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Bioaccumulation of Perfluoroalkyl Substances by *Daphnia magna* in Water with Different Types and Concentrations of Protein

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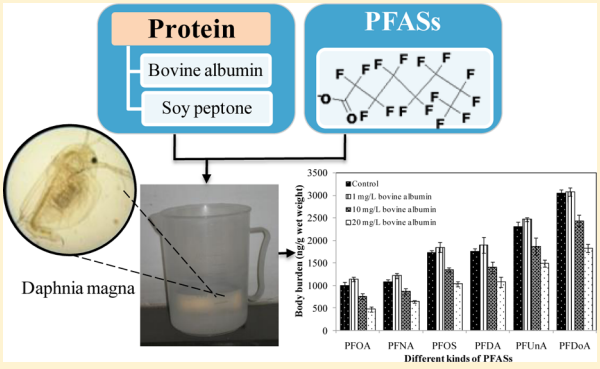
Supporting Information

ABSTRACT: Perfluoroalkyl substances (PFASs) are sometimes regarded as proteinophilic compounds, however, there is no research report about the effect of environmental protein on the bioaccumulation of PFASs in waters. In the present study we investigated influences of protein on the bioaccumulation of six kinds of PFASs by *Daphnia magna* in water; it included perfluorooctane sulfonate, perfluorooctanoic acid, perfluorononanoic acid, perfluorodecanoic acid, perfluoroundecanoic acid, and perfluorododecanoic acid. Two types of protein including bovine albumin from animal and soy peptone from plant were compared and the effects of protein concentration were investigated. Both types of protein at high concentrations (10 and 20 mg L^{-1}) suppressed the bioaccumulation of PFASs. When protein concentration increased from 0 to 20 mg L^{-1}, the decreasing ratios of the PFAS body burden (35.3−52.9%) in *Daphnia magna* induced by bovine albumin were significantly higher than those (22.0−36.6%) by soy peptone. The dialysis bag experiment results showed that the binding of PFASs to protein followed the Freundlich isotherm, suggesting it is not a linear partitioning process but an adsorption-like process. The partition coefficients of PFASs between bovine albumin and water were higher compared to soy peptone; this resulted in higher reducing rates of freely dissolved concentrations of PFASs with increasing bovine albumin concentration, leading to a stronger suppression of PFAS bioaccumulation. However, the presence of both types of protein with a low concentration (1 mg L^{-1}) enhanced the bioaccumulation of PFASs. Furthermore, the water-based bioaccumulation factor based on the freely dissolved concentrations of PFASs even increased with and the depuration rate constants of PFASs from *Daphnia magna* decreased with protein concentration, suggesting that protein would not only reduce the bioavailable concentrations and uptake rates of PFASs but also lower the elimination rates of PFASs in *Daphnia magna*. Because these two opposite effects would change with different protein concentrations in water, the net effect of protein on PFAS bioaccumulation would also vary with protein concentration.



1. INTRODUCTION

Perfluoroalkyl substances (PFASs) are used in a multitude of consumer products because of their ability to repel water and oil, resistance to heat, and chemical inertness. The carbon−fluorine bond is one of the strongest bonds in organic chemistry, thus, these fully fluorinated hydrocarbons are exceedingly stable and resistant to degradation by natural processes, such as metabolism, hydrolysis, photolysis, and biodegradation.[1,2] These properties of PFASs also make them potentially harmful to organisms when they leak into the environment. Toxicological studies have demonstrated that the most two prevalent PFASs in the environment, perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA), may induce liver, reproductive and developmental toxicity as well as potential carcinogenicity on test organisms.[3,4]

PFASs are widely present in the environment; a lot of data are published regarding the levels of PFASs in water, soil, and air.[5−9] Despite being amphiphilic, PFASs demonstrate bioaccumulation tendencies, and broad distribution of PFASs in wildlife animals and humans has been observed.[10,11] Some controlled laboratory studies have demonstrated that PFASs can be bioaccumulated in aquatic organisms such as *Chironomus plumosus* larvae, *Daphnia magna*, and *Oncorhynchus mykiss*, and the bioaccumulation generally increases with perfluoroalkyl chain length.[12−14] Furthermore, both field monitoring and laboratory studies have shown that PFASs tend to bind to proteins in organisms and accumulate in blood and protein rich tissues of exposed organisms.[12,15−17] For example, Jones et al.[16] confirmed that PFOS has been strongly associated with serum protein of birds and fishes, and Houde et al.[18] also reported the similar result. Therefore, PFASs are sometimes regarded as proteinophilic compounds.[19,20]

Because protein compounds not only exist in organisms but also are ubiquitous in aquatic environment, we hypothesize that protein compounds in aquatic environment will also combine

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with PFASs, affecting the bioavailability and bioaccumulation of PFASs in aquatic organism, and the influence of protein will depend on their types and concentrations. But there is no report about the effect of protein in water on the bioaccumulation of PFASs in organisms. Hundreds of different proteins exist in natural water and wastewater. For instance, the protein component can account for more than 40% of the total chemical oxygen demand of a dairy wastewater stream.[21] Other processing industries such as abattoir, whey, casein, fish, and some vegetable processing also typically produce wastewater containing significant amounts of proteins with the concentration ranging from 1 to 20 mg L^{-1}.[22] Feng et al.[23] reported that the protein concentration ranged from 1.8 to 30.2 mg L^{-1} in domestic wastewater of a typical region in Zhejiang province of China.

The main objectives of this study were to study the effect of protein on the bioaccumulation of six PFASs in *Daphnia magna*; it included PFOS, PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), and perfluorododecanoic acid (PFDoA). Two types of protein including bovine albumin from animal and soy peptone from plant were compared and the effects of protein concentration were investigated. In addition, the binding of PFASs with protein as well as the bioaccumulation and depuration kinetics of PFASs were examined to explore the effect mechanism of protein.

2. MATERIALS AND METHODS

2.1. Reagents. PFOA (99.9%) and PFDA (99.9%) were from Aldrich Chemical Co. (Milwaukee, WI); PFUnA (95%), PFNA (97%), and PFDoA (95%) from Acros Organics (New Jersey, US); PFOS (98%) from Tokyo Chemical Industries (Tokyo, Japan). A purity corrected equimass stock standard solution containing these PFASs was prepared in a 80:20 (v/v) methanol/water solution with the concentration of 200 mg L^{-1} for each PFAS. Methanol of chromatography grade was purchased from J.T. Baker Phillipsburg, NJ. [1,2,3,4-$^{13}C_4$] perfluorooctanoic acid (MPFOA) (purity >99%) and -[1,2,3,4-$^{13}C_4$] perfluorooctane sulfonate (MPFOS) used as recovery indicators were obtained from Wellington Laboratories (Guelph, Canada). Ammonium acetate (98%), methyl-tert-butyl ether (MTBE, 99.5%), and tetrabutylammonium hydrogensulfate (TBA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and they were used to extract PFASs from *Daphnia magna*. Bovine albumin and soy peptone were purchased from Sigma-Aldrich and Organotechnie (La Courneuve, France), respectively. Anhydrous sodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) were from Fisher Chemical (Fairlawn, NJ). Dialysis bag of spectra6 7000 Da molecular weight was purchased from Sigma -Aldrich Chemical Co..

2.2. *Daphnia magna* Cultivation. *D. magna* were cultured under the conditions described in the guideline of Organization for Economic Cooperation and Development for the testing of chemicals.[24] Briefly, the *D. magna* were cultured in the artificial freshwater (AFW) and maintained at 21 ± 0.5 °C under a 16: 8 (light: dark) photoperiod. Cultured daphnids were fed with a suspension of the *Scenedesmus subspicatus* twice daily. The detailed culture procedure was described in our previous study.[14]

2.3. Bioaccumulation of PFASs in *Daphnia magna* with the Presence of Protein. The bioaccumulation experiments were conducted in 500 mL polypropylene beakers. Each kind of protein was prepared with the concentrations of 1, 10, and 20 mg L^{-1} in AFW, respectively. A total of 200 mL protein solution was added into each beaker; then 0.02 mL of PFAS solution (50 mg L^{-1}) was added into each beaker with the nominal concentration of 5 μg L^{-1} for each PFAS. Methanol was used as a carrier and its amount in the final test medium was less than 0.1 mL L^{-1}. The beakers were shaken at 95 rpm in darkness at 21 °C for 72 h before the exposure experiment. For the kinetic experiment of bioaccumulation, a total of 150 *D. magna* (6−24 h old) were added into each beaker and cultured at 21 ± 0.5 °C under a 16: 8 (light: dark) photoperiod, and *D. magna* were sampled at the time points of 1, 3, 7, 11, and 24 h. Then the rest of the *D. magna* was transferred to the same protein solution without PFASs to start the depuration experiment, and *D. magna* were sampled at 1, 3, 7, 11, and 24 h, respectively. At each time point, 10 *D. magna* were transferred from each beaker by pipet to polystyrene culture dish and rinsed with AFW. Then *D. magna* were dried by filter paper and transferred to a 10 mL polypropylene (PP) centrifuge tube, and a wet weight was obtained, with an average of 7.62 mg for 10 *D. magna*. The *D. magna* were stored at −20 °C until extraction. A control group was set to study the bioaccumulation of PFASs in the absence of protein, and a blank experiment without spiking of PFASs and protein was also conducted. Each experiment set was conducted in triplicate. The pH and hardness of exposure solutions were measured at the beginning and end of all tests, and the results indicated that their variations were ≤5%. The kinetic model expressed in eq 1[25] was used to fit the uptake and elimination rate constants of PFASs in *Daphnia magna*,

$$C_b = k_u C_w \left(\frac{1 - e^{-k_e t}}{k_e} \right) \quad (1)$$

where C_b is the PFAS concentration in *D. magna* (μg kg^{-1}) at time t (d); C_w is the PFAS concentration in water phase (μg L^{-1}), which was considered as an constant because the variation of PFAS concentration was within 2% in the bioaccumulation experiments of the present study; k_u is the uptake rate constant of PFASs from water (L kg^{-1} d^{-1}) and k_e is the elimination rate constant of PFASs from *D. magna* (d^{-1}). The depuration rate constant (k_d, d^{-1}) was obtained by fitting the depuration phase data with the following equation:

$$C_b = A \cdot \exp(-k_d t) \quad (2)$$

For the steady state experiment of bioaccumulation, the procedure was the same as kinetic experiment except that 10 *D. magna* were added into each beaker to start the exposure, and the *D. magna* were sampled after 3 d exposure. The bioaccumulation factors (BAF_{ss}, L kg^{-1}) of PFASs based on the steady state experiment were calculated with eq 3

$$BAF_{ss} = \frac{C_B}{C_w} \quad (3)$$

Where C_B is the PFAS concentration in *D. magna* (μg kg^{-1}) at the end of experiment; C_w is the PFAS concentration in water phase (μg L^{-1}).

2.4. Dialysis Bag Experiments. Dialysis bag experiment was conducted to investigate the binding of PFASs with protein (Supporting Information (SI) Figure S1). Before the experiment, the dialysis bag was cut into 8 cm length and placed in the boiling water for 5 min; then it was washed by hot deionized water (60−80 °C) and room temperature deionized

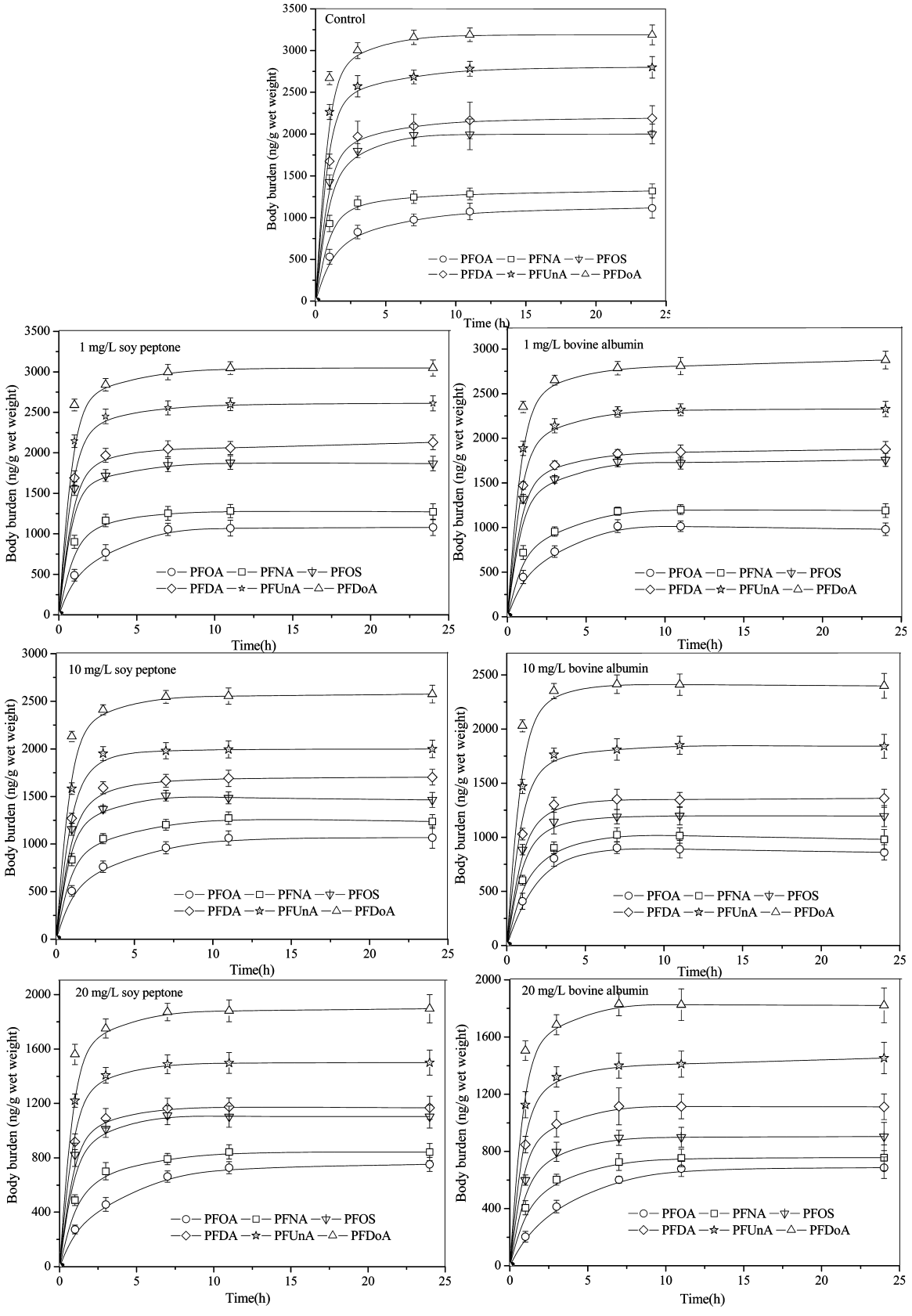

Figure 1. Bioaccumulation kinetics of PFASs in *Daphnia magna* in the presence of different concentrations and types of protein (mean ± standard deviation, $n = 3$).

water twice. A total of 20 mL of bovine albumin or soy peptone stock solution with the concentrations of 1, 10, and 20 mg L^{-1} was filled in the dialysis bags, respectively. The bags were sealed and placed into 200 mL deionized water in 500 mL

Table 1. Bioaccumulation Kinetic Parameters of PFASs in *Daphnia magna* under the Effect of Different Concentrations and Types of Protein (The Error Is the 95% Confidence Interval Obtained from the Regression)

PFASs	PFOA	PFNA	PFOS	PFDA	PFUnA	PFDoA
	Uptake Rate Constants (k_u, L·kg^{-1}·d^{-1})					
control	131 ± 66.12	335 ± 36.19	724 ± 32.72	763 ± 37.87	1067 ± 103.66	1279 ± 113.8
1 mg L^{-1} Soy peptone	112 ± 19.79	301 ± 42.52	638 ± 38.97	701 ± 77.90	933 ± 39.14	1220 ± 93.23
10 mg L^{-1} Soy peptone	100 ± 47.40	273 ± 24.36	444 ± 19.90	521 ± 6.18	665 ± 20.10	953 ± 22.55
20 mg L^{-1} Soy peptone	52 ± 18.31	135 ± 27.82	297 ± 26.95	368 ± 22.10	507 ± 26.90	690 ± 31.14
1 mg L^{-1} Bovine albumin	101 ± 27.99	189 ± 11.92	499 ± 18.69	601 ± 28.51	795 ± 51.58	1055 ± 41.01
10 mg L^{-1} bovine albumin	85 ± 13.36	156 ± 11.48	329 ± 11.23	423 ± 10.84	612 ± 15.98	896 ± 36.47
20 mg L^{-1} bovine albumin	43 ± 33.05	103 ± 55.27	189 ± 24.48	332 ± 72.53	460 ± 52.19	644 ± 60.46
	Elimination Rate Constants (k_e, d^{-1})					
control	0.63 ± 0.07	1.34 ± 0.10	1.73 ± 0.26	1.79 ± 0.12	2.05 ± 0.05	2.11 ± 0.49
1 mg L^{-1} soy peptone	0.53 ± 0.07	1.22 ± 0.22	1.68 ± 0.39	1.73 ± 0.28	1.84 ± 0.27	2.06 ± 0.44
10 mg L^{-1} soy peptone	0.48 ± 0.12	1.15 ± 0.23	1.51 ± 0.29	1.69 ± 0.32	1.74 ± 0.25	1.91 ± 0.30
20 mg L^{-1} soy peptone	0.35 ± 0.03	0.82 ± 0.09	1.36 ± 0.17	1.62 ± 0.16	1.71 ± 0.18	1.87 ± 0.14
1 mg L^{-1} bovine albumin	0.49 ± 0.05	0.80 ± 0.13	1.49 ± 0.24	1.69 ± 0.37	1.75 ± 0.19	1.93 ± 0.33
10 mg L^{-1} bovine albumin	0.47 ± 0.08	0.77 ± 0.13	1.40 ± 0.04	1.66 ± 0.058	1.70 ± 0.07	1.87 ± 0.06
20 mg L^{-1} bovine albumin	0.31 ± 0.01	0.69 ± 0.08	1.06 ± 0.10	1.56 ± 0.25	1.66 ± 0.12	1.79 ± 0.21

polypropylene beakers, respectively; they were shaken at 90 rpm in darkness for 72 h to remove the component with molecular weight less than 7000 Da. A preliminary experiment results showed that 4.3% of the soy peptone and no substantial bovine albumin could pass through the dialysis bag; this has been taken into account in the calculation of the partition coefficients of PFASs between protein and water. The pretreated dialysis bags containing the protein solution were placed into beakers with 200 mL AFW, and they were opened and added with certain amounts of PFAS solution, with the nominal PFAS concentrations of 5, 10, 20, and 50 μg L^{-1} in these dialysis bags, respectively. Then the dialysis bags were sealed and the beakers were shaken at 120 rpm in darkness at 21 °C for 72 h; a preliminary experiment showed that 48 h was enough for PFASs to reach the equilibrium between the protein inside of the dialysis bag and the water outside of the dialysis bag. A control experiment was conducted with dialysis bag but without protein. Each of the treatment had three replicates. After equilibrium, water samples were collected from the inside and outside of the dialysis bags, respectively for the determination of PFASs. The partition coefficients (K_p, L kg^{-1}) of PFASs between protein and water were calculated with the following equation:

$$K_p = \frac{C_s}{C_{free}} \quad (4)$$

where C_{free} (μg L^{-1}) is the freely dissolved concentration of each PFAS in the solution of PFASs and protein and it was equal to the PFAS concentration outside of the dialysis bag; C_S (μg kg^{-1}) is the PFAS concentration bound with protein, which was calculated based on the difference of PFASs between inside and outside of the dialysis bag.

2.5. Extraction and Analysis of PFASs. The PFASs in *D. magna* and water samples of the dialysis bag experiments were extracted by ion-pairing agent extracted method with some modification.[26,27] Briefly, 2 mL of Na_2CO_3 (0.25 M), 1 mL of the ion-pairing agent TBA (0.5 M, adjusted to pH 10), 2 mL of MTBE and 100 μL (10 ng) of MPFOA and MPFOS, the internal standard, were added into each PP centrifuge tube containing *D. magna* or water sample. The detailed procedure has been described in our previous study.[14] PFASs were analyzed using liquid chromatography−tandem mass spectrometry (LC−MS/MS; Dionex Ultimate 3000 and Applied Biosystems API 3200) in electrospray negative ionization mode. Briefly, a 10 μL aliquot of sample was injected into a 4.6 × 150 mm Acclaim 120 C18 Column with 50 mM ammonium acetate and methanol as mobile phase, at a flow rate of 1 mL min^{-1}. Detailed procedure can be found in Xia et al. (2012).[13]

2.6. Data Analysis. All statistical analyses were performed using SPSS 18.0 for windows (SPSS Inc., Chicago IL). Analysis of the variance (ANOVA, one factor) was carried out to test differences between each two compared groups. Difference was considered significant when the significance level was smaller than 0.05. The Pearson correlation coefficient was calculated and used to test the significance of correlation between each two variables.

3. RESULTS AND DISCUSSION

3.1. QA/QC Results. The determined limits of quantification (LOQs) (S/N=10) for the target analytes with LC-MS/MS were in the range of 0.01−0.05 μg L^{-1}, and all procedural blank areas were less than half LOQs. The correlation coefficients of standard calibration curves were higher than 0.99, and repeatability of these calibration curves were confirmed prior to each set of determination. Recoveries of the target analytes from the *D. magna* were between 81% and 95%, and the recoveries of MPFOS and MPFOA, mass spectrometric isotope spiked to the *D.magna* samples, were between 90% and 95%. Recoveries of the target analytes, MPFOS, and MPFOA from the water samples and protein solutions were between 86% and 108% (SI Table S1). The detailed procedure for quality assurance and quality control is shown in SI. The sample concentrations below the LOQs were set to zero, and all data were corrected according to the recovery indicators. PFASs were not detected in the *D. magna* samples of the blank bioaccumulation experiments. In addition, each PFAS accumulated in *D. magna* was less than 2% of the whole spiked in the system, indicating that the bioaccumulation of PFASs in the organisms did not significantly change the nominal concentrations of PFASs in the water system and break the equilibrium of PFASs between protein and water. The mass balance results of the dialysis bag experiments showed that the variations of PFASs in the system were less than 10%, and the control experiment results showed that the

difference in PFAS concentration between inside and outside of the dialysis bag was less than 8% in the absence of protein.

3.2. Effect of Protein Concentration on the Uptake and Elimination Rates of PFASs in *D. magna*. According to the results shown in Figure 1, the bioaccumulation of PFASs in *D. magna* almost achieved equilibrium within 24 h, and the bioaccumulation kinetic parameters of PFASs under the effects of soy peptone and bovine albumin were obtained (Table 1). No matter in the presence or absence of protein, the uptake rate constants (k_u) of PFASs increased with increasing perfluoroalkyl chain length, indicating that the uptake of PFASs in the *D. magna* becomes faster as the perfluoroalkyl chain length increases. This is probably due to the fact that PFASs with long hydrophobic chain would exhibit stronger interactions with protein in organisms than those with shorter carbon chain.[12,28] For both types of protein, the uptake and elimination rate constants of each PFAS decreased with increasing protein concentration. For example, when the soy peptone concentration increased from 0 to 1, 10, and 20 mg L^{-1}, the uptake rate constant of PFOA decreased from 131 to 112, 100, and 52 L kg^{-1} d^{-1}, with the decreasing ratios of 15%, 24%, and 60%, respectively; the elimination rate constant of PFOA decreased from 0.63 to 0.53, 0.48, and 0.35 d^{-1}, with the decreasing ratios of 16%, 24%, and 44%, respectively.

3.3. Effect of Protein Concentration on the Body Burden of PFASs in *D. magna*. After exposure for 3 days, the effect of protein on the body burden of PFASs in *D. magna* varied with the protein concentration. As shown in Figure 2, for both types of protein, when their concentrations increased from 1 to 10, and 20 mg L^{-1}, the body burden of all kinds of PFASs decreased significantly with the increase of protein concentration ($P < 0.05$). For instance, after exposure for 72 h, the body burden of PFOA decreased from 1133 to 758 and 464 ng g^{-1} when the bovine albumin concentration increased from 1 to 10, and 20 mg L^{-1}. Correspondingly, the BAF_{ss} values of PFASs based on the nominal concentrations of PFASs also decreased with increasing protein concentration (Table 2). The body burden and BAF_{ss} values of PFASs were decreased by 39.8–59.0% when bovine albumin concentration increased from 1 to 20 mg L^{-1}. Similar results were also observed in the bioaccumulation of benzo[*a*]pyrene and pyrene in *D. magna*; they decreased when dissolved organic carbon concentration increased from 1.4 to 18.5 mg L^{-1}.[29] This suggests that similar to the effect of dissolved organic matter on traditional persistent organic pollutants, the bioaccumulation of PFASs can also be reduced by the presence of high levels of protein.

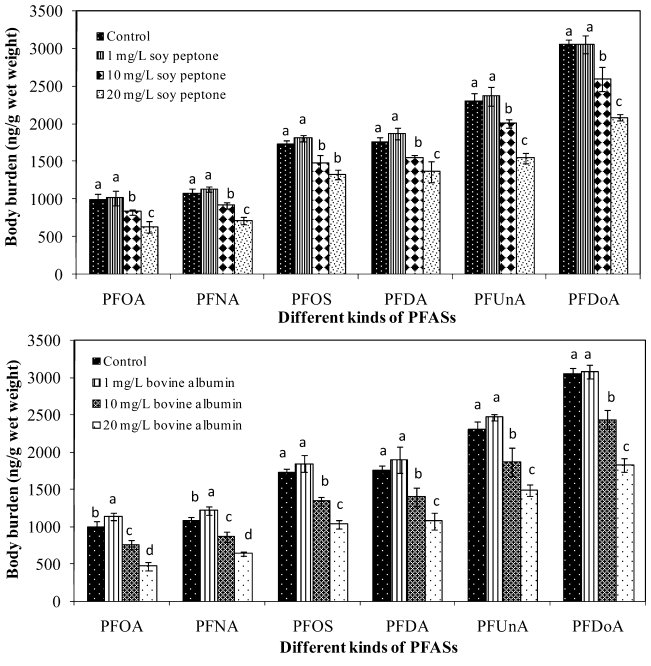

Figure 2. Effect of different concentrations of soy peptone and bovine albumin on the body burden of PFASs in *Daphnia magna* after 3 days exposure (mean ± standard deviation, $n = 3$). The a, b, c, and d denote the significant differences among the four groups determined by Duncan's multiple range test.

However, the body burden of all kinds of PFASs was elevated when the concentrations of both types of protein increased from 0 to 1 mg L^{-1}, and the increase was significant for PFOA and PFNA ($P < 0.05$) in the presence of 1 mg L^{-1} bovine albumin. For instance, the body burden of PFOA increased from 985 ± 86 to 1133 ± 50 and 1012 ± 93 ng g^{-1} when the bovine albumin and soy peptone concentrations increased from 0 to 1 mg L^{-1}, with the increasing ratios of 15.0% and 2.6%, respectively. Some studies have also reported enhancements in bioaccumulation of certain contaminants in the presence of low levels of DOM. For example, Leversee et al.[30] found that if adding 2 mg L^{-1} Aldrich humic acid to the test medium, water fleas accumulated 3-methylcholanthrene up to three times as much as in the control. Also, Muir et al.[31] observed that if the tests were performed in natural lake water or in solutions containing low concentrations (0.5–4 mg L^{-1}) of Aldrich humic acid, the bioconcentration factors (BCFs) of pyrethroids and DDT in rainbow trout could be up to four times as high as the respective BCFs in synthetic freshwater.

No matter with or without the presence of protein, as shown in Figure 2 and Table 2, the bioaccumulation was positively correlated with the carbon chain length for perfluorocarboxylic acids; the body burden of PFASs in *D. magna* was in the order of PFDoA> PFUnA> PFDA > PFOS > PFNA> PFOA throughout the exposure. PFDoA was the most accumulative PFAS in *D. magna* among the six kinds of PFASs studied; its BAF_{ss} value was approximately 3.1, 2.8, 1.8, 1.7, and 1.3 times that of PFOA, PFNA, PFOS, PFDA, and PFUnA, respectively in the absence of protein. The similar relationship between bioaccumulation and fluorinated carbon chain length as well as end functional groups has been observed in many aquatic organisms such as rainbow trout,[12] mussels,[32] *lumbriculus variegates*,[33] and *Chironomus plumosus* larvae.[13] In the present research, even with the presence of a high concentration (20 mg L^{-1}) of protein, the bioaccumulation was still positively correlated with the carbon chain length for perfluorocarboxylic acids, suggesting that the presence of protein would not change the order of PFAS bioaccumulation in *D. magna*.

3.4. Comparison of the Effects of Soy Peptone and Bovine Albumin on PFAS Bioaccumulation. The effect of bovine albumin on PFAS bioaccumulation was more pronounced than that of soy peptone (SI Figure S2). When protein concentration increased from 0 to 1 mg L^{-1} in the systems, the increasing ratios of the body burden of PFASs caused by bovine albumin (1.1–15.0%) were higher than that caused by soy peptone (0.3–6.7%). When protein concentration increased from 0 to 20 mg L^{-1} in the systems, the decreasing ratios of the body burden of PFASs induced by bovine albumin (35.3–52.9%) were also significantly higher than that induced by soy peptone (22.0–36.6%) ($p < 0.05$). In

Table 2. BAF_{ss} Values (L kg^{-1}) of PFASs Based on the Nominal Concentration of PFASs (5μg L^{-1}) under the Effect of Different Concentrations and Types of Protein (Mean±Standard Deviation, n = 3)

	PFOA	PFNA	PFOS	PFDA	PFUnA	PFDoA
control	197 ± 17	214 ± 13	344 ± 12	349 ± 15	458 ± 22	609 ± 15
1 mg L^{-1} soy peptone	202 ± 18	225 ± 7	361 ± 8	373 ± 15	473 ± 25	611 ± 24
10 mg L^{-1} soy peptone	165 ± 6	183 ± 8	296 ± 21	309 ± 6	400 ± 12	518 ± 33
20 mg L^{-1} soy peptone	125 ± 14	142 ± 9	265 ± 11	272 ± 27	308 ± 14	416 ± 9
1 mg L^{-1} bovine albumin	227 ± 10	243 ± 12	366 ± 21	378 ± 35	492 ± 5	614 ± 17
10 mg L^{-1} bovine albumin	152 ± 12	173 ± 13	266 ± 11	278 ± 24	372 ± 38	486 ± 25
20 mg L^{-1} bovine albumin	93 ± 10	127 ± 6	205 ± 10	214 ± 22	296 ± 15	365 ± 18

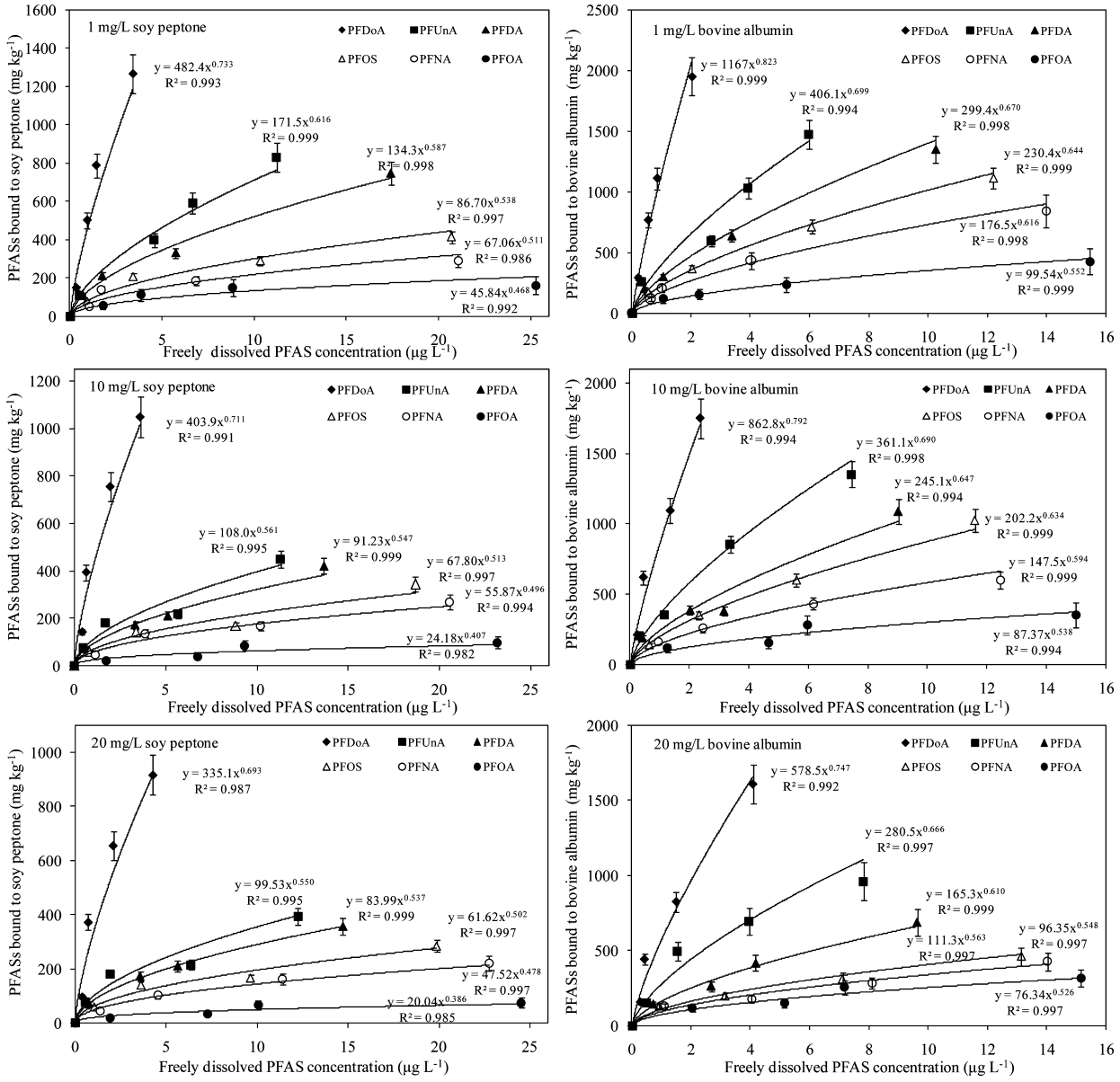

Figure 3. Sorption isotherms of PFASs to different types of protein with different concentrations (mean ± standard deviation, n = 3).

addition, according to the results shown in Table 1, the decreasing ratios of the uptake rate constants of PFASs caused by the presence of 20 mg L^{-1} bovine albumin (49.6–73.9%) were also higher than that caused by soy peptone (46.0–60.3%). It suggests that bovine albumin with a higher molecular weight has a stronger effect on the bioaccumulation of PFASs by *D. magna* than soy peptone in water.

3.5. Influencing Mechanisms of Protein on PFAS Bioaccumulation.

3.5.1. Partition of PFASs between Protein and Water. As shown in Figure 3, the binding of PFASs to soy peptone and bovine albumin could be well expressed by Freundlich sorption isotherm as follows,

$$C_S = K_F C_{free}^n \quad (5)$$

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Table 3. Partition Coefficients (K_p, L/kg) of PFASs (Represent As Log Value) Between Protein and Water When with the Initial PFAS Concentration of 5μg L^{-1} (The Error is the 95% Confidence Interval Obtained from the Regression)

	PFOA	PFNA	PFOS	PFDA	PFUnA	PFDoA
1 mg L^{-1} soy peptone	4.47 ± 0.022	4.65 ± 0.042	4.92 ± 0.044	5.17 ± 0.041	5.30 ± 0.024	5.60 ± 0.028
10 mg L^{-1} soy peptone	4.11 ± 0.023	4.59 ± 0.049	4.85 ± 0.043	5.01 ± 0.007	5.17 ± 0.015	5.49 ± 0.024
20 mg L^{-1} soy peptone	4.02 ± 0.007	4.51 ± 0.034	4.76 ± 0.032	4.96 ± 0.01	5.13 ± 0.022	5.41 ± 0.039
1 mg L^{-1} bovine albumin	5.04 ± 0.013	5.27 ± 0.022	5.49 ± 0.041	5.67 ± 0.032	5.92 ± 0.008	6.13 ± 0.038
10 mg L^{-1} bovine albumin	4.96 ± 0.024	5.22 ± 0.014	5.33 ± 0.026	5.64 ± 0.006	5.80 ± 0.005	5.89 ± 0.032
20 mg L^{-1} bovine albumin	4.74 ± 0.012	5.06 ± 0.014	5.16 ± 0.013	5.39 ± 0.004	5.55 ± 0.011	5.71 ± 0.019

Table 4. Freely Dissolved Concentrations of PFASs ($\mu g \cdot L^{-1}$) in the Exposure Systems with Different Concentrations and Types of Protein (Error is Caused by the *K*p Values Shown in Table 3)

	PFOA	PFNA	PFOS	PFDA	PFUnA	PFDoA
control	5.00	5.00	5.00	5.00	5.00	5.00
1 mg L^{-1} soy peptone	4.86 ± 0.01	4.79 ± 0.02	4.62 ± 0.03	4.36 ± 0.04	4.17 ± 0.03	3.58 ± 0.06
10 mg L^{-1} soy peptone	4.43 ± 0.03	3.60 ± 0.10	2.93 ± 0.08	2.47 ± 0.02	2.02 ± 0.04	1.22 ± 0.05
20 mg L^{-1} soy peptone	4.13 ± 0.01	3.04 ± 0.08	2.32 ± 0.10	1.77 ± 0.02	1.35 ± 0.05	0.81 ± 0.06
1 mg L^{-1} bovine albumin	4.51 ± 0.01	4.22 ± 0.03	3.82 ± 0.08	3.41 ± 0.06	2.73 ± 0.03	2.13 ± 0.10
10 mg L^{-1} bovine albumin	2.62 ± 0.06	1.88 ± 0.04	1.59 ± 0.06	0.93 ± 0.01	0.68 ± 0.01	0.57 ± 0.03
20 mg L^{-1} bovine albumin	2.38 ± 0.03	1.52 ± 0.03	1.29 ± 0.02	0.84 ± 0.01	0.61 ± 0.02	0.44 ± 0.02

where C_s ($\mu g\ kg^{-1}$) is the PFAS concentration bound to protein; K_F ($(\mu g\ kg^{-1})\cdot(\mu g\ L^{-1})^{-n}$) is the Freundlich sorption coefficient of PFAS on protein; C_{free} ($\mu g\ L^{-1}$) is the freely dissolved PFAS concentration; n is the Freundlich exponent. The sorption of PFASs by both soy peptone and bovine albumin showed highly nonlinearity with the Freundlich exponent n ranging from 0.443 to 0.823. It suggests that the sorption of PFASs on the protein is not a linear partitioning process but an adsorption-like process. The sorption quantity of PFASs on bovine albumin was higher than that on soy peptone (Figure 3), suggesting that the binding sites of the former are more than the latter. This might be caused by the different characteristics of these two types of protein (SI Table S2). The molecular weight of bovine albumin is larger than the soy peptone, and the contents of total nitrogen and amino acids of the former are also higher than the latter. Therefore, the bovine albumin can provide more binding sites for PFASs through electrostatic and van der Waals' force.

The partition coefficients (K_p) of PFASs between protein and water increased with increasing perfluoroalkyl chain length except for PFOS. For example, as shown in Table 3, when the initial PFAS concentration was 5 $\mu g\ L^{-1}$ and the bovine albumin concentration was 1 mg L^{-1}, the K_p values were $10^{5.04}$, $10^{5.27}$, $10^{5.49}$, $10^{5.67}$, $10^{5.92}$ and $10^{6.13}$ L kg^{-1} for PFOA, PFNA, PFOS, PFDA, PFUnA, and PFDoA, respectively, and the log K_p values of PFASs are positively correlated with their *n*-octanol/water partition coefficients (log K_{ow}) ($r = 0.97$, $p < 0.01$). The K_p values of PFASs between protein and water obtained in this research are much higher than other compounds with similar K_{ow} values, such as 1,3,5-trichlorobenzene ($K_{ow} = 10^{4.19}$, $K_p = 10^{2.14}$), pentachlorobenzene ($K_{ow} = 10^{5.18}$, $K_p = 10^{2.64}$), and perylene ($K_{ow} = 10^{6.25}$, $K_p = 10^{5.23}$).[34] Also, they are much higher than their organic carbon/water partition coefficients (K_{oc}) shown in SI Table S1, and the K_p value of PFOS is higher than its distribution coefficient between multiwalled carbon nanotubes and water.[35] However, the K_p values of PFASs between protein and water are comparable to the K_p value ($10^{5.27}$) of pentachlorobenzene ($K_{ow} = 10^{5.18}$) between lipid and water .[34] This suggests that the binding of PFASs to protein is very strong, which is comparable to that of hydrophobic organic compounds to lipids. In addition, as shown in Table 3, the K_p values of PFASs decreased with increasing protein concentration ($p < 0.05$). This is similar to the particle concentration effect as known for sorption of many compounds.[36,37]

3.5.2. Effects of Protein on the Freely Dissolved Concentrations of PFASs. Because the nominal concentration of each PFAS was 5 $\mu g\ L^{-1}$ in the exposure system, the K_p values with the initial PFAS concentration of 5 $\mu g\ L^{-1}$ (Table 3) were used to calculate the freely dissolved concentrations of PFASs in the exposure system with the following equations:

$$C_{total} = C_{free} + C_{free} K_p C_p \quad (6)$$

$$C_{free} = \frac{C_{total}}{1 + K_p C_p} \quad (7)$$

where C_{total} ($\mu g\ L^{-1}$) is the total PFAS concentration in the system; C_{free} ($\mu g\ L^{-1}$) is the freely dissolved PFAS concentration; K_p (L kg^{-1}) is the protein/water partition coefficient; C_p (kg L^{-1}) is the protein concentration in the system. As shown in Table 4, the freely dissolved concentrations of PFASs in the exposure system decreased with increasing protein concentration, and the reduction caused by bovine albumin was higher than those by soy peptone.

Further analysis showed that the uptake rate constants of PFASs were positively correlated with their freely dissolved concentrations in water system ($r > 0.89$; $p < 0.05$). This is because the complex of PFASs and protein is either too large, too polar or both to pass through the membrane of *D. magna* at an appreciable rate; only the freely dissolved compound is left available for uptake. McCarthy[38] also suggests that most likely, the impaired uptake of organic compounds involves a charge-exclusion or size-exclusion mechanism. As shown in Table 1, the uptake rate constants of PFASs to *D. magna* in the presence of soy peptone were higher than those in the presence of bovine albumin. This is due to the lower freely dissolved concentrations of PFASs with the presence of bovine albumin (Table 4), which is caused by the higher partition coefficients of PFASs between bovine albumin and water.

In addition, if the reduction of freely dissolved concentrations of PFASs caused by the presence of protein was the sole reason for the decreased bioaccumulation of PFASs, the $BAF_{ss, free}$ based on the freely dissolved concentrations of PFASs should be constant for each PFAS in the presence of different concentrations and types of protein. However, as shown in SI Table S3, the $BAF_{ss, free}$ did not keep constant, and it even increased significantly ($p < 0.05$) in the presence of protein for all PFASs except PFOA. This suggests that protein might also enhance the bioaccumulation of PFASs through other mechanism. As shown in Table 2, the elimination rate constants of PFASs also decreased with protein concentration. This might lead to the elevation of $BAF_{ss, free}$ value for each PFAS. Because protein can act as a food for *D. magna*,[39] the ingested protein would combine with PFASs in *D. magna* and resulted in the reduction of elimination rates of PFASs. This is demonstrated by the results shown in SI Table S4; the depuration rate constants (k_d) of PFASs decreased with the increase of bovine albumin concentration. For example, the k_d value of PFOA decreased from 3.40 to 2.30, 1.72, and 1.53 d^{-1} when bovine albumin concentration increased from 0 to 1, 10, and 20 mg L^{-1}. Therefore, the presence of protein would exert influences not only on the uptake but also on the elimination rates of PFASs in *D. magna*.

When protein concentration increased from 0 to 1 mg L^{-1}, the decrease of PFAS elimination rates from *D. magna* caused by the protein might prevail over the decrease of their uptake rates, resulting in the elevation of body burden of PFASs. However, when protein concentration increased from 1 to 10 and 20 mg L^{-1}, the decrement of depuration rate constant was lower than that caused by the increase of protein concentration from 0 to 1 mg L^{-1}. For example, the depuration rate constant of PFOS reduced by 0.21 d^{-1} when bovine albumin increased from 0 to 1 mg L^{-1}, whereas it only reduced by 0.12 d^{-1} when bovine albumin increased from 1 to 20 mg L^{-1} (SI Table S4). Therefore, when protein concentration increased from 0 to 10 and 20 mg L^{-1}, the influence of protein on the bioavailable concentrations and uptake rates of PFASs played a dominant role in PFAS bioaccumulation by *D. magna*, leading to the reduction of body burden of PFASs with increasing protein concentration.

In addition, possible explanation for the occurrence of enhanced bioaccumulation in the presence of low levels of protein could be that "PFAS loaded" protein is adsorbed to the surface of the organisms, leading to a higher body burden of PFASs than in the control treatments without protein. It has been shown that DOM can adsorb to biological surfaces, such as phytoplankton or fish gills.[40] Traina et al.[41] speculated that the observed enhancement of the bioconcentration of alkylbenzenesulfonates by DOM may have been due to an accumulation of contaminant-DOM aggregates in the mucous layer of the test fish, rather than to a real increase of contaminant uptake into the fish. In the present research, if the sorption of "PFAS loaded" protein was the reason, the body burden of PFASs in *D. magna* in the presence of 1 mg L^{-1} protein should be higher than those in the absence of protein in the beginning period of exposure because the sorption rates of PFASs must be higher than their uptake rates in *D. magna*. However, as shown in SI Figure S4, after exposure for 1, 3, and 24 h, the body burden of PFASs in *D. magna* in the presence of 1 mg L^{-1} protein was lower than those in the absence of protein, whereas after 3 days exposure, the former was higher than the latter. This infers that the enhancement of PFAS bioaccumulation in *D. magna* with the presence of 1 mg L^{-1} protein was not caused by the sorption of "PFAS loaded" protein but mainly caused by the reduction of elimination rates of PFASs from *D. magna*. The bioaccumulation factors of PFASs ($BAF_{kinetic}$, k_u/k_e, L kg^{-1}) based on kinetic method were determined from the kinetic parameters. As shown in SI Table S5, the $BAF_{kinetic}$ values were in agreement with the BAF_{ss} values obtained by steady state method (Table 2), and they increased in the presence of 1 mg L^{-1} protein and decreased gradually when protein concentration increased from 1 to 10 and 20 mg L^{-1}. This further demonstrates that protein would exert influences not only on the uptake but also on the elimination rates of PFASs in *D. magna*.

Because low concentrations of DOM including protein and humic acid commonly exist in most natural waters, their influences are of important environmental significance. Therefore, more experimental evidence are needed to investigate the influences of low levels of DOM from different origins on pollutant bioaccumulation in aquatic organisms.

■ ASSOCIATED CONTENT

Supporting Information

Additional information includes the QA/QC procedures, physicochemical and analytical parameters of PFASs, $BAF_{kinetic}$ values, depuration rate constants and comparison of PFAS body burden with and without 1 mg L^{-1} protein after different exposure time, etc. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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