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New Perspectives on Perfluorochemical Ecotoxicology: Inhibition and Induction of an Efflux Transporter in the Marine Mussel, *Mytilus californianus*

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The toxicological effects of perfluoroalkyl acids on the p-glycoprotein (p-gp) cellular efflux transporter were investigated using the marine mussel *Mytilus californianus* as a model system. Four of the perfluoroalkyl acids studied exhibit chemosensitizing behavior, significantly inhibiting p-gp transporter activity. The inhibitory potency is maximal for the longer chain acids perfluorononanoate (PFNA) and perfluorodecanoate (PFDA), with average IC_{50} values of 4.8 and 7.1 μM, respectively. Results indicate that PFNA inhibits p-gp by an indirect mechanism, and this inhibition is reversible and accompanied by a rapid loss of PFNA from the tissue. In addition, PFNA induces expression of the p-gp transporter after a 2-h exposure, a stress response that may result in a metabolic cost to the organism. Given that most organisms, including humans, share efflux transporters as a first line of defense against toxicants, the results of this study may have broader implications for the ecotoxicology of perfluoroalkyl acids.

Introduction

Perfluorochemicals (PFCs) are used in a variety of industrial and household products, such as fire-fighting foams, textile and paper coatings, and insecticides (*1*). They are globally pervasive chemical pollutants that are largely resistant to bio- and photodegradation (*2*) and have been detected in marine environments (*3*, *4*), wildlife (*5*, *6*), and humans (*7*). Additionally, longer-chain perfluoroalkyl-based compounds such as fluorotelomer alcohols (*8*) and perfluoroalkyl sulfonamide derivatives (*9*) have been shown to degrade to produce stable perfluoroalkyl acid and sulfonate end products. Once released or formed in the environment, these PFCs are transported to marine environments (*3*) where some may bioaccumulate. These concerns and others led to a voluntary phase-out of the perfluorooctane sulfonyl-based product line (*10*), such as that used in Scotchgard, and the United States Environmental Protection Agency has recently called for voluntary reductions of industrial emissions of perfluorooctanoic acid (PFOA) by 95% by 2010 with an elimination of residual PFOA in consumer products by 2015 (*11*).

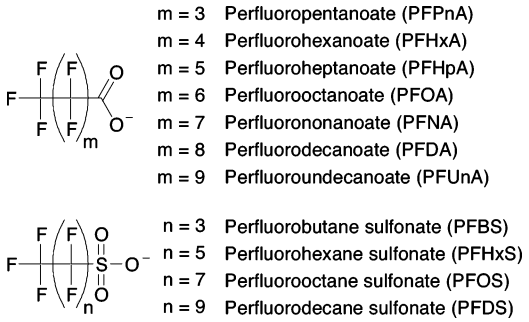

FIGURE 1. Perfluoroalkyl acids tested in this study.

Perfluoroalkyl acids (PFAs) are unique among persistent organic pollutants because they do not preferentially accumulate in fatty tissues yet are globally distributed and can be found in marine organisms distant from human activity, such as polar bears (*12*). These chemicals also have variable half-lives in different organisms, ranging from hours to years (*13*−*15*). The reasons for bioaccumulation and variable elimination rates are unclear but might relate to the combination of lipophobic/hydrophobic perfluoroalkyl tails of varying lengths with charged and polar headgroups, which allow these chemicals to interact with biological membranes as well as proteins. Several PFAs can affect membrane fluidity (*16*) and have also been shown to bind to proteins (*17*). Toxicological research on PFAs to date is limited and has focused primarily on PFOS (*2*, *18*), its derivatives (*2*, *18*), and PFOA (*13*).

In this study, we investigate the toxicity of eleven PFAs (Figure 1) with respect to the multixenobiotic resistance (MXR) mechanism. This mechanism acts as a cellular first line of defense against broad classes of xenobiotics (*19*), exporting moderately hydrophobic chemicals from the cell (*20*) via ATP-dependent, transmembrane transport proteins belonging to the ATP Binding Cassette (ABC) super-family of cellular membrane-spanning proteins (*19*).

The most studied transporter involved in the MXR mechanism is the p-glycoprotein (p-gp), which, in humans, is active in the kidney, adrenal gland, liver, blood−testes barrier (*21*), and blood−brain barrier (*22*). P-glycoprotein orthologues have also been identified in many other organisms by gene homology, activity measurements, and immunological probes, and are active in aquatic invertebrates (*23*−*27*) such as the marine mussel, *Mytilus californianus* (*28*).

The MXR defense mechanism is fragile and can be compromised by some xenobiotics, such as synthetic musks (*29*, *30*), thus sabotaging this cellular defense mechanism. This increased sensitivity, referred to as chemosensitization (*19*, *31*), arises from the ability of p-gp to recognize and bind to multiple xenobiotic substrates, resulting in the saturation of the binding capacity; even nontoxic substances can be chemosensitizers and cause adverse effects on an organism by allowing normally excluded toxic substrates to accumulate in the cell.

Here we examine the chemosensitizing effects of PFAs on efflux transport in the gill tissue of the marine mussel *M.*

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californianus, which is known to have high p-gp transport activity (*28*). We found that certain PFAs—PFOA, PFNA, PFDA, and PFHxS—inhibit transporter activity and thus are chemosensitizers. Our study also reveals that this inhibition is reversible, and from a detailed study of PFNA, we find that this reversibility results from a rapid loss of PFNA from the tissue. The loss is not mediated by p-gp since pharmacological inhibition of p-gp activity has no effect on the kinetics of PFNA loss. Irrespective, exposure to PFNA does inhibit transporter activity as well as induce the subsequent synthesis of more p-gp. The results of this study provide new insights about the toxicity of PFAs and also raise important questions about the biological accumulation and loss of these enigmatic chemicals.

Experimental Section

Chemicals. Perfluoropentanoic acid (PFPnA, 99.1%), perfluoroheptanoic acid (PFHpA, 99.8%), perfluorooctanoic acid (PFOA, 99.9%), perfluorodecanoic acid (PFDA, 99.9%), perfluoroundecanoic acid (PFUnA, 102.2%), and ammonium perfluorodecanesulfonate (PFDS, 25%) were from Aldrich Chemical Co. (Milwaukee, WI). Perfluorohexanoic acid (PFHxA, 98.8%), perfluorononanoic acid (PFNA, 99.2%), and potassium perfluorooctanesulfonate (PFOS, 98%) were from Fluka through Sigma-Aldrich (St. Louis, MO). Potassium perfluorobutanesulfonate (PFBS, 88%) and potassium perfluorohexanesulfonate (PFHxS, 97%) were provided by 3M Co. (St. Paul, MN). The internal standards [1,2-$^{13}C_2$]-perfluorooctanoic acid ([1,2-$^{13}C_2$]PFOA, 97.5%) and [1,2-$^{13}C_2$]-perfluorodecanoic acid ([1,2-$^{13}C_2$]PFDA, 99%) were from Perkin-Elmer Life Sciences (Boston, MA) and Wellington Laboratories (Guelph, ON), respectively. The standards used were of the highest grade available at a reasonable cost. Optima grade methanol and HPLC grade acetonitrile were from Fisher Scientific (Pittsburgh, PA). Ammonium acetate and glacial acetic acid were from Mallinckrodt (Phillipsburg, NJ). Supelclean ENVI-Carb 120/400 was from Supelco (Bellefonte, PA). Rhodamine B and verapamil were from Sigma (St. Louis, MO).

Sample Collection and Tissue Preparation. Mussels (*Mytilus californianus*, Conrad, 1837) were collected from the rocky intertidal zone at Hopkins Marine Station, Pacific Grove, CA, (valve length range: 60−85 mm) and were maintained in outdoor tanks with running seawater (12 °C) for 1−5 days before experiments. Mussel gills were excised and placed in filtered seawater (FSW, 12 °C). For transporter activity assays, biopsy punches (Acuderm, Fort Lauderdale, FL) were used to excise tissue pieces of equal size (5 mm diameter).

Transporter Activity Assay. A detailed account of the transporter activity assay is available in the Supporting Information. Rhodamine B, a fluorescent dye and p-gp substrate, was used as a proxy for efflux transporter activity. Inhibition of the transporter by a test compound results in increased fluorescence due to greater accumulation of rhodamine B within the cell (*32*). Transporter activity assays were performed according to the procedure by Luckenbach and Epel (*30*) with some modifications: test solutions were prepared in polystyrene dishes with 5 mL of FSW, 1 μM rhodamine B, and test chemicals or solvent only (0.5% DMSO). Equally sized discs of mussel gill tissue were incubated with rhodamine B and a test chemical for 90 min at 12 °C, washed in FSW to remove dye from the tissue surface, and immediately frozen on dry ice. The amount of rhodamine B in the tissue was quantified by extracting 3 combined discs per tested chemical or control into hypotonic lysis buffer by sonication. Tissue fragments were removed from the homogenate by centrifugation, and the amount of dye in the supernatant was determined spectrophotometrically (emission 590 nm, excitation 530 nm). Transporter activity assays were repeated at least three times using a different mussel for each experiment. The rhodamine B assay was also used to determine dose−response relationships for PFNA, PFDA, and verapamil, a known p-gp inhibitor (*33*) used as a positive control.

Duration of Transporter Inhibition and Induction. A detailed account of the experiments used to determine the duration of transporter inhibition and induction was according to Luckenbach and Epel (*30*) and is available in the Supporting Information. Gill tissue discs were exposed for 2 h to either 50 μM PFNA, 50 μM PFDA, 10 μM verapamil (positive control), or 0.5% DMSO, which was used as a solvent for the test compounds (negative control). Transporter activity and induction of p-gp synthesis in gill tissue were determined in one portion of the discs directly after exposure (0 h) and in two other portions of the discs after 24 and 48 h washing periods in FSW (50 mL, 12 °C).

For protein analyses, another sub-portion of the discs sampled after each of the washing time points was frozen in liquid nitrogen and stored at −80 °C until analysis. P-gp titers were determined using a Western blot analysis according to Kurelec et al. (*34*) with minor modifications. The long-term transporter inhibition experiment was conducted three times using tissue from a different mussel for each experiment, and the Western blot analysis was conducted twice.

Tissue Accumulation Studies. Tissue accumulation studies were performed using whole excised mussel gills. Gills were placed in 10 mL of FSW at 12 °C in plastic wells and gently agitated on a rocker table to ensure consistent exposure. Each well contained 2 gill lobes. A PFA was added to each well as a concentrated stock solution in DMSO; the DMSO concentration did not exceed 0.25% in the final experimental solution, and within each experiment the DMSO concentration was the same in each well. Following each exposure, tissues were rinsed vigorously in 3 successive beakers containing 500 mL of fresh FSW. Tissues were frozen at −80 °C until analysis, at which time they were freeze-dried at −80 °C. Freeze-dried tissue was homogenized with a glass mortar and pestle.

Sample Extraction. A detailed account of the sample extraction method is available in the Supporting Information. Briefly, ground, freeze-dried gill tissue was extracted with acetonitrile (3 × 3 mL) in a 50-mL polypropylene tube. For each addition of acetonitrile, the tube was vortexed, sonicated (60 °C, 10 min), centrifuged (1500*g*, 10 min), and decanted into 15-mL glass tubes. Extracts were acidified (1% v/v glacial acetic acid) and purified using a dispersed sorbent (ENVI-Carb, 25−50 mg) as described previously (*35*). Spike/recovery experiments were used to determine the efficiency of the extraction procedure, and for all analytes reported recoveries were between 80 and 100%.

Sample Analysis by Liquid Chromatography−Tandem Mass Spectrometry. A detailed description of the sample analysis is available in the Supporting Information. Briefly, concentrations of PFAs in mussel gill tissue were determined using high-performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). Chromatography was performed using an aqueous ammonium acetate (2 mM) and methanol gradient (initial and final eluent condition of 35% methanol) delivered at a flow rate of 250 μL/min. A Targa Sprite C18 column (40 mm × 2.1 mm, 5-μm pore size, Higgins Analytical, Mountain View, CA) equipped with a C18 Guard Column (Higgins Analytical) was used. A Sciex API 3000 triple quadrupole mass spectrometer (MDS Sciex, Ontario) operating in negative electrospray ionization multiple reaction monitoring (MRM) mode was employed for sample analysis. The MRM transition yielding the greatest sensitivity was monitored for each analyte. All other analysis parameters were similar to those previously described (*36*). The internal standards used in this study were analyte-

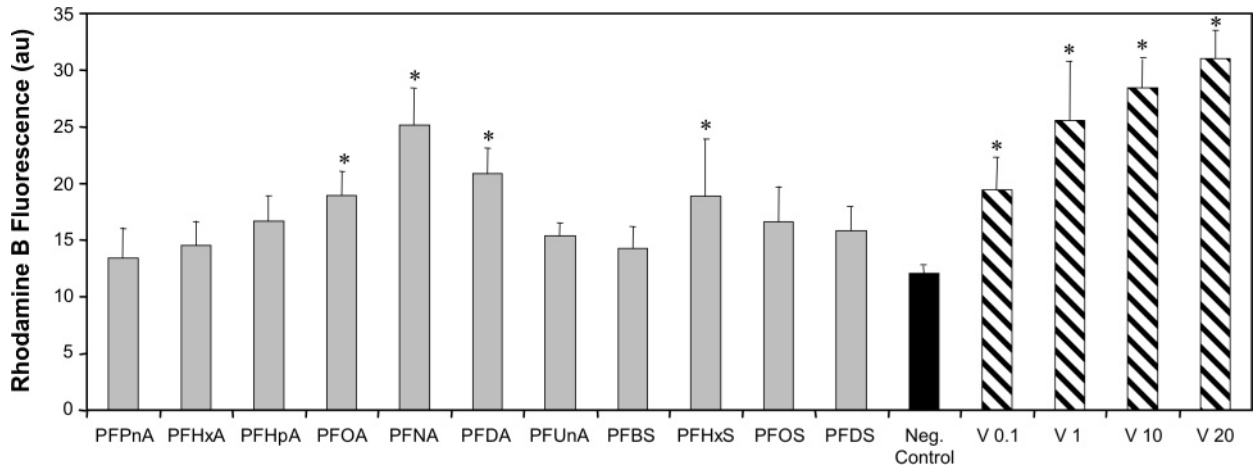

FIGURE 2. Inhibition of p-gp transporter activity by PFAs (50 μM) compared to a negative control (0.5% DMSO) and positive controls (0.1−20 μM verapamil, V). Increased rhodamine B fluorescence levels indicate p-gp inhibition. Asterisks indicate significant inhibition compared to the negative control ($p \leq 0.05$). Data are reported as the average and standard deviation of four experiments using gill tissue from four different mussels.

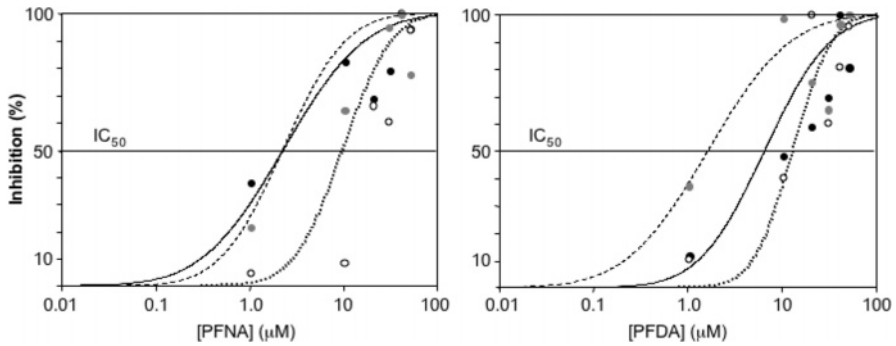

FIGURE 3. Dose−response curves for inhibition of p-gp transporter in mussel gill tissue by PFNA (left panel) and PFDA (right panel). Black (solid curve), gray (dashed curve), and white (dotted curve) circles represent data from three individual mussels. Curve fits were obtained by probit regression. Correlation coefficients (R^2) range from 0.4 to 0.6.

dependent: [1,2-$^{13}C_2$]PFOA was chosen for PFPnA, PFHxA, PFHpA, PFOA, PFBS, PFHxS, and PFOS; [1,2-$^{13}C_2$]PFDA was chosen for PFNA, PFDA, PFUnA, and PFDS. Quantitation and QA/QC are described in detail in the Supporting Information.

Data Analysis. Transporter activities in treated gill discs were compared to untreated controls for each mussel using Dunnett's test (JMP software, version 6, SAS Institute Inc., Cary, NC). The increase in fluorescence (compared to the negative control) was used as a measure of inhibition by each chemical at the specified concentration. Probit analysis was used to calculate the inhibitory potencies of test chemicals as concentrations causing 50% of the maximal inhibition observed for that chemical (IC_{50}) (*30*). All experiments were performed at least three times using gill tissue from different mussels. Data from the kinetic uptake/loss experiment were compared using Student's *t*-test.

Results and Discussion

Inhibitory Effects of Perfluorochemicals on p-gp. The effects of eleven PFAs on p-gp transporter activity were assessed as the inhibition of efflux of the fluorescent dye rhodamine B (a p-gp substrate) from *M. californianus* gill tissue; inhibition is seen as an enhanced accumulation of the dye. Preliminary studies showed that a 90-min exposure to 50 μM concentration showed the clearest difference in inhibitory effects of the various PFAs. Among the PFAs tested, PFOA, PFNA, PFDA, and PFHxS were identified as p-gp inhibitors and, therefore, chemosensitizers (Figure 2). Their effects were determined to be significant using Dunnett's test ($p \leq 0.05$). A chain-length-dependent inhibition pattern was observed, with PFNA and PFDA (9 and 10 carbons) causing the greatest inhibition ($p \leq 0.0001$ and 0.01, respectively). Subsequent studies focused on these two compounds since they had the greatest inhibitory effect.

To determine the inhibitory potency of PFNA and PFDA, dose−response curves were constructed (Figure 3). From these curves IC_{50} values (concentrations at which the chemical exerts 50% of its total inhibitory effect) were determined. The IC_{50} values were similar for PFNA (4.8 $\pm$ 4.3 μM) and PFDA (7.1 $\pm$ 5.8 μM). Variations in IC_{50} values reflect a natural variability among individual mussels, which has been observed previously (*30*). While the variability in the dose−response data result in significant error in determining the IC_{50} values, the data shown in Figures 2 and 3 clearly demonstrate the reproducible inhibitory effects of the PFAs. Effective concentrations of PFNA and PFDA are in the same range as for synthetic musks, another set of emerging contaminants with IC_{50} values of ~1−3 μM (*30*).

Duration and Reversibility of Inhibition. Immediately after exposing gill tissue to PFNA or PFDA for 2 h, inhibition of p-gp transporter activity was evident but was no longer apparent after 24 h in clean seawater (Figure 4a), indicating a reversible effect. A similar reversibility has been observed for the inhibition of gap junctional intercellular communication by longer-chain PFAs (C6−C10), with complete recovery in less than 1 h (*37*, *38*). In contrast, the long-term p-gp inhibitor verapamil (*30*, *33*) shows no dissipation of inhibitory effects after 24 h and still shows inhibitory effects after 48 h in clean seawater (Figure 4a).

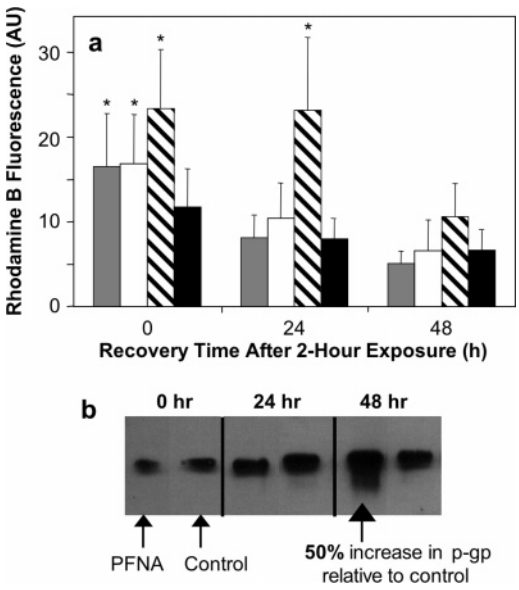

FIGURE 4. Duration of effects of PFNA and PFDA on the p-gp transporter. (a) After a 2-hr exposure to 50 μM PFNA (gray bars) or 50 μM PFDA (white bars), p-pg transporter activity returns to control levels (black bars) within a 24-hr recovery period in clean FSW; in contrast, verapamil (1 μM; striped bars) shows long-term inhibition. Data are reported as the average and standard deviation of three experiments using gill tissue from three different mussels. Asterisks indicate significant inhibition compared to the negative control ($p \leq 0.05$). (b) The Western blot shows significant induction of the synthesis of p-pg by PFNA over 48 h.

Induction of p-gp Synthesis. Despite the reversible nature of p-gp inhibition, the exposure to PFNA results in a long-term increase in total p-gp activity in the gill. Tissue that had originally been exposed to PFNA for only 2 h showed a 24% reduction in rhodamine B accumulation compared to the control after 48 h of washing in clean seawater. Such a decrease in dye uptake suggests increased transporter activity relative to the control, and possibly a long-term induction of synthesis of the transporter. A Western blot assay using a p-gp specific antibody was therefore performed on the same mussels used in the inhibition duration study to determine if there was indeed any induction of the transporter. As seen in Figure 4b, there was on average a 50% increase in the amount of p-gp after 48 h (relative to the control at 48 h, replicate analysis) in the tissue exposed to PFNA. In the case of PFDA, there was no consistent increase in the amount of p-gp after 48 h. This induction of p-gp could indicate that PFNA induces a general stress response, in which the syntheses of other stress proteins are also induced (*39*). This response may create a metabolic cost to the organism.

Mechanistic Studies of p-gp Inhibition: Differential Uptake or Differential Inhibition? The results presented in Figure 2 show that certain PFAs affect the transporter more than others. This could be due to differences in inhibitory potencies, but it could also mirror differences in the uptake rates of the different PFAs. To assess differential uptake, gills were exposed to the suite of PFAs at 50 μM and 100 nM to determine if the tissue accumulation pattern matched the transporter inhibition pattern. At both concentrations, the longer-chain PFAs—PFNA, PFDA, PFUnA, and PFOS—accumulated in the gill tissue to the greatest extent (results for 100 nM shown in Figure 5). Two of the longer-chain PFAs, PFNA and PFDA, also cause significant inhibition of the transporter (Figure 2). However, comparing Figures 2 and 5, there is no clear correlation between the extent of tissue accumulation and the magnitude of the effect on the transporter. Notably, the pattern of tissue accumulation illustrated in Figure 5 is in agreement with other data indicating that longer-chain carboxylates and PFOS bioaccumulate in wildlife (*12*). It is interesting to note that, while PFOS is bioaccumulative, it does not significantly inhibit p-gp at 50 μM. However, without further studies of the detailed mechanism of p-gp inhibition by PFAs, this observation remains unexplained.

Mechanistic Studies of p-gp Inhibition: Direct or Indirect Inhibition? Another mechanistic question is whether PFAs inhibit the transporter directly or indirectly. An inhibitor can act directly, behaving as a substrate and thus competing with the rhodamine dye used to monitor activity or binding irreversibly to the transporter. Alternatively, an inhibitor can act indirectly, by blocking the ATPase activity of the transporter or changing the membrane environment and thus disrupting the conformation and function of the transporter (*40*).

To determine if PFNA is a p-gp substrate, its uptake was measured in the presence and absence of the p-gp inhibitor verapamil. Increased uptake of PFNA in the tissue with blocked transporter function (+ verapamil) would indicate that PFNA is a substrate that binds to p-gp, competitively inhibiting its activity. No difference in uptake between experiments with blocked vs nonblocked transporter activity would indicate it is not a substrate and that inhibition is via an indirect mechanism.

In the experiment depicted in Figure 6, gill tissue was exposed to a low concentration of PFNA that was minimally inhibitory (100 nM, see Figure 3) for 60 min either with or without 1 μM verapamil and was subsequently allowed to recover in clean seawater for 4.25 h. Tissue concentrations were compared at each time point, and there is no significant difference ($p = 0.1$) in the uptake of PFNA in the absence or presence of verapamil. This indicates that PFNA is not a competitive substrate and that it is not recognized by the transporter.

One explanation for the effects of PFNA on p-gp activity may be nonspecific detergent-like effects on the membrane, which have been proposed by Hu et al. (*16*) to explain PFOS-induced changes in membrane fluidity and permeability. Given the surface-active nature of PFAs and their affinity for proteins (*17*, *41*), a possible mechanism for such membrane effects may be association with trans- and integral-membrane proteins (*16*). An alteration of membrane fluidity could lead to the observed inhibition of p-gp, a transmembrane protein, either through an effect on transporter tertiary structure or through alteration of the lipid environment around the transporter (*40*).

Mechanism of PFNA Loss. Gills exposed to PFNA for 60 min (with or without verapamil during the uptake phase) were allowed to recover in fresh seawater with no PFNA but with or without 1 μM verapamil. No significant effect of verapamil (present during uptake or recovery) on the rate or extent of loss of PFNA was apparent. The finding that verapamil has no significant effect on the uptake or loss of PFNA indicates that the p-gp transporter or other verapamil-sensitive transporters are not involved in these processes.

The kinetics of loss shown in Figure 6 reveal that the disappearance of PFNA is described best by a biexponential decay model. Initially, the rate of loss of PFNA is comparable to that of uptake. The fast rate of loss suggests that the majority of the PFNA is weakly associated with the tissue. The prolonged, slow release of some of the PFNA indicates that a fraction is retained within the tissue. It is possible that the observed loss of PFNA could result from conjugation or metabolism in the gill, which would be measured as a loss of PFNA by the detection method used in this study. However, other studies indicate that metabolism (*15*, *42*) and conjugation (*41*) of perfluorocarboxylates are unlikely.

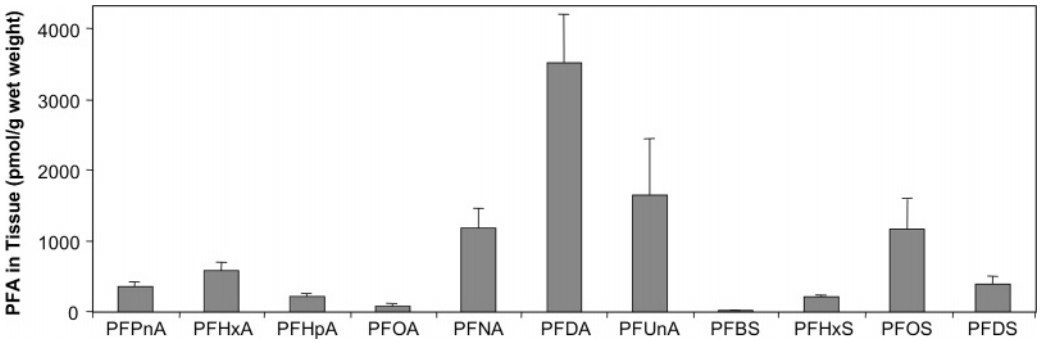

FIGURE 5. Concentrations of PFAs in gill tissue after a 60-min exposure at 100 nM. Each PFA was tested independently. Data are reported as the average and standard deviation of three experiments using gill lobes from three different mussels.

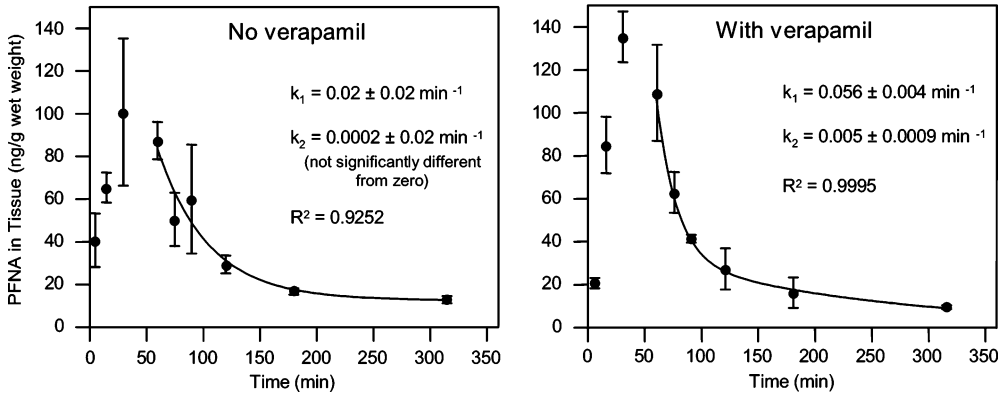

FIGURE 6. Time-dependent uptake and loss of PFNA in mussel gills. Gills were exposed to 100 nM PFNA for 60 min, either with (right panel) or without (left panel) 1 μM verapamil (a p-gp inhibitor). In both cases, the uptake was followed by a 4.25-h recovery in clean FSW without verapamil. Data are reported as the average and standard deviation of three experiments using gill lobes from three different mussels.

These results strongly suggest that the reversal of inhibitory effects of PFNA on the p-gp transporter shown in Figure 4a ensues from the rapid loss of the bulk of the PFNA from the tissue within a few hours after exposure. Perhaps a similar loss could account for other reports of reversible effects of PFAs on cell function (*37*, *38*). The fraction of PFNA that remains associated with the tissue is most likely the component that affects the signaling systems leading to the observed late induction of p-pg synthesis. This may also be the component that is retained in biological tissue and is measured as bioaccumulation.

Several PFAs inhibit the p-gp transporter after an acute exposure at 1−50 μM. Questions remain, however, concerning the chronic effects of these PFAs on the MXR mechanism (chemosensitization) at concentrations commonly measured in the blood and tissue of wildlife and humans (∼1−500 ppb, or ∼2 nM−1 μM). The availability of PFAs in blood serum or tissue to affect MXR transporter proteins is also unknown, given the possibilities of reversible/irreversible binding to serum and tissue proteins (*17*) and to integral membrane proteins.

The results presented here for PFNA also raise questions concerning the observed bioaccumulation and bioconcentration of PFAs. For instance, how do PFAs bioaccumulate if a large fraction is rapidly lost/eliminated from biological tissue (*13*−*15*)? The biphasic nature of the loss observed here suggests two associated fractions of PFNA characterized by low and high affinity binding. Could similar variations in binding affinity partially explain the wide variation seen in elimination half-lives of PFAs in different organisms (*13*−*15*), as well as the bioaccumulation of PFAs in wildlife and humans? The loss of PFNA could also ensue from the activity of another efflux transporter (*43*). Further research is needed to answer these questions.

This study also brings a new perspective to the toxicology of some PFAs, adding them to the spectrum of known chemosensitizers that can affect efflux transporters. At the concentrations observed in some wildlife, this chemosensitization may cause an increased sensitivity to other environmental toxicants. In addition, we have shown that an acute exposure to PFNA causes a later induction of p-gp transporter synthesis, a stress response that may result in a metabolic cost to the organism. Given that most organisms, including humans, share efflux transporters as a first line of defense against toxicants, the results of this study may have broader implications for the ecotoxicology of perfluorochemicals.

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Supporting Information Available

Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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