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Immunotoxicity in Green Mussels Under Perfluoroalkyl Substance (PFAS) Exposure: Reversible Response and Response Model Development

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Abstract: The immunotoxicity of 4 commonly detected perfluoroalkyl substances (PFASs), namely, perfluorooctanesulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) was investigated by measuring biomarkers of the immune profile of green mussels, *Perna viridis*. The biomarkers included neutral red retention, phagocytosis, and spontaneous cytotoxicity, all of which were tested on mussel hemocytes. Hemocytes are an important component of the invertebrate immune system. We found that exposure to PFASs could lead to reduced hemocyte cell viability and suppress immune function by up to 50% of normal performance within the experimental exposure range. The results indicate that PFASs have an immunotoxic potential and thus could pose severe health risks to aquatic organisms. The reported immunotoxicity is likely to result from the compounds' direct and indirect interactions with the hemocyte membrane, and therefore likely to affect the functionality of these cells. The immunotoxic response was found to be related to the organism's burden of PFASs, and was reversible when the compounds were removed from the test organisms. Based on this relationship, models using an organism's PFAS concentration and bioaccumulation factor (BAF) as the independent variables were established to quantify PFAS-induced immunotoxicity. The models help us to gain a better understanding of the toxic mechanism of PFASs, and provide a tool to evaluate adverse effects for the whole group of compounds with one mathematical equation. *Environ Toxicol Chem* 2018;9999:1–8. © 2018 SETAC

Keywords: Perfluoroalkyl substances; Immunotoxicity; Toxic response model; Marine invertebrates

INTRODUCTION

Perfluoroalkyl substances (PFASs) have been applied in a broad range of industrial and commercial products as surfactants, coatings, and lubricants because of their excellent water- and oil-repellent properties (Chen et al. 2017; Liu et al. 2014). Their wide application also resulted in widespread contamination: varying levels of PFASs have been detected in air, water, soil, wildlife, and even human bodies (Corsini et al. 2014; US Department of Health and Human Services 2016). These compounds are of concern not only because of their ubiquitous presence in the environment, but also because they are chemically and physically stable, nonbiodegradable, and therefore highly persistent (US Department of Health and Human Services 2016). Although the production of some common PFASs has been phased out, these compounds will remain in our environment for a long time. It is therefore important to understand the environmental and ecological impacts of PFASs. Data from animal studies have demonstrated a series of adverse effects including liver degeneration, hepatomegaly, developmental and reproductive toxicity, oxidative toxicity, and gene modulation (Bjork and Wallace 2009; Corsini et al. 2012; Guruge et al. 2009; Hagenaars et al. 2011). The ocean is the final sink for many persistent pollutants including PFASs. Some PFASs have been detected at a level as high as hundreds of pg/L in coastal waters (Hu et al. 2011; Wang et al. 2012). However, to date, environmental toxicity data for the effects of PFASs on marine organisms are very limited.

Organisms rely on their innate defense mechanism to identify and protect against foreign materials (Hannam et al. 2009). The ability of organisms to respond effectively against pollutants is important to their general health, and disruptions of their immune systems can result in high health risks, even at sublethal doses (Giannapas et al. 2012). Previous studies have confirmed that the immune system is a sensitive target of PFASs (Corsini et al. 2012,

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2014; Mogensen et al. 2015). However, those studies focused on human health effects, and the data are limited to human and rodent species (Dewitt et al. 2012). To date, there are no data available on the immunological effects of PFASs on invertebrates, which represent more than 90% of extant species and thus play important roles in ecosystem function (Binelli et al. 2009). Considering the widespread contamination of PFASs and their health risks, it is necessary to extend investigations of the ecological impacts of these contaminants. Hemocytes are an important component of invertebrate immune systems (Sheir and Handy 2010). Hemolymph has also been suggested to be an appropriate tissue for biomonitoring because it is directly exposed to environmental contaminants and its plays a physiological role in the transport of toxic materials (Villela et al. 2006).

The aim of the present study was to conduct an in vivo investigation into the immunotoxicity of PFASs in the green mussel *Perna viridis*. The immunological effects of 4 long-chain PFASs (C7–C9) were examined by measuring biomarkers of the immune profile, which included neutral red retention, phagocytosis, and spontaneous cytotoxicity. Mussels have been used as sentinel organisms for contamination monitoring because they are sessile, filter-feeding, and have the capacity to accumulate contaminants (Villela et al. 2006). The circulatory system of mussels is continuously exposed to the mussels' living environment and contaminants (Thiagarajan et al. 2006). Previous studies have already shown that mussels can accumulate PFASs to a much higher level than their ambient environment (Fernandez-Sanjuan et al. 2010; Liu et al. 2011). To our knowledge, the present study is the first to examine the immune health effects of PFASs on marine invertebrates in an attempt to reveal the health status of organisms under PFAS exposure.

MATERIALS AND METHODS

Chemicals

Potassium perfluorooctanesulfonate (PFOS, 98% purity), perfluoroocanoic acid (PFOA, 96% purity), perfluorononanoic acid (PFNA, 97% purity), and perfluorodecanoic acid (PFDA, 98% purity) were purchased from Sigma-Aldrich. Other test chemicals used in the toxicity tests were also purchased from Sigma-Aldrich unless otherwise specified.

Mussel source and exposure

Green mussels obtained from a local fish farm (Yun Lee Fish Farm, Lim Chu Kang) were first acclimated to laboratory conditions for 7 d prior to transfer to the exposure system. They were maintained in artificial seawater (salinity 30 ppt, pH 8, dissolved oxygen 6 mg/L) in polypropylene tanks where the temperature was maintained at 25 °C. Two sets of 70-L duplicate polypropylene tanks were used for each exposure concentration and the control. A total of 60 to 65 mussels were raised in each tank. The exposure concentrations were 0.1, 1, 10, 100, and 1000 μg/L of individual PFASs for 7 d, followed by another 7-d depuration period, during which no PFASs were added to the tanks. The tanks were cleaned and refilled every 2 d, and 4 mussels and 100-mL water samples were taken every 24 h. During water changes, the tanks were drained and refilled with fresh artificial seawater spiked with PFASs. Commercial dense algae Shellfish Diet 1800 (Reed Mariculture) was used to feed the mussels every 2 d, 2 h before the water change. The feeding density was approximately 1.25×106 cells/L.

Sample preparation

For biomarker tests, mussel hemolymph was taken from the posterior adductor muscle of the mussel using a syringe. Details of the extraction method and recovery tests are described elsewhere (Liu et al. 2011). In brief, homogenized dry mussel tissues were extracted using 30 mL of potassium hydroxide solvent (KOH 0.01 mol/L in methanol) in 50-mL polypropylene tubes. The mixture was vortexed and centrifuged (4000 rpm, 15 min). The supernatant was diluted (1:100 with Milli-Q water) and extracted by using Oasis HLB cartridges (0.2 g, 6 cm^3; Waters). Cartridges were vacuum-dried before elution using 15 mL of methanol, and elutes were dried by nitrogen gas and reconstituted to 2 mL with methanol.

Biomarker testing

Trypan blue exclusion. Trypan blue exclusion was employed to measure cell viability. The method has been described elsewhere (Liu et al. 2007). In brief, 0.4% trypan blue was added to the hemocyte suspension in 1:2.5 (v/v). The cells were then examined under a microscope at ×400 magnification. An average of 150 cells was counted in 4 different fields per culture (per aliquot of hemocyte suspension). Cell viability was evaluated based on the percentage of the stained cells that were considered nonviable.

Neutral red retention. The neutral red retention assay assesses lysosome membrane stability by measuring the neutral red retention time. The hemolymph suspension was transferred to a poly-L-lysine–coated microscope slide, and the slides were immediately placed into a light-proof humidity chamber for 15 min. After incubation, excess hemolymph mixture was removed. Neutral red working solution was added to the slides, which were examined under a light microscope using a ×40 objective every 15 min. When not examined, the slides were kept in a humidity chamber. The time at which 50% of the cells showed stress was recorded as the retention time. The neutral red working solution was prepared by mixing neutral red stock solution (20 mg/mL in dimethyl sulfoxide [DMSO]) with physiological saline at 1:200 (v/v).

Spontaneous cytotoxicity. The test method has been described previously (Sheir and Handy 2010). In brief, sheep red blood cells (RBCs) were washed with phosphate-buffered saline (PBS) and resuspended in trishydroxymethylaminomethane-buffered saline with calcium (TBS-Ca) to 2×10^6 cells/mL. The mussel hemocytes were washed with TBS and also resuspended in TBS-Ca to 2×10^6 cells/mL. Then 100 μL of hemocytes was mixed with 100 μL sheep RBCs and incubated at 25 °C for 60 min

on a shaker. The samples were centrifuged at 100 *g* for 5 min after the incubation. Then 100 μL of the supernatants was transferred to 4 replicate wells in a microplate, and the absorbance was read at 405 nm. The specific cytotoxicity was calculated as a percentage relative to the maximum release (100 μL sheep RBCs in 100 μL H_2O) and the minimum or the spontaneous release (100 μL sheep RBCs in 100 μL TBS-Ca). The assay determines the ability of hemocytes to kill foreign cells by measuring the release of hemoglobin from sheep RBCs as they are lysed by the hemocytes.

Phagocytosis. The phagocytosis assay measures the ability of hemocytes to engulf foreign material, which in this test is zymozan particles (*Saccharomyces cerevisiae*). The zymozan particles (Cell Biolabs) were first stained with neutral red and resuspended in TBS at a final concentration of 1×10^7 particles/mL. Mussel hemolymph was diluted with TBS at 1:1 dilution. Then 50 μL of each hemolymph sample was aliquoted to 4 replicate tubes followed by addition of 50 μL of red-stained zymosan suspension. Zymosan with fixed hemocytes and zymozan in buffer were used as blank and negative controls, respectively. After a 30-min incubation at 10 °C, 100 μL of Baker's formol calcium (with 2% NaCl) was added to stop the reaction. The tubes were centrifuged and supernatant was discarded. The samples were then resuspended in 100 μL of TBS. The previously described procedures were repeated approximately 6 times until there was no evidence of zymosan remaining in the negative controls. The hemocytes were solubilized by adding 100 μL 1% acetic acid in 50% ethanol and incubating them for 30 min. They were then transferred to a microplate and read at 550 nm.

Biomarker scoring

The transformation of measured biomarker responses into one general index was performed as previously described with modifications (Liu et al. 2013). Details of the biomarker score calculation are included in the Supplemental Data. The normalized score of each biomarker response (B_i) was computed as:

$$B_i = \frac{Y_i + |\min_i|}{|\min_i|} \quad (1)$$

where Y_i is the standardized biomarker response, and $\min_i$ and $\max_i$ are the minimum and maximum value for each biomarker. The enhanced integrated biomarker response value (EIBR) was calculated as the average summation of the individual biomarker score:

$$EIBR = \sum_{i=1}^{n} B_i/n \quad (2)$$

Statistical analysis

Data were checked for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene's tests. Once the data had passed these tests, one-way analyses of variance followed by Tukey's post hoc tests were performed to compare variables between the control and the exposure samples. The significance level was set at $p < 0.05$. Statistical analysis was performed using SPSS 19 (IBM). Pearson correlation was applied in correlation analysis.

RESULTS AND DISCUSSION

PFAS-induced immunotoxicity

Biomarker results. The selected immunological biomarkers provided an assessment of the status of the internal defense system of the test organisms. Our biomarker results showed that exposure to PFASs resulted in measurable reductions in the immune fitness of green mussels, as indicated by the significant decrease in the biomarker response as the exposure level increased (Figures 1 and 2).

The hemocyte cell viability was measured by trypan blue exclusion assay. Immune activity depends on viable cell-to-cell interaction, and therefore hemocyte cell viability is directly related to immune mechanisms. The results showed that the tested compounds can decrease cell viability in a concentration-dependent manner (Figure 2). Loss in cell viability after PFAS exposure has also been observed in rodent and human cell cultures (Kraugerud et al. 2011; Slotkin et al. 2008; Watanabe et al. 2009).

A significant reduction in neutral red retention time was observed at 100 μg/L for most PFAS compounds, and a further reduction was observed at 1000 μg/L (Figure 1). The results suggest that PFAS exposure can lead to lysosome membrane instability. Lysosomes are cellular organelles that can engulf foreign substances and break down wastes and cell debris. They constitute the main sites of toxic metal sequestration and detoxification (Dailianis et al. 2003; Giannapas et al. 2012). We also observed that only a minor adverse response occurred in mussels after PFOS exposure compared with the other perfluorinated carboxylates (PFCAs). Because lysosomal instability is likely the result of organelle membrane damage (as discussed in *Possible causes of immunotoxicity*), the observed result may be explained by a previous finding that only minor inductions of cellular membrane damage were obtained as a result of PFOS exposure, compared with other PFCAs (Nobels et al. 2010).

Decreases in phagocytosis and spontaneous cytotoxicity were also observed with increases in PFAS exposure concentration (Figure 1). Phagocytosis and spontaneous cytotoxicity are the major mechanisms in the immune system of invertebrates that suppress infection. Phagocytosis is the cellular process of engulfing solid particles and is considered an important mechanism to remove pathogens and bacteria. Inhibition of phagocytosis induced by pollutants could suppress the immunocompetence of organisms. Previous studies have reported depression in phagocytosis in marine bivalves exposed to oil-contaminated seawater and organic pollutants (Auffret et al. 2004; Hannam et al. 2009). The present study shows that most of the PFASs tested induce inhibition of phagocytosis, as

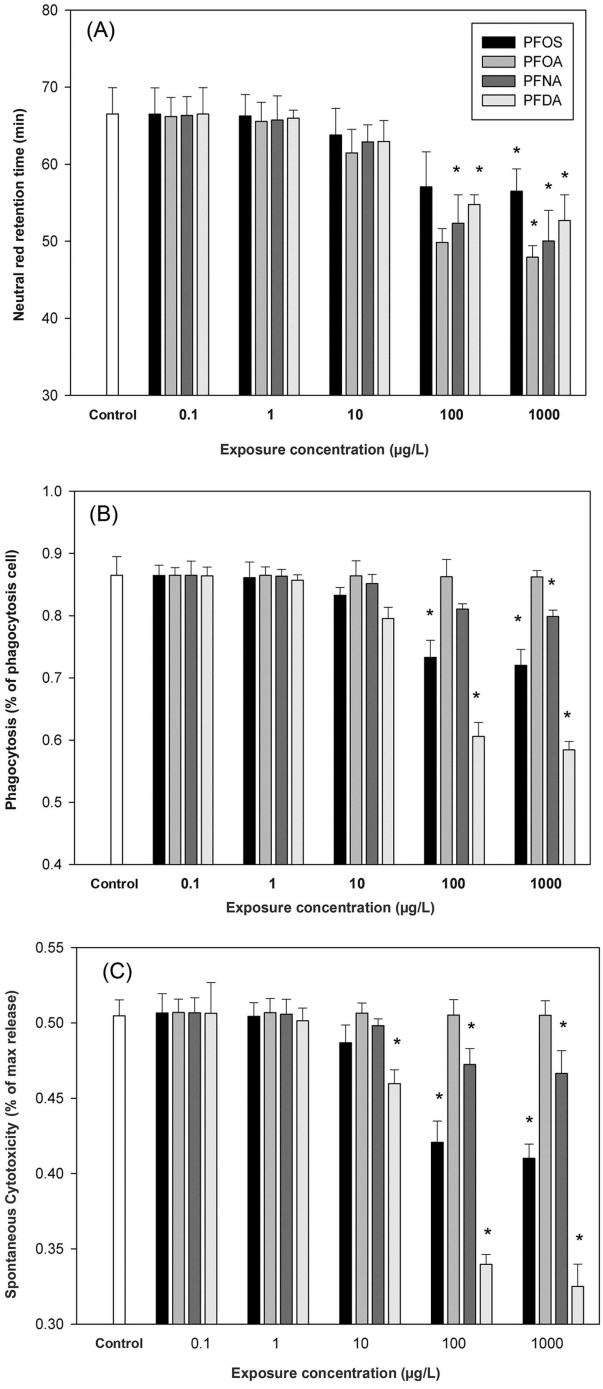

FIGURE 1: Response of immunotoxicity biomarkers in green mussels under perfluoroalkyl substance exposure. (**A**) Neutral red retention time. (**B**) Phagocytosis. (**C**) Spontaneous cytotoxicity. Significant responses ($p < 0.05$) are marked with *. Error bar stands for standard deviation. PFOS = perfluorooctanesulfonate; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFSA = perfluorodecanoic acid.

demonstrated by the decrease in the particular biomarker response. This inhibition of phagocytosis could result from reduced hemocyte cell viability under PFAS exposure, as demonstrated by the trypan blue exclusion test. The spontaneous cytotoxicity assay measures the ability of mussel hemocytes to recognize and kill foreign cells. Similar trends in the response pattern were observed for spontaneous cytotoxicity and

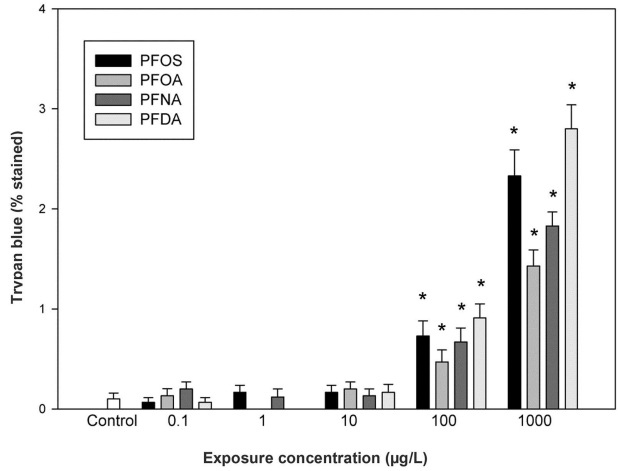

FIGURE 2: Hemocyte cell viability test result under perfluoroalkyl substance exposure. Significant responses ($p < 0.05$) are marked with *. Error bar stands for standard deviation. PFOS = perfluorooctanesulfonate; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFSA = perfluorodecanoic acid.

phagocytosis (Figure 1). The reason for this could be that both spontaneous cytotoxicity and phagocytosis are closely related to cellular membrane viability, where functioning of these immune mechanisms relies on viable cell-to-cell contact between hemocytes and foreign cells (Hannam et al. 2009).

Implications for organism health. The PFAS-induced inhibition of these immunological biomarkers suggests a weakened internal defense system in the presence of these contaminants. As observed in the present study, statistically significant decreases in normal immune function were associated with elevated exposure concentrations of PFASs, especially at the 1000 µg/L level, where the immune activities were suppressed by up to 50% of their normal performance. These results suggest that PFASs have an immunotoxic potential and may pose health risks to organisms at high exposure concentrations, for example, during an accidental spill. Immune modulation has been reported in mussels from polluted seawater (Hannam et al. 2009), and also after exposure to toxic metals and organic pollutants such as polycyclic aromatic hydrocarbons (PAHs; Giannapas et al. 2012; Thiagarajan et al. 2006). The PFASs have also been reported to modulate immune function in rodent and marine fish (Son et al. 2009; Yang 2010). In cultured human cells, PFASs were found to directly affect immune cell activation and reduce cytokine production (Corsini et al. 2012). Because the immune system protects organisms from foreign substances and pathogens, a weakened immune system implies that the organism becomes vulnerable in its living environment. Although some studies have shown that PFASs do not induce lethal toxic effects, our results suggest that sublethal effects induced by PFASs could be problematic, because the health of an organism will be compromised by a susceptible immune system, especially in the presence of other toxic pollutants or pathogens. Moreover, in some cases, contaminant exposure could induce elevated immune responses with sequestration of potentially toxic contaminants, especially at low concentrations

(Hannam et al. 2009; Luengen et al. 2004). However, similar inductions were not observed in the present study.

Possible causes of immunotoxicity. An impact on the cellular and organelle membrane is likely to be a major pathway through which PFASs affect the immune function of an organism, as shown by the biomarker results of trypan blue exclusion and neutral red retention, which measured cellular and organelle membrane integrity, respectively. Several hypotheses have been put forward to explain how PFASs cause membrane disturbance and lead to immunotoxicity.

One possible cause is baseline toxicity, which is the minimal toxicity as a result of intercalation of the chemicals into biological membranes, where they disturb structures, resulting in decreased activity and diminished ability to react to stimuli (Escher et al. 2017; van Wezel and Opperhuizen 1995). Previous studies have shown that some PFASs can cause increased cellular membrane fluidity through direct interference with the membrane (Hu et al. 2003). Lysosomal membrane stability can also be affected by a compound's direct interaction with the organelle's membrane (Hannam et al. 2009). In fact, the neutral red assay can be used as an indicator of baseline toxicity (Escher and Schwarzenbach 2002). Moreover, PFAS-induced immunotoxicity was found to be reversible, as discussed in the *Reversible responses and model* section. Therefore it is possible that immune function is affected through baseline toxicity and the resulting membrane perturbation in cells and cellular organelles that are involved in an organism's innate defense system.

The interaction of PFASs with functional proteins in membranes may also be responsible for immune function susceptibility, possibly because of the proteinophilicnature of the compounds (Kelly et al. 2009). The immune function susceptibility may be the result of alterations in cytoskeletal proteins that would then affect cell viability and functionality (Thiagarajan et al. 2006). These alterations could be caused by direct interaction with PFASs binding with these cytoskeletal proteins, such as peroxisome proliferator-activated receptor (PPAR) protein, which has been found to play an important role in PFAS immunotoxicity (Dewitt et al. 2012). The receptor proteins regulate important physiological processes that impact homeostasis, inflammation, adipogenesis, wound healing, and carcinogenesis (Corsini et al. 2011). The PFASs can bind and activate PPARs, which then lead to various adverse effects. Previous studies also showed that phagocytosis of bivalve hemocytes can be modulated by specific membrane receptors (Wang et al. 2011); in the present study, it was found that the membrane receptor could be bounded and activated or deactivated by PFASs.

Oxidative stress may also play a role in immunotoxicity. Oxidative stress induced by PFASs has been reported in several studies (Arukwe and Mortensen 2011; Liu et al. 2007). It has been suggested that immune susceptibility in hemocytes of bivalves exposed to organic toxicants is probably related to the depletion of nonenzymatic antioxidant molecules (Giannapas et al. 2012). Depletion of antioxidant components can cause depletion of antioxidant capacity and lead to oxidative stress. Oxidative damage (such as lipid peroxidation) to the membrane structure of hemocytes will thus affect the normal functionality of these cells. It has been suggested that the loss of cell viability could also be mediated by excess reactive oxygen species (ROS) production (Liu et al. 2007).

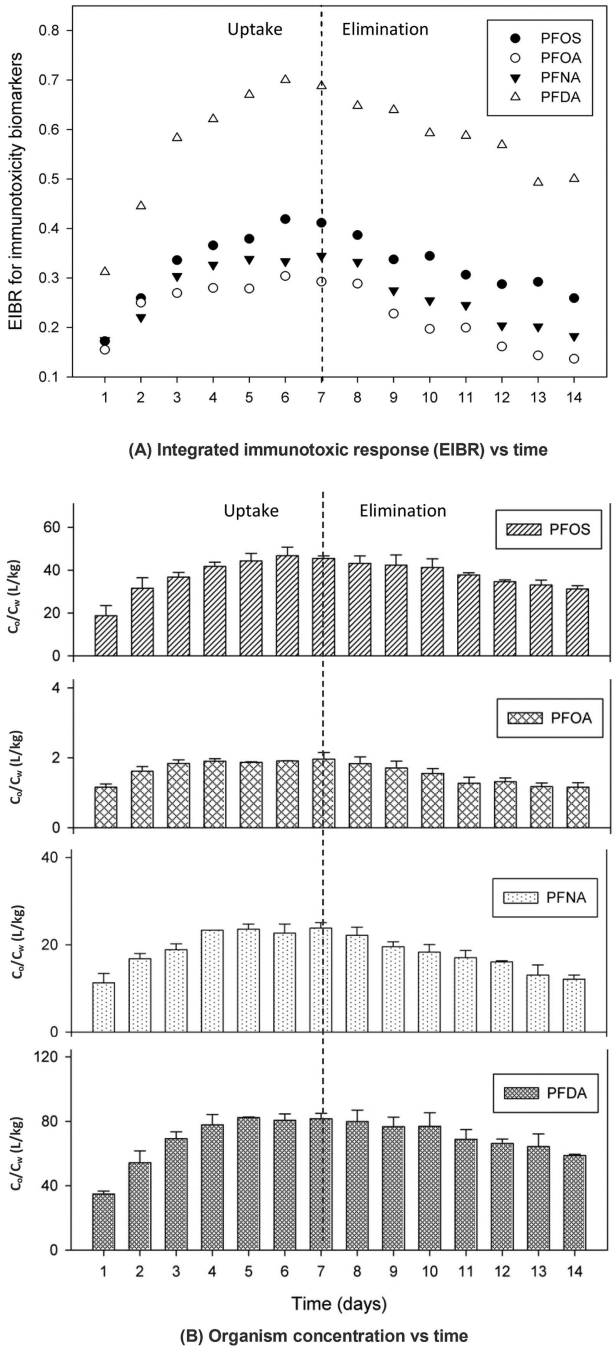

FIGURE 3: Relationships among integrated immunotoxicity, organism concentration, and time ($C_w = 100\ \mu g/L$). (**A**) Integrated immunotoxic response (enhanced integrated biomarker response [EIBR]) versus time. (**B**) Organism concentration versus time. Error bar stands for standard deviation. C_o = organism perfluoroalkyl substance concentration in ng/kg; C_w = exposure concentration; PFOS = perfluorooctanesulfonate; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFSA = perfluorodecanoic acid.

In addition to membrane disturbances, PFASs may also induce immunotoxicity through gene regulation. As mentioned, the defense mechanism of phagocytosis and spontaneous cytotoxicity relies on effective cell attachment. Previous studies have demonstrated that PFASs can cause suppression of genes that relate to immunity and especially cell adhesion (Cui et al. 2009).

Reversible responses and model

We found that exposure duration was another significant factor influencing the immune fitness of green mussels. At the same exposure concentration, adverse responses increased with exposure time (Figure 3). The magnitude of the adverse response was associated with the burden of PFASs inside the organism. During the depuration phase, toxic responses began to decrease when exposure to PFASs stopped (Figure 3). The immune fitness demonstrated a tendency to return to its original baseline state during the depuration period. Recovery of immunotoxicity when exposure stops has been reported in studies of different environmental stresses, such as high temperature and oxygen deficiency (Wang et al. 2011). Reversible effects of PFASs accompanied by depuration of the organism's burden of the compounds have also been reported in fish, rodents, and mammals (Du et al. 2009; Hu et al. 2002; Stevenson et al. 2006). This could be a result of reversible binding to integral membrane proteins and tissue proteins.

Our immunotoxicity results suggest that the toxic effects observed were closely related to the concentration of PFASs accumulated by the organism. For organisms exposed to different levels of PFASs or with different exposure durations, approximately the same toxic response was obtained when the concentration of PFASs reached the same level. Correlation analysis was performed, and the results further confirmed the correlations between an organism's PFAS concentration and integrated toxic response (Figure 4). Models using an organism's PFAS concentration and the bioaccumulation factor (BAF) as the

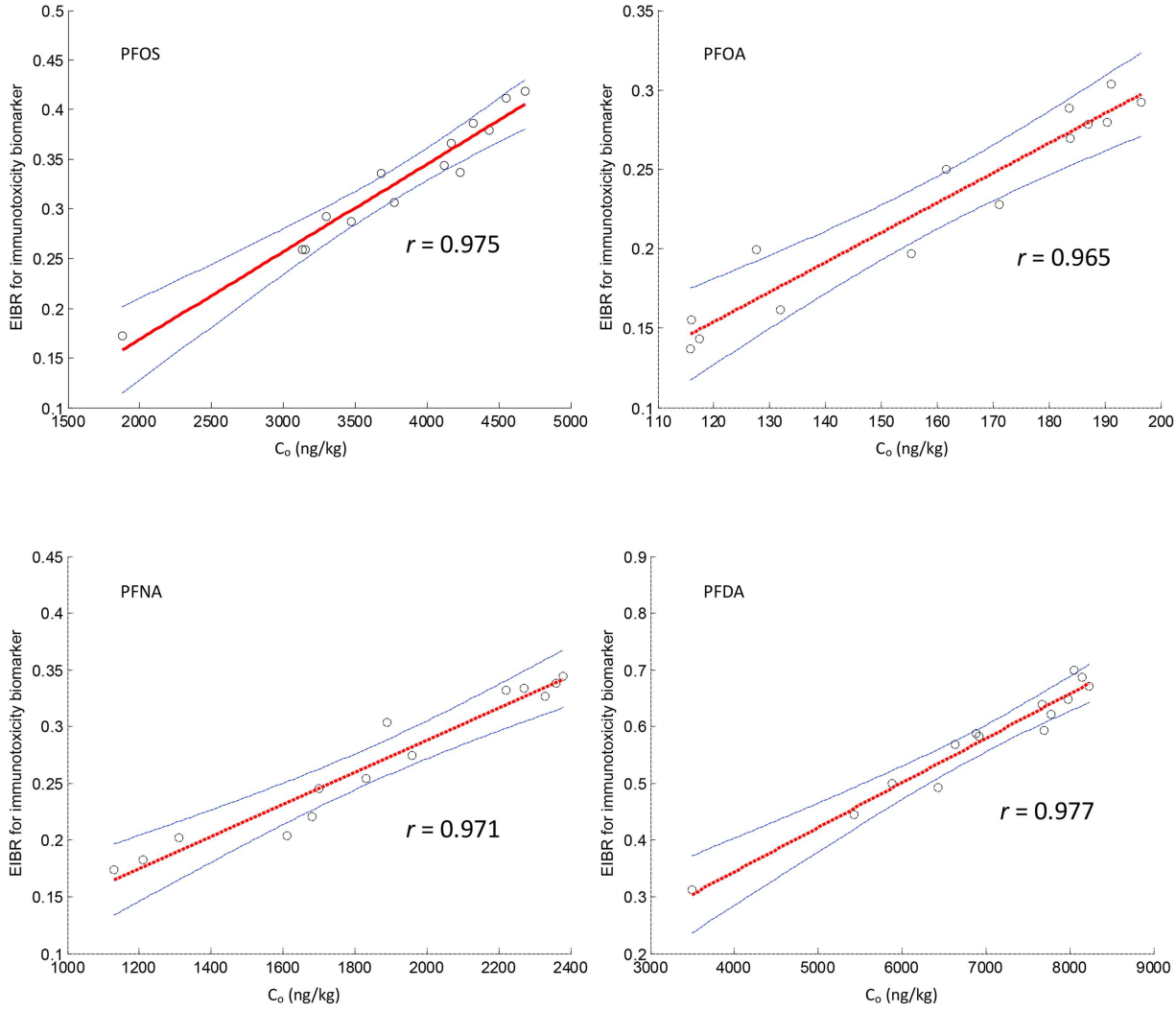

FIGURE 4: Correlation analysis of immunotoxicity enhanced integrated biomarker response (EIBR) with organism perfluoroalkyl substance concentration in ng/kg (C_o). Dashed line represents 99% confidence interval. PFOS = perfluorooctanesulfonate; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFSA = perfluorodecanoic acid.

independent variables were accordingly developed, to describe/quantify the integrated immunotoxicity as follows:

$$\log EIBR = a \log C_0 + b \log BAF + c$$
$$n = 4, r^2 = 0.877, s = 0.142, F = 596, Q^2 = 0.872 \quad (3)$$

where EIBR is the integrated immunotoxicity biomarker response value (Equation 2); C_o is the organism's PFAS concentration in ng/kg; and BAF is the bioaccumulation factor in L/kg measured in a previous bioaccumulation study (Liu et al. 2011). Parameters *a*, *b*, and *c* are constant for all the tested compounds. Based on the present biomarker results, the values of these constants were found to be 0.983, –0.712, and –2.070 for *a*, *b*, and *c*, respectively. Equation 3 provides a unified model to evaluate toxicity of different PFASs at various C_o by using a single set of *a*, *b*, and *c* values. The model shows that for the same compound, immunotoxicity increases with the organism's concentration of the compound. The toxic effects also vary among compounds as a factor of the bioaccumulation potential, where the larger the BAF, the more severe the immunotoxicity exhibited.

The concentration–response relationship demonstrated that there is an effective exposure concentration, approximately 10 to 100 μg/L, at which a significant response can be observed. Therefore results from test exposure concentrations of 0.1 and 1 μg/L were omitted for the model development.

The toxicity model provides a tool to evaluate and predict adverse effects induced in organisms under PFAS exposure when organism concentration is known. However, in reality, it is more conventional and easier to measure the contaminant concentration in the water phase. In this case, a toxic response model using aqueous exposure concentration would be more convenient. In PFAS bioaccumulation studies, a number of bioaccumulation models have been established that can be used to transform the organism concentration, C_o, to the exposure concentration C_w. For example, in a recent study, Liu et al. (2011) demonstrated that bioaccumulation of PFASs can be modeled through the following equation:

$$C_o = f(C_w, t) = \frac{nk_u C_w}{k_u C_w + k_e}\{1 - \exp[-(k_u C_w + k_e)t]\} \quad (4)$$

By substituting C_o in Equation 3, the immunotoxicity model can be further modified as a function of C_w, the exposure concentration:

$$EIBR = 10^c\, BAF^b \left(\frac{nk_u C_w}{k_u C_w + k_e}\{1-\exp[-(k_u C_w + k_e)t]\}\right)^a \quad (5)$$

With this equation, toxicity of PFASs can be estimated when the exposure concentration and exposure duration are known. It should be noted that for biomonitoring purposes, a direct measurement of organism concentration and application of Equation 3 may be more appropriate. This is because in natural environments, the contamination level may fluctuate, and the estimation of exposure duration may not be accurate or may be unknown.

CONCLUSION

In conclusion, the present study demonstrated the immunotoxic potential of selected PFASs: PFASs can induce inhibition of the organism's immune mechanism, which may lead to a weakened internal defense system in the presence of these contaminants. The immunotoxicity induced by PFASs is likely to result from the compounds' direct and indirect interactions with the hemocyte membrane, which thus affects the functionality of these cells. The observed immunotoxicity was also found to be reversible and correlated with the organism's concentration of the compounds. The immunotoxicity response can be evaluated using mathematical models when the accumulated internal tissue concentration of compounds is known. The environmental concentrations of the tested compounds are generally less than those causing the effects seen in the present study and thus are outside of the valid range of the immunotoxicity model, except for PFOA, for which the model indicates that the present levels may already affect the health, especially the immune health, of marine organisms. Although not specified by the results of the present study, it should be noted that in addition to the low threshold, there might also be a high threshold at which the immune system is totally destroyed by the compounds and not able to recover. In either case, the proposed model may not be appropriate.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4060.

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Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (ceeginyh@nus.edu.sg).

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