches⁴⁰ (Text S1 and Figure S5A) using RZT gene set and whole transcriptome. The POD_t values estimated for the RZT gene set were compared with those for the whole transcriptome, and the lowest observed adverse effect level (LOAEL) of apical end points.

2.3 Experimental evaluation of RZT

Zebrafish embryo culture and exposure—Embryos at 1 hpf were obtained from group spawns and incubated in buffered embryo medium at 28 until 8 hpf. Then, 1ml of exposure solution was prepared in buffered embryo medium with a series of concentrations of single chemical or mixture (Table 2) with a final vehicle concentration of 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). Ten embryos were added to each well of 24-well microtiter plates. Plates were sealed with parafilm and incubated at 28 on a 14 h/10 h, light/dark cycle. At 32 hpf, the zebrafish embryos were collected for RNA isolation. The basal transcriptional expression of RZT gene set were evaluated on three replicates of 0.1% DMSO treatment sampled from six batches of culture.

Single chemical—Stock solutions of BPA (Sigma-Aldrich, USA) were prepared in DMSO. First, a single concentration experiment (0.1 μ M and 10 μ M BPA) with three replicate was conducted to validate the gene expression profiling of RZT-ampliseq platform by comparing with the whole transcriptome evaluated using microarray. Second, embryos were dosed to a serial 10-fold dilutions of BPA (10~10E⁻⁵ μ M) with a single replicate, in addition to three vehicle control (Table 2)

Environmental mixture—Four water extract samples (Table 2) previously characterized as representing high, medium, and low toxic potencies²⁵, were tested by the RZT-ampliseq-embryo approach. The dried extracts were stored at -80 °C until analysis. Stock solutions of samples were prepared in DMSO with relative enrichment factor (REF) of 10,000, followed by 8–32 hpf embryo exposure. The REF represents the concentration in the ambient water samples (e.g. REF of 10 means 10-fold concentrated sample; REF of 0.1 means 10-fold diluted sample).

2.4 RNA ampliseq profiling of RZT and bioinformatics

mRNA expression profiling by ampliseq—At 32 hpf, zebrafish embryos were collected for total RNA extraction using RNeasy mini kit (QIAGEN, GmbH, Hilden). RNA Quantification was performed by using Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA). 10 ng total RNA from each sample were reverse transcribed into cDNA by poly-A priming followed by PCR pre-amplification (15 cycles) according to the protocol supplied with the Ion AmpliSeq™ RNA Library Kit (Life Technologies, Carlsbad, USA). The library was amplified, purified and stored at -20°C. Amplicon size and DNA concentration were measured using Agilent High Sensitivity DNA Kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's recommendation. The resulting libraries were sequenced by Ion Proton (Life Technologies, Carlsbad, USA).

Gene expression analysis pipeline—Raw reads were automatically quantified by using Torrent Mapping Alignment Program as previously described.²³ Then log2 fold change of each gene was calculated using edgeR package.⁴¹ Differentially expressed genes (DEGs) of a single BPA concentration were identified by the threshold of absolute fold change 1.5 and Benjamini-Hochberg adjusted $p < 0.05$. For full dose-response profiling, log2 fold changes of genes against concentrations were submitted to dose-response modeling analysis using drc⁴² and DoseFinding.⁴³ Briefly, nine dose-response models (Table S2) were fitted for each gene. The best-fit model, with the lowest Akaike's information criterion (AIC) value, was used for calculation of effect concentration (EC) values (p -value < 0.05). Finally, genes whose EC LOEC (BPA), or 30 REF (water samples, NO effect concentration²⁵) were defined as DEGs.

Robustness analysis of RZT-ampliseq—The zebrafish embryos treated with 0.1% DMSO was used to evaluate the basal expression of RZT genes, since 0.1% DMSO is frequently used as a control in toxicological studies. To evaluate the optimum sequencing depth of RZT-ampliseq-embryo for consistently detecting most genes, mRNA sample of 32 hpf zebrafish embryo exposed to 0.1% DMSO with 300,000 sequencing counts of RZT-ampliseq (coverage of ~200 counts for each gene) were used. The optimum sequencing

depth of samples was determined by Monte Carlo simulation conducted using R. Each sequenced depth was repeated 100 times to calculate the detected number of genes (Reads>0, Reads > 20) and the coefficient of variation (CV) (SD/Mean) of each gene's expression abundance. For each gene, the relationship between CV and sequencing depth was fitted with loess model and then the minimum sequencing depth of ensuring CV<15% was calculated. To evaluate the mRNA profiling performance of ampliseq on the RZT gene set, the number of detected and undetected genes, as well as the each gene expression abundance measured by RZT-ampliseq were compared to that by microarray platform (GSE43186) and RNA-seq⁴⁴ platform on 36 hpf zebrafish embryo. Correlations of the gene expression abundance between different technologies were calculated using number of reads per amplicon for RZT-ampliseq, RPKM (reads per kilo base per million reads) values for RNA-seq and signal intensity values for microarray technology. Finally, to evaluate the repeatability of RZT-ampliseq, the CV of RZT gene set in zebrafish embryos of 0.1% DMSO (n=3) from six batches were analyzed using the edge package.⁴¹

Pathway-level and biological process validation—For single dose experiment, functional enrichment analysis of identified DEGs was performed using a one-sided Fisher's exact test on GO of Biological Process (BP), and KEGG pathways with RZT gene list (Table S5) as background. For full dose experiment, the EC values of GO terms and KEGG pathways were calculated as the geometric mean of EC values of matched DEGs. Only GO terms or KEGG pathways matched by at least three genes were included in EC calculation and further analysis. Finally, to analyze overall biological potency of each sample, the proportionally ranked distribution of GO and KEGG of EC values was fitted with a four-parameter dose-response curve using GraphPad Prism 5.0 software (San Diego, CA, U.S.A.).

The molecular responses profiling (DEGs, KEGG pathways of DEGs) of 0.1 μ M BPA treatment by RZT-ampliseq were compared with whole transcriptome analysis of BPA archived in NCBI.⁴⁵ To compare RZT-ampliseq-embryo approach with existing Toxcast high throughput *in vitro* assays with regard to biological activities associated with BPA exposure, the responsive gene endpoints and molecular pathways (KEGG, GO BP terms) identified by the both methods were evaluated. The responsive molecular gene endpoints were DEGs captured by dose-response model analysis of RZT-ampliseq-embryo. The responsive genes of Toxcast *in vitro* assay were download from (<https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data>). The responsive molecular endpoints were converted to zebrafish orthologous genes.

2.5 Comparison of RZT with *in vitro* bioassays & RHT method on mixtures

A supervised approach was used to assess the RZT representation of the previous *in vitro* bioassays. First, gene sets associated with cellular toxicity pathways tested by *in vitro* bioassays were manually curated from Wiki Pathways and Gene Ontology, KEGG (Table S6). Then the EC of each pathway was calculated by the geometric mean of the ECs of matched DEGs. Pathway patterns identified by the RZT approach was shown by heatmap using gplot package.⁴⁶ The hierarchical clusters of water samples identified by RZT analysis were compared with the results of *in vitro* bioassays.

To evaluate the sensitivity and specificity of RZT-ampliseq-embryo in identification of bioactivity of mixtures, the results of RZT-ampliseq-embryo were compared to that by RHT-ampliseq using human HepG2 and MCF7 cells²³ on the same sample set. Briefly, the sensitivity of 50% biological potency of water samples identified by RZT were compared with those identified by RHT in HepG2 and MCF7 cells in terms of KEGG or GO. In addition, linear regression was conducted on values of 50% biological potency identified by RZT and RHT. Finally, the coverage of most sensitive pathways (top 20 sensitive KEGG pathways) of Eff2, the sample with potential highest and broadest bioactivity were compared between RZT and RHT approaches.

3. RESULTS AND DISCUSSION

3.1. Development and *in silico* validation of RZT gene set

The developed RZT gene set consists of 1637 zebrafish Entrez ID genes, including a list of 1000 genes with greatest pathway centrality scores and a list of 724 toxicology-relevant genes. The 1000 pathway-central genes were shown to be the minimum number of genes representing the maximum biological pathways in terms of GO BP terms and KEGG pathways (Figure S1). Toxicology-relevant genes (n=724) were selected to provide linkages between molecular mechanism and apical endpoints (Table 1). Then 44 genes were removed by the online designer either because their background expression was too high or too low, or because effective multiplexed primers could not be designed (Table S7). This resulted in 1637 genes as the final RZT gene set (Table S5).

The RZT gene set showed a broad coverage of biological pathways, where 95% KEGG pathways and 94% GO BP terms were represented by at least one gene in RZT gene set (Figure S2A and B). The uncovered pathways were mainly associated with basic metabolic processes (Table S8). Furthermore, the RZT gene set of 1637 genes were significantly enriched in 29 KEGG pathways and 839 GO BP terms (adjusted $p < 0.05$), respectively, which was 48-fold and 17-fold more than the average number of KEGG pathways and GO BP terms enriched by randomly selected genes from zebrafish transcriptome (Figure S3A and B).

The RZT gene set could faithfully represent the global expression patterns and sensitivities of zebrafish whole transcriptome to toxicants. The similar clustering patterns of samples were revealed by PCA analysis using RZT gene set and whole genome on five transcriptomic studies across 24 hpf to adult zebrafish (Figure S4A to E). Furthermore, RZT gene set quantitatively represented the sensitivity of whole transcriptome for estimating POD_t in response of toxicants. The POD_t estimated by the RZT gene set was similar to that of whole transcriptome, where the overall ratios of POD_t between RZT to whole genome were less than 1.5 more than 80% of the time. (Figure S5B and C). For the dataset with recorded LOAEL, the overall POD_t values calculated by RZT gene set were within 3-fold of whole transcriptomic LOAEL (Figure S5B). For datasets without LOAEL, the overall POD_t values calculated by RZT gene set were still within 10-fold ranges of LOAEL retrieved from other literature (Figure S5C). The POD_t values calculated from pathway-based approaches (approach 1, 2, 3 and 5) showed robust consistency between whole transcriptome and RZT

gene set, suggesting that pathway-based approaches may be applicable for estimating POD_t by RZT.

3.2. mRNA profiling of zebrafish embryo by RZT-ampliseq

The RZT incorporated with RNA-Ampliseq technology (RZT-ampliseq) can provide a cost-effective approach for multiplexed sequencing analysis on zebrafish mRNA. Monte Carlo simulations revealed that sequencing depth of 1,000,000 reads was sufficient for detecting the transcriptional expression of RZT gene set in one sample (Figure S6A, B). All sequence data of present paper was deposited in the NCBI BioProject database (accession no. [PRJNA396480](#)). Increasing sequencing depth beyond one million reads failed to result in much increase of detection of genes. Moreover, one million reads could detect 75% of RZT genes at CV below 0.15, based on the relationship between CV and the sequence depth of each gene (supplementary material S2, Figure S6B). Given the current output of 144 million reads from one Ion Proton PI chip under optimal conditions, up to 150 samples can be analyzed in a single run with RZT-ampliseq approach. This not only significantly reduces cost but also increases throughput.

RZT-ampliseq platform, with a sequencing depth of one million reads, could effectively profile the mRNA expression of RZT gene set in zebrafish at 32hpf. 1458 gene transcripts (counts>0) were detected in all 32hpf embryo RNA samples (n=18) under sequencing depth of one million (Figure S7A). Of the 179 genes not detected (count=0) in 32 hpf embryo by RZT-ampliseq, 47 genes were consistently undetected in 36 hpf embryo in a previous RNA-seq study⁴⁴. Among the other 108 genes detected by RNA-seq instead of RZT-ampliseq platform, 86% (93 out of 108) had normalized reads less or equal than 10 counts (RPKM), among which 72% (79 out of 108 genes) had a sequence read below 1 count. Nine genes weren't detected by RNA-seq but were detected by RZT-ampliseq platform, among which 5 genes had a normalized read below 10. The mRNA expression abundances quantified by RZT-ampliseq and RNA-seq platform showed a nearly linear relation ($R=0.81$) between two platforms for the 1270 gene transcripts detected by both (counts>0) (Figure S7B). Moreover, out of 82 transcripts that were not detected by RZT-ampliseq but were detected by microarray, only 29 were above 8 on log2 scale (Figure S7C, D). A similar linear relationship ($R=0.77$) was observed for the 1254 common genes between the RZT-ampliseq approach and a microarray platform (Figure S7D).

3.3. Molecular profiling of zebrafish exposed to BPA

The RZT approach showed good repeatability for quantifying transcriptional response to chemical by zebrafish embryo. Common CV of 32 hpf embryo mRNA samples exposed to 0.1% DMSO from 8–32 hpf were 13% (biological replication within one batch), 14% (biological replication between 6 batch) for RZT-ampliseq-embryo (Figure S8). This variation was acceptably low when compared with other RNA profiling technology, such as qPCR (CV: 1% ~ 15%)⁴⁷, microarray (CV: 5% ~ 15%)⁴⁸ or RNA-seq (CV: 10% ~ 15%)⁴⁹. After exposure of two independent batches of embryos to a single dose of 10 μ M BPA, 67, 45 DEGs (ANOVA, $p<0.05$), respectively, were identified by RZT-ampliseq-embryo and 26 DEGs were common to both batches (Figure S9A). Moreover, the fold change values of all

expressed genes showed significant correlation between batches ($R^2=0.62$) and the difference among the DEGs of two batches was nearly within 2-fold (Figure S9B).

The full dose-response analysis of an RZT profile following exposure to BPA provided a distinct DEGs profile compared to that detected following single doses. Following 24 h exposure to 0.1 or 10 μM BPA, 67 and 58 DGEs, respectively, were captured by RZT-ampliseq-embryo and 31 DGEs were common expressed. (Figure S9A) Eight GO: BP term and 5 KEGG term were shared between the 10 μM and 0.1 μM BPA concentration groups (Table S4). Transcriptional changes associated with DNA damage (DNA repair, cellular response to DNA damage stimulus) and central nervous system development were only captured after 0.1 μM BPA exposure. Oocyte meiosis were only identified after 10 μM BPA exposure. Moreover, there were no common DEGs in RZT-ampliseq-embryo and the published microarray data⁴⁵ in embryo exposed to 8–32 hpf 0.1 μM BPA. Three common KEGG pathways (counts of mapped gene ≥ 3) were identified in two platforms, but these were only involved in fundamental apoptosis process (FoxO signaling pathway, p53 signaling pathway) and regulation of actin cytoskeleton (Figure S10). However, 98 DEGs were identified by dose-response analysis of the embryo exposed to the seven, 10-fold, dilutions of BPA ($10\text{ E}^{-5} \sim 10\text{ }\mu\text{M}$). Three and five of the DEGs identified by dose-response analysis were also detected as DEGs in embryos exposed to 10 and 0.1 μM , respectively (Figure S9A).

One significant advantage of dose-response analysis by RZT-ampliseq is the sensitivity analysis of genes and biological pathways in response to chemicals, which could aid inference regarding the potentially sensitive apical endpoints effects. The responsive DEGs were mainly fitted with U-shaped models, which suggest that the mode of hormesis dominates the low dose response of transcriptome (Figure 2A). There are other interpretations other than true Hormesis. For example, the time-course of dynamic transcriptional response may be different at different doses, such that at higher doses, the transcript abundance may have peaked earlier but has fallen by 32 h. Alternatively, perhaps there is a developmental delay at the higher doses, associated with triggering more and more AOPs in the organism, thereby causing more and more disruption of normal development. What is effectively a monotonic response to the chemical may produce a non-monotonic dose-response for a given snap-shot in time. The response genes ($\text{EC} < 0.001\text{ }\mu\text{M}$) (foxa3, dhhrs3a, src, rpa3, myb, csf1ra, flt4) were mainly fitted with gaussian model (Figure 2B) and were primarily associated with pathways of cell based process, responses to external stimuli, immune system and neurogenesis. The significant enrichment of neurogenesis-related processes (central nervous system development, nervous system development, locomotion) at very low concentrations of BPA ($\text{EC} < 0.001\text{ }\mu\text{M}$) were corroborated by hyperactivity behavior ($0.0068\text{ }\mu\text{M}^{50}$) and increased neuronal development⁵⁰ observed in previous study. Moreover, hyperactivity behavior showed a non-monotonic concentration-dependent response and was induced only at very low effect concentration, which were consistent with gaussian model of the relevant DEGs. For the DEGs with EC between $0.001 \sim 0.01\text{ }\mu\text{M}$, only apoptosis relevant FOXO signaling pathway ($\text{FDR} = 0.057$, covered 4 genes) was enriched. Previous research reported BPA could induced apoptosis of mice spermatocytes⁵¹ and zebrafish embryo⁵². A larger number of pathways, which included liver development, regulation of nucleobase-containing compound metabolic process, sensory organ

morphogenesis, and DNA binding, were associated with the DEGs with EC between 0.01~0.1 μ M. The DEGs with EC (0.1~10 μ M) closed to LOEC (10 μ M) were mainly associated with carbohydrate metabolic process and blood vessel development process.

The coverage of enriched pathways and the corresponding sensitivity detected following full dose-response profiling using RZT-ampliseq-embryo were compared with ToxCast *in vitro* results for BPA. Not only was there consistency with ToxCast in terms of the coverage of biological pathways, the RZT-ampliseq-embryo method appeared more sensitive (Figure 3). Bisphenol A was tested with regard to 821 ToxCast assay endpoints. Out of 96 genes that aligned with the 390 relevant assays, 78 genes had a corresponding orthologous zebrafish gene. In total, 5 KEGG pathways and 15 GO: BP terms were commonly identified by ToxCast and RZT, although only five genes were identified by the both approaches (Figure 3). Furthermore, the sensitivity rank of common genes (ptgre2a, tcf7l1b, src, xbp1, tfap2b) and KEGG pathways (metabolic pathways, MAPK signaling pathway, Focal adhesion, endocytosis, adhere junction) were similar across the two platforms (Figure 3B). However, RZT-ampliseq-embryo was more sensitive than in-vitro tests in ToxCast with 2.3, 2.9, 2.8 magnitude difference at gene, KEGG, GO: BP level, respectively.

3.4 RZT profiling of mixtures in zebrafish embryo

The RZT-ampliseq-embryo was able to discriminate the relatively clean water samples from the polluted environmental samples. The number of DEGs selected by the nine dose-response models across all 4 samples ranged from 78 to 300 (Figure S11). Although the number of DEGs didn't directly correlate with decreasing pollution level, the sensitivity distribution curve of biological pathways indicated the decrease of pollution level (Eff2>MF>RW>DW). The EC values of the most sensitive KEGG or GO pathways of DW samples were 1–2 orders of magnitude higher than those of effluent samples, suggesting relative weak biological effects were induced by DW. (Figure 4)

The enriched pathways in RZT analysis could be used to prioritize potential biological endpoints for future assessment. Specifically, the most sensitive KEGG or GO (BP) pathways may be linked with adverse outcome. For instance, the top 20% sensitive KEGG or GO (BP) pathways of Eff2 were mainly associated with adverse reproductive outcomes, genotoxicity and development outcomes (Figure S12). The most sensitive RZT profile of MF samples was associated with the adverse outcome of endocrine system disturbance and development. For DW, the most sensitive responses were also associated with genotoxicity, which might be due to the fact that the samples of metropolitan drinking water treatment plant contained toxic “byproduct” chemicals from chlorination⁹. All development relevant pathways (GO terms, each covered at least 3 DEGs suggested Eff2 and MF samples might induce potential development toxicity while RW and DW samples may not (Figure S13). The predicted adverse outcomes were corroborated by zebrafish embryo 48hpf lethality and 120hpf sub-lethal development experiment⁹ (Figure S13).

The RZT-ampliseq-embryo method provided more sensitive pathway profiles for the four water samples comparable to the previous 103 *in vitro* bioassays. (Figure S14) First, the RZT profiling analysis revealed similar pattern although with greater sensitivity than the *in vitro* bioassays. Similar to the *in vitro* cell-based bioassays, RZT-ampliseq-embryo approach

could clearly distinguish wastewater (Eff2, MF) from reclaimed or clean water samples (DW, RW). Biological responses associated with genotoxicity and oxidative stress responses, xenobiotic metabolism, PR, RAR/RXR were identified using both RZT-ampliseq-embryo and *in vitro* bioassays. However, the RZT method identified weak TR and hypoxia-related responses (RPE: 0–10), which were not detected in the *in vitro* assays. The sensitive detection on the thyroid hormone signaling pathways by the RZT-ampliseq-embryo method might be contributed by the active involvement of TR pathway during zebrafish embryo development. However, the RZT profile showed low sensitivity for ER and GR, which comes as no surprise, because ER and GR would show high sensitivity at 48hpf or later phase zebrafish embryo⁵³.

The RZT approach provided a broader biological coverage than *in vitro* cell-based bioassays which focused on preselected biomarkers assessment. Thus, the RZT approach could support more comprehensive assessment of biological effects of environmental samples. Beside the responsive endpoints *in vitro* bioassays, the RZT approach also identify other biological responses such as development and reproduction-related pathways, some of which might be linked to established AOPs of regulatory concerns⁵⁴. For example, profiling analysis on the enriched KEGG pathways (Figure S16) indicated that Eff2, MF samples other than RW, DW samples existed potential development toxicity by inducing several relevant pathway responses, including VEGF signaling pathway, Hedgehog signaling pathway and Vascular smooth muscle contraction. An established AOPs “disruption of VEGFR signaling leading to developmental defects”(<https://aopwiki.org/wiki/index.php/Aop:43>) might be used to guide the prediction of developmental risk by the observed molecular event. Additionally, Hedgehog pathway was verified to play an essential role in cardiomyocyte differentiation and heart morphogenesis in model species including mouse⁵⁵ and zebrafish⁵⁶. Furthermore, wastewater Eff2 and MF samples showed prominent pathways effect involved in the reproductive axis including oocyte meiosis, TGF-beta signaling pathway, progesterone-mediated oocyte maturation, GnRH signaling pathway, ErbB signaling pathway. However, inlet and outlet samples of metropolitan drinking water treatment plant (RW, DW) showed very weak reproduction-related effects.

3.5 Comparison between RZT-embryo and RHT cells profiles of water samples

Although only four common water samples were tested by RZT and RHT approaches, the water quality of four water samples ranked by 50% biological potency identified by RZT-embryo were consistent with those by RHT in MCF7 and HepG2 cells (Figure 4). The values of 50% biological potency by RZT-embryo correlated well with those by RHT in MCF7 ($R^2 = 0.95$) in terms of GO (Figure S17), and correlated well with those by RHT in HepG2 ($R^2 = 0.95$) in terms of KEGG ($R^2 = 0.99$) (Figure S18).

RZT-embryo also provided different profiles of altered genes & pathways of the four water samples from that by RHT approach, which might be due to their biological nature of origins. The most sensitive pathways of water samples identified by RZT was distinct with those by RHT in HepG2 and MCF7. Take Eff2 for example (Figure S19), only four KEGG pathways were overlapped between the 20 most sensitive pathways (with lowest EC values) identified by RZT-embryo and RHT in HepG2 and MCF7 cells. Nine of the 20 most

sensitive pathways uniquely identified by RZT-embryo were associated with basic biological process, which may be due to that zebrafish embryo at rapid developmental stage was specifically sensitive to alterations of basic process, such as oxidative phosphorylation, than the single cell-type *in vitro* system. Moreover, the top sensitive KEGG pathways identified by RHT in HepG2 and RHT in MCF7 showed cell-type responses, such as pathways involved in immune response and cellular communication, which were not presented in the top sensitive KEGG pathways by RZT-embryo. However, some cell-type specific responses, including endocrine response in MCF7 and metabolism response in HepG2, were also identified by RZT as top sensitive KEGG pathways of Eff2.

The RZT-embryo approach was more sensitive than RHT in HepG2 and MCF7 cells in terms of estimating 50% biological potency of water samples. The value of 50% biological potency of each sample identified by RZT was generally one order of magnitude less than that identified by RHT in either HepG2 or MCF7 (Figure 4, Table S3A, B). Furthermore, a distinct curve of pathways sensitivity distribution by MF sample was identified by RZT-embryo comparing to RHT in HepG2 and MCF7 cells. Although the potency of the median sensitive pathway (50% biological potency) of by MF was lower than that of RW and DW in RZT-embryo, MF was more potent than that of RW and DW at the most sensitive pathways, which was profiled by the RZT-embryo. (Figure 4A, B) These highly sensitive biological responses induced by MF was primarily related to embryo development (eg. heart jogging, embryo pattern specification, notochord development, determination of left/right symmetry) (Figure S12), which might be related to pollutants of developmental toxicity presented in MF sample. MF was water sample taken after microfiltration using filters disinfected by chlorination to avoid biofouling in a water reclamation plant, in which micro-pollutants of such as carbamazepine, a teratogen, with the highest detected concentrations (1.9 µg/L) out of ten sample²⁴. Carbamazepine has been reported to disturb embryonic development with increasing hatching rate, body length, swim bladder appearance and yolk sac absorption rate at 1µg/L.⁵⁷ However, knowledge gaps still exist in toxicological assessment of these environmental samples, such as response from unknown chemicals and a combined effect. An effect-directed analysis (EDA) integrating extract fractionation and instrument analysis with the sensitive RZT approach can be used to identify the chemicals responsible for the observed effect in future.

In conclusion, we developed a RZT approach by integrating reduced transcriptome, RNA-ampliseq technology and zebrafish embryo test as a novel approach to assess environmental toxicant. First, the RZT approach by multiple dose-response analysis could identify early molecular response and molecular mechanism of single chemical which would help to predict apical effect. The RZT approach has potential to be used to evaluate and prioritize chemicals for further testing and potentially to predict adverse outcomes. Second, RZT-ampliseq-embryo effectively discriminated relatively clean from more polluted environmental samples, and the responsive pathways revealed by RZT analysis could help to prioritize targeted biological end points for future assessment. Future studies should explore the utility of other stages, such as 48hpf (sensitivity for endocrine disrupting effects), in the identification of early molecular response for assessing the hazard potencies of environmental toxicants in early developmental stage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design and workflow of the reduced zebrafish transcriptome (RZT) approach. RZT-ampliseq, RZT genes were quantified by Ion Ampliseq Technology; Akaike's Information Criterion; EC, effect concentration; DEGs, differentially expressed genes; REF, relative enrichment factor; SDC, sensitivity distribution curve; AOPs: adverse outcome pathways.

Figure 2.

Concentration-dependent differentially expressed genes (DEGs) identified by a chemical test protocol integrating RZT-ampliseq in zebrafish embryo (RZT-ampliseq-embryo) in 8–32hpf exposure of BPA ($10 \sim 10E^{-5} \mu M$). (A) Histogram of the log 10 (effective concentration, EC) values of DEGs in full concentration range of BPA exposure. (B) Network interaction of DEGs ($p < 0.05$) across different ranges of EC value (μM) excluding those with EC value higher than LOEC ($10 \mu M$).

Figure 3.

Coverage and sensitivity of biological pathways enriched by BPA in RZT-ampliseq-embryo (blue, log10 EC value) and Toxcast in vitro assays (orange, log10 AC50 value) at gene level (A), pathway level (B: KEGG, and C: GO: BP term). For KEGG pathways and GO: BP terms, only those with counts of mapped genes ≥ 3 were included in this analysis. Common molecular endpoints were labeled by red triangle. Pathway scores (EC or AC50 value) were the geometric mean of the effect concentration (EC) (RZT-ampliseq-embryo) or AC50 (Toxcast) values of the relevant genes. The horizontal distance of 50% biological potency between RZT-ampliseq-embryo and Toxcast in vitro assays was labeled in red.

Figure 4.

Sensitivity distribution curve of biological pathways by the four water samples using RZT-ampliseqembryo approach (A, GO terms, B, KEGG) and reduced human transcriptome (RHT) in HepG2 (C, GO terms; D, KEGG)

Table 1

Sources of reduced zebrafish transcriptome (RZT) gene panel.

Category of genes	Number of zebrafish orthologs	Sources
Public databases	1019	Pathway reporter genes
	1022	L1000 landmark genes
	4260	KEGG database
Core genes	1000	Central roles of public databases
Toxicology-relevant genes	326	ToxCast
	173	AOP
	176	Graphical gene model
	152	Retrieved from references ^{31–38}
RZT gene panel	1637	Core genes and toxicology-relevant genes

Table 2

Samples and exposure design for the validation of reduced zebrafish transcriptome (RZT) approach using embryo test with exposure time of 8–32hpf.

Type	Treatment	Description	Exposure level
Solvent	DMSO	Dimethyl Sulfoxide	0.1% DMSO (6 bathes, n=3)
Single Chemical	BPA	Bisphenol A	10~10E ⁻⁵ (μM) with 10-fold dilution, 0.1, 10 μM (n=3) ^b , 10 μM (n=3 of second batch)
Mixture ²⁵	Eff2	Secondary sewage effluent	10~6.4E ⁻⁴ (REF ^a) with 5-fold dilution
	MF	Microfiltration treated effluent	
	RW	River water	
	DW	Drinking water	

^aREF: relative enrichment factor.

^bIf not otherwise stated, all treatment groups were with a single replicate in addition to three vehicle control (0.1% DMSO)