The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.37, No.2, 245-254, 2012

Original Article

Toxicity evaluation of glyphosate agrochemical components using Japanese medaka (*Oryzias latipes*) and DNA microarray gene expression analysis

Masaya Uchida[1,2], Shota Takumi[3,4], Keiko Tachikawa[3], Ryoko Yamauchi[3], Yoshiyuki Goto[1], Hiromi Matsusaki[3], Hiroshi Nakamura[2], Yoshihiro Kagami[2], Teruhiko Kusano[2] and Koji Arizono[3]

[1]Graduate School of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100 Tsukide, Kumamoto, 862-8502 Japan
[2]Ecogenomics, Inc., 1-1 Hyakunenkouen, Kurume, Fukuoka, 839-0864 Japan
[3]Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100 Tsukide, Kumamoto, 862-8502 Japan
[4]Environmental Health Sciences Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, 305-8506 Japan

(Received October 7, 2011; Accepted December 13, 2011)

ABSTRACT — Using glyphosate agrochemical components, we investigated their acute toxicity to juvenile Japanese medaka (*Oryzias latipes*) as well as their toxic impact at gene expression level on the liver tissues of adult medaka using DNA microarray. In our acute toxicity test, juvenile medaka were exposed for 96 hr to each of the following glyphosate agrochemical components: 10~160 mg/l of glyphosate, 1.25~20 mg/l of fatty acid alkanolamide surfactant (DA), and 12~416 mg/l of a fully formulated glyphosate herbicide. As a result, LC_{50} values of glyphosate, DA, and the glyphosate herbicide were > 160 mg/l, 8.5 mg/l, and 76.8 mg/l, respectively. On the other hand, adult male medaka fish were exposed to each of the glyphosate agrochemical components for 48 hr at the following concentrations: 16 mg/l of glyphosate, 0.5 mg/l of DA, and 16 mg/l-glyphosate/0.5 mg/l-DA mixture. Interestingly, DNA microarray analysis revealed that there were no significant gene expression changes in the medaka liver after exposure to glyphosate. Nevertheless, 78 and 138 genes were significantly induced by DA and the glyphosate/DA mixture, respectively. Furthermore, we identified five common genes that were affected by DA and glyphosate/DA mixture. These results suggested that glyphosate itself possessed very low toxicity as previously reported by some researchers at least to the small laboratory fish, and the major toxicity of the glyphosate agrochemical resided mainly in DA and perhaps in unintentionally generated byproduct(s) of glyphosate-DA mixture.

Key words: Glyphosate, Fatty acid alkanolamide (DA), Japanese medaka (*Oryzias latipes*), Acute toxicity, DNA microarray, Gene expression

INTRODUCTION

Glyphosate agrochemicals are widely used herbicides in the world, and their component residues often become pollutants of rivers and surface waters (Cox, 1998; IFEN, 2006). They consist of glyphosate and one or more surfactants, and it was reported that these components often act synergistically to be the cause of environmentally toxic effects (Benachour *et al.*, 2007).

Glyphosate is weak organic acid consisting of one glycine molecule with a single phosphonomethyl moiety. Its empirical formula is $C_3H_8NO_5P$, and it is usually forming salt of de-protonated acid and a cation such as isopropylamine or trimethylsulfonium. The purity of technical grade glyphosate compound is generally above 90%, and it is an odorless white crystalline powder with specific gravity of 1.704. Its vapor pressure is very low, and it is highly soluble in water. The octanol-water partition coef-

Correspondence: Masaya Uchida (E-mail: uchida@ecogenomics.co.jp)

ficient (log K_{ow}) is -2.8, and thus glyphosate compound is amphoteric and may exist as a different pH-dependent ionic species. Identification of glyphosate is generally laborious, complex, and costly, and derivatization with fluorogenic substances is the most common method being applied pre- or post-column (IPCS, 1994). The glyphosate that is formulated in agrochemicals inhibits plant growth by the enzymatic inhibition of enolpyruvylshikimate phosphate synthase (Williams *et al*., 2000).

Surfactants and other minor substances are included in various formulations of commercial agrochemical herbicides. Although each of them as a single chemical may be considered "inert", these surfactants and substances often become toxicologically effective when they are combined with one another. However, these less toxic and minor ingredients are not often identified as toxic ingredients, because in most cases only the active major ingredients are assessed for the product's toxicity for chemical registration (Peixoto, 2005; Cox and Surgan, 2006).

Increased use of glyphosate-based herbicides (glyphosate and fatty acid alkanolamide (DA) are usually two major components) in recent agriculture may be a part of the cause of ecological imbalances in the aquatic ecosystems. A few toxicity study reports in China showed that one glyphosate agrochemical and its major components were investigated extensively, and this particular agrochemical was concluded to be non-harmful in their environmental toxicity evaluation (Tsui and Chu, 2003, 2008). However, the other studies reported some adverse (and potentially toxic) effects of the same glyphosate agrochemical on various biological processes (e.g. energy metabolism, free radical formation, acetylcholine esterase activity, etc.) (Rendón-von Osten *et al*., 2005; Glusczak *et al*., 2006, 2007; Langiano and Martinez, 2008). Additionally, micronucleus test and Comet assay showed that this glyphosate agrochemical, in fact, affected the abovementioned biological processes in some fish species such as *Tilapia rendalii* (Grisolia, 2002), *Prochilodus lineatus* (Cavalcante *et al.,* 2008) and *Carassius auratus* (Çavas and Könen, 2007). Furthermore, glyphosate by itself once had shown crucial influences upon immune responses in *Tilapia nilotica* (el-Gendy *et al*., 1998), and produced histological changes in hepatocytes of *Oreochromis niloticus* (Jiraungkoorskul *et al.*, 2003) and *Cyprinus carpio* (Szarek *et al.,* 2000) (Table 1).

Thus, the toxicity studies of glyphosate-based agrochemicals have been carried out so far in many laboratories using fish and other aquatic organisms, and quantity of those studies are still increasing. Nevertheless, studies with genomics technology application for toxicity evaluation and risk assessment/prediction have barely been reported. Consequently, the main purpose of this study was to investigate toxicological effects of the major components of a glyphosate-based agrochemical: glyphosate and fatty acid alkanolamide (DA), first by performing acute toxicity test using toxicologically more sensitive juvenile medaka to determine LC_{50} for each of the chemicals, and subsequently by gene expression analysis with medaka DNA microarray and the chemical-exposed adult medaka liver samples. Choosing glyphosate-, DA-, and glyphosate/DA-exposed liver as the samples for microarray gene expression analysis was based upon the liver being a major detoxification-metabolic organ and on our expectation of hepatic gene expression patterns which would provide us some insights for better understanding molecular toxicology of the glyphosate agrochemicals.

Table 1. Recent published toxicity studies on glyphosate agrochemical formulation

Toxicity	Organism*	Concentration (exposure time)	Assay	Auther (publication)
DNA damage	*Pprochilodus lineatus*	10 mg/l (6, 96 hr)	comet assay	Cavalcante *et al.* (2008)
	Carassius auratus	5, 10, 15 mg/l (48, 96 hr)	comet assay	Çavas and Könen (2007)
Oxidant stress	*Rhamdia Quelen*	0.2, 0.4 mg/l (96 hr)	AChE activity TBARS activity	Glusczak *et al.* (2006)
	Pprochilodus lineatus	10 mg/l (6, 24, 96 hr)	Catalase activity	Langiano and Martinez (2008)
Histopathological change	*Oreochromis niloticus*	5, 15 mg/l (3 months)	histological observation	Jiraungkoorskul *et al.* (2003)

* : scientific name

MATERIALS AND METHODS

Chemicals

N-(phosphonomethyl) glycine (a.k.a. glyphosate, > 99.3% purity: Cas No. 1071-83-6) was obtained from Wako pure Chemical Industries, Osaka, Japan. The surfactant component of a herbicide used in this study was fatty acid alkanolamide (DA), and it was purified from the herbicide using Sep-Pak® Vac C18 cartridge (Waters Corporation, Milford, MA, USA). The analysis of DA was carried out by high-performance liquid chromatography/mass spectrometry (LC/MS). The LC/MS instrument that we used was Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) connected to Agilent 6410 triple quadrupole mass spectrometer. The LC/MS analysis conditions were shown in Table 2.

Test fish

Japanese medaka (*Oryzias latipes*) was obtained from National Institute for Environmental Studies (Ibaraki, Japan). They were maintained in 5-liter glass aquaria with flow-through system of water temperature at 24 ± 1°C and light:dark cycle of 16 hr : 8 hr. Fish were fed twice daily with brine shrimp (within 24 hr post-hatch) (El-Alfy *et al*., 2002).

Acute toxicity test for juvenile medaka

For each treatment, fifteen juvenile medaka (within 24 hr post-hatch) were placed in glass beakers containing 100 ml of each test solution at 24 ± 1°C with a 16 hr : 8 hr light:dark cycle (Ishibashi *et al*., 2005). Each group of juvenile medaka was exposed to each concentration (glyphosate: 10~160 mg/l, DA: 1.25~20 mg/l, a glyphosate-based herbicide: 12~416 mg/l) prepared by diluting each of the stock solutions with control water (de-chlorinated, activated charcoal-filtered, and UV-sterilized). Each treatment was conducted at 24 ± 1°C for 96 hr. Juvenile medaka fish were not fed during the entire test period, the fish were observed daily under stereoscopic microscope, and the dead fish (identified by the absence of heartbeat) were removed daily. The test was performed in triplicate, and 96-hr median lethal concentration (LC_{50}) was calculated by Probit method.

Exposure design for microarray analysis

Based on 96-hr LC_{50} data obtained from the juvenile medaka acute toxicity test, chemical exposure concentration in the test for microarray analysis was determined. Adult male medaka fish (3-month-old Orange-red strain, body length 280 ± 37 mm, body weight 29 ± 1 mg) exposed to each chemicals at nominal concentration (16 mg/l of glyphosate, 0.5 mg/l of DA, and the mixture of 16 mg/l glyphosate + 0.5 mg/l of DA) in 2-l glass tanks for 48 hr at 24 ± 1°C. None of the tested fish was fed during the entire exposure period. After 48 hr of exposure, the tested fish were sacrificed and their livers were removed. This liver sample collection was performed in Petri dish filled with ice-cold RNA*later*® solution (Sigma-Aldrich Corporation, St. Louis, MO, USA), and the samples were immediately stored frozen at -30°C until total RNA extraction and purification process was carried out.

Table 2. Instrumental condition of liquid chromatography mass spectrometer

HPLC instrument: Ultimate3000 (Dionex corporation, Osaka, Japan)
Column: Xterra MS C18 (C18, 2.5 μm, 2.1 mm × 50 mm, Waters, Milford, USA)
Column temperature: 40°C
Mobile phase A: 10 mmol/l ammonium acetate (pH 5) B：methanol
Gradient elution program: 0 min (A:B = 90:10) → 45 min (0:100) → 60 min (0:100)
Flow rate: 0.2 ml/min
Injection volume: 10 μl
MS instrument: microTOF II (Bruker Daltonics, Billerica, MA, USA)
Ionization mode: ESI (positive)
Capillary voltage: 4000 V
Dry gas: Nitrogen (180°C, 8 l/min)
Capillary exit voltage: 100 V
Scan range: m/z 50-1000

Medaka DNA microarray gene expression analysis

In order to understand molecular toxicity of the major components of a glyphosate-based herbicide, we carried out gene expression analysis with medaka DNA microarray on adult medaka liver samples (9 fish per condition, and 3 fish were pooled to make n = 3 per condition) where those fish were exposed to glyphosate, DA, and the mixture of these two chemicals.

Total RNA samples were extracted from the exposed medaka liver samples with RNeasy Mini Kit (Qiagen, Hilden, Germany). Integrity of the extracted total RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and quality assured total RNA samples were then used in DNA microarray analyses. For DNA microarray hybridization, antisense RNA (aRNA) was prepared by Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX, USA), and Cy5 fluorescent dye (GE Healthcare, Little Chalfont, UK) was used to label the purified aRNA samples.

The DNA microarray that we used for this study was Medaka EG 6000 oligo DNA microarray, which was manufactured by Ecogenomics, Inc. This microarray contained 6000 oligo DNA probes (35-base-long, duplicated spots) that were designed from the medaka cDNA sequences available at NRBP medaka database (http://www.shigen.nig.ac.jp/medaka/). Cy5-labeled aRNA samples were used in hybridization with the oligo DNA probes on the microarray, and it was carried out for 16 hr at 45ºC in a hybridization solution (6 x SSPE, 0.05% Tween-20, 20 mM EDTA, 25% formamide, 100 ng/µl salmon sperm DNA, 0.04% SDS). After the hybridization reaction, washing was performed in the following order and frequency of wash solutions: twice with 6 x SSPE and 0.05% Tween-20 at 45ºC; twice with 3 x SSPE and 0.05% Tween-20 at room temperature; twice with 0.5 x SSPE and 0.05% Tween-20 at room temperature; twice with 2 x PBS and 0.1% Tween-20 at room temperature; and finally twice with 2 x PBS at room temperature. Each of the post-wash microarrays was scanned with GenePix 4000B scanner at 5 µm resolution (Axon Instruments, Union city, CA, USA), and expression signal intensities were digitally processed using Microarray Imager (Combimatrix Corp., Mukilteo, WA, USA).

Statistical analysis

Statistical analysis of the microarray data was carried out as follows: the expression signals obtained from each of the 6,000 genes probes were background signal-subtracted and normalized by the expression signal of ribosomal protein L7 within each microarray's dataset (Zhaobin and Jianying, 2007). Then, statistics was performed using ArrayStat z-test (Imaging Research Inc, Piscataway, NJ, USA) with offset correction and $p < 0.05$ (significance cutoff) to obtain average expression ratio of the exposure group (n = 3) to the control group (n = 3) for each of the genes on the microarray.

RESULTS

Acute toxicity for juvenile medaka

Acute toxicity tests were carried out using glyphosate-, DA-, and glyphosate-based herbicide-exposed juvenile medaka (24 hr post-hatch, n = 10 fish per condition), and their results were shown in Fig. 1. The toxicity data were processed by Probit method, and 96-hr-exposure LC_{50} of glyphosate, DA, and Roundup® herbicide were >160 mg/l, 8.5 mg/l, and 76.8 mg/l, respectively (Table 3).

Gene expression analysis

Using medaka EG 6000 DNA microarray, we assessed molecular toxicity effects of glyphosate, DA, and glyphosate/DA mixture exposure to the livers of adult medaka. Male medaka fish (9 fish per condition, and 3 fish were pooled to make n = 3 per condition) were exposed for 48 hr to each chemical (16 mg/l of glyphosate, 0.5 mg/l of DA, and mixture of 16 mg/l of glyphosate and 0.5 mg/l of DA), and then gene expression profiles in their liver tissues were analyzed.

Interestingly, no statistically significant ($p < 0.05$) differential expression was observed in the liver samples exposed by glyphosate. On the other hand, we observed significant ($p < 0.05$) expression changes (expression ratio of exposed to control > 2 or < 0.5) in 78 genes (44 genes were up-regulated and the 34 genes were down-regulated) by the exposure of DA (Table 4), and furthermore, 138 genes (125 genes were up-regulated and 13 genes were down-regulated) were observed to be affected by the exposure to the glyphosate/DA mixture (Table 5). The number of genes responded to the mixture exposure was detected more than the number responded either to glyphosate or to DA. There were merely 5 genes found responding to both exposures of DA and glyphosate/DA mixture (Fig. 2).

DISCUSSION

World Health Organization (WHO) reported in 2005 that glyphosate agrochemical and its components possessed very low acute toxicity by oral and dermal administration routes. However, many of the glyphosate agrochemical products contained not only the active ingredients but also surfactants as one of the major ingre-

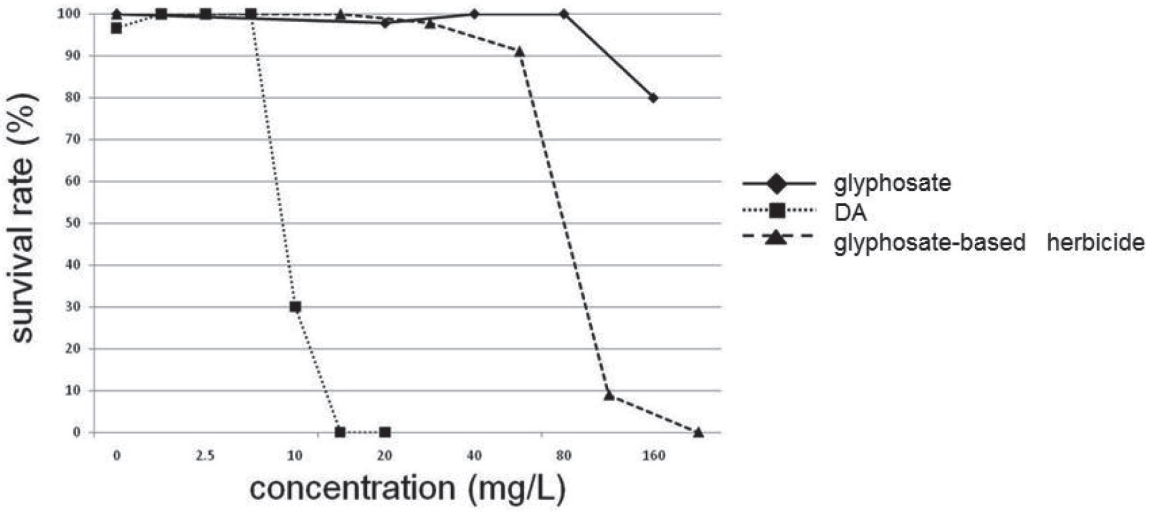

Fig. 1. Survival rate of juvenile medaka during 96 hr of acute toxicity test using glyphosate, fatty acid alkanolamide (DA), and a glyphosate-based herbicide (composed mainly of glyphosate and DA).

Table 3. LC_{50} values of pure glyphosate alone, DA alone, and Roundup® herbicide that were exposed to juvenile medaka for 96 hr.

Chemicals	96 hr LC50 values (mg/l)
glyphosate	> 160
DA	8.5
glyphosate-based herbicide	76.8

dients, and in some cases, the surfactant chemicals had more toxicity to the target organism than the active ingredients (Amarante *et al*., 2002). In fact, there was a report showing that the surfactants in agrochemical formulations were often more toxic than the major active chemicals (Tatum *et al*., 2011).

In this study we investigated toxicological effects of glyphosate agrochemical components by an acute toxicity test using juvenile medaka fish and by DNA microarray gene expression analysis in adult medaka liver tissues.

One glyphosate-based herbicide was the subject of our toxicological study, and in its formulation it contained glyphosate and fatty acid alkanolamide (DA) as a surfactant. According to Mayer and Ellersieck (1986), 96 hr LC_{50} values of glyphosate for various fish species at their various growth stages were 1~220 mg/l, and from our study 96-hr LC_{50} values of glyphosate, DA, and glyphosate-based herbicide on juvenile medaka fish measured

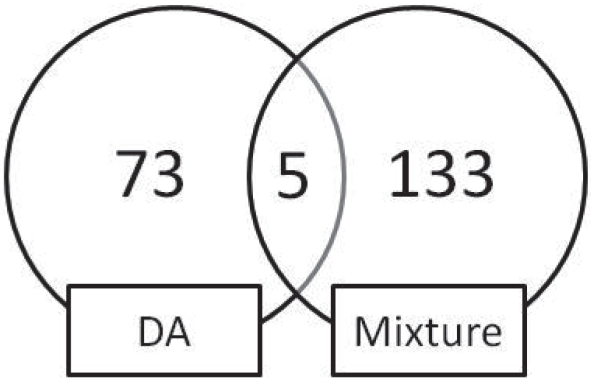

Fig. 2. Number of adult medaka hepatic genes that responded to 96-hrs exposure by DA and by the mixture of glyphosate and DA.

in this study were > 160 mg/l, 8.5 mg/l, and 76.8 mg/l, respectively. The acute toxicity of glyphosate on juvenile medaka appeared to be very low, and it was placed at the lowest toxicity end of the observed LC_{50} range reported by Mayer and Ellersieck. Also, our acute toxicity result showing that the observed LC_{50} of DA was more toxic than the glyphosate-based herbicide, and the fact that this glyphosate-based herbicide was more toxic than glyphosate supported the above mentioned reference studies of agrochemical surfactants' high toxicity (Amarante *et al*., 2002, Tatum *et al*., 2011). According to our analysis, the amount of DA in the glyphosate-based herbicide was esti-

Table 4. List of the significantly affected adult medaka hepatic genes by DA alone exposure

Gene Name		Ratio
protein binding		
	WAS protein family, member 2	9.3
	dystroglycan 1	8.7
	matrilin 4	4.8
	myosin, heavy chain 9, non-muscle	4.4
	ATPase, Na+/K+ transporting, beta 3 polypeptide	3.1
	mitogen-activated protein kinase 8 interacting protein 3	2.7
	kelch-like 20 (Drosophila)	2.0
	frizzled-related protein	0.5
	Tax1 (human T-cell leukemia virus type I) binding protein 1	0.4
	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	0.4
metal ion binding		
	sirtuin (silent mating type information regulation 2 homolog) 6 (S. cerevisiae)	7.4
	early B-cell factor 1	4.9
	tissue inhibitor of metalloproteinase-3	2.5
	peptidase M20 domain containing 1	2.4
	splicing factor 1	0.5
	cytochrome P450, family 2, subfamily U, polypeptide 1	0.5
	zinc finger protein 622	0.5
	hepatocyte nuclear factor 4, alpha	0.4
transporter activity		
	major intrinsic protein of lens fiber 2	10.3
	dihydrolipoamide branched chain transacylase E2	5.7
	FGGY carbohydrate kinase domain containing	0.4
	3-oxoacid CoA transferase 1a	0.4
	xylulokinase homolog (H. influenzae)	0.4
receptor activity		
	scavenger receptor class B, member 2	0.5
	signal sequence receptor, alpha	0.4
	progesterone receptor membrane component 1	0.3
oxidoreductase activity		
	alcohol dehydrogenase, iron containing, 1	0.4
	3-hydroxybutyrate dehydrogenase, type 1	0.4
	retinol dehydrogenase 1, like	0.4
catalytic activity		
	fumarylacetoacetate hydrolase domain containing 2A	0.5
	transaldolase 1	0.5
	glycine C-acetyltransferase	0.3
others		
	ADP-ribosylation factor interacting protein 2	12.8
	calcium channel, voltage-dependent, gamma subunit 8	4.0
	family with sequence similarity 83, member G	3.8
	l(3)mbt (lethal(3)malignant brain tumor protein)-like 4 (Drosophila)	3.7
	bromodomain containing 7	3.6
	myotubularin related protein 10	3.4
	Double-stranded RNA-binding protein A	3.3
	chromobox homolog 7	3.1
	BTG family, member 3 (BTG3)	2.8
	KFH-G protein	2.3
	follistatin-like 5	2.2
	mitogen-activated protein kinase 3	2.1
	syntaxin 1A (brain)	2.1
	solute carrier family 25, member 36	0.5
	MAF1 homolog (S. cerevisiae)	0.5
	ARP3 actin-related protein 3 homolog (yeast)	0.5
	phospholipase A2, group XIIB	0.5
	discoidin, CUB and LCCL domain containing 2	0.5
	acyl-Coenzyme A binding domain containing 5	0.5
	caudal type homeobox 1 b	0.4
	death-associated protein	0.4
	cell division cycle 16 homolog (S. cerevisiae)	0.4
	nicalin homolog (zebrafish)	0.4
	solute carrier family 38, member 4	0.4
	abhydrolase domain containing 4	0.4
	RAB18, member RAS oncogene family	0.3
	kininogen 1	0.3

The expression ratio (DA alone-exposed to control) > 2 and < 0.5 with significance at $p < 0.05$ were listed in the Table.

Table 5. List of the significantly affected adult medaka hepatic genes by pure glyphosate-DA mixture exposure.

Gene Name		Ratio
protein binding		
	complement component 8, alpha polypeptide	3.1
	secreted immunoglobulin domain 4	2.7
	solute carrier family 25, member 5	2.6
	complement component 8, beta polypeptide	2.6
	insulin-like growth factor binding protein, acid labile subunit	2.6
	kin of IRRE like (Drosophila)	2.5
	protein kinase C binding protein 1, like	2.4
	mitogen-activated protein kinase 8 interacting protein 3	2.4
	enolase 1, alpha non-neuron	2.4
	SWI/SNF related, actin dependent regulator of chromatin, subfamily b, member 1	2.3
	myotrophin	2.3
	neural cell adhesion molecule 1	2.3
	potassium voltage-gated channel, shaker-related subfamily, member 2	2.2
	collagen, type I, alpha 3	2.1
	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulatory factor 2	2.1
	protein kinase C and casein kinase substrate in neurons 1	2.1
	translin-associated factor X	2.0
	SWI/SNF related, actin dependent regulator of chromatin, subfamily c, member 1	2.0
	coronin, actin binding protein, 1B	0.4
metal ion binding		
	ferric-chelate reductase 1	3.4
	metallothionein	2.6
	metallophosphoesterase 1	2.4
	zinc finger and BTB domain containing 5	2.4
	ring finger protein 115	2.3
	cytochrome b5 type B (outer mitochondrial membrane)	2.3
	inhibitor of growth family, member 5	2.3
	B-cell CLL/lymphoma 11B (zinc finger protein)	2.2
	ubiquitin specific peptidase 39	2.2
	fibrinogen C domain containing 1	2.1
	carboxypeptidase N, polypeptide 1	2.1
	PHD finger protein 14	2.0
nucleotide binding		
	TRAF2 and NCK interacting kinase	2.4
	misato homolog 1 (Drosophila)	2.2
	splicing factor, arginine/serine-rich 15	2.2
	ubiquitin-conjugating enzyme E2D 2 (UBC4/5 homolog, yeast)	2.2
	RAB27A, member RAS oncogene family	2.1
	ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, yeast)	0.5
	torsin family 2, member A	0.4
	ras homolog gene family, member Q	0.4
catalytic activity		
	fucosidase, alpha-L- 2, plasma	3.1
	ATPase, Na+/K+ transporting, alpha 1a.4 polypeptide	2.8
	protease, serine, 35	2.6
	phosphatidic acid phosphatase type 2A	2.4
	pyruvate kinase, muscle	2.1
	lactate dehydrogenase D	2.1
	coagulation factor XI	0.5
transferase activity		
	alpha3-fucosyltransferase	3.2
	fibronectin type III and SPRY domain containing 1	3.1
	discoidin domain receptor family, member 2	2.6
	polymerase (DNA directed), delta 1, catalytic subunit	2.2
	NUAK family, SNF1-like kinase, 2	2.1
	NOL1/NOP2/Sun domain family, member 6	2.1
oxidoreductase activity		
	isovaleryl Coenzyme A dehydrogenase	2.8
	malate dehydrogenase 1b, NAD (soluble)	2.4
	aldo-keto reductase family 1, member A1 (aldehyde reductase)	2.3
	aldehyde dehydrogenase 3 family, member B1	2.2
	acyl-Coenzyme A dehydrogenase, long-chain	2.2
	delta-9-desaturase 2	0.4
hydrolase activity		
	proteasome (prosome, macropain) subunit, beta type, 7	2.6
	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	2.6
	dual specificity phosphatase 26 (putative)	2.0

Table 5. (Continued).

Gene Name		Ratio
	abhydrolase domain containing 4	0.5
	patatin-like phospholipase domain containing 4	0.5
	dual specificity phosphatase 2	0.4
others		
	major intrinsic protein of lens fiber 2	4.9
	ADP-ribosylation factor interacting protein 1 (arfaptin 1)	3.2
	matrilin 4	3.2
	purinergic receptor P2Y, G-protein coupled, 5	3.0
	KFH-G protein	3.0
	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)	2.8
	purinergic receptor P2X, ligand-gated ion channel, 1	2.8
	SH3KBP1 binding protein 1 (SH3 domain-containing kinase-binding protein 1)	2.7
	clathrin interactor 1	2.6
	hepatoma-derived growth factor, related protein 2	2.5
	C1q and tumor necrosis factor related protein 4	2.5
	ninjurin 2	2.5
	phenylalanine hydroxylase	2.4
	neurocan	2.4
	Mhc class I A	2.4
	inositol(myo)-1(or 4)-monophosphatase 1	2.4
	leucine-rich repeats and immunoglobulin-like domains 3	2.3
	Double-stranded RNA-binding protein A	2.3
	transmembrane protein 195	2.2
	solute carrier family 6 (neurotransmitter transporter, GABA), member 1	2.2
	angiopoietin-like 7	2.2
	coagulation factor VII precursor	2.2
	mitochondrial ribosome recycling factor	2.2
	MAD homolog 2 (Drosophila)	2.2
	predicted protein	2.2
	SEC22 vesicle trafficking protein homolog A (S. cerevisiae)	2.2
	golgi autoantigen, golgin subfamily a, 7	2.2
	collagen, type I, alpha 2	2.1
	ATPase family, AAA domain containing 5	2.1
	retinal pigment epithelium-specific protein 65kDa	2.1
	cadherin 2, neuronal	2.1
	phosphatidylinositol transfer protein, cytoplasmic 1	2.1
	cerebellin 2 precursor	2.1
	nucleobindin 2a	2.1
	myocilin	2.1
	CD68 molecule	2.1
	serine proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	2.1
	arsenate resistance protein 2	2.0
	N-myc downstream regulated gene 1	2.0
	tetraspanin 1	2.0
	FK506 binding protein 5	2.0
	microtubule-associated protein 7 domain containing 1	2.0
	nuclear pore complex glycoprotein p62	2.0
	nitrogen fixation gene 1 (S. cerevisiae)	2.0
	ADP-ribosylation factor-like 6 interacting protein 2	2.0
	complement component C9	2.0
	keratin 5	2.0
	DEAQ box polypeptide 1 (RNA-dependent ATPase)	2.0
	mediator complex subunit 17	2.0
	LECT2 neutrophil chemotactic factor	2.0
	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	0.5
	alcohol dehydrogenase Class VI	0.5

The expression ratio (the mixture-exposed to control) > 2 and < 0.5 with significance at $p < 0.05$ were listed in the Table.

mated to be roughly 10% (w/v) , in other words, about 7.7 mg/l of DA was present in 76.8 mg/l (LC_{50}) of the glyphosate-based herbicide. Since this value was very close to the LC_{50} (8.5 mg/l) of the DA exposure in this acute toxicity study, it was likely to assume that the acute toxicity of the glyphosate-based herbicide on juvenile medaka was derived almost fully from DA.

Nevertheless, despite the DA's toxicity contribution that we estimated in the acute toxicity study, DNA microarray gene expression analysis showed that the gene expression pattern in liver of medaka by glyphosate/DA mixture exposure was quite different from the expres-

sion pattern by the DA exposure. The number of overlapping genes between these two exposure conditions was only five (Fig. 2). But these genes turned out to be matrilin 4 (involved in protein binding at the cellular surface), alpha-beta hydrolase domain containing 4 (involved in protein structure/conformation), and a gene that is similar to mitogen-activated protein kinase 8 interacting protein 3 (involved in stress-response/signal transmission), and it seems reasonable to consider that they in hepatic tissues responded to the cellular stress/damage caused by the surfactant (DA). Furthermore, the number of genes showing differential expression, induced by the glyphosate/DA mixture exposure (133 genes), was almost twice as many as the one by DA exposure (73 genes) (Fig. 2). These results suggested that the gene pathway of the mixture of glyphosate and DA could be very different from the gene pathway of the DA alone. It was very possible to think that glyphosate with DA in the mixture exposure was causing synergistic toxicity or some other influence to induce almost completely different gene responses (gene expression pattern) in adult medaka liver tissue.

Meanwhile, it could have been said that the same or similar synergistic toxicity to the glyphosate/DA mixture were actually present at all time in our juvenile medaka acute toxicity test condition with the glyphosate-based herbicide formulation, although it contained minor ingredients potentially interacting to the other ingredients to cause or not to cause variety of toxicity. Even though there were large differences in this study's test structures and sensitivity between the acute toxicity test and DNA microarray gene expression analysis: e.g. test fish's growth stage and endpoint (dead or alive vs. gene responses of 6,000 genes), these two seemingly contradicting toxicity test results, i.e. "DA appeared to be the major cause of toxicity in both DA exposed- and the glyphosate-based herbicide exposed-juvenile medaka (acute toxicity test)" vs. "DA exposure and glyphosate/DA mixture exposure caused quite different hepatic gene responses in adult medaka (gene expression analysis)", may correlate to each other when further quantitative data validation and life stage-wide and systematic understanding of toxicity endpoint evaluation will be performed in the near future.

Lastly but not least, no statistically significant gene expression change was detected in adult medaka liver tissues that were exposed to glyphosate alone. This observation was well accorded with the lowest toxicity result of glyphosate exposure obtained in our juvenile medaka acute test. Moreover, it was very informative to have learned that on one hand single chemical exposure by DA seemed to induce a set of genes that were involved in intracellular signaling (ADP-ribosylation factor interacting protein 2, WAS protein family member 2, calcium channel voltage-dependent gamma subunit 8, and so on), and on the other hand the multiple chemical exposure of glyphosate and DA appeared to induce the genes that were involved in cellular metabolism (ferric-chelate reductase 1, alpha3-fucosyltransferase, fucosidase alpha-L- 2 plasma etc.) (Tables 4 and 5). These genes unfortunately did not show any distinct correlation among their functions, but alpha3-fucosyltransferase in particular showed its expression increase by DA-exposure and more significant increase by glyphosate/DA mixture-exposure. Since this gene is known to be involved in the fucosylation of sugar chains, it was possible to speculate that forced increase by the synergistic effect of glyphosate/DA mixture in the expression of alpha3-fucosyltransferase gene might have affected cell surface structuring. These findings altogether at gene expression level strongly suggested that glyphosate in the glyphosate-based herbicide formulation possessed very low toxicity to medaka fish, but DA alone or DA with glyphosate appeared more toxic at both individual and molecular (gene expression) levels with different set of target gene groups and toxicity mechanisms.

ACKNOWLEDGMENTS

This work was supported by the members of Arizono Laboratory (Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto) and by the members of Ecogenomics, Inc.

REFERENCES

Amarante, O.P.Jr., Santos, T.C.R., Brito, N.M. and Ribeiro, M.L. (2002): Glifosato: propiedades, toxicidade, uso e legislac¸a˜o. Quı´m Nova, **25**, 589-593

Benachour, N., Moslemi, S., Sipahutar, H. and Seralini, G.E. (2007): Cytotoxic effects and aromatase inhibition by xenobiotic endocrine disrupters alone and in combination. Toxicol. Appl. Pharmacol., **222**, 129-140.

Cavalcante, D.G., Martinez, C.B. and Sofia, S.H. (2008): Genotoxic effects of Roundup on the fish Prochilodus lineatus. Mutat. Res., **655**, 41-46.

Çavas, T. and Könen, S. (2007): Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (Carassius auratus) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis, **22**, 263-268.

Cox, C. (1998): Glyphosate (Roundup). J. Pest. Reform., **18**, 3-17.

Cox C. and Surgan M. (2006): Unidentified inert ingredients in pesticides: implications for human and environmental health. Environ. Health Perspect., **114**, 1803–1806.

El-Alfy, A.T. and Schlenk, D. (2002): Effect of 17beta-estradiol and testosterone on the expression of flavin-containing monooxygen-

ase and the toxicity of aldicarb to Japanese medaka, *Oryzias latipes*. Toxicol. Sci., **68**, 381-388.

el-Gendy, K.S., Aly, N.M. and el-Sebae, A.H. (1998): Effects of edifenphos and glyphosate on the immune response and protein biosynthesis of bolti fish (Tilapia nilotica). J. Environ. Sci. Health. B, **33**, 135-149.

Glusczak, L., dos Santos Miron, D., Crestani, M., Braga da Fonseca, M., de Araújo Pedron, F., Duarte, M.F. and Vieira, V.L. (2006): Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (Leporinus obtusidens). Ecotoxicol. Environ. Safe., **65**, 237-241.

Glusczak, L., dos Santos Miron, D., Moraes, B.S., Simões, R.R., Schetinger, M.R., Morsch, V.M. and Loro, V.L. (2007): Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (Rhamdia quelen). Comp. Biochem. Physiol. C Toxicol. Pharmacol., **146**, 519-524.

Grisolia, C.K. (2002): A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides. Mutat. Res., **518**, 145-150.

IFEN (2006): Les pesticides dans les eaux. Dossier, **5**, 15-20.

IPCS (1994): Glyphosate. Geneva, World Health Organization, International Programme on Chemical Safety (Environmental health Criteria 159).

Ishibashi, H., Watanabe, N., Matsumura, N., Hirano, M., Nagao, Y., Shiratsuchi, H., Kohra, S., Yoshihara, S. and Arizono, K. (2005): Toxicity to early life stages and an estrogenic effect of a bisphenol A metabolite, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene on the medaka (*Oryzias latipes*). Life Sci., **77**, 2643-2655.

Jiraungkoorskul, W., Upatham, E.S., Kruatrachue, M., Sahaphong, S., Vichasri-Grams, S. and Pokethitiyook, P. (2003): Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (Oreochromis niloticus). Environ. Toxicol., **18**, 260-267.

Langiano, V.C. and Martinez, C.B. (2008): Toxicity and effects of a glyphosate-based herbicide on the Neotropical fish Prochilodus lineatus. Comp. Biochem. Physiol. C, **147**, 222-231.

Mayer, F.L. and Ellersieck, M.R. (1986): Manual of acute toxicity: Interpretation and data base for 410 chemicals and 66 species of freshwater animals. US Fish and Wildlife Service Resource, Washington, **160**, 439.

Peixoto, F. (2005): Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere, **61**, 1115-1122

Rendón-von Osten, J., Ortíz-Arana, A., Guilhermino, L. and Soares, A.M. (2005): *In vivo* evaluation of three biomarkers in the mosquitofish (Gambusia yucatana) exposed to pesticides. Chemosphere, **58**, 627-636.

Szarek, J., Siwicki, A., Andrzejewska, A., Terech-Majewska, E. and Banaszkiewicz, T. (2000): Effects of the herbicide Roundup on the ultrastructural pattern of hepatocytes in carp (Cyprinus carpio). Mar. Environ. Res., **50**, 263-266.

Tsui, M.T. and Chu, L.M. (2003): Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere, **52**, 1189-1197.

Tsui, M.T. and Chu, L.M. (2008): Environmental fate and non-target impact of glyphosate-based herbicide (Roundup) in a subtropical wetland. Chemosphere, **71**, 439-446.

Tatum, V.L., Borton, D.L., Streblow, W.R., Louch, J. and Shepard, J.P. (2011): Acute toxicity of commonly used forestry herbicide mixtures to Ceriodaphnia dubia and Pimephales promelas. Environ. Toxicol. DOI 10.1002/tox 20686. [Epub ahead of print].

Williams, G.M., Kroes, R. and Munro, I.C. (2000): Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul. Toxicol. Pharm., **31**, 117-165.

World Health Organization (2005): Glyphosate and AMPA in drinking-water. Background document for development of WHO guidelines for drinking-water quality. WHO, Geneva.

Zhaobin, Z. and Jianying, H. (2007): Development and validation of endogenous reference genes for expression profiling of medaka (*Oryzias latipes*) exposed to endocrine disrupting chemicals by quantitative real-time RT-PCR. Toxicol. Sci., **95**, 356-368.