Studies on phenotypic and genotypic effects of xenoestrogens for integrated toxicity evaluation of environmental water

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ABSTRACT

Xenoestrogens, are ubiquitous in the environment covering wide chemicals (e.g. pesticides, pharmaceuticals, endocrine disruptors, heavy metal), contribute high risk to the ecosystem and human health. Xenoestrogens induced various toxicities resulted from the mode of actions (MoAs) (e.g. estrogenic effect, membrane transporter/ion channel, G protein-coupled receptor protein, DNA damage, neuroscience, etc.) rose the importance of integrated toxicity evaluation and the challenges for toxicity identification in the environmental matrix.

Various *in vitro* (e.g. MCF-7 cells) and *in vivo* (e,g, zebrafish embryo, magma, etc.) bioassay technologies were developed for evaluation of the estrogenic activates of xenoestrogens and environmental samples based on estrogen receptor pathways. However, it is difficult for a single conventional method to cover more toxicity pathways related to MoAs due to various disadvantages: 1) lack of toxicity-pathways for biological models; 2) lack of information-rich and toxicity-based endpoints; 3) lack of multivariate data analysis approaches. Therefore, test batteries (a group of assays conducted together for a specific purpose) and tiered test schemes (testing approaches based on sequential assessments) were applied for integrated toxicity evaluation and identification, however, the combined testing processes reduced the high-throughput capabilities. Therefore, bioassays with information-rich, toxicity-pathway-related, and high-throughput capability are needed for integrated toxicity evaluation and identification.

In this dissertation, three emerging technologies involved in *vitro* phenotype-based bioassays and in vivo genotype-based bioassays were developed to increase the high-throughput capability for toxicity assessment, and multiple phenotypic and genetic

parameters addressing abundant toxicity pathways were applied for integrated toxicity evaluation of xenoestrogens and environmental water samples including estrogenic effect, neurotoxicity, DNA methylation, etc.

In chapter 3, *in vitro* phenotypic analysis using MCF-7 cells was developed for integrated evaluation of estrogenic activities and other possible toxicity of whole water samples. Chapter 3 exhibited our original study applying phenotypic analysis using MCF-7 cells for integrated toxicity evaluation of xenoestrogens, their mixture, and environmental water samples. Morphological parameters of nuclei and cells (intensity, perimeters, major, minor, minor/major, area, form factors) were applied as multiple endpoints to catch more toxicity-pathways. A phenotypic database was constructed firstly exposed to 26 organic chemicals with various toxicity pathways, the multivariate data analysis indicated that the morphological parameters dressed diverse biological pathways, exhibiting high-potential for integrated toxicity evaluation. Mixture effects on cellular morphologies were classified by toxicity-pathway, and the mixture with morphology-sensitive xenoestrogen induced similar morphological variation, indicating the potential for toxicity identification using morphology-based analysis. Finally, we applied the morphology-based analysis for integrated evaluation and identification of whole environmental samples in combination with conventional bioassays (cell viability, E-screen, ELISA) and non-target chemical strategies (UPLC-qTOFMS). Phenotypic effects also showed significant diverse exposed to whole water samples and exhibited high relation to organic chemicals structures based on non-target chemical analysis. A high correlation between nuclei intensity and estrogenic effect; morphological variation and overall chemicals structures of environmental water samples indicated the high potential for further chemical and toxicity identification. In all, In vitro

morphology-based analysis showed correlation with toxicity-pathway and provided rich information for integrated toxicity evaluation and identification of xenoestrogens in environmental water samples.

In chapter 4, in vivo genotypic analysis was performed using zebrafish for integrated evaluation of estrogenic effects and neurotoxicity of insecticides and environmental water samples. Multiple genetic parameters including *ache, gabra1, gad2, vtg1* were selected to provide an integrated evolution of the estrogenic effect and neurotoxicity. Firstly, genetic effect of each pesticide and their mixture were performed under no observed effect concentration (NOEC) expressed a mechanism insight and the interactions of pesticides based on MoAs, then integrated toxicity of environmental water samples taken from Indonesia were evaluated based on multivariate data analysis approaches comparing with the mixture toxicity evaluation. The genotypic effect of insecticide exhibited diverse. All the insecticide decreased expression of *ache*, and chlorpyrifos induced the highest impact on ache expression due to the AchE inhibition mechanism. *gabra1* and *gad2* expression significantly increased after exposure to permethrin. Imidacloprid and chlorpyrifos exhibited high estrogenic effects due to the significant active on *vtg1* expression. Moreover, genotypic effect exhibited diverse after exposed to water samples, agricultural wastewater induced neurotoxicity, and river water exhibited the significantly estrogenic effect. In conclusion, the multiple genetic parameters covered various toxicity pathways and provided an information-rich approach for integrated evaluation of neurotoxicity and estrogenic effects.

In chapter 5, In vitro morphology-based analysis of enhanced green fluorescent protein fused (EGFP) with methyl CpG-binding protein (MBD) was developed for evaluation of DNA methylation effect of xenoestrogens. The multiple morphological

parameters of fluorescent EGFP were characterized and exhibited positive correlations between DNA methylation status and the phenotypic parameters comparing with the conventional DNA methylation analysis. Then, the DNA methylation effect of xenoestrogens including pesticides and estrogens were evaluated and classified with multivariate data analysis approaches. This study revealed novel insight for integrated toxicity evaluation of xenoestrogens. (5-aza-dc) induced a decrease in intensity, area, and count of EGFP-assembled granules, demonstrating the positive correlations between DNA methylation status and the phenotypic parameters. With a high-throughput screening of environmental chemicals, we found that imidacloprid, carbaryl, and o.p-DDT increased the parameters, indicating the induced DNA methylation. In conclusion, the granular sensitivity and area exhibited high correlations with DNA methylation, and the phenotypic analysis provided a high-throughput approach for DNA methylation evaluation.

This dissertation is the first study to develop in vitro phenotype-based and in vivo genotype-based bioassay for integrated toxicity evaluation of xenoestrogens and indicated the usefulness for toxicity identification of xenoestrogens and environmental water samples including estrogenic effect, neurotoxicity, DNA methylation.

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Chapter 1

Introduction

1.1 Research background

Xenoestrogens, also known as endocrine disrupting chemicals, covered categories of xenoestrogens such as pharmaceutical and personal care products (PPCPs), pesticides, plasticizer, acting on diverse toxicity pathways(Diamanti-Kandarakis et al. 2009). They were detected in surface water and drinking water from ng to ug due to the insufficient treatment process in wastewater treatment plant or agricultural runoff (Blair et al. 2015; Vanderford et al. 2011). Based their mechanism of action, xenoestrogens caused estrogenic effect, neurotoxicity and epigenetic effect contributing eco-toxicity and health risks in the environmental aquatic matrix (Chen et al. 2013; Pham et al. 2017; Vilahur et al. 2014). Therefore, it is important for evaluation and identification of toxicities of xenoestrogens in complex water samples.

Toxicity identification evaluation (TIE) and effect-directed analysis (EDA) provided an integrated approach to address toxicity and the causative toxicant in complicated environmental matrices by combining biological responses and chemical analyses (Brack et al. 2007; Yi et al. 2015). Various in vitro and in vivo bioassays were well applied for toxicity characterization in the first phase of TIE and EDAs (e.g. E-screen for estrogenic effect evaluation; AchE activity for neurotoxicity evaluation) (Sancho et al. 2000; Schiliro et al. 2011). However, the conventional bioassays in TIE and EDA have various disadvantages: ambiguity between adverse effects and modes of action; simplex endpoint which is difficult to cover more toxicity pathways; relative low high-throughput capability and so on (Hongxia et al. 2004; Lei and Aoyama 2010). It is

difficult for a single bioassay to cover aboudant toxicities. Therefore, biological pathway-abundant and time-efficient bioassays could provide robust and high-throughput approaches for toxicity evaluation and identification in environmental samples

The Toxicity in the 21st Century (Tox21) program suggested the application of high throughput screening and high content screening using lower mammal cells or lower animal species (e.g. zebrafish embryo) for in-depth toxicological evaluation (Phillips et al. 2009; Roggen 2011; Sipes et al. 2011). Various in vitro and in vivo bioassays were developed and applied for the toxicity evaluation. The methods in Tox21 are needed to achieve the following goals: 1) identification of environmental chemicals posing biological responses to clarify the mechanism of actions; 2) Development models which can predict adverse health effect to humans; 3) Annotation more toxicity pathways and measure the response of chemicals to the pathways(Phillips et al. 2009).

The U.S. Tox21 collaborative program reported an in vitro assay with a high-content screen platform based environmental chemical toxicity mechanisms (Attene-Ramos et al. 2013). Image-based chemical screening is a powerful and cost-efficient approach for identification of small molecules using cellular phenotypes related to specific mechanisms of action (Moffat et al. 2006; Xia et al. 2010). Recent RNA interference (RNAi) research established the theory for correlations between loss-of-function phenotypes and genes, thus making it feasible to predict chemical mechanisms or functions using phenotypic similarity (Fuchs et al. 2010). Construction of morphological database illustrated the relevance of chemical structure similarity and phenotypic similarity (Young et al. 2008) and in turn, supplied a novel method to identify chemicals using morphological profiles. However, this advanced technology

was restricted to drug discovery due to the small scale of the chemicals database and its unclear biological relevance (Futamura et al. 2012). Cellular receptors, such as G protein-coupled receptors (GPCRs), the acetylcholinesterase receptor, estrogen receptors (ERs) are frequently used as an endpoint to detect environmental contaminants, such as pharmaceuticals and pesticides(Ihara et al. 2015). According to the RNAi database, genes related to cellular receptors (GPCRs, GTPases, etc.) or growth (e.g., apoptosis and cellular proliferation) induced specific variation in cell morphology(Young et al. 2008).

Zebrafish embryo was also applied as one of the optimal species for toxicology evaluation in Tox21 due to its transparency, low cost, similarity genotype with humans (Sipes et al. 2011). The abundance of toxicity pathways in zebrafish embryo supply the feasibility platform for toxicity evaluation and identification with can achieve the goals in Tox21.

1.2 Research Objective

In the dissertation, three emerging technologies based on phenotypic and genotypic analysis were applied for evaluation of estrogenic effect, neurotoxicity and DNA methylation of xenoestrogens and complex environmental waters. The main objective of this dissertation is the development of rapid and toxicity-pathway-based bioassay technologies for integrated toxicity evaluation with multiple phenotypic and genotypic endpoints in both *in vitro* and *in vivo* platforms.

To achieve this objective, the main procedures were researched as follow:

1) Phenotypic and genotypic effects of xenoestrogens to investigate the correlations of multiple phenotypic and genotypic parameters with toxicity pathways.
2) Phenotypic and genotypic effects of the xenoestrogens mixture to investigate the

feasibility of phenotypic and genotypic parameters for toxicity characterization.

3) Phenotypic and genotypic effects of complex environmental waters for toxicity identification and evaluation with multiple phenotypic and genotypic parameters.

In detail, multi-parameter phenotypic profiling in MCF-7 and GFP-MBD-nls MSCs and multi-parameters genotypic profiling in zebrafish embryo were performed, respectively, for evaluation of estrogenic effect, neurotoxicity, and DNA methylation. We would like to supply multiple phenotypic and genotypic parameters related to abundant toxicity pathways to achieve a simultaneous evaluation of toxicities in a water sample, which increases the high-throughput capability for toxicity evaluation and identification.

1.3 Research Structure

The structure of this dissertation is organized as follow (Figure 1.1).

Chapter 2 gives a brief introduction of xenoestrogen occurrence, their mode of mechanism, and their environmental risks. The current bioassay and promising bioassay for toxicity evaluation of xenoestrogens were also summaries for comparison.

In chapter 3, multi-parameter phenotypic profiling of xenoestrogens and whole water were applied for integrated toxicity evaluation using MCF-7 cells. Firstly, we investigated phenotypic effects of xenoestrogens on MCF-7 cells, and make the correlation of the phenotypic effects with toxicity-pathway. Then, mixture effects of xenoestrogens on phenotypic effects were researched to investigate the viability using similarity of phenotypic effects for toxicity identification. Finally, phenotypic effects of whole water sample were investigated for toxicity evaluation and identification comparing with conventional bioassay and chemical analysis.

In chapter 4, Multi-parameter genotypic analysis with zebrafish embryo was

performed for integrated toxicity evaluation of both estrogen effect and neurotoxicity in environmental waters. The genotypic effects of xenoestrogens and their mixture were investigated, and then we applied such approach for evaluation and identification of estrogenic effect and neurotoxicity in environmental waters.

In chapter 5, we performed multi-parameter phenotypic analysis with GFP-MBD-nls Mouse ES cells for evaluation of DNA methylation effect of xenoestrogens. Multiple phenotypic parameters related to DNA methylation were selected for DNA methylation evaluation after comparison with conventional bioassay.

In chapter 6, conclusions of this research and recommendations for further study were summarized.

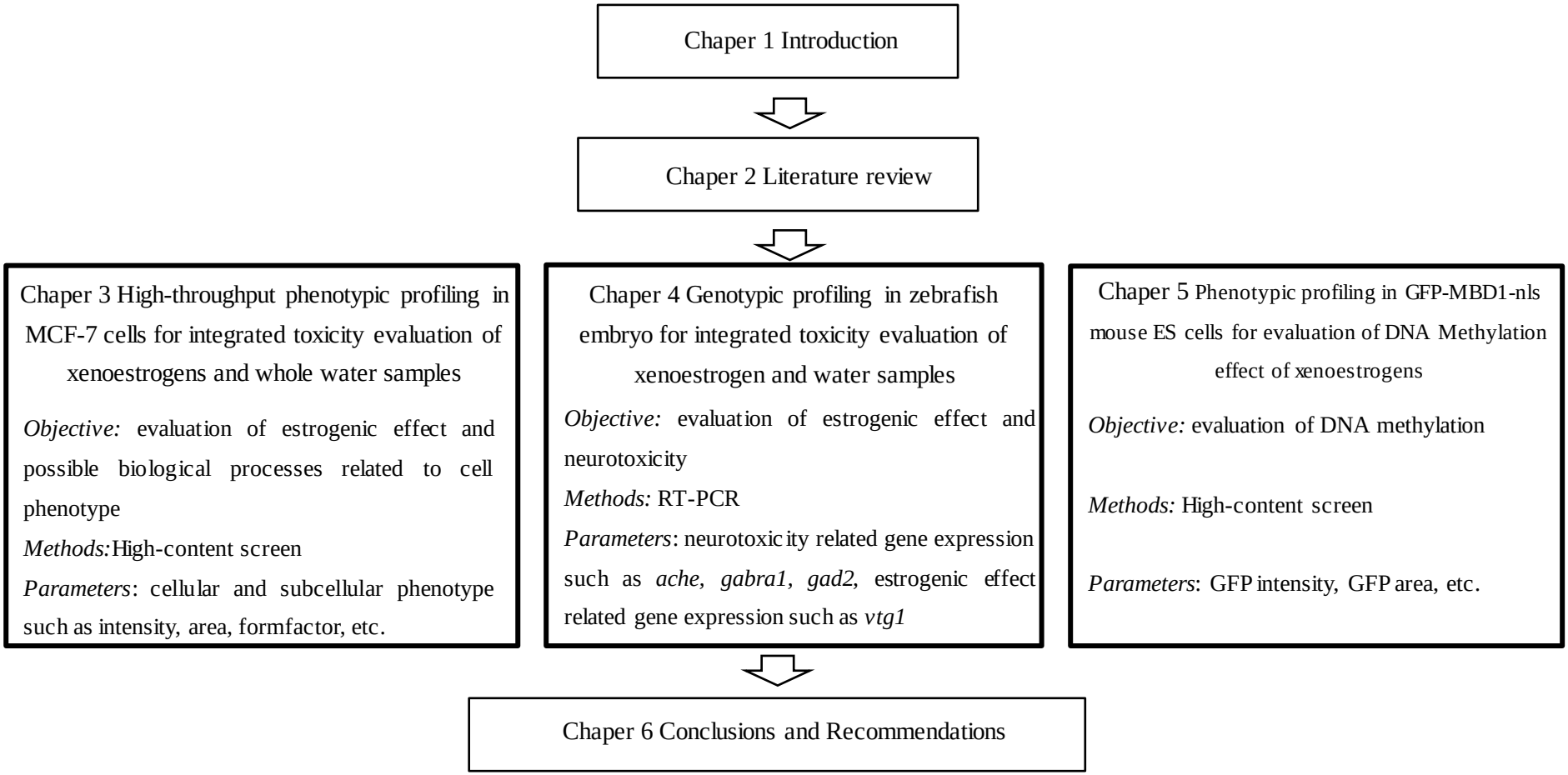

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Figure 1.1 Structure of this dissertation.

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Chapter 2

Literature Review

2.1 Xenoestrogens

2.1.1 Xenoestrogens definition and classification

Xenoestrogens, also known as endocrine disrupting chemicals, were defined agents interferes the actions of the natural hormone in the body that evoke estrogenic responses as well as neurological, metabolic, reproductive outcomes (Kavlock et al. 1996; Singleton and Khan 2003). The xenoestrogens exhibited high heterogeneity and covered wide categories of chemicals which are classified based on their origins and applications as bellow(Diamanti-Kandarakis et al. 2009):

i. Natural chemicals in humans or food (e.g coumestrol)
ii. Pharmaceuticals and Personal Care Products (PPCPs) with hormone effect (e.g. diethylstilbestrol (DES))
iii. Industrial chemicals and their by-products(Polychlorinated biphenyls (PCBs))
iv. Household chemicals in consumer products (Bisphenol A (BPA), phthalates, phenol))
v. Pesticides (DDT, chlorpyrifos, atrazine, 2,4-dichlorophenoxyacetic acid, glyphosate)

2.1.2 Occurrence of xenoestrogens in environmental water

Xenoestrogens have been detected in surface water and groundwater ranging from ng to μg/L covering the wide type of chemicals such as PPCPs, pesticides, plasticizer (Kuch and Ballschmiter 2001; Vanderford et al. 2011). They can reach the aquatic environment through point resources such as wastewater treatment plant effluent which

contains PPCPs. The xenoestrogens can be removed during the wastewater treatment process, however, conventional treatment processes (e.g. activated sludge) failed to achieve a high removal efficiency (Blair et al. 2015; Grassi et al. 2013). Moreover, evidence indicated that agricultural runoff contained various pesticides, herbicides, heavy metals, which were considered as other main resources of xenoestrogens (Klett et al. 2010).

Table 2.1 listed common and emerging xenoestrogens detected in surface water. Estrone(E1), estradiol(E2) and estriol (E3) as common natural hormones discharged from livestock and the human body, and was detected in surface water with max concentration at 175 ng/L (Sornalingam et al. 2016). DDT was well known as pesticides xenoestrogens were banned due to this association with endocrine-related diseases such as breast cancer, preterm birth and so on. However, it was still detected up to 368 ng/L due to its high persistence capability. Imidacloprid was also banned in Europe(Gross 2013), and chlorpyrifos and other neonicotinoid rose the concern due to evidence of estrogenic effect. The plasticizer such as Bis(2ethylhexyl) phthalate (DEHP) and Bisphenol A (BPA) was detected with high concentration, and they were replaced by di-isononyl phthalate (DiNP) and Biphenol S (BPS), gradually. However, their still huge amount of consumption posing the importance of the chemical analysis and toxicity evaluation.

Table 2.1 Common and emerging xenoestrogens in surface water: description and concentration (Lecomte et al. 2017)

Classification	Compounds	Use	Concentration
Pharmaceuticals	EE2	Contraceptive pill	nd–34[a]
DDT	DDT	Insecticide	nd-368[b]
Organophosphate	Chlorpyrifos		nd-10800[c]
Neonicotinoid	Acetamiprid		20.6–23[d,e]
	Imidacloprid		20–10,400[d,e]
Phthalate	DEHP	Plasticizers	nd–197,000[f]
Phenolic compounds	Bisphenol A		0–56,000[g]
Natural hormones	E2	Natural hormones	nd–175[h]
Heavy metal	Cadmium (Cd)	Batteries	9000–15,500[i]

[a] Aris et al. 2014 [b] Gao et al. 2008 [c] Marino and Ronco 2005 [d] Morrissey et al. 2015

[e] Struger et al. 2017 [f] Gao and Wen 2016; [g] Im and Loffler 2016 [h] Sornalingam et al. 2016

[I] Yusta-Garcia et al. 2017

2.2 Mode of mechanisms and adverse effect of xenoestrogens

Due that xenoestrogens cover various chemicals including pharmaceuticals, pesticides, heavy metals, plasticizers. Therefore, they have the divers mode of mechanisms, and estrogenic effect, neurotoxicity, and DNA methylation seemed as major toxicity mechanism for ecosystems and humans.

Xenoestrogens were an original thought to act on nuclear hormone receptors (e.g. estrogen receptor (ERs), androgen receptors (ARs), thyroid receptors (TRs), etc.)(Ishihara et al. 2003; Luccio-Camelo and Prins 2011; Shanle and Xu 2011). Xenoestrogens exhibited estrogenic effect through ER binding (ERα and ERβ) due to the structural similarity with estrogenic hormones in the human body, this induced impact of DNA sequences referred to as estrogen response elements (e.g.

AREG, P21,

IGF,etc.,), which induced cellular outcomes such as apoptosis, growth, survival, and cell cycle (Figure 2.2). More evidence indicated xenoestrogens may also act on other reporters such as neurotransmitter reporters, aryl hydrocarbon receptors (AhR) which induced cross-talk actions to ER. For instance, TCDD acting on Cytochrome P450 (CYPs) may reduce estrogenic response through enhancement of E2 metabolism. Moreover, xenoestrogens affect the enzymatic pathway with the role of functions of endocrine systems such as steroid synthesis (Chen et al. 2013).

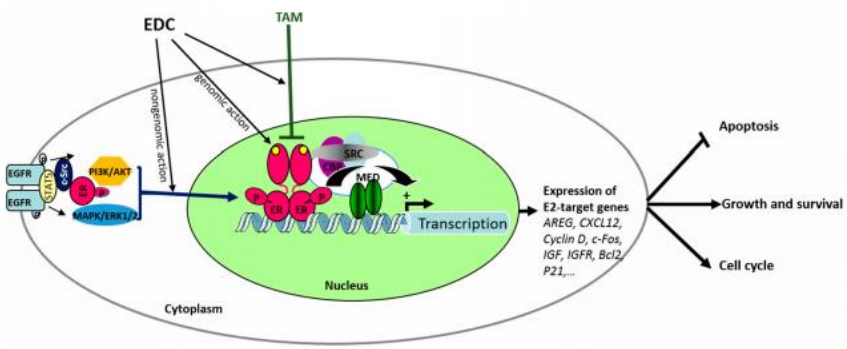

Figure 2.2 Ability of xenoestrogens to alter estrogen signaling(Lecomte et al. 2017)

Xenoestrogens involved in wide types of chemicals including pesticides, PPCPs, heavy metal included the amount of biological/toxicity pathways. Therefore, the original mode of mechanisms of xenoestrogens posed the concern for consideration of integrated toxicity evaluation. For instance, most of the pharmaceuticals were designed targeting on G protein-coupled receptor (GPCR), otherwise, most of the insecticides acting on neurotransmitter systems (e.g. Acetylcholinesterase (AchE) receptors(Pham et al. 2017), gamma-aminobutyric acid (GABA) receptors(Nakao 2017), etc.,) of insects may also make the adverse effect to wildlife and human. Table 2.2 summarized mode of action of common xenoestrogens and their adverse effects on human health. Although DDT was banned last century, however, the residue in the

environmental matrix may induce adverse effects to the human body through directly intake or food chain. Therefore, DDT with neurotoxicity and estrogenic effects may injure the nervous system, immune and endocrine system inducing birth defect, cancer, etc.(Mansour 2004). Based on the mode of mechanisms, xenoestrogens almost make acute or chronic impacts on nervous systems, endocrine systems (Pelclova et al. 2006), which indicated the importance for the evaluation of neurotoxicity, estrogenic effects, and carcinogenic effects of xenoestrogens and the environmental matrix.

Table 2.2 Mode of action of common xenoestrogens and adverse effects

Compounds	Potential mechanisms	Adverse effect
DDT	Neurotoxic as sodium channel modulator, neuroendocrine by estrogenic effect [a]	injury to the nervous system, lung damage, dysfunction of the immune and endocrine systems, birth defects, and cancer [f]
BPA	Androgen Receptor agonist; Estrogen receptor agonist [b]	reproductive health [g]
PCB	Actions on estrogen receptors, neurotransmitter receptors [c]	lymphocytic leukemia, immune system, cardiovascular disease, Asthma [h]
TCDD	Aryl hydrocarbon receptor agonist- estrogen receptor (ER) cross-talk [d]	Chloracne, transient hepatotoxicity, neuropsychological impairment [i]
DEHP	Androgen receptor antagonist [e]	reproductive health, carcinogenesis, and respiratory system [j]

a (Rasier et al. 2007) b(Acconcia et al. 2015) c(Safe et al. 1985) d(Tanaka et al. 2007) e (Takeuchi et al. 2005) f (Mansour 2004) g (vom Saal et al. 2005) h (Brown et al. 1991) i (Pelclova et al. 2006) j (Zarean et al. 2016)

In summary, xenoestrogens rose public concern due to the potential to make an adverse effect on human health and ecology. The abundance of evidence indicated that xenoestrogens existed in the environmental matrix such as environmental water, soil and so on. Their health risk and environmental risk may be amplified through directly or indirectly exposure such as drinking water intake and food chain. Based on the mode of mechanism, most of the xenoestrogens exhibited neurotoxicity and estrogen effect and act on two more toxicity-pathways, which rose the importance for integrated toxicity evaluation of xenoestrogens and the environmental matrix.

2.3 Current bioassays for toxicity evaluation of xenoestrogens

Estrogen effect, neurotoxicity, and epigenetic effect of xenoestrogens contributed adverse effect on human health and ecosystems (Ankley et al. 2009). Therefore, various bioassay methods were developed based on the mode of mechanisms for toxicity evaluation of xenoestrogens through different biological platforms(Hamilton et al. 2012). Therefore, current bioassays methods for evaluation of estrogenic effect, neurotoxicity, and epigenetic effects were summarized, it can improve the understanding of current situation and the development of bioassay methods.

2.3.1 Current bioassays for estrogenic effect evaluation of xenoestrogens

Exoestrogen mimic ER ligand and bind to the receptors, thus, various bioassays were developed based on this receptor-mediated mechanism of action. *In vivo* bioassay applied various endpoints such as organ weigh, cell differentiation, protein expression, and enzyme activities, however, it is difficult for large-scale screening of estrogenic effect due to the cost, complex mode of mechanism, low sensitivities. Therefore, In vitro bioassay methods were developed using ER-sensitivity cell lines (e.g. MCF-7 cells,

yeast), and their remarkable endpoint such as cell proliferation, gene expression increased the throughput capability for large-scale screening. The well-applied methods for estrogenic activity evaluation are summarized as follows:

Cell proliferation assays

Cell proliferation assays, referred as E-SCREEN, were well applied for estrogenic activity evaluation using ER-sensitive MCF-7 breast cancer cells lines(Lange et al. 2014). Cell proliferation after 7-days exposure was characterized by estrogenic activities. However, it has drawbacks due that the MCF-7 cells also express other receptors such as androgen, progesterone, glucocorticoid and retinoid receptors, which may also affect on cell proliferation of MCF-7 cells(Okubo et al. 2003). Therefore, the simplex endpoint can suggest but cannot unequivocally verify estrogenic effects when test compound with anti-estrogenic effects or complex chemicals mixture.

Protein Expression/Enzyme Activities assay

Due to that xenoestrogen mimic ER ligand and bind to the receptors, according to this mechanism, Enzyme-Linked Immunosorbent Assay (ELISA) was developed and exhibited high-throughput capacities for estrogenic effects. The antigen was immobilized on a solid surface in the plate and then complexed with the antibody which linked to ER. This method is fast and sensitive to quantify estrogenic activities, however, the cost of this method inhibited for the large-scaled screen.

Yeast estrogen screen (YES)

Yeast estrogen screen (YES) was well applied for evaluation of estrogenic activities of chemicals and complex environmental samples. In YES, the yeast cells are transfected with the ER-a gene and an expression plasmid containing the estrogen response elements (ERE) that control the *b*-galactosidase-encoding reporter gene Lac-z and can

be stained by red-b-D-galactopyranoside (CPRG)(Nakama et al. 2007).

Report gene expression assay

Report gene assay both in vitro and in vivo was also developed for rapid evaluation of estrogenic activities. The expression of ER subunits such as ERα, ERβ in mammal cells or zebrafish were applied as endpoints(Legler et al. 2002), Moreover, various study applying vitellogenin (*vtg*) expression in both zebrafish embryo and adult for estrogenic activities evaluation of complex environmental samples(Versonnen and Janssen 2004). However, the abundance of active genetic subunit induced the difficulty to construct the benchmark of different biological platforms.

In all, there is no perfect single bioassay method for estrogenic effect evaluation due to diverse drawback, and can be classified as follows:

(1) The simple endpoint which cannot cover complex mode of the mechanism of chemicals or complex environmental samples. For instance, cell proliferation assay can suggest an estrogenic activity, however, other toxicity-pathway in MCF-7 cells may affecting on cell proliferations interfered quantification of estrogenic activities.

(2) Cost efficiency and labor consuming. For instance, ELISA assay is sensitive for estrogenic activity evaluation, however, the high cost inhibits this method for large-scale screening. Moreover, some *in vivo* bioassay using rodent exhibited labor and time consuming for evaluation.

(3) Low relationship with human health or ecosystems. For instance, YES assay using yeast cells provide a rapid and sensitive platform for estrogenic effect evaluation, however, the result is difficult to link human health risk or eco-toxicity.

2.3.2 Current bioassays for neurotoxicity evaluation of xenoestrogens

Xenoestrogens such as pesticides were well-known having neurotoxicity (Alavanja et al. 2004). To evaluate the neurotoxicity, various in vitro and in vivo bioassays were developed applied various endpoints such as cell proliferation(Golstein and Kroemer 2007), neurite outgrowth(Christen et al. 2017), and neurotransmitter gene expression (Munoz and Inestrosa 1999). Acetylcholinesterase (AChE) activity was well applied as a biomarker for evaluation of development neurotoxicity of xenoestrogens especially organophosphorus and carbamate pesticides (Hobbiger 1961; Veronesi et al. 1990). However, more neurotoxicity pathways such as GABA receptor, nAChE were neglected using the single AchE biomarker. Therefore, a single endpoint cannot satisfy the complex neurotoxicity pathways.

2.3.3 Current bioassays for DNA methylation evaluation of xenoestrogens

In general, methods for analyzing DNA methylation are classified into three techniques: bisulfite conversion; digestion with methylation-sensitive restriction enzymes; and affinity purification of methylated DNA (Zilberman and Henikoff 2007). Each approach uses different parameters to characterize DNA methylation with different sensitivity levels. Bisulfite conversion approaches apply sodium bisulfite to convert unmethylated cytosine to uracil, whereas methylated cytosines are protected. DNA methylation levels are then measured using PCR-amplified bisulfite-treated DNA (Brena et al. 2006; Ehrich et al. 2005). Another global methylation high-throughput quantification method visualizes DNA methylation by fluorescence polarization (FP). In this approach, DNA cleavage is achieved using methyl-sensitive Hall and methyl-insensitive Mspl restriction enzymes. Cleaved DNA is treated with fluorescent

deoxycytidine monophosphate to bind the non-methylated DNA segments. DNA methylation is then analyzed using FP to determine the difference between Hall- and MspI-treated DNA. Another study using MBD1 as a readout signal is similar to our method, except that it joins MBD1 with either the N-terminal or C-terminal fragment of firefly luciferase (Badran et al. 2011). However, the method is time and labor consuming for large-scale screening.

2.4 Toxicology in 21 century for toxicity evaluation

The concept of "toxicology in 21 century" (Tox21) was proposed aiming at deploy understand toxicity processes with non-animals during toxicity evaluation in order to fulfill large-scale screening for chemicals (Pamies and Hartung 2017). Up to now, various in vitro and in vivo bioassay methods were developed based on high-throughput and toxicity-pathway related endpoints (e.g. cell morphology, genome-wide genes), the bioassays were improved along with the development of screening technologies (e.g. robotic image technologies, next-generation sequencing)(Stephens et al. 2012).

2.4.1 Principles of Toxicology in 21 century

Conventional bioassay exhibited various drawbacks such as simplex endpoint with the less toxicity-pathway relationship, time and labor consuming, low throughput capacity as summarized previously. Therefore, Tox21 should fulfill principles as following(Rowlands et al. 2014):

(1) Deeply understanding of physiological and toxicity processes. The conventional bioassay applied cell proliferation or cytotoxicity for toxicity evaluations, however, it is failed to understand the physiology (e.g. cell division, cell apoptosis) and toxicity processes, thus, it posed difficulty for "up-bottom" understanding of toxicities.

(2) Covering much relevant toxicity pathways. The conventional bioassays such as report gene or enzyme analysis focused on single toxicity pathway showing limitation to understanding the "bottom-up" processes through toxicity pathways. It made the data difficult for extrapolations to the human health.

(3) Less animal usage. The Tox21 suggested applying cells for toxicity evaluation in order to reduce the animal usage. Moreover, aquatic species such as zebrafish, water flea were considered as a good platform for toxicity test due to their easy handle, an abundance of offspring, fast life cycle, etc(Bugel et al. 2014).

To catch the principle of Tox21, various bioassay technologies were developed, and we introduce a novel concept for toxicity test: integrated test strategies (ITS) and emerging technology: in vitro phenotype-based bioassay.

2.4.2 Integrated test strategies (ITS) for toxicity evaluation

The concept of integrated test strategies (ITS) was proposed in order to integrate the maximum toxicity information, ITS combined different toxicity results, or non-test toxicity information to make an integrated results. Test battery (a group of bioassays) or tiered test scheme (sequencing bioassays) was applied to provide more information for decision-makers referring more toxicities. However, the combination of different bioassays exhibited time and labor consuming. (Blaauboer 2015). Therefore, multiple endpoints such as comics and phenotypic analysis) were applied to provide the maximum information for toxicity identification or risk characterization.

2.4.3 In vitro phenotype-based bioassay

RNA interference (RNAi) research indicated that cellular phenotype exhibited relations with functional genes, therefore, cellular phenotype rose concern as the endpoints for toxicity evaluation(Moffat et al. 2006). Daniel et. al. indicated that 6547

compounds exhibited concordance between chemical structure similarity and cellular phenotypic similarity, which indicated the ambition for toxicity identification and chemical screen using this method(Young et al. 2008). Due to the development of technologies including an automatic microscope, image analysis systems, it made feasible to evaluate the small molecular effects on cellular phenotype with high-throughput capacity(Carpenter 2007) and identify the mode of mechanism according to the phenotypic similarity through statistical analysis.

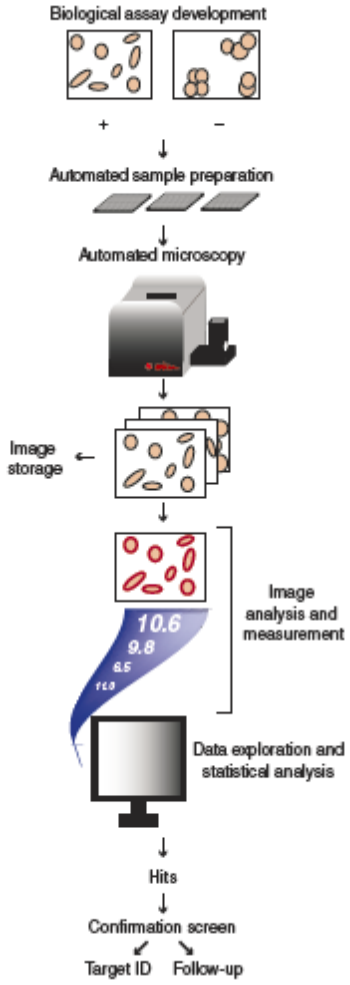

Figure 2.3 the process of the high-content screen(Carpenter 2007).

The high-content screen generally has three steps including exposure process, image acquisition, image analysis, data mining, chemical or toxicity identification (Figure 2.3) It has various advantages: (1) multiple phenotypic parameters relevant to toxicity pathways. In general, cellular (cell body) and subcellular (nuclei) phenotype were characterized with multiple parameters such as area, length, perimeter, form factor, intensity and so on. These parameters were sensitive and exhibited high relation with toxicity pathways such as cell death, cell apoptosis, etc. It provides an information-rich pipeline to cover more toxicity pathways. (2) information-rich. High-content screen systems were available to scan thousands of cells and analyze the phenotype simultaneously. It provided several TB of data which involved in huge cellular phenotypic characteristic, the big data from abundant cells made it sensitive for the perception of effect on the single cell. (3) High-throughput capacity. High-content screen identifies toxicity or compounds through phenotypic similarity, the automatic microscope, image analysis system made the screen robotics. Moreover, the data mining process can be automatically after machining learning and statistical programming, which made it feasible for the large-scale screen. (4) Cost efficiency. Compared with omic analysis using Microassay, high-content screen reduced the cost efficiently due to only staining spend.

2.5 Summary

- ✓ Xenoestrogens have been detected in surface water and groundwater covering the wide type of chemicals such as PPCPs, pesticides, plasticizer.
- ✓ Xenoestrogens posed risks for the ecosystem and human health due to their mode of mechanism mainly involved in estrogenic effect, neurotoxicity, and epigenetic effect.

- ✓ Various in vitro and in vivo bioassays were developed to evaluate toxicities of xenoestrogens in environmental samples, however, drawbacks of the conventional bioassays made it difficult to cover more toxicity pathways.
- ✓ Toxicology test in 21 century was proposed to achieve following goals: improving the high-throughput capacity; covering more toxicity pathways; reducing animal usage.
- ✓ Integrated test strategies and the high-content screen showed high potential to achieve these goals for toxicity evaluation and identification.

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Chapter 3

High-throughput phenotypic profiling in MCF-7 cells for integrated toxicity evaluation of xenoestrogens and whole water samples

3.1 Introduction

Xenoestrogens in environment posed estrogenic activities inducing risks to the ecosystem and human health (Ribeiro et al. 2017). Various bioassay technologies were developed, E-screen using MCF-7 cells were well applied for evaluation the estrogenic effect (Isidori et al. 2010), however, the simplex endpoint (e.g. cell proliferation) and algorithm induces the boundedness to cover multiple biological pathways of complex chemicals, and make it difficult to understand the in-depth toxicity of chemicals or environmental samples.

The U.S. Tox21 collaborative program reported an in vitro assay with a high-content screen platform based environmental chemical toxicity mechanisms (Attene-Ramos et al. 2013). Image-based chemical screening is a powerful and cost-efficient approach for identification of small molecules using cellular phenotypes related to specific mechanisms of action (Moffat et al. 2006; Xia et al. 2010; Zanella et al. 2010). Recent RNA interference (RNAi) research established the theory for correlations between loss-of-function phenotypes and genes, thus making it feasible to predict chemical mechanisms or functions using phenotypic similarity (Fuchs et al. 2010; Horn et al. 2007; Young et al. 2008). Construction of morphological database illustrated the relevance of chemical structure similarity and phenotypic similarity (Young et al. 2008) and in turn, supplied a novel method to identify chemicals using morphological profiles.

However, this advanced technology was restricted to drug discovery due to the small scale of the chemicals database and its unclear biological relevance(Futamura et al. 2012b). Cellular receptors, such as G protein-coupled receptors (GPCRs), the acetylcholinesterase receptor, estrogen receptors (ERs) are frequently used as an endpoint to detect environmental contaminants, such as pharmaceuticals and pesticides (Ihara et al. 2015; Zhang et al. 2013). According to the RNAi database, genes related to cellular receptors (GPCRs, GTPases, etc.) or growth (e.g., apoptosis and cellular proliferation) induced specific variation in cell morphology(Young et al. 2008). Therefore, we hypothesized that environmental chemicals with different modes of action exhibit specific phenotypic features and that toxicants can be preliminarily screened and identified using image-based analyses.

The objective of this chapter was to develop a high-throughput method using image-based analyses for integrated evaluation of estrogenic effect and other possible toxicity of xenoestrogens, their mixture, and whole water. Multiple phenotypic parameters of MCF-7 cells were firstly used for characterization of toxicity pathways including estrogenic effect, cell apoptosis, etc.

To investigate the cellular phenotype and toxicity pathways, phenotypic profiling in MCF-7 cells of environmental chemicals was studied. 26 organic chemicals including pharmaceuticals, pesticide, and estrogens and 4 inorganic chemicals were selected and exposed to MCF-7 cells based on various toxicity pathways (e.g. GPCR, Membrane Transporter/Ion Channel, DNA damage, neurotoxicity, Endocrinology/ Hormones, etc.). Then, phenotypic profiling in MCF-7 cells of the chemical mixture was performed to evaluate the feasibility of such method for toxicity identification in complex mixtures based on phenotypic variations related to active cell receptors or biological processes

with completed with a principal component analysis (PCA). Finally, we applied the image-based analyses for whole water toxicity evaluation compared with conventional bioassays (e.g MTT assay, direct count, E-screen, ELISA) and chemical analysis (e.g. UPLC-qTOF-MS) to deeply understand the feasibility of integrated evaluation of estrogenic activities and other potential toxicities of whole water samples.

3.2 Material and methods

3.2.1 Chemicals

Environmental chemicals including pharmaceuticals, pesticides, endocrine disruptors, ions were selected based on biological pathways and showed as Table 3.1. All pharmaceuticals were purchased from SCREEN-WELL® Cardiotoxicity library (BML-2850, ENZO Life Sciences, Farmingdale, USA). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DMSO was used as the primary solvent, with solutions further diluted in cell culture medium prior to use. The final concentration of DMSO in the medium did not exceed 0.1% (v/v).

3.2.2 Cell culture

Human breast cancer cells (MCF-7) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham (DMEM-F12) (Sigma-Aldrich, Japan) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA). Cells were maintained at 37 ℃ in a 5% CO2 humidified incubator.

3.2.3 Sampling site, preparation, and treatment

The water samples (500 mL) were collected at 8 river locations and 6 wastewater treatment plants (WWTP) within one river basin in the Kanto area of Japan from July 3rd to August 7th, 2014. The 15 water samples included wastewater treatment plant

effluents (W1-Eff, W2-Eff, W3-Eff), wastewater treatment influents (W1-Inf, W2-Inf, W3-Inf), upstream river water (T2, T7), middle stream river water (T3, T8, T9), downstream river water (T4), and river water from the estuary (T5, T6) (Figure 3.1). For cell exposure experiments, 50 mL of each sample was filtered through a 0.22 μm polyethersulfone (PES) membrane (Millipore, Germany) and stored at −20 °C before use. Filtered water samples and 10× concentrated DMEM-F12 medium (1:9) were mixed, and the pH was adjusted to 7.2–7.4 with a sodium bicarbonate solution [7.5% w/v] after adding 10% fetal bovine serum (FBS). The remaining water samples (400 mL) were extracted by Autoprep EDS-1 (SHOWA DENKO, Japan) and eluted with methanol. The eluate was then evaporated to dryness with a nitrogen stream and the residue was dissolved with 1mL of DMSO for Enzyme-linked immunosorbent assays (ELISA) and high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-qTOFMS).

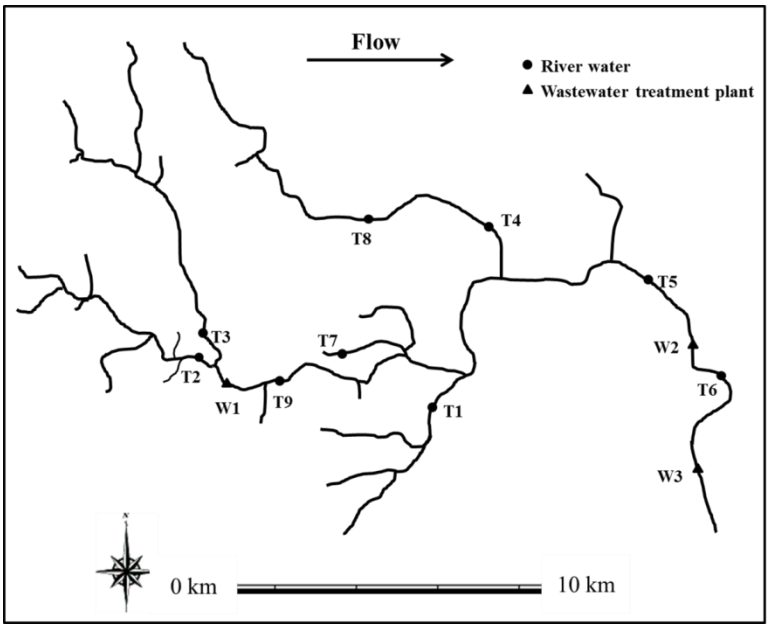


Figure 3.1 Sampling sites.

Table 3.1 List of selected chemicals

Name	Abbreviation	CAS Number	Main Pathways	Target	Ref.
Amoxicillin	AMO	26787-78-0	Microbiology & Virology	PHPA inhibitor, CYP2C19 inhibitor, SLC15A1 inhibitor	(Jariyawat et al. 1999;
Ranitidine HCl	RAN	66357-35-5	GPCR/G protein	Adrenergic receptor agonist	(Khilnani and Khilnani
Lisinopril	LIS	83915-83-7	Angiogenesis	Angiotensin-converting enzyme (ACE) inhibitor	(Andujar-Sanchez et al.
2-phenylphenol	OPP	90-43-7			
Digitoxin	DGT	71-63-6	Membrane Transporter/Ion Channel	Na^+/K^+ ATPase inhibitor	(Ravikumar et al. 2000)
Quinidine HCl H2O	QUI	6119-47-7	Membrane Transporter/Ion Channel	AChR antagonist, Histamine antagonist, Potassium Channel inhibitor	(Stokoe et al. 2007)
Flecainide acetate	FLE	54143-55-4	Membrane Transporter/Ion Channel	Voltage-gated sodium channel inhibitor	(Liu et al. 2003)
Amiodarone HCl	AMI	1951-25-3	Membrane Transporter/Ion Channel; GPCR/G Protein	Potassium Channel; Adrenergic Receptor antagonist	(Kodama et al. 1997)
Acebutolol HCl	ACEB	34381-68-5	GPCR/G Protein	Adrenergic Receptor antagonist	(van den Meiracker et al.
Propranolol HCl	PH	318-98-9	Neuroscience; GPCR/G protein	5-HT receptor antagonist; Adrenergic Receptor antagonist	(Rouget et al. 2006)
Bepridil HCl H2O	BEP	74764-40-2	Membrane Transporter/Ion Channel	Voltage-gated calcium channel activity inhibitor	(Bezprozvanny and Tsien
Carbamazepine	CARB	298-46-4	Membrane Transporter/Ion Channel	Sodium Channel inhibitor	(Willow and Catterall 1982)
Tamoxifen citrate	TAM	10540-29-1	Endocrinology/ Hormones	Estrogen/Progestogen Receptor agonist	(Sasson 1991)
Ibuprofen	IBF	15687-27-1	Angiogensis; Metabolism; Neuroscience; Apoptosis	Thrombomodulin; PPAR activator; COX inhibitor;	(Noreen et al. 1998)
Erythromycin	ERM	114-07-8	Microbiology & Virology	Antibiotic	(Warrilow et al. 2010)
Busulfan	BUS	55-98-1	DNA Damage/DNA Repair	DNA Alkylating	(Meng et al. 2003)
Estradiol	E2	50-28-2	Endocrinology/ Hormones	Androgen Receptor antagonist; Estrogen receptor agonist	(Takabe et al. 2010)
Prednisone	PSE	53-03-2	Endocrinology/ Hormones	Glucocorticoid Receptor agonist; MRP inhibitor	(Lanza et al. 1996)
Acetamiprid	ACE	135410-20-7	Neuroscience	Nicotinic acetylcholine receptor (NACHR) modulator	(Kimura-Kuroda et al. 2012)
Bisphenol A	BPA	80-05-7	Endocrinology/ Hormones	Androgen Receptor antagonist; Estrogen receptor agonist	(Acconcia et al. 2015)
Carbaryl	CAR	63-25-2	Neuroscience	Acetylcholinesterase (AChE) inhibitor	(Colovic et al. 2013)
Chlorpyrifos	CPS	2921-88-2	Neuroscience	Acetylcholinesterase (AChE) inhibitor	(Colovic et al. 2013)
O,p'-Dichlorodiphenyltrichl	o,p'-DDT	789-02-6	Neuroscience /Ion Channel	Sodium Channel inhibitor	(Dong 2007)
Di(2-ethylhexyl)phthalate	DEHP	117-81-7	Endocrinology/ Hormones	Androgen receptor antagonist	(Takeuchi et al. 2005)
2,3,7,8-Tetrachlorodibenzodi	TCDD	1746-01-6	Metabolism/Membrane Transporter	Aryl hydrocarbon receptor agonist	(Garrett and Gasiewicz
Thalidomide	THD	50-35-1	Neuroscience; Immunology/Inflammation	NF-κB inhibitor; TNF inhibitor; COX;FGFR inhibitor	(Zhu et al. 2013)

3.2.4 Image-based chemical screening

1) Exposure setup. MCF-7 cells (1000 cells/well in 200 μL DMEM-F12) were plated in 96-well plates. After 24 h, triplicate samples of cells were exposed to E2 (10 × 10-8 M to 10 × 10-10 M) or whole water medium for 6 days prior to staining. 2) Immunofluorescence. After a 6-day exposure, the cells were fixed with 4% paraformaldehyde for 15 min, treated with 0.1% TritonX-100 for 30 min, and then incubated with 1% BSA-PBS for 30 minutes at room temperature. Samples were stained with 2% Phalloidin (546 A2228; Red) (Life Technologies, USA) for 1 h and 2 μg/ml Hoechst (33342; Blue) (DOJINDO, Japan) for 15 minutes at room temperature. 3) Image Acquisition. Typical microphotographs were obtained using an Olympus LV1200 High-Performance Laser Scanning Microscope (Olympus, Japan). For image-based analysis, immunofluorescence images (9 fields per well of a 96-well plate) were acquired automatically on an IN Cell Analyzer 1000 (GE Healthcare, UK) using a 10× objective. A laser autofocus system analyzed at least 1000 cells in each well. Hoechst-positive nuclei and phalloidin-positive cell cytoskeletons were recognized using IN Cell Developer Software (GE Healthcare, UK.). 4) Data processing. To characterize the phenotypic response of cells, morphological parameters including 6 cellular and nuclear parameters (intensity, perimeter, major, minor, minor/major, area, and form factor) were quantified with the IN Cell Developer Tool Box 1.7 (GE Healthcare, UK). The morphological parameters of cells ($n > 2000$) were averaged and normalized to a negative control. The fold changes of multi-parameters were determined with a PCA, and the resulting principal components were displayed in a 2D score plot. The “Euclidean distance” of each treatment from the control was calculated using a

distance formula. The detailed procedure is described in Figure 3.2.

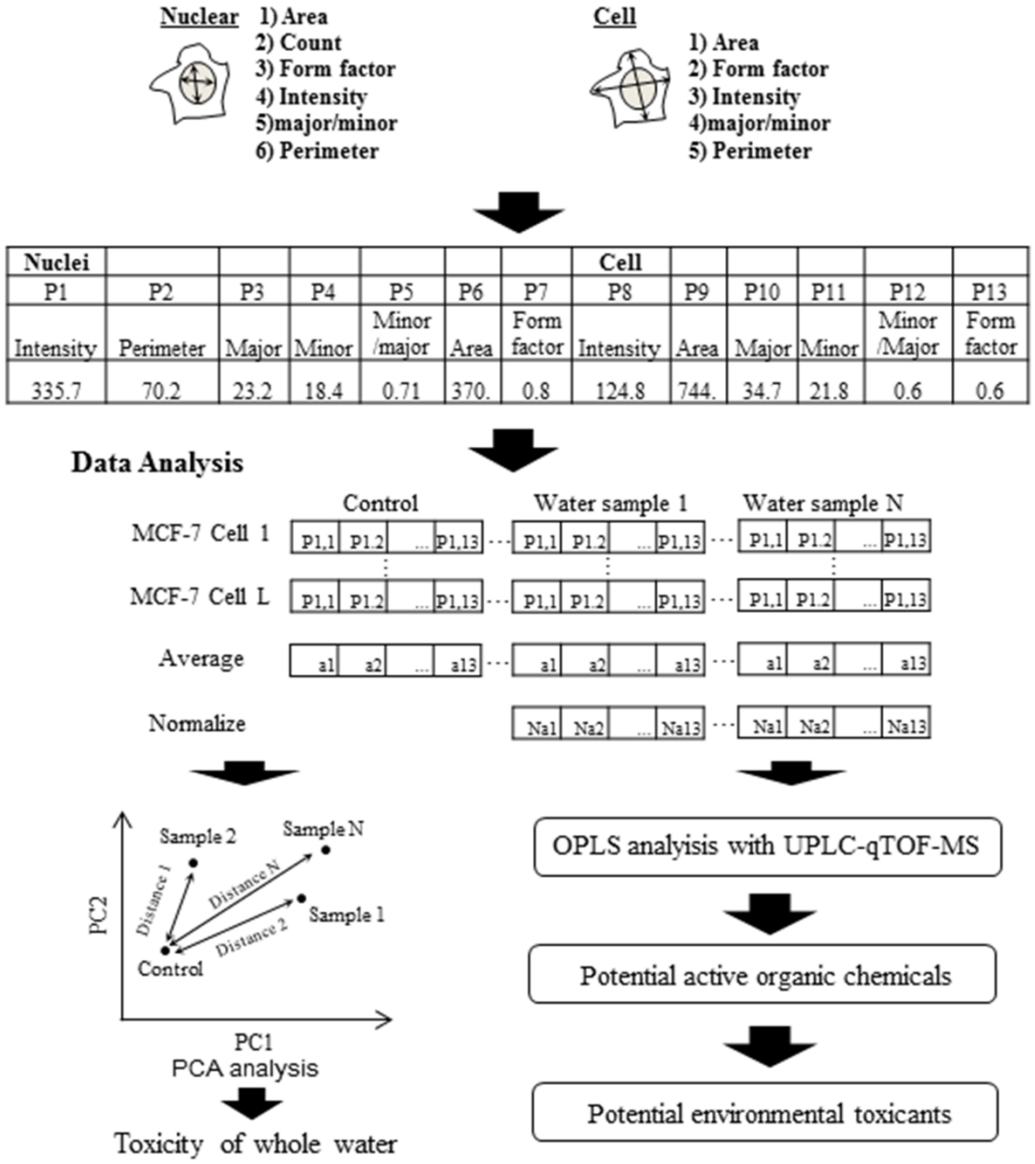


Figure 3.2 Data processing for image-based phenotypic analysis. Stochastic MCF-7 cells (n > 1000) in each well were collected and averaged. Multiple phenotypic parameters were calculated as the fold change vs. control. The normalized data were analyzed with statistical methods including a PCA analysis and OPLS-DA.

3.2.5 Conventional Bioassay

Two methods, an MTT assay, and direct cell counting, were used to assess cell viability. The E-screen assay, based on the protocol developed by Soto (1995), was used to calculate the estrogen equivalent concentration (EEQ) in river water and wastewater during a 7-day exposure in MCF-7 cells28. The 17ß-estradiol (E2) concentration was measured with estradiol enzyme immunoassay (EIA) kits (Cayman Chemical, USA). Five-hundred microliters of a concentrated solution equivalent to a 200-mL water sample was used for triplicate ELISAs.

Cell viability analyses included the MTT assay and a direct cell count. MCF-7 cells were seeded into 96-well plates and exposed to whole river water medium for 7 days. Next, the medium in each well was replaced with 100 μL of fresh medium with 10 μL of an MTT solution, incubated at 37 ℃ for 4 h. The medium was removed and sediment formazan crystal was dissolved in dimethyl sulfoxide (DMSO) for optical density testing using a Microplate Reader at 570 nm. The cell viability was calculated using normalized absorbance values. A cell count was performed with the IN cell analyzer 1000 (GE Healthcare, UK). After a 7 day exposure, cells were fixed, and nuclei were stained with the Hoechst 33342 nucleic acid stain. Immunofluorescence images (9 fields per well of a 96-well plate) were acquired automatically on an IN Cell Analyzer 1000 (GE Healthcare, UK) using a 4× objective. The number of nuclei in each well was counted with the IN Cell Developer Tool Box 1.7(GE Healthcare, UK), and the normalized cell viability was calculated.

E-screen assay

The E-screen assay was performed to evaluate estrogenic activities in water samples. Water samples underwent solid phase extraction (SPE) and were eluted with 2 mL of

ethyl acetate after nitrogen drying. Next, 0.5 mL of ethyl acetate (125 mL water sample equivalent) was dried and dissolved with 50 μL of DMSO (99.5%, Sigma) for the E-screen assay. MCF-7 cells were exposed to chemicals or concentrated water (0.001-10 fold concentrations) in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM-F12) (Sigma Aldrich, Japan) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) (DMSO < 0.01%) for 6 days. After exposure, cell viability was assessed using direct cell count. The proliferative effect (PE) and relative proliferative effect (RPE) was calculated to distinguish the agonistic and agonistic epigenetic activity of water samples, and the estradiol equivalency quantity (EEQ) was calculated:

PE: (maximum cell number)sample/(cell number)negative control;

RPE% : [(PE-1)sample/(PE-1)E2]* 100;

EEQ: (EC50)E2/(EC50)sample.

Enzyme-linked immunosorbent assay (ELISA)

Water samples underwent solid phase extraction (SPE) and were eluted with 2 mL of ethyl acetate after nitrogen drying. Next, 0.2 mL of ethyl acetate (50 mL water sample equivalent) was dried and dissolved with 200 μL of enzyme immunoassay (EIA) buffer from a commercial EIA kit (Cayman Chemical, USA). After the reaction of reagent and a concentrated water sample, the absorbance was determined with a Microplate Reader at 405 and 420 nm.

3.2.6 Non-target LC-qTOFMS analysis and Physical-chemical properties

A non-target chemical analysis was completed with an LC (Agilent 1200 series, USA) coupled to a Q-TOF-MS (Agilent 6540 UHD Accurate-Mass, USA) and

electrospray ionization (ESI) source with positive and negative modes. Chromatography was performed with a reversed-phase column (ZORBAX Extend-C18, 5 μm, 2.1 × 150 mm, Agilent, USA) and 0.2 mL/min flow rate at 40 ℃. The injection volume was 5 μL. Mass spectra were collected in full-scale mode from 50–2000 m/z. The data was assessed with MassHunter Qualitative analysis (MPP, version 12.0, Agilent, USA) software for molecular feature detection followed by Agilent Mass Profiler Professional Software. The raw data were normalized with a quantile algorithm using GeneSpring v14.5 (Agilent, USA), and chemicals with a significant difference (fold change > 2, $P < 0.05$, n=500) were selected for multivariate statistical analyses, including hierarchical clustering and PCA.

After water sampling, the following physicochemical parameters were measured: total organic carbon (TOC, mg/L), pH, electrical conductivity (EC, μs/cm) and major cation content (K^+, Na^+, Ca^{2+}, Mg^{2+}, mg/L). The pH and electrical conductivity of water samples were measured using a pH meter and conductivity meter (HORIBA, Japan). Water samples with major cation content including K^+, Na^+, Ca^{2+}, Mg^{2+} were acidified to a pH<2 with HNO_3 (Wako, Japan) and then were measured with inductively coupled plasma atomic emission spectrometry (ICP-AES; IRIS Intrepid II, Thermo Scientific, UK) in triplicate.

3.2.7 Statistical Analysis.

A compound database search was performed in ChemSpider (http://www.chemspider.com/). The data from phenotypic and non-target analyses were used in a PCA to evaluate the differences in phenotypic and chemical profile among samples. An OPLS-DA was used to identify chemical candidates that exhibited high correlations with phenotypic parameters. The PCA and OPLS-DA were performed using

SIMCA 13 software (Umetrics, Sweden). Quantitative data are expressed as the fold change vs. the control value ± standard deviation (SD). Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Dunnett's test for pairwise comparisons. Differences were considered statistically significant when $P < 0.05$.

3.3 Results and Discussions

3.3.1 Phenotypic profiling in MCF-7 cells of xenoestrogens

To verify the feasibility of such phenotypic approach for toxicity evaluation, we firstly investigate the xenoestrogens impacts on the phenotype of MCF-7 cells. Therefore, 26 organic chemicals including pharmaceuticals, pesticides, dioxins were selected for the evaluation. Then, the correlations between phenotypic parameters and toxicity pathways were evaluated through statistical method (e.g. PCA, OPLS-DA).

3.3.1.1 Phenotypic profiling in MCF-7 cells of 17β-estradiol (E2)

To construct image-based phenotypic processes and investigate the morphological relevance of estrogenic activities, E2 was selected due that it was the typical estrogen and exhibited sensitive to ER in MCF-7 cells(Huan et al. 2014). The image-based phenotypic analysis was performed after 6 days exposure following with data mining process (normalization, heat map, PCA analysis). The morphological dissimilarity was calculated as "distance to control" to characterize the phenotypic effect of E2 on MCF-7 cells.

In result, MCF-7 cells exhibited shrinkage along with cell growth. The 13 phenotypic parameters including intensity, perimeter, major, minor, major/minor, form factor were quantified and showed as a heat map (figure. 3.2B). Nuclei intensity, cell intensity, cell form factor increased, adversely, perimeter, major, minor, major/minor, area of nuclei

and cell decreased after E2 exposure. The decrease of nuclei area and cell area indicated cell shrink during the cell growth. The multiple phenotypic data were analyzed with PCA (Figure 3.2C), and the distance to control indicated phenotypic variation degree. The distance score showed a dose-dependent relationship with an EC50 (-12.02 Log M) similar to the cell proliferation analysis (-12.13 LogM) (Figure 3.2D). Moreover, nuclei intensity and nuclei area exhibited high correlations with cell proliferation (R^2=0.90, R^2=0.89, respectively) indicated their high potential for estrogenic activity evaluation. The result after E2 exposure suggested the feasibility of such phenotypic analysis for estrogenic effect evaluation.

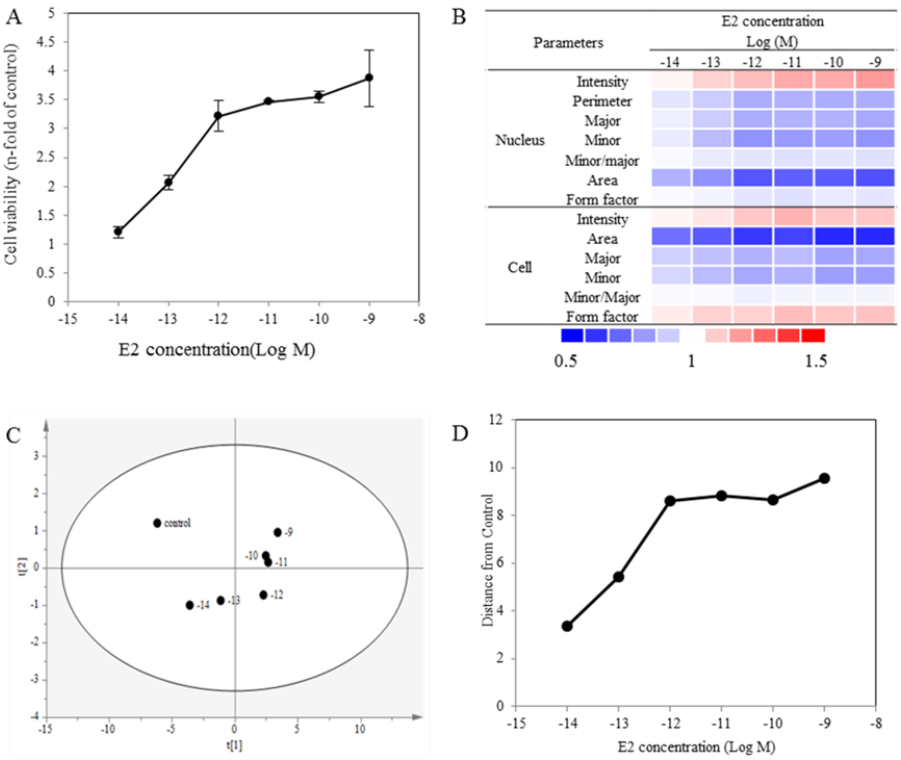


Figure 3.2 Image-based phenotypic analysis of 17β-estradiol. A) Dose-dependent effects of 17β-estradiol on the proliferation of MCF-7 cells. B) Effect of 17β-estradiol on the morphology of MCF-7 cells. C) PCA of 17β-estradiol-induced morphological variations. D) Correlation between morphological variation scope and E2 concentration.

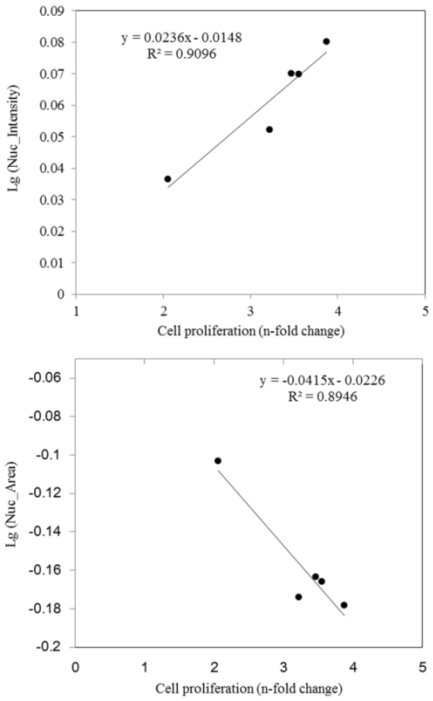


Figure 3.3 Correlations between cell proliferation and morphological parameters after E2 treatment.

3.3.1.2 Cell viability effects of xenoestrogens on MCF-7 cells

E2 binding to estrogen receptor seemed as a paradigmatic model for evaluation of estrogenic effect(Marino et al. 2006). The exposure concentrations of E2 (-14 M to -9 M) in the previous section referred to low-dose ranges and was considered as physiological doses for estrogenic effects (Welshons et al. 2003). In the other word, the estrogen receptor occupancy act in low-dose ranges, however, may be occupied by another toxicity pathways in high-dose ranges. Dose-response relations of estrogenic effect generally exhibited nonmonotonic (Figure 3.4). Therefore, in order to in-depth understand toxicity of xenoestrogens, it is important to identify the toxicities in high-dose range.

Moreover, xenoestrogens covered various toxicity pathways such as GPCR,

neurotoxicity, Membrane Transporter/Ion Channel except with estrogenic pathways, which posed the difficulty for estrogenic effect evaluation and toxicity identification due to the possible crosstalk actions. Therefore, we applied the multiple phenotypic parameters which may relate to more toxicity pathways.

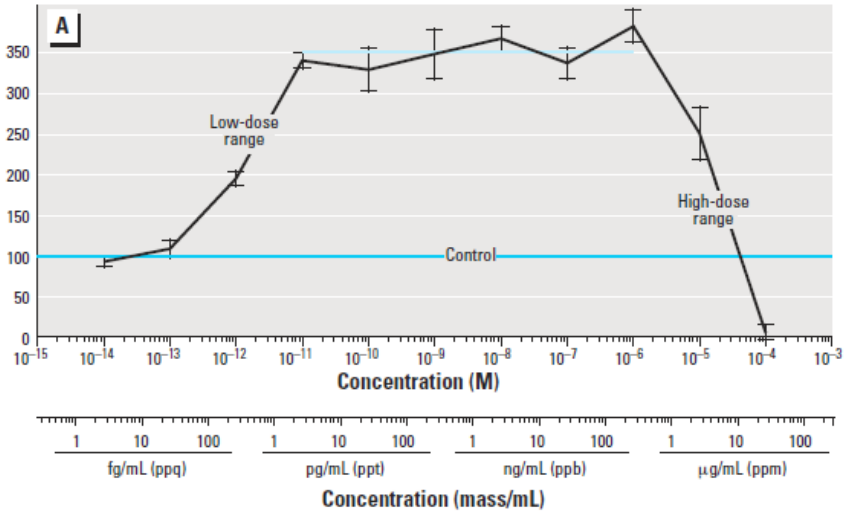
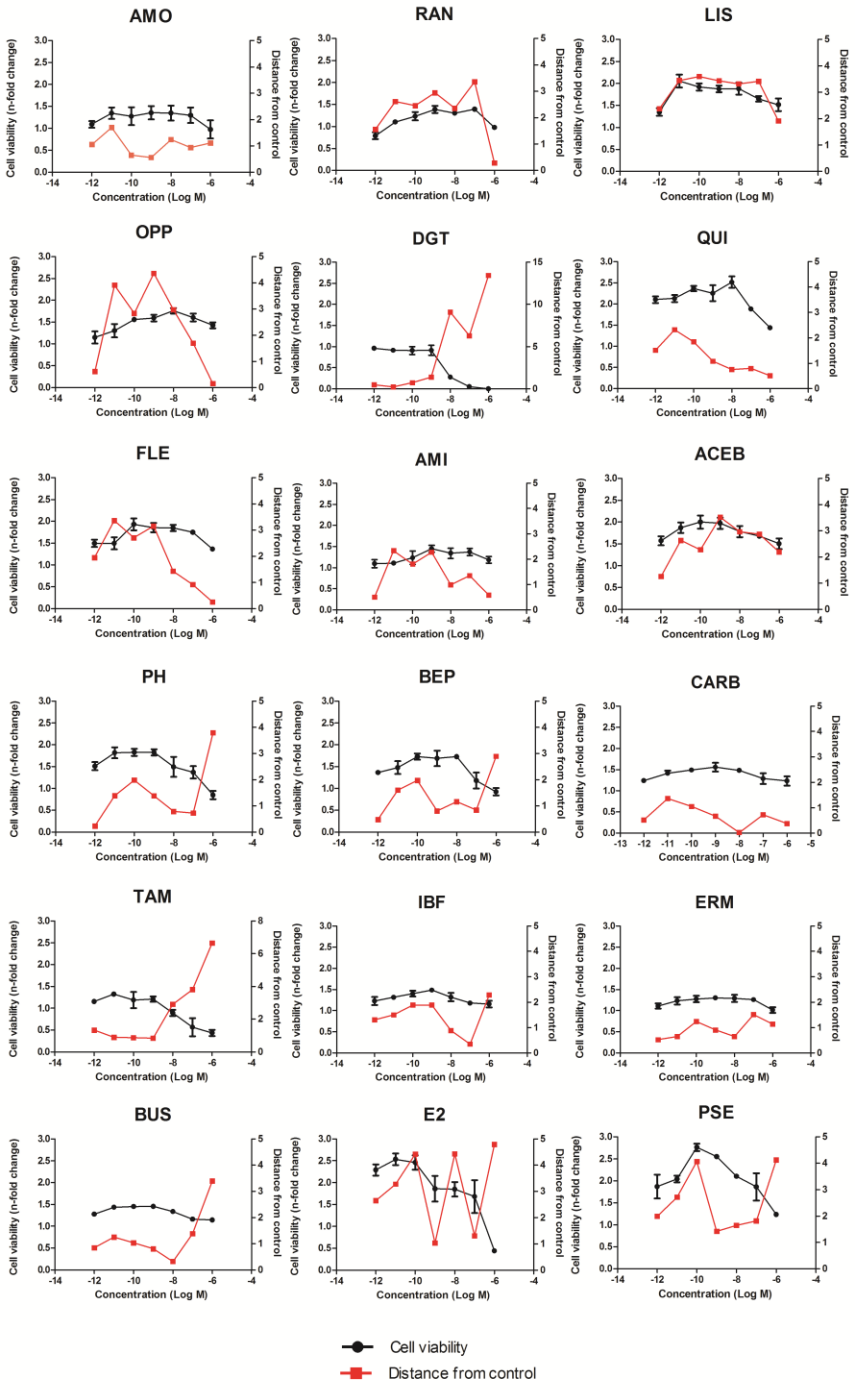


Figure 3.4 nonmonotonic dose-response relations of estrogenic effect

To verify our hypothesis, 26 organic chemicals with the wide mode of mechanism were selected and performed with both cell proliferation analysis and phenotypic analysis. Figure 3.5 showed dose-response curves of cell proliferation. According to the nonmonotonic dose-response mode, pharmaceuticals Lisinopril (LIS), Quinidine (QUI), Acebutolol (ACEB), Estradiol (E2), Prednisone (PSE), environmental contaminants Acetamiprid (ACE), Bisphenol A (BPA), Di(2-ethylhexyl)phthalate (DEHP), Thalidomide THD exhibited an estrogenic effect in low-dose range. Digitoxin (DGT) induced cytotoxicity after exposed with -8M. In the active organic chemicals, E2, BPA, PSEm DEHP were considered acting on Endocrinology/ Hormones pathways (Table 3.1), other chemicals such as acetamiprid were evaluated having potential estrogenic effects. Interestingly, increasing evidence indicated that neonicotinoids such as acetamiprid were considered as emerging estrogenic pollutants which should be

concerned(Lecomte et al. 2017). The chemicals exhibited acute toxicity in high-dose range, however, it is failed to identify the toxicity using cell viability parameters.



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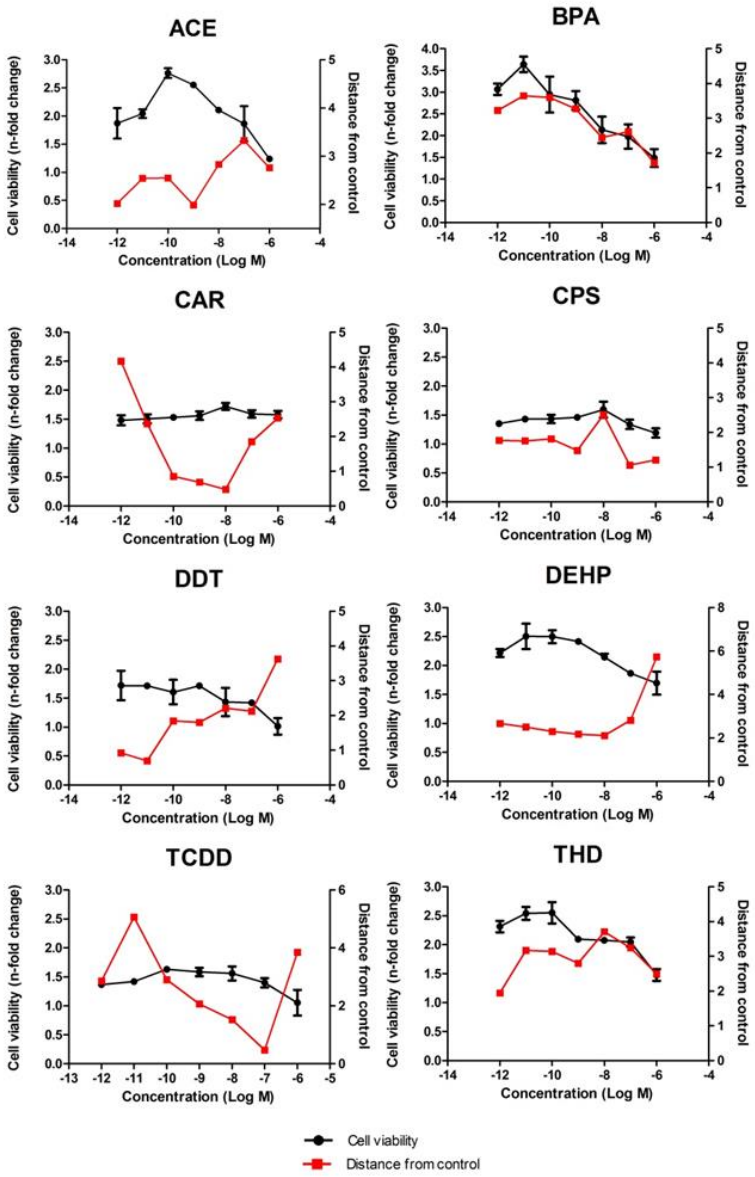


Figure 3.5 Viability and morphological variation of MCF-7 cells exposed to pharmaceuticals and environmental contaminants.

3.3.1.3 Phenotypic effects of xenoestrogens on MCF-7 cells

The phenotypic profiling in MCF-7 cells of xenoestrogens (doses range from -12M to -6M) was performed with the constructed phenotypic analysis procedures. Due to the whole water exposure in this study, it is necessary to investigate the phenotypic effects of inorganic chemicals and osmotic pressure, therefore, common ions (Ca, K, Mg, Na) and phosphate buffered saline (PBS: 10%~100%) were exposed to MCF-7 cells (Figure 3.6).

Based on the results in chapter 3.3.1.1, intensity and area of cell and nuclei pose the most sensitivity and showed positive and negative relations to cell proliferation, respectively. It suggested a high potential for estrogenic effect evaluation with the two phenotypic parameters. Therefore, we firstly focus on these phenotypic parameter parameters to hit the potential estrogenic active chemicals. In results, RAN, LIS, QUI, AMI, PSE, and THD exhibited the similar phenotypic characteristics with previous E2 exposure (increasing intensity but decreasing area of both nuclei and cell), which indicated their potential estrogenic effects. Compared with cell proliferation analysis, diverse phenotypic effects of ACE (increased cell area), BPA (decreased nuclei intensity), DEHP (decreased nuclei intensity and increased cell area) may result from their action of different pathways in MCF-7 cells. BPA was well known as androgen receptor antagonist and estrogen receptor agonist (Kim et al. 2001; Teng et al. 2013). Evidence indicated that PBA inhibited nuclear translocation of androgen receptor via various mechanisms, which decreased the nuclei intensity (Teng et al. 2013). In this study, the phenotypic variation after exposed with BPA exhibited estrogenic active (e.g. decreased nuclei area and cell area, increased cell intensity), however, the decreased

nuclei intensity may result from the coexist of anti-androgenic mechanisms of BPA. DEHP, known as androgen receptor antagonist, also reduced the nuclei intensity. Due to that whole water samples would be addressed with the phenotypic analysis, therefore, inorganic chemicals and osmotic pressure should be considered. It was reported that cellular concentration of ions act on ion channels and exhibited a significant positive response to cell proliferation (Cameron and Smith 1989; Rao et al. 2015). In this study, Ca, K, Mg increased cell proliferation of MCF-7 cells (data not shown), therefore, the cellular intensity and area showed positive and negative, respectively. To investigate the phenotypic effects of physical factors (osmotic pressure), PBS was diluted (100%-6.25%) and exposed to MCF-7 cells. In results, the cell shape increased remarkably at high-dose ranges (-7M and -6M) (Figure 3.7B). Therefore, the results indicated that multiple-phenotypic parameters were powerful to cover multiple toxicity pathways.

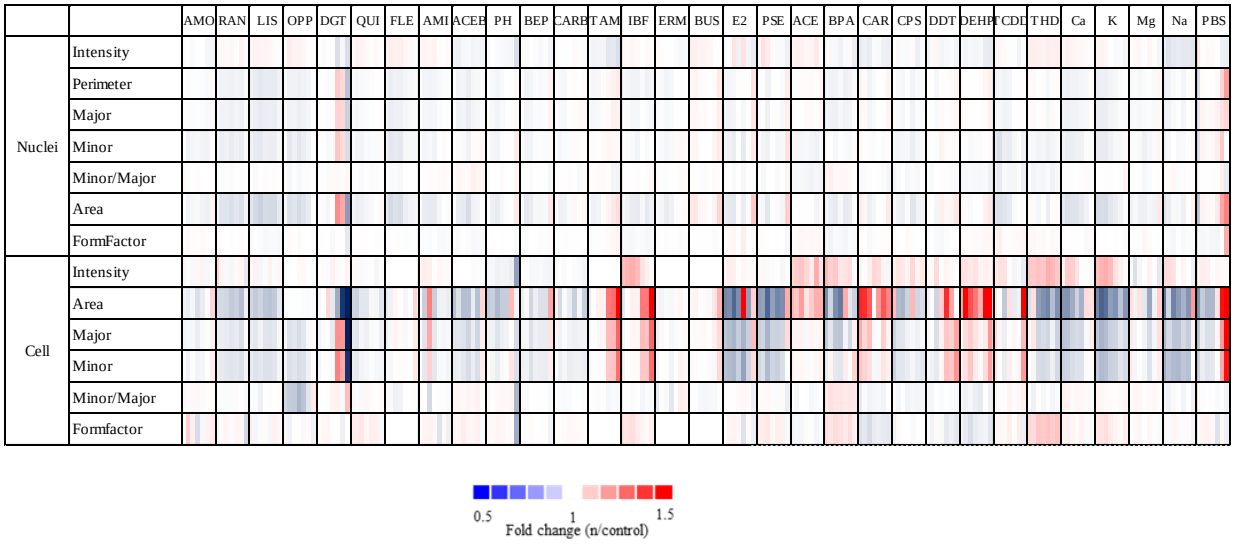


Figure 3.6 Phenotypic effects of xenoestrogens on MCF-7

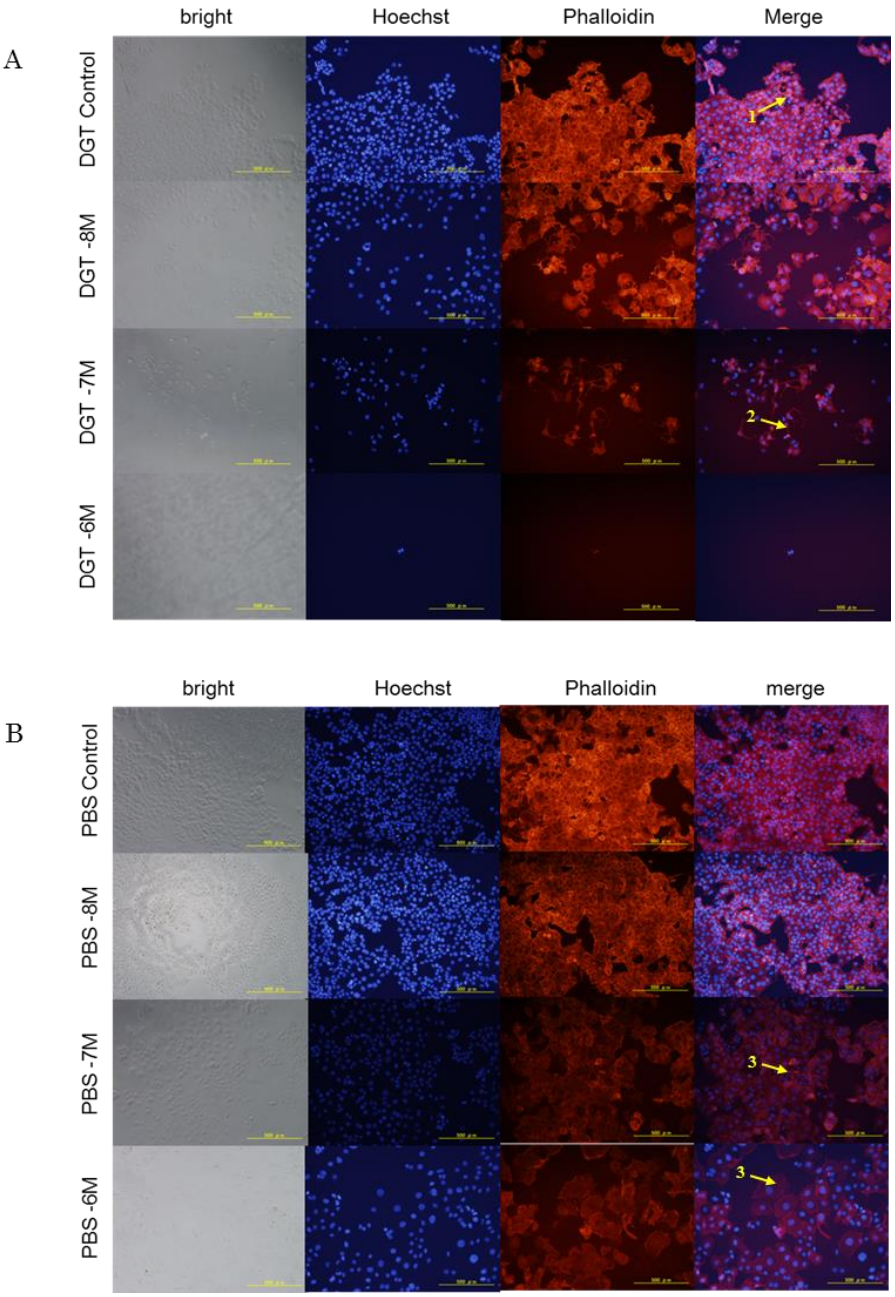


Figure 3.7 Confocal images of the MCF-7 exposed to DGT (A) and PBS (B). Arrows indicate (1) control cells; (2) echinoid spikes cells; (3) enlarged nuclei and cells.

To visualize and classify the phenotypic characteristics of each chemicals, the phenotypic effects were analyzed by PCA analysis (Figure 3.8). In results, chemicals with a different mode of mechanisms were separated in PCA score plot, in which DEHP, ACE, OPP, BPA, K, Ca were well clustered due to the specific phenotypic variation. Estrogenic against chemicals were distributed near BPA but DEHP and showed significate diverse of phenotypic effects. E2, TAM, DEHP, TCDD, DGT, PBS, PSE, ACBE in high-dose ranges induced remarkable and specific phenotypic effect but low effects of cell proliferation, which indicated that multiple phenotypic parameters covering various toxicity-pathways had a high potential for toxicity evaluation and identification in high-dose ranges. Phenotypic dissimilarity was applied to hit candidate toxicity or chemicals based on the constructed phenotypic databases (Antczak et al. 2012), this study was the first study to construct a phenotypic database of xenoestrogens, the multiple phenotypic parameters provided a novel sight to toxicities, the phenotypic analysis would be a powerful tool for toxicity identification along with the improvement of phenotypic databases.

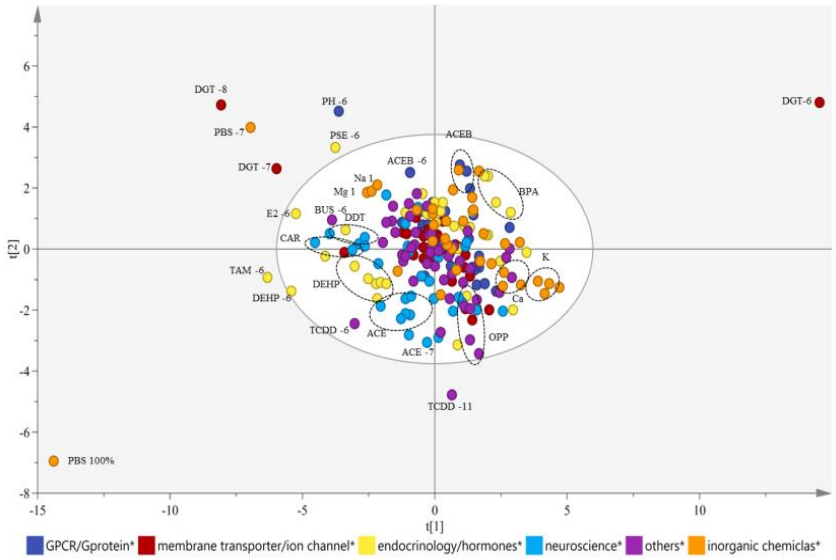


Figure 3.8 Multivariate analysis of phenotypic variation in MCF-7 cells exposed to xenoestrogens

3.3.1.4 Correlation evaluation of morphological parameters with estrogenic effect

In this study, MCF-7 cell lines were first applied as the platform for phenotypic analysis. Due to the ER sensitivity of MCF-7 cells(Holliday and Speirs 2011), estrogenic effects was preferred to be considered. According to E2 exposure, it was found that cellular intensity and area exhibited high correlations with cell proliferation (Figure 3.3). However, it increased the difficulty for estrogenic effect evaluation of xenoestrogens due to the possible interactions of complex mechanisms. Therefore, it is important to investigate the correlations of phenotypic parameters with estrogen effect of xenoestrogens. According to the PCA score plot of each phenotypic parameter, cell intensity and nuclei intensity exhibited relatively high positive correlations, in adverse, nuclei area showed a negative correlation with cell proliferation (Figure 3.9). Therefore, the three phenotypic parameters could be considered as a potential biomarker for estrogenic effect evaluation.

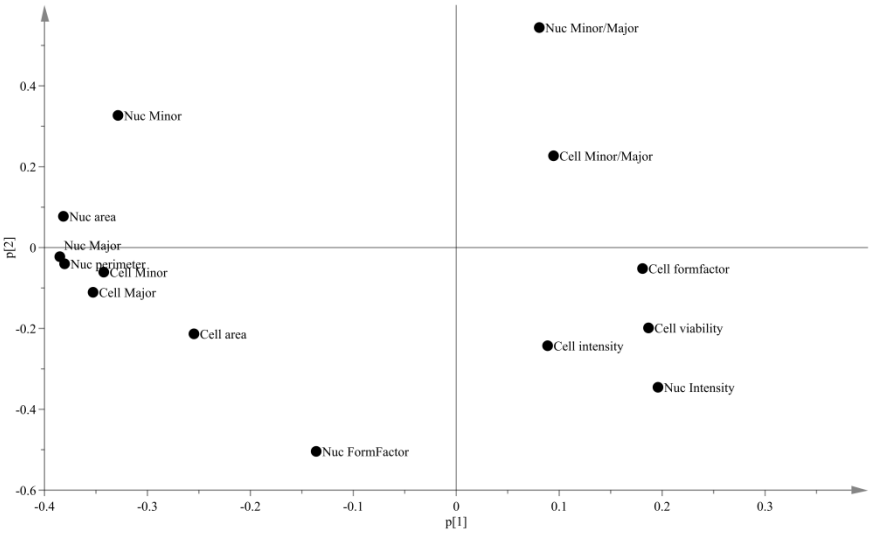


Figure 3.9 Multivariate analysis of phenotypic parameters in MCF-7 cells exposed to xenoestrogens

3.3.1.5 Relations of morphological parameters with other toxicity-pathways

The phenotypic parameters provided multiple pipelines to characterize the various toxicity pathways and made it possible for integrated toxicity evaluation and identification in the single bioassay. To our best knowledge, biological processes such as cell death, cell proliferation, cell communication were the main factor to induce phenotypic effects(Morgan and Liu 2013). The possible phenotypic effects of different biological processes were summarized as Table 3.2. Cell death such as cell apoptosis induced chromatin condensation and nuclear fragmentation which were shown in Figure 3.10B. The apoptosis cells exhibited high nuclei intensity but low nuclei area by the phenotypic analysis. Cell mitosis could be visualized (Figure 3.10A) and showed high nuclei intensity, low nuclei area, and low nuclei form factor. Moreover, the nuclei area in M-phase increased significantly as showed in Figure 3.10C. Cell shape showed abnormal (synaptic cell membrane, large cell body) resulting from cell communication (e.g. cell migration, cell adhesion). In chapter 3.3.1.2, DGT induced synaptic cell due to the possible inhibition of cell migration. For the estrogenic effect of xenoestrogens, cell proliferation was an important endpoint, but not the only outcome. Xenoestrogens targeted on ER impacting on the expression of various genes (e.g. *p21, bcl2*), thus it induced cellular outcome including apoptosis, growth and survival and cell cycle (Lecomte et al. 2017). Therefore, integrated evaluation of biological processes was desirable to understand the overall toxicities. In all, the multiple phenotypic parameters were sensitive to various biological processes and exhibited powerful for toxicity identification.

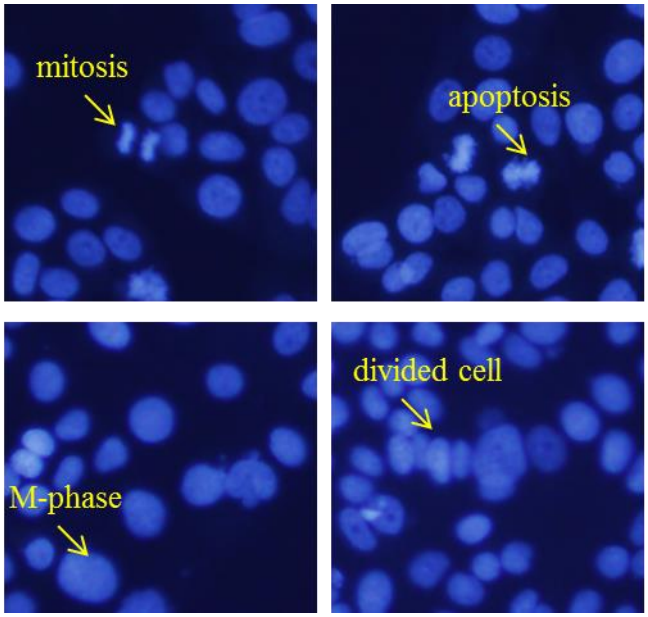


Figure 3.10 Nuclear shape variations by cell cycle and apoptosis

Table 3.2 Possible biological processes that induce phenotypic variations

	Biological process	**Possible morphology variation**	**Ref.**
Cell death	Apoptosis	chromatin condensation, nuclear fragmentation	a,b,c
	Necrosis	cell size increases, nucleus disintegration, increased nuclei fluorescence	d
Cell proliferation	Cell division	Cell division, cell adhesion, tapered daughter cell or triangular mother cells	e
	Nuclei division	Elongated, multiple nuclei	f
	M-phase	Enlarged nucleus, decreased nuclei fluorescence	g
Cell communication	Cell Adhesion	Synaptic cell membrane, large cell body	h
	Transport	Abnormal cell shape	i
Inorganic chemicals	Loss of cooper	Spindle-shaped cells	j
Others	Hydrocarbon receptor	loosening cell-cell contacts, increased cell area, cell scattering	k

a,(Futamura et al. 2012a)b,(Dix et al. 2007)c,(Elumalai et al. 2012)d,(Narayanan et al. 2015)e,(Eidet et al. 2014)f,(Van Cruchten and Van Den Broeck 2002)g,(Neumann et al. 2010)h,(Edens et al. 2013)i,(Wang et al. 2014)j,(Frixen et al. 1991)k,(Lauffenburger and Horwitz 1996)

3.3.2 Effect of xenoestrogens mixtures on MCF-7 cell morphology for toxicity identification

3.3.2.1 Mixture effects of xenoestrogens based on toxicity-pathways

It is difficult to evaluate the mixture toxicity due to the complex toxicity pathways. To investigate the feasibility of phenotypic analysis for toxicity evaluation of complex mixture, the chemicals with a different mode of actions were mixed for phenotypic analysis (M1: Mixture of chemicals acting on neuro pathway including IMI, ACE, CAR, CPS, THD, DDT; M2: Mixture of chemicals acting on endocrinology/hormones pathway including E2, BPA, TAM; M3: Mixture of chemicals acting on membrane transporter/ion channel pathway including AMI, BEP, FLE, CARB). In chapter 3.1.1.2, DGT induced specific phenotypic effect (echinoid spikes cells). To investigate the feasibility of phenotypic analysis for toxicity identification of complex mixtures, mixtures with or without DGT were made for phenotypic analysis (M4: Mixture of environmental chemicals without DGT; M5: Mixture of pharmaceuticals with DGT; M6: Mixture of all chemicals with DGT; M7: Mixture of pharmaceuticals without DGT; M8: Mixture of all chemicals without DGT).

In results, the phenotypic variations of each mixture were different (Figure 3.11). M2 with estrogenic active chemical induced similar phenotypic effect with estrogens (e.g. increased nuclei intensity, decreased cell area), which proved the feasibility of phenotypic analysis for estrogenic effect evaluation. Interestingly, mixtures with DGT (M5 and M6) also induced cellular abnormal (echinoid spikes), whoever, the cells exhibited normal after exposure to the mixture without DGT (M7 and M8). This evidence indicated that chemicals or toxicities with specific phenotypic effects also

induce similar phenotypic effects in their mixture. In turn, it can be identified based on the phenotypic similarity with phenotypic analysis.

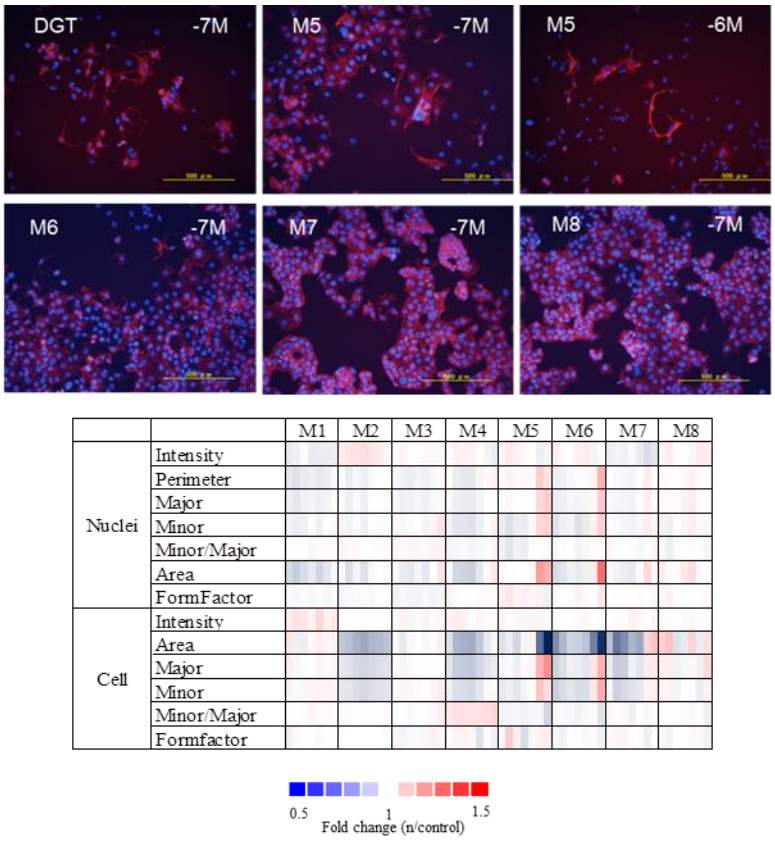


Figure 3.11 Confocal images of MCF-7 cells exposed to xenoestrogens and an image-based chemical screen using 13 parameters

3.3.2.2 Toxicity identification of xenoestrogens mixtures

To visualize and identify phenotypic effects of chemical mixture, phenotypic results were analyzed by PCA (Figure 3.12). In results, chemicals mixtures were well separated in PCA score plot based on the mode of the mechanism. Interestingly, the mixtures with DGT (-6M and -7M) induced the similar phenotypic effects and distributed near with DGT (-7M) in PCA score plot (phenotypic dissimilarity=0.12 and 0.35, respectively). It indicated that toxicity or chemicals can be identified with the dissimilarity of cell phenotype.

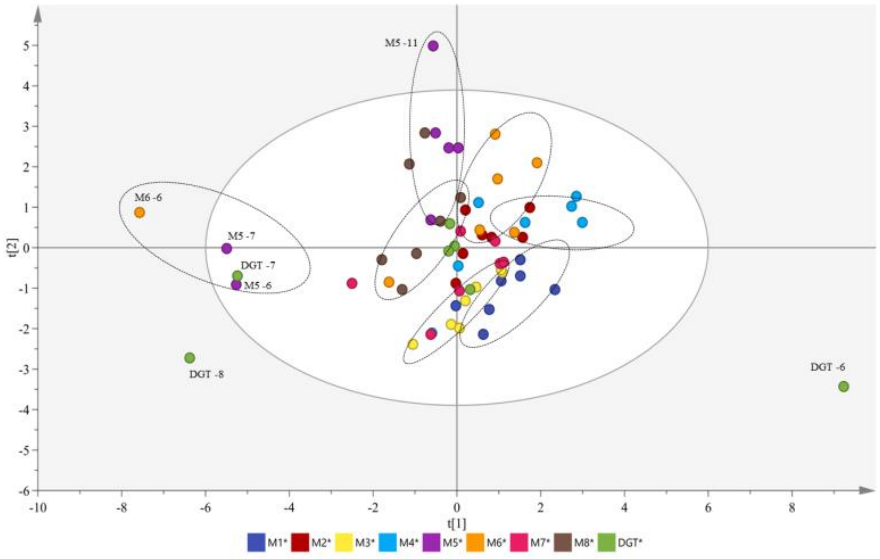


Figure 3.12 Multivariate analysis of phenotypic variation in MCF-7 cells exposed to xenoestrogens mixtures

3.3.3 Integrated toxicity evaluation of whole water sample using phenotypic profiling

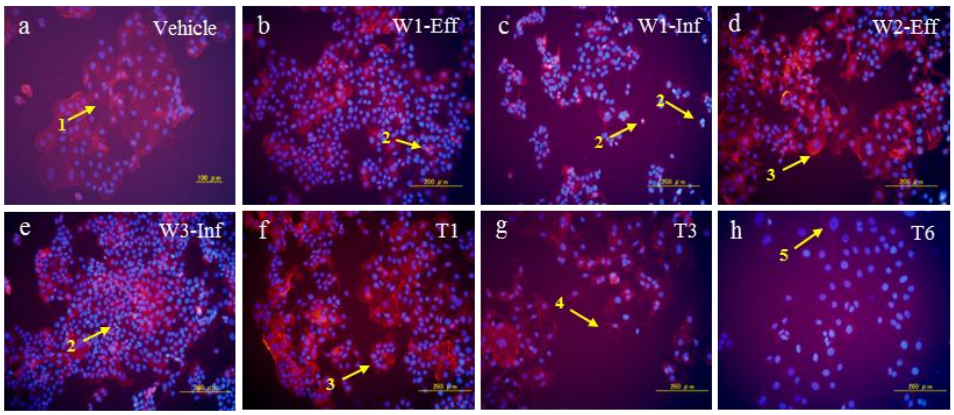
Various bioassays were developed for integrated toxicity evaluations of whole water using both *in vivo* and *in vitro* platform(Magdeburg et al. 2012). For instance, zebrafish embryo toxicity test was well applied for whole water toxicity test, however, the simple endpoint failed to the in-depth understanding of toxicity pathways. Moreover, the benchmark researches combine in vitro and in vivo made it difficult to extrapolate to human health. Omics approaches using microarray were applied for toxicity evaluation an identification of whole water samples (Hara-Yamamura et al. 2013), thousands of a gene related to various toxicity pathway (cell death, cell proliferation, cell communication) were selected to evaluate and identify whole water toxicities. This technology was a high-throughput and toxicity-pathway based approach, however, the

cost made it difficult for the large-scale screen. Therefore, the phenotypic analysis was applied for integrated toxicity evaluation of whole water samples.

3.3.3.1 Morphological effects of whole water samples on MCF-7 cells

The morphology of MCF-7 cells showed various change after exposure to wastewater and river water (Figure 3.13A). The vehicle control cells showed around and equipotent shape (Figure 3.13A-a), whereas exposure to wastewater or river water induced a malformed cellular shape, including shrunk and aggregated cells (e.g., W1-Eff and W3-Inf exposure) (Figure 3.13A-b, e), synapse cells (e.g., W2-Eff, T1, and T3 exposure) (Figure 3.13A-d, f, g), and enlarged nuclei and cell bodies. (e.g., T6 exposure) (Figure 3.10A-h). The morphology changes of MCF-7 cells were classified into 3 categories: 1) shrunk or enlarged cells; 2) synapse or polygonal cells, and 3) rounded cells.

A high-content image analysis was performed to evaluate phenotypic responses. These responses were characterized using 13 parameters including the intensity, perimeter, major, minor, area, and form factor of both nuclei and cells. Most of the wastewater induced an increase in the nuclei intensity of MCF-7 cells, but negatively affected other parameters. Across river water samples, the morphological variations were markedly different, such as increased nuclei intensity (T1-T3) and cell area (T4), increased nuclei area (T6), and decreased nuclei form factor (T8) (Figure 3.13B). The statistical analyses of parametric data and cellular images showed that the image-based analysis had good sensitivity and a high-throughput capacity. T6 induced increased nuclei area and cell area, T6 was taken in estuary region with high osmotic pressure (Figure 3.1) Based on the phenotypic effect of BPS, osmotic pressure in T6 took an important role for the enlarged cells. Moreover, T3 induced echinoid spike cells may result from the inhabitation of cell migration by chemicals.



Parameters		Whole water sampling														
		W1-Eff	W1-Inf	W2-Eff	W2-Inf	W3-Eff	W3-Inf	T1	T2	T3	T4	T5	T6	T7	T8	T9
Nuclei	Intensity	1.08	1.47	1.03	1.22	1.10	1.30	1.12	1.12	1.16	0.87	1.03	0.86	0.95	1.02	1.04
	Perimeter	0.84	0.89	0.92	0.88	0.92	0.88	0.89	0.92	0.86	1.05	0.94	1.13	1.01	1.05	1.03
	Major	0.84	0.88	0.92	0.87	0.91	0.88	0.89	0.92	0.85	1.05	0.94	1.13	1.01	1.05	1.02
	Minor	0.81	0.82	0.92	0.83	0.90	0.82	0.87	0.91	0.83	1.08	0.92	1.18	1.03	1.08	1.06
	Minor/major	0.97	0.94	1.01	0.96	0.99	0.95	0.97	1.00	0.98	1.03	0.99	1.05	1.03	1.02	1.05
	Area	0.69	0.76	0.84	0.75	0.83	0.75	0.77	0.84	0.72	1.11	0.87	1.31	1.04	1.11	1.06
	Form factor	0.98	0.95	1.00	0.97	0.99	1.09	0.98	1.00	0.98	1.02	1.00	1.04	1.01	0.66	1.03
Cell	Intensity	1.02	0.97	1.02	1.00	1.02	0.98	1.01	0.99	1.04	0.99	1.01	0.96	0.98	1.03	1.00
	Area	0.56	0.54	0.71	0.54	0.68	0.55	0.57	0.67	0.57	1.26	0.71	1.19	0.99	0.85	0.84
	Major	0.76	0.73	0.84	0.74	0.82	0.73	0.76	0.79	0.76	1.10	0.84	1.00	0.96	1.09	1.06
	Minor	0.74	0.74	0.84	0.74	0.83	0.73	0.77	0.81	0.75	1.08	0.84	1.04	0.99	1.08	1.06
	Minor/Major	0.99	1.01	1.01	1.01	1.01	1.02	1.01	1.03	1.00	0.99	1.01	1.07	1.04	0.99	1.00
	Form factor	0.93	0.89	0.91	0.93	0.91	0.92	0.94	0.89	0.93	0.83	0.90	0.85	0.88	1.02	1.02

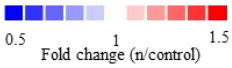


Figure 3.13 Phenotypic analysis of MCF-7 after exposure with whole water samples. Confocal images of MCF-7 cells exposed to wastewater and river water (A) and an image-based chemical screen using 13 parameters (B). The arrows indicate (1) control cells, (2) condensed cells, (3) scattering cells, (4) echinoid spike cells, and (5) enlarged nuclei

A PCA was performed to visualize and classify the phenotypic responses induced by whole water exposure (Figure 3.14). The PCA score plot showed a significant difference between the morphological profiles of wastewater and most river water samples, which may be due to the presence of different sample constituents. T6, T8, and W1-Inf samples exhibited the strongest effect on morphology. The similarity between T5 and W2-Eff suggests a similar chemical profile. Moreover, most WWTP effluents exhibited similarity to control samples than WWTP influents, which illustrates the efficiency of wastewater treatments at reducing chemical contaminants.

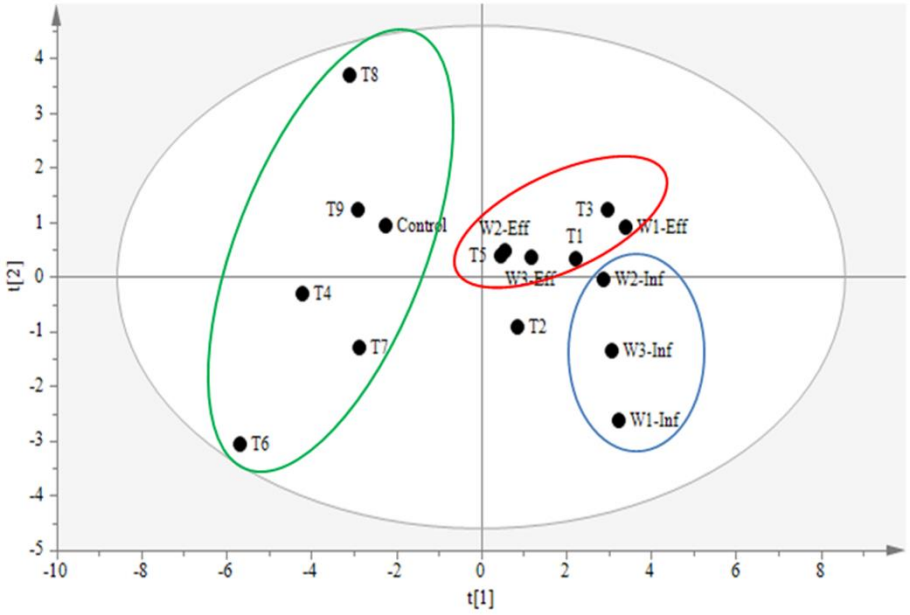


Figure 3.14 Multivariate analysis of phenotypic variation in cells exposed to wastewater and river water

3.3.3.2 Comparison phenotypic analysis with conventional bioassay

For whole water effluent toxicity elevation, *in vitro* bioassay with fish cells (Dayeh et al. 2002), mammal cells (Hara-Yamamura et al. 2013) were developed targeting on cell proliferation using MTT assay or direct nuclei count. However, chemicals in water may pose both antagonistic and antagonistic effect on the biological processes, therefore, single parameters may be difficult for integrated toxicity test. To optimize the evaluation, a phenotypic analysis combining statistical analysis was applied for the integrated toxicity of whole water sample.

The morphological dissimilarity with control was defined as a "score" calculated with Euclidean distances (Figure 3.15A). Wastewater treatment plant influents W1-Inf, W2-Inf, and W3-Inf exhibited a relatively high score, whereas after treatment, these scores were reduced by 13.7%, 45.2%, and 40.3%, respectively (W1-Eff, W2-Eff, and W3-Eff). T9 showed the smallest score indicating a minimum effect on cell morphology. The results indicated that phenotypic analysis was sensitive and provided a novel insight for toxicity reduction evaluation (TRE).

Cell viability was analyzed using an MTT assay and direct cell counting. The results of these two methods were conflicting. W1-Inf induced cytotoxicity (0.54 fold change) in MCF-7 cells in MTT assay but increased cell proliferation (1.34 fold change) in the direct cell count assay, suggesting the cell death occurred after exposure. Cell viability in the MTT assay after W2-Inf and W3-Inf exposure was 1.01 and 1.06, respectively, may indicate the counteraction agonist and antagonist actions of estrogen receptor (ER) for MCF-7 cells. T6, which had the highest E2 concentration (2.26 ng/L), had little effect on cell viability, suggesting the presence of an ER antagonist in the T6 samples.

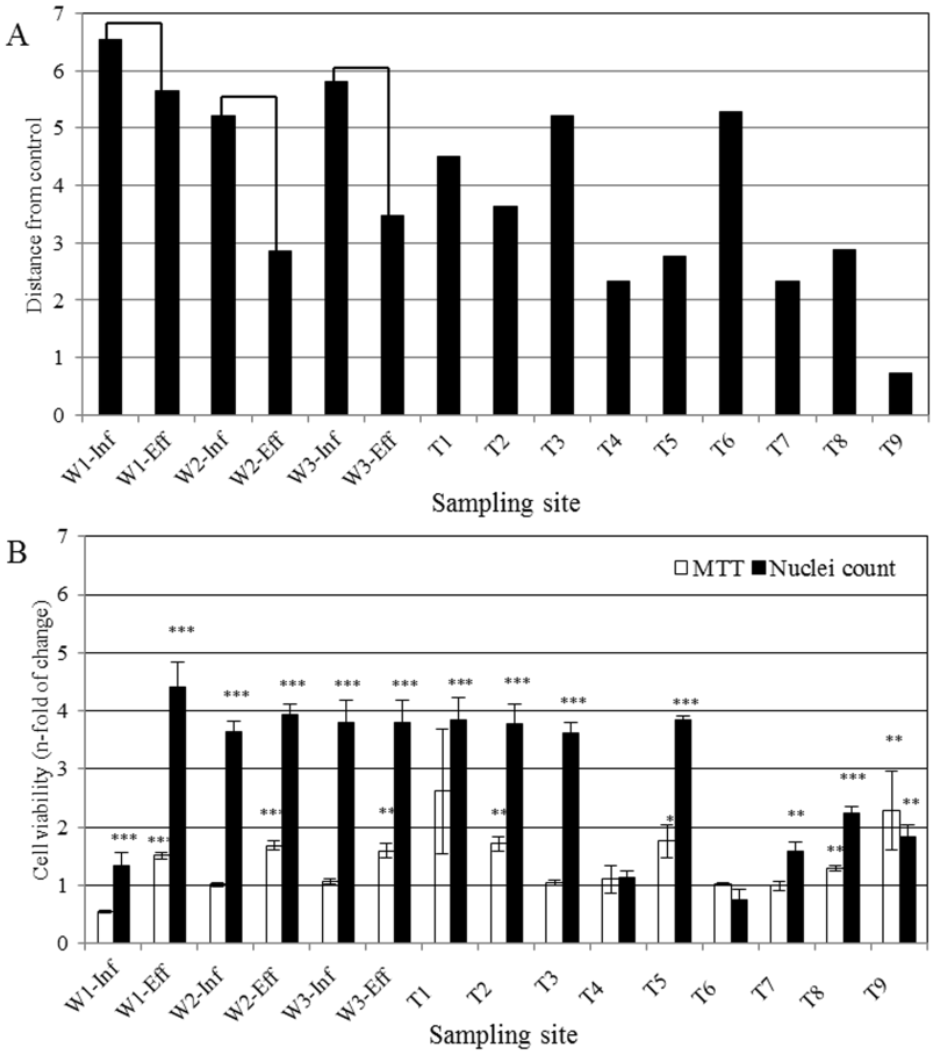


Figure 3.15 Comparison of the image-based chemical screen (A) and conventional cell viability analyses (B) for whole water toxicity evaluation. * $P<0.05$ was considered to be a statistically significant difference

3.3.3.3 Organic and inorganic chemicals in environmental water samples

Thousands of chemicals existed in water samples posed adverse effect MCF-7 cells through complex toxicity pathways. According to the previous researchers, multiple parameters made correlations with various biological processes (e.g. cell proliferation, cell communication, cell death), and the mixture toxicity researchers indicated a high potential of phenotypic analysis for chemical and toxicity identification. To investigate its feasibility for identification of complex whole water samples, we detected organic chemicals and ions in the water sample

The variation in cell morphology caused by the water samples may be due to the diverse chemical structure in each sample. To investigate the relationship between phenotypic variation and chemicals profile, we selected 6 samples that induced distinct cell morphological change (T2, T8, T9, W1-Inf, W1-Eff, and W3-Inf) for non-target chemical analysis using LC-qTOFMS. A total of 3,061chemicals were extracted, and 500 chemicals (fold change > 2) were used to generate a heat map with a hierarchical clustering analysis to highlight general differences in each sample (Figure 3.16). PCA was performed to identify differences in chemical profiles of each sample. Clear separation of T2, T8, and W1-Inf from other samples indicated a significant difference in chemicals profiles (Figure 3.16B). A compactness of distribution was observed among T2, W3-Inf, and W1-Eff, indicating similar chemical profiles. The identified chemicals in each sample were compared using a Venn diagram (Figure 3.16C): 79 chemicals exhibited overlap in all water samples, 92 chemicals were common to W1-Inf and W1-Eff indicating untreated chemicals, and 20 chemicals were unique to W1-Eff, which may indicate by-products from the treatment process. Moreover, 227, 12, 43, and

8 chemicals were unique to W1-Inf, W1-Eff, T8, and T2, respectively, indicating that these chemicals may contribute to specific changes in cell morphology. Interestingly, the T2, W3-Inf, W3-Inf with similar organic chemical structure (Figure 3.16B) induced the similar phenotypic effects (Figure 3.14). Moreover, the diverse phenotypic effects of whole water sample may result from the particular chemicals based on the Venn diagram (Figure 3.16C).

For inorganic chemicals, T5 and T6 were detected with high electrical conductivity (1450μs/cm and 5900μs/cm, respectively) and high concentration of ions. The high osmotic pressure in T6 resulted in the enlarged cell and nuclei, and the increased cellular area can be applied for identification of osmotic pressure of water sample.

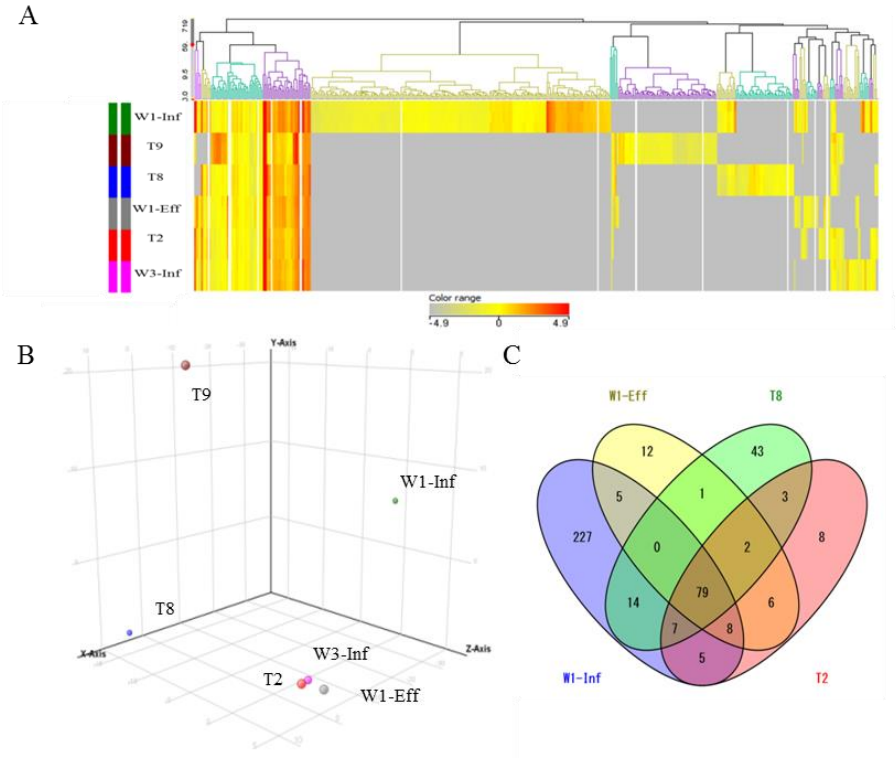


Figure 3.16 Hierarchical clustering analysis of chemicals in wastewater and river water. Five-hundred chemicals (fold change > 2, $p < 0.05$) were selected for the clustering analysis of 20 groups using chemical features

Table 3.3 Physical-chemical properties of wastewater and river water

	W1-Eff	W1-Inf	W2-Eff	W2-Inf	W3-Eff	W3-Inf	T1	T2	T3	T4	T5	T6	T7	T8	T9
pH	7.3	8.5	7.2	8.4	6.9	7.4	7.2	7.3	6.8	7.9	6.9	6.8	6.9	7.9	7.4
Electrical conductivity (µs/cm)	410	470	280	370	600	410	410	250	370	290	1450	5900	210	290	260
Ca (mg/L)	24.8 ±1.5	17.5 ±0.1	18.1 ±0.1	25.5 ±0.1	39.3 ±0.1	22.7 ±0.1	34.8 ±0.2	30.5 ±0.1	32.1 ±0.1	26.2 ±0.1	40.5 ±0.1	137.5 ±0.5	16.1 ±0.1	27.5 ±0.2	32.3 ±0.3
K (mg/L)	11.6 ±0.9	12.6 ±0.1	7.9 ±0.1	6.8 ±0.1	20.9 ±0.1	12.0 ±0.1	13.0 ±0.1	7.1 ±0.1	6.8 ±0.1	2.5 ±0.	35.0 ±0.1	236. ±3.7	8.0 ±0.1	8.2 ±0.0	2.7 ±0.0
Mg (mg/L)	6.4 ±0.7	7.1 ±0.1	6.2 ±0.1	6.57 ±0.03	11.0 ±0.1	7.4 ±0.0	7.7 ±0.1	9.1 ±0.1	9.8 ±0.1	13.4 ±0.0	53.0 ±0.2	246. ±1.5	4.0 ±0.0	8.1 ±0.01	14.6 ±0.1
Na (mg/L)	54.2 ±0.5	51.9 ±1.6	40.4 ±0.3	37.0 ±0.3	86.7 ±2.2	44.5 ±0.9	60.7 ±1.7	33.8 ±0.3	29.8 ±0.1	14.9 ±0.1	305.9 ±12.0	1279 ±42.4	40.1 ±0.2	37.9 ±0.4	16.5 ±0.6

3.3.3.4 Estrogenic activities of water sample using conventional bioassay

Conventional bioassay for estrogenic effect was evaluated and compared with the phenotypic analysis. In results, cell proliferation of whole water samples induced higher positive effect that pretreated water samples indicated the possible effects of inorganic chemicals. Moreover, E-screen and ELISA assay were performed to evaluate the estrogenic effect of water samples. W1-Inf was detected with the highest E2 concentration in both bioassays (5.87 ng/L in E-screen; 13.49 ng/L in ELISA). For phenotypic analysis, W1-Inf induced the highest phenotypic effect (phenotypic dissimilarity score = 6.56) especially for nuclei intensity (1.47 fold change). However, it is difficult to characterize the estrogenic effect of W1-Inf due to the induced cytotoxicity to MCF-7 cells(0.54 fold change). It indicated that nuclei intensity would be potential for estrogenic effect evaluation in whole water samples.

Table 3.4 Cell viability of whole water and SPE water, 17ß-estradiol Concentration, and EEQs

Sample Number	MTT assay	IN cell assay (Whole water)		IN cell assay (SPE water)		E-screen	ELISA assay
	PE	PE	RPE (%)	PE	RPE (%)	EEQ (ng/L)	(ng/L)
W1-Eff	1.51±0.03	4.41±0.42	118.61±14.8	2.70±0.18	59.17±6.21	0.87	2.93±0.06
W1-Inf	0.54±0.01	1.35±0.21	12.08±7.27	3.14±0.30	74.43±10.69	5.87	13.49±0.27
W2-Eff	1.69±0.05	3.93±0.17	102.01±6.13	2.21±0.26	41.98±9.11	0.79	0.53±0.01
W2-Inf	1.01±0.19	3.64±0.18	91.82±6.15	1.56±0.02	19.48±0.86	1.25	1.19±0.02
W3-Eff	1.59±0.07	3.78±0.40	97.12±14.93	2.69±0.20	58.93±6.86	0.96	2.25±0.45
W3-Inf	1.06±0.02	3.56±0.12	97.12±13.93	1.42±0.26	23.98±9.21	0.76	0.88±0.02
T2	1.71±0.07	3.78±0.35	96.64±12.01	3.28±0.90	79.33±31.41	0.70	0.45±0.01
T4	1.10±0.12	1.13±0.11	4.57±3.72	1.87±0.10	30.14±3.32	0.93	0.33±0.01
T5	1.76±0.15	3.83±0.07	98.68±2.51	1.55±0.07	19.02±2.43	0.88	0.15±0.002
T6	1.02±0.01	0.75±0.18	-8.60±6.37	1.89±0.09	31.09±3.24	0.72	2.31±0.03
T8	1.28±0.04	2.24±0.11	43.27±3.86	1.14±0.06	4.95±2.28	1.02	0.06±0.001
T9	2.28±0.68	1.86±0.24	29.75±8.30	1.20±0.16	6.87±5.54	1.96	0.31±0.005

3.3.3.5 Integrated comparison of chemical analysis, estrogenic activities, and morphology variation

To investigate the correlations of phenotypic parameters with chemicals and estrogenic effect in whole water, the phenotypic results were analyzed with OPLS-DA model combing with conventional bioassay (cell proliferation assay, E-screen assay, and ELISA assay) and non-target strategies (UPLC-qTOF-MS) (Figure 3.17). In results, Nuclei intensity, EEQ, and E2 concentration exhibited well clustered in score plot, which indicated that nuclei intensity posed relatively higher correlations to estrogenic effects. Moreover, the chemicals distributed near these regions were suggested with highly potential of estrogenic effects, which may provide a novel approach for chemical identification combing with bioassay and chemical analysis.

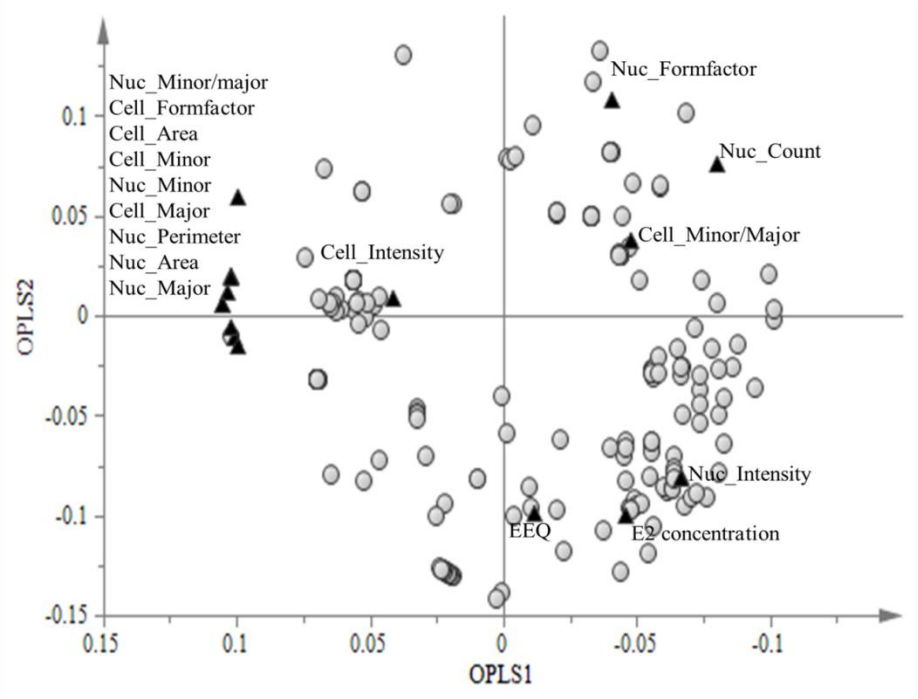


Figure 3.17 Orthogonal partial least-squares discrimination analysis (OPLS-DA) loading plot of chemicals to multiple phenotypic parameters. (●) X, chemicals; (▲) Y, phenotypic parameters

3.4 Conclusion

For the Multi-parameter phenotypic profiling in MCF-7 cells for integrated toxicity evaluation of whole water samples in chapter 3, the main results and conclusion are shown as following:

According to phenotypic profiling in MCF-7 cells of 26 xenoestrogens, we got main results:

- ✓ Chemicals with each toxicity pathways induced difference morphological variation of MCF-7 cells which can be classified with principal component analysis (PCA).
- ✓ Nuclei intensity exhibited high correlations with cell viability of MCF-7 cells demonstrating its high potential for estrogenic effect evaluation.
- ✓ Other morphological parameters and characteristics could address other toxicity-pathways such as cell apoptosis, cell division, M-phase, cell adhesion and transport, Membrane Transporter/Ion Channel.
- ✓ Digitoxin targeting on Na+/K+ ATPase induced echinoid spikes cells.

The results indicated that In vitro morphology-based analysis, providing information-rich and toxicity-pathway-related parameters, exhibited high-potential for integrated toxicity evaluation.

According to phenotypic profiling in MCF-7 cells of xenoestrogens mixtures, we got main results:

- ✓ Xenoestrogens mixture based on different toxicity-pathways exposure induced diverse effects of cell morphologies.
- ✓ A mixture with or without morphology-sensitive xenoestrogen (Digitoxin) induced echinoid spikes cells or normal cells.

The results indicated that the multi-parameter phenotypic approach is potential for toxicity identification using morphology-based analysis.

According to phenotypic profiling in MCF-7 cells of whole water samples comparing with conventional bioassay and non-target strategies, we got main results:

- ✓ Phenotypic variations showed a significant difference (e.g., cell body and nucleus

shrinkage, synapse cell, a rhomboid cell shape, and cell body and nucleus enlargement) exposed to whole water samples, and exhibited similarity with chemical profiles in PCA.

- ✓ These phenotypic variations could be induced by biological processes (e.g., cell death, cell communication) or toxicity related to chemicals in water samples.
- ✓ Compared with conventional cell viability analyses (MTT assay and direct cell counting), image-based chemical screens provide information-rich toxicity-based endpoints for whole water toxicity analysis.
- ✓ After a combination of E-screening, ELISA assay, we found that the nuclei intensity could be the parameter for estrogenic effect evaluation.
- ✓ The similarity of distribution in PCA analysis of morphological results and overall chemicals indicated the correlations between morphology variations and chemical structures in environmental water samples

In all, we conclude that in vitro morphology-based analysis showed correlation with toxicity-pathway and provided rich information for integrated toxicity evaluation and identification of xenoestrogens in environmental water samples. We also believe that this method is a promising approach for environmental toxicant screening as well as studying toxicity-phenotype-chemical interactions and constructing cell morphology database for environmental contaminants.

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Chapter 4
Genotypic profiling in zebrafish embryo for integrated toxicity evaluation of xenoestrogen and water samples

4.1 Introduction

Insecticides as common xenoestrogens contributed high risks to ecosystems and human health. Most insecticides were designed to act the neuro systems in insects functioning on various neurotransmitter pathways (e.g AchE, GABA, etc.)(Nakao et al. 2013; Pham et al. 2017), therefore, the neurotoxicity of insecticides in the environment was concerned. The conventional methods for neurotoxicity based on AchE activities or AchE gene expressions failed to cover more neurotoxicity pathways (Zhao et al. 2016), which induced insufficient for the understanding of neurotoxicity in environmental waters.

Zebrafish embryo was well applied as an integrative model in Tox 21 for toxicity evaluation (Sipes et al. 2011) due to various advantages such as transparent, easy for the handle, small size, and genetic similarity with humans(Braunbeck et al. 2005). A lot of research applied omics analysis for integrated toxicity evaluation of water samples with zebrafish embryo (Babic et al. 2017; Na et al. 2017). Multiple-endpoints gene alteration-based (MEGA) assay was developed using zebrafish embryo for integrated toxicity evaluation of wastewater(Fukushima et al. 2017). However, there was little research focusing on the neurotoxicity of environmental waters samples with zebrafish. Neurotransmitter took an important role for neurotoxicity and was detected as biomarkers for neurotoxicity evaluation(Kofke et al. 2000; Slikker et al. 2005). Neurotransmitter profiling in zebrafish focusing on various neuro systems (dopaminergic–adrenergic, glutaminergic–GABAnergic, serotoninergic, histaminergic, and cholinergic systems) were performed using LC/MS/MS for neurotoxicity evaluation of various pesticides(Tufi et al. 2016a). However,

In chapter 4, *in vivo* gene-based approach were developed using zebrafish embryo

for integrated evaluation of estrogenic effect and neurotoxicities of insecticides and environmental water samples. Multiple genetic parameters including *ache, gabra1, gad2, vtg1* were selected for integrated evolution of the estrogenic effect and neurotoxicity. Firstly, genetic effect of each pesticide was performed under observed effect concentration (NOEC) expressed a mechanism insight and the interactions of pesticides based on Mode of actions (MOAs). Then, the integrated toxicity of environmental water samples taken from Indonesia was evaluated based on multivariate data analysis approaches comparing with the previous mixture toxicity evaluation. We would like to develop a fast and cost-efficient bioassay method for integrated evaluation of neurotoxicity and estrogenic effect in environmental water samples.

4.2 Material and methods

4.2.1 Chemicals

Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and imidacloprid, acetamiprid, permethrin, carbaryl were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DMSO was used as the primary solvent, with solutions further diluted in cell culture medium prior to use. The final concentration of DMSO in the medium did not exceed 0.1% (v/v).

4.2.2 Zebrafish maintenance

Adult zebrafish, progeny from wild-type fish supplied from Department of Growth Regulation, Institute for Frontier Medical Sciences of Kyoto University in the flow-through system (IWAKI, Japan). The temperature was kept at 28℃ and the photoperiod was 14:10h light: darkness. Fish were fed daily with brines shrimp. Fish were placed in breeding tank two hours before darkness with the female/male ratio in 1:1 or 2:1. The fishes were allowed to spawn at the beginning of following morning. Eggs collected within one hour.

4.2.3 Acute embryo toxicity test of xenoestrogens and the mixture

The acute embryo toxicity test was performed based on FET-test (OECD, 2006).

The 60X stock medium zebrafish embryo medium (E3 medium) was prepared with 17.3 g/L NaCl, 0.8 g/L KCl, 2.4 g/L CaCl2 2H2O, and 4.89 g/L MgCl2 6H2O. Fresh medium was diluted and aerated overnight before exposure. The embryo with 2 hpf was selected and exposed in 96-well plates with 300 μL zebrafish embryo medium (one embryo per well). The zebrafish eggs were within 2h after collection exposed to a series of concentrations of the test substance dissolved in zebrafish medium. Fertilized eggs, which were at least in the four-cell stage, were selected and individually transferred to wells in 96-well plate (IWAKI, Japan) along with 300μL of the exposure medium. Two plates were used for each test, and the plates were left in an incubator with a temperature of 26°C. The embryo was observed and collected at 96h, for phenotypic and genotypic analysis.

4.2.4 Gene selection and gene expression analysis

Total RNA was extracted from 20 treated or control embryos after homogenization using Trizol reagent (Invitrogen, France). The concentration of total RNA was determined using Thermo Scientific™ NanoDrop™ spectrophotometer (Thermo Scientific, USA) and the sample purity was examined based on the OD_{260}/OD_{280}. The sample was dissolved in 100μL RNase-free water and stored at -80°C.

The cDNA was reverses transcribed with 1μg RNA using cDNA Synthesis Kit according to the manufacturer's introduction. Samples were placed in a Veriti Thermal Cycler (Applied Biosystem, USA) for cDNA generation. Real-time quantitative PCR (q-PCR) was carried out in an ABI Real-Time PCR system. SYBR Green PCR Master Mix was used for the real-time PCR analysis. The expression of *b-actin* as internal standard and fold change was calculated relative to the untreated control using the ΔΔt method. The target genes were selected as Table 4.1. The cycle conditions used were: an initial denaturation step of 95 °C for 10 min; 40 cycles of 95 °Cfor 30 s, 57 °C for the 40s, 72 °Cfor 30 s; and the last cycle of 95 °Cfor 15 s for 60 s to generate the dissociations curve. The efficiency of each gene were approximately 100% (*ache*: eff %=98.562, R^2=0.998; *gabra1*: eff %=99.523, R^2=0.999; *gad2*: eff%=98.782, R^2=0.998; *vtg1*: Eff%=80.442, R^2=0.964, *β-actin*: Eff%=97.676; R^2=0.998).

Table 4.1 Specific primer pairs matching with D. rerio genes used for qPCR.

Gene name	Gene symbol		Primer sequence (5'–3')	Ref.
ACHE receptor	*ache*	F	GCTAATGAGCAAAAGCATGTGGGCTTG	(Pereira et al. 2012)
		R	TATCTGTGATGTTAAGCAGACGAGGCAGG	
GABA receptor, α1	*gabra1*	F	GTCTGTTACG CCTTTGTCTTCT	(Raftery and Volz 2015)
		R	GCGGTGTAGGTGTTGTTCTT	
Glutamate decarboxylase 2	*gad2*	F	CAAGAAGCATGACGTCTGGA	(Wirbisky et al. 2014)
		R	CTGCATCAGTCCCTCCTCTC	
Vitellogenin 1	*vtg1*	F	CTGCGTGAAGTTGTCATGCT	(Chen and Chan 2016)
		R	GACCAGCATTGCCCATAACT	
ACTB actin beta	*β-actin*	F	CGAGCTGTCTTCCCATCCA	(Ling et al. 2017)
		R	TCACCAACCTAGCTGTCTTTCTG	

4.2.5 Sampling site, preparation, treatment, and exposure

The water samples (1000 mL) were collected at 10 river locations water samples on January 10th, 2017 in two villages in Yogyakarta, Indonesia. The 10 water samples included agricultural wastewater (S1, S2), river water in domestic region (S3) , river water in downstream (S4), and fish pond water (S5). The water samples were extracted by Sep-Pak PS2 (Waters, Japan) and eluted with methanol. The eluate was then evaporated to dryness with a nitrogen stream and the residue was dissolved with 1mL of DMSO. For bioassay, the concentrated samples were diluted with E3 zebrafish embryo medium for 1000 times and exposed to zebrafish in 96-well plate, the zebrafish embryo was taken after 96h exposure for gene expression analysis.

4.2.6 Statistical Analysis.

The data from genotypic analyses were used in a PCA to evaluate the differences in genotypic effects. The PCA was performed using SIMCA 13 software (Umetrics, Sweden). Quantitative data are expressed as the fold change vs. the control value ± standard deviation (SD). Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Dunnett's test for pairwise

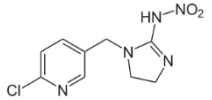
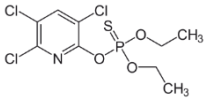
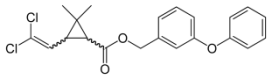
comparisons. Differences were considered statistically significant when $P < 0.05$.

4.3 Results and Discussions

4.3.1 Acute toxicity of single xenoestrogens to fish larvae

Various insecticides including neonicotinoid, organophosphate, carbamate, pyrethroids were selected based on their mode of actions. The acute toxicity, mode of action, physical properties were summarized as table 4.1. The consumption of neonicotinoid insecticides was rapidly growing in agriculture (Jeschke and Nauen 2008). Neonicotinoid act on nicotinic acetylcholine receptor (nAchE) of insect and exhibited low toxicity to ecosystems. However, the high polarity (e.g. Log Kow of Imidacloprid:-0.41) induced the high persistent in water and soil, which poses a high risk to ecosystems and human health (Haith 2010). Organophosphate and carbamate were the biggest class of pesticide exhibited inhibition of acetylcholinesterase (AchE). Therefore, AchE activities were well applied as a biomarker for pesticide identification in water samples. The LC50 of Chlorpyrifos and Carbaryl to zebrafish embryo was 13.03 and 14.46 mg/L, respectively. Permethrin posed the highest toxicity to zebrafish (LC50: 0.3 mg/L). In this study, no observed effect concentrations (NOEC) of phenotype were performed to evaluate the genotypic effects of insecticide on zebrafish embryo.

Table 4.2 Literature review of physical properties, mode of action, and acute toxicity of selected insecticides

Compounds	Category	molecular formula	Log Kow	Chemical Structure	Mode of action	LC50 (mg/L)	NOEC on phenotype (mg/L)
Imidacloprid	neonicotinoid	C9H10ClN5O2	-0.41		nAchR modulator	241a	25.5e
Chlorpyrifos	organophosphate	C9H11Cl3NO3PS	4.66		AchE inhibitor	13.03b	2.0e
Carbaryl	carbamate	C12H11NO2	2.35		AchE inhibitor	14.46c	2.0e
Permethrin	pyrethroids	C21H20Cl2O3	7.43		Sodium channel modulator	0.3d	0.8e

nAchR: Nicotinic acetylcholine receptor

AchE: Acetylcholinesterase

NOEC : no observed effect concentrations

a (Tisler et al. 2009) b(Wang et al. 2017)c(Pandey and Guo 2015)d(Yang et al. 2014)e(Tufi et al. 2016b)

4.3.2 Expression of target genes exposed to xenoestrogens on NOEC level

Neuronal activity is related to the excitatory or inhibitory of various neurotransmitters. Evidence indicated that insecticide posed effect on the behavior of organism via neurotransmitters (Grue et al. 1997). Therefore, AchE activities were well applied as an important biomarker for evaluation of development neurotoxicity (Abreu-Villaca and Levin 2017). However, various neurotoxicity pathways also act on the neurotransmitters expression such as gamma-aminobutyric acid (GABA) pathway pathways. Therefore, gabra1 and gad2 were selected for integrated neurotoxicity evaluation. Moreover, vitellogenin (Vtg) was applied as a biomarker for estrogenic effect evaluation of environmental samples (Ihara et al. 2015). We selected four target genes as biomarkers for integrated evaluation of neurotoxicity and estrogenic effect.

In results, all the insecticides induced inhibition of *ache*. Permethrin dramatically increased *gabra1* expression (3.21 n-fold change), but the imidacloprid, chlorpyrifos, and carbaryl posed the adverse effect of *gabra1*. Sara Tuf et al. also indicated that permethrin made a positive effect on GABAnergitic proteins expression, imidacloprid, chlorpyrifos, and carbaryl exhibited negative effects. For estrogenic activities, imidacloprid and chlorpyrifos significantly increased the vtg1 expression, indicating the high potential of estrogenic effects.

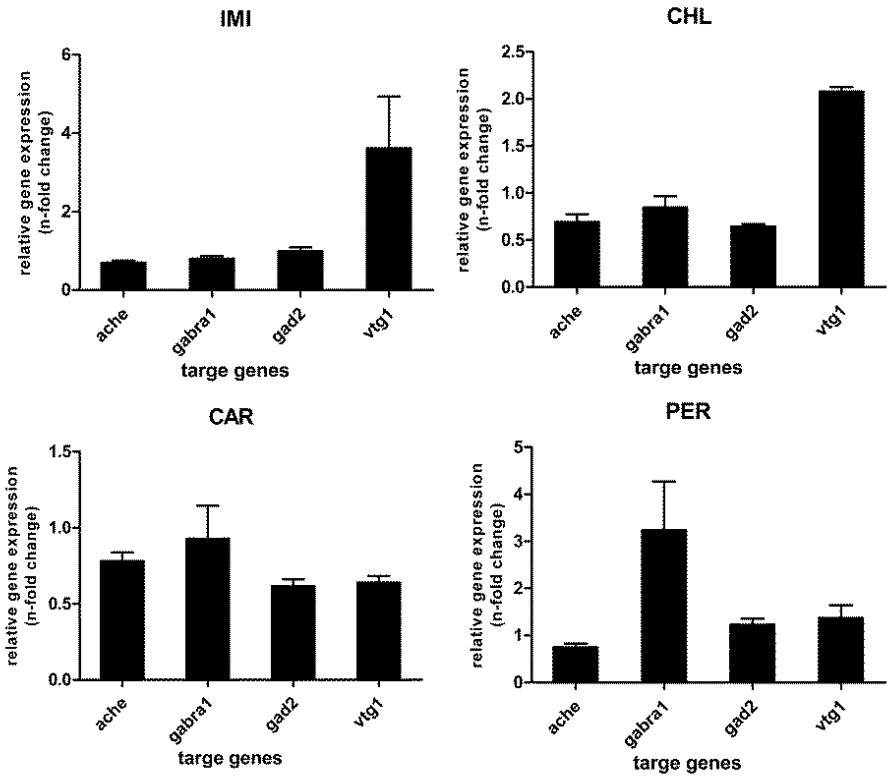


Figure 4.1 Genotypic effects of insecticide on zebrafish embryo.

4.3.3 Expression of target genes exposed to environmental water samples

6 water samples were selected including agriculture wastewater (S1, S2), river water (S3, S4), water in a fish pond (S5). The multiple genotypic profiling was performed to investigate the neurotoxicity and estrogenic effect of water samples.

In results, genotypic exhibited divers after water sample exposure. Agricultural wastewater S1 and river water S3 decreased ache, *gabra1*, *gad2* indicating the potential neurotoxicity. Vtg1 expression significantly increased after S3 exposure indicated its estrogenic effects. The water in fish pond induced significantly increased expression of *gabra1*(Figure 4.2).

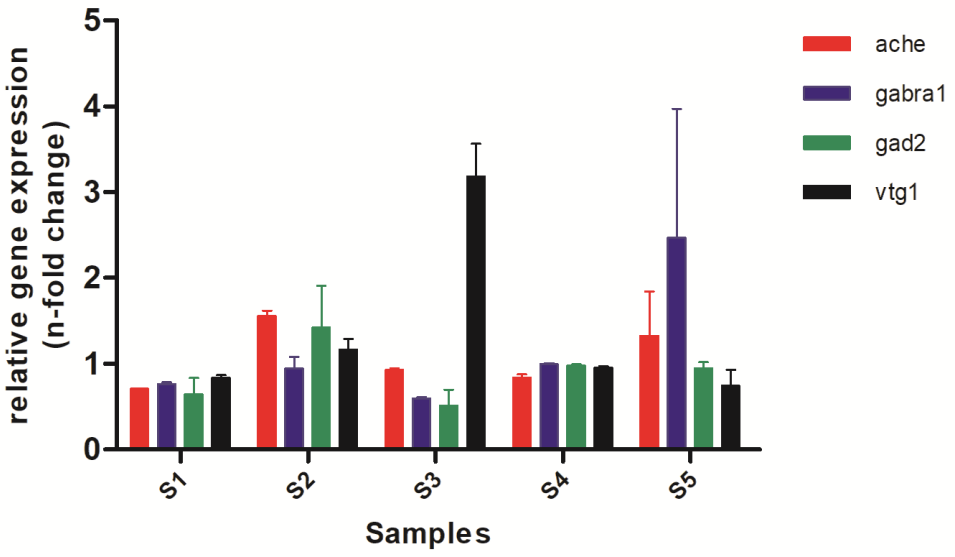


Figure 4.2 Genotypic effects of water samples on zebrafish embryo.

4.4 Conclusion

In vivo gene-based approach were developed using zebrafish embryo for integrated evaluation of estrogenic effect and neurotoxicities of insecticides and environmental water samples. Multiple genetic parameters including ache, gabra1, gad2, vtg1 were selected to provide an integrated evolution of the estrogenic effect and neurotoxicity. We found that zebrafish acute toxicity of insecticide is permethrin (PER) > carbaryl (CAR) > chlorpyrifos (CHL) > imidacloprid (IMI), and all the insecticide decreased expression of ache, and CHL induced highest impact on AchE due to the ache inhibition mechanism. *gabra1*and *gad2* expression decreased exposed to IMI, CHL, CAR, however, significantly increased exposure to PER. IMI and CHL exhibited high estrogenic effects according to *vtg1* expression. Moreover, phenotypic effect exhibited diverse after exposed to water samples, agricultural wastewater S1 induced neurotoxicity, and river water S7 exhibited the significantly estrogenic effect. In conclusion, the multiple genetic parameters covered various toxicity pathways and provided an information-rich approach for integrated evaluation of neurotoxicity and estrogenic effects.

4.5 Reference

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Chapter 5

Phenotypic profiling in Fluorescent Methyl CpG-binding Mouse Embryonic Stem Cells for evaluation of DNA Methylation effect of xenoestrogens

5.1 Introduction

Detecting the epigenetic effect of environmental chemicals is important for understanding the risks and in preventing cancer and other related diseases. Epigenetic influences have been observed with environmental contaminants such as nickel (Costa et al. 2005b), cadmium (Takiguchi et al. 2003), arsenic (Zhao et al. 1997), benzene (Bollati et al. 2007), and bisphenol A (Ho et al. 2006; Yaoi et al. 2008). These chemicals may lead to adverse health outcomes such as cancer or neurodegenerative disorders (Esteller 2008; Jones and Baylin 2007; Kaminsky et al. 2006; Obeid et al. 2009). Furthermore, major environmental factors influence the epigenome through phenotypic variation but do not modify DNA sequences. Therefore, detecting phenotypic variations arising from changes in the epigenome may be a useful way to monitor epigenetic effects induced by environmental chemicals.

DNA methylation is a common epigenetic modification. Several approaches detect methylation using enzyme digestion, such as methylation-sensitive arbitrarily primed polymerase chain reaction (MS–AP–PCR) (Liang et al. 2002), affinity enrichment approaches such as methylated DNA immunoprecipitation (MeDIP) (Costa et al. 2005a; Down et al. 2008; Pelizzola et al. 2008), and sodium bisulphite approaches such as combined bisulphite restriction analysis (COBRA) (Xiong and Laird 1997). However, these approaches do not visualize dynamics changes in epigenetic states, which is a

drawback in understanding the hazard or risk of environmental chemicals for human health. Moreover, the labor and expense involved in methods using polymerase chain reaction (PCR) or gene microarray assays inhibit processing of large numbers of samples. Therefore, high-throughput and cost-effective technology for detecting DNA methylation are needed for large-scale evaluation of epigenetic changes induced by environmental chemicals.

Methylated DNA binding domain (MBD) proteins bind specifically to symmetrically methylated CpG motifs (Hendrich and Bird 1998), and have been applied as biomarkers in high-throughput methods for assessing DNA methylation (Badran et al. 2011; Hiraoka et al. 2012; Rauch et al. 2006; Yu et al. 2010). Most approaches using MBD sensors harbor gene sequences from signaling proteins such as green fluorescent protein (GFP), β-lactamase (Porter et al. 2007), and luciferase-fused zinc finger protein (Hiraoka et al. 2012). Such sensors have been validated for the direct determination of genomic DNA methylation levels in vitro. However, genomic DNA extraction and PCR process are labor- and time-consuming (Corbisier et al. 2007).

In our system, the MBD coding sequence confers methylated DNA specific binding. Enhanced green fluorescent protein (EGFP) is fused to the MBD1 binding domain linked to the MBD-nls to yield EGFP-MBD-nls. The EGFP–MBD–nls protein can then be used to track DNA methylation and nuclear visualization is assessed by green fluorescence, such that analysis can be performed by fluorescence- or image-based analyses (Carpenter et al. 2006; Pinkel et al. 1986). Mouse embryonic stem cells (ESCs) have a unique nuclear organization in that methylated centromeric heterochromatin coalesces to form large clusters around the nucleoli. Therefore, we used heterochromatin granule staining intensity, area, and number for high-throughput

quantification of genome-wide DNA methylation. High-content screening (HCS) combines the efficiency of high-throughput analysis with an evaluation of cellular phenotype (Zanella et al. 2010). Several successful genome-scale HCS approaches have been reported (Brass et al. 2008; Moffat et al. 2006). Therefore, HCS of mouse ESCs harboring EGFP–MBD–nls should provide a rapid approach for evaluating DNA methylation status.

In this study, we developed an HCS approach that allows one to target proteins to heterochromatin regions, assess DNA methylation status, and follow dynamic heterochromatin interactions in mouse ESCs. The DNA demethylating agent 5-aza-2′-deoxycytidine (5-aza-dc) is a common anticancer drug that induces DNA demethylation and subsequent activation of tumor-suppressor gene expression. We first assessed our method by investigating the effects of 5-aza-dc on mouse ESCs expressing EGFP–MBD–nls to demonstrate that the approach detects well-known epigenetic effects. The cells were exposed to chemicals with or without leukemia inhibitory factor (LIF) to investigate the effect of cell differentiation on DNA methylation status. We then sought to evaluate its application for detecting the presence of xenoestrogens. We investigated the epigenetic effects of 7 environmental chemicals including estrogen and pesticides such as 17β-estradiol (E2), bisphenol A (BPA), and pesticides such as imidacloprid (IMI), acetamiprid (ACE), permethrin (PER), carbaryl (CAR), p.p'-DDT , and o.p'-DDT.

5.2 Material and methods

5.2.1 Chemicals

Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and 5-aza-dc, TSA, E2, BPA, permethrin, carbaryl, p.p'-DDT , and o.p'-DDT

were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DMSO was used as the primary solvent, with solutions further diluted in cell culture medium prior to use. The final concentration of DMSO in the medium did not exceed 0.1% (v/v).

5.2.2 Cell culture and exposure

EGFP–MBD–nls cells (mouse embryonic stem cells) were provided by the RIKEN cell bank (Tsukuba, Japan). Cells were cultured on 0.1% gelatin-coated 60-mm dishes or 48-well plates (1×10^4 cells/well). ESCs were cultured with or without leukemia inhibitory factor (LIF)-supplemented Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37 ℃ under 5% CO_2 in the humidified air. Cells were induced to differentiate by replacing of LIF supplemented DMEM containing 5-aza-dc, TSA, BPA, imidacloprid, acetamiprid permethrin, carbaryl, p.p'-DDT , and o.p'-DDT 10^{-8} to 10^{-6}M) or E2 (1×10^{-13} to 1×10^{-9} M) in DMSO for 48 h (Figure 5.1A).

5.2.3 Immunofluorescence.

Immunostaining with 4,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA, USA) was performed after 48 h exposure to reagents (Figure 5.1A). The differentiated cells were fixed with 4% paraformaldehyde for 15 min and then treated with 0.1% Triton X-100 (Sigma-Aldrich) for 30 min. After incubation with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature, cells were incubated with DAPI for 15 min at room temperature.

5.2.4 Image Acquisition and Analysis.

Microphotographs were obtained using an Olympus LV1200 High-Performance Laser Scanning Microscope (Olympus Optical, Tokyo, Japan). To quantify variations in cellular and subcellular components, immunofluorescent images (4 fields per well of a

48 well plate) were obtained by an automated IN Cell Analyzer 1000 (GE Healthcare UK Ltd., Buckinghamshire, UK) using a 10 × objective. Fluorescence emissions were recorded separately for green (475-535 nm) channels. In addition, nuclei (n = 100) were counted in each well for confocal image analysis.

The localization of hypermethylated centromeric heterochromatin reflects the nuclear organization and global patterns of DNA methylation (Kobayakawa et al. 2007). To localize centromeric heterochromatin and heterochromatin granule numbers, granule intensity and area were quantified by the high-throughput assay. Mouse ESCs were cultured in glass-bottomed dishes in parallel to observe heterochromatin variations directly (Figure 5.1B).

Cells were analyzed using image analysis algorithms generated using the IN Cell Developer Tool Box software (v1.7) in four steps: 'nuclear segmentation'; 'cell segmentation', 'granular segmentation', and 'measure nodes'. Because a vast array of parameters can be calculated, we selected the following parameters to characterize DNA methylation status: intensity, area, and numbers of nuclear heterochromatin granules. Data are expressed as the mean per cell divided by the value of the control. The control values were set at 100%.

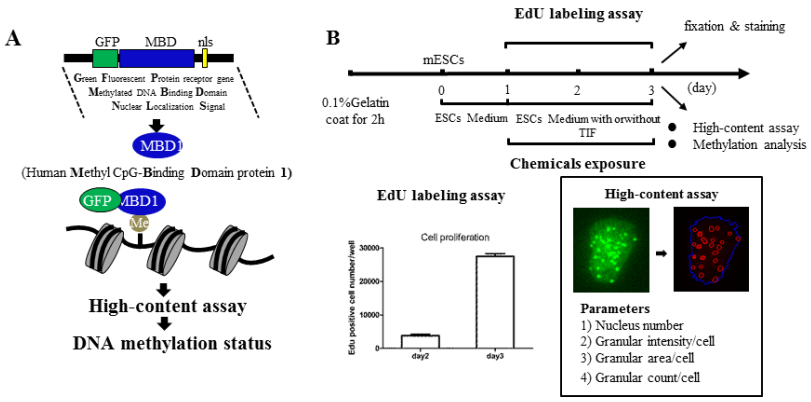

Figure 5.1 Experiment set-up

5.2.5 Statistical Analysis.

Quantitative data are expressed as the mean percentage of the control value ± standard deviation (SD) of at least three independent experiments. Analyses were performed using IBM SPSS statistics (IBM Corp., Armonk, NY, USA). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's test for pairwise comparisons. Differences were considered statistically significant when $P < 0.01$.

5.3 Results and discussions

5.3.1 Effect of 5-aza-dc on the phenotype of EGFP–MBD–nls cells.

Cells were treated with varying doses of 5-aza-dc and analyzed for cytotoxicity and phenotype variation. DAPI-stained nuclei images were acquired by fluorescence microscopy. Images showed that the EGFP–MBD–nls product localized specifically to nuclei and hypermethylated centromeric heterochromatin, which exhibited green fluorescence as granules (Figures 5.2A). Evidence indicated that LIF played a positive role during the differentiation of ES cells into neuronal cells(He et al. 2006). Then phenotypic effects of the chemical were an evaluation with or without LIF.

In results, the cell proliferation decreased in ESCs treated with 5-aza-dc, with cytotoxic effects more noticeable after cell differentiation. Control ESCs and those exposed to low 5-aza-dc concentration (1×10^{-8}) exhibited tight heterochromatin clusters. However, open heterochromatin clusters were seen after exposure to higher concentrations of 5-aza-dc. Notably, nuclear granule staining intensity decreased, suggesting fewer hypermethylated regions due to 5-aza-dc (Figure 5.2C). To investigate 5-aza-dc cytotoxicity, ESC viability was evaluated by direct cell counting following DAPI staining. Cell viability was reduced to 77% ($P < 0.05$) and 49% ($P < 0.001$) of

controls when treated with 10^{-7} M or 1×10^{-6}M, respectively (Figure 5.2B).

Nuclear organization changes are associated with cell differentiation such that the changes in heterochromatic granule count can reflect differentiation status (Kobayakawa et al. 2007). To evaluate the effect of each chemical on the epigenetic status and cellular differentiation, we analyzed granule numbers and granule count. After 48 h of treatment, 5-aza-dc (10^{-7}) substantially decreased granular area and count ($P < 0.05$) (Figure 5.2D, E). This confirmed that 5-aza-dc simultaneously affected ESC differentiation and DNA methylation.

We further analyzed granular staining intensity to investigate the effect of 5-aza-dc on methylation, as there is a strong association between EGFP signal intensity and methylation status in this model system (Kobayakawa et al. 2007). Granular intensity decreased after exposure to higher concentrations of 5-aza-dc (10^{-6} M) ($P < 0.05$) consistent with the demethylation activity (Figure 5.2C).

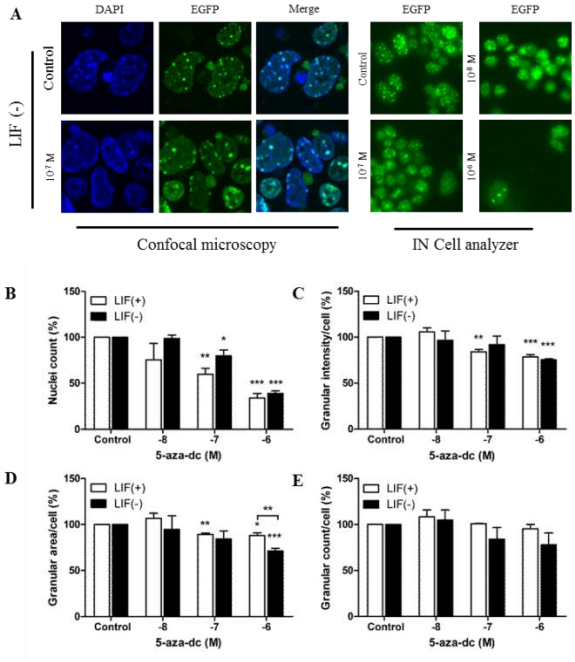

Figure 5.2 Phenotypic effect of 5-aza-dc on mouse ESCs

To the best of our knowledge, this study is the first to construct a high-throughput method to evaluate the epigenetic effects of environmental chemicals using EGFP–MBD–nls cells. This transgenic cell line was established to enable simultaneous visualization of epigenetic dynamic changes and nuclear structures (Kobayakawa et al. 2007; Yamazaki et al. 2007), which provides a basis for epigenetic and phenotypic profiling. Here, we constructed a multiple phenotype profiling model using mouse ESCs harboring EGFP–MBD–nls to not only observe dynamic DNA methylation but also improve its sensitive and throughput capability for detection of DNA methylation status.

In EGFP-MBD-nls cells, nuclear granules exhibiting MBD1 signals represent an aggregation of centromeric or pericentric heterochromatin, which reflects epigenetic status (Kobayakawa et al. 2007). Therefore, granular intensity, count, and area were assayed and served as indicators. ESCs exposed to 5-aza-dc exhibited shrinking or disappearance of these granules, indicating that granule variation was readily characterized by phenotypic profiling.

5-aza-dc prevents DNA methyltransferase 1 (DNMT1) from replicating DNA during cell division, which decreases DNA methylation (El Kharroubi et al. 2001). It thus has a dramatic effect on chromosomes, leading to an open chromatin structure (Haaf et al. 2003). EGFP–MBD–nls binds to methylated DNA located in DAPI-stained heterochromatin, which then emits green fluorescence. After exposure to 5-aza-dc, heterochromatin decrease in both count and intensity, resulting from the 5-aza-dc demethylation (Figure 5.2A). Multiple HCS parameters correspondingly exhibited decreases in granular intensity, area, and count (Figure 5.2C, E). These results are consistent with other approaches using genome-wide analyses of DNA methylation (Takeshima et al. 2015; Wang et al. 2009; Zhang et al. 2004).

In general, methods for analyzing DNA methylation are classified into three techniques: bisulfite conversion; digestion with methylation-sensitive restriction enzymes; and affinity purification of methylated DNA (Zilberman and Henikoff 2007). Each approach uses different parameters to characterize DNA methylation with different sensitivity levels (Table 5.1). Bisulfite conversion approaches apply sodium bisulfite to convert unmethylated cytosine to uracil, whereas methylated cytosines are protected. DNA methylation levels are then measured using PCR-amplified bisulfite-treated DNA (Brena et al. 2006; Ehrich et al. 2005). This approach may produce different results for DNA methylation levels with different cell lines and exposure times (table 1). For instance, DNA methylation in the A549 cell line decreased to 35% of controls following 0.1 μM 5-aza-dc treatment, whereas levels increased after exposure to 0.5 μM 5-aza-dc (Brena et al. 2006). DNA methylation levels in MCF-7 and MDA-MB-435 cell lines decreased to 86% and 83% of controls with 0.5 μM 5-aza-dc treatment (Jiao et al. 2014). Use of DNMT1 to characterize DNA methylation levels showed 96% and 72% methylation relative to controls in murine embryonic fibroblasts treated with 0.1 μM and 1 μM 5-aza-dc, respectively (Maslov et al. 2012). Another global methylation high-throughput quantification method visualizes DNA methylation by fluorescence polarization (FP). In this approach, DNA cleavage is achieved using methyl-sensitive HaII and methyl-insensitive MspI restriction enzymes. Cleaved DNA is treated with fluorescent deoxycytidine monophosphate to bind the non-methylated DNA segments. DNA methylation is then analyzed using FP to determine the difference between HaII- and MspI-treated DNA. Using this method, 5 μM 5-aza-dc treatment inhibited DNA methylation up to 67% of controls (Zhao and Xue 2012). Another study using MBD1 as a readout signal is similar to our method, except that it joins MBD1 with either the

N-terminal or C-terminal fragment of firefly luciferase (Badran et al. 2011). This approach is slightly more sensitive than our method, for low concentration exposures (e.g., 0.1 and 0.25 μM 5-aza-dc), although our method was more sensitive in detecting DNA methylation with 1 μM 5-aza-dc treatment (Table 5.1). This discrepancy may result from cytotoxicity following exposure to higher 5-aza-dc concentrations.

Table 5.1 Summary of methods available for the large-scale analysis of DNA methylation

Parameter	General basis	Cell	5-aza-dc (uM)	DNA methylation statues (%)	Refs
Bisulfite-treated DNAs	Sodium bisulfite converts unmethylated cytosine to uracil, whereas methylated cytosines are protected	A549	0.05	36	Brena, 2006
			0.1	35	
			0.5	46	
			0.75	45	
			1	57	
			2	80	
		MCF-7	-	74.6	Jiao, 2014
			0.5	64.9	
			5	52.5	
			10	19.0	
		MDA-MB-435	-	27.6	
			0.5	23.1	
			5	15.8	
			10	10.0	
Fluorescent dCMP treated DNAs	Fluorescent dCMP to cooperate with the non-methylated DNA segments.	HEK293T cells	-	100	Zhao, 2012
			5	67.7	
			10	45.1	
Variation of DNMT1	Methylation and Demethylation are accompanied by production and degradation of DNA methyltransferase (DNMT1)	MEFs	-	85	Maslov, 2012
			0.1	82	
			1	62	
			10	79	
MBD1	Luciferase analysis to combination of MBD1-NFluc and CFluc-MBD1	Hela	-	100	Badran, 2011
			0.1	80	
			0.25	73	
			1	62	
MBD1	High throughput screening of structure of GFP-MBD1-nls proteins in heterochromatin	mESCs	-	100	This study
			0.01	94.8	
			0.025	97.8	
			0.1	91.4	
			0.25	89.1	
			1	56.8	

5.3.2 Multiple phenotypic profiling for DNA Methylation of xenoestrogens

5.3.2.1 Effect of 17ß-estradiol (E2) on the phenotype of EGFP–MBD–nls cells

Endocrine disrupting chemicals (EDCs) exhibit intergenerational effects by altering epigenetic regulation (Xin et al. 2015). Notably, negative effects on DNA methylation have been observed with exposure to EDCs such as bisphenol A (BPA), and 2,3,7,8-tetrachlorodibenzo-[p]-dioxin (TCDD) (Sales et al. 2013). In addition, E2, as both a synthetic and a natural EDC, is present in a wide range of environments. To investigate the viability of the high-throughput approach for EDC evaluation, we exposed ESCs to E2 as described above. In result, E2 slightly improved life proliferation ESCs, conversely, granule count and granule intensity showed slightly decreases (Figure 5.3 A). E2 (1×10^{-9} M) stimulated cell viability up by 28% compared with controls ($P < 0.01$). The granular intensity, area, and count were inhibited to 81%–90%, 73%–84%, 67%–80% of controls, respectively (at 1×10^{-13} M to 1×10^{-9} M E2).

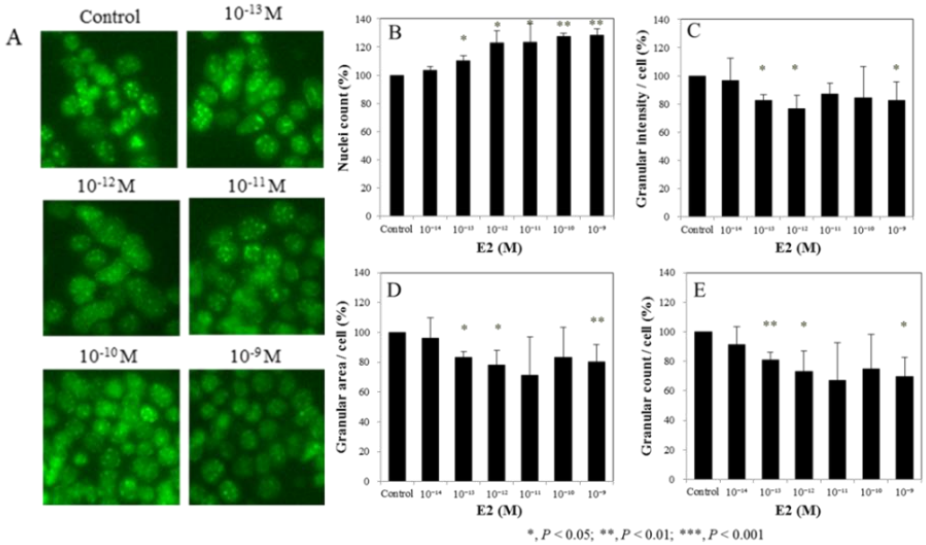

Figure 5.3 Phenotypic effect of E2 on mouse ESCs

How E2 induces epigenetic changes is not well understood. Some studies identified a relationship between estrogen receptor alpha (ERα) activity and DNA methylation in breast cancer cells, indicating downregulation of ERα gene expression, as observed in various tissues (Issa et al. 1994; Sasaki et al. 2002). There is evidence that E2 treatment increases ERα gene expression in cancer cell lines such as MCF-7 (Lonard et al. 2000). In endothelium, E2 may decrease DNA methylation levels by upregulating ERα gene expression (Ihionkhan et al. 2002), which was reflected in decreasing phenotypic parameters in our study. A potential role for E2 inhibiting DNA methylation was also observed through direct sequencing of bisulfite-treated DNA using PCR approach and mouse ESCs (Jung et al. 2010). Moreover, decreased expression levels of DNMT1, DNMT3a, MBD1, and MBD4 in cells was observed after E2 treatment (Treas et al. 2012). This evidence is consistent with the decreasing nuclear intensity resulting from the binding of EGFP to MBD1 protein in our present study (Figure 5.3 C-E). We also investigated BPA to verify its ability to detect E2-induced DNA methylation. The results indicated that BPA also decreased granular intensity, area, and count, but exhibited relatively fewer effects than E2 (Figure 5.4). Additional evidence indicates that BPA moderately decreases global DNA methylation in placenta, kidney, and liver in the human fetus (Nahar et al. 2015). The BPA-induced hypomethylation may result from ER or other signaling pathways (Kundakovic and Champagne 2011).

5.3.2.2 Effect of neonicotinoids on the phenotype of EGFP–MBD–nls cells

Neonicotinoids as novel insecticide posed the publish concern due to the reported risks to various species such as honey bee(Baines et al. 2017), earthworm(Chevillot et al.

2017), mice(Tomizawa 2004), etc. Evidence indicated that neonicotinoids posed reproductive toxicity, neurotoxicity, and genotoxicity resulting from various mechanisms such as ER agonist, nicotinic acetylcholine receptors (nAChRs) inhibitor, and epigenetic effects(Han et al. 2018). The result previous also indicated that neonicotinoids exhibited estrogenic effect and neurotoxicity based on both *in vitro* and *in vivo* assays. Therefore, we highlighted the DNA methylation effect of neonicotinoids using the phenotypic analysis.

In results, imidacloprid (-8M and -7M) slightly increased cell viability of mouse ESC cells, but it induced slightly cytotoxicity in -6M. The granular intensity significantly increased after the exposure, indicated that possibility of DNA methylation.

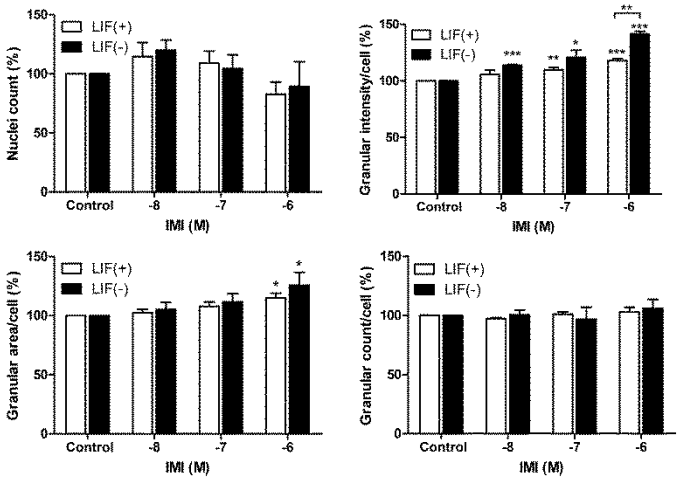

Figure 5.4 Phenotypic effect of imidacloprid (IMI) on mouse ESCs

Compared with imidacloprid, acetamiprid induced cytotoxicity after exposure. The granular intensity and granular area slightly increased after exposure with -7M and -6M. Acetamiprid induced slightly higher positive effect without LIF than with LIF, which indicated that DNA demethylation process may exist during cell differentiation.

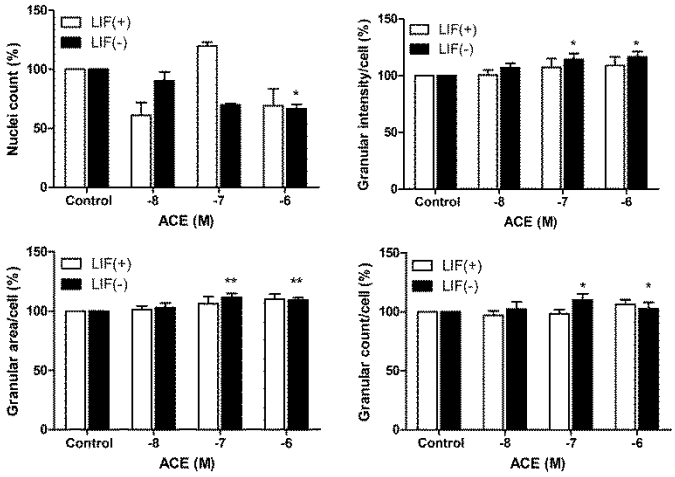

Figure 5.5 Phenotypic effect of acetamiprid (ACE) on mouse ESCs

5.3.2.3 Effect of other xenoestrogens on the phenotype of EGFP–MBD–nls cells.

To investigate the effect of other environmental chemicals on DNA methylation, we tested for DNA methylation following treatment with BPA and 5 pesticides: imidacloprid, permethrin, carbaryl, p.p'-DDT, and o.p'-DDT, using the same experimental procedure. Granular area and count decreased slightly after BPA and permethrin exposure but exhibited substantial effects at high concentration (10^{-6} M, $P<0.05$). In contrast, the parameters increased after treating with imidacloprid and carbaryl ($P<0.01$). o'.p-DDT also slightly increased granular area at high concentration ($P<0.05$). However, p'.p-DDT did not exhibit any noticeable effects (Figure 5.6).

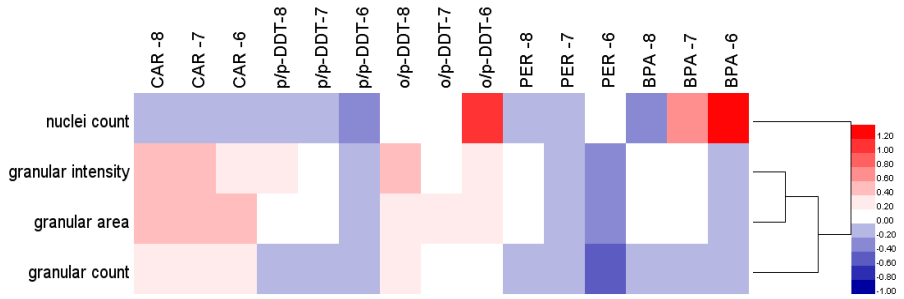

Figure 5.6 Multiple phenotypic profiling for DNA Methylation of xenoestrogen

Pesticides are widely used and readily detected in the environment, which increases the risk to human health (Weichenthal et al. 2012). Increasing evidence suggests that pesticides induce cancer and neurodegenerative disorders through epigenetic effects. Therefore, there is concern that DNA methylation may provide a new mechanism for disease progression. DDT, an organochlorine insecticide, has been detected in soil throughout the world (Daly et al. 2007). New research demonstrates that DDT may function on CAR (constitutive active/androstane receptor) and ERα, simulating cell cycle (Kazantseva et al. 2013). In our study, both o,p'-DDT and p.p'-DDT increased cell proliferation of GFP-MBD-nls cells, presumably resulted from ERα activation. Evidence indicates that low-dose DDT induces DNA hypomethylation in rat liver even though the mechanism occurs unclear (Shutoh et al. 2009). However, recent research suggests that DDT induces DNA methylation through down-regulation of P53 and P16 genes in rat liver (Kostka et al. 2014). Our results indicate that DNA methylation status increases slightly after o.p'-DDT, but remains unchanged after p,p'-DDT treatment.

Imidacloprid and carbaryl induce neurotoxicity by inhibiting the enzyme acetylcholine esterase (Lee et al. 2015; Millar and Denholm 2007). However, little is known about the effect on DNA methylation, although our results indicate that both imidacloprid and carbaryl induce DNA methylation with relatively high efficiency. Few studies have examined pesticide effect on DNA methylation. Our HCS method may help to close the gap and allow rapid detection DNA methylation of pesticide-induced DNA methylation.

5.4 Conclusion

Our present study is the first to evaluate DNA methylation of environmental chemicals by detecting MBD protein using its fluorescence signature. The results indicated that fluorescent methyl CpG-binding proteins in Mouse Embryonic Stem Cells were well characterized with multiple phenotypic parameters, and were a sensitive endpoint for detecting DNA methylation. Through high-throughput screening of the parameters, we found that pesticides imidacloprid, acetamiprid, carbaryl, and o.p-DDT may induce DNA methylation, in contrast, permethrin induced slightly hypomethylation may result from a different mechanism which should be deeply researched. Our present study provided a rapid way for toxicity evaluation and provided data basis for further mechanism research of pesticides in DNA methylation. E2 and BPA also induced slightly hypomethylation which may result from the action of ER. We believe the in silico approach will facilitate to cover knowledge gaps on the epigenetic effects of much more environmental chemicals.

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Chapter 6.

Conclusions and Recommendations

6.1. Main results and conclusions

In this dissertation, we focused on estrogenic effects, neurotoxicity and DNA methylation of xenoestrogens and developed three bioassay methods for integrated toxicity evaluation through multiple phenotypic and genotypic parameters. Our results indicated that the phenotypic and genotypic parameters in each bioassay provided rich information related to abundant toxicity pathways including estrogenic effects, neurotoxicity and DNA methylation. Moreover, the phenotypic and genotypic approaches exhibited as promising tools for toxicity evaluation and identification in complex environmental water.

In chapter 3, In vitro phenotype-based analysis using MCF-7 was develop for integrated evaluation of estrogenic activities and other possible toxicity of whole water sample. Morphological parameters of nuclei and cells (intensity, perimeters, major, minor, minor/major, area, form factors) were applied and analyzed by PCA analysis, phenotypic dissimilarity was first characterized with “distance to control” in PCA score plot for toxicity evaluation.

The phenotype-based analysis was performed to evaluate the phenotypic effects of 26 chemicals with the various mode of mechanisms firstly. It was found that the phenotypic parameters exhibited diverse variations by a different mode of mechanism, especially in high-doses range. For instance, phenotypic parameters exhibited dose-independent response (decreased cell area and increased nuclei intensity) exposed to 17ß-estradiol (E2), and the EC50 of phenotypic variation (EC50=12.1 LogM) exhibited similar EC50

with cell proliferation (EC_{50}= 12.3 LogM). Moreover, Nuclei intensity and Nuclei area exhibited high correlations (R^2=0.90 and R^2=0.89) with cell proliferation indicated the possibility of phenotypic analysis for estrogenic effect evaluation. For other chemicals, cell exhibited abnormal (echinoid spikes) exposed to Digitoxin (DGT) due to its inhibition of cell migration, and the phenotypic dissimilarity was 14.8 in PCA score plot indicating the remarkable phenotypic effect. Moreover, compared with cell proliferation assay, busulfan, DEHP, DDT, TCDD induced higher phenotypic dissimilarity scores (3.48, 5.95, 4.74, 3.96, respectively) but low cell proliferation (1.21, 1.57, 1.01, 1.05, respectively) in high-doses (-6M) which may result from actions of the toxicity pathways other than estrogenic effect. In conclusion, phenotypic parameters exhibited correlations with various biological processes (cell proliferation, cell communication, cell death) and physical factor (osmotic pressure), and provided an information-rich approach for integrated toxicity evaluation.

The phenotypic effects of chemical mixtures were studied to investigate the feasibility of phenotypic analysis for toxicity identification based on the various mode of the mechanism (GPCR, membrane transporter, estrogenic effect, neurotoxicity, etc.). In result, the phenotype exhibited diverse, mixture with estrogenic active chemicals induced similar phenotypic effect with estrogens such as increased nuclei intensity and decreased cell area. Moreover, cells exhibited echinoid spikes exposed to DGT. The mixtures with DGT (-6M and -7M) induced the similar phenotypic effects and distributed near with DGT (-7M) in PCA score plot (phenotypic dissimilarity=0.12 and 0.35, respectively). It indicated that toxicities or chemicals can be identified with the dissimilarity of cell phenotype. In conclusion, the phenotypic analysis is possible for toxicity characterization based on specific phenotypic effects of different mode of

actions.

Finally, we applied the morphology-based analysis for integrated evaluation and identification of whole environmental samples in combination with conventional bioassays (cell viability, E-screen, ELISA) and non-target chemical strategies (UPLC-qTOFMS). In results, cell proliferation exposed to wastewater influent exhibited lower than effluent (e.g. W2-Inf: n=1.01 fold change, W2-Eff: n=1.59 fold change). The single parameter was failed to evaluate whole water toxicity resulting from the coexist of antagonist and agonist for cell proliferation in wastewater. However, phenotypic analysis covered more biological pathways (cell proliferation, cell death, cell communication). The resulted indicated that phenotypic variation of wastewater influent was higher than effluent(e.g. W2-Inf: phenotypic dissimilarity=5.25, W2-Eff: phenotypic dissimilarity =2.78), which indicated that phenotypic analysis was sensitive and provided a novel insight for toxicity reduction evaluation (TRE). Moreover, compared to conventional bioassays for estrogenic activity evaluation with statistical analysis (OPLS-DA), it was found that nuclei intensity exhibited high correlations with the estrogenic activity of whole water samples. In conclusion, the phenotypic analysis provided an information-rich platform for whole water toxicity evaluation compared with the conventional bioassay.

In chapter 4, in vivo gene-based approach were developed using zebrafish embryo for integrated evaluation of estrogenic effect and neurotoxicity of insecticides and environmental water samples. Multiple genetic parameters including ache, gabra1, gad2, vtg1 were selected to provide an integrated evolution of the estrogenic effect and neurotoxicity. We found that zebrafish acute toxicity of insecticide is Permethrin (PER) > Carbaryl (CAR) > Chlorpyrifos (CHL) > imidacloprid (IMI), and all the insecticide

decreased expression of ache, and CHL induced highest impact (0.56 fold change) on AchE due to the ache inhibition mechanism. Gabra1and gad2 expression decreased exposed to IMI, CHL, CAR, however, significantly increased exposure to PER (3.06 fold change and 1.35 fold change). IMI and CHL exhibited high estrogenic effects according to vtg1 expression (3.37 fold change and 2.07 fold change). Moreover, genotypic effect exhibited diverse after exposed to water samples, agricultural wastewater induced neurotoxicity, and river water exhibited the significantly estrogenic effect. In conclusion, the multiple genetic parameters covered various toxicity pathways and provided an information-rich approach for integrated evaluation of neurotoxicity and estrogenic effects.

In chapter 5, In vitro morphology-based analysis using enhanced green fluorescent protein fused (EGFP) with methyl CpG-binding protein (MBD) binding mouse ES cells was developed for evaluation of DNA methylation effect of xenoestrogens. The multiple morphological parameters (intensity, area, number of granular) of fluorescent EGFP were characterized and exhibited positive correlations between DNA methylation status. 5-aza-2-deoxycytidine (5-aza-dc) induced a decrease in intensity, area, and count of EGFP-assembled granules, demonstrating the positive correlations between DNA methylation status and the phenotypic parameters. Among these environmental chemicals, granular area and count decreased after bisphenol A and permethrin exposure ($P<0.05$), but increased after treating with imidacloprid, carbaryl ($P<0.01$) and o.p'-DDT ($P<0.05$). p.p'-DDT did not exhibit any noticeable effects. With a high-throughput screening of environmental chemicals, we found that imidacloprid, carbaryl, and o.p-DDT increased the parameters, indicating the induced DNA methylation. In conclusion, the granular sensitivity and area exhibited high correlations

with DNA methylation, and the phenotypic analysis provided a high-throughput approach for DNA methylation evaluation.

6.2 Recommendations

Environmental samples contained complex contaminants including legacy pollutants such as DDT, dioxin which remained in the environment for decades (Letcher et al. 2018). Moreover, emerging chemicals raised the concern including personal care products (PPCPs), pharmaceuticals, novel insecticides neonicotinoids (Tri et al. 2016). Due to the high risk for human health and ecosystems, it is important to identify the contaminants in environmental samples. However, it is difficult for conventional chemical analysis (LC/MS, LC/qTOFM/MS, etc) to cover such wide range of chemicals and to identify the toxicity of environmental samples. Therefore, Toxicity Identification Evaluation (TIE) and Direct-Effect Analysis (EDA) were developed to identify the toxicant in environmental samples combing various bioassays with chemical analysis.

For TIE, three steps were performed, TIE phase I: toxicity characterization; TIE phase II: toxicity identification; TIE phase III: toxicity confirmation. The objective of phase I aimed to characterize the toxicity of environmental samples and to predict the possible class of toxicant which induced such toxicity. Then, the specific toxicants were identified combing the bioassay results with chemical analysis (LC/MS, LC/qTOFM/MS) in phase II, and the toxicants were confirmed by the target chemical analysis in Phase III(Burgess et al. 2013; Li et al. 2017).

In chapter 3, a high-throughput phenotypic analysis was applied for toxicity evaluation and identification of whole water samples. The results indicated that multiple phenotypic parameters exhibited high potential for toxicity and chemical identification. For instance, nuclei intensity showed high correlations with estrogenic effects.

Therefore, it was a powerful tool for toxicity characterization in TIE phase I. In our research, conventional bioassays (E-Screen, ELISA assay) were performed for estrogenic effect evaluation, the non-target chemical analysis (UPLC-qTOFM-MS) combined with such bioassay for toxicity identification in phase II. Based on the statistical analysis (OPLS-DA), Chemicals exhibited diverse relations with each bioassay parameters in chapter 3 (Figure 3.17). It provided a novel approach for toxicity identification in TIE phase II.

For identification of phenolic EDCs, candidate chemicals were classified using OPLS-DA (Table 6.1) and then performed database search using ChemSpider. We reduced the number of candidate chemicals using phenolic structure, hydrophobicity (Log *Kow*), and phenotypic parameters or EEQ criteria (Table 6.2). For example, $C_{21}H_{20}O_{15}S$ exhibited a positive effect on nuclei count suggesting it may be an estrogen agonist. Our database search retrieved 5 candidate phenolic chemicals, including 2 estrogen agonist: centabractein and quercetin 3-(3''-sulfatoglucoside). With the same procedure, benzaldehyde, tropolone, phenyl formate, and 3-hydroxy-2,4,6-cycloheptatriene-1-one were identified as candidate chemicals associated with EEQ. For phenotypic parameters, $C_{18}H_{24}O_3$ with high VIP value showed a negative effect on nucleus which may result from nucleus shrink during cell growth. Moreover, an increase in W1-Eff (Table S4) indicated that it may be by-product during the wastewater treatment processes, suggesting that estriol (E3) exhibits a high possibility of candidate chemicals. According to OPLS-DA, we selected top 7 chemicals with high VIP scores (VIP score >1) and listed their relations with each phenotypic parameter (Table 6.3). VIP scores in OPLS model were well applied to identify the active component in complex samples (Morita et al. 2015; Oussama et al. 2012). In

results, C6H5Cl2N, C18H24O3, C10H15NO2S, C8H8ClNO, C9H18O2, C12H14O4, C7H5NOS had high VIP scores (n > 1.4), and mostly affect nucleus phenotypic parameters (Nuclei area and nuclei intensity), suggesting their sensitivity to ER of MCF-7 cells.

Table 6.1 Candidate chemicals that correlate with phenotypic parameters and EEQ based on OPLS analysis

Phenotypic parameters	Candidate Chemicals	Concentration (Fold change)					
		T2	T8	T9	W1-Eff	W1-Inf	W3-Inf
Nuc_Formfactor	$C_9H_{10}Cl_2N_2O$	0.56	0.32	1.16	1.59	0.84	0.86
	$C_7H_7ClN_2O_2$	1.24	1.25	0.95	1.32	1.08	1.25
Nuc_Intensity	$C_{15}H_{13}O_4S$	<LOD	<LOD	<LOD	0.32	0.84	0.21
	$C_{16}H_{28}O_2$	1.06	<LOD	<LOD	<LOD	2.13	1.03
	$C_{20}H_{30}O_2$	1.18	<LOD	<LOD	<LOD	6.07	0.76
Nuc_count	$C_{21}H_{20}O_{15}S$	0.20	<LOD	<LOD	0.49	0.90	<LOD
	$C_{24}H_{30}O_8$	0.74	<LOD	<LOD	0.64	0.62	<LOD
	$C_{21}H_{30}N_6OS_2$	0.41	<LOD	<LOD	0.60	<LOD	0.33
	$C_7H_8N_4O_2$	<LOD	<LOD	<LOD	<LOD	6.32	0.76
	$C_{14}H_{18}O_2$	<LOD	<LOD	<LOD	<LOD	2.68	0.43
	$C_{14}H_{24}O_2$	<LOD	<LOD	<LOD	<LOD	0.76	0.71
	$C_{12}H_7Cl_3O_2$	<LOD	<LOD	<LOD	<LOD	5.97	0.70
	$C_{15}H_{31}OS_2$	<LOD	<LOD	<LOD	<LOD	0.51	0.34
	$C_{23}H_{46}O_6$	<LOD	<LOD	<LOD	<LOD	1.29	0.51
	$C_{20}H_{16}NO_9S$	0.29	<LOD	<LOD	0.63	2.09	<LOD
Cell_Minor/Major	$C_9H_{14}N_2O_2$	<LOD	<LOD	<LOD	<LOD	<LOD	0.25
	$C_6HO_{11}S$	<LOD	<LOD	<LOD	<LOD	<LOD	0.73
	$C_{12}H_{11}NO_2S$	<LOD	<LOD	<LOD	<LOD	<LOD	0.74
	$C_{16}H_{19}O_7$	<LOD	<LOD	<LOD	<LOD	<LOD	2.25
	$C_{16}H_{34}NO_5S$	<LOD	<LOD	<LOD	<LOD	<LOD	0.27
	$C_{15}H_{18}Cl_2N_2O_2$	<LOD	<LOD	<LOD	<LOD	<LOD	0.60
	$C_{19}H_{36}O_2$	<LOD	<LOD	<LOD	<LOD	<LOD	0.92
Cell_Intensity	$C_{16}H_{26}O_3S$	1.04	1.55	1.42	1.38	1.46	1.17
	$C_{17}H_{28}O_3S$	2.26	3.42	3.01	2.78	3.01	2.63
Nuc_Perimeter; Nuc_Major; Nuc_Minor Nuc_Aera; Nuc_Minor/major; Cell_Area Cell_Major; Cell_Minor; Cell_Formfactor	$C_6H_5Cl_2NO$	<LOD	0.32	0.23	<LOD	<LOD	<LOD
EEQ	C14H28O2	23.3	30.8	26.8	25.2	39.9	24.5
	C14H26N6O2	1.34	1.67	1.38	1.43	1.93	1.40
	C9H11NO3	1.90	2.07	2.41	2.06	2.57	2.23
	C12H24O2	4.22	4.30	4.11	4.31	7.91	4.45

LOD: limit of detection

Table 6.2 Identification of candidate chemicals using an image-based chemical screen, and database search

Formula	Impacted phenotype	ChemSpider hits	Alkylphenol or phenol hits	Log *Kow* Hits	Candidate chemicals
C7H7ClN2O2	Nuclei form factor (+)	382	4	4	`3-Chloro-4-hydrazinobenzoic acid 5-Chloro-N,2-dihydroxybenzenecarboximidamide 4-Chloro-N',2-dihydroxybenzenecarboximidamide
C21H20O15S	Nuc count (+)	5	5	3	Centabractein Quercetin 3-(3"-sulfatoglucoside)
C7H6O2	EEQ (+)	71	7	7	Benzaldehyde Tropolone Phenyl formate 3-hydroxy-2,4,6-cycloheptatrien-1-one
C16H26O3S	Cell Intensity (+)	130	3	3	7-oxodehydroabietic acid 2-[(2,6-Dimethoxy-4-methylphenoxy)methyl]-2-ethyl-1-butanethiol 2-[(2,6-Dimethoxy-4-methylphenoxy)methyl]-3,3-dimethyl-1-butanethiol
$C_6H_5Cl_2NO$	Nuc Perimeter Nuc Major; … Cell Major Cell Minor; Cell Formfactor (+)	87	13	13	4-Amino-2,6-dichlorophenol (ADCP) 4-Amino-2,5-dichlorophenol 5-Amino-2,4-dichlorophenol 4-amino-2,3-dichlorophenol
C18H24O3	Nuc area (-)	1303	7	6	Estriol Estra-1,3,5(10)-triene-3,16,17-triol Estra-1(10),2,4-triene-2,3,17-triol 3-(6-Methoxy-3,4-dihydro-2-naphthalenyl)-2,2-dimethylpentanoic acid Estra-1(10),2,4-triene-3,4,17-triol (17β)-Estra-1,3,5(10)-triene-1,3,17-triol

(+): Positive effects; (-): Negative effects

Table 6.3 Candidate chemicals with significant VIP scores, associated phenotypic parameters, composite spectrum and concentration in river water and wastewater

Candidate Chemicals	VIP	Associated parameters	Composite Spectrum	Concentration (Fold change)					
				T2	T8	T9	W1-Eff	W1-Inf	W3-Inf
C6H5Cl2NO	1.64	Nuc_Area (+)	(159.9724, 6468.5835)(160.97542, 607.89667) (161.96959, 4061.015)(162.9739, 200.36002)	<LOD	0.32	0.23	<LOD	<LOD	<LOD
C18H24O3	1.61	Nuc_Area (-)	(287.16516, 32823.45)(288.16843, 6533.641)(289.17282, 1477.36) (290.177, 172.17499)	2.05	<LOD	<LOD	3.81	0.45	0.80
C10H15NO2S	1.45	Nuc_Intensity (+)	(212.07512, 12169.185)(213.07764, 1766.0898)(214.0727, 744.79944)	0.40	0.25	0.39	0.50	0.84	0.5
C8H8ClNO	1.44	Nuc_Area (-)	(168.02223, 66645.53)(169.02536, 6404.813) (170.01926, 21623.326)(171.02235, 2143.1006)	5.26	4.59	3.77	5.13	5.42	5.68
C9H18O2	1.41	Nuc_Intensity (+)	(157.12361, 127959.336)(158.12686, 13248.276) (159.12909, 1246.2332)(160.13098, 50.57667)	5.61	5.19	4.13	7.05	9.56	6.53
C12H14O4	1.41	Cell_Formfactor (-)	(221.08127, 12438.906)(222.08449, 1952.9291)	0.56	0.52	0.36	0.66	0.68	0.68
C7H5NOS	1.38	Nuc_Intensity (-)	(150.00192, 19558.623)(151.005, 1747.2091)(151.99986, 1095.8812)	0.44	0.65	1.02	0.41	<LOD	<LOD

LOD: limit of detection

In this part, we suggested a novel approach for chemicals identification combing with phenotypic analysis, chemical analysis, and databases. The methods satisfied with the principle of Tox21. However, the predicting process was rude for chemical identification due to the following knowledge gaps:

The linkage of phenotypic parameters with toxicity pathways

In this study, MCF-7 cell lines were applied to evaluate the estrogenic effect of whole water samples. Our results indicated that nuclei intensity, nuclei area, cell intensity, and cell area exhibited high relations with estrogenic effects. However, the relations of other phenotypic parameters such as nuclei form factor to toxicity pathways were not known. Moreover, the results indicated that phenotypic analysis using MCF-7 cell lines were sensitive to estrogenic activities, however, it was failed to catch other toxicities such as neurotoxicity. It may result from the lack of toxicity pathways in MCF-7 cell lines. Therefore, a morphological database of MCF-7 cell lines of more environmental chemicals should be constructed. Moreover, the phenotypic analysis should be developed to cover another toxicity pathway such as neurotoxicity using neurotoxic sensitive cells such as PC12.

The rude data mining processes

In this study, PCA analysis and OPLS-DA model were applied for integrated toxicity evaluation and identification. However, due to the complex multivariable elements such as hundreds of chemicals and multiple phenotypic parameters, it is difficult to make reliable relations with each chemical to phenotypic parameters. Therefore, more advanced data mining process using bioinformatics tool should be performed to make the correlations of chemicals to phenotypic parameters such as Bayesian analysis.

Fractionation processes

To predict the candidate phenotype active chemicals, the candidate chemicals were abstained based on chemicals database ChemSpider. Physical properties such as Log Kow and chemicals categories (e.g. phenolic compounds) were selected as the parameters to hit candidate chemicals. In this research, solid phase extraction was performed with Autoprep EDS-1 (SHOWA DENKO, Japan) which is sensitive to extract phenolic compounds in water samples. Therefore, the phenolic chemicals were targeted as candidate chemicals. However, the simplex chemicals category made it difficult to catch more chemicals. Moreover, physical properties were also selected for chemical prediction. However, the SPE without fractionation (e.g. 100% solvent, 50% solvent, etc) made it difficult to classify the chemicals via physical properties. Therefore, the phenotypic analysis after fractionation process should be performed for chemicals identification.

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