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Evaluation of Whole Wastewater Effluent Impacts on HepG2 using DNA Microarray-based Transcriptome Analysis

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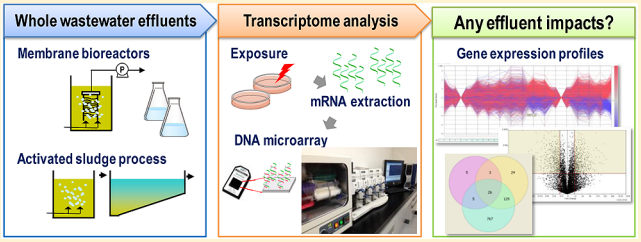
Supporting Information

ABSTRACT: DNA microarray-based transcriptome analysis with human hepatoma HepG2 cells was applied to evaluate the impacts of whole wastewater effluents from the membrane bioreactors (MBRs) and the activated sludge process (AS). In addition, the conventional bioassays (i.e., cytotoxicity tests and bioluminescence inhibition test), which were well-established for the evaluation of the overall effluent toxicity, were also performed for the same samples. Transcriptome analysis revealed that 2 to 926 genes, which were categorized to 0 to 225 biological processes, were differentially expressed after exposure to the effluents and the raw wastewater. Among the tested effluents, the effluent from a MBR operated at a relatively long solid retention time (i.e., 40 days) and small membrane pore size (i.e., 0.03 μm) showed the least impacts on the HepG2 even at the level comparable to tap water. The observed gene expression responses were in good agreement with the results of cytotoxicity tests, and provided additional molecular mechanistic information on adverse effects occurred in the sublethal region. Furthermore, the genes related to "lipid metabolism", "response to endogenous stimulus", and "response to inorganic substance" were selected as potential genetic markers, and their expression levels were quantified to evaluate the cellular impacts and treatability of wastewater effluents. Although the harmful impacts and innocuous impacts could not be distinguished at present, the results demonstrated that the DNA microarray-based transcriptome analysis with human HepG2 cells was a powerful tool to rapidly and comprehensively evaluate impacts of whole wastewater effluents.



INTRODUCTION

The reclaimed wastewater has been served as an alternative water source in some countries, for irrigation water, industrial water, recreational water, and even for indirect and direct potable water.[1,2] Although growing water stress, cost, and energy constraints have prompted a call for more widespread utilization of wastewater reclamation and reuse practices, uncertain impacts of vast categories of micropollutatns (e.g., pesticide, endocrine disrupters, pharmaceuticals),[3−5] poorly characterized or even unknown chemicals in wastewater effluents (e.g., degradation products),[6,7] remains as a challenge to increase the public acceptance. Due to such complex nature of wastewater effluents, it is not feasible that chemical-specific analysis covers all the constituents potentially appear in the effluent, and thereby, the importance of whole effluent evaluation has been recognized.[8]

Various types of bioassays have been used to evaluate overall impacts of wastewater effluents. The test organisms used in the previous studies include Japanese medaka, *Daphinia magna*, algae, luminescent marine bacteria, yeast, and mammalian cells.[9−12] However, most of the conventional bioassays targeted on the impacts on nonhuman organisms, and those on humans have not been fully developed for potable or any other type of reuse practice with a contact with human bodies. Furthermore, since a set of bioassays targeting on different end points are usually adopted to evaluate the overall effluent toxicities, the test procedure tends to be laborious and time-consuming. Thus, a single more rapid, comprehensive, and sensitive approach needs to be developed.

The recent advances in genomics have provided clues to understand the responses of biota to the environmental pollutants and its associated mechanisms of actions.[13,14] The DNA microarray can detect the genome-wide gene expression. The patterns of such gene expression responses represent the primary interactions between the environmental contaminants and biota. Some researchers have applied this technology to characterize the toxicity of mixed chemicals or environmental samples such as polyfluorinated and perfluorinated compounds,[15] diesel exhaust particles,[16] oil contaminated waters,[17,18] and wastewaters from factories.[19,20] Those studies demonstrated that DNA microarray analysis could detect the

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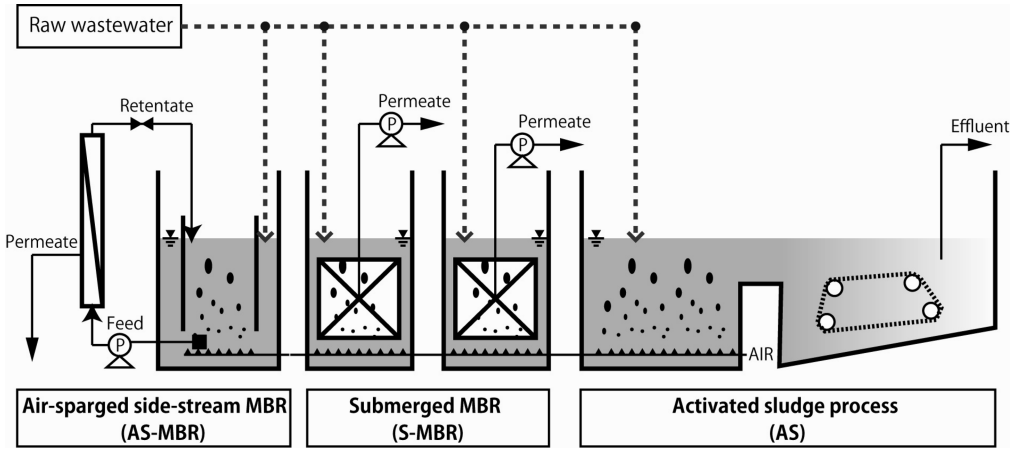

Figure 1. Flow scheme of selected treatment processes. The raw wastewater and effluents were taken from October 2011 to February 2012 at a full-scale activated sludge process (AS) and three pilot-scale membrane bioreactors (MBRs) treating the same domestic wastewater.

impacts of the samples even without prior concentration, in a relatively short-term exposure from several hours to a couple of days. In addition, the observed gene expression responses were found to be associated with various biological processes. However, since there is still a gap between gene expression responses and the actual toxicity, accumulation of microarray data along with the well-established bioassay data is necessary.

Thus, due to its rapid, sensitive, and comprehensive nature, the DNA microarray-based transcriptome analysis was applied to evaluate the impacts of whole wastewater effluents from the membrane bioreactors (MBRs) and the activated sludge process (AS), on the biological processes of human hepatoma HepG2 cells. The conventional bioassays (i.e., cytotoxicity tests and bioluminescence inhibition test) were conducted in parallel since they were well-established evaluation methods for the overall effluent toxicity. HepG2 was selected because it has been frequently used in vitro model for human detoxification of chemicals, is easy to handle, and provides a reproducible human system. However, since it is known that expression of specific genes were extremely low in the cancer derived HepG2, the results could not be simply extrapolated into the normal cells and in vivo.[21] The integration of all data obtained at molecular and cellular levels suggested the potential applicability of DNA microarray-based transcriptome analysis to evaluation of whole wastewater effluent impacts.

MATERIALS AND METHODS

Cell Culture. Human hepatoma HepG2 cells were provided by Cell Bank, RIKEN BioResource Center (Tsukuba, Japan). Cells were grown in Eagle's minimal essential medium (MEM) (Nissui, Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS) and 60 mg/mL kanamycin. Cells were maintained at 37 °C in the 5% CO_2 humidified incubator.

Sampling Site. Sampling was carried out from October 2011 to February, 2012 at the full-scale municipal wastewater treatment plant (Soseigawa Municipal Wastewater Treatment Plant, Sapporo, Japan), which daily received ca. 130 000 m^3 of wastewater via combined sewage system. The plant uses an activated sludge system (AS), which consists of aeration tank and secondary sedimentation basin. Three pilot-scale MBRs were also installed at the plant: two submerged MBRs (S-MBRs) equipped with hollow-fiber polytetrafluoroethylene (PTFE) membrane with 0.3 μm nominal pore size, and one air-sparged side-stream MBR (AS-MBR) equipped with tubular polyvinylidene difluoride (PVDF) membrane with 0.03 μm nominal pore size (Figure 1).[22] Two S-MBRs (S-MBR_A and S-MBR_B) were operated at different sludge concentration (mixed liquor suspended solids: MLSS) and solid retention times (SRTs). The operational conditions of selected processes are listed in Table 1. Raw wastewater was fed through a grit chamber to the MBRs.

Table 1. Operational Condition of the Selected Treatment Processes[a]

	AS-MBR	S-MBR_A	S-MBR_B	AS
HRT (h)	4	5	5	12
MLSS (g/L)	12	1	8	1.7
SRT (day)	40	10	50	7

[a]Two S-MBRs (S-MBR_A and S-MBR_B) were operated at different MLSS and SRT. HRT: Hydraulic Retention Time; MLSS: Mixed Liqor Suspended Solids; SRT: Solid Retention Time.

Grab samples were taken around 10:00 a.m., from the effluents of the grid chamber (considered as raw wastewater), the secondary sedimentation basin of the AS, and the three MBRs. As a reference, two types of drinking waters were also collected: the tap water of our laboratory and the bottled water (commercial dechlorinated drinking water). In terms of general water quality, effluents from AS-MBR, S-MBRs and AS all satisfied the 40 national effluent standards (see Supporting Information A). Characteristics of the raw wastewater of this plant can be found elsewhere.[23]

Sample Preparation and Treatment. Immediately after sampling, 400 mL of collected samples were filtered with 0.22 μm polyethersulfone (PES) membrane using an aseptic vacuum filter system (Corning Japan K.K., Tokyo, Japan) for sterilization, and stored at 4 °C in sterilized muffled bottles until they were used for the assays (up to 48 h). Prior to exposure, 36 mL filtered water was combined with 4 mL of ×10 concentrated MEM, and supplemented with 1% fetal bovine serum (FBS) and 60 mg/mL kanamycin. Finally, pH was adjusted to 7.2−7.5 by adding 7.5% sodium hydrogen carbonate solution. Cells were seeded onto culture vessels in 10^5 cell/mL and incubated overnight to grow 40−50% confluent. The normal culture medium was replaced by the prepared water samples and incubated for 48 h.

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DNA Microarray Analysis. Cells were lysed on the 60 mm culture dishes, and total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). For duplicate, two dishes were prepared for each sample and individually treated in parallel. Total RNAs were quantified using the NanoVue Plus (GE Healthcare, Buckinghamshire, UK). The integrity of the RNA samples was also checked by microchip electrophoresis using the Cosmo Eye with the i-RNA Kit (Hitachi Chemical, Tokyo, Japan). RNA with a A260/A280 ratio 1.8 or higher was used for DNA microarray analysis using Human Genome Focus Array (Affymetrix, Santa Clara, CA), which represents 8795 verified human sequences from the NCBI RefSeq database. Target preparation and hybridization were carried out as described in the manufacturer's instruction. After hybridization, the array was washed, stained with streptavidin-phycoerythrin using the Affymetrix GeneChip Fluidics Station 450 (Affymetrix), and scanned on a GeneChip Scanner 3000 7G (Affymetrix). The raw data files [cell intensity (CEL) files] were created from scanned images by AGCC (GeneChip Command Console Software, Affymetrix). The files are available on Gene Expression Omnibus (GEO; http://www.ncbi.nlm.gov/geo) of the National Center for Biotechnology Information (NCBI) (accession number: GSE 45605).

Quantitative Real-Time Reverse-Transcriptase PCR (qPCR). Based on the microarray data, seven genes (i.e., *CYP39A1*, *AKR1D1*, *STS*, *ADM*, *DUSP1*, *GCLC*, and AQP3) were selected as marker genes from its differentially expressed genes associated with one or more of the biological processes such as "lipid metabolism" (i.e., *CYP39A1*, *AKR1D1*, *STS*, *ADM*), "response to endogenous stimulus" (i.e., *STS*, *ADM*, *DUSP1*, *GCLC*), and "response to inorganic substance" (i.e., *DUSP1*, *GCLC*, *AQP3*). *IGFBP7* was used as the negative marker, which was differentially expressed only in AS exposure in the microarray analysis. The expressions of candidate marker genes were quantified by the quantitative real-time reverse-transcriptase polymerase chain reaction (qPCR) assay for the same RNA sample used for DNA microarray analysis, and those taken on different sampling days between October 2011 and August 2012 to confirm the reproducibility. Briefly, single-strand cDNA was synthesized from 25 ng/μL total RNA in reaction cocktail of PrimeScript RT reagent Kit (Takara, Shiga, Japan). The qPCR was carried out with ABI prism 7500 (Applied Biosystems, Foster City, CA) using SYBR Ex *Taq*II (Takara, Shiga, Japan), according to manufacturer's instruction. The amplification reaction consisted of initial incubation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at specific temperatures for 30 s, and extension at 72 °C for 60 s. The primer sequences and annealing temperatures are shown in Supporting Information D. Data were analyzed by the relative quantification method using the Ct values normalized with the endogenous reference, *GAPDH*, which was measured in all runs. The fold induction/suppression of each target gene was calculated by dividing the normalized Ct value in a sample with that of the control. All assays were tested for presence of unspecific amplicons and primer dimers by melting curve analysis.

Statistics. For microarray analysis, probe-level data in CEL files were normalized by MAS5 using AGCC and transferred to the Subio Platform ver. 1.15 (Subio, Inc., Tokyo, Japan). Reproducibility of the overall gene expressions of sample pairs were confirmed by hierarchical clustering analysis and principal component analysis (PCA) (Supporting Information B). The features which were NOT flagged "Present" in any duplicates of the samples were rejected. We identified differentially expressed genes based on the two criteria: (1) a *t* test *p*-value threshold of 0.05 and (2) a minimum fold change of 2 between the controls and exposed data sets. Gene ontology (GO) assignments and clustering into functional groups were performed using web-accessible programs provided by the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/).

Conventional Bioassays. The three bioassays well-established for the evaluation of the overall effluent toxicity were performed in parallel with the DNA microarray analysis: bioluminescence inhibition test based on ISO 11348 and the two cytotoxicity tests using direct cell counting and MTT [3-(4,5-dimethylthial-2-yl)-2,5-diphenyltetrazalium bromide] assay. The procedures of the three bioassays are found in Supporting Information C.

RESULTS

Gene Expression Analysis Using DNA Microarray. The DNA microarray analysis identified 2 to 926 genes which were differentially expressed after exposure to the effluents and raw wastewater (Table 2). The numbers of the altered genes were far large in the raw wastewater (926 genes) compared to the effluents (<200 genes), indicating the reduction of cellular impacts after treatments. Among the effluents, however, the extent of alteration of the gene expression was relatively large for S-MBR$_B$ (181 genes), moderate in S-MBR$_A$ (39 genes) and AS (20 genes), and the lowest for AS-MBR (2 genes), which was even comparable to the tap water (TAP; 2 genes). For the bottled water (BOT), differentially expressed genes were not identified. More than half of the altered genes of S-MBR effluents were common with those of the raw wastewater.

Table 2. Number of Differentially Expressed Genes in HepG2 Cells After 48 h Exposure to Effluents, The Raw Wastewater, The Tap Water (TAP), and the Bottled Water (BOT)[a]

	AS-MBR	S-MBR$_A$	S-MBR$_B$	AS	RAW	TAP	BOT
total	2	39	181	20	926	2	0
induction	1	17	115	9	432	0	0
suppression	1	22	66	11	494	2	0

[a]Differentially expressed genes were selected based on the two criteria: (1) a *t*-test *p*-value threshold of 0.05 and (2) a minimum fold change of 2 between the controls and exposed data sets.

Biological functional analysis was conducted by DAVID to link altered genes and related biological processes and to identify the specific processes consisting of significant number of linked genes (Table 3). The smaller *p* value represents the higher significance of alteration of the corresponding biological process. In AS-MBR effluent and the real drinking waters (i.e., TAP and BOT), any biological process was not identified at the statistical significance ($p < 0.05$). For the rest of effluent samples and the raw wastewater, altered genes were categorized into 7 to 225 biological processes, which were further summarized into major nine groups of closely related processes: cell growth, cell division, lipid metabolism, lipid transport, response to endogenous stimulus, response to inorganic substance, acute inflammatory response, response to nutrients, and cell death. The biological processes identified for S-MBR$_A$ exposure were common with those for S-MBR$_B$ exposure, and the significance of alteration was mostly higher

Table 3. Gene Ontology (GO) Categories of Differentially Expressed Genes. Biological Functional Analysis Was Conducted by the Web-Accessible Program, DAVID (http://david.abcc.ncifcrf.gov/) to Assign GO Terms to the Altered Genes and Cluster the Related Biological Processes

Related Process Groups	Biological Process	p value (log10)						
		AS-MBR	S-MBR$_A$	S-MBR$_B$	AS	RAW	TAP	BOT
Cell growth	regulation of cell growth				-2.37	-1.50		
Lipid metabolismth	regulation of lipid metabolic process				-1.75	-1.16		
	lipid catabolic process		-1.88	-1.95				
	steroid metabolic process		-2.23	-3.02		-6.02		
	cholesterol metabolic process		-1.33	-1.05		-2.80		
	bile acid catabolic process			-2.87		-1.49		
Lipid transport	lipid transport			-1.73		-1.48		
	carboxylic acid transport		-1.95	-1.12				
Response to endogenous stimulus	response to endogenous stimulus			-3.35	-1.39	-2.66		
	response to hormone stimulus			-2.96		-2.77		
	response to corticosteroid stimulus			-3.56		-1.68		
	response to glucocorticoid stimulus			-3.07		-1.68		
	response to estradiol stimulus					-2.07		
Response to inorganic substance	response to inorganic substance			-1.40				
Acute inflammatory response	leukocyte mediated immunity					-2.93		
Cell deth	cell death					-2.45		
	apoptosis					-2.49		
Response to nutrient	response to nutrient					-2.45		
	response to vitamin					-3.33		
Cell division	mitosis					-10.01		
	M phase					-9.37		
	cell cycle					-8.24		
	spindle organization					-3.47		
	microtubule-based process					-3.31		

Legend: -2.0 < log[p] ≤ -1.0; -3.0 < log[p] ≤ -2.0; -4.0 < log[p] ≤ -3.0; log[p] ≤ -4.0

in the latter. The process groups such as "lipid metabolism" and "response to endogenous stimulus" were commonly identified in S-MBR effluents, AS effluent, and the raw wastewater exposures, while "response to inorganic substance" and "cell growth" were unique to S-MBR$_B$ effluent and AS effluent, respectively. Although the significance of alteration of the gene expression was relatively high in "acute inflammatory response", "cell death", "response to nutrient", and "cell division", these process groups were only identified in the raw wastewater.

The altered genes in the selected process groups and their fold change (FC) (relative expression ratios to the control) for the effluents and the raw wastewater are listed in Table 4. Most of the genes related to "lipid metabolism" were shared between S-MBR effluents and raw wastewater, with the increase of FCs in the latter sample. In AS effluent, however, the altered genes such as *AVPR1A*, *IGFBP7*, and *PPARA* were unique to its exposure. The genes categorized in "response to endogenous stimulus" exhibited a similar trend as "lipid metabolism", reflecting some overlaps of the genes and probably close relationship of the two functions in the cellular response to the effluents. In S-MBR$_B$ effluent, the genes categorized in "response to inorganic substance" (i.e., *DUSP1*, *FGB*, *SERPINE1*, *GCLC*, and *AQP3*) were largely altered by 4- to 13-fold, while in AS effluent, the genes categorized in "cell growth", (i.e., *IGFBP7*, *CYR61*, *OSGIN1*, and *AVPR1A*) were moderately altered by 2- to 4-fold.

Quantification of Candidate Marker Genes Using qPCR. Eight genes were selected as representative of gene expression responses observed for the S-MBR$_B$ effluent exposure, and their expression levels were quantified by qPCR assay (data shown in Supporting Information D). The FCs in the base-2 logarithm for all the selected genes varied from −3.3 to 3.6 in the microarray data, while those for qPCR were somewhere between −4.1 and 4.0. The direction of regulation (induction or suppression) was identical between the qPCR data and the microarray data. Although the more samples from different days are needed for meaningful statistical analysis, similar trend of the overall gene expression patters was observed for all the effluents taken on different days (Figure 2). The gene expression level of each gene varied day to day possibly due to the variation of water quality of effluents and the raw wastewater.

Bioluminescence Inhibition. Bioluminescence intensity of *V. fisheri* after exposure to effluent samples, the raw wastewater and drinking water samples is shown in Figure 3a. Clear bioluminescent inhibition was observed for raw wastewater (>50% of the control). However, in AS-MBR, S-MBR$_A$, and AS effluents, the bioluminescence intensity was unchanged as compared to that of the control. Only S-MBR$_B$ effluent showed a slight reduction (i.e., 10%). Similarly, significant bioluminescence inhibition was not observed for the drinking water samples.

Cytotoxicity. Cell viability was evaluated by direct cell counting with tripan blue dye exclusion method and the MTT assay (Figure 3b and c). The direct cell counting exhibited a significant reduction of cell viability in S-MBR$_B$ and raw wastewater by 13% and 55%, respectively, whereas AS-MBR and S-MBR$_A$ effluents did not affect the cell viability. Cell viability increased by 26% after exposure to the AS effluent. The results of MTT assay were consistent to those of the direct cell counting except for AS, which on the contrary marked 23% reduction in the cell viability. The relative MTT activity divided by the cell numbers was smaller in AS effluent exposure (i.e., 0.58) than in raw wastewater exposure (i.e., 0.74), suggesting its lower activity level of the HepG2.[24]

DISCUSSION

Previous studies have demonstrated that global gene expression analysis using DNA microarray is a powerful tool to characterize the biological impacts of various environmental

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Table 4. Differentially Expressed Genes in the Selected Process Groups and Their Relative Expression Ratios (In Base-2 Logarithm)

(a) Lipid metabolism

Symbol	Gene name	Fold change (log2) AS-MBR	S-MBR$_A$	S-MBR$_B$	AS	RAW
ADM	adrenomedullin			2.24		3.06
AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)		2.43	2.27		2.07
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)		1.68			2.18
APOL1	apolipoprotein L, 1			1.45		
AVPR1A	arginine vasopressin receptor 1A				2.11	
CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1		1.02	1.06		2.03
LEPR	leptin receptor			1.48		1.42
PPARA	peroxisome proliferator-activated receptor alpha				1.14	
AKR1D1	aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)		-1.16	-1.24		-3.32
APOC3	apolipoprotein C-III			-1.97		-3.78
EHHADH	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase			-1.19		-3.20
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2		-1.85	-2.12		
IGFBP7	insulin-like growth factor binding protein 7				-1.15	-1.81
LDLR	low density lipoprotein receptor		-1.00			-1.52
NR1H4	nuclear receptor subfamily 1, group H, member 4			-1.01		-1.35
PLA2G1B	phospholipase A2, group IB (pancreas)		-1.34	-1.76		-6.01
SERPINA6	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6			-1.10		-3.02
STS	steroid sulfatase (microsomal), isozyme S			-1.65		-2.36
SULT2A1	sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1			-1.28		-2.29
WWOX	WW domain containing oxidoreductase			-1.60		-1.53

Legend: 3<log[FC]; 2≤log[FC]<3; 1≤log[FC]<2; -2<log[FC]≤-1; -3<log[FC]≤-2; log[FC]≤-3

(b) Response to endogenous stimulus

Symbol	Gene name	Fold change (log2) AS-MBR	S-MBR$_A$	S-MBR$_B$	AS	RAW
ADCY7	adenylate cyclase 7		1.06	1.39		2.29
ADM	adrenomedullin			2.24		3.06
AVPR1A	arginine vasopressin receptor 1A				2.11	
CPN1	carboxypeptidase N, polypeptide 1			1.32		1.50
DUSP1	dual specificity phosphatase 1			2.00		2.63
ENO2	enolase 2 (gamma, neuronal)			1.63		3.73
FHL2	four and a half LIM domains 2			1.04		2.11
FOSL1	FOS-like antigen 1			1.22		2.33
GCLC	glutamate-cysteine ligase, catalytic subunit			1.98		1.86
GNAO1	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O				2.36	
IGFBP1	insulin-like growth factor binding protein 1			1.96		2.18
IL6R	interleukin 6 receptor			1.02		1.70
LDLR	low density lipoprotein receptor			1.48		1.42
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)			3.51		7.10
PLA2G1B	phospholipase A2, group IB (pancreas)				2.20	
PRSS8	protease, serine, 8			1.80		2.03
AGXT	alanine-glyoxylate aminotransferase			-1.37		-3.16
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)			-1.03		-1.91
IGFBP7	insulin-like growth factor binding protein 7				-1.15	-1.81
PPARA	peroxisome proliferator-activated receptor alpha		-1.34	-1.76		-6.01
SERPINA7	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7		-1.36	-1.77		-4.19
SLC1A2	solute carrier family 1 (glial high affinity glutamate transporter), member 2			-1.01		
STS	steroid sulfatase (microsomal), isozyme S			-1.65		-2.36

(c) Response to inorganic substances

Symbol	Gene name	Fold change (log2) AS-MBR	S-MBR$_A$	S-MBR$_B$	AS	RAW
AQP3	aquaporin 3 (Gill blood group)			2.17		3.61
AVPR1A	arginine vasopressin receptor 1A				2.11	
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)			1.32		1.50
DUSP1	dual specificity phosphatase 1			2.00		2.63
FOSL1	FOS-like antigen 1			1.22		2.33
GCLC	glutamate-cysteine ligase, catalytic subunit			1.98		1.86
GNAO1	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O				2.36	
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1		1.72	3.68		5.80
FGA	fibrinogen alpha chain		-1.51	-1.51		-5.75
FGB	fibrinogen beta chain		-1.08	-2.49		-6.01
FGG	fibrinogen gamma chain					-4.92
MT1X	metallothionein 1X			-1.50	-1.24	2.07

(d) Cell growth

Symbol	Gene name	Fold change (log2) AS-MBR	S-MBR$_A$	S-MBR$_B$	AS	RAW
AVPR1A	arginine vasopressin receptor 1A				2.11	
OSGIN1	oxidative stress induced growth inhibitor 1	2.14			1.10	1.08
IGFBP7	insulin-like growth factor binding protein 7				-1.15	-1.81
CYR61	cysteine-rich, angiogenic inducer, 61				-1.31	

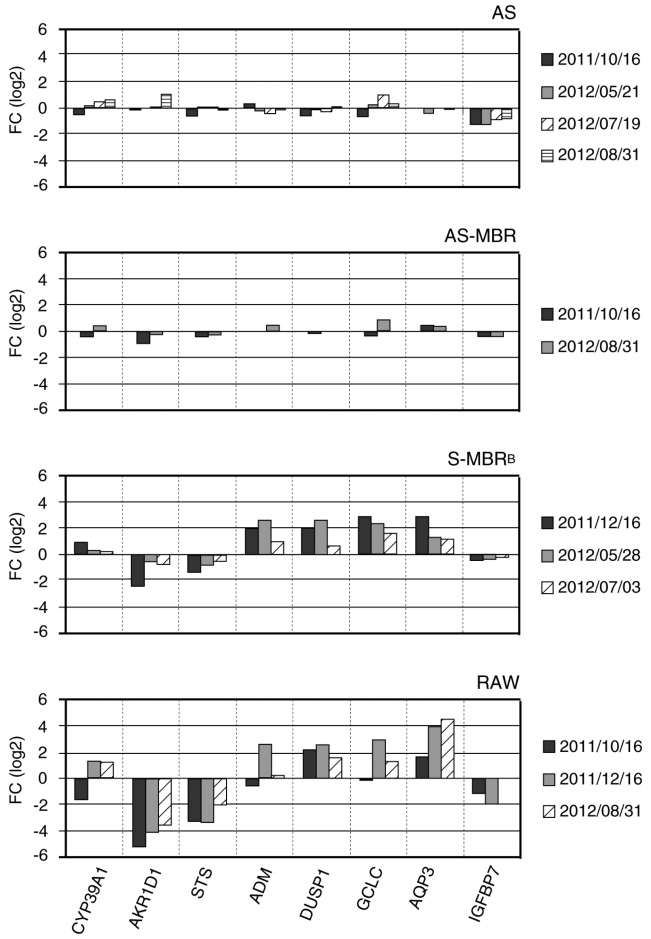

Figure 2. Change of relative expression ratios (in base-2 logarithm) of eight selected genes in HepG2 cells exposed to effluents and the raw wastewater taken on different days. The expression level of eight genes was quantified by qPCR.

contaminants. These contaminants include benzene,[25] dioxins, and PCB,[26] VOC,[27] estrogens,[28] pesticides,[29] nanomaterials,[30,31] arsenic,[32,33] and heavy metals.[34−36] However, applications of this technology to environmental samples or complicate mixtures of the contaminants are still limited. Up to now, only three studies have been reported the applications to wastewaters: industrial wastewater studied with common carp,[19] wastewater from oil and gas production with zebrafish,[18] and textile mill effluents with yeast.[20] Previous studies mainly targeted on the ecological impacts of industrial wastewaters, and the impacts of municipal wastewaters on humans have not been investigated. In the present study, we applied for the first time this DNA microarray-based transcriptome analysis using human HepG2 cells exposed to treated and untreated municipal wastewaters for evaluation of whole effluents impacts on humans from the viewpoint of possible potable reuse.

Table 5 summarizes the results of the transcriptome analysis and the three conventional bioassays applied to the MBR effluents, the AS effluent, the raw wastewater, and the real drinking waters (i.e., TAP and BOT). Despite that all the treated effluents satisfied the 40 national effluent standards; transcriptome analysis of HepG2 exposed to the effluents demonstrated a variation in the effluent quality in terms of their impacts on cellular processes (Table 3). The degree of impacts on the gene expression was roughly evaluated based on the number and the degree of differentially expressed genes and the

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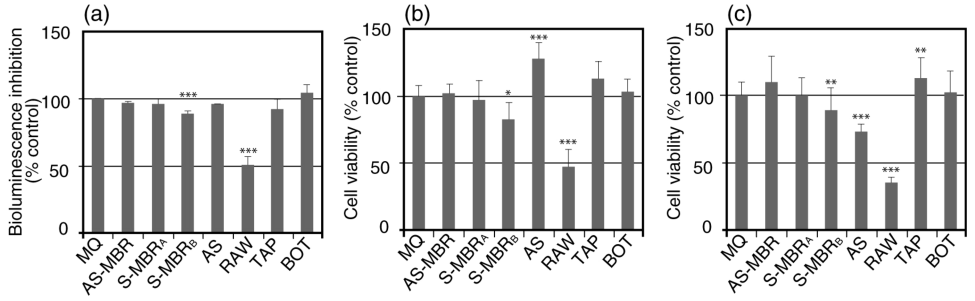

Figure 3. Results of effluent toxicity evaluation by conventional bioassays: bioluminescence inhibition test (a) and the two cytotoxicity tests using direct cell counting (b) and MTT assay (c). Values were expressed as mean ± standard deviation of replicated experiments (n > 3). Statistical test was performed by using Tukey's multivariate analysis; *: <0.05; **: <0.01; ***: <0.001.

Table 5. Comparison of Results for Transcriptome Analysis, Conventional Bioassays, And Chemical Analysis of Effluent Standards[a]

	AS-MBR	S-MBR$_A$	S-MBR$_B$	AS	RAW	TAP	BOT
① transcriptome analysis							
no. of differentially expressed genes	2	39	181	20	926	2	0
no. of affected biological processes	0	12	57	7	225	0	0
② conventional bioassays							
bioluminescence inhibition test	—	—	+	—	++	—	—
direct cell counting	—	—	+	—	++	—	—
MTT assay	—	—	+	+	++	—	—
③ chemical analysis							
effluent standards	—	—	—	—	N/A	N/A	N/A

[a]—: satisfactory or <10% reduction, +: 10-30% reduction, ++:>50% reduction, N/A: not applied.

affected biological processes as S-MBR$_B$ > S-MBR$_A$ > AS > AS-MBR. This result suggested that the effluent from S-MBR$_B$ had the largest impacts on the HepG2 cells and the AS-MBR had the least. The three conventional bioassays (i.e., direct cell counting, MTT assay, and the bioluminescence inhibition test) supported the result of transcriptome analysis. In both direct cell counting and MTT assay, only S-MBR$_B$ effluent showed a reduction of cell viability. These results suggested that the overall or a part of observed gene expression responses could be related to adverse effects on the cell proliferation in the sublethal region.

Although we currently cannot exclude the genetic response derived from the innocuous matters, if any, the gene expression responses which have been suggested as a response to the toxic chemicals or environmental stresses were considered as a potential indication for the toxic effects. The alteration of genes related to "lipid metabolism" was commonly observed for the effluents and the raw wastewater (Table 3). Those genes were also identified when HepG2 cells were exposed to the organic toxicants such as aflatoxin,[37] polycyclic aromatic hydrocarbons (PAHs),[38,39] cationic amphiphilic drugs,[40] and the common carp exposed to the oil contaminated water.[18] Based on these results, it was inferred that the alteration of "lipid metabolism" might not be a chemical-specific response but a general response to a wide range of organic chemicals. The drastic alteration of those genes in the raw wastewater supported this hypothesis since the raw wastewater was likely to contain the largest variety of organic chemicals as indicated in its highest total organic carbon (TOC) (i.e., 34 mg/L). The alteration of cellular function related to "lipid metabolism", therefore, could be proposed as a potential indication of organic-derived effluent impacts in general, and the genes categorized in this process (i.e., *CYP39A1*, *AKR1D1*, *STS*, *ADM*) may serve as its marker genes.

Besides, the functional analysis of the altered genes indicated that the S-MBR$_B$ effluent had its own unique impacts on the cells. In the S-MBR$_B$ exposure, the group of genes categorized in "response to inorganic substance" such as *DUSP1*, *FGB*, *SERPINE1*, *GCLC*, and *AQP3* were significantly altered 4−13 fold (Table 4). Previous studies, for example, have reported *AQP3* was related to the transport of arsenite into mammalian cells,[41,42] while the glutathione level affected by *GCLC* expression has been demonstrated to play a pivotal role in excretion of heavy metals.[43] Induction of the genes associated with ion transport was also reported for yeast and zebrafish exposed to industrial wastewaters.[18,19] Alteration of the genes associated with "response to inorganic substance" along with those associated with "lipid metabolism" in the S-MBR$_B$ exposure suggested the existence of residual organic and inorganic contaminants in the effluent, while AS, AS-MBR, and S-MBR$_A$ exhibited the gene expression response only related to the organics.

To be noted, transcriptome analysis together with the conventional bioassays indicated the smaller cellular impacts of S-MBR$_A$ with shorter SRT (or lower MLSS) than S-MBR$_B$ with longer SRT (or higher MLSS) even though all the tested MBRs were treating the same domestic wastewater. Previous researches have reported that the accumulated organic matters in the MBRs varied with SRTs such as the increase in hydorophobic humic or humin-like substances with longer SRT.[44,45] Although a limited number of reports are currently available for the toxicity of effluent organic matters (EfOM),[46]

we hypothesize that a certain type of EfOM produced only under the longer SRT (S-MBR_B) posed adverse effects on the tested cells, and currently the physicochemical properties of the biologically active fractions in the S-MBR_B are being investigated. Compared between MBRs with similar SRTs, the AS-MBR with 0.03 μm membrane pore size exhibited much smaller impact than S-MBR_B with 0.3 μm membrane pore size, whose gene expression pattern was very similar to that of the raw wastewater (Table 4). These results suggested that the tighter membrane was effective to reject the responsible fraction for the biological impacts, verification of which is also the focus of our ongoing research.

In conclusion, the DNA microarray-based transcriptome analysis with human HepG2 cells was suggested as a powerful tool to rapidly and comprehensively evaluate impacts of whole wastewater effluents. The potential genetic markers were also proposed to quantitatively evaluate the cellular impacts and the treatability of the wastewater effluents, which can be used to search for the physicochemical properties of the biologically active fraction of effluents. However, since the harmful impacts and innocuous impacts could not be distinguished at present, continued efforts are indispensible to identify some key biological processes and establish their relationship with some important end points.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Asano, T., Burton, F. L., Leverenz, H., Tsuchihashi, R., Tchobanoglous, G. *Water Reuse: Issues, Technologies, and Applications*; McGraw-Hill: New York, 2007.

(2) Grant, S. B.; Saphores, J.; Feldman, D. L.; Hamilton, A. J.; Fletcher, T. D.; Cook, P. L. M.; Stewardson, M.; Saners, B. F.; Levin, L. A.; Ambrose, R. F.; Deletic, A.; Brown, R.; Jiang, S. C.; Rosso, D.; Cooper, W. J.; Marusic, I. Taking the "Waste" out of "wastewater" for human water security and ecosystem sustainability. *Science* **2012**, *337* (6095), 681−686.

(3) Watkinson, A. J.; Murby, E. J.; Costanzo, S. D. Removal of antibiotics in conventional and advanced wastewater treatment: Implications for environmental discharge and wastewater recycling. *Water Res.* **2007**, *41* (18), 4164−4176.

(4) Kimura, K.; Hara, H.; Watanabe, Y. Elimination of selected acidic pharmaceuticals from municipal wastewater by an activated sludge system and membrane bioreactors. *Environ. Sci. Technol.* **2007**, *41* (10), 3708−3714.

(5) Hollender, J.; Zimmermann, S. G.; Koepke, S.; Krauss, M.; McArdell, C. S.; Ort, C.; Singer, H.; von Gunten, U.; Siegrist, H. Elimination of organic micropollutants in a municipal wastewater treatment plant upgraded with a full-scale post-ozonation followed by sand filtration. *Environ. Sci. Technol.* **2009**, *43* (20), 7862−7869.

(6) Escher, B. I.; Fenner, K. Recent advances in environmental risk assessment of transformation products. *Environ. Sci. Technol.* **2011**, *45* (9), 3835−3847.

(7) Feron, V. J.; Cassee, F. R.; Groten, J. P.; van Vliet, P. W.; van Zorge, J. A. International issues on human health effects of exposure to chemical mixtures. *Environ. Health Perspect.* **2002**, *110*, 893−899.

(8) Brack, W. Effect-directed analysis: A promising tool for the identification of organic toxicants in complex mixtures? *Anal. Bioanal. Chem.* **2003**, *377* (3), 397−407.

(9) Cao, N.; Yang, M.; Zhang, Y.; Hu, J. Y.; Ike, M.; Hirotsuji, J.; Matsui, H.; Inoue, D.; Sei, K. Evaluation of wastewater reclamation technologies based on in vitro and in vivo bioassays. *Sci. Total Environ.* **2009**, *407* (5), 1588−1597.

(10) Kontana, A.; Papadimitriou, C. A.; Samaras, P.; Zdragas, A.; Yiangou, M. Bioassays and biomarkers for ecotoxicological assessment of reclaimed municipal wastewater. *Water Sci. Technol.* **2008**, *57* (6), 947−953.

(11) Petala, M.; Samaras, P.; Kungolos, A.; Zouboulis, A.; Papadopoulos, A.; Sakellaropoulos, G. P. The effect of coagulation on the toxicity and mutagenicity of reclaimed municipal effluents. *Chemosphere* **2006**, *65* (6), 1007−1018.

(12) Macova, M.; Toze, S.; Hodgers, L.; Mueller, J. F.; Bartkow, M.; Escher, B. I. Bioanalytical tools for the evaluation of organic micropollutants during sewage treatment, water recycling and drinking water generation. *Water Res.* **2011**, *45* (14), 4238−4247.

(13) Mahadevan, B.; Snyder, R. D.; Waters, M. D.; Benz, R. D.; Kemper, R. A.; Tice, R. R.; Richard, A. M. Genetic toxicology in the 21st Century: Reflections and future directions. *Environ. Mol. Mutagen.* **2011**, *52* (5), 339−354.

(14) Aardema, M. J.; MacGregor, J. T. Toxicology and genetic toxicology in the new era of "toxicogenomics": Impact of "-omics" technologies. *Mutat. Res., Fundam. Mol. Mech. Mutagen.* **2002**, *499* (1), 13−25.

(15) Wei, Y.; Shi, X.; Zhang, H.; Wang, J.; Zhou, B.; Dai, J. Combined effects of polyfluorinated and perfluorinated compounds on primary cultured hepatocytes from rare minnow (Gobiocypris rarus) using toxicogenomic analysis. *Aquat. Toxicol.* **2009**, *95* (1), 27−36.

(16) Omura, S.; Koike, E.; Kobayashi, T. Microarray analysis of gene expression in rat alveolar epithelial cells exposed to fractionated organic extracts of diesel exhaust particles. *Toxicology* **2009**, *262* (1), 65−72.

(17) Lie, K. K.; Meier, S.; Olsvik, P. A. Effects of environmental relevant doses of pollutants from offshore oil production on Atlantic cod (*Gadus morhua*). *Comp. Biochem. Physiol. C* **2009**, *150* (2), 141−149.

(18) Olsvik, P. A.; Lie, K. K.; Stavrum, A. K.; Meier, S. Gene-expression profiling in gill and liver of zebrafish exposed to produced water. *Int. J. Environ. Anal. Chem.* **2007**, *87* (3), 195−210.

(19) Moens, L. N.; Smolders, R.; van der Ven, K.; van Remortel, P.; Del-Favero, J.; De Coen, W. M. Effluent impact assessment using micro array-based analysis in common carp: A systems toxicology approach. *Chemosphere* **2007**, *67* (11), 2293−2304.

(20) Kim, H. J.; Rakwal, R.; Shibato, J.; Iwahashi, H.; Choi, J. S.; Kim, D. H. Effect of textile wastewaters on Saccharomyces cerevisiae using DNA microarray as a tool for genome-wide transcriptomics analysis. *Water Res.* **2006**, *40* (9), 1773−1782.

(21) Wilkening, S.; Stahl, F.; Bader, A. Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. *Drug. Metab. Dispos.* **2003**, *31* (8), 1035−1041.

(22) Hoque, A.; Kimura, K.; Miyoshi, T.; Yamato, N.; Watanabe, Y. Characteristics of foulants in air-sparged side-stream tubular. membranes used in a municipal wastewater membrane bioreactor. *Sep. Purif. Technol.* **2012**, *93*, 83−91.
(23) Kimura, K.; Yamato, N.; Yamamura, H.; Watanabe, Y. Membrane fouling in pilot-scale membrane Bioreactors (MBRs) treating municipal wastewater. *Environ. Sci. Technol.* **2005**, *39* (16), 6293−6299.
(24) Ezendam, J.; Staedtler, F.; Pennings, J.; Vandebriel, R. J.; Pieters, R.; Harleman, J. H.; Vos, J. G. Toxicogenomics of subchronic hexachlorobenzene exposure in Brown Norway rats. *Environ. Health Perspect.* **2004**, *112* (7), 782−791.
(25) Gerlier, D.; Thomasset, N. Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods* **1986**, *94* (1−2), 57−63.
(26) Vezina, C. M.; Walker, N. J.; Olson, J. R. Subchronic exposure to TCDD, PeCDF, PCB126, and PCB153: Effect on hepatic gene expression. *Environ. Health Perspect.* **2004**, *112* (16), 1636−1644.
(27) Yim, W. C.; Min, K.; Jung, D.; Lee, B. M.; Kwon, Y. Cross experimental analysis of microarray gene expression data from volatile organic compounds treated targets. *Mol Cell Toxicol.* **2011**, *7* (3), 233−241.
(28) Olsvik, P. A.; Lie, K. K.; Sturve, J.; Hasselberg, L.; Andersen, O. K. Transcriptional effects of nonylphenol, bisphenol A and PBDE-47 in liver of juvenile Atlantic cod (*Gadus morhua*). *Chemosphere* **2009**, *75* (3), 360−367.
(29) Kitagawa, E.; Takahashi, J.; Momose, Y.; Iwahashi, H. Effects of the pesticide thiuram: Genome-wide screening of indicator genes by yeast DNA microarray. *Environ. Sci. Technol.* **2002**, *36* (18), 3908−3915.
(30) Kawata, K.; Osawa, M.; Okabe, S. In vitro toxicity of silver nanoparticles at noncytotoxic doses to HepG2 human hepatoma cells. *Environ. Sci. Technol.* **2009**, *43* (15), 6046−6051.
(31) Kim, J. S.; Song, K. S.; Lee, J. K.; Choi, Y. C.; Bang, I. S.; Kang, C. S.; Yu, I. J. Toxicogenomic comparison of multi-wall carbon nanotubes (MWCNTs) and asbestos. *Arch. Toxicol.* **2012**, *86* (4), 553−562.
(32) Hamadeh, H. K.; Trouba, K. J.; Afshari, C. A.; Germolec, D. Coordination of altered DNA repair and damage pathways in arsenite-exposed keratinocytes. *Toxicol. Sci.* **2002**, *69* (2), 306−316.
(33) Su, P. S.; Hu, Y. J.; Ho, I. C.; Cheng, Y. M.; Lee, T. C. Distinct gene expression profiles in immortalized human urothelial cells exposed to inorganic arsenite and its methylated trivalent metabolites. *Environ. Health Perspect.* **2006**, *114* (3), 394−403.
(34) Andrew, A. S.; Warren, A. J.; Barchowsky, A.; Temple, K. A.; Klei, L.; Soucy, N. V.; O'Hara, K. A.; Hamilton, J. W. Genomic and proteomic profiling of responses to toxic metals in human lung cells. *Environ. Health Perspect.* **2003**, *111* (6), 825−838.
(35) Kawata, K.; Yokoo, H.; Shimazaki, R.; Okabe, S. Classification of heavy-metal toxicity by human DNA microarray analysis. *Environ. Sci. Technol.* **2007**, *41* (10), 3769−3774.
(36) Kawata, K.; Shimazaki, R.; Okabe, S. Comparison of gene expression profiles in HepG2 cells exposed to arsenic, cadmium, nickel and three model carcinogens for investigating the mechanisms of metal carcinogenesis. *Environ. Mol. Mutagen* **2009**, *50*, 46−59.
(37) Ratajewski, M.; Walczak-Drzewiecka, A.; Salkowska, A.; Dastych, J. Aflatoxins upregulate CYP3A4 mRNA expression in a process that involves the PXR transcription factor. *Toxicol. Lett.* **2011**, *205* (2), 146−153.
(38) Castorena-Torres, F.; de Leon, M. B.; Cisneros, B.; Zapata-Perez, O.; Salinas, J. E.; Albores, A. Changes in gene expression induced by polycyclic aromatic hydrocarbons in the human cell lines HepG2 and A549. *Toxicol. in Vitro* **2008**, *22* (2), 411−421.
(39) Staal, Y. C. M.; van Herwijnen, M. H. M.; van Schooten, F. J.; van Delft, J. H. M. Modulation of gene expression and DNA adduct formation in HepG2 cells by polycyclic aromatic hydrocarbons with different carcinogenic potencies. *Carcinogenesis* **2006**, *27* (3), 646−655.
(40) Sawada, H.; Takami, K.; Asahi, S. A toxicogenomic approach to drug-induced phospholipidosis: Analysis of its induction mechanism and establishment of a novel in vitro screening system. *Toxicol. Sci.* **2005**, *83* (2), 282−292.
(41) Wang, J. L.; Yin, D. P.; Liu, Y. X.; Baer, R.; Yin, Y. Dual specificity phosphatase 1/CL100 is a direct transcriptional target of E217−1 in the apoptotic response to oxidative stress. *Cancer Res.* **2007**, *67* (14), 6737−6744.
(42) Naranmandura, H.; Ogra, Y.; Iwata, K.; Lee, J.; Suzuki, K. T.; Weinfeld, M.; Le, X. C. Evidence for toxicity differences between inorganic arsenite and thioarsenicals in human bladder cancer cells. *Toxicol. Appl. Pharmacol.* **2009**, *238* (2), 133−140.
(43) Aiba, I.; Hossain, A.; Kuo, M. T. Elevated GSH level increases cadmium resistance through down-regulation of Sp1-dependent expression of the cadmium transporter ZIP8. *Mol. Pharmacol.* **2008**, *74* (3), 823−833.
(44) Liang, S.; Liu, C.; Song, L. F. Soluble microbial products in membrane bioreactor operation: Behaviors, characteristics, and fouling potential. *Water Res.* **2007**, *41* (1), 95−101.
(45) Kimura, K.; Naruse, T.; Watanabe, Y. Changes in characteristics of soluble microbial products in membrane bioreactors associated with different solid retention times: Relation to membrane fouling. *Water Res.* **2009**, *43* (4), 1033−1039.
(46) Barker, D. J.; Stuckey, D. C. A review of soluble microbial products (SMP) in wastewater treatment systems. *Water Res.* **1999**, *33* (14), 3063−3082.