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Combined Effects of PFOS and PFOA on Zebrafish (*Danio rerio*) Embryos

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Abstract Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are two kinds of emerging contaminants most studied in recent years. However, there is limited information about their combined toxicity to aquatic organisms. In the present study, the single and combined toxicity of PFOA and PFOS to zebrafish (*Danio rerio*) embryos were investigated. PFOS was more toxic than PFOA for the single toxicity. In four mixtures, PFOS and PFOA showed complex interactive effects that changed from additive to synergistic effect, then to antagonistic effect, and at last turnover to synergic effect again, with increased molar ratios of PFOS. Neither the concentration-addition model nor the independent-action model could predict the combined effects when strong interactive effects existed. Although the interactive effects of PFOS and PFOA affected their combined toxicity, the trend of mixture toxicity still showed an increase with increasing molar ratios of PFOS in the mixture.

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Perfluorinated compounds (PFCs) are a large class of man-made compounds and used widely in commercial and industrial applications as surfactants, paper and textile coatings, and food packaging (Kissa 2001). Because of their worldwide application, PFCs have been released to the environment all over the world by various ways during manufacture, distribution, use, and disposal processes (Paul et al. 2009). Recently, numerous studies have showed that PFCs exist in nearly all environmental matrices, wildlife, and humans around the world (Giesy and Kannan 2002; Kannan et al. 2005; Houde et al. 2006; Fromme et al. 2009; Butt et al. 2010). Among them, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are two typical and predominant PFCs, and they are of greatest concern due to their persistence, potential bioaccumulation, and toxicity to humans and animals.

To assess health impacts of PFOS and PFOA, mammalian toxicity has been assessed on rodents, nonhuman primates, and human cell lines (Lau et al. 2007; Andersen et al. 2008). In addition, toxicological studies have been performed on aquatic organisms, such as algae, water flea, frog, and fish, to evaluate their ecological risk (Ankley et al. 2004; Liu et al. 2008; Li 2009; Huang et al. 2010; Jeon et al. 2010; Ding et al. 2012a, b). However, most of the toxicological studies available are focused on assessing the single toxicity of PFOS and PFOA, and there are few reports on their mixture toxicity with each other or with other pollutants (Jernbro et al. 2007; Hu and Hu 2009; Watanabe et al. 2009; Wei et al. 2009; Rodea-Palomares et al. 2012). In the real environment and organisms, PFOS and PFOA have often been shown to commonly coexist (Houde et al. 2006; Suja et al. 2009). Therefore, it is necessary to test their combined toxicity to better assess their ecological risk and human health risk.

To determine mixture toxicity, Hu and Hu (2009) investigated combined effects of PFOS and PFOA on

hepatoma Hep G2 cells. The study showed that the combined effect of PFOA and PFOS was a summation effect that was neither synergistic nor antagonistic. Wei et al. (2009) tested the combined effects of six polyfluorinated and perfluorinated compounds, including PFOS and PFOA, on primary cultivated hepatocytes from rare minnows using a custom cDNA microarray. The study showed that 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. Furthermore, certain genes were regulated by the mixture, whereas they were not affected by the individual substances. Rodea-Palomares et al. (2012) examined toxicological interactions of PFOA and PFOS using a combination-index method. PFOA and PFOS showed an antagonistic interaction at the whole range of effect levels. Therefore, PFOS and PFOA may have complicated toxicological interactions and thus have different effects on different organisms.

Due to the temporal and spatial variability of the mixture composition, a direct effects measurement is, although desirable, not feasible in most cases. When the mixture components are fixed, prediction of mixture toxicity from the toxicity of the individual compounds is a promising alternative. Several approaches for the prediction of mixture toxicity have been reported (Rider and LeBlanc 2005). Among them, concentration addition (CA) and independent action (IA) are mostly used for the predictive assessment of combination effects.

Because the CA and IA models assume that no interactions are present in the analyzed mixtures, they might overestimate or underestimate the combined toxic effects when interactions exist. To describe and quantify such deviations, many methods have been introduced, such as toxic unit summation (TU), additivity index, and mixture toxicity index (MTI) (Altenburger et al. 2003; Koutsaftis and Aoyama 2007). Among them, MTI is a popular method for mixture toxicity assessment. Therefore, in the present study, the single and mixture toxicity of PFOA and PFOS were first tested on zebrafish (*Danio rerio*) embryos, and then mixture toxicity was predicted by CA and IA models and assessed by MTI.

Materials and Methods

Test Chemicals

Salts of PFOS and PFOA were used in the present study to test the toxicity of PFOS and PFOA. PFOS potassium salt (PFOSK; CAS no. 2795-39-3, purity 98 %) and ammonium perfluorooctanoate (CAS no. 3825-26-1, purity 98 %) were purchased from Sigma-Aldrich. The chemicals were dissolved in reconstituted water for the tested concentrations, and no solvents were used. The stock solutions were kept at 26 °C for use.

Zebrafish Maintenance and Embryo Collection

Adult zebrafish (*D. rerio*) maintenance and embryo collection were performed according to the guide in the zebrafish book (Westerfield 2000). Briefly, adult zebrafish were kept in aerated and biologically filtered reconstituted freshwater at 26 ± 1 °C with a photoperiod of 14 h of light to 10 h of dark. Water was totally renewed, and aquaria were cleaned each week. The fish were fed twice daily with either the zebrafish diet (Zeigler, Aquatic Habitats, Apopka, FL) or live *Artemia* (Jiahong Feed Co., Tianjin, China).

The day before a test, male and female zebrafish, at a ratio of 1:1, were placed in spawning tanks before the onset of darkness. Mating, spawning, and fertilization take place within 30 minutes after light onset in the morning. Eggs were collected from spawn traps and washed with clean water (Organisation for Economic Co-operation and Development 1992). Unfertilized or abnormal eggs were removed under a stereomicroscope.

Toxicity Bioassays

For a toxicity test, six exposure concentrations were performed with three replicates. Twenty normally fertilized eggs/exposure concentration were divided into a 24-well plate with 1 embryo/well containing 2 mL test solution. The remaining four wells were filled with control water and fertilized eggs used as the control. An embryo was considered dead when 1 of 4 end points (i.e., coagulation of the embryo, nondetachment of the tail, nonformation of somites, and nondetection of the heart beat) was observed. The survival rates were monitored and documented at 72 and 96 h postfertilization (hpf). The test solutions were half renewed every 24 h.

The mixture toxicity of PFOS and PFOA was tested with fixed mixture ratios (1:1, 1:3, 1:6, and 1:10) of individual chemicals. Although the mixture ratio was kept constant, the total concentration of the mixture was varied so that a complete concentration–response relationship of the mixture could be determined experimentally.

Data Analysis

The concentration–response relationships of the individual chemicals and their mixtures were fitted using a general best-fit method (Scholze et al. 2001). A set of ten different two or three-parametric nonlinear regression models (including probit, logit, and Weibull models) were chosen. For each individual set of data, the best-fitting model was

chosen based on the residual sum of squares and adjusted *R*-square. The observation-based 95 % confidence limits of dose–response curves were calculated according to the study of Zhu et al. (2009). The calculations were performed using Origin 8.0 and Matlab 7.1.0 software.

Prediction and Assessment of Mixture Toxicity

The CA and IA models were used to predict mixture toxicity. For calculation of the effect concentrations by CA, Eq. 1 was used as follows (Eq. 1):

$$EC_{x,\mathrm{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x,i}} \right)^{-1}, \quad (1)$$

where $EC_{x,i}$ and $EC_{x,\mathrm{mix}}$ are the individual concentration and the total concentration of the mixture provoking x effect, respectively, and p_i denotes the fraction of component i in the mixture.

Equation 2 was used as a starting point to calculate the mixture effects according to IA. The concentration–response relationships F_i of the individual components ($i = 1, \ldots, n$) were used to calculate their effects $E(c_i)$:

$$E(c_{\mathrm{mix}}) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] = 1 - \prod_{i=1}^{n} [1 - F_i(c_i)]. \quad (2)$$

The MTI method was chosen to assess the interactions in the mixture toxicity. First, TU of component i, TU_i, was calculated as $TU_i = \frac{c_i}{EC_{50,i}}$, where c_i and $EC_{50,i}$ are concentration and EC_{50} of component i, respectively. Then M and M_0 were calculated as $M = \sum TU_i$, and $M_0 = \frac{M}{\max(TU_i)}$, where $\max(TU_i)$ is the maximum value of TU_i. Finally, MTI was determined as $MTI = 1 - \frac{\log M}{\log M_0}$.

When the value of MTI is <0, mixture potency is defined as antagonism; when it equals 0, there is no addition (IA); when it is >0 but <1, mixture potency is regarded as partial addition; when it equals 1, mixture potency is defined as addition; and when it is >1, mixture potency is defined as synergism.

Results and Discussion

Single Toxicity of PFOS and PFOA

According to the 10 nonlinear regression models, concentration–response relationships for single toxicity of PFOS and PFOA on zebrafish embryos were determined. On the basis of the residual sum of squares and adjusted *R*-squares of the models, best-fit models were chosen for PFOS and PFOA. The best-fit models for the concentration–response relationships of PFOA and PFOS were the Generalized Logit I model and the Aranda–Ordaz model, respectively. Model parameters and the fitted LC_{50} values are listed in Table 1. For the single toxicity of PFOA, the 72- and 96-h LC_{50} values were determined to be 1.448 and 0.896 mM, respectively. The obtained 72- and 96-h LC_{50} values for PFOS were 0.102 (54.9 mg/L) and 0.101 mM (54.4 mg/L), respectively.

For the single toxicity of PFOS, Shi et al. (2008) exposed 4-hpf zebrafish embryos to 0.1, 0.5, 1, 3, and 5 mg/L PFOS until 132 hpf. Before 84 hpf, there was no significant difference in the percentage of survivorship in any of the exposed groups compared with the control group. At PFOS concentrations of 0.1 and 0.5 mg/L, no significant increase in mortality was observed over the

Table 1 The best-fit models and the toxicity of PFOS and PFOA

	End point	The best-fit model	Model parameters			R^2_{adj}	RSS	LC_{50} (mM)	MTI	Interactive effect
			β_1	β_2	β_3					
PFOA	72-h LC_{50}	Generalized Logit I	119.0	44.34	0.100	0.951	0.0287	1.448		
	96-h LC_{50}	Generalized Logit I	171.2	63.34	0.0317	0.989	0.00617	0.896		
PFOS	72-h LC_{50}	Aranda-Ordaz	4.618	1.367	−0.673	0.859	0.0283	0.102		
	96-h LC_{50}	Aranda-Ordaz	4.689	1.384	−0.674	0.857	0.0298	0.101		
M1 (1:10)	72-h LC_{50}	Generalized Logit I	1032	371.0	5.28E−03	0.958	0.0167	0.733	1.00	ADD
	96-h LC_{50}	Generalized Logit I	1046	367.7	5.26E−03	0.991	0.00375	0.628	0.81	PAD
M2 (1:6)	72-h LC_{50}	Generalized Logit I	901.4	303.6	7.99E−03	0.848	0.0693	0.556	1.05	ADD/SYN
	96-h LC_{50}	Generalized Logit I	1466	483.5	4.03E−03	0.842	0.0706	0.409	1.21	SYN
M3 (1:3)	72-h LC_{50}	Aranda-Ordaz	7.208	2.468	−0.734	0.942	0.0105	0.568	−0.67	ANT
	96-h LC_{50}	Aranda-Ordaz	3.988	1.581	−0.375	0.975	0.00633	0.561	−0.42	ANT
M4 (1:1)	72-h LC_{50}	Generalized Logit I	1209	350.4	5.92E−03	0.992	0.0163	0.164	3.99	SYN
	96-h LC_{50}	Generalized Logit I	1158	335.3	3.25E−03	0.972	0.0407	0.081	7.80	SYN

RSS residual sum of squares, *ANT* antagonistic effect, *SYN* synergistic effect, *ADD* additive effect, *PAD* partial addition, *INT* no addition (IA)

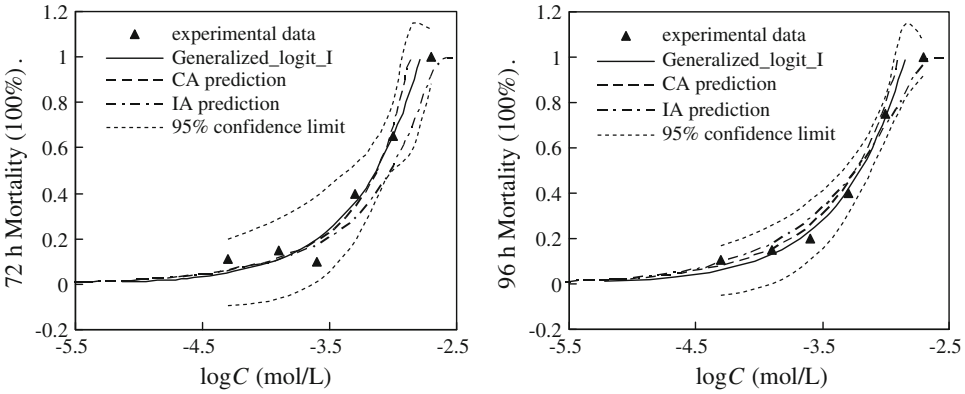

Fig. 1 Observed and predicted mixture toxicity of PFOS and PFOA with a c_{PFOS}:c_{PFOA} ratio of 1:10

whole exposure time, whereas a significant increase in mortality was observed at PFOS concentrations of 1, 3, and 5 mg/L at 132 hpf. Huang et al. (2010) also tested the single toxicity of PFOS to zebrafish embryos from 6 to 120 hpf. It was reported that PFOS ≥1.0 mg/L was lethal to embryos, whereas embryos exposed to 0.25 and 0.5 mg/L PFOS showed no increased mortality over the exposure time. The calculated LC_{50} value of PFOS at 120 hpf was 2.20 mg/L. These two previous studies showed more lethal effects of PFOS to zebrafish embryos. Differences between these studies could be related to different PFOS chemicals and solvent used. The previous studies both used PFOS for exposure and dimethylsulfoxide as a solvent, whereas this study used PFOSK for exposure without the use of solvents.

Mixture Toxicity of PFOS and PFOA

For mixture M1 with a c_{PFOS}:c_{PFOA} (mg/L:mg/L) ratio of 1:10, the combined toxicity was best characterized by the Generalized Logit I model (Fig. 1). The model parameters and the fitted LC_{50} values are also listed in Table 1. From the model, the 72- and 96-h $LC_{50,mix}$ values were calculated to be 0.733 and 0.628 mM, respectively.

The CA and IA models were both used to predict the mixture toxicity of PFOS and PFOA based on the effects of the individual compounds. The observed and predicted mixture toxicity is presented in Fig. 1. It could be seen that the predictions of the CA and IA models all fell in the range of 95 % confidence limit of the dose–response estimation, although there were some deviations. For 72-h mortality, the predictions of the CA model agreed well with the observed effects. The predictions of the IA model also agreed with the observed effects at lower concentrations, whereas it underestimated the combined effects at greater concentrations. In general, the CA and IA models were used to predict the mixture toxicity of compounds with similar or different modes of actions, respectively. The results of M1 might suggest that PFOS and PFOA act on similar modes of action at 72 h of exposure. For 96-h mortality, both models slightly overestimated the observed mixture toxicity for most of the concentration range. At greater concentrations, the IA model corresponded with the observed experimental data, which might suggest that PFOS and PFOA have different modes of actions at greater concentrations after 96 h of exposure.

Table 1 also lists MTI values for M1. Because the MTI for the 72-h toxicity tends toward 1.00, PFOS and PFOA show an additive effect after 72 h of exposure. For the 96-h toxicity, MTI is 0.81, suggesting that PFOS and PFOA have a partial additive effect. Therefore, the CA model could also predict the combined toxicity at 96 h for M1 with some deviations.

For mixture M2 with a c_{PFOS}:c_{PFOA} (mg/L:mg/L) ratio of 1:6, the joint toxicity was also best fitted by the Generalized Logit I model (Fig. 2). Based on the fitted model, 72- and 96-h $LC_{50,mix}$ values were determined to be 0.556 and 0.409 mM, respectively.

For the mixture toxicity of M2, the predictions of the CA and IA models all fell in the range of the 95 % confidence limit of the dose–response estimation, but the predictions of the IA model tended to be closer to the lower confidence bounds at greater concentrations. It could be seen that the IA model underestimated the observed mixture toxicity of M2 at greater concentrations after 72 and 96 h of exposure, whereas the CA model performed better than the IA model for the effects at 72 and 96 h. This might suggest that PFOS and PFOA showed more or less similar modes of actions when the c_{PFOS}:c_{PFOA} ratio equaled 1:6.

As the MTI value for the 72-h toxicity of M2 is 1.05, PFOS and PFOA mainly show an additive effect after 72 h of exposure. For the 96-h toxicity, MTI increased to 1.21, indicating that PFOS and PFOA tend to have a weak synergistic effect. Therefore, the CA model slightly underestimates the combined toxicity at 96 h.

When Hu and Hu (2009) investigated the effects of PFOS and PFOA on hepatoma Hep G2 cells, they found that cells exposed to a mixture of PFOA and PFOS and individual compounds did not show a significant difference on the apoptotic rate and then suggested that the combined

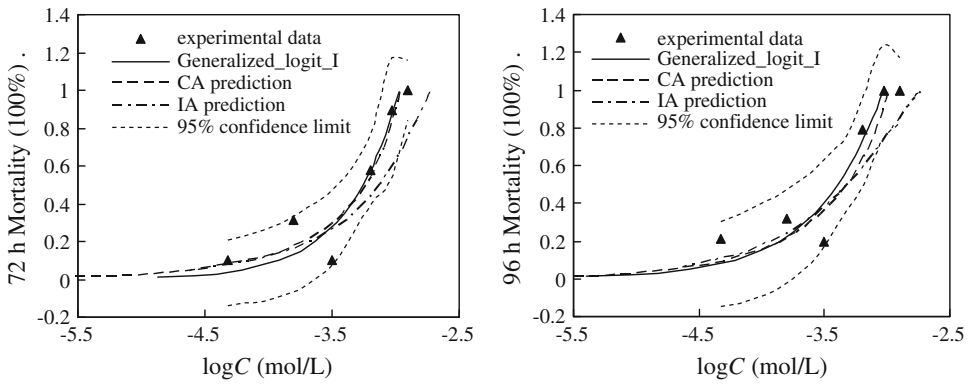

Fig. 2 Observed and predicted mixture toxicity of PFOS and PFOA with a c_{PFOS}:c_{PFOA} ratio of 1:6

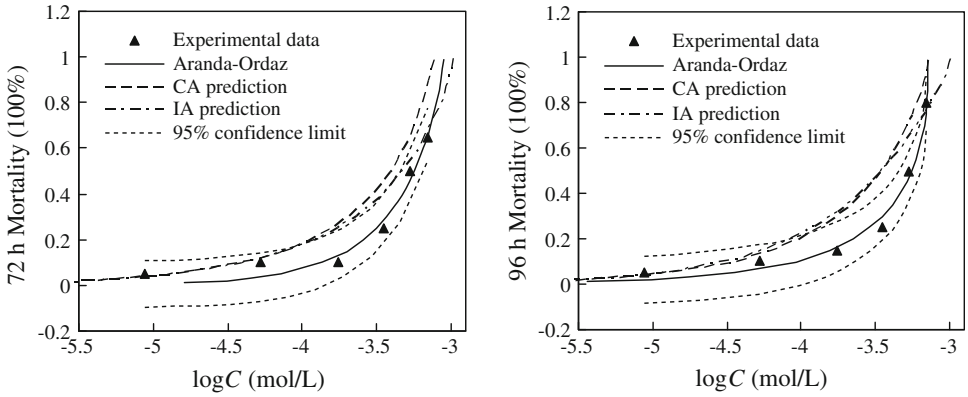

Fig. 3 Observed and predicted mixture toxicity of PFOS and PFOA with a c_{PFOS}:c_{PFOA} ratio of 1:3

effect of the two compounds was an additive effect. These findings coincide with part of the results of M1 and M2 that PFOS and PFOA acted independently and the combined effect was the sum of the effects of the individual compounds.

For mixture M3 with a c_{PFOS}:c_{PFOA} (mg/L:mg/L) ratio of 1:3, the joint toxicity was best fitted by the Aranda–Ordaz model (Fig. 3), which gave 72- and 96-h $LC_{50,mix}$ values of 0.568 and 0.561 mM, respectively. For prediction of mixture toxicity, the CA and IA models both overestimated the combined effects at 72 and 96 h of exposure with the predictions above the upper bounds of the 95 % confidence limit of the dose–response estimation. Because the experimental data did not correspond with the prediction of the CA and IA models, PFOS and PFOA might have complex interactions in the mixture.

For M3, the MTI values for 72- and 96-h toxicity were −0.67 and −0.42, respectively. As the values were both <0, PFOS and PFOA show an antagonistic interaction. Therefore, the CA and IA models overestimated the combined effects of M3. This result agreed with the results of Rodea-Palomares et al. (2012), which indicated that PFOA and PFOS had an antagonistic interaction at the whole range of effect levels on bioluminescent cyanobacterium *Anabaena* CPB4337.

For mixture M4 with a c_{PFOS}:c_{PFOA} (mg/L:mg/L) ratio of 1:1, the joint toxicity was again best fitted by the Generalized Logit I model (Fig. 4). From the fitted model, 72- and 96-h $LC_{50,mix}$ values of M4 were 0.164 and 0.081 mM, respectively. For prediction of 72-h mixture toxicity, the CA and IA models both underestimated the combined effects at greater concentrations. However, they greatly underestimated 96-h mixture toxicity for all of the concentration range.

The MTI values for 72- and 96-h toxicity of M4 were 3.99 and 7.80, respectively. The values were both >1, which suggests that PFOS and PFOA have a synergistic effect for M4. Therefore, the CA and IA models underestimated the combined effects at 72 and 96 h of exposure. Wei et al. (2009) assessed the combined effects of PFOS and PFOA on primary cultured hepatocytes from rare minnow (*Gobiocypris rarus*) using a custom cDNA microarray. Their results showed that the mixture of PFOS and PFOA with equal ratios regulated 52 genes (28 upregulated and 24 downregulated), which were not affected by either of the two individual compounds. Therefore, PFOS and PFOA could induce synergistic effects that individual compounds did not cause.

In addition, the results of Wei et al. (2009) also showed that a total of 334 genes regulated individually by PFOA or PFOS were not affected by their mixture, and 21 genes displayed consistent increases or decreases in the expression responses among the single and mixture exposure. Therefore, PFOS and PFOA could have complex toxic

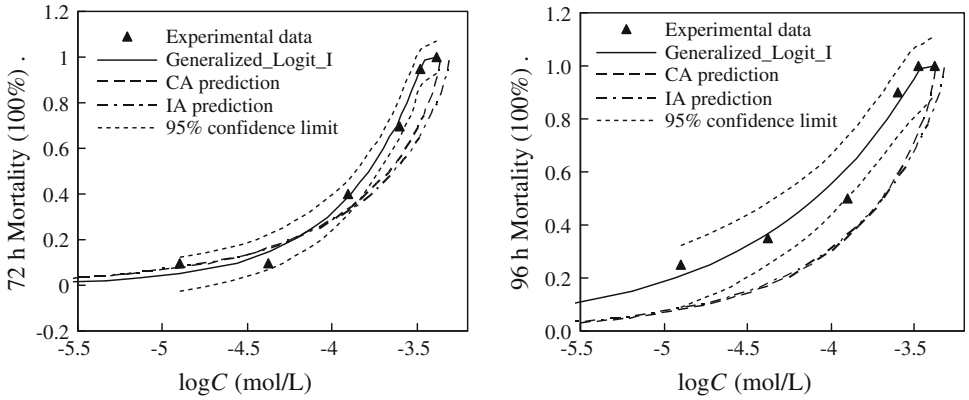

Fig. 4 Observed and predicted mixture toxicity of PFOS and PFOA with a c_{PFOS}:c_{PFOA} ratio of 1:1

effects when they are coexposed to organisms. In the present study, PFOS and PFOA showed a variation of combined toxic effects with different mixture ratios over different time frames, which agreed with the results of Wei et al. (2009).

Relationship Between Mixture Toxicity and Molar Ratios of PFOS in the Mixture

Figure 5 gives the linear regression between $\log LC_{50,mix}$ and $c_{PFOS}/(c_{PFOS} + c_{PFOA})$ values. From the figure, it can be seen that the mixture toxicity increases with increasing molar ratios of PFOS in the mixture. However, PFOS and PFOA have complex interactive effects with variable molar ratios of PFOS in the mixtures. The interactive effects changes from addition to synergistic effect, then to antagonistic effect, and at last turnover to synergic effect again, with increased molar ratios of PFOS in the mixture.

The toxicity of PFOS and PFOA has been extensively investigated, and modes of action have been analyzed (Andersen et al. 2008; Hu et al. 2002, 2003; Kleszczyński and Składanowski 2009; Kleszczyński et al. 2009; Lau et al. 2007; Liu et al. 2008, 2009; Shi et al. 2008; Wei et al. 2008, 2009). These previous studies showed that the toxicity of PFOS and PFOA was involved in multiple biological processes, including lipid metabolism and transport, membrane integrity, oxidative stress, hormone action, immune responses, and mitochondrial functions. There are both similarities and differences in toxicological effects and modes of action between PFOS and PFOA, and for the same toxicity effect, the potential is different for these two chemicals (Hu et al. 2003; Lau et al. 2007; Liu et al. 2008, 2009; Kleszczyński and Składanowski 2009; Shi et al. 2008; Wei et al. 2008, 2009). Therefore, when PFOS and PFOA are mixed at a certain concentration ratio, they might interact and show an antagonistic effect based on the same mode of action of individual chemicals. Otherwise, they will represent an additive or synergistic effect.

In the present study, the interactive effects of PFOS and PFOA in the mixture affected their combined effects, which could deviate the mixture toxicity from the relationship between $\log LC_{50,mix}$ and $c_{PFOS}/(c_{PFOS} + c_{PFOA})$ values. Although the mixture toxicity was affected by the interactive effects, the trend of the mixture toxicity still showed an increase with increasing molar ratios of PFOS in the mixture.

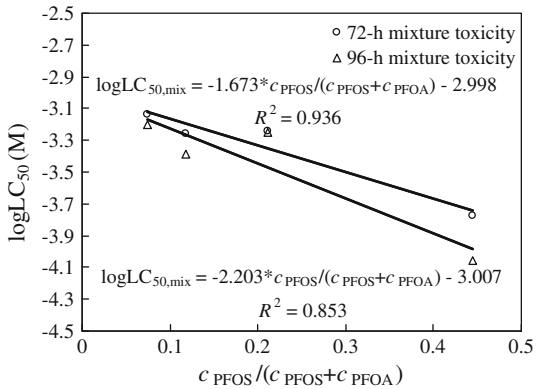

Fig. 5 The relationship between log LC_{50} values of the mixture and molar ratios of PFOS in the mixture

Conclusion

In the present study, single and mixture toxicity of PFOS and PFOA on zebrafish embryos were investigated. PFOS showed stronger single toxicity than PFOA. In four different mixtures, PFOS and PFOA showed complex interactive effects that changed from addition to synergistic effect, then to antagonistic effect, and at last turnover to synergic effect again, with increased molar ratios of PFOS. Neither the CA nor the IA model could predict the combined effect when strong interactions existed. Although the interactive effects of PFOS and PFOA affected their mixture toxicity, the trend of the mixture toxicity still showed an increase with increasing molar ratios of PFOS in the mixture.

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