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Combined effects of polyfluorinated and perfluorinated compounds on primary cultured hepatocytes from rare minnow (*Gobiocypris rarus*) using toxicogenomic analysis

Yanhong Wei[a], Xiongjie Shi[b], Hongxia Zhang[a], Jianshe Wang[a], Bingsheng Zhou[b], Jiayin Dai[a,*]

[a] *Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China*
[b] *State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, PR China*

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ABSTRACT

Polyfluorinated and perfluorinated compounds (PFCs) are used in numerous commercial products and have been ubiquitously detected in the environment as well as in the blood of humans and wildlife. To assess the combined effects caused by PFCs in mixtures, gene expression profiles were generated using a custom cDNA microarray to detect changes in primary cultured hepatocytes of rare minnows exposed to six individual PFCs (perfluorooctanoic acid, perfluorononanoic acid, perfluorodecanoic acid, perfluorododecanoic acid, perfluorooctane sulfonate, and 8:2 fluorotelomer alcohol) and four formulations of the PFCs mixtures. Mixtures as well as individual compounds consistently regulated a particular gene set, which suggests that these conserved genes may play a central role in the toxicity mediated by PFCs. Specifically, a number of genes regulated by the mixtures were identified in this study, which were not affected by exposure to any single component. These genes are implicated in multiple biological functions and processes, including fatty acid metabolism and transport, xenobiotic metabolism, immune responses, and oxidative stress. More than 80% of the altered genes in the PFOA- and PFOS-dominant mixture groups were of the same gene set, while the gene expression profiles from single PFOA and PFOS exposures were not as similar. This work contributes to the development of toxicogenomic approaches in combined toxicity assessment and allows for comprehensive insights into the combined action of PFCs mixtures in multiple environmental matrices.

1. Introduction

Polyfluorinated and perfluorinated compounds (PFCs), which consist of fluorine-containing chemicals with unique properties, have been used in a variety of commercial and industrial applications since the 1950s (Giesy and Kannan, 2001; Houde et al., 2006; Lau et al., 2007). The widespread use of PFCs has resulted in the ubiquitous detection of these contaminants in a wide variety of environments, wildlife, and human populations from around the world (Dai et al., 2006; Giesy and Kannan, 2001; for reviews, see Houde et al., 2006 and Lau et al., 2007). Many data have indicated that perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluorododecanoic acid (PFDoA), perfluorooctane sulfonate (PFOS), and fluorotelomer alcohol (FTOH) are the most commonly detected PFCs. PFOS and PFOA are predominant in environmental matrices and human blood (Monroy et al., 2008; Weihe et al., 2008; Yeung et al., 2009; Young et al., 2007).

A range of studies have shown that the liver is an important target organ of PFCs (Adinehzadeh and Reo, 1998; Lau et al., 2007; Liu et al., 2008; Wei et al., 2008a,b), and disturbance in fatty acid metabolism, lipid transport, cholesterol synthesis, cytochrome P-450 (CYP) mediated reactions, and oxidative stress are of the predominant effects in the liver (Biegel et al., 2001; Seacat et al., 2002; Haughom and Spydevold, 1992; Kennedy et al., 2004). Although a variety of studies have investigated the individual effects and mechanisms of PFCs, few studies have assessed the combined effects caused by PFCs in complex mixtures. Various PFCs have been demonstrated to commonly coexist in the environment as well as in organisms (Monroy et al., 2008; Weihe et al., 2008; Yeung et al., 2009; Young et al., 2007). The uncertainty of the combined effects and interactions between different PFCs has been a barrier in hazard identification and risk assessments of PFCs. Therefore, there is a need for studies investigating the combined effects of PFCs under co-exposure.

Recently, toxicogenomics based on microarray analysis have been increasingly applied in the characterization of the cellular

* Corresponding author. Tel.: +86 10 64807185.
E-mail address: daijy@ioz.ac.cn (J. Dai).

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effects of single and mixed chemical exposures (Bae et al., 2002; Finne et al., 2007; Hendriksen et al., 2007; Hook et al., 2008; Bjork et al., 2008; Rosen et al., 2008, 2007). The pattern of genes altered by exposure to a contaminant mixture may include a set of genes characteristic of exposure to a particular compound, thereby allowing identification of that compound as a component of the chemical mixture (Finne et al., 2007). In addition, non-additive interactions may be discerned by comparing the gene expression patterns resulting from exposure to the mixture with those identified from the individual components. These expression patterns yield novel insights into the effects of potential compound combinations. Possible additive or interactive combined actions could be addressed by the comparison between multiple chemicals yielding similar or different toxicities.

In this study, a primary cell culture of hepatocytes from rare minnows (*Gobiocypris rarus*) was established, taking advantage of minimization of the inter-individual variation from pooled samples and the small size of animal population for tests. Transcriptional profile analysis of the *in vitro* primary cell culture was utilized to delineate both the individual and combined effects of six common PFCs. The results from this analysis will provide useful data for development of toxicogenomic approaches in combined toxicity and the assessment of the potential effects involved in the co-exposure to PFCs.

2. Materials and methods

2.1. Chemicals and animals

PFOA (98%) was purchased from Acros Organics (Geel, Belgium). PFNA, PFDA, PFDoA, PFOS, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) culture medium (with L-glutamine and 15 mM HEPES, without phenol and sodium bicarbonate), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and collagenase IV were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). 8:2 FTOH (97%, CAS No. 678-39-7) was purchased from Alfa Aesar (MA, USA). Leibovitz's L15 medium and insulin–transferrin–selenium (ITS) were supplied by Gibco Life Technology (Paisley, UK). Each six of the compounds stock solution was prepared by dissolving it in dimethylsulfoxide (DMSO; Amresco, Solon, OH, USA). All other chemicals used in this study were of analytical grade.

Mature male rare minnows (about 9 months old) were generously provided by Prof. J.W. Wang of the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, People's Republic of China). Fish were maintained at $26 \pm 0.5\,^{\circ}C$ in a 16:8 h light:dark cycle in a continuous flow-through system in dechlorinated tap water and fed with *Artemia nauplii* twice daily. Since the induction of vitellogenin have been reported *in vivo* as well *in vitro* (Wei et al., 2007; Liu et al., 2007a), we speculated that PFCs mixture exposure may display the possible endocrine disruption *in vitro*. Thus the male fish were selected in the present study.

2.2. Cell culture and treatments

The isolation and primary culture of rare minnow hepatocytes methods were modified from Zhou et al. (2006). Briefly, fish were anesthetized on ice and then the hepatic tissues were carefully excised and transferred onto a glass petri dish, and rinsed with DMEM/F12 medium twice to remove the most of blood cells. The tissue was dissected into small pieces followed by collagenase IV digestion at room temperature for 30 min on a shaker (0.1% collagenase in PBS, pH 7.4, without Ca^{2+}). Then the hepatic tissues were softly triturated by pipetting with a narrow tip and filtered through 70 μm nylon mesh (Falcon, NJ, USA). The cell suspensions were then centrifuged at $150 \times g$ for 10 min at $10\,^{\circ}C$ and rinsed twice with DMEM/F12 medium. After the last wash, the cells were re-suspended in mixture cultured medium (50% DMEM/F12, 50% Leibovitz's L15, 200 i.u. mL^{-1} penicillin, 200 μg mL^{-1} streptomycin and 1% insulin–transferrin–selenium) and seeded at a density of 1.5×10^6 cells/mL (200 μL per well) in a 96 well "Primaria" plate (Falcon, NJ, USA). The cell culture was maintained at $26 \pm 0.5\,^{\circ}C$. Hepatocytes isolated from sixteen individual fish were used for one batch of experiments.

After 24 h culture, 100 μL medium together with unattached cells were removed per well. DMSO was used as the vehicle to make the stock solution for single PFCs that were concentrated solution more than 10,000-fold (that is the final concentration of DMSO was less than 0.1%). Chemicals in stock solution were diluted with cultured medium, and the PFCs mixtures were made by mixing the single PFCs solutions as the specific ratio. Then 100 μL cultured medium contained twofold of designed concentration of chemicals was added to each wells. A summary of the exposure design is presented in Table 1. The concentration used in this study was based on the previous *in vitro* studies on the effects of PFCs using hepatocytes from fish (Liu et al., 2007a,b). The M1 comprises equal ratios of mass of six PFCs and the M4 consists of equal ratios of mass of PFOA and PFOS. The M2 or M3 is mixed by 50% of PFOA or PFOS and the other five PFCs based on the fact that PFOA and PFOS are two of the most frequent and dominant PFCs detected in different environments and wildlife (Dai et al., 2006; Giesy and Kannan, 2001). Every treatment consists of six wells of cells and was performed in triplicate.

2.3. Cell viability assay

Hepatocytes viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after 44 h exposure, 20 μL of MTT (5 mg/mL in PBS) was added and the plates were incubated for additional 4 h at $26 \pm 0.5\,^{\circ}C$. Subsequently, the culture medium was removed and 100 μL DMSO was added to each well in order to dissolve the formazan crystals formed by mitochondria. After incubation for another 20 min, the absorbance was determined using a microplate reader (Molecular Device, SpectraMax M2, Union City, CA) at 570 nm. Each treatment was tested in triplicate. Hepatocyte viability data are presented as the percentage of living cells compared with the control. Differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison using SPSS v13.0 software. The criterion for statistical significance was $p < 0.05$.

2.4. RNA extraction

After 48 h exposure, total RNA was extracted from eighteen wells of cells in each treatment using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. To remove genomic DNA contamination, total RNA was digested by RNase-free DNaseI (Qiagen, Hilden, Germany) and then purified. Isolated RNA was quantified based on the A_{260} value. The purity of the RNA was assessed by determining the A_{260}/A_{280} ratio as well as visual inspection of 1.0 μg RNA on 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining.

2.5. Microarray analysis

The rare minnow cDNA microarray which contains 1773 unique genes was used in this experiment. In each array, total RNA from the pooled samples in each treatment was co-hybridized with that from the control. The microarray experiments were performed as described in the previous study (Wei et al., 2008b). Briefly, 1 μg

Table 1
Exposure design and concentrations used in treatment.

Group	No.	PFCs and concentration	
Single	H1	PFOA	15 mg/L
	H2	PFNA	15 mg/L
	H3	PFDA	15 mg/L
	H4	PFDoA	15 mg/L
	H5	PFOS	15 mg/L
	H6	FTOH(8:2)	15 mg/L
Mixture	M1	1/6 H1+ 1/6 H2+ 1/6 H3+ 1/6 H4+ 1/6 H5+ 1/6 H6	
	M2	1/2 H1+ 1/10 H2+ 1/10 H3+ 1/10 H4+ 1/10 H5+ 1/10 H6	
	M3	1/2 H5+ 1/10 H1+ 1/10 H2+ 1/10 H3+ 1/10 H4+ 1/10 H6	
	M4	1/2 H1+ 1/2 H5	

of high quality total RNA was transcribed into cDNA and labeled with fluorescent dye (Cy5 or Cy3-dCTP) via Eberwine's linear RNA amplification method. Arrays were hybridized in a CapitalBio BioMixer^{TM}II Hybridization Station overnight at 42 °C followed by washed with two consecutive solutions at room temperature to remove nonspecifically or unhybridized cDNAs. Arrays were scanned with a confocal LuxScan^{TM} scanner (CapitalBio, China), and the images were then analyzed using LuxScan^{TM}3.0 software (CapitalBio, China). Data were normalized using LOWESS program (Berger et al., 2004). Fold changes were calculated relative to controls. The statistical method of significance analysis of microarrays (SAM) was applied to evaluate the differences. The false discovery rate (FDR) lower than 1% combined with an average fold change greater than or equal to 1.5 was used to identify significantly differentially expressed genes. Only differentially expressed genes were selected for discussion in the present study. Hierarchical clustering of the expression data from the discriminatory gene set (FDR is lower than 1% as well as the average fold change greater than or equal to 1.5) of individual exposures as well as the M1 mixture exposure was performed.

2.6. Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to validate the results from the microarray analysis. The RNA for each treatment was the same pooled sample in microarray analysis. Synthesis of cDNA was performed using 1 μg of total RNA from each sample mixed with the oligo-dT primer using M-MuLV reverse transcriptase as described by the manufacturer (NEB, Beverly, USA). Quantitative real-time PCR was carried out using the QuantiTect SYBR Green PCR master mix kit (Takara, Dalian, China) and PCR amplification was conducted on a Stratagene Mx3000P qPCR system (Stratagene, USA). Gene names, accession numbers in GenBank, forward and reverse primer sequences, and amplicon sizes are listed in Table 2. Each sample was processed in triplicate. The expression level of each target gene was normalized to its glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content since the results of the microarray data indicated that expression levels of this gene were not significantly different between control and treatment groups. Fold differences in expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Cell culture and cytotoxicity assessment

A total of 3×10^6 single liver cells were obtained from 16 rare minnows by collagenase digestion. Freshly isolated hepatocytes were round and showed the morphology similar to that identified *in vivo* (results not shown). Most of the non-hepatocytes and dead cells were effectively removed by repeated low-speed centrifugation (Fig. 1(A)(a)). After 24 h of culturing, the majority of the hepatocytes aggregated and attached to the plate, forming a well-organized monolayer (Fig. 1(A)(b)). After 48 h of culturing, the hepatocytes were more rounded and formed clumps of living cells. After 96 h of culturing, the cells in the control group displaying as the big clumps were in good status and few were dead. After 120 h, a certain number of cells stop dividing and undergo the process of death. The 48 h exposure time was chosen to make sure that cells are healthy and gene expression profile changes are mainly response to chemicals exposure. Cells were visually inspected under a microscope to evaluate viability and cell number. Under the exposure, cells grown in medium supplemented with PFCs for 48 h displayed no significant morphological change, as compared to the control (Fig. 1(A)c and d).

Cytotoxicity assessment was performed using MTT reduction at 48 h after exposure. No obvious differences in MTT activity were detected from treatment with PFOA, PFNA, PFDA, 8:2 FTOH, M1, and M2, while a significant increase in formazan production was

Table 2
Gene-specific primers used in quantitative real-time PCR.

Gene	Accession no.	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	Amplicon size (bp)
GAPDH	EE397198	CGTGCTGCTGTCCAGTCCAA	GCCGCCTTCTGCCTTAACCT	138
Intelectin	EE394394	TGGGTTTGTGCTTCTGCTGT	CTGTTCCCCTCAAGGTCGTA	159
Calmodulin	EE393456	ACAAGGGAAAGTCTCCAGTC	GGAAGCCACTCACATAACAG	98
CYP3A	EE398218	AAGAAAGGGAATATGGGACT	TGTTTGGCTGTGACCACTAG	136
L-FABP	EE397881	GGAGGGTCACATATCAGGAA	TTGCCACTAAAACCAAGAGC	147
Dio2	EE396040	GACATCACCAGCCCAACATT	TGGAGCAGCATAGCGAAAGA	51
ANXA1	EE398297	TTCCTTCTCCAAGCGGTCTA	TGAAGTCGTTGCCGTTTTGT	200
ANXA4	EE397590	TTTGCGGATGTCGTTAGGTC	ATTCTCGCTCGTGATTGGTG	120
ApoB	EE397768	TATGCTCCATTTTGCTGCTT	TGACATTGCCGTGATACTTG	89
WTARP	EE396951	CCGTATTGACCCAAATGAGC	GAAAATGTTCCCAGCAGCCT	161
PA	EE393293	GGAGGGTCACATATCAGGAA	TTGCCACTAAAACCAAGAGC	147
SOX9b	EE397752	GACATCACCAGCCCAACATT	TGGAGCAGCATAGCGAAAGA	51

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L-FABP, fatty acid-binding protein 10, liver basic; Dio2: type II iodothyronine deiodinase; Annexin A1, ANXA1; Annexin A4, ANXA4; Apo B, apolipoprotein B; WTARP, warm-temperature-acclimation-related 65-kDa protein; PA, proliferation-associated 2G4 protein; SOX9b, transcription factor Sox9b.

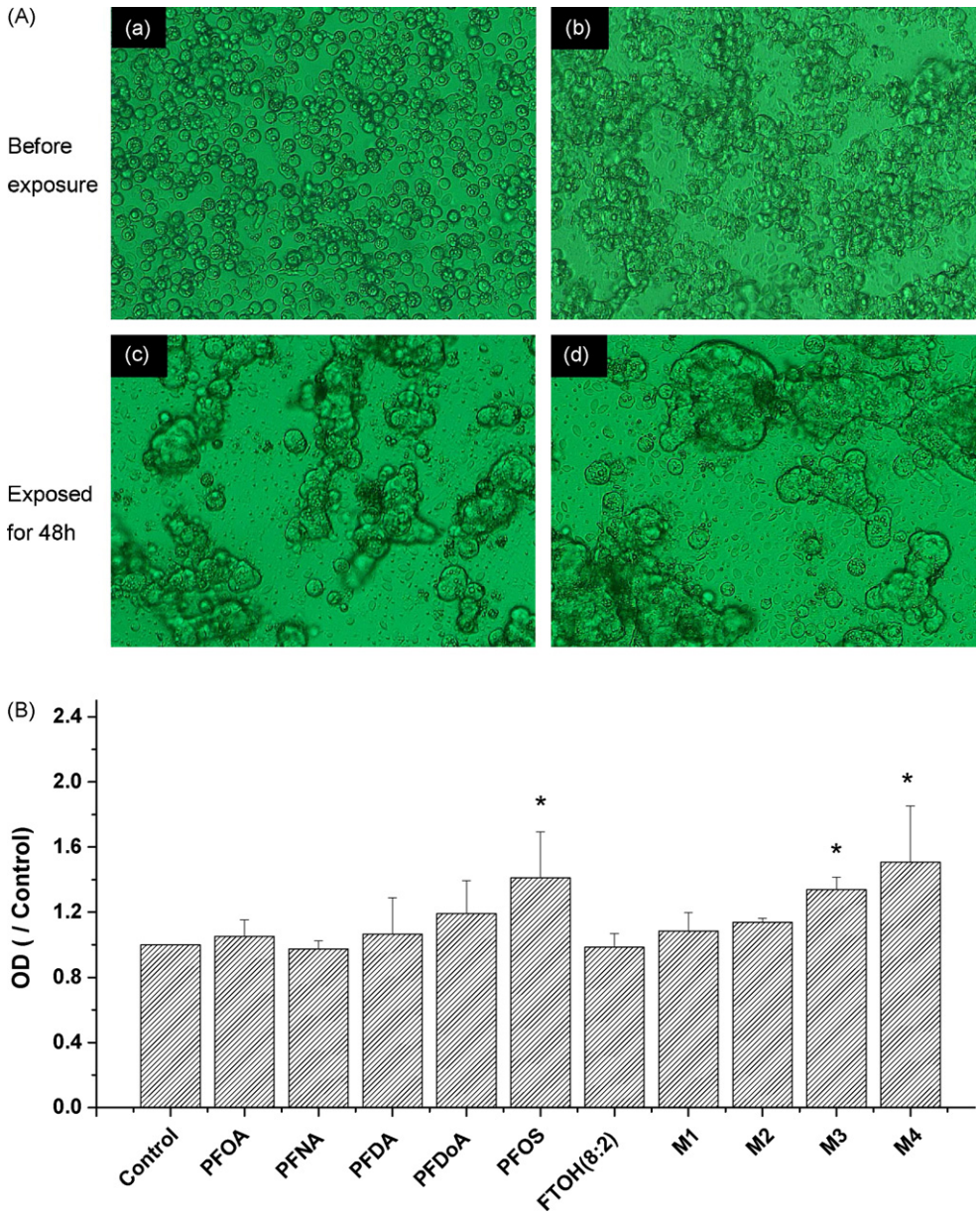

Fig. 1. Primary cultured hepatocytes from rare minnow and growth status under PFCs exposure. (A) The morphology of primary cultured hepatocytes. (a) 0 h, (b) 24 h, (c) controls, (d) PFCs exposure. (B) The viability of hepatocytes after PFCs exposure. Data were shown as divided by the control. *Significant difference as compared to the control ($p < 0.05$).

observed after exposure to PFOS, M3, and M4, as compared to the control (Fig. 1(B)). These results indicate that the compounds concentrations used in these experiments had no suppressive effects on cell survival.

3.2. Overview of gene expression profiles

Overviews of the number of regulated genes for each single and mixture exposure are presented in Fig. 2. The greatest numbers of gene expression responses were obtained for the M2 and M3 treatments, accounting for about 500 genes. These two groups are the PFOA- and PFOS-dominant groups, respectively. A total of 230 and 125 genes exhibited differential levels of expression in hepatocytes exposed to the M1 and M4 mixtures, which contained equal ratios of PFOA or PFOS and the other five PFCs, respectively. For single exposure experiments, the group of PFOA exposure, which included 159 upregulated genes and 204 downregulated genes, exhibited a greater number of altered genes than the other five groups. Only ten

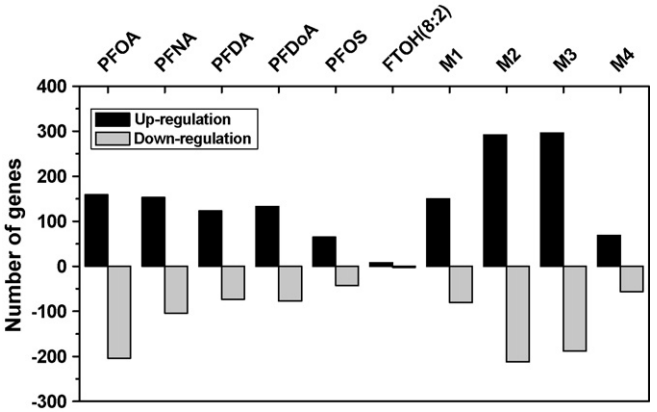

Fig. 2. Overviews of the gene expression under single and mixture PFCs exposures.

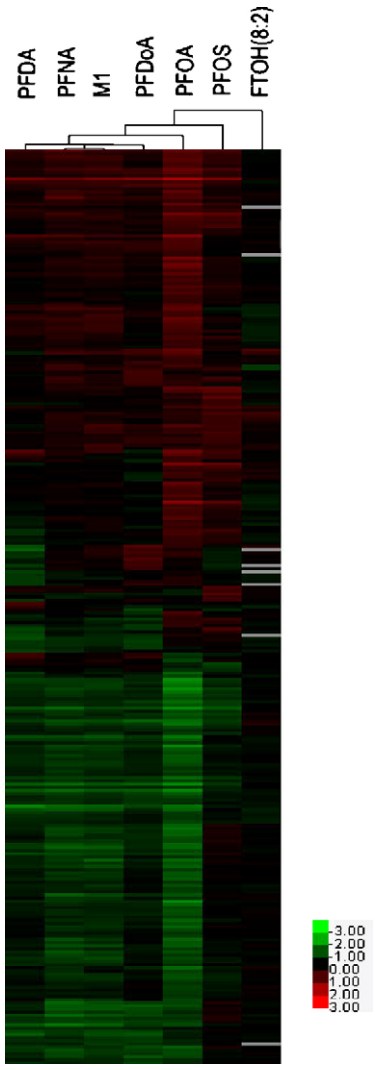

Fig. 3. Hierarchical clustering of the altered expressed gene data from the individual exposures as well as the M1 mixture exposure in terms of the fold-change.

alterations in gene expression were observed under exposure to 8:2 FTOH, including eight genes that were induced and two genes that were suppressed, as compared to the control. Overall, the number of upregulated genes was generally equal to that of the downregulated genes.

3.3. Gene expression analysis for individual exposures

Clustering analysis revealed that PFNA, PFDA, PFDoA, and PFOA have closer associations in altered genes profiles, as compared to PFOS and 8:2 FTOH (Fig. 3). The degree of overlap in differential expression for each individual PFCs exposure was analyzed (Fig. 4). The gene expressions altered by 8:2 FTOH were not affected by any of the five other PFCs. These five compounds resulted in the consistent upregulation of 11 genes, while 7 genes were downregulated. These consistently altered genes were assigned to known annotation groups (Table S1, supplementary materials), including CYP 3A, thioredoxin interacting protein, and fatty acid binding protein 10, liver basic (L-FABP).

3.4. Gene expression analysis for the M1 mixture

Exposure to the equal ratio of six PFCs induced the upregulations of 150 genes and the downregulation of 80 genes (Fig. 2). The gene expression profiles of M1 were compared to the gene responses of all of the individual PFCs (Fig. 4(A) (a) and (b)). Based on this comparison, 141 upregulated genes and 80 downregulated genes in M1 were consistent with those of the single exposure groups. However, 184 significantly upregulated genes and 222 significantly downregulated genes were affected by the individual compounds, but not by the M1 mixture. While nine genes were upregulated by the M1 mixture, as compared with the single exposures, which may suggest the combined effects of the mixture exposure. All of the genes altered by 8:2 FTOH exposure were not affected by M1. The alterations of genes in the M1 group, including the directions and fold changes, were more similar to those of the PFNA single exposure group, which well matches the clustering analysis results (Fig. 3).

Of the altered genes in the M1 group, 99 significantly altered genes were assigned to known annotation clusters (Table S2), with 13 genes consistently altered under exposure to the M1 mixture and all genes altered for the individual PFCs, except for 8:2 FTOH. Fourteen known genes altered by the M1 mixture were only affected by only one of the individual compounds.

3.5. Gene expression analysis for the M2 mixture

The expression of 292 genes was induced and 212 genes were suppressed under the exposure to a PFOA-dominant mixture (Fig. 2). The transcriptional responses to M2, PFOA and the other five single PFCs exposures were compared (Fig. 4(A) (c) and (d)). Among the altered genes, 84 upregulated genes and 44 downregulated genes exhibited common responses between M2 and PFOA. Additionally, 59 upregulated genes and 30 downregulated genes affected by at least one of the single compound were consistently regulated by M2. Interestingly, a number of genes were specifically affected following exposure to M2. Among these, 149 genes were uniquely upregulated and 138 genes were uniquely downregulated by the M2 mixture. The altered genes in the M2 group that were assigned to known annotation clusters are listed in Table S3.

3.6. Gene expression analysis for the M3 mixture

The present study also investigated gene expression patterns associated with the PFCs mixture consisting of 50% PFOS. Overall, 484 genes exhibited altered expression following exposure to M3, with 296 significantly upregulated and 188 significantly downregulated (Fig. 2). The comparisons of genes expressed profiles have been conducted among M3, PFOS and the other five single PFCs exposures (Fig. 4(A) (e) and (f)). Thirty-four upregulated genes and eighteen downregulated genes affected by M3 displayed similar directional responses with PFOS. A total of 152 genes changed (105 genes unregulated and 47 downregulated) in M3 relative to at least one of the other individual PFCs. M3 exposure uniquely affected 157 upregulated genes and 123 downregulated genes. These results demonstrated a significantly different alteration in hepatic gene expression between PFOS and M3. The altered genes in the M3 group assigned to known annotation clusters are listed in Table S4.

3.7. Gene expression analysis for the M4 mixture

In order to characterize the effect of the PFOS and PFOA combined toxicity, the alteration of gene expression in the M4 mixture, which consisted of equal ratios of these two PFCs, was analyzed (Fig. 4(A) (g) and (h)). The results demonstrated that exposure to the M4 mixture resulted in the upregulation of 69 genes and the downregulation of 56 genes (Fig. 2). Among these, 13 and 9 genes displayed consistent increases or decreases, respectively, in the expression responses among the PFOA, PFOS single exposure, and M4 groups. These results also demonstrated that the M4 mixture

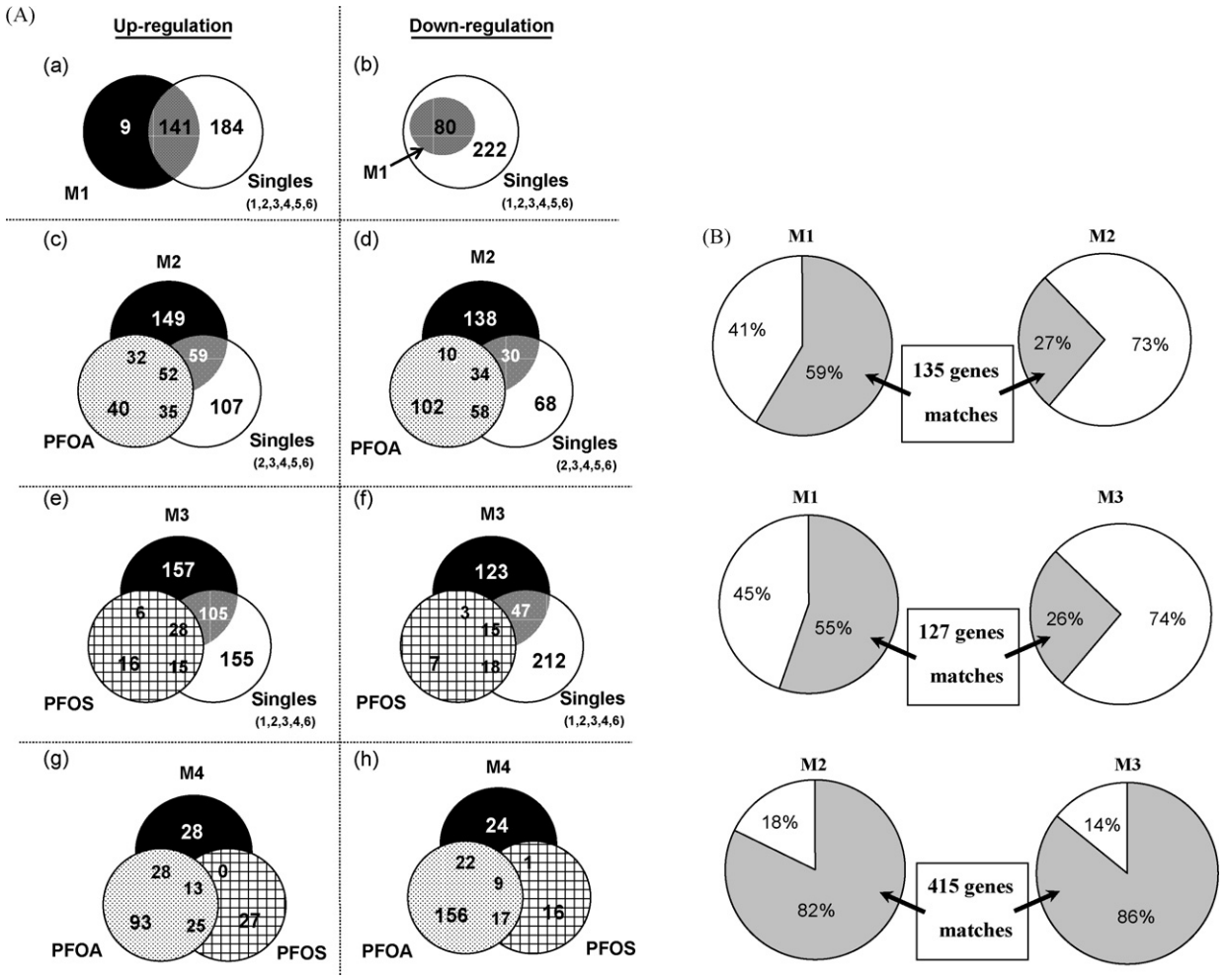

Fig. 4. The comparisons of gene expressions in the PFCs mixture groups. (A) Comparisons with the single exposure groups; (B) comparisons of mixtures consisting of six PFCs. M1 (equal ratio of PFOA, PFNA, PFDA, PFDoA, PFOS, and 8:2 FTOH), M2 (PFOA-dominant mixture), and M3 (PFOS-dominant mixture).

regulated 52 genes (28 upregulated and 24 downregulated), which were not affected by either of the two individual compounds. In addition, a total of 334 genes regulated by PFOA or PFOS was not affected by the M4 mixture. The altered genes in the M4 group assigned to known annotation clusters are listed in Table S5.

Additionally, the results demonstrate that the gene expression profiles induced by different component ratios of PFCs exhibited both similarities and discrepancies (Fig. 4(B)). A similar directional pattern of regulation was displayed for 135 genes in both M1 (59%) and M2 (27%). Similar directional responses were exhibited for 172 genes in both M1 (55%) and M3 (26%). In contrast, M2 and M3 revealed more similar action patterns, with 415 genes consistently regulated by M2 (82%) and M3 (86%).

Table 3
Comparison of fold change values of selected genes analyzed using microarray analysis and real-time PCR[a].

Gene name[b]	PFOA		PFNA		PFDA		PFDoA		PFOS		M1		M2		M3		M4	
	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR	Array	PCR
Intelectin	1.88	−1.32	2.33	3.71	2.63	6.77	2.08	4.32	–	–	2.20	3.39	2.77	10.27	2.59	9.00	1.92	4.38
Calmodulin	1.80	1.11	2.91	1.32	1.88	1.78	2.16	1.69	–	–	2.59	1.18	2.90	3.36	2.66	3.61	1.64	1.62
CYP3A	2.36	1.89	2.10	2.75	1.67	3.03	2.90	3.66	1.58	4.20	1.74	2.45	1.83	5.98	2.02	5.50	1.64	3.61
L-FABP	−1.73	−1.68	−2.22	−1.93	−2.32	−1.58	−2.02	−1.56	−1.74	−1.51	−2.04	−2.89	−1.59	1.53	−1.52	1.73	–	–
Dio2	–	–	2.81	2.17	2.57	2.07	2.14	1.82	–	–	2.72	1.65	−2.37	−5.03	−1.96	−4.76	–	–
Annexin A1	−1.66	−2.14	−1.63	−1.27	−1.33	−1.13	−2.04	−1.39	−1.80	−1.69	−1.82	−1.52	−1.86	−1.21	−1.72	−1.26	–	–
Annexin A4	−2.43	−2.03	−1.87	−1.80	−1.62	−1.33	−2.18	−1.72	−1.59	−2.08	−1.86	−1.89	1.73	4.44	1.67	3.78	−1.84	−2.04
ApoB	–	–	–	–	–	–	–	–	–	–	1.86	1.84	3.01	11.47	2.47	8.34	–	–
WTARP	–	–	–	–	1.70	1.02	1.64	1.77	–	–	–	–	4.43	3.18	4.89	3.18	–	–
PA	–	–	1.50	1.48	–	–	–	–	–	–	1.62	1.74	1.57	5.10	1.62	5.06	–	–
SOX9b	−1.82	−6.77	−1.91	−6.82	−1.98	−6.77	−1.73	−4.63	−1.74	−5.90	−2.24	−6.68	−1.54	3.20	–	–	−1.57	−10.13

[a] The fold changes were based on the comparison with the controls. The genes with fold changes lower than 1.5, as determined by microarray analysis, or with an FDR higher than 1%, as determined by SAM analysis, were not processed by real-time PCR and are noted by "–". Values >1 indicate upregulations, and values <1 indicate downregulations.

[b] L-FABP: fatty acid-binding protein 10, liver basic; Dio2: type II iodothyronine deiodinase; Apo B: apolipoprotein B; WTARP: warm-temperature-acclimation-related 65-kDa protein; PA: proliferation-associated 2G4 protein; SOX9b: transcription factor Sox9b.

3.8. Verification of gene expression data with quantitative real-time PCR

In order to validate the gene expression profiles measured by cDNA microarray analysis, 11 genes were selected and quantified by qRT-PCR. These genes may play the important roles and/or be implicated in the mechanism of PFCs effects according to the previous studies (Martin et al., 2007; Guruge et al., 2006; Hu et al., 2005). Based on the results from the microarray analysis, these genes were regulated not only by the individual compounds exposures, but also by the PFCs mixtures exposure groups. The genes selected for qRT-PCR validation are involved in immune response (intelectin), cellular regulation (calmodulin), xenobiotic metabolism (CYP3A), membrane trafficking (annexin A1, annexin A4), fatty acid metabolism and transport (L-FABP, ApoB, PA), warm-temperature-acclimation-related 65-kDa protein (WTARP), and hormone action (Dio2, Sox9b). These genes were regulated in most of the exposed treatments and may play important roles in the response to PFCs exposure (Table 3). The results from qPCR were in agreement with that from microarray except 4 cases showing the different direction of alteration between the two methods (Table 3).

4. Discussion

The present study utilized a toxicogenomic approach to assess the combinatory toxic actions of PFCs. Four perfluorinated carboxylic acids (PFCAs) differing in carbon chain length (PFOA, PFNA, PFDA, and PFDoA) and PFOS and 8:2 FTOH, were selected. These six PFCs are ubiquitous in the environment and are found in the blood of humans and wildlife. The experiment was performed using mixtures of these six PFCs at four specific ratios with comparisons to single compound exposures. The results demonstrated that the PFCs mixtures exposures revealed similar cellular responses to that by single chemical exposures as well as novel molecular responses.

Although PFCs levels in surface waters range from 0.011 to 2270 μg/L, total PFCs downstream of spills can range up to 17 mg/L (Moody et al., 2001, 2002). Besides, the bioaccumulation has been detected in the monitoring studies of PFCs. For example, the average concentrations of PFOS in the fish from New York State in the United States (ranging from 9 to 315 ng/g wet weight) were 8850-fold greater than those in surface water (Sinclair et al., 2006). Likewise, higher concentrations of PFOS have been detected in the liver (7760 ng/g wet weight) of plaice (*Pleuronectes platessa*) (Hoff et al., 2003) and feral gibel carp (*Carassius auratus gibelio*) (up to 9031 ng/g wet weight) in Belgium (Hoff et al., 2005). In the studies of chemicals regarding the possible modes of function, relatively higher concentrations of compounds are often used to elicit clearly distinguishable effects that allow possible toxic mechanisms to be determined (Lema et al., 2007). In the present study, we used the 15 mg/L PFCs and the different ratios of PFCs mixtures based on the constitution of environmental PFCs (PFOS and/or PFOA are predominant) to investigate the combined effects of PFCs as well as the potential modes of action. Based on the MTT results, the PFCs treatments under the dosages and duration in this study had no suppressive effects on cell survival.

Clustering analysis revealed that PFNA, PFDA, PFDoA, and PFOA have closer associations in altered genes profiles, as compared to PFOS and 8:2 FTOH. The result may be attributed to the variances in chemical structure, as the four PFCAs only differ in the length of the carbon chain and PFOS and 8:2 FTOH are different subtypes of PFCs with different functional groups on the carbon chain. The study demonstrated a common set of genes with expression regulated in not only the individual compounds exposure groups, but also the four PFCs mixtures exposure groups. The results suggested that this gene set may play an important role in PFCs toxic mechanisms. For example, CYP3A was significantly induced in all four PFCs mixture groups as well as in the individual PFCs groups, except for the exposure to 8:2 FTOH. Although few data demonstrated the implications of CYPs in the metabolisms of PFCs, studies have confirmed that the expressions of CYPs were altered under the exposure to PFOA or PFOS (Guruge et al., 2006; Hu et al., 2005).

In the present study, the M2 and M3 mixture, consisting of 50% PFOA or PFOS and 10% of each of the other PFCs, was designed to investigate the combined effects in the case in which PFOA or PFOS is the major component. Several genes exhibited conserved expression responses between single (PFOA or PFOS) and mixtures exposures, suggesting that effects mediated by the major components of PFOA or PFOS in mixtures are associated with the actions of mixtures. Whereas a number of genes affected by mixtures were not altered relative to any of the individual components, indicating differential modes of action of mixture as compared with single PFCs exposures.

The primary effects of PFOA- or PFOS-dominant exposure were associated with fatty acid beta-oxidation, biosynthesis and transport, xenobiotic metabolism, immune response, and cell death. Besides, the effects of PFOA-dominant exposure were also reflected by the decrease in the expression of WTARP (1.67-fold, shown in Table S3 in supplementary materials), which is related to acute phase response, and genes involved in mitochondrial oxidative phosphorylation. The effect of PFOS-dominant exposure was represented by the upregulation of superoxide dismutase 2 (1.90-fold, shown in Table S4 in supplementary materials) (Fig. 5A).

Because of the physical and chemical properties of PFCs, they might act as structural analogues of endogenous fatty acids in cells that then affect genes involved in transport of lipids, particularly fatty acids (Guruge et al., 2006). Using microarray technique, PFOS and PFOA was found induced fatty acids metabolism genes like acetyl-coenzyme A dehydrogenase, acly-CoA synthetase, and acly-CoA oxidase in rat (Guruge et al., 2006; Hu et al., 2005). In our previous *in vivo* study, rare minnows exposed to PFOA for 28 d exhibited the suppression of genes related to fatty acid biosynthesis and mitochondrial fatty acid beta-oxidation (Wei et al., 2008a). PFDoA exposure changed several genes involved in lipogenesis and lipid transport in rat (e.g. HMGR, stearoyl-CoA desaturase 1, and acetyl-CoA carboxylase 1) (Zhang et al., 2008). In this study, hepatocytes from rare minnow exposed to PFCs for 48 h did not show significant alteration in gene expression associated with fatty acid metabolism, while the upregulations of apolipoprotein B and fatty acid synthase and the downregulations of C-4 to C-12 straight chain acyl-coenzyme A dehydrogenase were displayed in PFOA- and PFOS-dominant mixture groups (Fig. 5A). Likewise, the effects on fatty acid biosynthesis and beta-oxidation also occurred under the exposure to equal ratio of PFOA and PFOS (Fig. 5B). Moreover, the genes expression related to fatty acid biosynthesis and transport showed the antagonistic effects of PFCs. The combined effects of PFCs co-exposure complicate the modes of action in disturbance of fatty acid metabolism and transport.

Unlike CYP3A, CYP1A was not induced in any of the 15 mg/L individual PFCs within 48 h but demonstrated a dramatic upregulation in the PFOA- or PFOS-dominant mixtures exposure groups (M2 and M3). Previous studies observed that CYP1A expression showed a 2-fold increase in Atlantic salmon (*Salmo salar*) primary cultured hepatocytes after exposure to 25 mg/L PFOS for 24 h, and CYP1A showed a pattern differing from that of PFOS after 48 h of exposure (Krøvel et al., 2008). These results suggest higher induction of CYP1A transcription by PFCs mixtures rather than by a single exposure.

Exposure to equal ratios of PFOA and PFOS in the M4 group enhanced and diminished expression of different gene clusters. Expression of gene clusters related to fatty acid biosynthesis and beta-oxidation (C-4 to C-12 straight chain acyl-coenzyme A dehydrogenase, delta-6 fatty acyl desaturase),

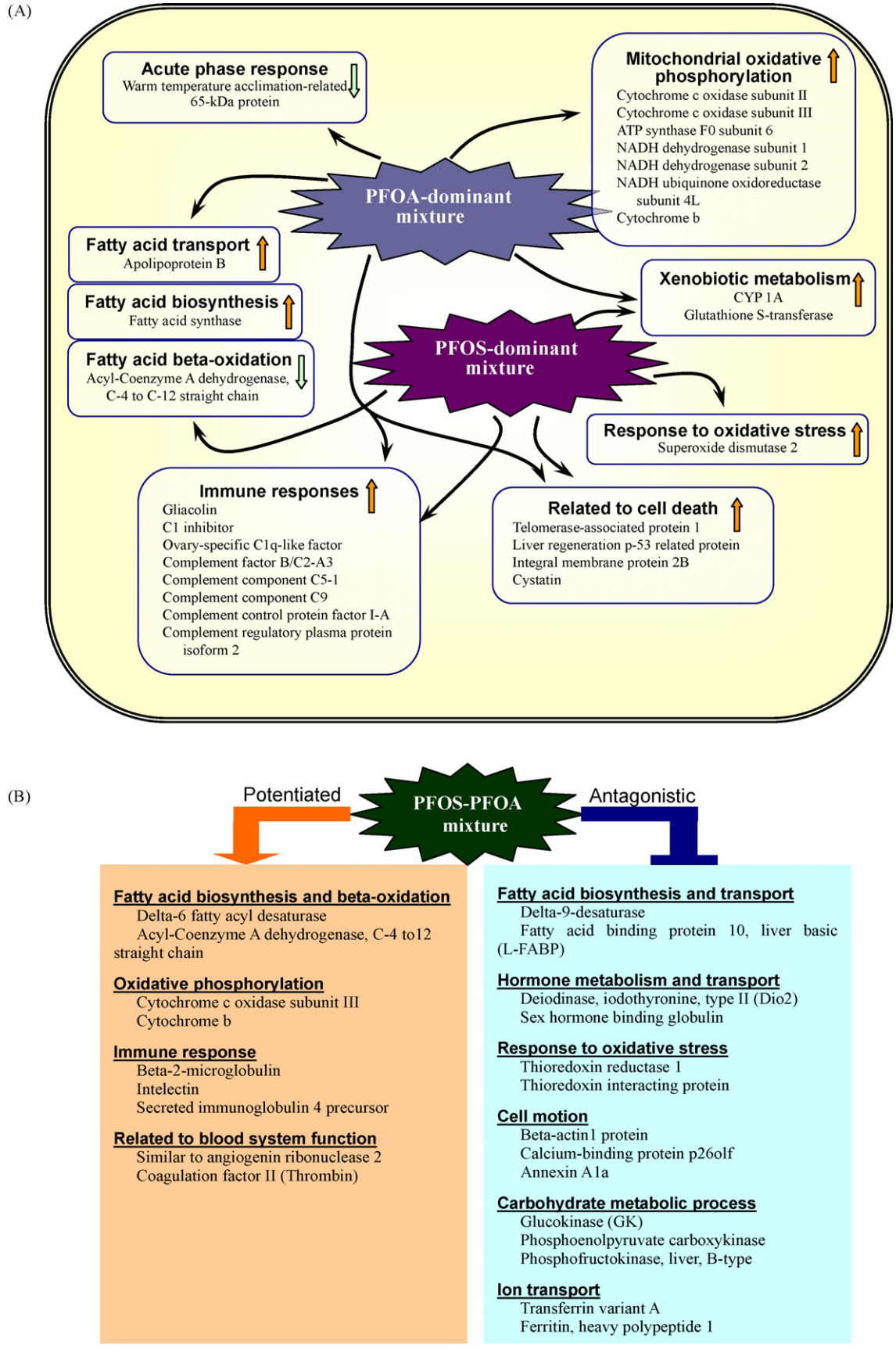

Fig. 5. The combined effects of PFCs mixtures on hepatocytes from rare minnow. (A) The novel effects of PFOA- or PFOS-dominant mixtures which were not detected in single PFCs exposure groups. (B) Alteration of genes under the PFOA–PFOS mixture, but not individual compounds.

oxidative phosphorylation (cytochrome *c* oxidase subunit III and cytochrome b), immune response (beta-2-microglobulin, intelectin and secreted immunoglobulin 4 precursor), and related to blood system function (similar to angiogenin ribonuclease 2 and coagulation factor II) were increased. Expression of gene clusters involved in oxidative stress, the metabolism of carbohydrates and fatty acid, and translation were diminished. The genes altered in M4 provided reasonable targets for investigating the mechanism elicited by the mixture of PFOA and PFOS. Notably, CYP1A and cytochrome *c* oxidase were induced in the M2 and M3 mixture but were not affected by the M1 and M4 mixture. The results indicate that distinct mechanisms may exist in PFCs mixtures relative to the different component ratios.

The results from comparisons of the genes altered by M1, M2, and M3 exposure suggest that although M2 and M3 consist of different major components of PFOA or PFOS, similarity was shown in the gene expression profiles, whereas the gene expression profiles from single PFOA and PFOS exposure were not as similar as those of the M2 and M3. These results further complicate the demonstration of the combined effects and mechanisms of PFCs co-exposure.

The likely interpretation of the combined effects is that the co-exposure to PFOA or PFOS in combination with other five PFCs affect distinctly different functions and then actives multiple cellular response targets. Subsequently, it generates complex cross-talk influences which may cause cascades of events in the biologic system. In a previous study, Finne et al. (2007) suggest a AhR-ER cross-talk may be occurred in the 17α-ethinylestradiol (EE2), 2,3,7,8-tetrachloro-di-benzodioxin, paraquat and 4-nitroquinoline-1-oxide mixture exposure. In addition, combined effects could take place at various levels, including metabolism, signal-transduction, regulation of gene transcription and post-transcriptional modification. Besides, individual PFCs may temporally and spatially regulate expression of distinct sets of genes. When multiple PFCs are present, they may elicit novel signaling patterns affecting global gene regulation. It has been reported that sequential exposure to N-methyl-N′-nitro-N-nitrosoguanidine, arsenic, and metal mixture results in a differential gene expression response pattern as compared to exposure to a single agent (Bae et al., 2002). In the setting of PFCs, chemical interactions among PFCs of different structure or length of carbon chain may occur, giving rise to differential effects on the exposed cells. Although the possible mechanisms of combined effects of PFCs have not been previously reported, the present study indicates that PFC mixtures can elicit effects differing from exposure to a single PFC. Therefore, more attention should be paid to the effects of particular PFC combinations in the assessment of PFC exposure.

The primary cultured hepatocyte model from rare minnow has been successfully established and utilized in toxicological tests in the current study. The *in vitro* cell culture model has many advantages, particularly the minimization of inter-individual variation by collecting cells from pooled samples. Accordingly, the sample size will be conserved, which benefits the assessments of combined toxicity and effects. Moreover, the construction of primary cultured hepatocytes from rare minnow will allow for the further development of the use of rare minnow in toxicological assessments.

In summary, our custom cDNA microarray studies identified a large number of transcriptional changes in rare minnow primary cultured hepatocytes exposed to six common PFCs, specifically PFOA, PFNA, PFDA, PFDoA, PFOS, and 8:2 FTOH, as well as combinations of these PFCs at four specific ratios. The results in the present study suggest that the modes of action of PFC co-exposure may be complex. The genes implicated in our microarray studies are involved in multiple biological functions and processes, including fatty acid metabolism and transport, xenobiotic metabolism, immune response, and oxidative stress. These genes provide clues to the molecular modes of action of PFC co-exposure and may allow for the formation of a set of PFC-responsive genes in order to monitor and assess toxicological risks of PFC exposure in different environments. The present study investigated the effects of PFCs at relatively high concentrations compared to actual environmental levels. Further research will be required to analyze the PFC mixtures toxicology using concentrations reflecting environmental levels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.07.020.

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