Characterizing Environmental Per- and Polyfluoroalkyl Substance (PFAS) Exposure and Effects in North Carolina Communities

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals used in a wide array of products and applications (e.g., nonstick cookware, waterproof and water-repellent textiles, firefighting foam). Following their decades of use, PFAS have garnered concern as "forever chemicals" due to their extreme persistence in the environment and in humans. PFAS have further elicited concern because they have been linked to adverse health effects in humans, and their huge number (over 12,000 different chemicals) and complex chemistry make them very challenging to analyze and study for exposure and toxicology. Two particular PFAS chemicals, perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS), are drinking water contaminants that can be found in the blood of the vast majority of people. PFOA and PFOS are also linked to toxic effects like kidney and testicular cancer, increased blood cholesterol, and reproductive outcomes. These two chemicals are being phased out of use and federal drinking water standards are likely upcoming. However, the replacements for these two chemicals are much less well-characterized, and many of these newer, replacement PFAS chemicals can be found in the environment of North Carolina due (at least in part) to industrial pollution.

The overarching goal of this dissertation was to characterize the potential exposure and health effects of PFAS in North Carolina communities. The surface water

and drinking water in some areas of North Carolina have been found to be contaminated with PFAS; however, there are additional routes of PFAS exposure beyond drinking water, such as ingestion of house dust or placental transfer during pregnancy. This dissertation explores various routes of PFAS exposure and better characterizes the specific PFAS analytes that can be found in North Carolina and the concentrations in which they are present. Additionally, this dissertation evaluates this exposure and potential associations with some adverse health outcomes in a few North Carolina communities.

In Chapter 2, the relationships between PFAS exposure during pregnancy and birth outcomes are explored. This chapter includes data on PFAS concentrations in placenta samples from 120 participants in Durham, North Carolina and evaluates the subsequent associations between placental PFAS exposure and birth outcomes (e.g., infant birth weight, gestational age). A total of 11 PFAS were measured in placental tissues collected in 2010-2011, and the compounds PFOS, PFOA, PFNA, and PFDA were detected in all placenta samples. A few placental PFAS were associated with birth outcomes. The most striking result was that placental PFOS was associated with changes in birth weight, but the direction of change depended on the sex of the infant. For male infants, placental PFOS was associated with lower birthweight, and in female infants, placental PFOS was associated with higher birthweight.

In Chapter 3, the exposure to PFAS through drinking water is evaluated in a community with known PFAS water contamination. This chapter includes data on PFAS concentrations in blood serum and drinking water samples from 49 participants in Pittsboro, North Carolina. The community receives its drinking water from the Haw River, a part of the Cape Fear River watershed. Blood and water samples were collected at two different timepoints to explore temporal variability in contamination. This community was found to have blood levels of PFAS about two to four times higher than the U.S. average. This chapter also includes results on the associations between PFAS blood level and clinical chemistry measurements, such as serum lipids, as indicators of health. Negative associations were found between serum PFOS and PFHxA with decreased electrolytes and decreased liver enzymes. Positive associations were found between serum PFOA and PFHxS with increased total cholesterol and increased non-HDL cholesterol.

In Chapter 4, the effects and toxicokinetics of PFAS in a pregnant rabbit model are evaluated. This chapter includes data from an animal study of 21 pregnant rabbits provided with drinking water that is representative of the PFAS exposure observed in Pittsboro, North Carolina. Rabbits were exposed to this environmentally-relevant mixture of ten different PFAS during and before pregnancy. After exposure, the wastes and tissues were evaluated to measure the PFAS concentration that accumulated. This provided information on where PFAS are distributed in the body after exposure. The

liver of the pregnant rabbit was also evaluated to determine if there was an increase in lipids in the liver, or any changes in liver lipid metabolism. For this study, few differences were noted between treated animals and control animals, indicating that the environmentally-relevant dose had little effect on pregnant rabbits. However, due to the lack of PFAS accumulation in blood, tissue, or in wastes, it is likely that the dose of PFAS given through drinking water was too low.

In Chapter 5, the levels of PFAS in indoor house dust were evaluated. This chapter includes data on PFAS concentrations in indoor dust from 184 homes in Durham, North Carolina, as well as 49 fire stations across the U.S. and Canada. House dust and fire station dust PFAS concentrations were then evaluated for associations with characteristics of the building (e.g., square footage, amount of carpeting, age of building construction). Levels of precursor PFAS, such as fluorotelomer alcohols, were typically higher in dust than the perfluoroalkyl acids. This study, along with previous literature, shows that the legacy PFAS in dust has been decreasing, but the precursor PFAS has been increasing in U.S. house dust. Few associations were found between building characteristics and dust PFAS. However, one notable result was that higher 8:2 FTOH was found in dust from buildings with more carpeting, indicating that carpets may be an important source of exposure to fluorotelomer alcohols (possibly from stain-proofing treatment).

Collectively, this dissertation provides important information on the potential exposure and health effects of PFAS in North Carolina communities.

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1. Introduction

1.1 Overview of PFAS Chemicals

Per- and polyfluoroalkyl substances (PFAS) are a group of over 12,000 heavily fluorinated chemicals extensively used for their grease- and water-repellent properties. These chemicals can be found in nonstick cookware, food packaging, textiles, and carpeting (Wang *et al.*, 2017), including brand-name products such as Teflon™, Scotchgard ™, and Gore-Tex®. They are also an important component in firefighting foams, especially for foams used at airports and military installations. However, their high volume of use has led to widespread, persistent environmental contamination. The same physicochemical properties that make PFAS chemicals desirable (e.g., stability, non-reactivity) also make them very difficult to remove from the environment. Their many carbon-fluorine bonds make PFAS resistant to degradation and contributes to their environmental persistence (Lau *et al.*, 2007). In fact, PFAS have been given the moniker of "forever chemicals" because of their extreme persistence.

Today, PFAS are found in the blood of 95-100% of the U.S. population, a result of both the high prevalence of PFAS and their long half-lives in the human body. Biomonitoring studies in other countries have highlighted that PFAS are a global exposure concern (Glynn *et al.*, 2012; Gockener *et al.*, 2020; Nøst *et al.*, 2014; Sundström *et al.*, 2011; Toms *et al.*, 2009; Toms *et al.*, 2014; Yeung *et al.*, 2013a, 2013b). This extensive

exposure is a concern for human health because PFAS are multi-system toxicants linked to kidney cancer, thyroid disease, and pre-eclampsia, among many other health outcomes (ATSDR, 2021).

The class of PFAS chemicals has been estimated to include between 5,000 chemicals (OECD, 2018) to over 12,000 chemicals as of June 2022 (USEPA, 2022c). While there are thousands of different PFAS chemicals, there is very little toxicological information on the vast majority of them. However, two of the most well-known and well-studied PFAS are perfluorooctanoic acid (PFOA) and perfluorooctane-sulfonic acid (PFOS) and are known for their toxicity and extreme persistence. Both PFOA and PFOS have been added to the Stockholm Convention on Persistent Organic Pollutants, an international environmental treaty aimed at reducing or eliminating the use of the most harmful and persistent chemicals. Several companies have now phased PFOA and PFOS out of production in the United States, [1] but replacements for these chemicals are now also a concern for environmental and human health. While efforts are ongoing to better characterize the data on some of these lesser-known PFAS (Carlson *et al.*, 2022; Pelch & Kwiatkowski, 2022; Pelch *et al.*, 2019), it is a daunting task to study and synthesize

[1] The 2006 voluntary agreement to phase out the use of PFOA and PFOS in the U.S. (the PFOA Stewardship Program) was established between only the EPA and eight major companies. As early as 2000, the 3M Company said it would be phasing PFOS out of its Scotchgard products. PFOA and PFOS may still be present in products imported to the U.S.

information on thousands of chemicals.

In the environment, these man-made chemicals can be found in drinking water, surface water, groundwater, wastewater treatment plant (WWTP) effluent, biosolids, soils, sediment, air, and dust. PFAS can enter the environment through multiple routes. Industrial discharges of PFAS, such as wastewater discharge from a facility that manufactures PFAS or PFAS-coated products, can lead to environmental contamination. The use of PFAS in aqueous film-forming foams (AFFF) as a firefighting measure at airports and military installations has led to known and extensive PFAS emissions and environmental contamination. Drinking water, indoor house dust, and consumer products (e.g., carpets, food packaging) can also contain or be coated with PFAS. Land application of PFAS-laden biosolids can further lead to environmental PFAS contamination of soil and water.

1.2 Health Effects of PFAS

PFAS are multi-system toxicants that have been linked to a myriad of human health effects. The following sections summarize the epidemiological and toxicological studies conducted in both humans and animals to explore the effects of PFAS.

Epidemiological studies on PFAS have observed significant associations with adverse health outcomes, including cancer, immune impairment, liver injury, and thyroid disease, among others (Barry *et al.*, 2013; Costello *et al.*, 2022; Fenton *et al.*, 2021;

Grandjean *et al.*, 2012; Lau *et al.*, 2007; NTP, 2016; Pelch *et al.*, 2019; Rappazzo *et al.*, 2017; Sunderland *et al.*, 2019). PFAS have been associated in humans with effects on the liver, kidney, immune system, thyroid, cholesterol, and infant birthweight (ATSDR, 2021).

Toxicity data from animal studies have further evidenced the potential harmful effects of PFAS (ATSDR, 2021; Fenton *et al.*, 2021; Lau *et al.*, 2007; Lilienthal *et al.*, 2017). Animal studies have also elucidated that the placenta may be a target organ for the reproductive and developmental toxicity of PFAS (Blake *et al.*, 2020; Marchese *et al.*, 2021; Marinello *et al.*, 2020). Placental lipid dysfunction, placental insufficiency, and pre-eclampsia may be caused or affected by PFAS, leading to adverse birth outcomes in humans (Szilagyi *et al.*, 2020a).

While some PFAS have been studied for their potential for toxicity, many other PFAS have not been studied at all. As of June 2022, the USEPA lists 12,034 PFAS chemicals on their Master List of PFAS Substances (USEPA, 2022c). This highlights the need to better characterize PFAS exposure to prioritize which PFAS to study and possibly to regulate for human and environmental health.

1.3 PFAS Exposure and PFAS in the Environment

PFAS are ubiquitous in the environment. Due to their useful chemical properties, PFAS have been intentionally added to many different products. PFAS can be found in nonstick cookware and food packaging (Begley *et al.*, 2005; Schaider *et al.*, 2017; Trier *et*

al., 2011), in personal care products such as cosmetics and sunscreens (Fujii *et al.*, 2013), in carpets (Chen *et al.*, 2020), in paper and textiles (Ritter *et al.*, 2017; Robel *et al.*, 2017), in impregnation sprays and agents used for textile protection, such as stain repellant carpets and upholstery (Fiedler *et al.*, 2010; Kotthoff *et al.*, 2015), and in anti-fog sprays (Herkert *et al.*, 2022). PFAS are also found as unintentional contaminants in food, such as milk and dairy products (Sadia *et al.*, 2020; Still *et al.*, 2013).

The widespread production and use of PFAS has led to contamination in the environment and in wildlife (De Silva *et al.*, 2021). Use of PFAS in consumer products (Kotthoff *et al.*, 2015) has also led to the detection of PFAS in the indoor environment, such as in indoor air (Winkens *et al.*, 2017) and in house dust (Savvaides *et al.*, 2021; Winkens *et al.*, 2018). Because people spend a significant amount of their time in homes and indoor environments, exposure to dust through inadvertent ingestion or inhalation can be an important route of exposure to chemicals, including PFAS. Infants and young children are especially vulnerable to chemical exposure through dust because they crawl and play on or near the floor and have higher hand-to-mouth activity.

Water contamination with PFAS is an especially large concern for human exposure. PFAS have been found at numerous drinking water utilities across the U.S. (Crone *et al.*, 2019; Quinones & Snyder, 2009). It is estimated that 18-80 million people in the U.S. receive drinking water with 10 ng/L of combined PFOA and PFOS, and over 200

million may be receiving water with ≥ 1 ng/L (Andrews & Naidenko, 2020).[2] Drinking water contamination in North Carolina has also led to PFAS exposure above the national average (Kotlarz *et al.*, 2020).

In humans, PFAS have been detected in blood (Calafat *et al.*, 2019; Calafat *et al.*, 2006; Olsen *et al.*, 2004; Olsen *et al.*, 2003; Wu *et al.*, 2015), urine (Calafat *et al.*, 2019), breast milk (Abdallah *et al.*, 2019; Cariou *et al.*, 2015; Fromme *et al.*, 2010; Jin *et al.*, 2020a; Macheka-Tendenguwo *et al.*, 2018; Zheng *et al.*, 2021a), placenta (Bangma *et al.*, 2020a; Chen *et al.*, 2017), and umbilical cord blood (Chen *et al.*, 2017; Mamsen *et al.*, 2017; Zhang *et al.*, 2013a; Zheng *et al.*, 2021b).

Data from the National Health and Nutrition Examination Survey (NHANES) have shown that nearly all of the U.S. population has detectable levels of some PFAS in their blood (Calafat *et al.*, 2019; Calafat *et al.*, 2006; Calafat *et al.*, 2007a; Calafat *et al.*, 2007b). PFAS have also been detected in the blood of North Carolina residents at concentrations higher than the general U.S. population (Kotlarz *et al.*, 2020).

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[2] Discussion is ongoing about what water concentration of PFOA and PFOS should be considered health-protective. As of April 2022, the EPA has a health advisory level of 70 ng/L for combined PFOA and PFOS in drinking water; however, several states (e.g., Massachusetts, Michigan, New Hampshire, New Jersey, New York, Vermont) have enacted maximum contaminant levels that are more stringent, below 20 ng/L.

1.4 PFAS Nomenclature

PFAS terminology can quickly become confusing as not all terms are standardized in the field. The below sections clarify some of the ways PFAS are classified and described.

An additional note: previous literature on PFAS prior to 2011 will refer to "PFCs" as an abbreviation for "perfluorinated chemicals" or "polyfluoroalkyl chemicals." This usage has now been mostly superseded with the term PFAS. Buck *et al.* (2011)b urged the scientific community to use the term PFCs exclusively for "perfluorocarbons," which do not have a functional group and contain only carbon and fluorine atoms.[3] The use of the terms "PFC" or perfluorochemicals should be avoided if possible and replaced with more specific terminology.

1.4.1 Precursor PFAS vs. Terminal PFAS

"Terminal PFAS" are the ultimate and final degradation products of other PFAS. Terminal PFAS are very stable and resistant to degradation and oxidation; no further transformations can occur to these compounds under normal environmental conditions. Perfluoroalkyl acids (PFAAs), such as PFOA and PFOS, are common terminal PFAS of concern. For PFAAs, no typical environmental process (i.e., transformation reactions

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[3] Adopting this usage would mean that PFCs are a subclass within the larger class of PFAS. Perfluorocarbons (PFCs) are also called fluorocarbons.

such as hydrolysis, photolysis, or microbial degradation) will break them down or degrade them, allowing PFAAs to accumulate in the environment indefinitely. Incineration may be a promising strategy to destroy and decompose these terminal PFAS into mineralized fluoride, but more research needs to be done.

Other PFAS can transform or degrade into different PFAS, including the terminal PFAS like PFAAs (Joudan *et al.*, 2020). These transforming PFAS are often referred to as "precursor PFAS." Precursor PFAS most commonly refers to the "PFAA precursors" that eventually lead to the production of persistent, harmful PFAAs such as PFOA and PFOS. Measurement of precursor PFAS can be accomplished through the total oxidizable precursor (TOP) assay, an assay that measures the amount of "potential PFAA" available in a sample (Houtz & Sedlak, 2012). The TOP assay will likely become important for future research and regulation, especially for quantifying and identifying the precursors that can generate PFOS and PFOA. For example, a contaminated site that currently has low levels of PFOS and PFOA may have large amounts of PFAA precursors; as these precursors transform over time, the PFOS and PFOA concentrations at the site could increase dramatically. Understanding the current PFAA contamination at a location as well as the potential PFAA contamination from existing precursor PFAS will be important in remediating PFAS issues.

1.4.2 "Per-"fluorinated vs. "Poly-"fluorinated

A perfluorinated chemical refers to an organic chemical where all the hydrogens of the hydrocarbon backbone (alkyl group) are substituted with fluorine. Thus, if fluorine atoms occupy all the spaces where hydrogen atoms would typically be, then the chemical is considered "perfluorinated." An alkyl group refers to a chain or carbon atoms bound together by single bonds and usually surrounded by hydrogen atoms; in the case of perfluorinated PFAS, these carbon atoms are completely surrounded by fluorine atoms instead of hydrogen.

If only some of the carbon atoms are substituted with fluorine (i.e., there are still some hydrogen atoms on the hydrocarbon backbone or alkyl chain), then the chemical is a "polyfluoroalkyl" substance. While polyfluoroalkyl substances have many fluorine atoms, they are not completely fluorinated. For example, 6:2 FTOH contains a polyfluoroalkyl chain of six fluorinated carbons (6) followed by two hydrogenated carbons (2). Different numbers of fluorinated carbons in the fluoroalkyl leads to prefixes like 4:2, 8:2, or 10:2; in each case, the 2 refers to the carbons that are bonded to hydrogen instead of fluorine.

1.4.3 Definitions by Chain Length

PFAS can also be defined by the lengths of their alkyl chains. Regardless of subclass, PFAS are frequently referred to by the number of overall carbon atoms in the

alkyl chain – PFOA and PFOS are known as "C8" compounds because they both have alkyl chains of eight carbons.[4]

The length of the fluoroalkyl chain within an individual PFAS can be an important indicator for potential PFAS toxicity and persistence. Longer fluoroalkyl chains like those in PFOA and PFOS often make those chemicals very persistent in the body and environment. Thus, shorter fluoroalkyl compounds are replacing the long-chain PFAS, with the hope that it will lead to decreased overall toxicity and persistence. Other compounds such as GenX are used as replacements because the fluoroalkyl groups are broken up by other atoms like oxygen; this replaces the straight-chain fluoroalkyl group with an ether group, making these compounds "ether" PFAS or "ether" acids.

Definitions of "short-chain" and "long-chain" PFAS will vary among references. Table 1 describes which PFAS are considered short- or long-chain in this dissertation. PFAS with carbon chain lengths of three or less (C2, C3) are rarely measured and have been called "ultra-short" chain compounds (Ateia *et al.*, 2019). Ultrashort-chain PFAS includes trifluoroacetic acid (TFA), perfluoropropanoic acid (PFPrA), perfluoroethane

[4] It may be worth noting that while PFOS has an alkyl chain of 8 fluorinated carbons, PFOS has an alkyl chain of 7 fluorinated carbons and 1 carbon from a carboxylic acid functional group.

sulfonate (PFEtS), and perfluoropropane sulfonate (PFPrS). TFA is abundant, but difficult to analyze.

PFBA and PFBS with carbon chain lengths of four carbon atoms (C4) are the shortest PFAS typically measured. "Short-chain" PFAS usually refers to the C4 to C6 compounds with "long-chain" referring to PFCAs with eight or more carbons and PFSAs with six or more carbons. PFOA (also called "C8") and PFOS are the two most well-known long-chain PFAS; they are known for their persistence and toxicity and are being replaced with shorter-chain compounds in hopes of reducing the toxicity and persistence, at least in the human body.

Table 1: Definitions of short- and long-chain PFAS as used in this work[5]

	Short-Chain PFAAs	**Long-Chain PFAAs**
PFCAs	(C4) PFBA, (C5) PFPeA, (C6) PFHxA, (C7) PFHpA	(C8) PFOA, (C9) PFNA, (C10) PFDA
PFSAs	(C4) PFBS	(C6) PFHxS, (C8) PFOS

[5] In the literature, PFHxS and PFHpA can be found referenced as both short- or long-chain by different references. This dissertation follows the classification according to OECD (2013).

1.4.4 Linear PFAS vs. Branched PFAS Isomers

An additional way to classify PFAS is by branching. When the alkyl group of a PFAS is arranged in a single straight-chain with no branching, it is considered a linear PFAS, or *n*-PFAS, or sometimes *L*-PFAS. Alternatively, PFAS can exist as structural isomers with branching among the alkyl groups, called branched PFAS or *br*-PFAS[6] (Figure 1). Branching can occur at any carbon along the alkyl chain, so PFAS with longer chain lengths (e.g., PFOA and PFOS) can have more branched isomers.

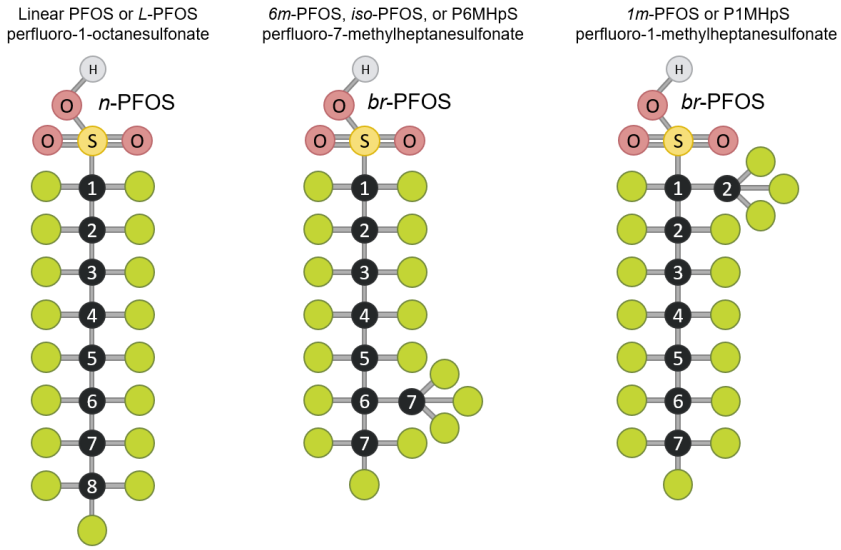

Figure 1: Isomers for PFOS.The linear isomer (*n*-PFOS) is compared to two examples of its many branched isomers (*br*-PFOS). The eight carbons are numbered to easily demonstrate where the branching occurs. While IUPAC nomenclature would classify the two branched isomers as perfluoromethyl-substituted perfluoroheptanoic

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[6] Additional abbreviations for branched PFAS: Sb-PFOA (the sum of branched PFOA isomers), Sm-PFOS (the sum of mono-methyl branched PFOS isomers), and Sm2-PFOS (the sum of di-methyl branched PFOS isomers).

acids, Benskin *et al.* (2007) proposed classifying each isomer with the position of its perfluoromethyl substitution (e.g., 1*m*-PFOS) for better clarity.

The different isomers of different PFAS can have implications for PFAS research. For example branched PFAS isomers may remain in water for longer than the linear versions and may preferentially accumulate in humans (Schulz *et al.*, 2020), and tissue-specific differences in PFOS isomer accumulation have been noted in wildlife (Greaves & Letcher, 2013). One study on PFOS isomers found that while linear PFOS had a half-life of 2.7 years in humans, the various branched PFOS isomers had half-lives of 2.7, 3.4, and 5.0 years (Li *et al.*, 2022).

Additionally, the ratio of branched and linear PFAS may provide important information on the source of contamination. Of the two major PFAS manufacturing processes, the electrochemical fluorination (ECF) process produced a higher proportion of branched PFAS isomers than the telomerization process (Schulz *et al.*, 2020).

This dissertation research reports all PFAS measurements as the sum of the linear and branched isomers; we did not quantify the levels of any branched isomers. Future work may endeavor to resolve and quantify some or all of the branched isomers for PFAS analysis, especially for the isomers of PFOA and PFOS.

1.5 PFAS Subclasses

PFAS as a group can refer to over 12,000 chemicals. An understanding of the various subclasses of PFAS is useful in parsing through the production, chemical

analysis, and health effects of PFAS. As groups push to regulate PFAS as an entire class, the similarities and differences between the subclasses will likely gain importance (e.g., one subclass should be regulated or monitored, but other subclasses should not).

Helpful resources for further understanding of PFAS subclasses include Buck *et al.* (2011) and Wang *et al.* (2017). The Interstate Technology and Regulatory Council (ITRC) also has extensive resources on PFAS.

The following subsections describe the PFAS subclasses that were evaluated in this dissertation research.

1.5.1 Perfluoroalkyl Acids (PFAAs)

The perfluoroalkyl acids (PFAAs) are the most commonly described PFAS in the literature and the primary focus of this dissertation. PFAAs include perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkane sulfonic acids (PFSAs). These are essentially singly-bonded carbon chains (i.e., an alkyl group) that end with either a carboxylic acid or sulfonic acid functional group. The carbon atoms are otherwise fully fluorinated (i.e., perfluorinated).

1.5.2 Fluorotelomer Sulfonates (FTSs)

Fluorotelomer sulfonates (FTS; also FtSs, FtSAs, or FTSAs) are very similar in structure to the PFSAs. However, for FTS compounds, an ethyl moiety (-CH2CH2-) is situated between the perfluoroalkyl tail and the sulfonate group. This makes the FTSs

polyfluorinated rather than perfluorinated. The FTSs would be included under "n:2 fluorotelomer-based substances" where 2 refers to the 2 carbons without fluorine and n refers to the number of carbons with fluorine.

Aqueous film-forming foams (AFFF) can contain FTSs. FTSs can potentially degrade and transform into PFAAs. In this work, 4:2 FTS and 6:2 FTS were measured in Pittsboro drinking water in Chapter 3.

1.5.3 Fluorotelomer Alcohols (FTOHs)

FTOHs are considered as "n:2 fluorotelomer-based substances" where "2" refers to the 2 carbons without fluorine and "n" refers to the number of carbons with fluorine. FTOHs can degrade and transform into PFAAs and are thus precursor PFAAs (Wang *et al.*, 2009).

Unlike the previous subclasses, FTOHs are relatively volatile and are commonly detected in air. Due to their volatility and neutral charge, they are routinely analyzed using gas chromatography instead of liquid chromatography.

Outdoor textiles, carpets, and shoes are sources of FTOHs; this is likely because these products are coated with water-repelling treatments or impregnating agents that contain FTOHs (Herzke *et al.*, 2012). Our research group has detected FTOHs in anti-fogging sprays (Herkert *et al.*, 2022) and in indoor dust, as described in Chapter 5 (Hall

et al., 2020). Others have similarly detected FTOHs in indoor air and dust (Winkens *et al.*, 2018; Winkens *et al.*, 2017; Zheng *et al.*, 2020).

1.5.4 Polyfluoroalkyl Phosphates (PAPs and diPAPs)

PAPs and diPAPs are mono-substituted and di-substituted polyfluorinated phosphate esters, respectively.[7] On a very simple level, a PAP is a phosphate group attached to one fluoroalkyl group while a diPAP is a phosphate group attached to two fluoroalkyl groups.

In Chapter 5, diPAPs were measured in indoor dust. The diPAPs are thought to be a source of PFCAs and are thus precursor PFAAs (De Silva *et al.*, 2012).

1.5.5 Perfluoroalkane Sulfonamides (FASAs) and Perfluoroalkane Sulfonyl Fluorides (PASFs), or PASF-based Substances

Chapter 5 includes data on MeFOSE and EtFOSE in indoor dust. MeFOSE and EtFOSE are examples of perfluoroalkane sulfonamido ethanols (FASEs). Others have detected MeFOSE and EtFOSE in air; MeFOSE has been used as a stain repellent on carpets and EtFOSE has been used to treat paper (Renner, 2004). Both MeFOSE and EtFOSE can transform into perfluorooctane sulfonamide (PFOSA, or FOSA) and ultimately PFOS (Benskin *et al.*, 2013; Buck *et al.*, 2011; Martin *et al.*, 2010; Olsen *et al.*, 2005). Perfluoroalkane sulfonamides (or fluoroalkyl sulfonamides, FASAs) such as

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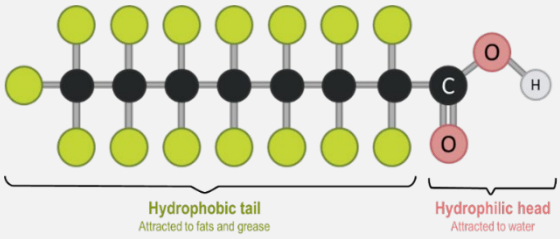
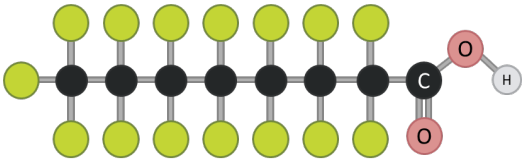
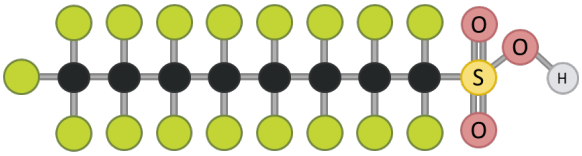
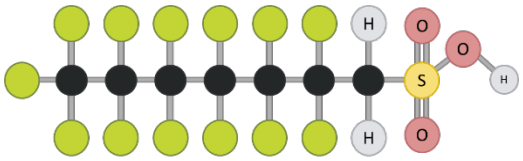
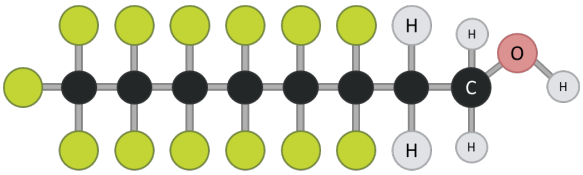
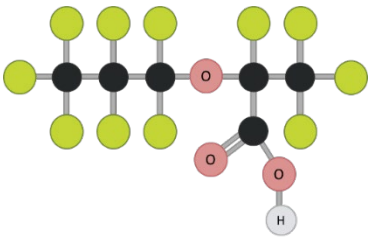
[7] Also referred to as polyfluoroalkyl phosphoric acid (di)esters or fluorotelomer phosphate (di)esters.

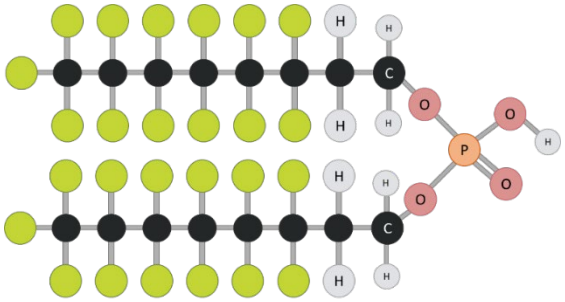
PFOSA are used as a raw material in the production process of PFAS-based surfactants and surface treatments.

1.5.6 Fluoroethers and Other Emerging PFAS

Fluoroethers include some PFAA replacement chemicals, such as GenX. GenX is one of the perfluoroalkyl ether acids (PFEAs) that can be detected in surface water in North Carolina (Strynar *et al.*, 2015; Sun *et al.*, 2016) and is a replacement for PFOA. The only fluoroether measured in this dissertation is GenX.

Table 2: Comparison of various PFAS chemical structures. Fluorine atoms are shown in green and carbon atoms are shown in black. Each subclass differs primarily by the functional group head (e.g., carboxylic acid, sulfonic acid, alcohol). Within each subclass, analytes will vary based on the number of fluorinated carbons are present in the fluoroalkyl tail.

GENERAL PFAS STRUCTURE		
Group	**Subclass**	**Representative Chemical Structure**
PFAAs	PFCAs, Perfluoroalkyl carboxylic acids	
PFAAs	PFSAs, Perfluoroalkane sulfonic acids	
PFAA Replace-ments	FTSs, Fluorotelomer sulfonates	
Precursor	FTOHs, Fluorotelomer alcohols	
PFAA Replace-ments	Fluoroethers	

Precursor	diPAPs, Polyfluoroalkyl phosphates	

1.6 Dissertation Research Aims

The overall goal of this work was to characterize the environmental exposure to PFAS and potential health effects of that PFAS in North Carolina communities. The specific aims of this dissertations are:

Aim 1: Characterize placental exposure to PFAS and explore associations with birth outcomes (Chapter 2). Human placental samples were collected from a cohort in North Carolina, United States and analyzed for PFAS. Associations between placental PFAS concentrations and birth outcomes (e.g., birth weight, gestational age) were evaluated.

Aim 2: Characterize exposure to PFAS through drinking water in an exposed community and explore potential health effects (Chapter 3). Pittsboro, North Carolina has been documented to have extensive historical and current PFAS contamination in its tap water and its drinking water source, the Haw River. Paired drinking water and blood samples were collected from a cohort in Pittsboro. Water and blood serum were

analyzed for PFAS concentration. Blood serum was further analyzed for clinical chemistry endpoints that could be potentially associated with PFAS (e.g., cholesterol, albumin, glomerular filtration rate).

Aim 3: Explore the mechanistic underpinnings of PFAS exposure through drinking water in a pregnant rabbit model (Chapter 4). In Chapter 4, rabbits were given drinking water formulated with the same PFAS concentrations as seen in Pittsboro, North Carolina. These rabbits were exposed before and during pregnancy, to better understand the reproductive and developmental effects of PFAS exposure. This exposure paradigm represents an environmentally-relevant mixture of PFAS because the exposure is provided through drinking water and with the same analytes and concentrations as seen in a real exposed community.

Aim 4: Evaluate the concentrations of PFAS found in indoor dust and associations with building characteristics (Chapter 5). Dust was collected from homes in North Carolina and fire stations in North America and analyzed for PFAS concentrations. This aim demonstrated the similarities and differences in dust PFAS composition and concentrations between residential homes and the living areas of fire stations.

Chapter 6 summarizes the conclusions and broader implications of this dissertation research and data gaps for future work.

2. PFAS Concentrations in Human Placenta and Associations with Birth Outcomes

This chapter was published under the title "Concentrations of per- and polyfluoroalkyl substances (PFAS) in human placental tissues and associations with birth outcomes" in *Chemosphere* in 2022. It is reprinted with permission from Hall, S. M.; Zhang, S.; Hoffman, K.; Miranda, M.L.; Stapleton, H.M. Concentrations of per- and polyfluoroalkyl substances (PFAS) in human placental tissues and associations with birth outcomes. *Chemosphere*. 2022, 295, 133873. DOI: 10.1016/j.chemosphere.2022.133873.

The accompanying supporting information is included in Appendix A.

2.1 Introduction

PFAS, or per- and polyfluoroalkyl substances, are a large group of chemical contaminants that can be found ubiquitously in the environment and in human bodies. Their occurrence is so frequent in part because PFAS are widely used for their water- and grease-repellant properties in a range of products such as non-stick cookware, textiles, carpeting, food packaging, firefighting foam, and consumer products (Wang *et al.*, 2017). In addition to being used in many products, many PFAS chemicals do not break down easily due to the strength of their multiple carbon-fluorine bonds (Lau *et al.*, 2007).

PFAS are frequently detected in human serum and urine, and over 95% of the U.S. population has PFAS in their blood according to data collected by the National Health and Nutrition Examination Survey (NHANES) within the Centers for Disease Control and Prevention (CDC) (Calafat *et al.*, 2007a; Calafat *et al.*, 2007b). PFAS can be persistent in the human body due to the long elimination half-lives of some PFAS, particularly longer-chain PFAS (Bartell *et al.*, 2010; Li *et al.*, 2018; Olsen *et al.*, 2007; Zhang *et al.*, 2013b). PFAS have been found in human serum (Calafat *et al.*, 2019; Calafat *et al.*, 2006), breast milk (Jin *et al.*, 2020a; Macheka-Tendenguwo *et al.*, 2018), and umbilical cord blood (Chen *et al.*, 2017; Mamsen *et al.*, 2017; Wang *et al.*, 2020). These findings are particularly concerning as they suggest the potential for placental and lactational transfer of PFAS to developing fetuses and infants; some PFAS are known to have developmental and reproductive toxicity (Bach *et al.*, 2015; Blake & Fenton, 2020; Chang *et al.*, 2018; Washino *et al.*, 2009).

These contaminants are multi-system toxicants with effects on the liver, kidney, immune system, thyroid, cholesterol, and infant birthweight (ATSDR, 2021). Epidemiological studies on PFAS have observed significant associations with adverse health outcomes, including cancer, immune impairment, and thyroid disease, among others (Barry *et al.*, 2013; Fenton *et al.*, 2021; Grandjean *et al.*, 2012; Lau *et al.*, 2007; NTP, 2016; Pelch *et al.*, 2019; Rappazzo *et al.*, 2017; Sunderland *et al.*, 2019). These observations

in humans have been further reinforced by data from animal studies (ATSDR, 2021; Fenton *et al.*, 2021; Lau *et al.*, 2007; Lilienthal *et al.*, 2017).

While the mechanisms of PFAS toxicity are still being elucidated, the placenta has come forward as a potential target organ of PFAS toxicity (Blake & Fenton, 2020). Placental effects have been observed after exposure to PFOA and GenX in mice (Blake *et al.*, 2020), and transplacental transfer of PFAS has been found experimentally in mice (Fenton *et al.*, 2009) and observationally in humans (Chen *et al.*, 2017; Mamsen *et al.*, 2019; Mamsen *et al.*, 2017; Yang *et al.*, 2016a; Yang *et al.*, 2016b). The placenta is a vital contributor to a healthy pregnancy as it mediates the exchange of nutrients, hormones, and other factors between mother and fetus. PFAS effects on the placenta may play a role in the mechanism of PFAS reproductive toxicity (Bangma *et al.*, 2020b; Blake *et al.*, 2020; Gao *et al.*, 2019; Lu *et al.*, 2021; Szilagyi *et al.*, 2020a; Szilagyi *et al.*, 2020b). For example, the placenta is a very important source of reproductive hormones, and prenatal exposure to short-chain PFAS such as PFBA and PFHpA has been associated with perturbations in fetal reproductive hormones (Nian *et al.*, 2020). A few prior studies have reported PFAS concentrations in placenta (Bangma *et al.*, 2020a; Chen *et al.*, 2017; Lu *et al.*, 2021; Mamsen *et al.*, 2019; Mamsen *et al.*, 2017; Martin *et al.*, 2016; Vela-Soria *et al.*, 2021; Zhang *et al.*, 2013a). However, the potential health implications of PFAS accumulation in the placenta remain largely unknown. To our knowledge, only one study (Bangma *et al.*, 2020a) has examined associations between placental PFAS with

health or birth outcomes in a high-risk cohort. In this study, researchers investigated whether PFAS placental levels were associated with gestational age at delivery, fetal growth, or hypertensive disorders of pregnancy; they did not find evidence for any associations.

In addition to being a potential target of toxicity, the placenta may also be a better measure of fetal PFAS exposure than maternal measures. While maternal serum PFAS is frequently used in studies focusing on birth outcomes, maternal serum PFAS concentrations during pregnancy can be impacted by other changes that occur during pregnancy. For example as pregnancy progresses, increases in blood volume, plasma volume, and glomerular filtration rate (GFR) can occur (Odutayo & Hladunewich, 2012; Peck & Arias, 1979). These changes may impact serum PFAS concentrations. The extent of change in blood volume can be highly variable, and normal pregnancies can show increases in blood volume of 20-100% of nonpregnant blood volume (Pritchard, 1965). It is unclear how these changes in blood volume and GFR affect the partitioning of contaminants between maternal serum and placental tissues.

The goals of this work were to evaluate the concentration of several PFAS in placenta collected from women living in central North Carolina in 2010-2011 and to further explore the associations between placental PFAS and birth outcomes.

2.2 Materials and Methods

2.2.1 Study Population and Sample Collection

Placental tissues were obtained from participants in the Healthy Pregnancy, Healthy Baby (HPHB) Study. This prospective cohort study aimed to explore racial disparities in pregnancy outcomes due to social, environmental, and maternal factors (Buttke *et al.*, 2013; Edwards *et al.*, 2015; Gona *et al.*, 2015; Grotegut *et al.*, 2017; Johnston *et al.*, 2014; Leonetti *et al.*, 2016a; Maxson *et al.*, 2012; Maxson *et al.*, 2016; Miranda *et al.*, 2015; Miranda *et al.*, 2011; Miranda *et al.*, 2010; Sanders *et al.*, 2014; Stapleton *et al.*, 2011; Swamy *et al.*, 2011). This study was carried out in accordance with a human subjects research protocol approved by the Duke University Medical Center Institutional Review Board (IRB), and all women provided informed consent prior to participation. In brief, pregnant women were enrolled from the Duke University Medical Center (DUMC) Obstetrics Clinic and the Durham County Health Department Prenatal Clinic. Women were excluded from participation if they were younger than 18 years old, were not English-literate, were less than 18 or greater than 28 weeks gestation at time of enrollment, lived outside of Durham County, North Carolina, had a multi-fetal gestation, had a known fetal genetic or congenital abnormality, or were not planning to deliver at DUMC (Miranda *et al.*, 2010). This population has previously been studied while investigating associations between brominated flame retardants and thyroid hormone levels in the placenta (Leonetti *et al.*, 2016a; Leonetti *et al.*, 2016b).

Our analyses include a subset of women from the HPHB study who delivered between March 2010 and December 2011 and from whom sufficient placental tissue was available (*n*=120). Placenta tissue subsamples (approximately 5-20 g) were taken at time of delivery at the Duke University Medical Center, and tissues were stored in Nalgene® polypropylene screw-top cryo-vials at −80 °C until analysis.

2.2.2 Sample Extraction and Purification

Extraction of PFAS from placenta was adapted from several methods previously described in the literature (Liu *et al.*, 2015; Martin *et al.*, 2016; Taniyasu *et al.*, 2005). Whole-thickness placenta tissue aliquots (~1.0 g wet weight) were thawed, lightly rinsed with ultra-pure water to remove blood, and minced with dissecting scissors in 50-mL polypropylene centrifuge tubes. Tissue was spiked with ^{13}C-mass-labeled internal standards (Wellington Laboratories, Guelph, Ontario, Canada), acidified with formic acid, and extracted with acetonitrile. Extracts (30 mL acetonitrile) were concentrated under nitrogen in an N-EVAP® on low heat (Organomation®, Berlin, Massachusetts) to ~1.0 mL. Extracts were then purified using solid-phase extraction (SPE) with weak anion exchange (WAX) cartridges (Oasis®, 6cc, 500 mg, 60 micron; Waters Corporation, Milford, Massachusetts) and eluted in 10 mL of methanol with 0.1% ammonium hydroxide in 15-mL polypropylene centrifuge tubes. Extracts were concentrated under nitrogen again to ~300 μL, spiked with 60 μL of 1 mM sodium hydroxide, and stored at −20 °C. Prior to LC-MS/MS analysis, 500 μL of 2 mM ammonium acetate in water was

added to each extract, and samples were transferred to 1-mL polypropylene push-top LC vials (Agilent Technologies, Santa Clara, California).

2.2.3 LC-MS/MS Analysis

Eleven PFAS (Table A1) were quantified in each extract using an Agilent 1260 Infinity II high-performance liquid chromatograph (HPLC) instrument coupled to an Agilent 6460A triple quadrupole mass spectrometer. The mass spectrometer was operated in negative electrospray ionization mode (HPLC-ESI-MS/MS). Separation of analytes by LC was performed using a 4.6 mm (I.D.) x 50 mm Agilent ZORBAX Eclipse XDB-C18 reversed-phase HPLC column (1.8 μm particle size) preceded by a 4.6 mm x 5 mm XDB-C18 guard cartridge.

Mobile phases were 2 mM ammonium acetate in water (mobile phase A) and 2 mM ammonium acetate in methanol (mobile phase B) using a flow rate of 0.4 mL/min. Gradient conditions for chromatographic separation were as follows: initial condition (30% B) was increased to 60% B over 1.5 minutes; then increased to 95% B over 2 minutes and held for 5.5 minutes. The gradient was then increased to 100% B over 3 minutes, before finally returning to initial conditions (30% B) over 0.5 minutes, and holding for 5.5 minutes. The column temperature was set at 45 °C and the injection volume was 20 μL. Data were acquired under multiple reaction monitoring (MRM) transitions using optimized parameters. Additional methods information, including transitions, is included in Tables A2, A3, and A4.

2.2.4 Quality Control and Quality Assurance (QA/QC)

Laboratory processing blanks were included in each batch and extracted alongside placenta tissue samples. A Lake Michigan fish tissue Standard Reference Material (SRM 1947) (NIST, 2017) of approximately 2.0 g was analyzed for PFAS alongside samples to examine accuracy. In total we included 7 lab processing blanks, 7 SRM extracts, and 12 duplicate samples (to assess precision) for our QA/QC assessment. Isotopically-labeled internal standards (^{13}C-mass-labeled, listed in Table A2) were used in all samples. Recovery of ^{13}C-labeled PFAS was calculated to assess the recovery efficiency of the extraction and clean-up methods; recovery averaged 133% for M3-GenX and ranged between 56-82% for all other mass-labeled standards as listed in Table A5. Our SRM values were extremely similar to the reference values for SRM 1947 as reported in Table A6.

2.2.5 Statistical Analysis

Method detection limits (MDLs) for each analyte were calculated as three times the standard deviation of the blank values. Concentrations of each PFAS in samples were blank-corrected using the average laboratory blank levels. Only PFAS that were detected in more than 50% of placenta samples (PFOS, PFOA, PFDA, PFNA) were included in subsequent statistical analyses. Values less than the MDL were imputed with MDL divided by two. Statistical analyses were performed using JMP Pro (version 14.0.0) and GraphPad Prism (version 9.0.2). The following data were abstracted from

medical records: gestational age at delivery, birth weight, infant sex, maternal age, parity, maternal tobacco use during pregnancy, and maternal race. Birth weight was normalized for gestational age using the INTERGROWTH-21st standards and is reported as a percentile (Stirnemann *et al.*, 2017). Preliminary analyses using the Shapiro-Wilk test indicated the PFAS concentration data were not normally distributed, so non-parametric tests were used for analyses. Spearman rank correlation coefficients were calculated to evaluate the associations between individual placental PFAS concentrations. To evaluate whether placental PFAS differed by demographic characteristics, the two-tailed Mann-Whitney test was performed.

Linear regression models were conducted to determine if placental PFAS and other factors were associated with continuous measures of birth outcomes, including birth weight, gestational age, and birth weight for gestational age. For these models, placental PFAS concentrations were categorized into tertiles. Each tertile included n=40 pregnancies. Pregnancies were grouped into tertile solely based on placental PFAS concentration, without regard for infant sex; previous analyses in Figure A1 showed no difference in placental PFAS by infant sex and the distribution of placental PFAS concentration data by infant sex was fairly even. Tertiles were approximately 55% female and 45% male, mirroring the sex distribution in the total study population. Gestational age in days, birth weight in grams, and birth weight for gestational age in percentile were treated as continuous variables for regression analyses.

The potential for demographic and behavioral factors to confound associations between PFAS and birth outcomes was also considered. We identified possible confounding variables based on a literature review and selected variables for inclusion in adjusted analysis if they were associated with both placental PFAS and birth outcomes.

The final adjustment set included maternal race (non-Hispanic black vs. other); parity: (nulliparous (first birth) vs. multiparous (second or later birth)); maternal smoking status (any tobacco use during pregnancy vs. no use during pregnancy) and maternal age at gestation (dichotomized at the median of 27 years for analysis). Maternal education was considered for inclusion in fully adjusted models, but it was not associated with any of the outcomes in this population and did not appreciably change models when included.

Previous work suggests sex differences in associations with PFAS and birth outcomes. Thus, analyses were stratified by infant sex to explore potential sex differences in birth outcomes. The threshold for statistical significance was $p<0.05$ for all analyses. Main analyses were performed on our full study population (n=120), including preterm births. However, to evaluate whether results were driven by the inclusion of premature babies, analyses were repeated excluding babies born before 37 complete weeks gestation (n=12); results were very similar in direction and magnitude (Table A9);

our conclusions and interpretations are unchanged by the inclusion or exclusion of preterm births.

2.3 Results and Discussion

2.3.1 Demographic Information

Placenta samples from 120 participants were included in this study. Table 3 summarizes the demographic characteristics of the women and children in this study cohort.

Of the 120 participants, 60% were non-Hispanic black, 22% were non-Hispanic white, 10% were Hispanic, and 8% were either non-Hispanic Asian, other, or multi-racial. Approximately 54% of the women were 27 years of age or older, and approximately 78% of women reported no tobacco use during pregnancy.

2.3.2 PFAS in Placenta

At least one PFAS analyte was detected in all placental samples (n=120) as reported in Table 4. The most frequently detected PFAS were PFOS, PFOA, PFNA, and PFDA, which were detected in more than 95% of placental samples. As displayed in Figure 2, PFOS and PFOA were the most abundant analytes with median concentrations of 0.95 and 0.27 ng/g, respectively. The concentrations of individual PFAS in placenta were correlated with the concentrations of other frequently-detected PFAS, with Spearman correlations ranging from 0.35 to 0.70, $p<0.05$ for all (Figure 3).

Table 3: Demographic characteristics of cohort (n=120 mother/infant pairs)

Characteristic		N	%
Total		120	100.0
Maternal age at gestation			
	18-19	11	9.2
	20-26	44	36.7
	27-34	46	38.3
	35-39	14	11.7
	40-46	5	4.2
Maternal race			
	Non-Hispanic black	72	60.0
	Non-Hispanic white	26	21.7
	Hispanic	12	10.0
	Other	10	8.3
Male infant		54	45.0
Gestational age <37 weeks		12	10.0
Tobacco use during pregnancy		26	21.7
Parity			
	Nulliparous	38	31.7
	Multiparous	82	68.3

Table 4: Placental PFAS concentration data. Detection frequency, method detection limit (MDL), minimum, median, 95th percentile, and maximum concentrations of PFAS in ng/g wet weight in placental tissues (n=120).

Analyte	Detection Frequency	MDL (ng/g)	Minimum (ng/g)	Median (ng/g)	95th percentile (ng/g)	Maximum (ng/g)
PFOS	99%	0.01	<MDL	0.95	2.5	7.2
PFOA	98%	0.02	<MDL	0.27	0.7	1.6
PFNA	100%	0.01	0.03	0.11	0.3	0.6
PFDA	96%	0.01	<MDL	0.06	0.2	0.3
PFBA	12%	2.36	<MDL	<MDL	19.2	29.4
PFPeA	0%	1.06	<MDL	<MDL	<MDL	<MDL
PFHxA	7%	0.03	<MDL	<MDL	0.1	0.1
PFHpA	0%	1.50	<MDL	<MDL	<MDL	<MDL
PFBS	4%	8.10	<MDL	<MDL	<MDL	10.3
PFHxS	19%	0.17	<MDL	<MDL	0.5	0.5
GenX	0%	0.06	<MDL	<MDL	<MDL	<MDL

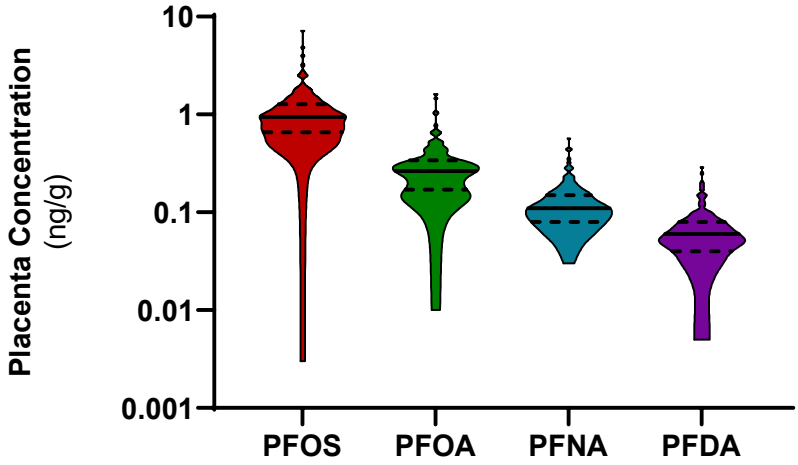


Figure 2: PFAS concentrations (ng/g) in placenta (n=120) for analytes detected in more than 95% of samples. Violin plots show the distribution of concentration data with solid lines and dotted lines demarcating median and quartiles, respectively. Concentrations are plotted on a logarithmic scale.

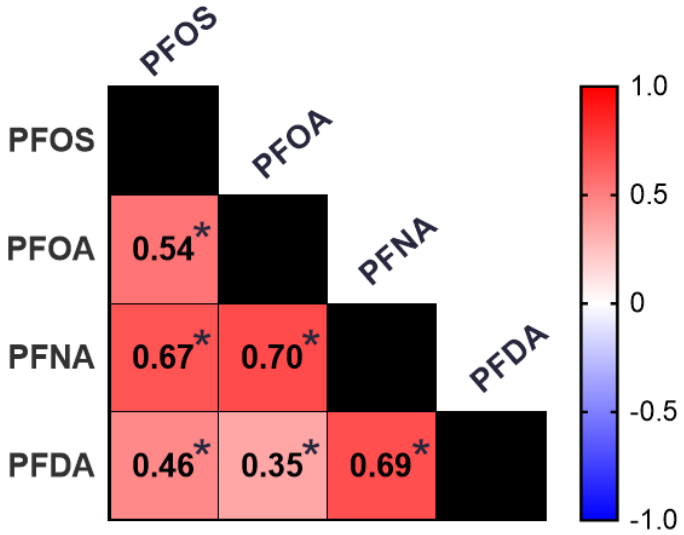


Figure 3: Spearman correlation coefficients for analytes detected in more than 95% of placental samples (n=120). Color indicates strength and directionality of correlation. *indicates p<0.05

Placental concentrations for PFOS, PFOA, PFNA, and PFDA were analyzed to determine if concentrations differed based on infant sex, but no statistically significant

differences were found. Figure A1 displays the concentrations of these four PFAS stratified by infant sex. However, placental PFOA, PFNA, and PFDA were significantly higher in placenta from nulliparous women compared to multiparous women; nulliparous women had 20-40% higher median concentrations (Figure 4). This is not a surprising result given the potential for transplacental and lactational transfer of these chemicals to reduce the PFAS body burden in the mother. Previous studies have found that PFAS concentration in the placenta may decrease with parity as lactational and placental transfer offloads the body burden of PFAS from the mother to the infant (Fei *et al.*, 2007; Kim *et al.*, 2011b; Lee *et al.*, 2013). For example, higher PFOA concentrations have been reported in nulliparous women compared to multiparous women (Lee *et al.*, 2013), and higher parity was associated with lower serum levels for PFOA and seven other PFAS (Shu *et al.*, 2018).

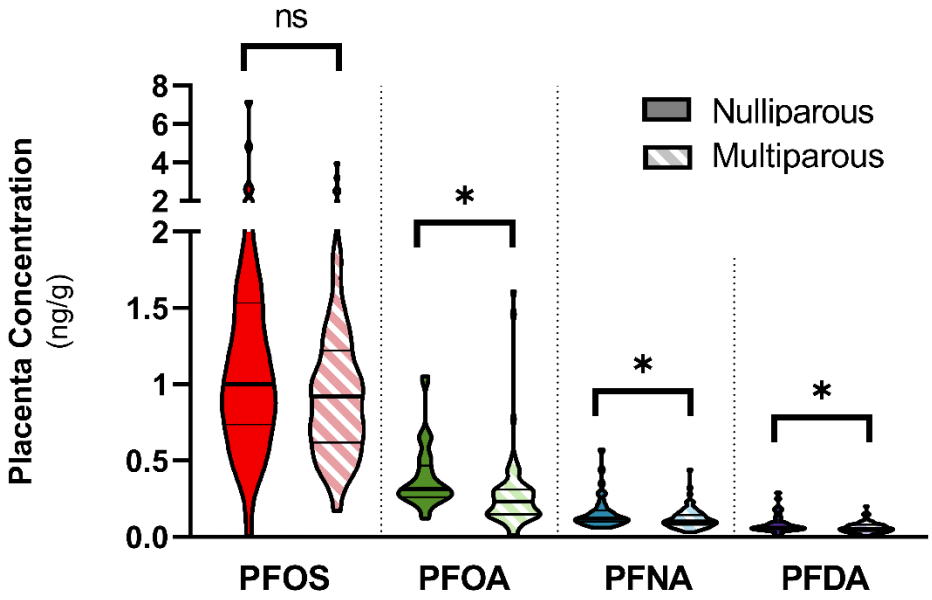


Figure 4: Parity and placental PFAS concentrations.Placenta samples from nulliparous (n=38) and multiparous (n=82) women were compared. PFOA, PFNA, and PFDA placental concentrations were significantly higher in nulliparous women. Violin plots show the distribution of concentration data with thick solid lines and thin solid lines demarcating median and quartiles, respectively. *indicates p<0.05, and "ns" indicates not significant.

We also examined differences in placental PFAS concentrations by maternal race. Previous studies have observed differences in PFAS serum concentrations based on race, with non-Hispanic whites having higher serum concentrations of PFOS, PFOA, and PFHxS than non-Hispanic blacks and Mexican Americans (Calafat *et al.*, 2006). In our study, we found no significant differences in placental PFAS by maternal race (Figure A2). However, it is important to note that the racial and ethnic breakdown of our cohort necessitated combining non-Hispanic white, Hispanic, and other races or ethnicities into

a single category for comparison with non-Hispanic black women. It is likely this limited our ability to detect differences between groups.

We also analyzed a small group of anonymous placenta samples (n=10) collected more recently in 2018 for PFAS. Methods for the collection, storage, and processing of these tissues from 2018 are included in Appendix A. Previous research in our lab has observed higher concentrations of brominated flame retardants in the fetal side of the placenta compared to the maternal side (Ruis *et al.*, 2019). To investigate whether PFAS would also partition preferentially to one side of the placenta, we sectioned these ten placenta samples into the maternal and fetal sides, using the same method reported in Ruis *et al.* (2019), and analyzed them for PFAS. No significant differences were observed between the maternal and fetal placenta concentrations of PFAS. These ten placenta samples were observed to have slightly lower PFAS concentrations than the 120 placenta samples collected in 2010-2011, though this comparison is limited due to the small sample size (Table A7).

2.3.3 Placental PFAS Concentrations and Birth Outcomes

Placental PFAS concentrations were analyzed for associations with birth outcomes using linear regression models and adjusted for maternal race, maternal age at gestation, parity, and tobacco use. For infant males, the highest exposure to PFOS was associated with lower birth weight for gestational age; the highest exposure exhibited a 13% decrease (95% CI: −23, −1.6) in birth weight percentile in comparison to the

reference (lowest exposure) tertile (Figure 5). To put that difference into context, for a baby born at 40 weeks gestation, a 13% decrease in percentile is equivalent to approximately a 130-gram decrease in birth weight. Conversely, for infant females, the highest exposure of PFOS was associated with higher birth weight for gestational age; the highest exposure group exhibited an 11% increase (95% CI: 2.8, 19) in birth weight percentile in comparison to the reference tertile. Placental PFNA had a complex association with birth weight for gestational age; while modeling results for female infants were null and showed no difference, male infants with the highest exposure had lower weight percentiles (14% percentile decrease; 95% CI: −24, −3.9) and male infants within the middle tertile of exposure had higher weight percentiles (11% percentile increase; 95% CI: 0.2, 21) in comparison to the reference tertile.

For models of gestational age, a significant association was observed in male infants exposed to the highest placental PFDA (Figure A3). Placental PFDA in males was associated with a shorter gestational age; male babies with the highest levels of exposure were born approximately ten days earlier on average. All other models of gestational age were null, suggesting no association between placental PFAS and the timing of parturition. Analyses of birth weight in grams, not normalized to gestational age, are included in Figure A4, and a significant association was observed only in female infants exposed to the highest placental PFOS.

A sensitivity analysis was performed to examine the influence of preterm births on the model outcomes. This restricted model excluded the samples from preterm births (gestational age less than 37 complete weeks, n=108). Analyses from the restricted model were very similar in direction and magnitude as results from the full model and conclusions were not altered; additional details are included in Table A9.

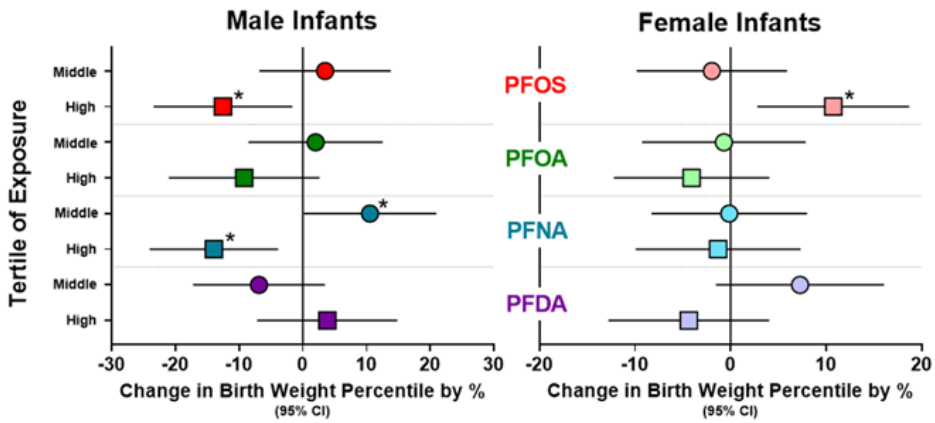


Figure 5: Results for regression models of birth weight for gestational age stratified by male and female infants. Analyses were performed using tertiles of placenta PFAS exposure with the lowest tertile as the reference group and were adjusted for maternal tobacco use, race, age, and parity. Horizontal bars reflect the 95% confidence interval (CI), *indicates p<0.05.

Many studies have explored associations between certain PFAS in maternal blood and birth weight or gestational age. Low birth weight has been associated with maternal serum PFOA and PFOS in previous studies (Darrow *et al.*, 2013; Fei *et al.*, 2007; Johnson *et al.*, 2014), particularly for male infants (Marks *et al.*, 2019). Manzano-Salgado *et al.* (2017) reported weak associations between maternal plasma PFOA, PFHxS, and PFNA and reduced birth weight in a Spanish birth cohort with samples collected from

2003-2008. Of note, they also report that higher PFOS in first-trimester maternal plasma (n=1,202) was associated with low birth weight in boys, similar to the results reported here (Manzano-Salgado *et al.*, 2017).

In a study comparing placental PFAS with maternal serum and fetal tissue PFAS, Mamsen *et al.* (2019) noted that the placenta:maternal serum ratio was higher in pregnancies with male fetuses compared to those with female fetuses. While we did not see differences in placental concentrations between male and female pregnancies, we also did not have maternal serum available to compare with this study or to determine the placenta:maternal serum ratio. Additionally, there are several reported sex differences in placental epigenetics that may also affect infant birth weight (Clark *et al.*, 2021; Martin *et al.*, 2017). This sexually-dimorphic epigenetic placental signature may explain the differential response to gestational PFAS exposure.

Impacts on birth weight and gestational age are not the only potential deleterious effects of PFAS exposure. Maternal PFAS exposure has been associated with adverse reproductive outcomes such as miscarriage (Wikström *et al.*, 2021) and earlier menopause (Ding *et al.*, 2020), though there is evidence of reverse causation with regards to PFAS and menopause (Dhingra *et al.*, 2017). Maternal PFAS exposure could also lead to disruptions in inflammatory pathways that impact pregnancy outcomes and reproductive health (Liu *et al.*, 2020).

2.3.4 Comparisons with Other Placenta Studies

Results from this study were compared with placenta PFAS concentrations reported in previous studies and are summarized in Table A8 and Figure A5. Overall, our PFAS concentration data are very similar to previously reported data for placental concentrations. Mamsen *et al.* (2017) reports PFAS concentrations in placental and fetal tissues from fetuses terminated in the first trimester of pregnancy. A second study by this group, reported in Mamsen *et al.* (2019), expands on their research by including tissues from the second and third trimester of pregnancy from fetuses that died *in utero*. Placental PFAS concentrations in their study were similar to fetal organ concentrations, and the placenta:maternal serum ratio for PFOS, PFOA, and PFNA increased in later trimesters, suggesting placental bioaccumulation or hemodilution due to plasma volume increase (Mamsen *et al.*, 2019). Given the similarity in PFAS concentrations between placenta and fetal organs seen in Mamsen *et al.* (2019), it is possible that our placenta data would be similar to the tissue concentration in the infants. However, Chen *et al.* (2017) measured PFAS in maternal serum, placenta, and cord serum from maternal-fetal pairs, and they found that PFAS concentrations in maternal serum and cord serum were higher than in placenta.

Zhang *et al.* (2013a) measured PFAS in maternal blood, placenta, cord blood, and amniotic fluid. They noted that maternal transfer efficiency (moving from maternal blood to cord blood) decreased with increasing PFCA chain length. Zhang *et al.* (2013a)

found that shorter-carbon chain PFAS (i.e., shorter than PFDA) partitioned more in the cord blood and maternal blood than in the placenta, possibly due to greater water solubility. In our study, longer-chain PFAS were abundantly detected in placenta, but shorter-chain PFAS were detected much less frequently. However, this absence of shorter-chain PFAS may also be reflective of a lack of exposure to shorter-chain PFAS in our study. The placenta may also not be the best matrix for measuring certain PFAS. While the longer-chain PFAS were frequently detected in our placenta, shorter-chain PFAS were not detected or detected very rarely. As these placenta samples were collected in 2010-2011, this may be due to a lack of exposure to the shorter-chain PFAS. It may also be an indication that these other PFAS do not accumulate in the placenta as efficiently. We were unable to compare our placenta PFAS to matched maternal serum PFAS. Having both the placenta and maternal serum, or fetal or cord serum, would have been valuable in determining whether the absence of shorter-chain PFAS was due to a lack of exposure or due to a limitation of the placenta matrix.

Bangma *et al.* (2020a) measured PFAS in placenta from a geographically similar cohort as our study, with placental tissues collected more recently (2015-2018 compared to 2010-2011). While their study did not find any significant associations between placental PFAS concentrations and fetal growth, gestational age, or hypertensive disorders of pregnancy, this may be explained by the fact that their study cohort was

comprised of women who were at increased risk for spontaneous preterm birth and included predominantly white women.

As seen in Table A8 and Figure A5, PFOS and PFOA are the most abundant PFAS measured in placental samples across the world. Over time, it appears that placental concentrations are slowly declining for PFOS, PFOA, PFNA, and PFDA. However, geographic location plays an important role in placenta PFAS concentration in addition to year of sample collection. Although both the present study (Hall *et al.*, 2022) and Zhang *et al.* (2013a) analyzed placenta samples collected in 2010-2011, our median and maximum concentrations were considerably lower. As Zhang *et al.* (2013a) collected placenta from healthy women at a hospital in Tianjin, China, the differences in placental PFAS exposure may be a result of differential use of consumer products and furnishings in each region (e.g., furniture, carpeting, stain-repellent).

2.3.5 Limitations

Limitations to our study include the fact that our study cohort consisted exclusively of women living in central North Carolina and may not be representative of other regions. Demographic differences in our cohort may also be a limitation; our cohort is unique as it consists primarily of non-Hispanic black women, an under-represented population in contaminant exposure studies. However, while this may limit the generalizability of our findings to other populations, we do not expect the demographic characteristics of the study population to limit the internal validity of our

findings; the homogeneity of our cohort may have helped to reduce unmeasured confounding. Additionally, exposures to PFAS have been changing over time as legacy PFAS such as PFOA and PFOS are phased out of use and replaced with newer, emerging PFAS. Our study examined associations with 120 placenta samples collected in 2010-2011. The placenta concentrations observed in these samples may not be consistent with current PFAS exposure as can be evidenced by the changing placental concentrations observed in more recent studies (Table A8) and in our limited observation of ten placenta samples collected in 2018 (Table A7). In addition, our results indicate that evaluating sex-specific associations between PFAS in placenta and birth outcomes is critical. However, our relatively small sample size (n=120) may have limited our power to detect more subtle associations.

2.4 Conclusions

Our present study shows that several PFAS are frequently detected in placenta, and our observed associations with birth outcomes indicate the potential concern for adverse health effects on infants exposed to the highest tertiles of PFAS exposure. Our concentrations are similar in magnitude to the few other studies on placenta PFAS conducted across the world, highlighting the widespread reach of PFAS exposure. In our study PFAS placental exposure was associated with sex-specific birth outcomes, similar to other studies that analyzed maternal serum PFAS. We found that the highest

exposure of placental PFOS was associated with lower birth weight for gestational age in infant males and higher birth weight in female infants.

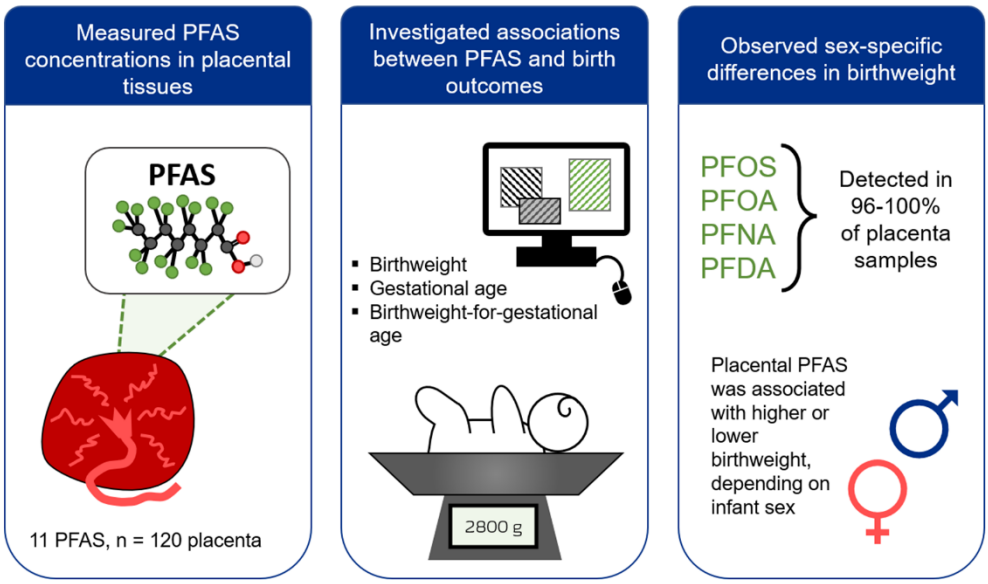


Figure 6: Graphical summary for Chapter 2

3. PFAS in Drinking Water and Serum in an Exposed Community in Central North Carolina

Many people must be acknowledged for their effort to this research aim. Samantha Hall performed the data analysis, performed the LC-MS/MS analysis of serum samples, and helped with study design and sample collection. George Tait performed the LC-MS/MS analysis of water samples. Sharon Zhang provided LC-MS/MS technical support. Dr. Kate Hoffman and Dr. Heather Stapleton helped with study design, sample collection, and data analysis and interpretation. Rachel Smith helped with study recruitment and management.

LabCorp performed the serum lipid and clinical chemistry measurements. Dr. David Collier at East Carolina University also shared expertise on interpreting serum lipid data. Emily Sutton and the Haw River Assembly provided support for sample collection, and Dr. Jane Hoppin at North Carolina State University provided insight, along with the rest of our Community Advisory Board.

Supporting information for this chapter is included in Appendix B.

3.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) are widespread environmental contaminants linked to adverse health effects in humans (ATSDR, 2021). PFAS are especially notable as recalcitrant contaminants in drinking water systems across the United States (Andrews & Naidenko, 2020; Hu *et al.*, 2016; Quinones & Snyder, 2009).

Due to their unique chemical properties and environmental persistence, PFAS are difficult to remove from water with standard drinking water treatment and filtration (Herkert *et al.*, 2020). These PFAS in drinking water are a significant contributor to human PFAS exposure (Hoffman *et al.*, 2011; Hu *et al.*, 2019; Post *et al.*, 2012; Sunderland *et al.*, 2019).

Much research has focused on the health effects of a specific legacy PFAS called PFOA or C8; PFOA has been well-established as a drinking water contaminant of health concern. In Parkersburg, West Virginia, drinking water was so highly contaminated with PFOA from an industrial facility that the nearby residents' serum PFOA levels were 500% higher than the general U.S. population (Frisbee *et al.*, 2009; Frisbee *et al.*, 2010). The subsequent epidemiologic investigation, the C8 Health Project, recruited over 69,000 participants in West Virginia and Ohio who had been drinking this PFOA-contaminated water; this study observed associations between PFAS exposure and adverse health outcomes, such as kidney and testicular cancer (Barry *et al.*, 2013), ulcerative colitis (Steenland *et al.*, 2013), increased serum lipids (Frisbee *et al.*, 2010; Steenland *et al.*, 2009) and changes in thyroid hormones (Knox *et al.*, 2011). Other communities have also been affected by PFAS contamination in their drinking water (Banzhaf *et al.*, 2017; Landsteiner *et al.*, 2014; Li *et al.*, 2018; Lindstrom *et al.*, 2011; Xu *et al.*, 2020; Zhang *et al.*, 2016b).

Previous research has heavily concentrated on the legacy chemicals PFOA and PFOS because of their toxicity and their persistence in both the environment and in people. However, there is a dearth of information on the newer, emerging PFAS that are now replacing PFOA and PFOS. While the shorter-chain PFAAs such as PFPeA, PFHxA, and PFHpA are thought to be less persistent in the human body, they are poorly-characterized in terms of their known exposure, potential toxicity, or environmental persistence. This data gap is a pertinent issue for people in North Carolina who are currently being exposed to these short-chain PFAAs through their drinking water.

Surface water across North Carolina has been impacted by PFAS contamination, particularly in the Cape Fear River watershed. In 2017, an emerging PFAS known as GenX was frequently detected in tap water in Wilmington, North Carolina and garnered considerable media attention. The source of Wilmington's tap water – the Cape Fear River – was found to be contaminated with a variety of legacy and emerging PFAS (Sun *et al.*, 2016).

Further testing of North Carolina surface water indicated that the Haw River, a tributary of the Cape Fear River, was also impacted by many of the same PFAS found in the Cape Fear River, including short-chain PFAAs (Pétré *et al.*, 2022). The Haw River serves as the source of drinking water to the community of Pittsboro, a small community in central North Carolina with a population of about 4,000 residents. As conventional water treatment does not remove PFAS, much of the PFAS in the Haw River can also be

found in Pittsboro's finished drinking water (Herkert *et al.*, 2020). This earlier study suggested that Pittsboro's drinking water is elevated in PFAS when compared to Pittsboro's immediate neighbors who draw their drinking water from other sources, apart from the Haw River or Cape Fear River (Herkert *et al.*, 2020).

Studies focused on the communities downstream of Pittsboro have noted elevated levels of blood PFAS in residents whose drinking water is sourced from the Cape Fear River watershed (Kotlarz *et al.*, 2020). There is also concern that the historical water exposure to harmful legacy PFAS in these communities may be substantial; the Cape Fear River was observed in 2006 to have concentrations of PFOS at 132 ng/L and of PFOA at 287 ng/L (Nakayama *et al.*, 2007). For context, the USEPA's current lifetime health advisory for combined PFOA and PFOS is 70 ng/L, much lower than what has historically been detected in Pittsboro's surface water.

In addition to the high levels of PFOA and PFOS, other studies have also detected high levels of PFPeA and PFHpA in the Cape Fear River watershed as early as 2012 (Strynar *et al.*, 2015). Research from our group has noted that the water concentrations of these shorter-chain PFAAs are temporally variable (Pétré *et al.*, 2022). This temporal variability in PFAS concentrations in Haw River water (and subsequently in Pittsboro tap water) is driven by seasonal changes in river discharge and flow, seasonal changes in evaporation and precipitation, and temporal and sporadic changes in effluent PFAS concentrations or industrial activities. For example, river discharge was

found to be negatively correlated with PFAS concentrations due to the diluting effect of increased river discharge (Pétré *et al.*, 2022). Variable levels of PFAS in drinking water may have implications for PFAS exposure in the Pittsboro community, especially if the shorter-chain PFAAs have human elimination half-lives on the order of days and months.

The Pittsboro community is thus unique because its residents are currently being exposed to temporally-variable levels of newer, short-chain PFAAs through drinking water. The goal of this study was to evaluate the concentrations of PFAS in both drinking water and blood serum of Pittsboro residents over time. By using paired samples collected at two different timepoints, we aimed to understand how the contribution of changing drinking water PFAS concentrations would affect blood PFAS concentrations. Additionally, a further goal was to evaluate whether blood PFAS was associated with any changes in health measures, such as increased serum cholesterol.

3.2 Materials and Methods

3.2.1 Study Population

Between fall 2019 and early 2020, participants were recruited into our study to evaluate the levels of PFAS in the drinking water and blood serum of Pittsboro residents. The Duke University Institutional Review Board reviewed and approved all study protocols prior to recruitment. Eligible participants were all: over 18 years of age; living in Pittsboro, North Carolina; and willing to provide a sample of their blood and

drinking water at two timepoints. A total of 49 participants were enrolled. Participants were also asked to complete a questionnaire asking about their age, sex, menstrual status, water source, water consumption, water filtration, and number of years they had lived in their current home. The study consisted of two timepoints, approximately 2-3 months apart (November 2019 and January/February 2020); at each timepoint participants provided paired samples of blood serum and drinking water.

3.2.2 Sample Collection, Extraction, Purification, and LC-MS/MS Analysis

3.2.2.1 Drinking Water Sample Collection and PFAS Analysis

Participants provided a sample of their primary household drinking water (e.g., tap water, filtered water, bottled water) at each timepoint. They were supplied pre-cleaned, labeled, wide-mouth, one-liter high-density polyethylene (HDPE) bottles (Catalog No. 414004-114, VWR International). Before use, sample bottles were pre-cleaned with rinses of methanol and ammonium hydroxide, methanol alone, and ultra-pure water. Participants were instructed to uncap the bottle, rinse the bottle three times with their drinking water, fill the bottle to ~0.5 inches below the bottom rim (~1.0 L), and return the water bottle immediately to the study team. Water samples were stored on wet ice or reusable ice packs until LC-MS/MS analysis.

Analysis of water for PFAS was performed as described in Herkert *et al.* (2020). The 13 PFAS measured are listed in Table B1. In brief, for each water sample, a volume of 800 mL was spiked with isotopically-labeled internal standards (Wellington

Laboratories, Guelph, Canada) and processed through an automated solid-phase extraction (SPE) system (Dionex AutoTrace 280 Solid-Phase Extraction instrument; Sunnyvale, California, USA).

On the AutoTrace, sample lines were cleaned with methanol and LC-MS grade water prior to loading samples. Oasis® WAX SPE cartridges (6 cc cartridge, 500 mg of sorbent, 60 μm; PN: 18600-4647; Waters Corporation, Milford, Massachusetts, USA) were used on the SPE instrument. SPE instrument methodology was as follows: condition cartridges with 6 mL 0.1% NH_4OH in methanol, condition cartridges with 6 mL methanol, condition cartridges with 6 mL NaOAc aqueous buffer, run 800 mL sample water through SPE column for 80 minutes at a rate of 10 mL/minute, wash cartridges with 6 mL NaOAc aqueous buffer, and wash cartridges with 6 mL methanol. Lastly, samples were eluted with 6 mL 0.1% NH_4OH in methanol. Samples were concentrated under gentle nitrogen (N-EVAP®, Organomation®, Berlin, Massachusetts).

After concentration of extracts to 250 μL, 500 μL of 2 mM ammonium acetate in water was added and a recovery standard (M2-PFOA; also $^{13}C_2$-PFOA; Wellington Laboratories) was spiked. Laboratory processing blanks (800 mL of LC-MS grade water) were analyzed alongside each batch of water samples to ensure there was no background contamination. Samples were transferred to 2-mL polypropylene screw-top vials with clear membrane caps (ThermoScientific, Waltham, Massachusetts, USA) and stored at −20 °C until LC-MS/MS analysis.

Samples were chromatographically separated under gradient conditions on an Agilent 1260 Infinity II high-performance liquid chromatograph (HPLC) instrument coupled to an Agilent 6460A triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, California, USA). The mass spectrometer was operated in negative electrospray ionization mode (LC-MS/MS ESI). The HPLC column used was a reversed-phase Agilent ZORBAX Rapid Resolution HT Eclipse XDB-C18, 1.8 μm particle size, 4.6 mm (I.D.) x 50 mm column (P.N. 927975-902; Agilent Technologies, Santa Clara, California, USA) preceded by corresponding guard cartridge (XDB-C18, 4.6 mm x 5 mm, 1.8 μm).

Mobile phases were (A) 2 mM ammonium acetate in water and (B) 2 mM ammonium acetate in methanol. The gradient program was as follows: initial condition 30% B, held for 1.5 minutes, increased to 95% B over 2 minutes, held for 5 minutes, increased to 100% B over 3.5 minutes, returned to initial condition 30% B over 0.5 minutes, and held for 5.5 minutes. The flow rate was 0.4 mL/minute, the column oven temperature was maintained at 45 °C, and the injection volume was 20 μL. Data were acquired under multiple reaction monitoring (MRM) conditions. All target compound parameters including precursor ion, product ion, collision energy, and fragmentor voltage were optimized for each compound. Additional methods information, including MS/MS conditions and transitions, are included in Tables B2 and B3.

3.2.2.2 Blood Serum Sample Collection and PFAS Analysis

Participants provided a non-fasting blood sample (approximately 10 mL) which was collected by a certified phlebotomist into a serum-separator tube (BD Vacutainer® SST™ tube, 10.0 mL, Becton-Dickinson (BD), Franklin Lakes, New Jersey). Whole blood was allowed to clot over wet ice. Blood tubes were stored on wet ice for no more than three hours before centrifugation to separate blood into serum. Centrifugation was performed in a swinging bucket centrifuge (Eppendorf™ 5810R with Rotor A-4-62, rotor radius of 18.0 cm) at room temperature for 5 minutes at 3500 rpm (2465 rcf). Serum was immediately aliquoted into polypropylene cryo-vials (Nalgene®, ThermoScientific, Catalog No: 5000-0020 and 5000-1020) and stored at −80 °C until analysis.

Serum samples (n=92) were prepared to extract and quantify the 13 PFAS analytes listed in Table B1. Approximately 1.0 mL of serum was transferred into 15-mL polypropylene centrifuge tubes (VWR International, Radnor, Pennsylvania), spiked with mass-labeled internal standards (Wellington Laboratories, Guelph, Canada), and extracted with 4 mL of 0.1 M formic acid using sonication in a water bath. Extracts were then purified by solid-phase extraction (SPE) using Oasis® weak anion exchange (WAX) SPE cartridges (3 cc, 60 mg, 60 μm; PN: 186002492; Waters Corporation). Cartridges were washed with 3 mL sodium acetate in acetic acid, then 3 mL methanol, and eluted with 4 mL of 0.1% ammonium hydroxide (Optima grade, Fisher Chemical) in methanol.

Extracts were concentrated to approximately 150 μL under gentle nitrogen and brought up to 500 μL with 2 mM ammonium acetate (>99.0%, Sigma-Aldrich) in water.

A recovery standard (M2-PFOA; also $^{13}C_2$-PFOA; Wellington Laboratories) was spiked into extracts immediately before LC-MS/MS analysis. Laboratory processing blanks (1.0 mL of LC-MS grade water) were analyzed alongside each batch of serum samples for quality control and assurance. Samples were transferred to 2-mL polypropylene screw-top vials with clear membrane caps (ThermoScientific, Waltham, Massachusetts, USA) and stored at −20 °C until LC-MS/MS analysis.

Samples were chromatographically separated under gradient conditions on an Agilent 1260 Infinity II high-performance liquid chromatograph (HPLC) instrument coupled to an Agilent 6460A triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, California, USA). The mass spectrometer was operated in negative electrospray ionization mode (LC-MS/MS ESI). The HPLC column used was a reversed-phase Agilent ZORBAX Rapid Resolution HT Eclipse XDB-C18, 1.8 μm particle size, 4.6 mm (I.D.) x 50 mm column (P.N. 927975-902; Agilent Technologies, Santa Clara, California, USA) preceded by corresponding guard cartridge (XDB-C18, 4.6 mm x 5 mm, 1.8 μm).

Mobile phases were (A) 2 mM ammonium acetate in water and (B) 2 mM ammonium acetate in methanol. The gradient program was as follows: initial condition 30% B, held for 1.5 minutes, increased to 95% B over 2 minutes, held for 5 minutes,

increased to 100% B over 3.5 minutes, returned to initial condition 30% B over 0.5 minutes, and held for 5.5 minutes. The flow rate was 0.4 mL/minute, the column oven temperature was maintained at 45 °C, and the injection volume was 20 μL. Data were acquired under multiple reaction monitoring (MRM) conditions. All target compound parameters including precursor ion, product ion, collision energy, and fragmentor voltage were optimized for each compound. Additional methods information, including MS/MS conditions and transitions, are included in Tables B2 and B3.

3.2.3 Quality Assurance and Quality Control (QA/QC)

For both serum and water samples, laboratory processing blanks of LC-MS grade water were included in each batch and extracted alongside samples. For quality assurance and control with the blood serum measurements, a serum standard reference material (SRM) from the National Institute of Standards and Technology (NIST) was used. Aliquots of approximately 1.0 mL of reconstituted SRM 1958 Fortified Freeze-Dried Human Serum (NIST, 2018a) were analyzed alongside samples. PFAS concentrations measured in SRM 1958 are reported in Table B4, and the observed serum SRM measurements have strong concordance with NIST reference values; PFNA in SRM was measured at 82-132% of the NIST value and all other PFAS were between 88-119% of the NIST values.

Isotopically-labeled internal standards (^{13}C- and ^{18}O-mass labeled, listed in Table B2) were used in all samples. A mass-labeled recovery standard (M2-PFOA; also $^{13}C_2$-

PFOA) was added to all samples immediately before LC-MS/MS analysis. Recovery of mass-labeled PFAS was calculated to assess the recovery efficiency of the extraction and clean-up methods; recovery for all analytes in serum and water at each timepoint are reported in Table B5.

3.2.4 Serum Lipid Panel and Comprehensive Metabolic Panel Measurements

Blood serum was analyzed by a clinical laboratory, LabCorp (Burlington, North Carolina), to obtain information on potential clinical health measures. After centrifugation, serum samples were fully-separated, clear, and straw-colored in appearance; no hemolysis was observed in any samples, indicating suitability for later clinical laboratory analysis (Lippi *et al.*, 2019). A few serum samples may have been lipemic as they were opaque, milky, and peach-colored in appearance, suggesting high levels of triglycerides in those samples as a result of non-fasting[1] (Nikolac, 2014). Before analysis by LabCorp, serum samples were stored for several months at −80 °C in aliquots in Nalgene® cryo-vials; samples were then transferred to LabCorp on dry ice. The minimum volume of 0.5 mL serum per test was provided to LabCorp to perform these analyses.

[1] This was corroborated by the later lipid results – these opaque and milky samples did indeed have the highest levels of serum triglycerides in our study.

Blood serum was analyzed for a panel of lipids (LabCorp Test: 303756, CPT: 80061). Total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were measured, and low-density lipoprotein (LDL) and very-low-density (VLDL) lipoprotein cholesterol were calculated as part of the blood lipid panel. Non-HDL cholesterol was calculated as:

$$\text{Non-HDL Cholesterol} = [\text{Total Cholesterol} - \text{HDL Cholesterol}]$$

A comprehensive metabolic panel (CMP) was also performed on the blood serum, also by LabCorp (LabCorp Test: 322000, CPT: 80053). A CMP is a broad medical screening tool that includes several different analytes related to liver and kidney function and other measures of health. Analytes included in this CMP included blood glucose, kidney tests (blood urea nitrogen or BUN, creatinine or Cre, BUN:Cre ratio, and estimated glomerular filtration rate or eGFR), electrolytes (calcium, chloride, sodium, potassium, and total carbon dioxide), liver tests (bilirubin and liver enzymes ALP, ALT, and AST), and protein (total protein, albumin, globulin, and the albumin:globulin or A:G ratio). The eGFR was calculated using the 2009 CKD-EPI equation for non-African Americans (Levey *et al.*, 2009).[2]

[2] As of 2022, LabCorp uses the 2021 CKD-EPI creatinine equation for eGFR, which updates the equation to not include a factor for patient's race (Inker *et al.*, 2021).

3.2.5 Statistical Analysis

For data analysis, all serum and water PFAS concentration values were blank-subtracted using the average of all the laboratory processing blank values, and method detection limits (MDLs) were calculated as three times the standard deviation of the laboratory processing blank values for each analyte. Analytes detected in less than 50% of water or blood samples at either timepoint were excluded from statistical analysis. For analytes with a detection frequency greater than 50%, values less than MDL were imputed with MDL divided by two.

We first examined the distributions of all data. Because both blood serum PFAS concentrations and water PFAS concentrations were lognormally distributed, we conducted non-parametric analyses as described below (e.g., Mann-Whitney, Spearman correlations) and log-10-transformed these data before inclusion in models. Some serum clinical measures were also lognormally distributed and similarly log-transformed.

Because samples were collected at two timepoints, analyses were performed as either repeated-measures in linear mixed effects models or stratified by timepoint. A total of 92 samples were collected. Timepoint 1 had a sample size of n=48 participants while Timepoint 2 had n=44; 43 participants attended both timepoints.

We evaluated how blood PFAS concentrations differed between variables such as sex and age by first averaging each participants' blood concentration values from both timepoints, if data were available. We then performed Mann-Whitney tests to

explore if blood concentration PFAS differed based on sex, menstrual status, age, or years in home. We dichotomized age into two categories at the median age of 58 years (58 or younger vs. 59 and older), and years in home into two categories (7 or more years vs. less than 7 years).

Because samples were collected at two timepoints, further statistical analyses were performed as either repeated-measures in linear mixed effects models or stratified by timepoint. A total of 92 samples were collected. Timepoint 1 had a sample size of n=48 participants while Timepoint 2 had n=44; 43 participants attended both timepoints.

We evaluated the correlations between water PFAS and serum PFAS at different times by performing Spearman correlations with all data stratified by timepoint. In order to further explore the associations between water PFAS and serum PFAS, we included other covariates such as age, sex, and years in home and performed repeated-measures mixed models analysis.

We also examined correlations between blood serum PFAS with age, serum lipids, and serum clinical measures by conducting Spearman correlations with all data stratified by timepoint. For serum lipid analysis, statistical analyses focused on total, HDL, and non-HDL cholesterol as these measures were independent of participants' fed state. Triglyceride measurements may be affected by fed state and since our participants were non-fasting, those data were dropped from further analysis; LDL and VLDL cholesterol were also dropped because they were not measured directly and were

calculated using participants' triglyceride measurements. For serum clinical chemistry measures, all 18 measured and calculated parameters were evaluated.

In order to more rigorously explore these associations between serum PFAS and health measure outcomes with other covariates such as sex, we performed repeated-measures mixed model analysis. Models for serum health measure outcomes were adjusted for age as a continuous variable and sex as a categorical variable (male vs. female). Models for serum PFAS concentration outcomes additionally included years in home (<7 or 7+ years) as a covariate of interest. A conceptual model is included in Figure 7 to depict the predictors, outcomes, and included covariates.

The effect of menstrual status (eumenorrhea vs. amenorrhea) on model results was evaluated as a categorical variable (male vs. non-menstruating female vs. menstruating female); a sensitivity analysis was performed and showed no qualitative changes in model performance (data not shown). Menstrual status did not qualitatively affect our results or conclusions and given the low sample size (n=6 participants with normal eumenorrhea), this variable was dropped from further analysis in mixed models.

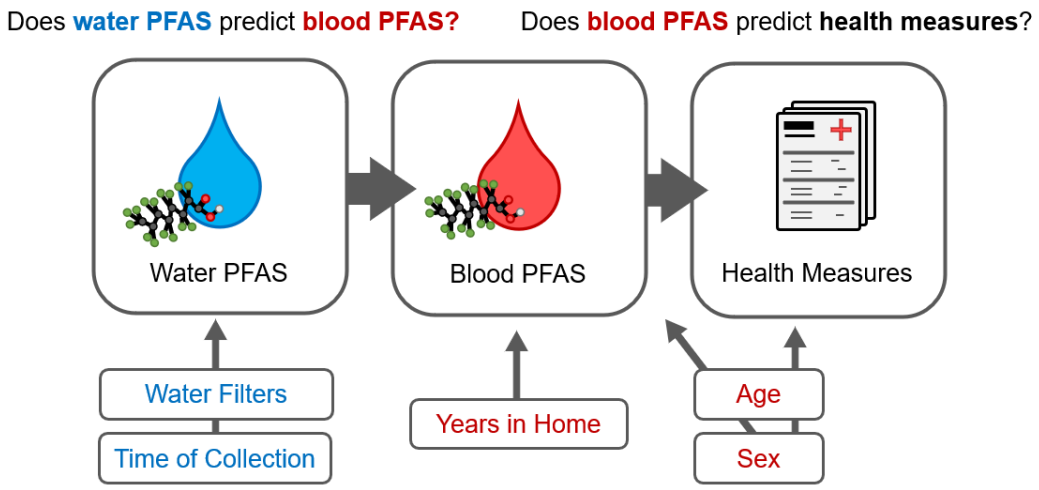


Figure 7: Conceptual model of the relationships between water PFAS, blood PFAS, and health measures as well as the other covariates of interest.

Data were log-transformed to improve normality where applicable and to reduce the potential influence of outliers. Outlier testing was performed with all measurements included in mixed models (ROUT method,[3] Q=1%) (Motulsky & Brown, 2006). No outliers were identified for any log-transformed serum PFAS concentrations or log-transformed water PFAS concentrations. However, some outliers were identified for some serum clinical endpoints even after log-transformation. We conducted sensitivity analyses excluding these potential outliers, and results and conclusions were unaffected (data not shown). Therefore, all presented results include all measured values.

[3] The ROUT method, or "Robust regression and OUTlier removal," is used to identify outliers and is based on robust nonlinear regression and an adapted false discovery rate approach to handling multiple comparisons. The Q value determines how aggressive or conservative the threshold is for defining outliers; a Q of 1% is a recommended default value.

Statistical analyses were performed using JMP Pro 16.0.0, GraphPad Prism 9.3.1, and Microsoft Excel. Mixed models were performed with JMP Pro.

3.3 Results and Discussion

3.3.1 Study Population

The study cohort was primarily female and older, with a median age of 58 years. Complete demographic data are included in Table 5 and visually depicted in Figure B1.

Table 5: Demographic characteristics of cohort (n=49 participants)

Characteristic	N	%
Total	49	100%
Sex		
Male	18	37%
Female	31	63%
Menstrual Status		
Still menstruating (normal eumenorrhea)	6	12%
Age		
30-39	8	16%
40-49	8	16%
50-59	10	20%
60-69	9	18%
70-79	12	24%
80 and older	2	4%
Timepoint Attendance		
Attended both timepoints	43	88%
Timepoint 1 (T1)	48	---
Timepoint 2 (T2)	44	---
Years Lived in Home		
Under 7 years	25	51%

7 or more years	24	49%

3.3.2 Drinking Water PFAS Concentrations

Drinking water was collected from each participant at two timepoints and measured for 13 PFAS via LC-MS/MS. Water PFAS concentration data are reported in Table 6.

Table 6: Drinking water PFAS concentration data. Detection frequency, method detection limit (MDL), median, and 90th percentile concentrations of PFAS in drinking water samples at both timepoints (T1 and T2). The range for Σ(13)PFAS for all 92 observations was <MDL to 458 ng/L. The nine analytes highlighted were used in later analyses because they were detected in more than 50% of samples.

Analyte	Timepoint 1 (T1) (n=48)				Timepoint 2 (T2) (n=44)				Averaged (n=49)	
	Detection Frequency	MDL (ng/L)	Median (ng/L)	90th percentile (ng/L)	Detection Frequency	MDL (ng/L)	Median (ng/L)	90th percentile (ng/L)	Median (ng/L)	90th percentile (ng/L)
PFBA	85%	0.11	14.3	50.7	68%	0.17	1.8	5.0	8.7	28.0
PFPeA	81%	0.18	28.5	98.5	64%	0.29	10.2	20.7	20.3	64.3
PFHxA	81%	0.16	34.3	161.1	66%	0.18	5.9	19.7	23.3	95.9
PFHpA	81%	0.09	12.6	72.7	61%	0.18	3.3	19.8	9.4	49.8
PFOA	90%	0.02	5.0	8.7	55%	0.38	0.7	6.8	2.8	7.9
PFNA	77%	0.04	0.5	0.7	46%	0.09	<MDL	0.7	---	---
PFDA	65%	0.09	0.3	0.5	36%	0.12	<MDL	0.2	---	---
PFBS	81%	0.29	4.1	6.0	66%	0.12	2.2	7.3	3.0	6.4
PFHxS	79%	0.04	1.7	3.1	57%	0.07	0.3	2.5	1.2	2.7
PFOS	75%	0.14	2.4	4.0	39%	0.72	<MDL	4.3	---	---
GenX	67%	0.01	0.04	0.09	80%	0.01	0.02	0.03	0.03	0.05
4:2 FTS	48%	0.002	<MDL	0.02	43%	0.01	<MDL	0.01	---	---
6:2 FTS	77%	0.04	0.7	1.2	57%	0.02	0.06	0.6	0.4	0.8

<MDL: less than method detection limit.

For analytes detected in both timepoints, a participants' averaged values over both timepoints are reported; before averaging, values less than MDL were imputed as MDL divided by two.

We observed some changes over time in drinking water PFAS concentrations. Water concentrations for several short-chain PFCAs decreased between T1 and T2 as shown in Figure 8. Four analytes (PFBA, PFPeA, PFHxA, and PFHpA) were significantly higher in the first timepoint, T1. Many samples at T2 were less than the MDL for most analytes.

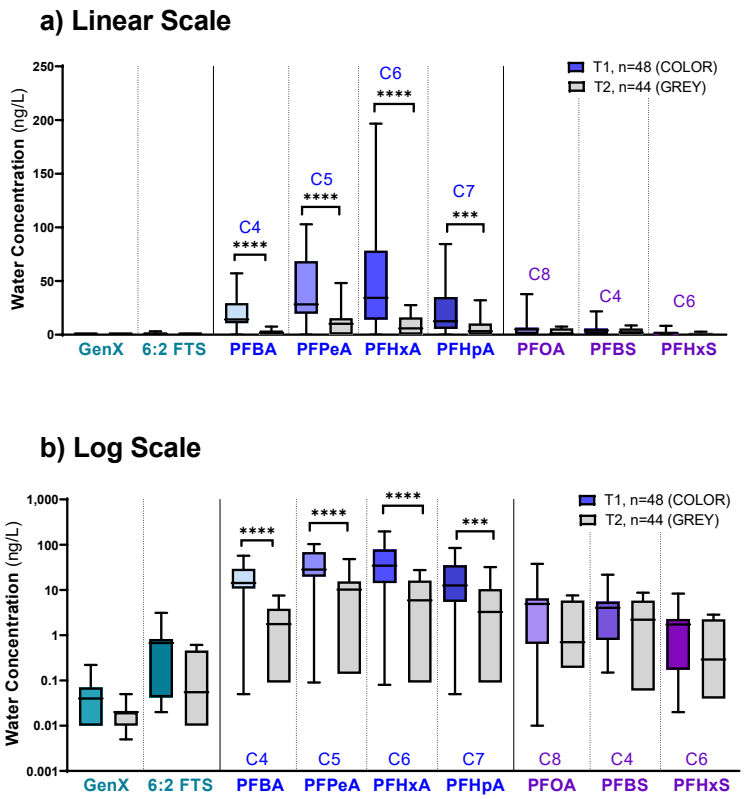


Figure 8: Drinking water PFAS concentrations at both timepoints. Several PFCAs were significantly higher in water at T1 than at T2 (Mann-Whitney, two-tailed). Boxplots depict medians and quartiles, including minimums and maximums. Values less than MDL were imputed with MDL divided by two. Data are plotted on a) linear scale and b) logarithmic scale.

The decline in PFCAs in drinking water between the timepoints may be driven by the decline in the source water's PFAS concentrations; PFAA concentrations in the Haw River declined between November 2019 and January 2020 (Pétré *et al.*, 2022) and Haw River PFAS concentrations are typically reflective of subsequent Pittsboro tap water PFAS. Alternatively, we cannot rule out the possibility that some of the decline in water PFAS concentration may also be due to more participants installing water filters, upgrading water filters, and switching to bottled water.

3.3.3 Serum PFAS Concentrations in Residents

Serum PFAS concentration data are reported in Table 7. Of the 13 analytes measured in blood serum, 6 PFAS were detected in 100% of participants at both timepoints (PFHxA, PFOA, PFNA, PFDA, PFHxS, and PFOS). An additional PFAS, PFHpA, was detected at a lower frequency; remaining analytes were detected rarely in <5% of serum samples.

Table 7: Blood serum PFAS concentration data. Detection frequency, method detection limit (MDL), median, and 90th percentile concentrations of PFAS in blood serum at both timepoints (T1 and T2). The six analytes highlighted were used in later analyses because they were detected in more than 50% of samples.

Analyte	Timepoint 1 (T1) (n=48)				Timepoint 2 (T2) (n=44)				Averaged (n=49)	
	Detection Frequency	**MDL** (ng/g)	**Median** (ng/g)	**90th percentile** (ng/g)	**Detection Frequency**	**MDL** (ng/g)	**Median** (ng/g)	**90th percentile** (ng/g)	**Median** (ng/g)	**90th percentile** (ng/g)
PFBA	4%	0.56	<MDL	0.5	0%	0.67	ND	ND	--	--
PFHxA	**100%**	**0.45**	**0.5**	**0.8**	**100%**	**0.36**	**1.3**	**2.7**	**0.94**	**1.8**
PFHpA	44%	0.43	<MDL	1.1	61%	0.09	0.2	0.9	0.24	0.8
PFOA	**100%**	**0.06**	**6.4**	**15.1**	**100%**	**0.51**	**5.3**	**11.6**	**6.1**	**16.1**
PFNA	**100%**	**0.14**	**1.3**	**2.9**	**100%**	**0.33**	**1.5**	**2.6**	**1.4**	**3.5**
PFDA	**100%**	**0.25**	**0.7**	**1.2**	**100%**	**0.29**	**0.7**	**1.1**	**0.7**	**1.2**
PFBS	0%	0.5	ND	ND	0%	0.5	ND	ND	--	--
PFHxS	**100%**	**0.02**	**2.9**	**5.6**	**100%**	**0.1**	**2.6**	**5.2**	**2.96**	**6.0**
PFOS	**100%**	**0.13**	**10.7**	**21.3**	**100%**	**0.15**	**9.8**	**21.3**	**11.6**	**21.6**
GenX	0%	0.5	ND	ND	0%	0.35	ND	ND	--	--
4:2 FTS	0%	0.06	ND	ND	0%	0.02	ND	ND	--	--
6:2 FTS	0%	0.5	ND	ND	0%	0.01	ND	ND	--	--

PFPeA was not able to be reliably quantified in serum and is not reported; ND: not detected; <MDL: less than method detection limit. For analytes detected in both timepoints, participants' averaged values over both timepoints are reported; before averaging for PFHpA, values less than MDL were imputed as MDL divided by two.

Serum PFHxA concentrations increased from the first timepoint to the second timepoint as shown in Figure 9. All other serum PFAS concentrations were stable between timepoints and did not significantly increase or decrease. This difference among analytes is likely because of the differences in elimination half-lives between different PFAS. Long-chain PFAS have serum elimination half-lives of years and are thus less likely to vary over the course of 2-3 months (Olsen *et al.*, 2007). In contrast, the short-chain PFHxA has an estimated human serum half-life of 14-49 days (Russell *et al.*, 2013). The short half-life of PFHxA, coupled with the temporally variable concentrations of PFHxA in drinking water (Figure 8) and in the Haw River, including a high spike in river PFHxA concentration in December (Pétré *et al.*, 2022), may explain the increase in serum PFHxA seen in Figure 9.

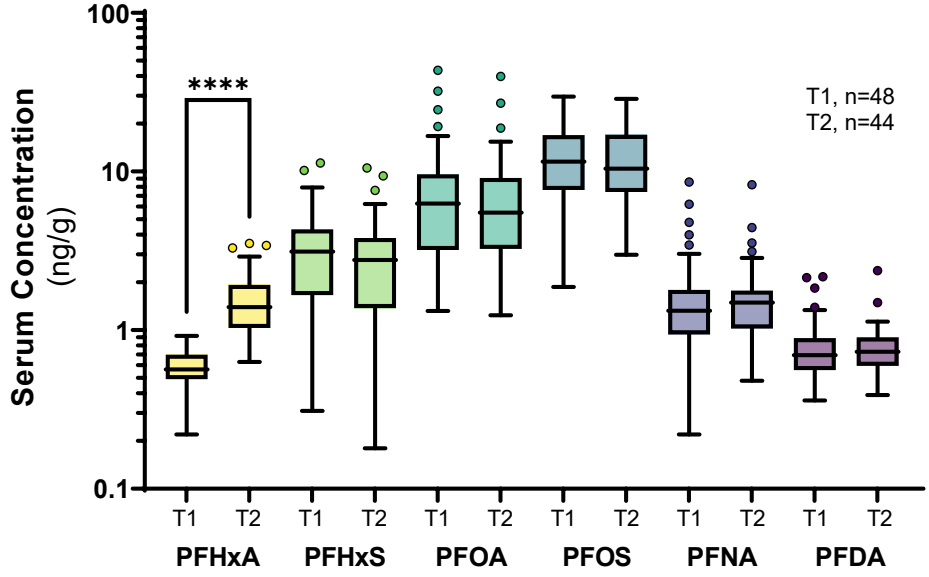


Figure 9: Serum PFAS concentrations in Pittsboro residents at both timepoints, T1 and T2. Timepoints were approximately 2-3 months apart. Serum concentrations are reported as Tukey boxplots. Only PFHxA serum concentration significantly changed between T1 and T2 (**p<0.0001 by Mann-Whitney test, two-tailed). Concentrations are plotted on a logarithmic scale.**

Comparing these data with other PFAS exposure studies, the concentrations of PFAS measured in our participants' serum are several times higher than those reported in the general U.S. population. Over 95% of the general U.S. population has PFAS in their blood according to data collected by the National Health and Nutrition Examination Survey (NHANES) within the Centers for Disease Control and Prevention (CDC) (Calafat *et al.*, 2007a; Calafat *et al.*, 2007b). The median PFAS serum concentrations in our cohort are 2-4 times higher than the median levels for the general U.S. population as reported by NHANES and as shown in Figure 10. However, the serum PFAS concentrations in our cohort are comparable to levels found in people living

downstream in Wilmington, North Carolina. Both Wilmington and Pittsboro source drinking water from surface water in the Cape Fear River Basin. The NHANES data plotted in Figure 10 are from adults during survey years 2017-2018,[1] and the blood from Wilmington residents was collected from adults in 2017-2018 (Kotlarz *et al.*, 2020).

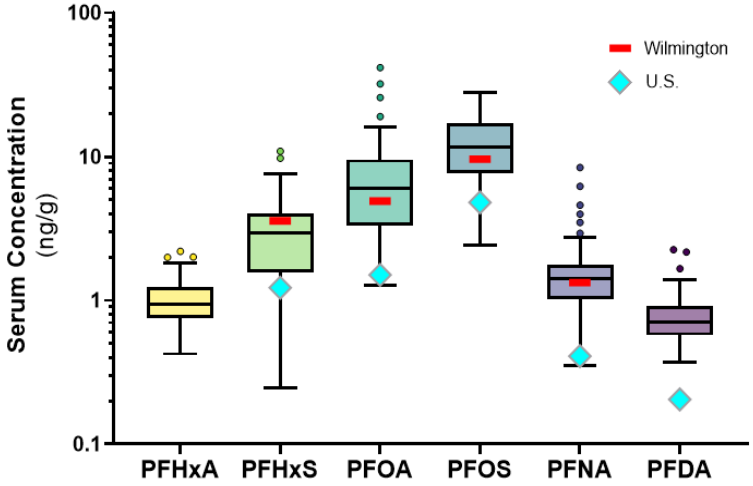


Figure 10: Averaged serum PFAS concentrations in Pittsboro residents (n=49). Data are compared with serum concentrations in the U.S. general population and residents of Wilmington, North Carolina. Data are represented in Tukey boxplots. The U.S. data (blue diamonds) are the median concentrations found in adults 20 years of age or older as reported in the CDC's NHANES survey years 2017-2018. The Wilmington data (thick red bars) are the median concentrations found in adults as reported in Kotlarz *et al.* (2020). PFDA and PFHxA were not reported in Kotlarz *et al.* (2020) and PFHxA was also not reported in NHANES data. Concentrations are reported on log scale.

[1] Blood from Pittsboro residents was collected in late 2019 and early 2020. The most appropriate comparison would typically be blood collected during the same time period, or NHANES survey years 2019-2020. However, the NHANES program suspended operations in March 2020 due to the coronavirus disease 2019 (COVID-19) pandemic. Thus, NHANES data from the 2019-March 2020 cycle are not nationally representative or generalizable to the U.S. population.

Previous literature has suggested that serum PFAS is lower in females (Ericson *et al.*, 2007; Kato *et al.*, 2011) and those who menstruate and that menstruation could be an elimination route for PFAS (Barton *et al.*, 2020; Harada *et al.*, 2005; Lorber *et al.*, 2015; Singer *et al.*, 2018; Wong *et al.*, 2014). We evaluated blood PFAS concentrations stratified by sex and menstrual status. When stratifying the data by sex alone, females had statistically lower levels of blood PFHxA than males (Figure B2). Further stratifying the data by menstrual status indicated that menstruating females had significantly lower levels of blood PFOS than males (Figure B3); however, our sample size for menstruating females was only n=6, limiting our ability to detect differences.

Blood PFAS concentrations were also evaluated by age and by the number of years a participant lived in their current home. We hypothesized that older participants and participants who had lived in their home longer would have experienced PFAS exposure for a longer time and would thus have higher serum PFAS. We found that older participants had significantly higher serum PFOA and PFNA than younger participants (Figure B4). We also found that participants who had lived in their homes for 7 or more years had significantly higher serum PFOA, PFNA, and PFDA (Figure B5). These results may suggest that residents who lived in Pittsboro for longer periods of time were exposed to more PFAS through water. However, it should be acknowledged that it is also possible that the serum PFAS increase seen in older individuals is independent of exposure through Pittsboro drinking water. Previous research has found

that serum PFNA generally increases with age, and serum PFOA increases with age in females (Kato *et al.*, 2011).

3.3.4 Water PFAS and Serum PFAS Comparisons and Associations

To evaluate the differences of PFAS composition in each matrix, we compared the profiles of PFAS analytes in each matrix in Figure 11. Despite the observation that PFOA and PFOS were relatively low in the drinking water samples (<5% of the total PFAS), PFOA and PFOS were most abundant in the serum PFAS (~75% of the total PFAS). Of the 9 PFAS frequently detected in Pittsboro drinking water, only 3 (PFOA, PFHxA, and PFHxS) were detected in more than 50% of the paired serum samples.

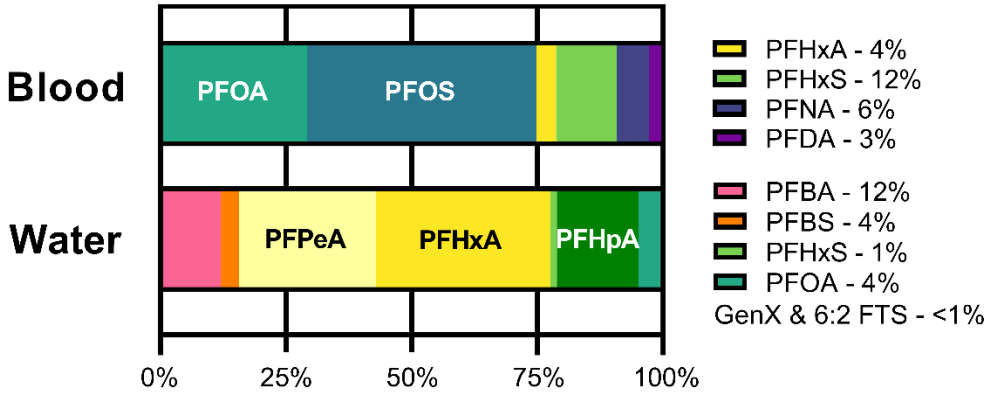


Figure 11: Proportion of each PFAS in serum and water from Pittsboro residents. PFAS profiles show the relative distribution of PFAS among samples. Values represent arithmetic mean concentrations. The Σ(6)PFAS mean serum concentration is 27 ng/g, and the Σ(9)PFAS mean water concentration is 95 ng/L, n=92 observations.

One goal of this study was to evaluate the relationship between drinking water PFAS exposure and corresponding blood serum PFAS. Spearman correlations between

water PFAS and serum PFAS indicated a relatively weak association between water and serum concentrations at either timepoint (Figure B6). While water PFAS are very well-correlated with other water PFAS, they are less correlated with serum PFAS. Most of the serum PFAS are very well-correlated with other serum PFAS; however, PFHxA is unique and notably less correlated with the other serum PFAS. There is also some indication that water PFAS are negatively correlated with serum PFHxS at the first timepoint, but these are weak correlations, are not statistically significant, and are not repeated at the second timepoint.

We additionally conducted repeated-measures analysis to determine if drinking water PFAS concentrations were associated with corresponding blood PFAS levels after adjusting for age (continuous), sex (male vs. female), and years in home (<7 or 7+ years).

Water PFAS concentration was not significantly associated with serum PFAS concentration in any models (Table 8). Blood PFAS was dominated by the more persistent PFAS with longer half-lives such as PFOA, possibly from previous drinking water exposure and not current water exposure. Bolstering this hypothesis, participants who had lived in their homes for 7 or more years were significantly more likely to have higher blood PFOA than those who had lived less than 7 years in their home (Figure B5). Additionally, years in home was a significant covariate in the model for PFOA (Table 8). Previous studies have detected high levels of PFOA in Pittsboro's drinking water source as far back as 2006 (Nakayama *et al.*, 2007; Sun *et al.*, 2016).

Table 8: Regression analysis of water PFAS predictors of PFAS in blood serum. Models were adjusted for age, sex, and years in home. The beta-coefficients (β), 95% confidence intervals, and adjusted p-values are reported. Both serum and water PFAS were log-transformed before analysis. Only PFHxA, PFHxS, and PFOA are included because these were the only analytes detected in more than 50% of both blood and water samples.

	Serum PFAS					
	PFHxA		PFHxS		PFOA	
Predictor	β	p	β	p	β	p
Respective PFAS Water Concentration[1,2]	−0.03 (−0.07, 0.02)	0.21	0.02 (−0.005, 0.05)	0.10	0.01 (−0.01, 0.03)	0.49
Age[3,4]	0.2 (−0.5, 1.0)	0.51	0.91 (−0.46, 2.31)	0.19	**1.5 (0.2, 2.8)**	***0.03**
Sex						
Males	Reference	---	Reference	---	Reference	---
Females[4]	**−13.6 (−23.2, −2.8)**	***0.02**	−18.0 (−33.7, 1.5)	0.07	−14.2 (−30.0, 5.2)	0.14
Years in Home						
7+ years	Reference	---	Reference	---	Reference	---
Less than 7 years[4]	−1.8 (−12.2, 9.7)	0.74	−17.1 (−32.4, 1.7)	0.07	**−28.5 (−41.2, −13.0)**	***0.001**

[1] Each analyte was compared to itself (i.e., water PFHxA was used in models for serum PFHxA).
[2] Beta-coefficients represent the % change in serum PFAS for each 1% increase in water PFAS.
[3] Age is defined as a continuous variable (in years).
[4] Exponentiated beta-coefficients represent the % change in serum PFAS relative to the reference group for categorical variables, or per 1-year increase for age.
*p<0.05

3.3.5 Serum Lipids

Associations between blood PFAS and serum lipids were evaluated. Non-parametric Spearman correlations in Figure 12 were performed with data stratified by timepoint (T1, n=48; T2, n=44; total, n=92). Age was positively associated with HDL

cholesterol at T1 ($p=0.02$, $R=0.33$) and PFOA, PFOS, PFNA, and PFDA at both T1 and T2 (all $p<0.05$). PFOA at T2 was positively associated with total cholesterol ($p=0.047$, $R=0.30$). PFHxS at T2 was positively associated with non-HDL cholesterol ($p=0.04$, $R=0.31$). PFHxA at T2 was negatively associated with HDL cholesterol ($p=0.01$, $R=-0.38$). PFHxA at T1 and T2, and PFHxS, PFOA, and PFNA at T2 were positively associated with triglycerides ($p<0.05$).

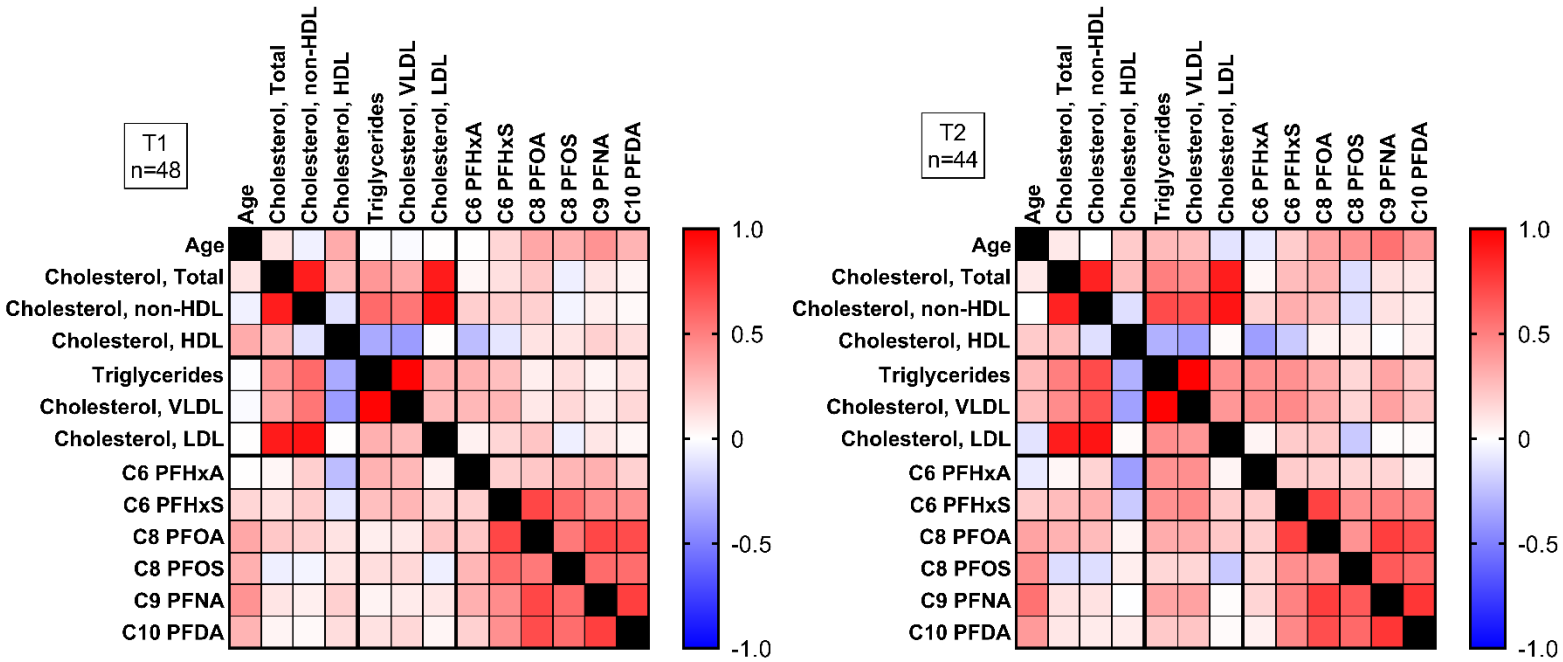


Figure 12: Spearman correlations for serum PFAS and serum lipids.

Study participants were not instructed to fast before blood was collected. Because triglyceride levels are sensitive to whether a person is fasting, analysis of triglyceride data in this study may be limited. This issue also impacts the LDL and VLDL cholesterol data as these are calculated using the triglyceride values; for example, VLDL cholesterol is simply estimated as 20% of the triglyceride value by the Friedewald formula. Previous human PFAS studies have found that fasting participants had markedly lower triglycerides than non-fasting participants (Steenland *et al.*, 2009).

However, total cholesterol and HDL cholesterol are independent of fasting or fed state. The HDL cholesterol is referred to as "good" cholesterol for its beneficial effect on atherosclerosis. Non-HDL cholesterol (the subtraction of HDL cholesterol from total cholesterol) is a representative measure of the negative, potentially atherogenic effects of higher levels of cholesterol while accounting for the beneficial effect of HDL cholesterol (Frost & Havel, 1998) and is a better predictor of coronary heart disease risk than LDL cholesterol (Liu *et al.*, 2006). Non-HDL cholesterol is also more robust than estimated LDL cholesterol as a health measure because it does not require any assumptions regarding VLDL cholesterol or triglycerides values (which are impacted by fasting) (Frost & Havel, 1998).

The associations between serum PFAS and serum lipids were further evaluated through repeated-measures mixed models. Analyses were adjusted for age and sex. Results indicated that total cholesterol and non-HDL cholesterol were positively

associated with both PFHxS and PFOA as shown in Figure 13 and Table B6. HDL cholesterol was not associated with any serum PFAS. The scale of HDL cholesterol is lower than for total or non-HDL cholesterol in Figure 13 because HDL cholesterol typically accounts for only 20-30% of the total cholesterol.

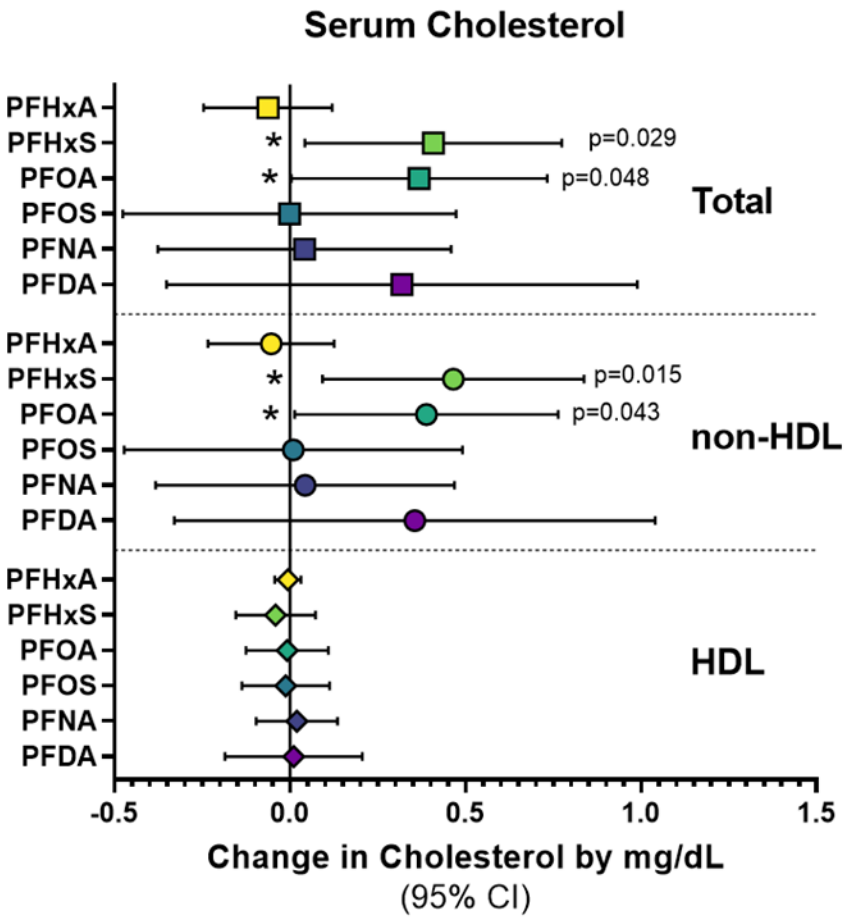


Figure 13: Associations between serum PFAS and serum cholesterol. A 95% confidence interval is reported. Depicts change in cholesterol for each 1% increase in serum PFAS. Analyses were adjusted for age and sex. PFAS serum concentrations were log-transformed before analysis. *p<0.05, n=92; repeated measures, linear mixed models.

Our results are similar to others that have found associations between PFOA and PFOS with increased total and non-HDL cholesterol and found no association between PFOA and PFOS with HDL cholesterol (Dong *et al.*, 2019; Nelson *et al.*, 2010; Steenland *et al.*, 2009). Conversely, our results for PFHxS conflict with some studies that have found negative associations between PFHxS and total and non-HDL cholesterol (Nelson *et al.*, 2010) though these associations have been inconsistent over time (Dong *et al.*, 2019). However, unlike other studies, we did not account for or exclude participants who were taking cholesterol-lowering medications, and our study population is orders of magnitudes smaller in number.

3.3.6 Serum Clinical Chemistry

Analytes included in the comprehensive metabolic panel (CMP) are listed with their abbreviations and units in Table 9.

Table 9: Health measurements included in the serum comprehensive metabolic panel. Abbreviation, units, and broad functional category of the test are listed. Values from each participant (n=49) were averaged from both timepoints; means and standard deviation (SD) for each endpoint are reported.

Function	Health Measure	Abbreviation	Units	Mean ± SD
Glucose	Glucose	---	mg/dL	92.3 ± 17.2
Kidney Tests	Blood urea nitrogen	BUN	mg/dL	16.4 ± 5.0
	Creatinine	Cre	mg/dL	1.0 ± 0.2
	Ratio of blood urea nitrogen to creatinine	BUN:Cre	unitless ratio	17.3 ± 4.9
	Estimated glomerular filtration rate	eGFR	mL / min / 1.73 m^2	76.5 ± 16.2
Electrolytes	Calcium	Ca	mg/dL	9.7 ± 0.4
	Chloride	Cl	mmol/L	101.1 ± 2.3
	Sodium	Na	mmol/L	138.2 ± 2.1
	Potassium	K	mmol/L	4.5 ± 0.4
	Carbon dioxide	CO2	mmol/L	22.7 ± 1.3
Liver Tests	Alkaline phosphatase	ALP	IU/L	76.0 ± 18.3
	Aspartate aminotransferase	AST (or SGOT)	IU/L	21.3 ± 6.5
	Alanine aminotransferase	ALT (or SGPT)	IU/L	20.8 ± 9.7
	Bilirubin	---	mg/dL	0.4 ± 0.2
Protein	Protein, Total	---	g/dL	7.0 ± 0.3
	Albumin	---	g/dL	4.5 ± 0.2
	Globulin, Total	---	g/dL	2.5 ± 0.3
	Ratio of albumin to globulin	A:G Ratio	unitless ratio	1.9 ± 0.3

The associations between blood PFAS and various clinical chemistry health measures were evaluated. Non-parametric Spearman correlations for serum PFAS and

serum clinical chemistry were performed in Figure B7 with data stratified by timepoint (T1, n=48; T2, n=44; total, n=92).

The relationship between serum PFAS and serum clinical chemistry was evaluated in repeated-measures linear mixed models. Measures that were lognormally distributed were log-transformed before analysis (i.e., A:G ratio, albumin, ALT, AST, bilirubin, calcium, creatinine, and glucose).

A summary of statistically significant model results is excerpted in Table 10 and beta-coefficients and full model results are reported in Table B6. Serum PFHxS, PFOA, PFNA, and PFDA were not statistically associated with any of the clinical chemistry measures. Serum PFHxA was statistically negatively associated with ALP, AST, carbon dioxide, chloride, and sodium; serum PFOS was statistically positively associated with BUN:Cre ratio and glucose. Associations between all outcomes and age or sex (unadjusted) are reported in Table B7.

Table 10: Summary of statistically significant model results for serum PFAS and serum clinical chemistry. Adjusted for age and sex. No statistically significant associations were seen for serum PFHxS, PFOA, PFNA, or PFDA. Full model results with all PFAS and all outcomes can be found in Table B6.

Function	Measure	Significant Predictor (β and 95% CI) [1]
Electrolytes	Sodium (Na)	▼PFHxA, −0.02, (−0.04, −0.01)
	Chloride (Cl)	▼PFHxA, −0.01 (−0.03, −0.001)
	Carbon Dioxide (CO2)	▼PFHxA, −0.01 (−0.02, −0.0002)
Liver Enzymes	ALP	▼PFHxA, −0.07 (−0.13, −0.02)
	AST[2]	▼PFHxA, −0.08 (−0.15, −0.001)
Glucose	Glucose[2]	▲PFOS, 0.10 (0.02, 0.19)
Kidney Tests	BUN:Cre	▲PFOS, 0.06 (0.004, 0.11)

[1] Beta-coefficients represent the unit change in serum concentration for each 1% increase in serum PFAS unless otherwise noted.
[2] Beta-coefficients represent the % change in serum concentration for each 1% increase in serum PFAS.

While we note these few statistically significant associations with serum PFOS and PFHxA, it is also worth noting the absence of other associations. Of the 6 PFAS in serum and 18 clinical health measures we evaluated, we only found 7 statistically significant associations. Our limited sample size and relatively homogeneous cohort may have limited our ability to detect associations. However, given that most of our

statistically significant associations were with PFHxA, it is worth highlighting this compound for future study in larger, more robust epidemiological studies.

3.3.7 Limitations

There are a few limitations to this work. This study measured drinking water concentrations at two timepoints, but there is extensive temporal variability in PFAS concentrations. This concurs with previous reports of seasonal PFAS concentration variability in the Haw River (Pétré *et al.*, 2022). If our study had measured drinking water concentrations at additional timepoints, it is possible we would have found greater correlation between drinking water and blood PFAS concentrations, especially for PFHxA.

Another limitation is the lack of information regarding historical drinking water consumption. We know Pittsboro's water previously contained higher concentrations of PFAAs (Nakayama *et al.*, 2007; Sun *et al.*, 2016), and we observed higher blood levels of PFOA in older participants and in those who had lived in their current home for 7 years or longer. Additional survey questions could have helped us better gauge historical drinking water exposure (e.g., how long have they lived in Pittsboro, what year did they regularly start filtering their water). Current drinking water exposure does not explain Pittsboro residents' blood levels, and while we strongly suspect that historical water exposure was higher for our participants, our evidence is limited and circumstantial.

Additionally, the small sample size and homogeneity of the cohort limits some analyses, such as analysis of participants with eumenorrhea or participants without water filtration. The health measures data are limited as measurements were obtained from non-fasting participants; associations with measures that can be affected by fed state (e.g., triglycerides, glucose) may be impacted as our participants were not fasting. We also did not account for other health conditions or medications such as cholesterol-lowering medications. This may limit our results regarding PFAS associations with health measures, likely decreasing our ability to detect associations.

3.4 Conclusions

Pittsboro residents in this study were found to have blood PFAS concentrations at significantly higher levels than the U.S. population and at levels similar to downstream communities that also have drinking water PFAS contamination. The Haw River has historically had considerable levels of PFOA and PFOS contamination, at least since 2006 (Nakayama *et al.*, 2007; Sun *et al.*, 2016), and bluegill fish collected from the Haw River in 2007 had median PFOS levels of 30 ng/g fillet (Delinsky *et al.*, 2009). This historical contamination may explain why the levels of serum PFAS are elevated in Pittsboro residents.

We also found some statistically significant associations for serum PFAS with health measures. Serum PFOA and PFHxS were both associated with increased total and non-HDL cholesterol. Serum PFOS and PFHxA were associated with changes in clinical

chemistry. While current drinking water was not found to be a predictor of serum PFAS, historical drinking water exposure may have been greater in the Pittsboro community resulting in the elevated serum PFAS levels observed in this study.

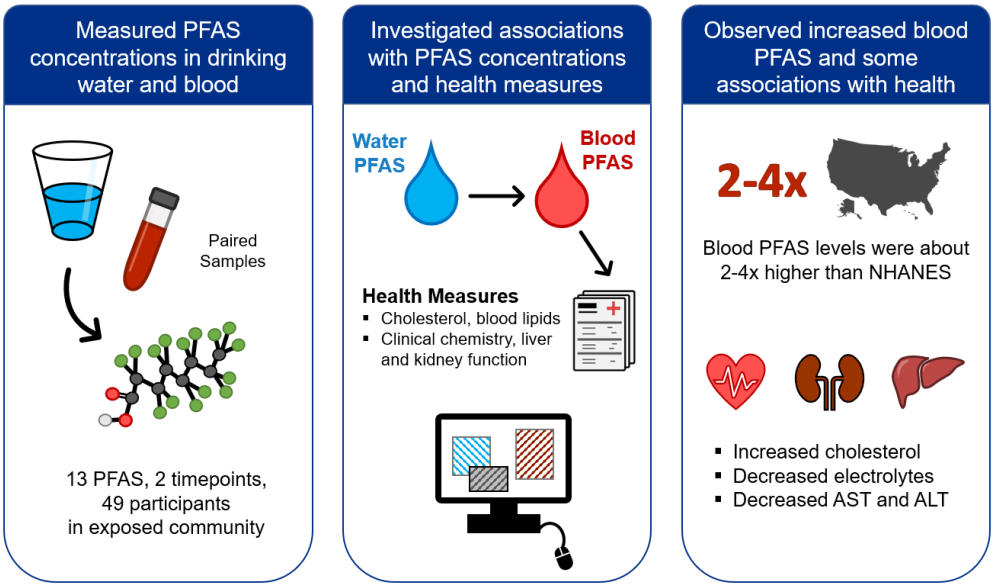


Figure 14: Graphical summary for Chapter 3

4. Toxicokinetics and Effects of an Environmentally-Relevant PFAS Drinking Water Mixture in a Pregnant Rabbit Model

Some of this work is included in manuscript submissions by lead author Dr. Christine Crute under the titles, "Evaluating maternal exposure to an environmental per and polyfluoroalkyl substances (PFAS) mixture during pregnancy: adverse maternal and fetoplacental effects in a New Zealand White (NZW) rabbit model" (published in *Sci Total Environ*, DOI: 10.1016/j.scitotenv.2022.156499) and "Maternal exposure to perfluorobutane sulfonate (PFBS) during pregnancy: evidence of adverse maternal and fetoplacental effects in New Zealand White (NZW) rabbits" (not yet submitted).

Samantha Hall is second and fourth co-author on these manuscripts and participated extensively in the study design, sample and data collection, data interpretation, and review. Sharon Zhang performed the LC-MS/MS analysis of maternal rabbit serum. George Tait performed most of the LC-MS/MS analysis of water samples. Other co-authors to be included on these manuscripts include: Dr. Chelsea Landon, Dr. Angela Garner, Dr. Jeffrey Everitt, Dr. Bevin Blake, Didrik Olofsson, Henry Chen, Dr. Susan Murphy, Dr. Heather Stapleton, and Dr. Liping Feng.

The qPCR experiments in this work were performed by Samantha Hall, with the generous technical expertise of Paige Varner, Sherry Coulter, Genevieve St. Armour, and Ian Ryde, and the equipment of Dr. Claudia Gunsch and Dr. Joel Meyer.

Fernando Orozco, Jesse DeGraff, Danielle Cecil, Dr. Mathias LeBlanc, and many others at the Duke University Division of Laboratory Animal Resources (DLAR) provided technical assistance and guidance with animal care and experiments. Students Shaza Gaballah, Taylor Hoxie, and Samantha Murphy assisted with necropsies. Dr. Suzanne Fenton and Dr. Collette Miller also provided support.

Supporting information for this chapter is included in Appendix C.

4.1 Introduction

Exposure to per- and polyfluoroalkyl substances (PFAS) through drinking water is an increasing concern throughout the U.S. and the world. As demonstrated in Chapter 3, PFAS contamination can be observed in drinking water, and some potential health effects are associated with drinking water PFAS exposure. However, there are data gaps in our understanding of PFAS toxicokinetics and mechanisms of toxicity. There is a particular data need for information on the short-chain PFCAs that predominate the surface water and drinking water of some communities (Herkert *et al.*, 2020; Pétré *et al.*, 2022) because these less-characterized chemicals are replacing other known toxicants.

Animal studies have elucidated some potential hypotheses for PFAS toxicity, particularly for PFOA and PFOS. However, no animal study to date has explored the effects of an environmentally-relevant mixture of PFAS through drinking water. Our rationale for this study was to provide animals with drinking water that reflected the PFAS levels found in Pittsboro, North Carolina and evaluate the effects and tissue

accumulation of PFAS after exposure. This drinking water contained a mixture of PFAS at the same composition and concentrations previously found in unfiltered Pittsboro tap water (Herkert *et al.*, 2020). Notably, this water is unique from other sites of PFAS drinking water contamination. Previous research sites, especially those near airports, military installations, or industrial facilities, have been contaminated primarily with PFOA, PFOS, and sometimes PFHxS (Babayev *et al.*, 2022; Glynn *et al.*, 2020). In contrast, the Pittsboro community's current drinking water is primarily contaminated with short-chain PFCAs such as PFHxA, and PFOS and PFOA make up a small percentage of the total PFAS contamination.

Another aim of this study was to evaluate the effects of PFAS exposure during pregnancy. The placenta is thought to be a target organ of PFAS toxicity (Blake *et al.*, 2020; Blake & Fenton, 2020), and associations have previously been found between placental PFAS concentrations and birth weight in humans, as further described in Chapter 2 (Hall *et al.*, 2022). To achieve this goal of evaluating the placental and developmental effects of PFAS, rabbits were chosen as the animal model. Rabbits are an appropriate model for the human placenta as they have more homology to human placenta than other species (Carter, 2007; Enders & Blankenship, 1999). Rabbits are also used by the USEPA in reproductive and developmental studies as a non-rodent model. In addition to the placenta, this study also focused on the liver as a target of toxicity. Previous literature has shown that PFAS affect the liver and may impact lipid

metabolism through effects on the liver (Armstrong & Guo, 2019; Costello *et al.*, 2022; Fenton *et al.*, 2021; Lau *et al.*, 2007; Schlezinger *et al.*, 2021).

This study was designed to replicate the real-world human exposure in Pittsboro, North Carolina by exposing rabbits to an environmentally-relevant dose of a PFAS mixture through drinking water before and during pregnancy. This chapter will summarize a few key findings: the distribution of PFAS in pregnant rabbits following exposure and the effects of exposure on the liver.

4.2 Materials and Methods

4.2.1 Animals and Animal Husbandry

Nulliparous 6-month-old female New Zealand White (NZW) rabbits (*Oryctolagus cuniculus*) were obtained from Envigo (formerly Covance Research Products; Envigo, Denver, Pennsylvania). Rabbits were approximately 3.0-4.0 kg in weight upon arrival.

Vendor surveillance reports were negative for any pathogens, including: rotavirus, PIV-1, PIV-5, cilia-associated respiratory bacillus, *Clostridium piliforme*, dermatophytes, *Pasteurella multocida*, *Pasturella pnemotropica*, *Salmonella spp*., *Streptococcus pneumoniae*, *Toxoplasma gondii*, *Treponema cuniculi*, ectoparasites, endoparasites, *Eimeria stiedae*, and *Encephalitozoon cuniculi*.

Rabbits were housed in an AAALAC-accredited facility within the Division of Laboratory Animal Resources (DLAR) at the Duke University Medical Center (DUMC). All rabbit procedures and experimental conditions were approved by the DUMC

Institutional Animal Care and Use Committee (IACUC; Protocol Registry Number A214-20-11). Rabbits were housed individually in standard, non-ventilated rabbit racks (Euro Cage, Allentown, LCC, Allentown, New Jersey). Rabbits were provided two cage units for a total 1,458 square inches of floor space per rabbit. Appropriate enrichment was provided by well-trained staff and rotated at regular intervals.

The room was kept on a 12:12 hour light-dark cycle, and conditions were monitored by a Watchdog environmental monitoring system (Watchdog V5, Avidity Science, formerly Edstrom Industries, Waterford, Wisconsin). Room temperature was maintained at 70 °F (± 1°F) or approximately 21 °C, and relative humidity was maintained at approximately 40% (± 1%).

Food and water were provided a*d libitum.* Food was provided via a J-feeder, and drinking water was provided via 0.5 L glass bottles with stainless steel ball-point sippers mounted to the cage. Rabbits were maintained on a standard laboratory rabbit diet (LabDiet 5321, LabDiet, St. Louis, Missouri)(LabDiet, 2022) following a week-long transition from the vendor diet. Hay was provided *ad libitum*. Additionally, a variety of vegetables were provided twice a week, and fruit was provided monthly. While the rabbits were acclimating to the facility prior to study, the drinking water was reverse-osmosis treated water provided by the animal facility (water sourced from City of Durham, North Carolina). This water was tested and confirmed to have no detectable levels of PFAS in it.

4.2.2 Study Design

A total of 23 rabbits (11 control, 12 PFAS-treated) were included in this study, which was conducted between fall 2020 and spring 2021. Groups of six rabbits were procured at staggered time intervals, and all six rabbits in each group were assigned to the same treatment. One rabbit from each treatment failed to become pregnant and one control rabbit suffered an adverse event prior to starting water treatment, leading to a final sample size of 10 control animals and 11 PFAS-treated animals. Upon arrival to the animal facility, the rabbits had an acclimation period of 3-5 days. They were provided with the animal facility's standard reverse-osmosis treated drinking water in glass bottles during this acclimation period.

After the acclimation period the study started (Day 0), and rabbits were switched to either control water (ultra-pure water) or PFAS-treated water (ultra-pure water dosed with a mixture of ten PFAAs). Further details on the PFAS-treated water are included in the next section, Chapter 4.2.3, and in Table 11. Water was provided *ad libitum* via glass bottles on the cage. Water intake was monitored; water levels were checked twice daily, and the mass of actual water consumed was measured once daily by weighing and recording the mass of the water bottles.

Total water exposure occurred over 32 days, including pre-conceptional and gestational exposure. Rabbits were provided water treatment for 7 days, bred with

males, and provided water treatment throughout pregnancy for an additional 25 days before necropsy on Gestational Day (GD) 25 (Figure 15).

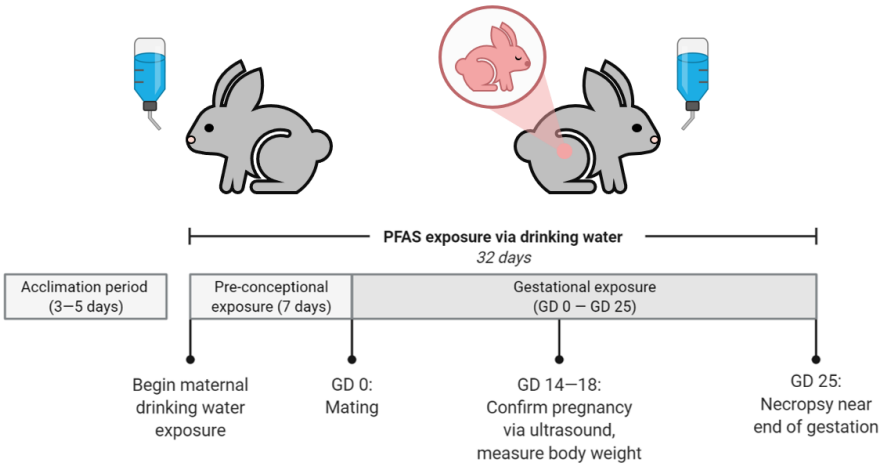


Figure 15: Diagram of rabbit study design.Parturition in rabbits is typically around GD 28 to GD 35.

After seven days of exposure (Day 7, also GD 0), rabbits were bred by live cover. Briefly, the doe was placed in a buck's cage for up to 5-10 minutes. The doe was removed from the cage once successful breeding was confirmed by vocalization or observation of collapse of the buck after mounting. Breeding was performed for all rabbits once in the morning and once in the afternoon on Day 7. On approximately GD 15, rabbits were sedated with acepromazine (1 mg/kg intramuscularly) and anesthetized with isoflurane by mask for pregnancy confirmation by ultrasound (Sonosite M-Turbo, FUJIFILM Sonosite, Inc., Bothell, Washington).

Blood was collected four times: before water treatment, during pre-conceptional exposure (GD −1), during gestational exposure (GD 15), and at necropsy (GD 25). For

blood collection during the study, rabbits were weighed and sedated with acepromazine (1 mg/kg intramuscularly). Following sedation, a local anesthetic of EMLA™ cream (a mixture of lidocaine and prilocaine) was applied topically to the ears, and rabbits were allowed to rest in their home cage for 15 minutes before being placed in a rabbit restrainer (TBJ, Inc., Chambersburg, Pennsylvania). For the first three collection timepoints, blood was collected from the ear via the central auricular artery, put in serum-separator tubes and EDTA tubes (BD Vacutainer® red- and lavender-cap plastic tubes; Becton-Dickinson (BD), Franklin Lakes, New Jersey), separated via centrifugation into serum and plasma, respectively, and immediately aliquoted into cryo-vials.

At study conclusion on GD 25, terminal blood was collected via cardiac stick following sedation with a combination of ketamine (30-40 mg/kg) and xylazine (3-5 mg/kg) administered either intramuscularly or subcutaneously. Blood was again put in serum-separator tubes and EDTA tubes (Becton-Dickinson, Franklin Lakes, New Jersey) and separated via centrifugation into serum and plasma, respectively. Whole blood was allowed to clot over wet ice during the necropsy, and blood samples were stored on wet ice for no more than six hours before centrifugation to separate blood into serum and plasma; serum and plasma were then immediately aliquoted into cryo-vials and stored at −80 °C.

After terminal blood collection, dams were humanely euthanized with an overdose of barbiturates (intracardiac, 1 mL/10 lbs Euthasol®, Virbac AH, Inc., Fort

Worth, Texas). The study was concluded on GD 25 (shortly before live birth or parturition) to ensure that kits with placenta could be collected. After removal from the uterus, fetuses were monitored for breathing and responsiveness to toe pinch. If these indications were observed, then supplemental isoflurane was administered prior to the terminal blood collection via cardiac stick and intracardiac administration of Euthasol® to confirm euthanasia.

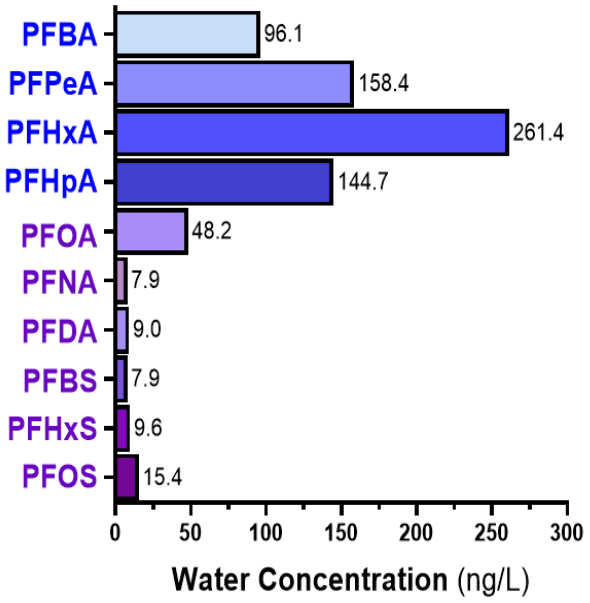
Tissues collected from the does included whole blood, serum, plasma, liver, kidneys, brain, uterus, ovaries, pancreas, spleen, peritoneal fluid, thyroid, quadriceps muscle, and adipose. Tissues collected from kits included whole blood, placenta, amniotic fluid, brain, liver, and kidney. Litter size, kit sex, kit body weight, and kit crown-rump length (CRL) were recorded.

4.2.3 Chemicals and Drinking Water Treatment

Female rabbits were supplied either control or PFAS-treated drinking water during the study. PFAS-treated water contained a mixture of ten different perfluoroalkyl acids (PFAAs) for a ΣPFAS amount of approximately 760 ng/L (Table 11). This mixture is environmentally-relevant as it reflects the same levels of PFAS measured in unfiltered Pittsboro, North Carolina tap water (Herkert *et al.*, 2020).

Table 11: Doses of PFAS in PFAS-treated drinking water for rabbits. Treated water contained a mixture of 10 PFAAs for Σ(10)PFAS of ~760 ng/L. This mixture is representative of the amounts and proportions of PFAS observed in unfiltered Pittsboro tap water (Herkert *et al.*, 2020).

PFAS	Treated Water Concentration (ng/L)	Percent of Σ(10)PFAS
PFBA	96.1	13%
PFPeA	158.4	21%
PFHxA	261.4	34%
PFHpA	144.7	19%
PFOA	48.2	6%
PFNA	7.9	1%
PFDA	9.0	1%
PFBS	7.9	1%
PFHxS	9.6	1%
PFOS	15.4	2%
Total	**757.9**	**100%**



PFBS was purchased as a potassium salt from SynQuest Laboratories (Alachua, Florida), and PFHxS and PFOS were purchased as potassium salts from MilliporeSigma (Sigma-Aldrich; Burlington, Massachusetts). Perfluoroalkyl carboxylic acids (PFCAs) were purchased as free acids from SynQuest Laboratories.[1] Purity was equal or greater than 98% for all PFAS compounds. Detailed chemical information with full chemical name, CAS registry number, and lot number is included in Table C1.

[1] Some PFAS are commercially available as ammonium, potassium, or sodium salts in addition to free acids; for analysis with LC-MS/MS, the anion is measured, and the identity of the cation is unimportant. The mass of salt added to the mixture was adjusted based on the amount of anion using the formula: mass(acid form) = mass(salt form) x (MW acid / MW salt), with "MW" being the molecular weight.

Control and treatment water were stored at room temperature in 50-L polypropylene carboys with spigots (VWR International, Radnor, Pennsylvania) during the study and used to refill the glass water bottles. Polypropylene was specifically chosen as it was an autoclavable material, unlike HDPE. Carboys were rinsed and autoclaved before use. Between exposures, carboys were thoroughly rinsed with methanol and ammonium hydroxide (Optima grade; Fisher Chemical, Chicago, Illinois), followed by successive rinses of methanol alone, hot tap water with Liquinox detergent (Alconox Inc., New York City, New York), tap water, deionized water, and finally ultra-pure water to remove any sorbed PFAS or other microbial contaminants before autoclaving. Glass water bottles and sippers were frequently changed, washed, and sterilized. Glass bottles were rinsed with methanol and ammonium hydroxide (to remove any sorbed PFAS), rinsed with at least ten volumes of tap water, and then washed and sterilized in the animal facility's dishwasher before next use.

4.2.4 Necropsy and Sample Collection

Gross necropsy was performed on GD 25 following terminal blood collection and euthanasia. The intact gravid uterus was weighed, and kits with placenta were removed and labeled according to their position within the left and right uterine horns. Amniotic fluid was collected with a syringe and needle from several kits per litter. Each kit was separated from its placenta, weighed for body mass, and measured with calipers for crown rump length (CRL). The CRL was measured from the top of the head (crown) to

the start of the tail (rump). Kits were sexed into male or female via internal gonad assessment, and whole blood, brain, liver, and kidneys were collected.

For the dam, the brain, liver, kidneys, and uterus were examined, weighed, and collected. Portions of adipose and quadriceps muscle were also collected. Small central portions of the left lateral lobe of the liver were processed and stored in 10% formalin for histologic evaluation and in RNA-*later* solution (Invitrogen) for gene expression analysis. For the kidneys, the right kidney was sectioned and stored in 10% formalin for histology while the left kidney was weighed and collected. Remaining liver (mostly whole), kidney (left), brain, muscle, adipose, and kit bodies were stored at −20 °C for later analysis of PFAS concentration. Blood serum was stored at −80 °C following separation by centrifugation.

4.2.5 PFAS Measurements

4.2.5.1 Water PFAS Measurements

Samples of drinking water were analyzed to confirm the absence of PFAS in control water and to confirm the appropriate PFAS concentrations in the mixture water. Samples were measured for each group of animals at the beginning and end of study exposure. Water samples from the animal facility were also analyzed for PFAS to confirm that the rabbits were not receiving PFAS exposure during their acclimation period. Briefly, water was collected in one-liter polypropylene bottles (Nalgene® Labware, ThermoScientific) from the carboys. Before use, sample bottles were cleaned

similarly to the carboys, with rinses of methanol and ammonium hydroxide, methanol alone, and ultra-pure water. Water was then analyzed for PFAS using the AutoTrace automated solid-phase extraction (SPE) method described previously in this dissertation in Chapter 3.2.2.1. The pH of the water was also measured and did not differ between control and treatment water; both water treatments had a pH of 6.

4.2.5.2 Serum PFAS Measurements

Maternal rabbit serum samples were analyzed for the ten PFAAs present in the PFAS-treated water: the C_4-C_{10} PFCAs and the C_4, C_6, and C_8 PFSAs. Serum was analyzed by solid-phase extraction (SPE) and LC-MS/MS quantification using modified methods (Liu *et al.*, 2015). In brief, 0.5 mL of maternal serum was spiked with isotopically-labeled internal standards (Wellington Laboratories, Guelph, Canada), acidified with 4 mL of 0.1 M formic acid, and sonicated for 15 minutes in a water bath without heat. After sonication, extracts were purified and concentrated using Oasis® weak anion exchange (WAX) SPE cartridges (6cc, 500 mg, 60 micron; Waters Corporation, Milford, Massachusetts).

SPE cartridges were preconditioned with 3 mL of 0.1 ammonium hydroxide (NH_4OH, Optima grade, Fisher) in methanol, 3 mL of methanol, and 3 mL of a pH 4 sodium acetate buffer. Samples were loaded on to the SPE cartridges and rinsed with 500 μL of 0.1 M formic acid. Cartridges were washed with 3 mL of the sodium acetate buffer and 3 mL of methanol. Analytes were then eluted from the SPE cartridge with 4

mL of 0.1 NH_4OH in methanol and concentrated to near dryness under a gentle nitrogen gas stream in a heated water bath (N-EVAP®, Organomation®, Berlin, Massachusetts). Extracts were reconstituted in 500 μL of a 30:70 volume:volume mixture of methanol and 2 mM ammonium acetate (Fisher). Extracts were centrifuged at 3500 rpm for 10 minutes and the supernatant was transferred to polypropylene LC vials (ThermoFisher, Waltham, Massachusetts). Samples were analyzed for PFAAs using negative electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS ESI–). Analytes were chromatographically separated under gradient conditions using an Agilent Zorbax Eclipse XDB-C18, 4.6 x 50 mm, 1.8 μm particle size column preceded by a C18, 4.6 x 5 mm guard cartridge (Agilent Technologies, Santa Clara, California). Mobile phases were methanol (A) and water (B), each with 2 mM ammonium acetate. Flow rate was 400 μL/min, injection volume was 20 μL, column temperature was 45 °C, and the gradient program for the 18-minute run was as follows: initial condition of 30% A, held for 1.5 min, increased to 95% A over 2 min, held for 5 min, increased to 100% A over 3.5 min, returned to initial condition of 30% A over 0.5 min, and held for 5.5 min. Data were acquired under multiple reaction monitoring (MRM) conditions using optimized parameters.

4.2.5.3 Liver Tissue PFAS Measurements

PFAS concentrations in dam liver tissue were measured. At necropsy, livers were weighed and mostly whole liver was frozen and stored at −20 °C in polypropylene

buckets until homogenization. Before homogenization, the tissue was set out to thaw and the gall bladder containing frozen bile was dissected away from the liver tissue. Thawed whole liver was then homogenized in a glass food processor (BLACK+DECKER™ Glass Bowl Chopper). All parts of the food processor were cleaned and rinsed with ultra-pure water and methanol between each sample. A similar volume of angus beef was also homogenized in the food processor as a quality control to ensure PFAS contamination did not occur during this homogenization step. Homogenized liver was then transferred to polypropylene tubes for storage at −20 °C until extraction.

Approximately 2.0 g of liver tissue was aliquoted into 50-mL polypropylene centrifuge tubes (VWR, Radnor, Pennsylvania), spiked with internal standards, spiked with 1 mL of 1.0 M formic acid, and extracted with acetonitrile. A total volume of 30 mL acetonitrile was used over three rounds. Extracts were then concentrated to dryness under gentle nitrogen in a heated water bath (N-EVAP®, Organomation®, Berlin, Massachusetts) and reconstituted in 1.0 mL methanol.

Extracts were then purified with solid phase extraction (SPE) using Oasis® weak anion exchange (WAX) SPE cartridges (6cc, 500 mg, 60 micron, for PFAS Analysis, PN: 186009347; Waters Corporation, Milford, Massachusetts). PFAS were eluted off SPE cartridges with 10 mL of 0.1% ammonium hydroxide in methanol.

Extracts were then filtered through Choice™ 0.22 micron, 13 mm nylon syringe filters (ThermoScientific, Rockwood, Tennessee) using a 3-mL plastic two-piece syringe

(ThermoScientific). After filtration, samples were transferred to 2-mL screw-top polypropylene LC vials (ThermoScientific) with blue vial caps with PTFE/silicone septa (Agilent).

4.2.5.4 Feces Collection and Feces PFAS Measurements

Dam fecal pellets were collected into polypropylene tubes from the cage pans during the afternoon before necropsy. As cage pans were changed at least twice a week, the fecal pellets collected were eliminated 12 to 96 hours prior to necropsy. Feces were stored at −20 °C until analysis.

Approximately 2.0 g of fecal pellets were ground in a ceramic mortar and pestle until powdery. As the feces were already dry, no sodium sulfate was added to facilitate grinding in the mortar. Approximately 2 g of fish tissue SRM (NIST 1947) was also aliquoted and ground in the mortar and pestle as a quality control. Homogenized feces were transferred to a polypropylene vial and processed for PFAS extraction using the same protocol for rabbit liver.

4.2.5.5 Urine Collection and Urine PFAS Measurements

Dam urine was collected from the cage pans on the morning of necropsy into 15-mL polypropylene tubes using glass Pasteur pipettes with rubber bulbs. Cage pans typically contained absorbent pads to catch waste from the cages; these pads were removed approximately 12 hours before urine collection. Collected urine was cloudy and contained fluffy, heavy white solids which settled to the bottom of the tube upon

refrigeration at −20 °C; these solids were likely excreted calcium. Rabbits renally excrete much more calcium than other mammals, mostly in the form of calcium-precipitated salts (Gallego, 2019). Urine was stored at −20 °C until analysis.

At analysis, specific gravity of rabbit urine was determined using a digital handheld refractometer (Palette series, UG-α, D 20/20, ATAGO ®). Specific gravity is an indicator of urine concentration and hydration status, with higher values indicating dehydration. The specific gravity of rabbit urine ranged between 1.05 and 1.06, but several urine samples were over the limit of detection (i.e., >1.0600). Because of that limitation, none of the urine PFAS concentrations included in this chapter are corrected for specific gravity. Other studies have corrected urine PFAS concentration for urine creatinine (Fenton *et al.*, 2009) because urine creatinine is an indicator of kidney function and GFR. While blood creatinine was measured for these animals (Crute *et al.*, 2022), urine creatinine was not.

Approximately 0.5 mL of urine was aliquoted into 15-mL polypropylene tubes and spiked with internal standards. To precipitate any proteins, 1.5 mL of acetonitrile was added to the urine samples. Samples were vortexed and stored overnight at 4 °C, then centrifuged. The supernatant was then transferred to clean polypropylene tubes. Extraction of PFAS from urine then proceeded as described in the serum PFAS extraction method described in Chapter 4.2.5.2 (i.e., a formic acid extraction and a WAX SPE cleanup).

4.2.5.6 Data Analysis for PFAS Measurements and Quality Assurance/Quality Control (QA/QC)

Laboratory processing blanks were extracted and run alongside all samples. All sample concentration values were blank-subtracted using the averages of their respective laboratory processing blanks. Method detection limits (MDLs) for each analyte were calculated as three times the standard deviation of the lab blank values. Values less than the MDL were imputed as the MDL divided by two for statistical analyses.

Standard reference materials (SRMs) were also extracted and run alongside samples for quality assurance and control. Tables C2, C3, and C4 report the PFAS concentrations extracted from SRM. Fish tissue NIST 1947 (NIST, 2017) is used as the SRM for rabbit liver tissue and feces in Table C2 and Table C3, respectively. Of the nine NIST SRMs with PFAS concentration data, this fish fillet tissue SRM is currently the best available surrogate for measuring PFAS in other animal tissues. Fish tissue is also the most similar matrix to rabbit feces, though admittedly not ideal.

Human serum NIST 1958 (NIST, 2018a) is used as the SRM in Table C4 as a surrogate for rabbit urine. There are currently no urine SRMs with PFAS concentration data available, so serum was chosen as the best alternative SRM. Even if a human urine SRM was available, it may still not be an ideal surrogate for rabbit urine; the specific gravity of the rabbit urine was much higher than what is typically found in human urine

(1.05 to >1.06 vs. 1.005-1.030), and rabbits excrete considerable amounts of calcium into their urine, unlike humans (Gallego, 2019).

For the fish tissue and serum SRMs, there are reference and information values for several, albeit not all, PFAAs included in this study's drinking water mixture (Table C7). No SRM was run alongside the rabbit blood serum samples included in this work, which is an unfortunate limitation and oversight. However, previous work in our lab with the 1958 serum SRM has indicated that our serum analytical method consistently works very well for measuring most PFAAs in human blood serum (and is evidenced in Chapter 3.2.3 and Appendix B).

Our observed experimental SRM PFAS measurements have strong concordance with the NIST reference values, indicating the reliability of our extraction methods. For the rabbit liver QA/QC, the SRM values were 77-150% of the NIST values. For feces, they were 73-215%, and for urine, they were 83-102%.

We used isotope dilution in all these samples with isotopically-labeled internal standards (^{13}C- and ^{18}O-mass labeled, listed in Table C5). Mass-labeled internal standards were added before extraction, and mass-labeled recovery standards (M2-PFOA, also $^{13}C_2$-PFOA; and M8-PFOS, also $^{13}C_8$-PFOS) were added at the end of extraction. Recovery of ^{13}C-labeled PFAS was calculated to assess the recovery efficiency of the extraction and clean-up methods. The recoveries of these internal standards for rabbit liver, feces, and liver are reported in Table C6. While a perfect recovery is 100%,

some PFAS had low recovery, such as M-PFDA in liver (7%) or M-PFBA in urine (5%). Other PFAS had very high recovery, such as M-PFBA in feces (322%). Generally acceptable recovery is between 50-150%. These discrepancies in recoveries indicate that our analytical methods may be limited or not optimal for measuring those PFAS in those matrices. PFAS with recovery outside the generally acceptable range of 50-150% include: in blood serum PFDA; in liver, PFDA, PFBS, and PFHxS; in feces, PFBA, PFBS, PFHxS, and PFOS; and in urine, PFBA, PFPeA, PFHxA, PFBS, PFHxS, and PFOS (Table C6). Denly *et al.* (2019) experienced a similar issue with low recoveries of internal standards when measuring PFAS in product leachates; however, these low recoveries did not adversely affect their final results. Denly *et al.* (2019) chose to use a conservative QA/QC cutoff of excluding any data with recovery less than 5%. All data included in this chapter had an internal standard recovery of 5% or higher, and thus, this chapter contains the data for all PFAS measurements in all matrices even if the QA/QC is not ideal. Further discussion on the analytical limitations can be found in Chapter 4.3.5.

One consistent finding of note was an abnormally high recovery of the labeled sulfonic acids in all of the rabbit liver, feces, and urine samples. For M3-PFBS, the recovery ranged from 212-728% and for M-PFHxS, the recovery ranged from 180-700%. The M-PFOS had slightly more normal recoveries, ranging from 65-487%. This finding was especially pronounced with the feces samples. The high recovery of mass-labeled sulfonates was not observed in the SRMs (Table C6) or in the blanks (data not shown).

The explanation for this high recovery is likely due to the use of only one recovery standard: M2-PFOA. While using only one recovery standard is typically sufficient for matrices like the SRM or the blanks, the rabbit liver, feces, and urine were much more challenging, more complex[2] matrices. Ion suppression can occur in these matrices, and the ion suppression in this case was apparently different for carboxylic acids than for sulfonic acids. Future work in these rabbit matrices should use a sulfonate recovery standard like M8-PFOS to calculate the recovery of mass-labeled sulfonic acids. Indeed, the blood serum used M8-PFOS as the recovery standard for the sulfonates, and the recovery of labeled sulfonates was much better in that matrix (Table C6). Other explanations for the high recovery of the mass-labeled sulfonic acids, such as interfering or poorly-resolved peaks, were ruled out as causes.

The high recovery of mass-labeled sulfonates (due to the dissimilar recovery standard) should not affect the accuracy of our unlabeled sulfonate measurements in the liver, feces, or urine. Each unlabeled sulfonate had its own respective ^{13}C- or ^{18}O-labeled standard (i.e., M3-PFBS for PFBS, MPFHxS for PFHxS, and MPFOS for PFOS), and any ion suppression should affect those compounds in a similar manner within the same sample.

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[2] These complex matrices might also be described as "dirtier" – there are far more other chemicals in these matrices, whether endogenous or xenobiotic, and some of these chemicals may be capable of interfering with the PFAS analysis. Some chemicals and particulates also need to be removed or filtered out of samples in order to allow the LC-MS/MS to run without clogging or blocking the LC analytical column.

4.2.6 Liver Lipid Measurements

To explore health effects of PFAS exposure, the lipid content in the rabbits' liver was measured. Lipids were extracted from aliquots of the maternal liver, and a gravimetric analysis was used to calculate the percent lipid in liver. In brief, approximately 2.0 g of liver tissue was aliquoted and ground with ~10 g cleaned sodium sulfate in a ceramic mortar and pestle until dry and powdery. Powdered liver tissue was transferred to 50-mL glass centrifuge tubes, extracted with a 50:50 volume of dichloromethane:hexane,[3] and sonicated in a water bath for 15 minutes. Extracts were decanted into a clean centrifuge tube, and the liver extraction was repeated two more times for a total solvent volume of 30 mL.

Extracts were blown down under gentle nitrogen to approximately 1.0 mL, then transferred to pre-weighed aluminum weigh boats (VWR International, Radnor, Pennsylvania). Weigh boats with extracts were moved to an oven heated to 90-100 °C and left overnight, allowing the solvent to fully evaporate and leaving behind the lipids coated on the weigh boat. After fully drying, weigh boats with the remaining mass of extracted lipids were weighed. The relative mass of extracted lipid to the mass of initial liver aliquot was then calculated to obtain the percentage of lipid in liver by weight:

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[3] A similar, commonly-used procedure called the Folch method uses a liquid-liquid extraction of chloroform and methanol to extract lipids from biological tissues. The Bligh and Dyer (B & D) method is also used to extract total lipids.

$$\text{Lipid in Liver (\%)} = \frac{\text{mass of extracted lipid in aluminum weigh boat after drying}}{\text{mass of initial liver aliquot extracted}}$$

4.2.7 Gene Expression Analysis of Maternal Rabbit Liver with Quantitative Real-Time PCR

Because some PFAS are hypothesized to disrupt lipid processes, gene expression in the maternal rabbit liver tissue was evaluated for several genes (Table 12) involved in lipid metabolism. A two-step reverse transcription quantitative real-time polymerase chain reaction (qPCR or RT-qPCR) was used to evaluate gene expression. Livers were collected from the control group (n=10) and experimental PFAS-treated group (n=11).

4.2.7.1 Liver Sample Processing

Pieces of the left lateral lobe of the liver were processed for histopathology and RNA expression. A central portion of the lobe was dissected with scissors or scalpel (Figure 16). The portion of liver intended for RNA expression analysis was further minced and stored in a cryo-tube (~0.5 mL of tissue), immediately put into RNA-*later* RNA stabilization solution (~700 μL; Invitrogen), and then placed in wet ice. Samples were stored at 4 °C in the RNA-*later* solution for over 48 hours to allow the RNA-*later* to fully penetrate the tissue, then the RNA-*later* solution was removed, and samples were transferred to a −80 °C freezer for long-term storage.

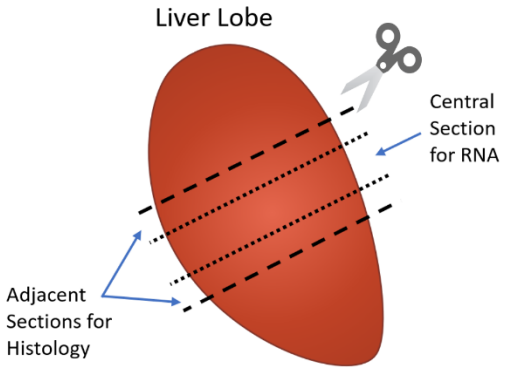

Figure 16: Diagram of maternal rabbit liver dissection

4.2.7.2 Nucleic Acid Isolation, Quantification, and Purity

The RNA was isolated from the rabbit liver using the QIAGEN RNeasy® Mini Kit (Catalog No: 74104; Germantown, Maryland) according to the manufacturer's protocol with no modifications. Approximately 25 to <30 mg of tissue was used. An on-column DNase I treatment step was included to purify RNA for qPCR analysis by removing any genomic DNA contamination (Catalog No: 79254, QIAGEN). The RNA was eluted from spin columns with 30 μL RNase-free water. Extracted RNA concentration was then quantified using a NanoDrop™ One spectrophotometer (ThermoFisher Scientific, Madison, Wisconsin). Absorbance was also measured with the NanoDrop™, and only RNA samples with A260/A280 absorbance ratios of 1.9-2.1 were accepted. Pure RNA samples uncontaminated by residual chemicals from extraction, DNA, or proteins should have a A260/A280 ratio of ~2.0. The RNA was stored at −80 °C until use.

4.2.7.3 Reverse Transcription

The cDNA was generated using 500 ng RNA (1-3 μL), 4 μL iScript™ Reverse Transcription Supermix for RT-qPCR[4] (Bio-Rad Laboratories, Hercules, California), and RNase-free water in 20 μL reactions. Reaction conditions for reverse transcription were as follows: 5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C. The cDNA was then diluted with 80 μL of RNase-free water and stored at −20 °C until use. Reactions without RNA template (no template controls or NTCs) were included as negative controls.

4.2.7.4 Quantitative Real-Time PCR Conditions

The qPCR reactions were run on a CFX96 Touch™ Real-Time PCR System (Bio-Rad) with Bio-Rad CFX Manager (version 3.1). TaqMan® Gene Expression Assays with FAM-labeled[5] oligonucleotide hydrolysis probes were used (Life Technologies Corporation, Pleasanton, California). Assay information is listed in Table 12. These genes were chosen from the literature to be assayed based on their previous perturbations in mouse liver or human liver cells with PFOA or PFOS (Behr *et al.*, 2020; Schlezinger *et al.*, 2020) and their commercial availability for the rabbit species.

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[4] Contains RNase H+ Moloney murine leukemia virus (M-MLV) reverse transcriptase
[5] FAM is a commonly-used fluorophore or fluorescent dye. FAM is an abbreviation for 'fluorescein amidites.' Complete reporter information for these assays is 6-FAM/MGB, and the quencher is NFQ.

Other equipment used for qPCR included hard-shell, low-skirted, thin wall, white/clear 96-well PCR plates (Catalog Number: HSP-9601, Bio-Rad) sealed with Microseal® 'B' adhesive seals (Bio-Rad).

Each assay was tested for PCR amplification efficiency and linear dynamic range with serial cDNA dilutions (Bustin *et al.*, 2009); cDNA was diluted with nuclease-free water. An eight-point standard curve with a 4-fold dilution series was generated with an x-axis of log(input RNA amount) and a y-axis of C_q value.[6] A line was plotted to this standard curve to obtain slope values and R^2 values. Efficiency of each assay was determined by the equation:

$$\text{PCR efficiency (\%)} = 10^{\frac{-1}{\text{slope}}} - 1 \times 100\%$$

All assays used had a reaction efficiency between 90-110%, and a R^2 greater than or equal to 0.99 within the linear range. A cDNA template diluted to contain ~100 ng reverse-transcribed total RNA was determined as suitable for all assays.

Each 20 μL reaction contained 2 μL cDNA template, 1 μL TaqMan® assay, 10 μL supermix (TaqMan® Fast Advanced Master Mix, Catalog Number: 4444963, ThermoFisher), and 7 μL DNase- and RNase-free water. TaqMan assays contained combined probe and primers, with probe concentrations at 250 μM and forward and

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[6] C_q or quantification cycle. This is also referred to as C_t or C_T or threshold cycle. The C_q term is preferred per the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009).

reverse primer concentrations each at 18 μM; final probe and primer concentrations in qPCR reactions is 5 μM and 900 nM per primer, respectively. Thermal conditions for qPCR were: 50 °C for 2 minutes (UNG[7] activation), 95 °C for 20 seconds (polymerase activation) and 40 cycles of 95 °C for 3 seconds followed by 60 °C for 30 seconds (denaturing and annealing/extending). Reactions were run in triplicate.

Table 12: Genes evaluated in qPCR experiments. All gene information is relevant to rabbit species (*Oryctolagus cuniculus* or Oc).

Gene	Gene Name	Entrez Gene ID	UniGene ID	TaqMan Assay ID	Chromosome Location on Build OryCun2.0	Amplicon Length (base pairs)
*GAPDH	glyceraldehyde-3-phosphate dehydrogenase	100009074	Ocu.87	Oc03823402_g1	Chr.8: 32580489 – 32584701	82
*ACTB	actin beta; β-actin	100009272	Ocu.734	Oc03824857_g1	Chr.Unknown: 124760 – 128215	106
PPARA; PPARα; PPAR-alpha; NR1C1	peroxisome proliferator activated receptor alpha	100356422	Ocu.2226	Oc06726505_mH	Chr.Unknown: 95601 – 164543	72
PPARG; PPARγ; PPAR-gamma NR1C3	peroxisome proliferator activated receptor gamma	100008892	Ocu.2191	Oc03397329_m1	Chr.9: 11495126 – 11632867	72
NR1H3; LXR-alpha; LXRα	nuclear receptor subfamily 1 group H member 3	100352900	Ocu.7812	Oc04252526_m1	Chr.1: 186607590 – 186616369	74

[7] UNG (and its superfamily UDG) are uracil-DNA glycosylases that remove uracil from DNA and are added to qPCR master mixes to prevent carryover contamination.

CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	100328551	Ocu.2032	Oc04250254_m1	Chr.3: 73947427 – 73958448	64
ABCA1; ABC1; CERP	ATP binding cassette subfamily A member 1	100356765	N/A	Oc00442663_g1	Chr.1: 8128430 – 8265889	91

*evaluated as reference genes for normalization

As the PCR efficiencies of all assays were within 10% of each other, the comparative Livak or $\Delta\Delta C_q$ method was used to measure relative gene expression (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Relative changes in expression were thus calculated using the following equations:

$$\Delta Cq = Cq_{\text{Target Gene}} - Cq_{\text{Reference Gene}}$$

$$\Delta\Delta Cq = \Delta Cq_{\text{Treated Group}} - \Delta Cq_{\text{Control Group}}$$

$$\text{Relative Fold Change in Gene Expression} = 2^{-\Delta\Delta Cq}$$

Target gene expression levels were normalized to the reference gene *Gapdh*. Expression in PFAS-treated animals (n=11) is reported as a difference in fold change relative to the average expression in control animals (n=10). No template controls (with 2 μL water in lieu of 2 μL cDNA template) were run alongside samples as negative controls and showed no PCR amplification – indicating no issues with reaction contamination.

4.3 Results and Discussion

4.3.1 Drinking Water Consumption

Water consumption was tracked throughout the study in order to quantify the total amount of PFAS consumed by the PFAS-treated rabbits. PFAS-treated rabbits

consumed approximately 258 g of water per day (Figure 17a). One concern for this study was that PFAS-treated animals would not drink or would drink less water than the control animals. Our results indicate that this was not an issue; PFAS-treated animals consumed equal or greater amounts of water when compared to controls (Figure 17).

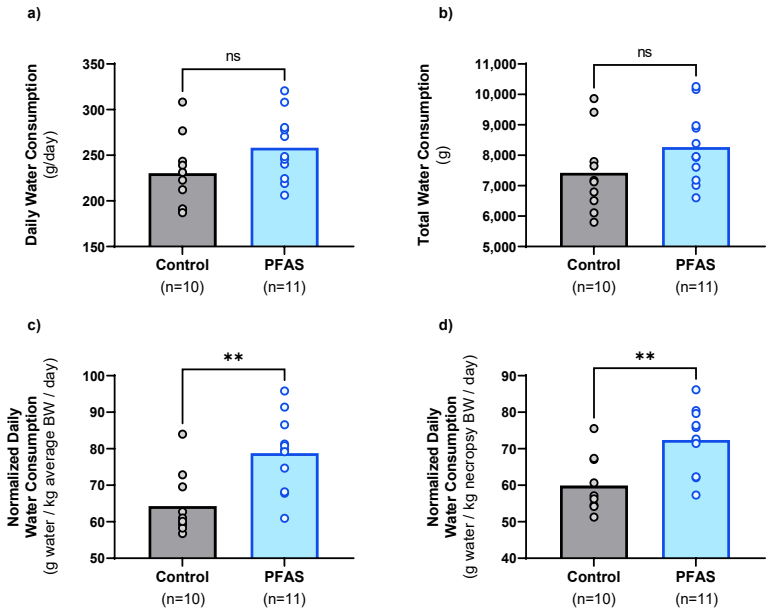

Figure 17: Drinking water consumption for both treatment groups. PFAS-treated animals consumed equal or greater amounts of drinking water when compared to controls (unpaired t test, two-tailed, **p<0.01). Both a) daily and b) total water consumption were analyzed. Water consumption was also normalized to body weight in c) and d). Because pregnant rabbits gain weight throughout gestation, data were normalized to both c) the average body weight during the study period and to d) the body weight at necropsy. ns, not significant

Rabbits drink 50–100 mL/kg BW/day[8] on average, so a 3.5 kg rabbit should be drinking 175–350 mL of water per day (Brewer & Cruise, 1994). All animals in our study consumed more than 50 g water/kg BW/day with controls consuming ~64 g/kg BW/day and treated animals consuming ~79 g/kg BW/day (Figure 17c).

4.3.2 PFAS Concentrations

4.3.2.1 Serum Concentrations

Blood serum was collected from the dams at necropsy (GD 25) and later analyzed for PFAA concentrations using LC-MS/MS. The blood serum PFAS concentrations were significantly higher in PFAS-treated dams for PFHpA, PFOA, PFHxS, and PFOS (Table 13). Another two analytes, PFHxA and PFDA, were detected in some PFAS-treated dams, but not in any controls. While PFBA, PFPeA, PFNA, PFBS were frequently detected in treated animals, they were not found at significantly higher concentrations than in control animals.

[8] Kilogram of body weight = kg BW. Doses of chemicals should be normalized to the body weight of the organism; for example, larger rabbits would be expected to drink more water than smaller rabbits.

Table 13: Blood serum PFAS concentrations in pregnant rabbit dams at necropsy (GD 25). The method detection limit (MDL), detection frequency (DF), range, median, and arithmetic mean concentrations for each analyte are reported for both treatment groups. Concentrations between treatment groups were compared (Mann-Whitney test, two-tailed) to generate p-values. Values less than MDL were imputed with MDL divided by two before the medians and means were calculated.

Analyte	MDL (ng/g)	Control (n=10)				PFAS (n=11)				p-value
		DF (%)	Range (ng/g)	Median (ng/g)	Mean (ng/g)	DF (%)	Range (ng/g)	Median (ng/g)	Mean (ng/g)	
PFBA	0.03	100	0.06 – 0.17	0.10	0.10	100	0.08 – 0.18	0.14	0.13	0.15
PFPeA	0.02	30	<MDL – 0.05	<MDL	N/A	73	<MDL – 0.06	0.03	0.03	0.20
PFHxA	0.02	0	ND	ND	ND	18	<MDL – 0.03	<MDL	N/A	ND
PFHpA	0.004	20	<MDL – 0.004	<MDL	N/A	91	<MDL – 0.02	0.01	0.01	***p<0.0001**
PFOA	0.04	50	<MDL – 0.08	0.04	0.04	100	0.04 – 0.41	0.08	0.13	***p=0.0004**
PFNA	0.11	90	<MDL – 0.20	0.14	0.14	91	<MDL – 0.67	0.18	0.21	0.09
PFDA	0.05	0	ND	ND	ND	36	<MDL – 0.06	<MDL	N/A	ND
PFBS	0.24	100	0.51 – 1.57	0.82	0.88	100	0.41 – 1.18	0.74	0.81	0.76
PFHxS	0.01	40	<MDL – 0.02	<MDL	N/A	82	<MDL – 0.13	0.09	0.08	***p=0.002**
PFOS	0.02	70	<MDL – 0.12	0.04	0.05	100	0.07 – 0.16	0.10	0.11	***p=0.002**

N/A: not applicable; arithmetic means were not calculated for any analyte with a DF below 50%.
ND: not detected

While some blood PFAS concentrations were significantly higher in treated animals than controls, the overall serum concentrations in PFAS-treated dams were low in magnitude – between 0.01 and 1.2 ng/g. The treated animals also have low blood levels relative to the dose of PFAS analytes to which they were exposed. The highest

blood level of any PFAS detected in treated animals was for PFBS at a concentration of 1.18 ng/g (and it should be noted that a control animal was higher at 1.57 ng/g PFBS). The next-highest blood level for the analytes measured was for PFNA, with a treated animal having a blood level of 0.67 ng/g. It is surprising that these two analytes were highest in magnitude in treated animals when PFNA and PFBS accounted for only 2% of the PFAS water mixture. Conversely, PFPeA accounted for over 20% of the PFAS water mixture yet there were not significantly elevated levels of PFPeA in the treated animals.

4.3.2.2 Liver Concentrations

Whole liver was collected from the dams at necropsy (GD 25), homogenized via food processor, and later analyzed for PFAA concentrations using LC-MS/MS. The liver PFAS concentrations were significantly higher in treated dams for PFHxS (Table 14). Surprisingly, the control animals had significantly higher concentrations of PFHxA in liver than the treated dams. It is unknown what could have been a source of PFHxA to the control rabbits.

For both the control and treated groups, PFOS was the most abundant and was detected at the highest median concentrations in liver (4-6 ng/g) compared to the other nine analytes. This is somewhat unexpected, as the blood levels of these animals do not reflect similarly high levels of blood PFOS (<0.2 ng/g, Table 13), and no known exposure to PFOS occurred to explain these levels. However, others have also found liver PFOS

concentrations to be several times higher than serum PFOS concentrations (Hundley *et al.*, 2006; Lau *et al.*, 2007; Seacat *et al.*, 2003; Seacat *et al.*, 2002).

The drinking water before and during the study was tested for PFOS. However, it is possible that these animals were exposed to PFOS through water before arrival at our animal facility, or through diet. Rabbit chow, vegetables, and hay were not measured for PFOS concentration. The difference of magnitude between liver PFOS and blood PFOS indicates that PFOS in rabbits may preferentially bioaccumulate in liver and that elimination of PFOS from rabbit liver is much slower than the elimination of PFOS from rabbit blood.

Table 14: Liver PFAS concentrations in pregnant rabbit dams at necropsy (GD 25). The method detection limit (MDL), detection frequency (DF), range, median, and arithmetic mean concentrations for each analyte are reported for both treatment groups. Concentrations between treatment groups were compared (Mann-Whitney test, two-tailed) to generate p-values. Values less than MDL were imputed with MDL divided by two before the medians and means were calculated.

Analyte	MDL (ng/g)	Control (n=10)				PFAS (n=11)				p-value
		DF (%)	Range (ng/g)	Median (ng/g)	Mean (ng/g)	DF (%)	Range (ng/g)	Median (ng/g)	Mean (ng/g)	
PFBA	2.10	0	ND	ND	ND	0	ND	ND	ND	ND
PFPeA	0.06	100	0.17 – 0.77	0.42	0.44	100	0.23 – 0.47	0.28	0.31	0.15
PFHxA	0.05	100	0.16 – 1.01	0.38	0.50	100	0.13 – 0.51	0.22	0.27	***p=0.04**
PFHpA	0.11	60	<MDL – 0.43	0.14	0.15	46	<MDL – 0.24	<MDL	N/A	N/A
PFOA	0.09	50	<MDL – 0.39	0.06	0.12	46	<MDL – 0.23	<MDL	N/A	N/A
PFNA	0.22	50	<MDL – 0.50	0.18	0.24	82	<MDL – 0.50	0.33	0.31	0.28
PFDA	0.06	60	<MDL – 1.64	0.07	0.25	82	<MDL – 0.26	0.12	0.14	0.49
PFBS	2.68	40	<MDL – 3.62	<MDL	N/A	0	ND	ND	ND	ND
PFHxS	0.02	100	0.18 – 0.87	0.58	0.54	100	0.53 – 4.68	0.94	1.56	***p=0.006**
PFOS	0.04	100	0.37 – 82.13	6.44	12.80	100	1.04 – 13.44	3.29	4.10	0.54

N/A: not applicable; arithmetic means were not calculated for any analyte with a DF below 50%.
ND: not detected

4.3.2.3 Feces Concentrations

Fecal pellets were collected from the dams before necropsy and later analyzed for PFAA concentrations using LC-MS/MS. The feces PFAS concentrations were not significantly higher in PFAS-treated dams for any analyte (Table 15).

Table 15: Feces PFAS concentrations in pregnant rabbit dams at GD 25. The method detection limit (MDL), detection frequency (DF), range, median, and arithmetic mean concentrations for each analyte are reported for both treatment groups. Concentrations between treatment groups were compared (Mann-Whitney test, two-tailed) to generate p-values. Values less than MDL were imputed with MDL divided by two before the medians and means were calculated.

Analyte	MDL (ng/g)	Control (n=10)				PFAS (n=11)				p-value
		DF (%)	Range (ng/g)	Median (ng/g)	Mean (ng/g)	DF (%)	Range (ng/g)	Median (ng/g)	Mean (ng/g)	
PFBA	0.31	60	<MDL – 14.22	1.5	4.0	91	<MDL – 12.44	2.1	4.9	0.51
PFPeA	0.29	70	<MDL – 11.05	1.5	3.3	82	<MDL – 10.92	2.4	3.9	0.60
PFHxA	0.19	80	<MDL – 4.51	1.3	2.1	82	<MDL – 4.68	1.5	2.0	>0.99
PFHpA	0.18	50	<MDL – 2.88	0.2	0.9	64	<MDL – 3.15	1.0	1.2	0.64
PFOA	0.10	40	<MDL – 0.94	<MDL	N/A	46	<MDL – 0.71	<MDL	N/A	N/A
PFNA	0.14	0	ND	ND	ND	0	ND	ND	ND	ND
PFDA	0.05	0	ND	ND	ND	0	ND	ND	ND	ND
PFBS	0.07	20	<MDL – 0.54	<MDL	N/A	46	<MDL – 2.48	<MDL	N/A	N/A
PFHxS	0.008	100	0.34 – 1.11	0.5	0.6	100	0.38 – 1.17	0.5	0.6	0.70
PFOS	0.02	100	0.23 – 1.98	0.8	0.8	100	0.42 – 4.76	0.9	1.3	0.39

N/A: not applicable; arithmetic means were not calculated for any analyte with a DF below 50%.
ND: not detected

There are a few prominent reasons why we may not have seen elevated levels of feces PFAS in treated dams. Firstly, the fecal elimination route may not be the primary route of elimination for these PFAS in rabbits. Secondly, rabbits excrete a large amount of feces per day, and this study only extracted 2 g of fecal pellets. This sample amount

may not have been large enough to detect any differences in PFAS, especially considering the low amounts of PFAS consumed by the rabbits through water in this study. However, the lack of detection in feces should not be interpreted to mean than the fecal elimination route is unimportant; previous work in rats has found that that the feces were the primary excretion route for PFDA and that it had notable differences between males and females (Vanden Heuvel *et al.*, 1991a).

4.3.2.4 Urine Concentrations

Urine was collected from the dams before necropsy (GD 25) and later analyzed for PFAA concentrations using LC-MS/MS. The urine PFAS concentrations were not significantly higher in PFAS-treated dams for any PFAS (Table 16). The analytes PFNA, PFDA, PFBA, and PFOS were not detected in any urine samples from either treatment group. For both the control and treated groups, PFHxA and PFHxS were detected at the highest median concentrations in urine.

Table 16: Urine PFAS concentrations in pregnant rabbit dams at GD 25. The method detection limit (MDL), detection frequency (DF), range, median, and arithmetic mean concentrations for each analyte are reported for both treatment groups. Concentrations between treatment groups were compared (Mann-Whitney test, two-tailed) to generate p-values. Values less than MDL were imputed with MDL divided by two before the medians and means were calculated.

Analyte	MDL (ng/g)	Control (n=5)				PFAS (n=5)				p-value
		DF (%)	Range (ng/g)	Median (ng/g)	Mean (ng/g)	DF (%)	Range (ng/g)	Median (ng/g)	Mean (ng/g)	
PFBA	1.59	0	ND	ND	ND	0	ND	ND	ND	ND
PFPeA	1.02	20	<MDL – 17.96	<MDL	N/A	0	ND	ND	ND	ND
PFHxA	0.38	60	<MDL – 5.64	0.38	1.36	100	1.20 – 1.70	1.51	1.50	0.13
PFHpA	0.21	80	<MDL – 1.95	0.32	0.61	100	0.45 – 0.87	0.72	0.69	0.17
PFOA	0.16	40	<MDL – 0.40	<MDL	N/A	40	<MDL – 0.18	<MDL	N/A	N/A
PFNA	0.17	0	ND	ND	ND	0	ND	ND	ND	ND
PFDA	0.07	0	ND	ND	ND	0	ND	ND	ND	ND
PFBS	0.01	80	<MDL – 0.40	0.16	0.20	100	0.17 – 0.60	0.40	0.42	0.06
PFHxS	0.01	100	1.09 – 1.65	1.20	1.33	100	1.12 – 2.06	1.72	1.66	0.15
PFOS	0.01	0	ND	ND	ND	0	ND	ND	ND	ND

N/A: not applicable; arithmetic means were not calculated for any analyte with a DF below 50%.
ND: not detected

There are a few plausible explanations for why we may not have seen elevated levels of urinary PFAS in treated dams. Firstly, the rabbit urine may be too challenging of a matrix to analyze for PFAS using our current methodology. Additional method development to optimize rabbit urine extraction may improve PFAS analysis. Secondly, the sample amount extracted may not have been large enough to detect any differences

in PFAS, especially considering the low amounts of PFAS consumed by the rabbits through water in this study. Normal urine output for rabbits is roughly equal to their volume of water consumed, somewhere between 75 mg/kg/day or 120-130 mg/kg/day.[9] We extracted 0.5 mL of urine; for context 0.5 mL of our dosed water would contain only 0.38 ng of Σ(10)PFAS. Thus, even if all of the PFAS in our dose were renally excreted into urine, it would be difficult to see any one individual PFAS elevated in the rabbit urine, especially given the background amounts of PFAS in the control animals.

4.3.2.5 Summary of PFAS Concentrations

Blood serum, liver, feces, and urine samples were all analyzed for PFAA concentration. Figure 18 visually compares these concentrations side-by-side. The PFAA concentrations of the drinking water mixture are also included for reference.

[9] Rabbits will produce more or less urine based on their diet; rabbits fed only grass hay will produce more urine than rabbits that are also fed pellets and vegetables. More or less urine should generally result in more dilute or more concentrated urine PFAS concentrations, respectively.

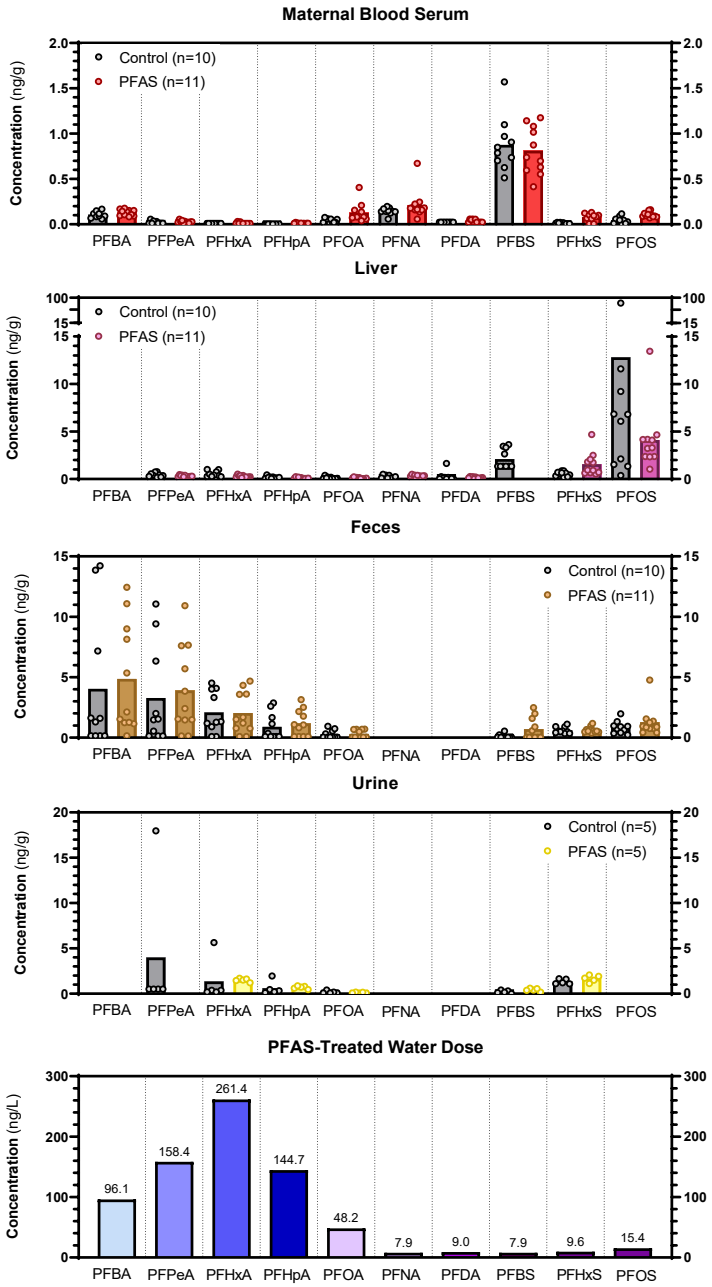

Figure 18: Summary of PFAS concentrations in rabbit tissues.

4.3.3 Liver Lipid Measurements

PFAS have been previously studied for their contribution to liver steatosis and non-alcoholic fatty liver disease (NAFLD) (Bassler *et al.*, 2019; Costello *et al.*, 2022; Das *et al.*, 2017; Jin *et al.*, 2020b; Sen *et al.*, 2022; Stratakis *et al.*, 2020). We hypothesized that the rabbits exposed to the PFAS mixture may have similar indicators of fatty liver and liver impairment, as measured by an increased amount of lipids in liver.

We extracted lipids from aliquots of homogenized liver and measured the mass of extracted lipid to mass of aliquoted tissue. There was no significant difference in liver percent lipid between treatment groups (Figure 19), indicating that PFAS did not lead to an accumulation of fat in the liver.

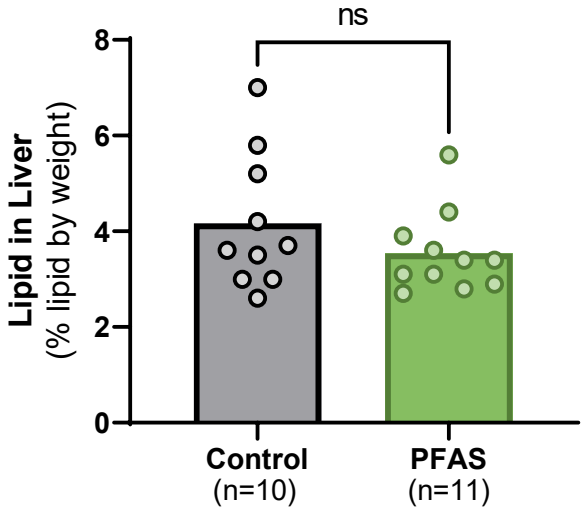

Figure 19: Lipid measurements in rabbit liver. The amount of lipid in liver (as a percentage of liver weight) did not significantly differ between treatment groups (unpaired t test, two-tailed). Means and individual values are plotted. ns, not significant.

Measuring the percentage of extractable lipid is a simple, albeit crude, measure for observing fatty liver disease or hepatic steatosis. Other histopathological indicators of fatty liver disease (e.g., fat vacuoles or liposomes, fatty cysts, and inflammation) were also not observed in any of the rabbit livers (Crute *et al.*, 2022). Serum liver enzymes (e.g., alanine transaminase (ALT) and aspartate transaminase (AST)) were not found to be elevated, further indicating an absence of liver injury (Crute *et al.*, 2022).

4.3.4 Quantitative Real-Time PCR Analyses in Rabbit Liver

The qPCR experiment explored whether PFAS treatment induced any changes in liver gene expression. For the five target genes tested, there were no significant differences in gene expression observed between the PFAS-treated and control groups (Figure 20). Target gene expression levels were normalized to the reference gene *Gapdh*. Expression in PFAS-treated animals (n=11) is reported as a difference in fold change relative to the average expression in control animals (n=10).

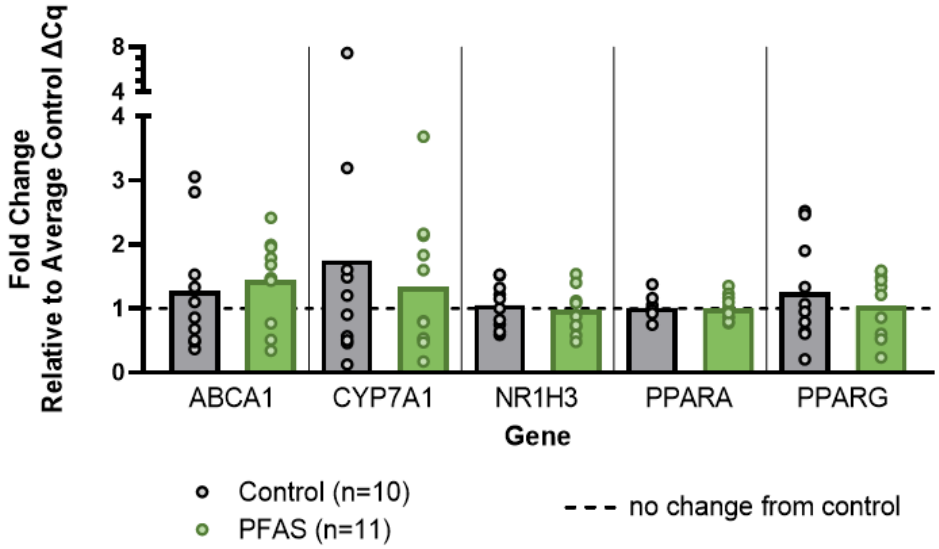

Figure 20: Relative gene expression in maternal rabbit liver. Control and PFAS-treated groups did not significantly differ in expression for any gene (unpaired t test, two-tailed, p>0.05). Means are plotted as bars, and individual animal values are plotted as scattered circles. Expression was calculated using the $\Delta\Delta C_q$ method using *Gapdh* as the reference gene and the average of the control ΔC_q.

Our results indicate that exposure to an environmentally-relevant PFAS water mixture did not lead to liver injury, fatty liver, or any changes in liver gene expression in pregnant rabbits. In contrast to our results, Das *et al.* (2017) found that PFAA-treated mice had increased expression of genes involved in lipid catabolism, fatty acid synthesis, and triglyceride synthesis, indicating increased triglyceride load and a shift in fatty acid metabolism to favor accumulation of fats in the liver. Several other studies have shown than PFAS exposures disrupt normal hepatic lipid metabolism to cause liver steatosis in animal models, usually with PPARα or PPARγ involvement (Bijland *et al.*, 2011; Kim *et al.*, 2011a; Tan *et al.*, 2013; Wan *et al.*, 2012; Wang *et al.*, 2013; Zhang *et al.*, 2019; Zhang *et*

al., 2016a). Because we did not see these effects on liver in our study, it is possible that drinking water exposure to short-chain PFAAs in rabbits does not exert toxicity through this mechanism or through these genes; however, it is also plausible that our rabbit study may be more limited when compared to previous studies that tested higher doses of PFAS exposure or in other species.

4.3.5 Limitations and Future Work

4.3.5.1 Animal Model

Rabbits were originally chosen as the animal model for this study for their suitability as a model for human placenta. As the placenta is a putative target organ for PFAS reproductive toxicity (Blake *et al.*, 2020; Blake & Fenton, 2020; Marinello *et al.*, 2020), it was important to use a small animal model that would be most analogous for human placentation. In normal human placentation, the single layer of syncytial trophoblast undergoes extensive trophoblast invasion of uterine arteries. Rabbits have a hemodichorial placenta with two trophoblast layers; in contrast, rodents have three trophoblast layers (i.e., hemotrichorial placenta) and very limited trophoblast invasion (Carter, 2007; Enders & Blankenship, 1999). Future work may consider using other animal models for exploring the role of PFAS in placental disruption. For example, the guinea pig is a rodent model that has a hemomonochorial placenta, like humans (Enders, 1965; Enders & Blankenship, 1999).

A significant limitation to this work is that the treated rabbits showed little accumulation of PFAS in blood. Treated rabbits consumed or absorbed too little PFAS or excreted PFAS very quickly, leading to our inability to detect PFAS in tissues at concentrations higher than the untreated animals. Many of the PFAAs that constituted our water mixture have short elimination half-lives, especially in animal models. It has been estimated that for PFHpA that the half-life in female rats is ~1 hour and the half-life in male rats is ~2.5 hours (Ohmori *et al.*, 2003). While longer-chain PFAS have been shown to have longer half-lives in rats (Ohmori *et al.*, 2003), these PFAS (i.e., PFOA, PFOS, PFNA, PFDA) were included in our water mixture treatment at much lower concentrations than the shorter-chain PFAS and make up a very small percentage of the total water PFAS dose.

A greater consideration should be given to the half-lives and toxicokinetic parameters of PFAS in animal models, including differences by sex. Our study tested an environmentally-relevant mixture as the dose of PFAS, and while that is an asset to this work, a more appropriate dose would have considered the different pharmacokinetics between humans and rabbits (Harada *et al.*, 2005). If the mixture dose had been simply increased (e.g., 10 or 100 times higher), we may have been able to detect where the PFAS distributed in the rabbit tissues while also maintaining the relevance of a real-world mixture. However, it must be noted that we do not have an abundance of data on the species-specific differences in half-lives for the shorter-chain compounds, limiting some

ability to choose an appropriate dose. Previous work in rabbits estimated that in order to reach and maintain a serum level of 1 ng/mL for PFOA and PFOS, the initial dose needed to be 180 ng/kg and 370 ng/kg, respectively (Gayrard *et al.*, 2021). Assuming a daily water consumption of 0.5 L[10] for a 3.5 kg rabbit, our water mixture of 760 ng/L would only have led to a daily total PFAS dose of 109 ng/kg, with the individual PFAS doses to the rabbits being far lower.

4.3.5.2 Analytical Methods

Another aspect of this work that is limiting is that the extraction and LC-MS/MS analysis of PFAS in rabbit tissues and wastes may not be optimal.

Analysis of short-chain PFAS such as PFBA and PFPeA via LC-MS/MS is particularly challenging. With these short-chain PFAS, only one fragmentation can be monitored (i.e., m/z 213→169 and m/z 263→219), and low-resolution mass spectrometry may not be sensitive enough for distinguishing between these PFAS and other compounds with similar molecular masses in complex matrices like rabbit feces (Abraham *et al.*, 2021). The use of mass-labeled internal standards and isotope dilution can somewhat help circumvent this issue because the unlabeled and labeled analytes should elute at the same retention time; this helps ensure that one is identifying the right

[10] This assumption for water consumption is optimistic. Our rabbits consumed between 200 to 350 g of water per day on average (see Figure 19).

analyte. However, at least one interfering compound for PFBA has been identified co-eluting at the same retention time as PFBA and M-PFBA in human tissue (Bangma *et al.*, 2021). Other PFAS have similar issues. PFHxS may be overestimated in human serum samples due to the interference of steroid sulfates[11] (Benskin *et al.*, 2007; Chan *et al.*, 2009), and the bile acid taurodeoxycholic acid (TDCA) can interfere with PFOS analysis (Benskin *et al.*, 2007) as can other tauro-conjugated bile acids like taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid (TUDCA), and taurohyodeoxycholic acid (THDCA) (Salihovic *et al.*, 2020).

Better extraction methods and clean-up methods can help minimize these interferences with PFAS analysis, but there is always a tradeoff – reducing interferences may also reduce sensitivity and ability to detect the compounds of interest. For PFAS, even with analytes of similar chemistries (e.g., among the PFCAs), there can be difficulty in finding a method that is optimal for all compounds. For example, the long-chain PFDA had low internal standard recovery in many of the rabbit samples (Table C6), and more work could be done to optimize the serum and tissue extractions to improve PFDA recovery. In particular our use of a nylon syringe filter may be partly to blame for the

[11] Chan *et al.* (2009) note that the less sensitive m/z 399→119 transition is interference-free when compared to 399→99 or 399→80; chromatographic conditions can also be changed to better separate interfering compounds away from PFHxS.

low PFDA internal standard recovery, and glass fiber filters may be a better choice (Chandramouli *et al.*, 2015).

The QA/QC from the SRM and from the internal standard recovery indicates that the rabbit matrices in this work are indeed challenging to analyze for all PFAAs. With the strong, sustained interest in PFAS by many stakeholders, better SRMs and analytical methods will hopefully be developed and improved. Additionally, future researchers may not wish to rely solely on LC-MS/MS techniques to determine PFAS tissue accumulation, distribution, and elimination. More robust toxicokinetic techniques such as exposing rabbits to ^{14}C- or ^{3}H-PFAS and measuring the subsequent radioactivity in wastes and tissues would be more conclusive than any of the data shown in this work (Vanden Heuvel *et al.*, 1991a, 1991b). This may be infeasible in some cases – exposure to radioactive isotopes via drinking water will likely be prohibitively impracticable, and rabbits would be a much less appealing model for toxicokinetic work than rats or mice, which are much smaller in size. Rats and mice would be also advantageous because they could be housed in metabolism cages, and urine and feces could be collected in full and analyzed at various timepoints; this is contrast to this work which was only able to collect and analyze a small sample of the wastes. It should be emphasized again though that the dose, in any model, should be carefully thought out prior to the study.

Additionally, background contamination from field equipment, sampling equipment, laboratory reagents, or analytical instruments is always possible (Denly *et*

al., 2019; Rodowa *et al.*, 2020), which poses a challenge to measuring PFAS accurately without any background contamination of samples. The use of PFAS in so many products may also explain why we see PFAS in the control animals. PFAS could have been present at low levels in the rabbits' diet (i.e., chow, vegetables, or hay) or in their environment (e.g., cages, enrichment, absorbent pads for waste collection), leading to some PFAS contamination in the control animals.

4.3.5.3 Future Work

Future work should further explore PFAS mixtures. People are exposed to mixtures, not single chemicals, and PFAS mixture exposure is especially relevant given the number of PFAS that can be found in different matrices. PFAS mixtures have also had intriguing effects in prior studies; in one recent *in vitro* study, mixtures of PFOA, PFPeA, and PFHpA increased the survival of ovarian cancer cells when compared to the chemicals alone, suggesting the PFAS mixtures had proliferative effects that might be relevant for cancer (Rickard *et al.*, 2022).

4.4 Conclusions

Our study explored the toxicokinetics and some effects of PFAS in drinking water in a pregnant rabbit model. Overall, we did not see any adverse changes in the maternal liver due to PFAS exposure. We saw no evidence for increased fatty liver or any changes in liver gene expression in PFAS-treated animals. While our dose was too

low in drinking water to make many conclusions about toxicokinetics in rabbits, our treated group did have higher levels of several serum PFAS than controls.

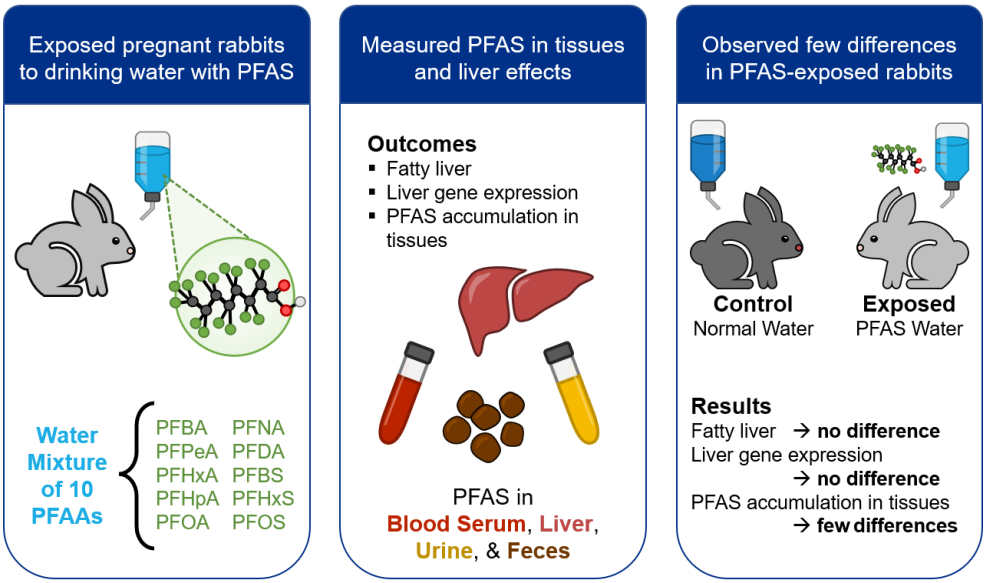

Figure 21: Graphical summary for Chapter 4

5. Per- and Polyfluoroalkyl Substances in Dust Collected from Residential Homes and Fire Stations in North America

This chapter was published under the title "Per- and Polyfluoroalkyl Substances in Dust Collected from Residential Homes and Fire Stations in North America" in *Environmental Science & Technology* in 2020. It is reprinted with permission from Hall, S. M.; Patton, S.; Petreas, M.; Hoffman, K.; Zhang, S.; Phillips, A.L.; Stapleton, H. M. Per- and Polyfluoroalkyl Substances in Dust Collected from Residential Homes and Fire Stations in North America. *Environ. Sci. Technol.* 2020, *54* (22), 14558–14567. DOI: 10.1021/acs.est.0c04869. Copyright 2020 American Chemical Society. The accompanying supporting information is included in Appendix D.

5.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) have garnered attention as "forever chemicals" that are widely detected in humans and the environment. PFAS are used for their water- and stain-repellant properties and can be found in many kinds of consumer products. However, concerns over the persistence, bioaccumulation, and toxicity of some PFAS have motivated additional research to better understand human PFAS exposure.

PFAS are a large group of chemicals that include approximately 5,000 different compounds (OECD, 2018) with several different subclasses such as perfluoroalkyl

carboxylic acids (PFCAs), perfluoroalkyl sulfonic acids (PFSAs), fluorotelomer alcohols (FTOHs), and polyfluoroalkyl phosphoric acid esters (PAPs) (Buck *et al.*, 2011; Wang *et al.*, 2017). The most persistent PFAS compounds are the perfluoroalkyl acids (PFAAs), which include the PFCAs and PFSAs. Some PFAAs, particularly PFOS and PFOA, are known to have long half-lives in humans and have been associated with a myriad of human health effects (Sunderland *et al.*, 2019). Other PFAS such as the FTOHs and PAPs are less persistent due to their physicochemical properties; however, these compounds can degrade into the more persistent PFAAs and have been referred to as PFAA precursors (Wang *et al.*, 2017).

PFAS are amphiphilic compounds with both hydrophobic and lipophobic properties; thus, they are frequently used as water and oil repellents. Various consumer products common to most homes contain PFAS (Kotthoff *et al.*, 2015) and can be sources of PFAS to the indoor environment and dust. For example, PFAS can be found in nonstick cookware and food packaging (Begley *et al.*, 2005; Schaider *et al.*, 2017; Trier *et al.*, 2011), in personal care products such as cosmetics and sunscreens (Fujii *et al.*, 2013), and in impregnation sprays and agents used for textile protection, such as stain repellant carpets and upholstery (Fiedler *et al.*, 2010; Kotthoff *et al.*, 2015).

Data for PFAS concentrations in the indoor environment are important for better understanding the main exposure pathways for PFAS in humans, particularly as indoor sources are less well-characterized than dietary sources. While a tolerable weekly intake

for four PFAS in food has been established by the European Food Safety Authority (EFSA CONTAM Panel, 2020), house dust ingestion and indoor air may be another major pathway of PFAS intake for some people (Poothong *et al.*, 2020). People spend a considerable amount of time indoors, leading to exposure to chemicals from indoor air and dust derived from building materials and consumer products in the home; exposure to dust has been shown to be an important exposure pathway for chemicals such as flame retardants (D'Hollander *et al.*, 2010; Jones-Otazo *et al.*, 2005; Stapleton *et al.*, 2012). The United States Environmental Protection Agency (USEPA) estimates adults ingest approximately 30 mg of indoor dust per day and children ingest approximately 60-100 mg (USEPA, 2011).

Previous studies have measured PFAS in dust from homes, daycares, and businesses across several countries (Ao *et al.*, 2019; Besis *et al.*, 2019; de la Torre *et al.*, 2019; Eriksson & Karrman, 2015; Fraser *et al.*, 2013; Karaskova *et al.*, 2016; Shoeib *et al.*, 2016; Winkens *et al.*, 2018; Zheng *et al.*, 2020). However, there are limited current data on PFAS in dust from homes in the United States, and no one to date has measured dust PFAS concentrations in fire stations as an occupational exposure pathway for fire fighters.

Studies have shown that fire fighters have higher exposure to PFAS compared to the general population (Dobraca *et al.*, 2015; Jin *et al.*, 2011; Laitinen *et al.*, 2014; Shaw *et al.*, 2013; Tao *et al.*, 2008; Trowbridge *et al.*, 2020). This is especially true for fire fighters

exposed to aqueous film-forming foams (AFFF), a type of firefighting foam that contains PFAS (Rotander *et al.*, 2015b). AFFF is used to quickly extinguish fires and contains varying types of PFAS depending on the formulation. AFFF is especially used to contain petroleum-fuel-based fires at airports and military sites (i.e., Class B fires); unfortunately, AFFF use has led to widespread PFAS contamination near these sites and at fire fighter training areas (McGuire *et al.*, 2014; Moody & Field, 2000a; Moody & Field, 2000b). In one study, fire fighters who trained with firefighting foams had increased serum PFHxS and PFNA following training in comparison to baseline serum concentrations taken two weeks prior (Laitinen *et al.*, 2014). Exposure to PFAS through dust and smoke inhalation during a fire event is also possible. For example, first responders to the World Trade Center collapse on September 11, 2011 were found to have plasma levels of PFOA and PFHxS that were two-fold higher than the general population, and especially high levels were found in first responders exposed to the greatest amounts of dust and smoke (Tao *et al.*, 2008). Similar results were seen in the plasma of California fire fighters, with PFOA and PFNA being two-fold higher than the general United States population (Shaw *et al.*, 2013). Additionally, protective turnout gear used by fire fighters may have PFAS coatings which could contribute to exposure (Peaslee *et al.*, 2020).

PFAS sources unique to fire fighters (e.g., AFFF and turnout gear) have raised questions about the extent of PFAS exposure in the fire stations where fire fighters spend

a substantial amount of their time while on duty. Therefore, the goal of this present study was to provide more information on the levels of PFAS in indoor dust, for both the general public and for fire fighters. To support this goal, we collected dust samples from fire stations (occupational exposure) and residential homes (general population exposure) and analyzed them for a suite of 17 PFAS (Table D1). Fire stations were hypothesized to have higher PFAS dust levels due to the likelihood of more frequent contact with potential PFAS sources (e.g., AFFF, turnout gear). A secondary goal of this study was to assess potential differences in PFAS levels between fire stations and residential settings and to determine if PFAS dust concentrations varied based on location, size, age of the buildings, or carpeting.

5.2 Materials and Methods

5.2.1 Dust Collection and Survey Data Collection

5.2.1.1 Residential House Dust

Dust samples from 184 residential homes in North Carolina, United States were collected between 2014 and 2016 as part of the Toddlers' Exposure to SVOCs in Indoor Environments (TESIE) study. The TESIE study population has been further described by previous literature (Hammel *et al.*, 2019; Hoffman *et al.*, 2018). Briefly, home visits were conducted with families enrolled in the TESIE study to collect dust samples and data about the home environment. Study protocols were approved by the Duke Institutional Review Board.

Dust extracts from homes were processed as previously described in Hammel *et al.* (2019) and Phillips *et al.* (2018). Briefly, the entire exposed floor area of the main living room was vacuumed with a Eureka® Mighty Mite® vacuum fitted with a cellulose thimble within the hose attachment (Stapleton *et al.*, 2012), and each thimble was wrapped in aluminum foil, placed in sealed plastic bags, and stored at –20 °C after collection until analysis.

Participants were asked to complete questionnaires during the home visits as also previously described in Hammel *et al.* (2019) and Phillips *et al.* (2018). Age of home and square footage of the home and main living area were collected as part of the questionnaire and verified with tax records where possible.

5.2.1.2 Fire Station Dust

Dust samples were collected from 49 fire stations in the United States in 2015 (Shen *et al.*, 2018) and in Canada in early 2018 (Gill *et al.*, 2020). Fire stations were selected for participation in the study through a collaboration with the International Association of Fire Fighters (IAFF). Stations were selected if they met certain criteria: location in urban or suburban areas, active engagement in urban or suburban fires, fire station as residence for on-duty fire fighters who return to station after deployment to a fire for duration of their shift, and fire stations and bays contained to one building.

The fire station dust samples have previously been analyzed for flame retardants (Gill *et al.*, 2020; Shen *et al.*, 2018). Additional methodology information for the U.S. fire

stations has been previously reported in Shen *et al.* (2018) and for the Canadian fire stations in Gill *et al.* (2020). In brief, participating stations were sent packets of information describing the study's purpose and instructions for collecting samples in only the living quarters areas (including kitchen and eating areas, sleeping areas, and TV viewing areas). Fire stations in the U.S. were asked to use their own vacuum cleaners with new vacuum bags and to collect dust over the course of one month. Canadian fire stations were provided with new vacuum cleaners (Dirt Devil®, Featherlite® Bagged Upright) and a set of new vacuum cleaner bags. Stations were instructed to use the vacuum cleaner only in the living quarters for the purposes of the study and to refrain from mopping, sweeping, or other cleaning techniques in the living quarters during the month of the study. Vacuum cleaner bags from fire stations were sealed in polyurethane bags and stored at 4 °C until analysis. Surveys were collected from fire stations to obtain information on building location, year of construction, square footage, and the percentage of the living quarters containing carpeting.

5.2.2 Dust Sample Preparation, Extraction, and Analysis

Full methods information and HPLC-MS/MS and GC-MS conditions are included in Appendix D and Table D2.

5.2.3 Quality Assurance and Quality Control (QA/QC)

Quality assurance and control were addressed by analyzing laboratory processing blanks alongside the samples and by analyzing house dust standard

reference material (SRM 2585, National Institute of Standards and Technology (NIST), Gaithersburg, MD). Laboratory blanks (n=5-6) and SRM 2585 (n=5) were analyzed in each batch of dust samples, and five field blanks (n=5) were processed along with the fire station dust samples. PFAS levels in samples were blank-corrected using the average laboratory processing blank. Method detection limits (MDLs) were calculated using three times the standard deviation of the average laboratory blank levels if the analyte was detected in the blanks, or by using a value equal to ten times the noise if absent in the lab blanks. MDLs were normalized to the average mass of dust extracted (0.1 g). If dust concentration values were below the MDL, the value was imputed with dust mass specific MDL values. PFAS measurements for the extracted SRM and reference values for house dust SRM 2585 (NIST, 2018b; Reiner *et al.*, 2015; Winkens *et al.*, 2018) are provided in Table D3.

[Note: Additional QA/QC information regarding the average percent recoveries of mass-labeled internal standards is included in Table D8. This table was not included in the original publication but is included here for additional supplemental information on the analytical methods. Recoveries in house dust ranged from 32% to 106%, with 8 of the 13 internal standards having an average recovery less than 50%. Recoveries in fire station dust were 227% for mass-labeled PFBA and 161% for mass-labeled 8:2 diPAP; recoveries for the seven remaining LC-amenable standards ranged from 52% to 90%. Recoveries for the GC-amenable compounds in fire station dust are not available.]

5.2.4 Statistical Analyses

Data were analyzed and graphed using GraphPad Prism (Version 8.4.0) and Microsoft Excel. Nonparametric statistical tests were used as the data were not normally distributed, as determined by the Shapiro-Wilk normality test. Statistical analyses were only performed on individual analytes that were detected in more than 50% of samples. The Mann-Whitney test (two-tailed) was used to determine statistical significance at $\alpha < 0.05$. Some survey questionnaires were incomplete or missing; thus, the sample size for some endpoints varied.

5.3 Results and Discussion

5.3.1 PFAS Concentrations in Dust Samples

In this study we report on PFAS concentrations measured in both residential dust samples and dust collected from living quarters of fire stations within the United States and Canada. PFAS were detected in all dust samples. Detection frequencies and method detection limits (MDLs) for each PFAS are presented in Table 17, along with maximum and median concentrations. In general, the shorter-chain PFAAs (PFBA, PFBS, and PFPeA) were less frequently detected than the longer-chain PFAAs and the precursor compounds. The FTOHs were the most abundant group of the PFAS quantified in both homes and fire stations. In contrast, the median concentrations for many of the PFAAs were below 20 ng/g dust.

There was considerable variation in the distribution of dust concentrations for individual PFAS analytes, as seen by the range of concentrations depicted in Figure 22. For example, the maximum concentrations for some PFAS (e.g., PFOS, FTOHs, and FOSEs) were orders of magnitude higher than the median concentrations. Based on median dust concentrations, the most abundant PFAS was 8:2 FTOH in residential homes (1,440 ng/g dust) and 6:2 FTOH in fire stations (760 ng/g dust). Of the PFAAs, PFHpA was the most abundant in homes (median concentration of 9.0 ng/g dust) while PFOS was the most abundant in fire stations (median concentration of 65 ng/g dust). Median levels for dust PFAS reported here ranged from tens to hundreds of nanograms per gram (ng/g), which are generally lower than the levels of organophosphate ester flame retardants (OPEs) (Phillips *et al.*, 2018) and phthalates (Hammel *et al.*, 2019) we found in these same house dust samples in previous studies.

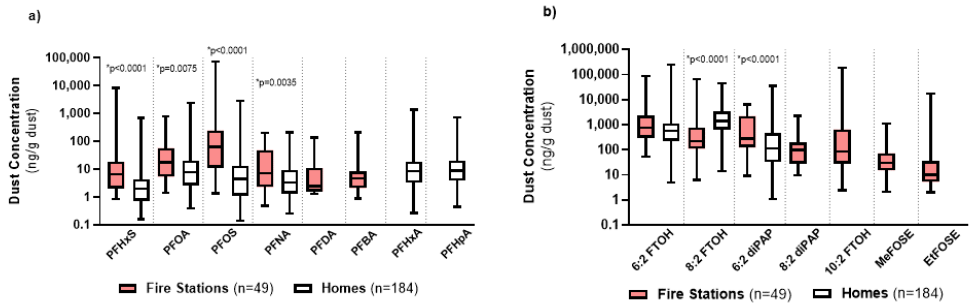

Figure 22: PFAS dust concentrations in fire stations and homes. Dust concentrations for a) legacy PFAAs and b) PFAA precursors in both fire stations and residential homes. Boxplots represent the minimum, 25th percentile, median, 75th percentile, and maximum concentrations for each analyte. Analytes are only represented as boxplots if they were detected in more than 50% of samples as

presented in Table 17. Concentrations are reported on a log scale. Significance determined by Mann-Whitney test.

Table 17: Dust PFAS concentration data. Detection frequency, method detection limit (MDL), median, and maximum concentrations for PFAS analytes in dust samples. Bolded compounds were significantly different between fire stations and homes.

	Class	Compound	Fire Stations (2015 and 2018, n=49)				Residential Homes (2014–2016, n=184)				p-value*
			Detection Frequency (%)	MDL (ng/g dust)	Median Concentration (ng/g dust)	Maximum Concentration (ng/g dust)	Detection Frequency (%)	MDL (ng/g dust)	Median Concentration (ng/g dust)	Maximum Concentration (ng/g dust)	
PFAA Precursors	diPAP	**6:2 diPAP**	**100**	**2.54**	**287**	**6,270**	**100**	**0.48**	**113**	**34,360**	***p<0.0001**
		8:2 diPAP	94	9.63	99.3	2,250	34	10.63	<MDL	6,890	-
	FOSE	MeFOSE	88	5.00	30.2	1,110	19	13.17	<MDL	7,980	-
		EtFOSE	65	5.00	9.97	17,540	15	15.45	<MDL	370	-
	FTOH	6:2 FTOH	96	48.90	756	86,060	97	6.44	569	248,920	p=0.1411
		8:2 FTOH	**92**	**7.23**	**216**	**65,170**	**99.5**	**15.80**	**1,435**	**44,220**	***p<0.0001**
		10:2 FTOH	82	5.00	84.9	183,730	N/A	N/A	N/A	N/A	-
Legacy PFAAs	PFCA	PFBA	84	1.03	4.6	213	9.2	1.72	<MDL	546	-
		PFPeA	41	1.20	<MDL	1,410	10	0.14	<MDL	135	-
		PFHxA	41	10.30	<MDL	1,150	97	0.42	8.5	1,380	-
		PFHpA	37	6.77	<MDL	382	97	0.51	8.9	713	-
		PFOA	**82**	**1.60**	**17.6**	**791**	**100**	**0.26**	**7.9**	**2,350**	***p=0.0075**
		PFNA	**96**	**0.47**	**7.2**	**203**	**99.5**	**0.15**	**3.3**	**208**	***p=0.0035**
		PFDA	63	1.49	2.5	137	41	0.06	6.2	4,130	-
	PFSA	PFBS	12	8.56	<MDL	2,650	1.1	22.28	<MDL	320	-
		PFHxS	**86**	**0.97**	**6.8**	**8,280**	**57**	**0.25**	**2.0**	**694**	***p<0.0001**
		PFOS	**96**	**1.44**	**64.5**	**74,370**	**84**	**0.20**	**4.4**	**2,810**	***p<0.0001**

5.3.2 Comparing Dust PFAS Concentrations in Fire Stations and Homes

Overall, precursor PFAS were found at higher concentrations than legacy PFAAs in both fire stations and homes, and both locations were dominated by diPAPs and FTOHs. This suggests that PFAS exposure via dust in fire stations versus residential settings is not drastically different, though a few analytes show some differences. PFOS, PFOA, PFHxS, PFNA, and 6:2 diPAP were significantly higher in fire station dust than residential dust. Median dust levels in fire stations were approximately 15 times higher for PFOS (*$p<0.0001$), approximately 3 times higher for PFHxS (*$p<0.0001$), and approximately 2.5 times higher for 6:2 diPAP (*$p<0.0001$) compared to median dust levels in homes. Only 8:2 FTOH was significantly higher in house dust than in fire station dust (approximately 7 times higher, *$p<0.0001$).

PFAS profiles in house and fire station dust were both dominated by diPAPs and FTOHs, as shown in Figure D1. Dust samples from the fire stations and homes were both collected from the living areas, so it is unsurprising that the overall relative proportions of PFAS in dust are similar. This likely suggests that the sources of PFAS to the living quarter dust are from similar products or similar construction materials used in both homes and in fire stations.

However, fire station dust PFAS levels did somewhat differ from homes as fire stations had higher median concentrations of PFOS, PFOA, PFHxS, PFNA, and 6:2 diPAP. The higher levels of PFOS and PFHxS in fire stations may be linked to the use or

storage of AFFF by fire fighters. PFOS and PFHxS have been noted to be components of some AFFF formulations (Backe *et al.*, 2013; Jin *et al.*, 2011; Moody & Field, 2000b; Rotander *et al.*, 2015b). High PFOS and PFHxS serum levels in fire fighters have been hypothesized by some to be due to use of these foams or exposure to burning stain-resistant carpeting (Barton *et al.*, 2020; Jin *et al.*, 2011; Rotander *et al.*, 2015a; Rotander *et al.*, 2015b). Information on the types of AFFF used by the various stations was not collected as part of this present study. There may also be differences between fire stations and homes because of how the living quarters are utilized in each environment. Fire stations likely have a greater flowthrough of people than homes in addition to frequent turnover as fire fighters arrive and leave for shifts; the fire stations in our study had an average of 7 fire fighters per shift.

5.3.3 PFAS Concentrations in Fire Station Dust by Country

A notable result from this present study is that fire stations in the United States had significantly higher ΣPFAS dust concentrations than fire stations in Canada for the subset of 17 PFAS analytes we measured. As displayed in Figure 23, U.S. fire stations (n=25) had a median ΣPFAS concentration of 10,600 ng/g dust, which is approximately 3.5 times higher than the Canadian fire stations' median ΣPFAS concentration of 2,900 ng/g dust (n=24).

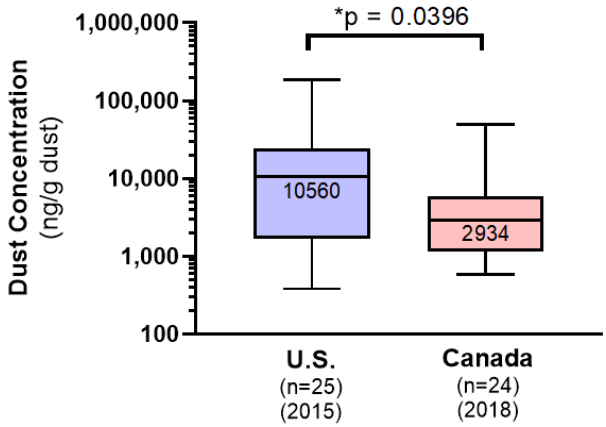

Figure 23: Dust ΣPFAS in fire stations by country. The ΣPFAS concentration in dust was significantly higher in U.S. fire stations than Canadian fire stations (Mann-Whitney test, two-tailed, *p=0.0396). The ΣPFAS concentration represents the sum of all 17 PFAS analytes measured in a dust sample. Boxplots represent the minimum, 25th percentile, median, 75th percentile, and maximum concentrations. Median concentration values are listed inside the box. Concentrations are reported on a log scale.

Different trends and regulations regarding building codes, construction materials, and fire station practices in the use of PFAS-containing products may result in different PFAS dust levels between the U.S. and Canada. However, it must be noted that the fire station samples in this present study were collected at different times (2015 for the U.S. stations and 2018 for the Canadian stations); thus, the Canadian fire station dust samples may have lower PFAS levels due solely because they were collected more recently. We also cannot rule out the possibility that differences in sample collection methodology may be contributing to the observed differences between countries.

Analyses were also conducted on individual PFAS to determine which PFAS were driving the difference in ΣPFAS between U.S. and Canadian fire stations. Median

concentrations of individual PFAS captured by our analytical methods were generally higher in U.S. fire station dust samples compared to Canadian fire station dust, as seen in Figure 24; 12 of 13 analytes had higher median dust concentrations in the U.S. samples with only 8:2 diPAP being higher in Canadian fire stations. PFOS, PFOA, PFHxS, PFDA, 10:2 FTOH, and EtFOSE were significantly higher in U.S. fire station dust compared to Canadian fire stations. The median PFOS dust concentration in the U.S. fire stations was 104 ng/g dust, which is 7 times greater than the Canadian concentration of 13 ng/g dust. Median and maximum concentrations by country are presented in Table D4.

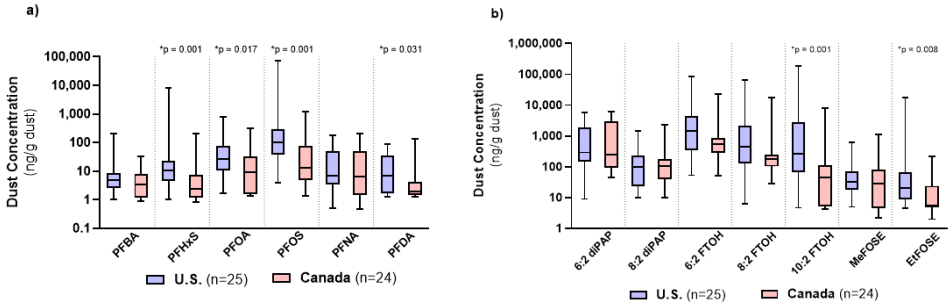

Figure 24: Dust PFAS concentrations by analyte between U.S. and Canadian fire stations. Results for a) legacy PFAAs and b) PFAA precursors. Significance determined by the two-tailed Mann-Whitney test. Boxplots represent the minimum, 25th percentile, median, 75th percentile, and maximum concentrations. Concentrations are reported on a log scale.

Fire station dust samples were collected from several different states and provinces. The ΣPFAS dust concentrations by region (state and province) are presented in Figure D2 to illustrate regional variability. The ΣPFAS for homes represents the sum of 16 PFAS compounds (without 10:2 FTOH) while ΣPFAS for fire stations represents

the sum of 17 PFAS compounds. The observed ranges of ΣPFAS dust concentrations displayed wide variability, even within a single region (e.g., within New Hampshire). No large differences in ΣPFAS dust levels were observed between regions in this limited assessment, although due to the low sample size per region for the fire station samples (n=4-9), statistical analysis by region was precluded.

5.3.4 PFAS Dust Concentrations and Survey Responses

Information on building characteristics was collected via surveys during the study. Table D5 describes survey data from fire stations, and Table D6 describes survey data from homes. The total number of samples was small, and there was wide variability in the recorded age of each building, so dust samples were grouped into two categories: construction pre- and post-1970. PFAS dust concentrations stratified by building age are presented in Figure 25a for fire stations and Figure D3a for U.S. homes. No significant differences in dust ΣPFAS were observed due to building age in this present study. However, it is important to note that we did not record whether any renovations might have occurred within the building.

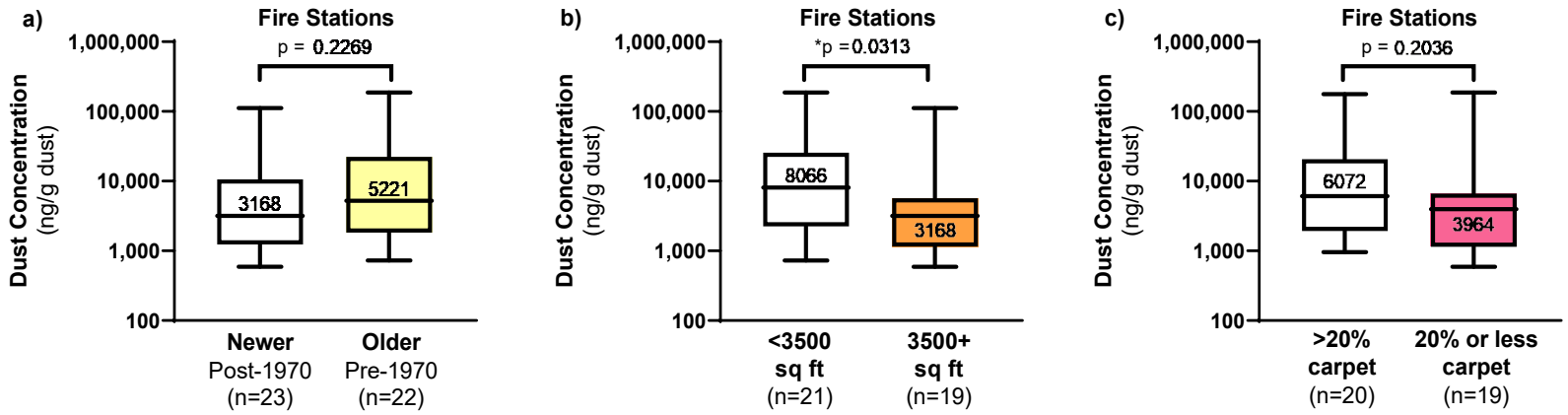

Figure 25: ΣPFAS dust concentration and survey responses for fire stations. Differences in dust ΣPFAS concentrations in fire stations due to: a) year of construction, b) square footage of fire station living quarters, and c) percentage carpeting in the living quarters. Significant differences were seen in ΣPFAS dust concentrations based on square footage in fire stations (*p=0.0313, two-tailed Mann-Whitney test). Sample sizes are variable due to missing survey data. Median concentrations are listed inside the box. Boxplots represent the minimum, 25th percentile, median, 75th percentile, and maximum concentrations. Concentrations are reported on a log scale. The ΣPFAS concentration represents the sum of all 17 PFAS analytes measured in an individual fire station dust sample.

Square footage of home and fire station living quarters was also recorded and analyzed. Due to the small sample size, square footage was dichotomized near the median and categorized into either less or greater than 3,500 square feet for fire stations and less or greater than 1,720 square feet for homes. Within fire stations, ΣPFAS dust concentrations were significantly higher in stations that had a living quarters with a smaller square footage (*p=0.03, Mann-Whitney test) (Figure 25b). This difference seems to be driven by differences in several PFAS, including PFBA, PFHxS, PFOA, PFOS, PFDA, 6:2 FTOH, and 10:2 FTOH (Figure D4). For homes, square footage was not significantly associated with ΣPFAS dust levels (Figure D3b).

Analyses were also conducted considering the amount of carpeting in the living quarters. For fire stations, we recorded the percentage of carpeting, and for homes, we recorded the presence of carpeting or rugs in the living quarters. Within fire stations, the percentage of carpeting in the living quarters was not significantly associated with ΣPFAS dust levels (Figure 25c); a similar non-significant result for ΣPFAS was found for homes (Figure D3c). However, when further delving into these data by individual analytes, several of the FTOHs were found to be significantly higher in the dust samples from carpeted areas in both fire stations and homes (Figure D5). This suggests that carpeting may be a source of the FTOHs observed in these dust samples.

Differences in building use and construction may be important factors for PFAS dust concentrations. For example, Zheng *et al.* (2020) recently reported PFAS dust

concentrations in U.S. childcare centers. Similar to this present study, Zheng *et al.* (2020) found that FTOHs were the most abundant class, and PFAAs were the least abundant class measured in dust. However, median PFAS dust levels in the childcare centers appear to be lower than the levels reported in this present study and may reflect differences in building use and construction.

5.3.5 Time Trends in PFAS Dust Levels and Comparisons to Previous Literature

Previous studies have measured PFAS in house dust from the United States (Fraser *et al.*, 2013; Karaskova *et al.*, 2016; Knobeloch *et al.*, 2012; Strynar & Lindstrom, 2008) and across the world (D'Hollander *et al.*, 2010; Ericson Jogsten *et al.*, 2012; Eriksson & Karrman, 2015; Goosey & Harrad, 2011; Harrad *et al.*, 2010; Haug *et al.*, 2011; Huber *et al.*, 2011; Karaskova *et al.*, 2016; Kato *et al.*, 2009; Kubwabo *et al.*, 2005; Shoeib *et al.*, 2011; Tian *et al.*, 2016; Winkens *et al.*, 2018). Studies reporting PFAS levels in U.S. house dust are summarized in Table D7 and compared to the present study. As shown in Figure 26, the median concentrations of PFOA, PFOS, and PFHxS in U.S. house dust samples appear to be decreasing over the last 20 years. In 2000, the 3M Company began phasing out its production of PFOS, and in 2006, several other companies pledged to phase out the use of PFOS and PFOA in the U.S. While PFOS and PFOA production have declined over the past two decades in the U.S., other PFAS are replacing them. We would expect to see increases in these newer replacement PFAS in dust as they are identified, and as analytical and sampling methods improve over time. Data on PFAS precursors in U.S.

house dust studies are limited and could not be examined as thoroughly for trends over time.

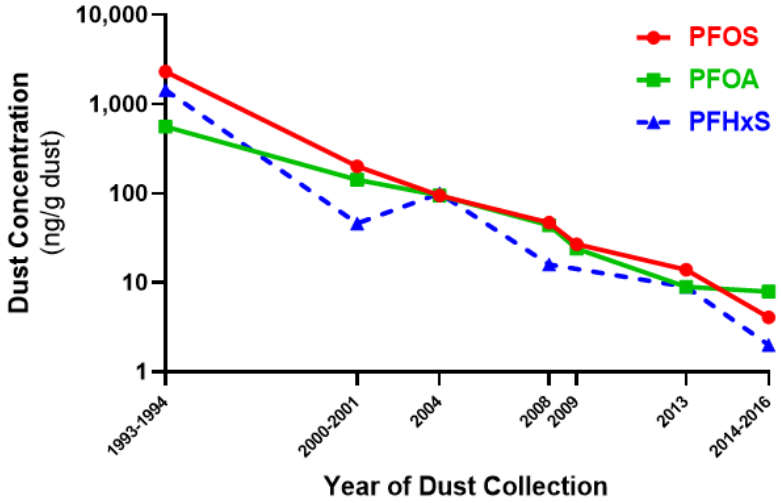

Figure 26: Decline in PFAS concentration in U.S. residential dust over time as reported in the studies cited in Table D7.

Karaskova *et al.* (2016) measured house dust collected in 2013 and found a higher median concentration for PFOS in U.S. homes than Canadian homes (14 vs. 9 ng/g), similar to our results in U.S. and Canadian fire stations. Strynar and Lindstrom (2008) analyzed U.S. house dust from 2000-2001 and found higher dust concentrations for PFAAs but lower 6:2 FTOH and 8:2 FTOH when compared to our house dust samples collected in 2014-2016. We compared house dust concentrations from the 102 U.S. homes in Strynar and Lindstrom (2008) to the house dust samples in our present study in Figure D6 and found that PFHxA, PFHxS, PFHpA, PFOA, and PFOS have significantly decreased over time. In contrast, 8:2 FTOH significantly increased and was detected

more frequently in our more recent house dust samples, and 6:2 FTOH followed a similar trend. Knobeloch *et al.* (2012) analyzed U.S. house dust collected in 2008 and measured lower median PFAA dust concentrations than in Strynar and Lindstrom (2008) and higher PFAA concentrations than the present study. These results, and their differences over time (Figure 26), may reflect the phase-out of PFOS and PFOA from consumer products since 2000.

Winkens *et al.* (2018) measured PFAS in floor dust from children's bedrooms in Finland and found that FTOHs and diPAPs dominated the dust samples, similar to the results of this current study. However, U.S. residential dust concentrations were higher than Finnish samples for 6:2 diPAP (113 vs. 54 ng/g) and for 8:2 FTOH (1,440 vs. 46 ng/g). FTOHs have been found at high levels in waterproofing agents, carpets, and textiles (Herzke *et al.*, 2012), and this may explain why FTOHs are the most abundant PFAS class measured in these dust samples collected from living quarters.

5.3.6 Limitations

There are several limitations to this present study. House dust samples were only collected in North Carolina, and it is possible that there are regional differences in home construction or building practices that influence PFAS dust levels. Furthermore, we measured 17 PFAS compounds using our LC and GC techniques in this study, but there are thousands of different PFAS (OECD, 2018). At the time when the house dust samples were analyzed, an analytical standard for 10:2 FTOH was not available in our

laboratory, and as a consequence, 10:2 FTOH was quantified in the fire station dust but not in house dust. Additionally, we did not measure 6:2 FTS, a PFAS that has been found in some kinds of AFFF concentrate (Schultz *et al.*, 2004). Another limitation is that PFBS had a relatively high detection limit in our study compared to the other analytes; this may explain why PFBS had a low detection frequency in our study despite PFBS being used to replace longer-chain PFAS.

Fire station dust was not retrieved using the same standardized techniques or equipment as the house dust. Fire station samples were collected by fire fighters rather than trained researchers, so we cannot guarantee that collection methodologies were consistent. For example, Canadian and U.S. fire stations used different vacuuming equipment, and a few fire stations in British Columbia collected combined dust from the interiors of their fire engine trucks as well as their living quarters. In contrast, the house dust was collected in a standardized way by a small team of researchers. A meta-analysis by Mitro *et al.* (2016) demonstrated the variability among indoor dust studies in collection method and storage conditions. Mitro *et al.* (2016) noted that methodological consistency may be improved by using extraction thimbles in the crevice tool of vacuum cleaners instead of sampling from used bags. Different brands of vacuum cleaners and bags could also contribute to dust data variability (Trakumas *et al.*, 2001).

In addition, house dust samples were collected at only a single point in time (i.e., during one home visit) while fire station dust was sampled and combined over the

period of one month. This present study also only examined dust found in the main living quarters of homes and fire stations, which may not represent PFAS exposure in other rooms of the buildings. While this present study explored some of the differences between PFAS dust concentrations in fire stations and homes, the relatively low number of fire stations somewhat limits generalizability. With these limitations, it is difficult to draw conclusive information regarding the factors influencing dust PFAS concentrations.

The lack of associations between the survey data and dust PFAS concentrations highlights a need for more research in understanding what drives PFAS dust concentrations. Survey data in this study was limited by participation, and questionnaires used at fire stations were originally designed to explore predictors of flame retardants, not necessarily PFAS. Our results suggest that fire stations in the United States have higher dust PFAS concentrations than Canadian fire stations. However, it is unclear if this difference could be driven by building materials or use of specific products used within those facilities (e.g., different types of AFFF) or by differences in how and when the dust was collected.

5.4 Conclusions

Moving forward it will be important to consider the impact of precursor PFAS on PFAS exposure as these were found at the highest concentrations in our dust samples. Identifying the sources of FTOHs in the dust is an important issue. Previous studies

have detected FTOHs in durable water repellant clothing (van der Veen *et al.*, 2020), so it is possible that firefighting turnout gear or other textiles are contributing FTOHs to indoor dust. Additionally, we detected FTOHs at significantly higher levels in samples with greater amounts of carpeting (Figure D5), suggesting that carpeting may be a source of FTOHs. FTOHs are used to treat paper and textiles and have been found at high levels in carpets, textiles, and waterproofing and cleaning agents (Herzke *et al.*, 2012; Kotthoff *et al.*, 2015). 6:2, 8:2, and 10:2 FTOH have been found in impregnating agents and some AFFF (Favreau *et al.*, 2017). Notably current commercial mixtures of AFFF contain 6:2 FTOH and 8:2 FTOH while AFFF produced before 2010 did not (Favreau *et al.*, 2017). Fluorotelomer alcohols are also used in the synthesis of fluorotelomer-based products, and some estimate that residual FTOHs may remain in those products at about 2% by weight (Prevedouros *et al.*, 2006). Fluorotelomer-based products (FTOHs and PAPs) are thought to be indirect sources of PFCAs in the environment through several means, namely by product degradation, by PFCA impurities, or through fluorotelomer-based AFFF (Prevedouros *et al.*, 2006). There is also evidence that atmospheric degradation of 8:2 FTOH and other FTOHs are a source for global PFCA pollution (Ellis *et al.*, 2004; Wallington *et al.*, 2006).

The dust concentrations reported in this present study show that FTOHs and diPAPs are abundant in residential dust and thus have the potential to lead to PFAA exposure. Additionally, the maximum values we report for the FTOHs, diPAPs, FOSEs,

and PFOS were orders of magnitude higher than the median concentrations, indicating greater exposure potential for some residents and fire fighters. Differentiating sources of PFAS in dust should also be explored in more depth in future studies. Comparing the relative amounts of different PFAS subclasses or branched vs. linear isomers (Beesoon *et al.*, 2011) could give better insight into which sources of PFAS are most contributive. More research is needed to understand the links between PFAA precursors in indoor environments and their contribution to PFAS in dust, and how this relates to overall PFAS exposure in people.

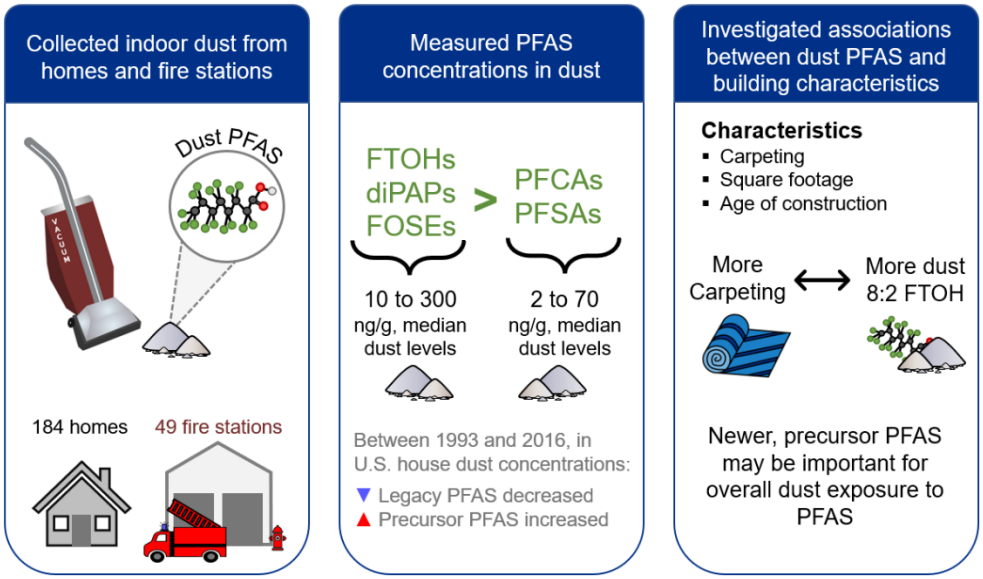

Figure 27: Graphical summary for Chapter 5

6. Overarching Conclusions and Discussion

6.1 Summary of Dissertation Aims

This dissertation aimed to characterize exposure and effects of PFAS in some North Carolina communities. This research provides important insights into many PFAS, particularly the persistent PFAAs.

A variety of different samples were chemically analyzed throughout this work. Water and house dust were measured for PFAS to understand the important sources of PFAS to humans. Blood and tissues were measured for PFAS to determine which PFAS are distributing and accumulating there. In our rabbit study, urine and feces were measured for PFAS to quantify how much PFAS were being eliminated.

Collectively, these PFAS measurements give us important insights into PFAS exposure. In Chapter 5, we observed that indoor house dust contains higher concentrations of precursor PFAS than of PFAAs, indicating that the exposure to precursor PFAS through dust may be an important pathway to consider for human risk assessment. This can be further evidenced by Kassotis *et al.* (2021), which studied the same house dust samples as in Chapter 5. They exposed cells to the same dust extracts and found that 8:2 FTOH in dust was positively associated with triglyceride accumulation in these dust-exposed cells. In Chapter 2, we noted that long-chain PFAAs could be detected in all placental tissues in our study population while short-chain PFAAs were detected much less frequently. In Chapter 3, we observed that many PFAS

could be detected in the drinking water and blood samples of an exposed North Carolina community and that some of these PFAS concentrations were higher than the national average.

This research also provides some understanding of potential health effects of PFAS. In Chapter 2, the placental PFAS concentration was associated with changes in infant birth weight, and some of these changes differed by infant sex. In Chapter 3, the blood PFAS concentration was associated with changes in serum cholesterol and various clinical chemistry measures. Blood PFOA and PFHxS were positively associated with total cholesterol and with non-HDL cholesterol, and blood PFOS and PFHxA were associated with changes in serum clinical chemistry (i.e., electrolytes, liver enzymes, glucose, and BUN:Cre). These findings provide evidence of potential adverse effects of PFAS exposure in NC communities. In Chapter 4, an experiment that purposefully exposed rabbits to PFAS through drinking water demonstrated that PFAS may cause some limited toxicity. However, no differences in the rabbit liver tissue or liver gene expression were observed, and the potential mechanisms of toxicity for PFAS are still to be elucidated.

A strength of this dissertation is its focus on developmental exposure. Development is a hugely important life stage for all organisms, and during this stage, organisms can be uniquely susceptible to toxic insults that lead to lifelong damage. PFAS have previously been found to negatively impact development, particularly in the

placenta (Blake *et al.*, 2020; Blake & Fenton, 2020). Chapter 2 focused on placental concentrations of PFAS, as a surrogate to quantify fetal exposure to PFAS throughout pregnancy. Chapter 2 also observed associations between PFAS and birth outcomes. In Chapter 4, rabbits were exposed before and during pregnancy to PFAS. While this particular dissertation was not able to quantify the levels of PFAS in rabbit placenta or offspring due to the low dose of PFAS, our collaborators were able to identify some adverse effects to both the placenta and the offspring due to PFAS exposure (Crute *et al.*, 2022).

An additional strength of this dissertation research was the consideration of chemical mixtures. While many toxicology studies focus narrowly on a single chemical, humans are regularly exposed to a plethora of chemicals at the same time. As the data in Chapter 3 show, people regularly drink water that contains many kinds of PFAS. Our study in Chapter 4 exposed rabbits to an environmentally-relevant mixture of PFAS. While the dose in the study likely should have been multiplied by an order of magnitude to account for differences in human and rabbit pharmacokinetics, its relevance to actual human exposure is a strength. In Chapter 3, associations were explored between health measures and blood PFAS, including the sum of all PFAS detected in blood (ΣPFAS). With more than 12,000 different PFAS in existence, it will be increasingly important to segue into studying PFAS holistically as a class or as subclasses instead of singly one at a time.

Overall, this dissertation accomplished its goal of providing important data and information into PFAS exposure and potential health effects in North Carolina communities.

6.2 Remaining Data Gaps and Future Directions

There are numerous avenues that should be explored with respect to PFAS. The issue of PFAS pollution will likely continue garnering attention as industries, governments, policymakers, and consumers discover how widespread and recalcitrant these contaminants are in our environment. Recent reviews have described many of the data gaps in PFAS research (Guelfo *et al.*, 2021; Ng *et al.*, 2021). The following topics are some of the most pressing key areas for future PFAS research relevant to this work and to North Carolina.

6.2.1 Analytical Methods

Analyzing for PFAS in environmental matrices is a challenge, not only because of the sheer number of PFAS but because of the diverse and complex chemistries associated with them. PFAS are also measured at very low concentrations, in the ng/L or parts per trillion levels, and the ubiquity of PFAS in products can make it challenging to avoid contaminating samples with PFAS from equipment (Denly *et al.*, 2019).[1]

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[1] Some researchers believe this is overstated and that field equipment is an unlikely source of significant PFAS contamination; see Rodowa *et al.* (2020) for one such perspective.

Developing better analytical methods for PFAS is essential. While all methods have limitations, and no single analytical method will be able to quantify all PFAS of interest or concern, improving PFAS methods will help in our understanding of PFAS environmental contamination.

Furthermore, in order to be used for federal regulation, these methods must be validated by the USEPA. Currently the only validated PFAS methods are for drinking water: EPA 533, EPA 537, and EPA 537.1. Using EPA 533 and EPA 537.1, labs can reliably quantify 31 unique[2] PFAS analytes in drinking water. While these drinking water methods have been successful in measuring some selected, targeted PFAS, there are many other PFAS present in drinking water; measuring this remaining PFAS will be a challenge (McDonough *et al.*, 2019).

There are currently no established EPA methods for measuring PFAS in other matrices like wastewater, surface water, groundwater, landfill leachate, soils, sediments, tissues, or biosolids.[3] These types of samples are challenging to analyze because they are not as clean as drinking water, can be much more variable, and can have more matrix

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[2] 25 PFAS through EPA 533 and 18 PFAS through EPA 537.1, version 2.0. Twelve PFAS can be measured by both methods. For UCMR 5, 29 of the 31 will be measured. PFHpS, PFMBA, and PFMPA will not be included in the UCMR 5.

[3] EPA SW-846 Test Method 8327 was finalized in 2021 for 24 PFAS in non-potable water matrices, in combination with Test Method 3512. This method has some substantial drawbacks, notably the lack of isotope dilution, and some groups say it should only be used for screening (Denly & Morin, 2022). Method 8327, which was developed as a RCRA method, is probably best described as suitable for screening for hazardous waste. In contrast, Draft Method 1633 is a method appropriate for the Clean Water Act and can be used for NPDES permits.

interferences (i.e., other chemicals or constituents interfere with the accurate analysis of PFAS). However, a Draft Method 1633 is in development for all these matrices (USEPA, 2021a), and a few commercial companies are now starting to offer this draft method for non-drinking water matrices. Draft EPA Method 1633 measures 40 PFAS and has been validated by a single laboratory thus far; the multi-laboratory validation is expected to be completed in 2022.

Measuring volatile PFAS are a further analytical challenge, so developing methods to test for PFAS in air and emissions will be a future priority. Though many PFAS of current concern are not very volatile, PFAS can be widely detected in indoor dust and air (Winkens *et al.*, 2018; Winkens *et al.*, 2017) and PFAS can still migrate via air. Other more volatile, precursor PFAS can also transform or degrade into more toxic, persistent PFAS. The USEPA is currently working to develop methods for PFAS in ambient air (ambient/near-source, semivolatile PFAS, and volatile PFAS) and in air emissions.

Targeted methods that only measure a few PFAS at a time will continue to be useful for quantifying the PFAS of most concern. Nontargeted methods will help to identify new and emerging unknown PFAS (Getzinger & Ferguson, 2020, 2021; Getzinger *et al.*, 2021). Other assays such as total oxidizable precursor (TOP)[4] and total

[4] Occasionally defined as Total Organic Precursors

organic fluorine (TOF) should be further explored to better characterize the aggregate hazards of PFAS. While targeted LC-MS/MS methods measure a few, specific, targeted PFAS chemicals, the TOP and TOF assays can show how much unknown PFAS or total PFAS could be in the sample (Figure 28). The USEPA is also in the process of developing TOP and TOF methods.

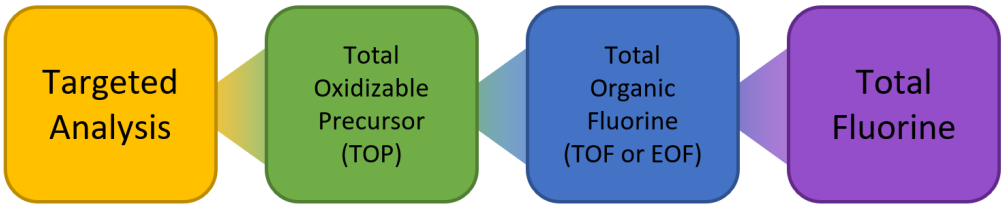

Figure 28: Targeted PFAS analysis and aggregate PFAS methods

The TOP assay (also TOPA) is intended to measure the amount of PFAA precursors in a sample (Houtz & Sedlak, 2012). This is especially useful because we do not have analytical standards for many precursor PFAS. Using the TOP assay, we can understand what amount of a sample constitutes a PFAA or a potential PFAA. The TOP assay has been used previously to characterize PFAA precursors in wastewater treatment; short-chain PFAA precursors were found to account for 30-60% of the total PFAS in effluent (Houtz *et al.*, 2016). It is also useful in understanding precursor transformation during water treatment (Glover *et al.*, 2018; Houtz *et al.*, 2018) or at AFFF sites (Harding-Marjanovic *et al.*, 2015).

However, the TOP assay is limited because its use of oxidation converts PFAA precursors into only PFCAs. The TOP assay will not replicate other environmental biotransformation processes. For example, while PFOSA and other compounds at AFFF sites under normal conditions could slowly biotransform into PFSAs, under the oxidative TOP assay they will transform into PFCAs instead (Mejia-Avendano *et al.*, 2016; Schaefer *et al.*, 2018; Yi *et al.*, 2018).

The adsorbable organic fluorine (AOF) assay can be used to look at aggregate PFAS in water, but common water constituents like fluoride or dissolved organic matter can adversely affect this method (Han *et al.*, 2021). The extractable organic fluorine (EOF) assay can be used in matrices beyond water, but may also miss capturing some cationic and neutral PFAS (Han *et al.*, 2021). Both the AOF and EOF assays measure more fluorine than targeted methods.

The TOF assay (also TOFA) is used to measure the total amount of organic fluorine in a sample. It is useful to compare the results from a TOF assay with how much PFAS was detected in a targeted method, such as EPA 537. This comparison can demonstrate that there are many unknown fluorinated chemicals present in a sample and that the targeted method may be insufficient to characterize all the PFAS.[5]

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[5] A limitation to note is that the TOF assay may detect fluorinated pharmaceuticals or herbicides; this could be problematic when testing wastewater or biosolids for PFAS from industrial sources.

As evidence for the importance of aggregate fluorine analytical methods, the EPA published a draft method in April 2022 for measuring adsorbable organic fluorine (AOF) in water matrices. This Draft Method 1621 screens for organofluorines in wastewater (USEPA, 2022a).

6.2.2 Animal Models

As reflected in Chapter 4, current animal models for PFAS can have significant limitations. In humans, PFAS such as PFOA and PFOS have elimination half-lives on the order of years; however, in animals, the half-lives are much shorter (Kudo *et al.*, 2006; Kudo *et al.*, 2001; Ohmori *et al.*, 2003; Russell *et al.*, 2015b). Further study of this discrepancy between human and animal toxicokinetics should be a priority (Table 18).

Table 18: Comparison of PFAS elimination half-lives in humans and animals

Analyte	Human Half-Life	Animal Half-Life	References
PFBA	3 days		(Chang *et al.*, 2008)
PFHxA	14 – 49 days		(Russell *et al.*, 2013)
PFHpA	62 days – 1.5 years	0.05 – 0.10 days (~1 – 2.5 hours)	(Nilsson *et al.*, 2013; Russell *et al.*, 2015a; Xu *et al.*, 2020) (Ohmori *et al.*, 2003)
PFOA	2.1 – 8.0 years	▪ 0.08 – 6 days (rat) ▪ 2.7 – 5.6 days (macaque) ▪ 3 days (rabbit)	(Bartell *et al.*, 2010; Li *et al.*, 2018; Olsen *et al.*, 2007) (Gayrard *et al.*, 2021; Harada *et al.*, 2005; Ohmori *et al.*, 2003)
PFNA	2.5 – 4.3 years	2.4 – 30 days	(Zhang *et al.*, 2013b) (Ohmori *et al.*, 2003)
PFDA		40 – 59 days	(Ohmori *et al.*, 2003)
PFBS	28 – 44 days		(Olsen *et al.*, 2009; Xu *et al.*, 2020)
PFHxS	4.6 – 9.2 years		(Li *et al.*, 2018; Olsen *et al.*, 2007)
PFOS	3.1 – 5.8 years	▪ 200 days (monkey) ▪ 50 days (rabbit)	(Li *et al.*, 2018; Olsen *et al.*, 2007) (Gayrard *et al.*, 2021; Harada *et al.*, 2005)

Additionally, there are sex differences in the elimination of some PFAS. Human females typically have lower PFCA serum levels than human males, and in a similar fashion, female rats have faster renal elimination of PFCAs than male rats (Kudo *et al.*, 2001; Ohmori *et al.*, 2003; Weaver *et al.*, 2010). Organic anion transporters (OAT) are key to this renal elimination of PFCAs. In rats, Oat1 and Oat3 are involved in renal secretion for PFHpA, PFOA, and PFNA, and Oatp1a1[6] is involved in reabsorption of PFOA,

[6] Oatp: organic anion transporting polypeptide. OAT1 and OAT3 are basolateral, and rat Oatp1a1 is apical. Most simply, basolateral transporters in the kidney move compounds from blood to the renal lumen for excretion, and apical transporters in the kidney move compounds from the renal lumen back to the blood. for reabsorption. OAT1 is also expressed in the brain and placenta. The human ortholog to rat Oatp1a1 is OATP1A2.

PFNA, and PFDA (Weaver *et al.*, 2010). However, other mechanisms for this sexual dimorphism may be yet to be elucidated.

6.2.3 Occurrence Data

While it is acknowledged that PFAS can be detected nearly everywhere, there are still gaps in our understanding of PFAS occurrence. For example, exactly which PFAS analytes are present and at what concentrations? PFAS levels can be high at sites with AFFF contamination or industrial pollution, but what about PFAS levels in the average American's or average North Carolinian's drinking water?

To help answer some of these questions, the USEPA will be generating occurrence data for 29 PFAS analytes in drinking water as part of their fifth Unregulated Contaminant Monitoring Rule (UCMR 5). The USEPA will mandate nationwide testing for PFAS of nearly all water utilities[7] in the United States from January 2023 to December 2025. The UCMR program generates data on chemicals that are not currently regulated in drinking water (e.g., contaminant occurrence, frequency, concentration). These data can be used to support the introduction of federal regulations for drinking water. Typically, the UCMR program monitors several different types of unregulated contaminants. However, UCMR 5 will focus almost exclusively on PFAS – of the 30

[7] All public water systems that serve more than 10,000 people will monitor for PFAS. All public water systems serving 3,300-10,000 people will monitor for PFAS, if appropriations and laboratory capacity allow. A representative sample of 800 smaller public water systems (serving fewer than 3,300 people) will also monitor for PFAS, if appropriations and laboratory capacity allow.

contaminants to be monitored, 29 of them are PFAS chemicals.[8] The UCMR 5 will generate data for many PFAS included in this dissertation, including PFBA, PFBS, PFHxA, PFHxS, PFHpA, PFOA, PFOS, PFNA, PFDA, and GenX.

Previously, the third UCMR (UCMR 3) monitored for six PFAAs[9] in drinking water from January 2013 to December 2015 (USEPA, 2012). However, the minimum reporting levels for these analytes was relatively high at 10 to 90 ng/L or parts per trillion (ppt). The UCMR 5 will have minimum reporting levels that are an order of magnitude lower at 2 to 8 ng/L[10] (USEPA, 2021b).

Though useful, the USEPA's UCMR program is limited because it only monitors public water systems; private wells and water systems are not subject to monitoring. Approximately 43 million people, or 13% of the U.S. population, were on a private water system for drinking water according to the U.S. Geological Survey in 2015 (Dieter *et al.*, 2018). This data gap for private well water concentration data is a particular problem for North Carolina. North Carolina has the second-largest state population[11] that relies on private wells – approximately 3.3 million residents or a third of the state population (MacDonald Gibson & Pieper, 2017; Maupin *et al.*, 2014). Though surface water

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[8] Lithium is the only non-PFAS contaminant that will be included in UCMR 5. A maximum of 30 contaminants are allowed to be monitored for each UCMR cycle.
[9] These 6 PFAAs were: PFBS, PFHxS, PFHpA, PFOA, PFOS, and PFNA.
[10] One PFAS analyte (NFDHA) has a higher minimum reporting limit of 20 ng/L or 0.02 μg/L.
[11] Only the state of Pennsylvania has a larger population on private water.

contamination with PFAS has been well-documented in North Carolina, less is known about PFAS contamination of its groundwater overall. Areas near known sites of contamination such as the DuPont Chemours Fayetteville Works plant have been tested, and thousands of wells were found to have high levels of groundwater PFAS due to this industrial pollution (NCDEQ, 2022).[12] Many more private wells could have PFAS contamination, but they are untested.

Additionally, many water utilities serving less than 800 people, and possibly some serving 3,300 people, will not be tested for PFAS at all during UCMR 5. Of the over 50,000 community water systems that provide water year-round in the U.S., about 43,000 or ~84% serve 3,300 or fewer people (GWI, 2009); more than 97% of the 156,000 public water systems serve 10,000 or fewer people (USEPA, 2021d). Smaller utilities have also previously been found to have more concerning PFAS contamination. Though PFAS were detected less frequently at smaller utilities during the UCMR 3, when PFAS were detected, the smaller utilities had higher median levels of PFAS than the larger utilities (Guelfo & Adamson, 2018). Statewide monitoring programs that included small utilities

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[12] NCDEQ says as of March 2022, 8,404 wells have been sampled near the Chemours Fayetteville Works site. Of those, 245 residences sampled had levels of GenX at or above 140 ppt, and 5,605 residences had either a total sum of 12 PFAS ≥ 70 ppt or any individual PFAS ≥ 10 ppt. The 12 PFAS tested are mostly emerging PFAS and PFEAs, and the only PFAA tested is PFHpA. The list of 12 PFAS tested at the residential wells can be found in Attachment C of the February 2019 Consent Order. "Attachment 3: Table 3+ SOP Compounds" of the October 2020 Addendum to Consent Order has a different list of 20 PFAS that are apparently attributable to Chemours operations, but this list does not appear to pertain to the residential well testing.

have uncovered issues with PFAS contamination that may have gone undetected otherwise. For example, monitoring by the NC PFAS Testing Network revealed that the small community of Maysville, NC had extensive PFAS contamination in its only drinking water well due to firefighting foam (NC PFAS Testing Network, 2019, 2021).

While the UCMR 5 will provide valuable PFAS occurrence data, there will still be extensive data gaps for tens of thousands of smaller water systems, especially in North Carolina.

6.2.4 Treatment Technologies and Cleaning Up PFAS Pollution

PFAS are pervasive in the environment and will continue to be for the foreseeable future. Their presence in people, water, wastewater, biosolids, and landfill leachate ensures that PFAS will continue to circulate in our environment even if we stopped the production of new PFAS. An important science and policy question has arisen at sites now polluted with PFAS: how do we begin to clean up this mess when there are thousands of PFAS and no proven options for effective PFAS destruction?

Many sites are contaminated with PFAS. AFFF currently contaminates the soil and water of many military sites. Wastewater treatment plants will have to wrestle with where to put their PFAS-laden biosolids (e.g., landfills or agricultural fields). Water treatment plants will also have to explore how to best remove PFAS from their influent as drinking water regulations for PFAS are implemented. While research is ongoing, there are currently no effective technologies for treating PFAS in water at scale.

Prevention of PFAS contamination by addressing upstream polluters could be one solution to implement, in addition to trying to remediate the already-present PFAS contamination.

Land application of municipal biosolids to agricultural fields is a known source of PFAS to the environment (Lindstrom *et al.*, 2011; Sepulvado *et al.*, 2011) and may be relevant to some of the PFAS contamination in the Haw River (Pétré *et al.*, 2022). Remediation techniques such as biosparging[13] (Nickerson *et al.*, 2021), bioremediation (Shahsavari *et al.*, 2020), persulfate oxidation or heat activated persulfate pretreatment (Shojaei *et al.*, 2021), and electrochemical treatment (Hou *et al.*, 2021) are being explored to recover and destroy PFAS at contaminated sites (Leung *et al.*, 2022).

6.3 Broader Implications: Current and Future Policy on PFAS

Currently as of May 2022, there are relatively few formal restrictions on PFAS in the United States. For drinking water, the USEPA has established a Health Advisory Level (HAL) for the combination of PFOS and PFOA at 70 ppt. Some states are going further and establishing enforceable maximum contaminant levels (MCLs) for their states' drinking water for PFOS, PFOA, GenX, and additional PFAAs, usually at levels lower than the 70 ppt.

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[13] Injecting oxygen and using indigenous aerobic microorganisms to remediate contaminants *in situ.*

The USEPA's PFAS Action Plan suggests that MCLs for PFOA and PFOS may be forthcoming, which would mark the first federal regulatory action on PFAS in drinking water. Additionally, the fifth cycle of the federal Unregulated Contaminant Monitoring Rule (UCMR 5) is almost exclusively focused on PFAS as 29 of the 30 chemicals to be monitored in drinking water are PFAS. This highlights the nationwide importance and relevance of the issue of PFAS in drinking water, notwithstanding the slow regulatory progress. The USEPA is similarly making progress on PFOA and PFOS in freshwater and set draft aquatic life ambient water quality criteria at 8.4 μg/L for PFOS and 94 μg/L for PFOA in April 2022 (USEPA, 2022b).

Some states are developing PFAS policies in areas besides drinking water. States are banning PFAS from use in food containers or food packaging, or proposing to ban the entire PFAS class from consumer products (Balan *et al.*, 2021). Manufacturers and corporations are also beginning to address PFAS in their products by committing to eliminating intentionally-added PFAS from their products and supply chains (Cousins *et al.*, 2019a). Manufacturers may also be responding to updates to the Toxics Release Inventory (TRI) program, a reporting program that monitors releases of certain toxic chemicals to the environment. In 2020, 172 PFAS were initially added to the TRI toxic chemical list, and eight more PFAS have been added since.

On the international front, some PFAS are being regulated by the Stockholm Convention. The Stockholm Convention on Persistent Organic Pollutants (POPs)[14] is a global environmental treaty to eliminate and restrict the use of POPs. Under the Stockholm Convention, pollutants are listed in various annexes, including Annex A for elimination and Annex B for restriction. PFOA was listed in Annex A in 2019 and PFOS was listed in Annex B in 2009, both with exceptions for some specified uses (Stockholm Convention, 2009, 2019a, 2019b).

Current regulatory policy on PFAS is rather piecemeal, focusing on only a few selected PFAS or subclasses. Some U.S. states have set maximum contaminant levels (MCLs) for a handful of PFAAs in drinking water. However, even a subclass approach may be inadequate. Regulating the PFAA subclass or regulating long-chain PFAS may be advisable given the known persistence and toxicity of some PFAAs, but many other PFAS are eventual precursors for PFAAs or may be toxic themselves. Regulating long-chain PFAS and transitioning to shorter-chain PFAS may be a form of "regrettable substitution" in which we replace known hazardous chemicals with very similar chemicals of unknown hazard (Maertens *et al.*, 2021; Scheringer *et al.*, 2014). Some groups have proposed that since thoroughly studying all 12,000 PFAS chemicals in a reasonable time frame is an impossible task, we should pivot to a more feasible option

[14] The United States is a signatory but not a party to the Stockholm Convention; the U.S. has never ratified the treaty since signing it in 2001.

like regulating PFAS as an entire class (Balan *et al.*, 2021; Cordner *et al.*, 2016; Kwiatkowski *et al.*, 2020) or regulating based on chemical persistence (Cousins *et al.*, 2020; Cousins *et al.*, 2019c).

An added nuance to this regulatory debate: what precisely is a PFAS? There are at least nine different definitions for what constitutes a PFAS, and broader definitions of PFAS can include widely-used fluorinated pharmaceuticals (e.g., atorvastatin, fluoxetine) (Hammel *et al.*, 2022). The USEPA Office of Pollution Prevention and Toxics (OPPT) defines a PFAS as a chemical with saturated carbon-carbon bonds that contains "R–(CF_2)–C(F)(R′)(R″)…and none of the R groups (R, R′, and R″) can be hydrogen" (USEPA, 2021c). Other definitions for PFAS could include all organofluorines (i.e., a –C–F) or all chemicals with a fully-fluorinated carbon (i.e., a –CF_3).[15]

A staggering number of resources and suggestions have been put forth by scientists to tackle the issue of PFAS, such as the Helsingør Statement (Scheringer *et al.*, 2014), the Madrid Statement (Blum *et al.*, 2015), and the Zürich Statement (Ritscher *et al.*, 2018) and others (Birnbaum & Grandjean, 2015; Cordner *et al.*, 2021; Ng *et al.*, 2021). The essential use concept is a frequently shared recommendation. Some uses of PFAS can be considered non-essential (Cousins *et al.*, 2021; Cousins *et al.*, 2019b), and limiting PFAS to

[15] See Wang *et al.* (2021) and OECD (2021) for full clarification on the revised OECD PFAS definition regarding what is considered a "fully-fluorinated carbon" and what exceptions are considered.

only essential uses can help reduce our collective exposure to potentially harmful PFAS. A summary of the most frequent general recommendations is included in Figure 29.

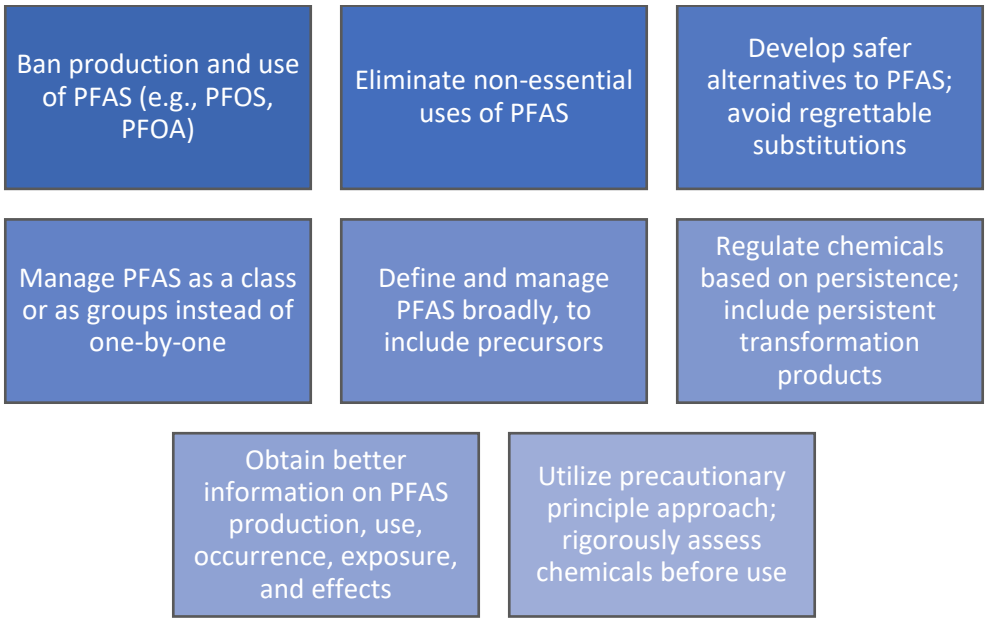


Figure 29: Summary of common strategies and recommendations put forth in the literature for preventing and reducing further PFAS contamination.

6.4 Final Insights

Per- and polyfluoroalkyl substances are not the first group of persistent chemicals to cause serious harm to human and environmental health. From dioxins to DDT to CFCs to PCBs to PBDEs, we have historically failed to anticipate and address the risks of the chemicals we put out into our products and environments. However, we can also look to these previous environmental health crises for solutions to the current PFAS pollution problem. Many of the strategies previously used to reduce, replace, and eliminate other harmful chemicals can be adapted to PFAS. Given the unprecedented

number and variety of PFAS and their extensive, sweeping presence and effects on our environment and our health, it will take a multipronged approach by many stakeholders to overcome PFAS pollution.

Appendix A

Appendix A contains supporting information for Chapter 2, published under the title, "Concentrations of per- and polyfluoroalkyl substances (PFAS) in human placental tissues and associations with birth outcomes," in *Chemosphere* in 2022. It is reprinted with permission from Hall, S. M.; Zhang, S.; Hoffman, K.; Miranda, M.L.; Stapleton, H. M. Concentrations of per- and polyfluoroalkyl substances (PFAS) in human placental tissues and associations with birth outcomes. *Chemosphere.* 2022, 295, 133873. DOI: 10.1016/j.chemosphere.2022.133873. Copyright 2022 Elsevier.

Additional Methods

Anonymous Placenta Samples Collected in 2018

Placenta samples described in Table A7 were collected from Duke University Medical Center in 2018 from women who underwent planned cesarean deliveries (indicated for reasons such as previous cesarean section). All births were singleton and had no known complications. These placenta samples were collected anonymously for the sole goal of quantifying contaminants in the placenta. All protocols were approved by the Duke University Medical Center Institutional Review Board prior to study initiation. Sections of the placenta were excised from the maternal side and from the fetal side using a scalpel, immediately after delivery of the placenta. The placental area within one centimeter around the umbilical cord, outer edge of placenta, and innermost part of the placenta were discarded at collection time in order to ensure accurate maternal and fetal

placenta sectioning. Placenta sections (approximately 2-3 g) were stored in amber glass jars at –20 °C and were processed for PFAS extraction as described in main paper.

Table A1: List of PFAS analytes measured in placenta

Analyte	Name	CAS Number
PFBA	Perfluorobutanoic acid	375-22-4
PFPeA	Perfluoropentanoic acid	2706-90-3
PFHxA	Perfluorohexanoic acid	307-24-4
PFHpA	Perfluoroheptanoic acid	375-85-9
PFOA	Perfluorooctanoic acid	335-67-1
PFNA	Perfluorononanoic acid	375-95-1
PFDA	Perfluorodecanoic acid	335-76-2
PFBS	Perfluorobutane sulfonic acid	375-73-5
PFHxS	Perfluorohexane sulfonic acid	355-46-4
PFOS	Perfluorooctane sulfonic acid	1763-23-1
GenX (also HFPO-DA)	Hexafluoropropylene oxide dimer acid or 2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-propanoic acid	13252-13-6; 62037-80-3

Table A2: Tandem mass spectrometry (MS/MS) parameters. Fragmentor voltage (Frag), collision energy (CE), retention time (RT) in minutes, and transitions (precursor and product ions) for quantifying PFAS in placenta samples. Analyses were performed with ESI in negative ion mode, using the multiple reaction monitoring (MRM) mode.

Analyte	Transition	Frag (V)	CE (V)	RT (min)	Mass-Labeled Internal Standard†	Transition	Frag (V)	CE (V)	RT (min)
PFBA	213.0 > 168.9	48.0	6.0	2.021	MPFBA	217.0 > 172.0	56.0	6.0	2.020
PFBS	298.9 > 98.9	136.0	38.0	4.169	MPFHxS	402.9 > 83.9	164.0	54.0	5.053
PFPeA	263.0 > 218.9	60.0	6.0	3.910	MPFHxA	315.0 > 269.9	52.0	6.0	4.677
PFHxA	313.0 > 268.9	48.0	6.0	4.677	MPFHxA	315.0 > 269.9	52.0	6.0	4.677
PFHxS	398.9 > 98.9	160.0	42.0	5.068	MPFHxS	402.9 > 83.9	164.0	54.0	5.053
PFHpA	363.0 > 318.9	60.0	6.0	5.040	MPFOA	417.0 > 371.9	60.0	6.0	5.328
PFOA	413.0 > 368.9* 413.0 > 168.9	64.0	6.0; 18.0	5.329	MPFOA	417.0 > 371.9	60.0	6.0	5.328
PFOS	498.9 > 98.9* 498.9 > 79.9	200.0	55.0	5.559	MPFOS	502.9 > 80.0	192.0	54.0	5.573
PFNA	463.0 > 418.9	68.0	6.0	5.575	MPFNA	468.0 > 422.9	60.0	6.0	5.589
PFDA	513.0 > 468.9	68.0	6.0	5.805	MPFDA	515.0 > 470.0	48.0	6.0	5.804
GenX	284.9 > 168.9* 284.9 > 118.9	144.0	6.0; 30.0	4.796	M3-GenX	286.9 > 168.9* 286.9 > 118.9	148.0	6.0; 30.0	4.795
					M2-PFOA	414.8 > 369.9	64.0	6.0	5.343

†all analytical standards sourced from Wellington Laboratories

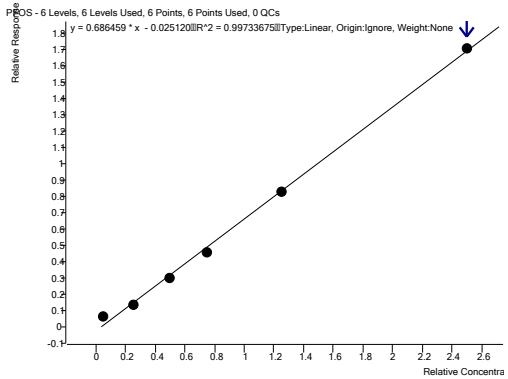
*quantitation ions for analytes with multiple transitions

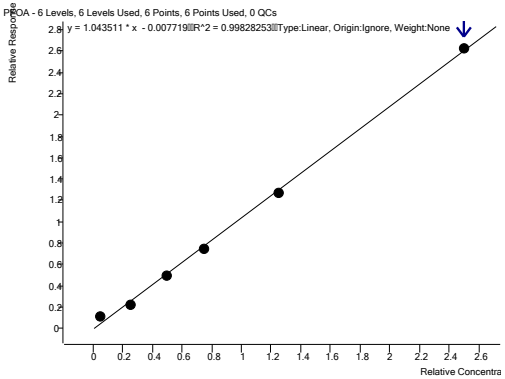
Table A3: LC and source parameters

LC Parameters	Value
Injection Volume	20 µL
Flow Rate	0.4 mL/min
Column Oven Temperature	45 °C
Source Parameters	**Value**
Gas Temp	250 °C
Gas Flow	8 L/min
Nebulizer	25 psi
Sheath Gas Heater Temp	350 °C
Sheath Gas Flow	12 L/min
Capillary – Negative	3,000 V
V Charging	0

Table A4: Calibration curves for PFOS, PFOA, PFNA, and PFDA. The linear range for all analytes was 1 to 50 ng. Curves represent response of analyte to its own mass-labeled isotope (MPFOS, MPFOA, MPFNA, and MPFDA, respectively).

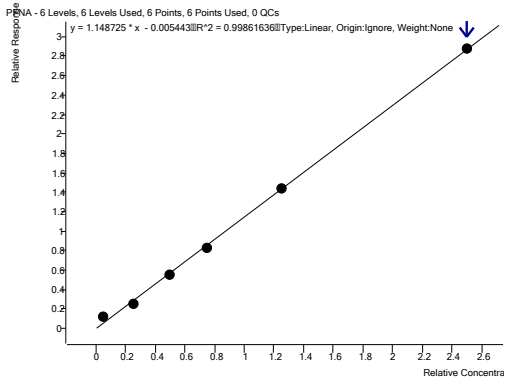
PFOS

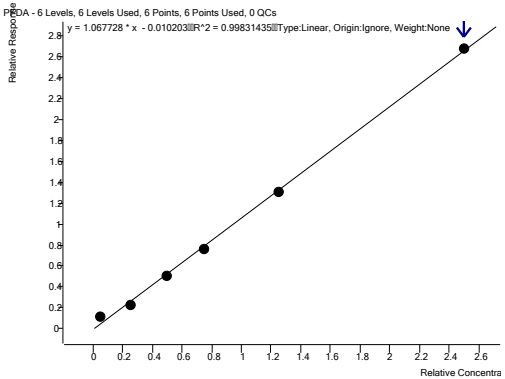




PFOA

PFNA





PFDA

Table A5: Percent recoveries of internal standards

Internal Standard	Average Percent Recovery (n=125[†])
MPFBA	72.0%
MPFHxA	69.2%
MPFHxS	56.4%
MPFOA	76.3%
MPFOS	62.1%
MPFNA	81.7%
MPFDA	61.5%
M3-GenX	132.5%

[†]5 placenta samples could not be associated with medical record data and were excluded from further analysis

Table A6: Average concentrations (ng/g) in NIST Standard Reference Material (SRM) 1947 Lake Michigan fish tissue and relative standard deviation (RSD) along with reference values and method detection limit (MDL)

Analyte	**MDL** (ng/g)	**SRM 1947** (n=7)	**SRM 1947 RSD** (%)	**SRM 1947 Reference Values**†	**Percent of NIST Value** (%)
PFOS	0.01	5.44	3.2%	5.90 ± 0.39†	92%
PFNA	0.01	0.20	4.1%	0.20† 0.18 ± 0.01**	100%
PFDA	0.01	0.25	5.3%	0.26† 0.28 ± 0.06**	96%
PFOA	0.02	0.15	4.2%	<0.297** <0.676**	N/A

†reference and information values as reported on the NIST Certificate of Analysis for SRM 1947 (NIST, 2017)
**values as reported in Reiner *et al.* (2012)
N/A: values are not reported in the NIST Certificate of Analysis

Table A7: PFAS concentrations observed in more recently collected human placenta samples (n=10 singleton pregnancies). Maternal and fetal portions of placenta were extracted separately for placental samples from 2018. Concentrations (ng/g wet weight placenta) reported are from the fetal section of placenta, and no differences were seen between concentrations in maternal and fetal placenta. Detection frequencies (DF) are also reported. Data are juxtaposed with PFAS concentration data from earlier samples described in main paper.

	2018 (n=10)			**2010-2011 (n=120)**			
Analyte	**DF**	**MDL** (ng/g)	**Placental Concentration Range** (ng/g)	**DF**	**MDL** (ng/g)	**Median Placental Concentration** (ng/g)	**Maximum Placental Concentration** (ng/g)
PFOS	40%	0.22	0.55 – 0.73	99%	0.01	0.95	7.2
PFOA	20%	0.06	0.15 – 0.23	98%	0.02	0.27	1.6
PFHxS	40%	0.07	0.19 – 0.22	19%	0.17	<MDL	0.5
PFNA	10%	0.04	0.17	100%	0.01	0.11	0.6
PFDA	0%	0.09	Not detected	96%	0.01	0.06	0.3

Table A8: Summary of placenta PFAS concentrations (ng/g) reported in the published literature and Hall *et al.* (2022).

Reference	Year of Sample Collection	Location	Sample Size (n)	Value	PFOS	PFOA	PFNA	PFDA	PFHxS
Zhang *et al.* (2013a)	2010	China	n = 29	Median	7.32	1.41	0.96	0.67	0.36
Martin *et al.* (2016)[1]	Unknown	Spain	n = 25	Range	<LOQ – 1.2	<LOQ – 0.37	-	-	-
Chen *et al.* (2017)	2015-2016	China	n = 32	Median	2.42	0.46	-	-	0.21
Mamsen *et al.* (2017)[2]	2014-2015	Denmark	n = 34	Mean	1.30	0.23	0.14	0.1	-
Mamsen *et al.* (2019)[3]	2015-2016	Sweden	n = 21	Median	1.42	0.36	0.17	0.24	-
Mamsen *et al.* (2019)[4]	2014-2016	Sweden & Denmark	n = 78	Median	1.24	0.30	0.15	0.18	-
Bangma *et al.* (2020a)	2015-2018	North Carolina, United States	n = 122	Median	0.48	<0.32	<0.16	<0.03	0.07
Vela-Soria *et al.* (2021)	Unknown	Spain	n = 20	Median	0.60	0.14	0.06	0.03	0.05
Lu *et al.* (2021)	2016	China	n = 54	Median	0.35	0.18	0.06	-	0.08
Hall *et al.* (2022)	2010-2011	North Carolina, United States	n = 120	Median	0.95	0.27	0.11	0.06	-

[1] Ranges reported in lieu of medians because very few samples had quantified concentrations.
[2] No median values were reported.
[3] Only placentae from third-trimester pregnancies are included.
[4] Placenta samples from all pregnancies are included.
Blank values were either not reported, below limit of quantification (<LOQ), or <MDL.

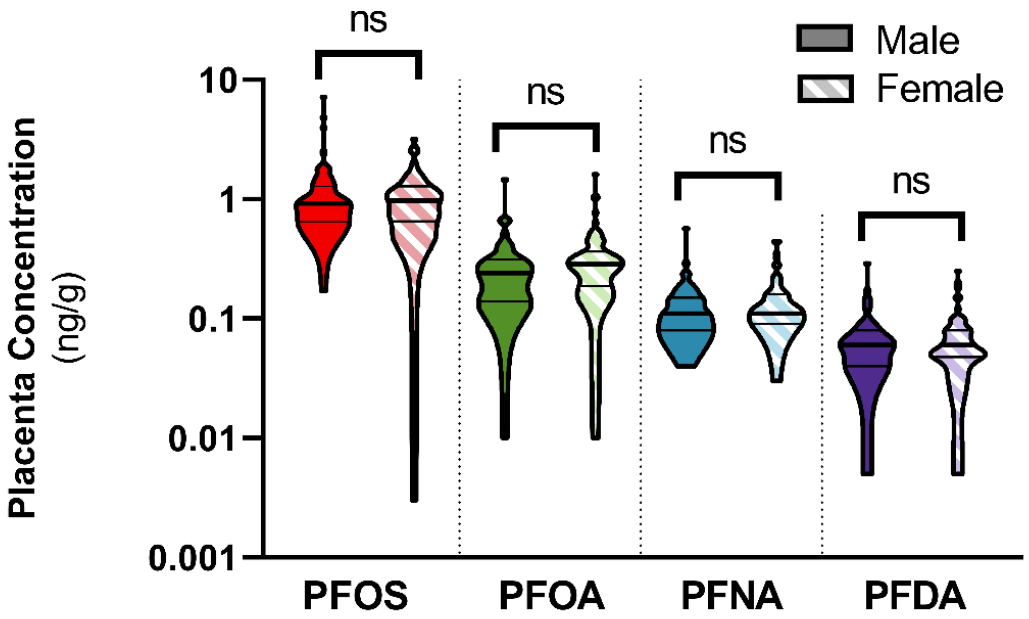


Figure A1: Placental PFAS concentrations (ng/g) stratified by infant sex; differences between sexes were not statistically significant (Mann-Whitney test, n=54 males, n=66 females, n=120 total). Violin plots show the distribution of concentration data with thick solid lines and thin solid lines demarcating median and quartiles, respectively. Concentrations are plotted on a logarithmic scale. ns, not significant

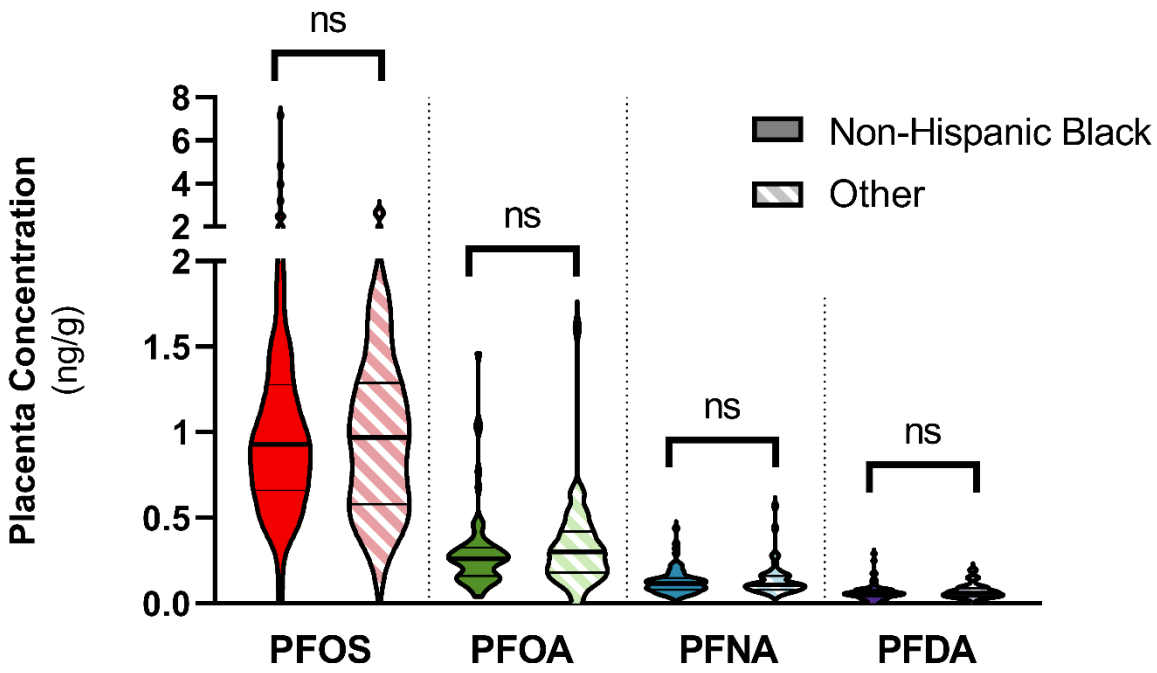


Figure A2: Maternal race and placental PFAS concentrations (ng/g). Maternal race was dichotomized into non-Hispanic black (n=72) and other (n=47). There were no significant differences between these two groups (Mann-Whitney test). Further analysis with four racial and ethnic groups (non-Hispanic white, non-Hispanic black, Hispanic, and other) was also not statistically significant (Kruskal-Wallis test). Violin plots show the distribution of concentration data with thick solid lines and thin solid lines demarcating median and quartiles, respectively. ns, not significant

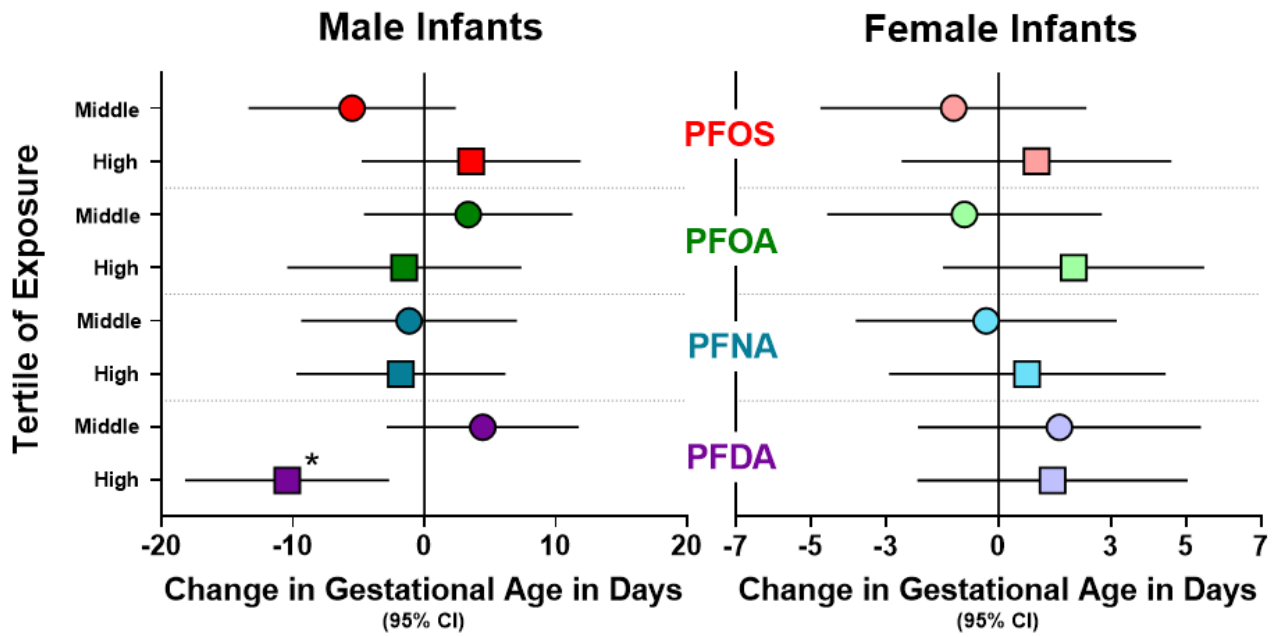


Figure A3: Gestational Age - Results for regression models of gestational age stratified by male and female infants. Analyses were performed using tertiles of placenta PFAS exposure with the lowest tertile as the reference group and were adjusted for maternal tobacco use, race, age, and parity. Horizontal bars reflect the 95% confidence interval, *indicates p<0.05.

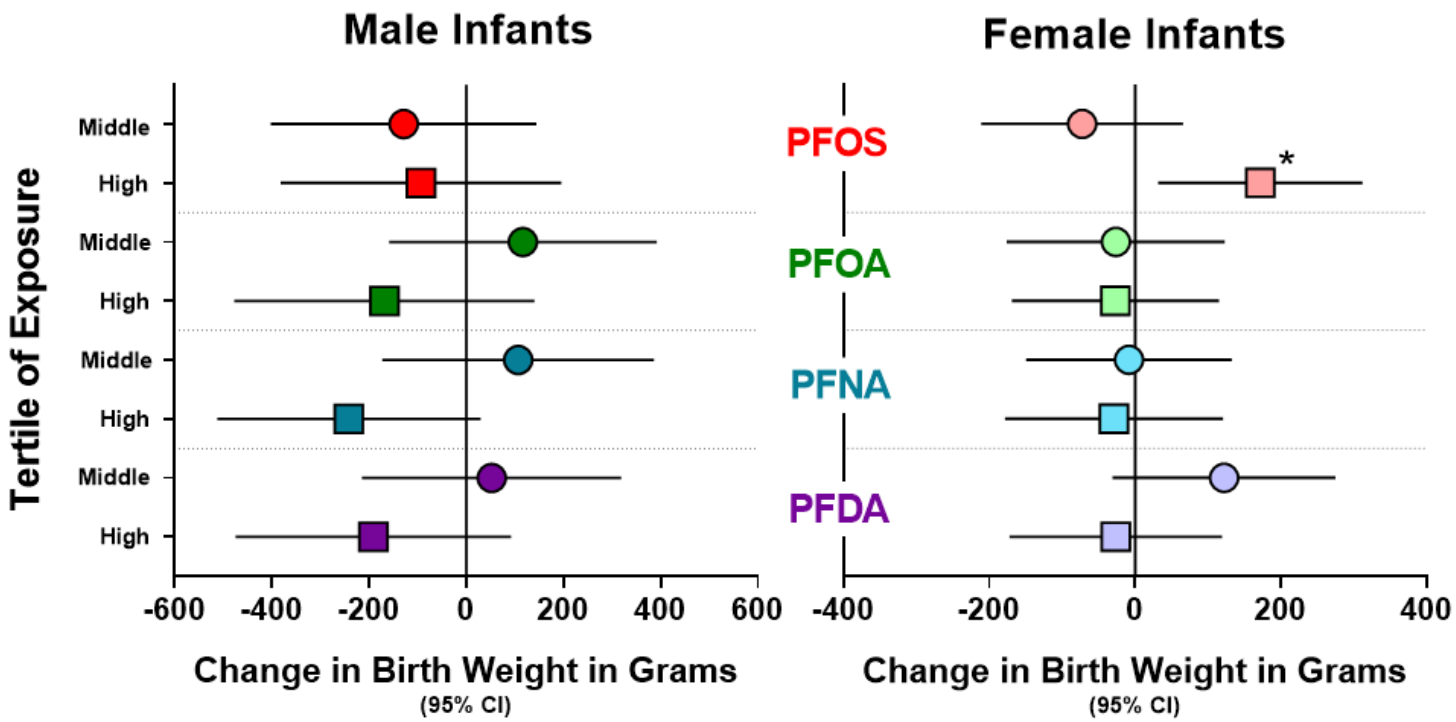


Figure A4: Absolute Birth Weight - Results for regression models for absolute birth weight stratified by male and female infants. Analyses were performed using tertiles of placenta PFAS exposure with the lowest tertile as the reference group and were adjusted for maternal tobacco use, race, age, and parity. Horizontal bars reflect the 95% confidence interval, *indicates p<0.05

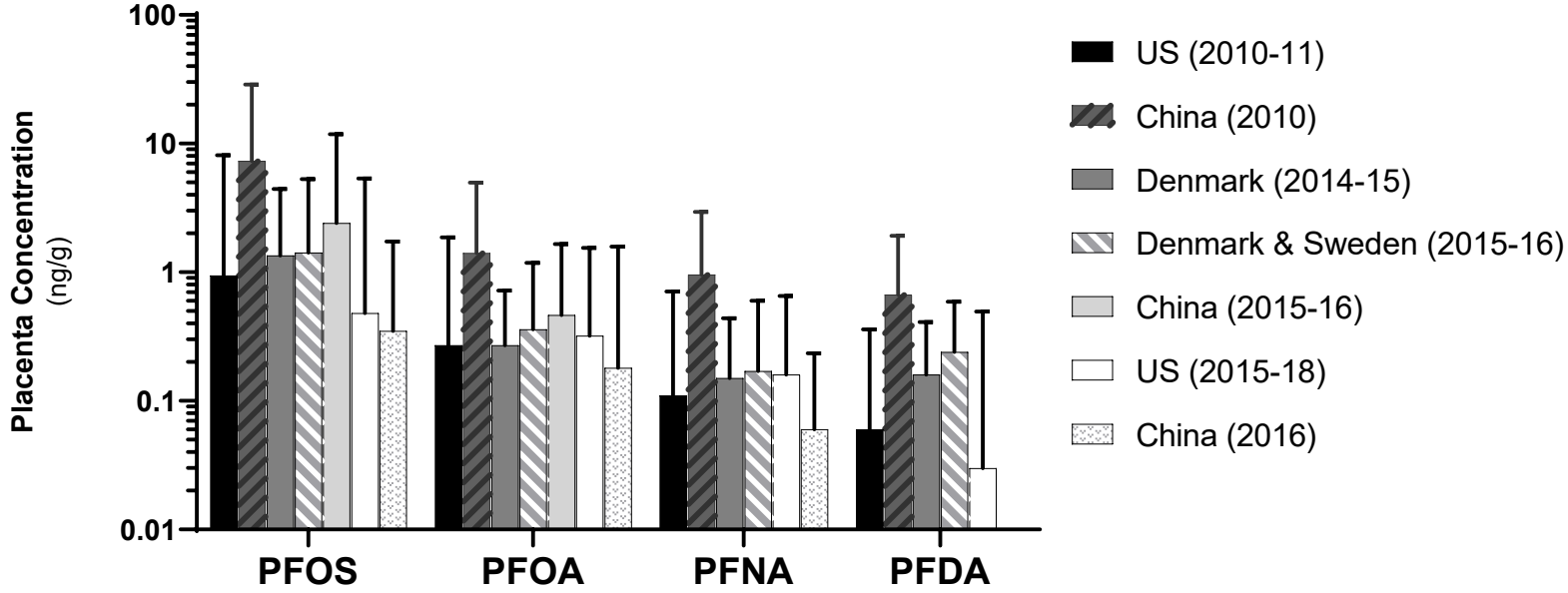


Figure A5: Median and maximum concentrations of PFAS (ng/g) in human placenta in Hall *et al.* (2022) and the literature. This plot includes the most frequently reported PFAS in placenta: PFOS, PFOA, PFNA, and PFDA. Concentrations are in ng/g wet weight placenta. Studies are referenced with their year of sample collection and study location. Studies referenced are cited in Table A8 along with information on study sample size. Means are reported in lieu of medians for the values from Denmark in 2014-2015 as Mamsen *et al.* (2017) did not report median concentrations.

Table A9: Image of the Supplemental Excel File. Excel spreadsheet contains the sensitivity analysis comparing models including or excluding preterm births. The Excel file as originally published is available at the publisher's website at DOI: 10.1016/j.chemosphere.2022.133873.

	Female (Full Model, n=120)			Female (Restricted Model, n=108)			Male (Full Model, n=120)			Male (Restricted Model, n=108)		
BIRTH WEIGHT PERCENTILE (%)												
Tertile	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence**	***p* Value**
High PFOS	10.7	(2.79, 18.7)	**0.01**	12.1	(3.58, 20.57)	**0.01**	-12.5	(-23.42, -1.64)	**0.03**	-8.8	(-20.98, 3.33)	**0.15**
Middle PFOS	-2.0	(-9.83, 5.9)	0.62	-1.9	(-10.33, 6.45)	0.65	3.5	(-6.82, 13.82)	0.50	2.1	(-9.52, 13.73)	0.72
High PFOA	-4.1	(-12.22, 4.06)	0.32	-3.5	(-12.15, 5.19)	0.43	-9.2	(-21.01, 2.63)	0.13	-8.8	(-22.34, 4.68)	0.19
Middle PFOA	-0.7	(-9.26, 7.86)	0.87	-1.3	(-10.4, 7.8)	0.78	2.0	(-8.53, 12.54)	0.70	2.9	(-8.33, 14.04)	0.61
High PFDA	-4.4	(-12.78, 4.03)	0.30	-4.0	(-12.95, 5)	0.38	3.9	(-7.14, 14.85)	0.49	-2.8	(-16.08, 10.53)	0.68
Middle PFDA	7.3	(-1.53, 16.07)	0.10	7.0	(-2.46, 16.4)	0.14	-6.9	(-17.22, 3.47)	0.19	-2.4	(-13.7, 8.95)	0.68
High PFNA	-1.3	(-9.92, 7.33)	0.77	-1.4	(-10.63, 7.76)	0.76	-13.9	(-24.03, -3.86)	**0.01**	-14.2	(-26, -2.47)	**0.02**
Middle PFNA	-0.1	(-8.29, 8)	0.97	0.1	(-8.55, 8.72)	0.98	10.6	(0.15, 20.96)	**0.05**	11.1	(-0.09, 22.31)	**0.05**
GESTATIONAL AGE (days)												
Tertile	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence**	***p* Value**
High PFOS	1.0	(-2.58, 4.6)	0.58	-0.3	(-3.16, 2.5)	0.82	3.6	(-4.76, 11.88)	0.40	-1.6	(-4.94, 1.65)	0.32
Middle PFOS	-1.2	(-4.75, 2.34)	0.50	-0.3	(-3.12, 2.47)	0.82	-5.5	(-13.38, 2.39)	0.17	3.1	(-0.07, 6.23)	0.06
High PFOA	2.0	(-1.48, 5.48)	0.26	2.4	(-0.23, 5.07)	0.07	-1.5	(-10.44, 7.38)	0.73	0.1	(-3.66, 3.88)	0.95
Middle PFOA	-0.9	(-4.57, 2.75)	0.62	-1.1	(-3.86, 1.71)	0.44	3.3	(-4.61, 11.27)	0.40	0.8	(-2.33, 3.91)	0.61
High PFDA	1.4	(-2.16, 5.04)	0.43	0.6	(-2.22, 3.41)	0.67	-10.4	(-18.21, -2.68)	**0.01**	-1.8	(-5.42, 1.88)	**0.33**
Middle PFDA	1.6	(-2.15, 5.39)	0.39	1.0	(-1.92, 4)	0.49	4.4	(-2.87, 11.75)	0.23	0.3	(-2.8, 3.41)	0.84
High PFNA	0.8	(-2.92, 4.45)	0.68	2.2	(-0.64, 4.98)	0.13	-1.8	(-9.74, 6.17)	0.65	0.5	(-2.95, 3.92)	0.78
Middle PFNA	-0.3	(-3.81, 3.15)	0.85	-1.4	(-4.05, 1.22)	0.29	-1.2	(-9.37, 7.04)	0.78	-1.3	(-4.56, 1.99)	0.43
ABSOLUTE BIRTH WEIGHT (grams)												
Tertile	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence Interval**	***p* Value**	**Estimate**	**Confidence**	***p* Value**
High PFOS	171.8	(31.8, 311.75)	**0.02**	149.0	(17.64, 280.42)	**0.03**	-93.2	(-381.81, 195.47)	0.52	-177.7	(-389.74, 34.35)	0.10
Middle PFOS	-72.8	(-211.21, 65.58)	0.30	-50.4	(-180.14, 79.38)	0.44	-128.9	(-402.36, 144.51)	0.35	97.7	(-105.08, 300.44)	0.34
High PFOA	-27.0	(-169.28, 115.2)	0.71	-12.6	(-143.61, 118.44)	0.85	-168.4	(-477.5, 140.64)	0.28	-131.4	(-369.2, 106.5)	0.27
Middle PFOA	-26.6	(-176.22, 122.94)	0.72	-33.5	(-171.05, 103.96)	0.63	116.5	(-158.96, 391.9)	0.40	72.3	(-124.63, 269.3)	0.46
High PFDA	-26.6	(-172.52, 119.41)	0.72	-50.3	(-185.28, 84.72)	0.46	-191.3	(-474.72, 92.09)	0.18	-73.2	(-307.16, 160.78)	0.53
Middle PFDA	121.9	(-30.9, 274.8)	0.12	103.0	(-38.95, 244.89)	0.15	52.2	(-214.54, 318.92)	0.70	10.6	(-188.54, 209.82)	0.91
High PFNA	-28.9	(-178.4, 120.53)	0.70	8.3	(-129.67, 146.27)	0.90	-241.5	(-512.59, 29.51)	0.08	-187.1	(-400.06, 25.8)	0.08
Middle PFNA	-8.5	(-149.69, 132.65)	0.90	-38.0	(-167.52, 91.55)	0.56	106.9	(-172.69, 386.47)	0.45	123.7	(-79.1, 326.48)	0.23

Analyses adjusted for maternal tobacco use, race, age, and parity

Appendix B

Appendix B contains supporting information for Chapter 3.

Table B1: List of 13 PFAS analytes measured in water or blood serum. CAS registry numbers are listed. InChIKey identifiers for the acid form of the analyte are also listed. Acid, anion, and common salt forms of each chemical are listed.

Abbreviation	Analyte Name	Formula	CAS Numbers	InChIKey
PFBA	Perfluorobutanoic acid	C4HF7O2	375-22-4 45048-62-2	YPJUNDFVDDCYIH-UHFFFAOYAI
PFPeA	Perfluoropentanoic acid	C5HF9O2	2706-90-3 45167-47-3	CXZGQIAOTKWCDB-UHFFFAOYSA-N
PFHxA	Perfluorohexanoic acid	C6HF11O2	307-24-4 92612-52-7	PXUULQAPEKKVAH-UHFFFAOYSA-N
PFHpA	Perfluoroheptanoic acid	C7HF13O2	375-85-9 120885-29-2	ZWBAMYVPMDSJGQ-UHFFFAOYSA-N
PFOA	Perfluorooctanoic acid	C8HF15O2	335-67-1 45285-51-6	SNGREZUHAYWORS-UHFFFAOYAQ
PFNA	Perfluorononanoic acid	C9HF17O2	375-95-1 72007-68-2	UZUFPBIDKMEQEQ-UHFFFAOYSA-N
PFDA	Perfluorodecanoic acid	C10HF19O2	335-76-2 73829-36-4	PCIUEQPBYFRTEM-UHFFFAOYSA-N
PFBS	Perfluorobutane sulfonic acid	C4HF9SO3	375-73-5 45187-15-3 ★ 29420-49-3 ✪ 60453-92-1 ✧ 68259-10-9	JGTNAGYHADQMCM-UHFFFAOYSA-N

PFHxS	Perfluorohexane sulfonic acid	C6HF13SO3	355-46-4 108427-53-8 ★ 3871-99-6 ✪ 82382-12-5 ✧ 68259-08-5	QZHDEAJFRJCDMF-UHFFFAOYSA-N
PFOS	Perfluorooctane sulfonic acid	C8HF17SO3	1763-23-1 45298-90-6 ★ 2795-39-3 ✪ 4021-47-0 ✧ 29081-56-9	YFSUTJLHUFNCNZ-UHFFFAOYAS
GenX (also HFPO-DA)	Hexafluoropropylene oxide dimer acid; 2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-propanoic acid; or perfluoro-2-propoxypropanoic acid	C6HF11O3	13252-13-6 ✧ 62037-80-3	CSEBNABAWMZWIF-UHFFFAOYSA-N
4:2 FTS	4:2 fluorotelomer sulfonic acid, or 1*H*,1*H*,2*H*,2*H*-Perfluorohexane sulfonic acid	C6H5F9SO3	757124-72-4 ✪ 27619-93-8	TXGIGTRUEITPSC-UHFFFAOYSA-N
6:2 FTS	6:2 fluorotelomer sulfonic acid, or 1*H*,1*H*,2*H*,2*H*-Perfluorooctane sulfonic acid	C8H5F13SO3	27619-97-2 ✪ 27619-94-9	VIONGDJUYAYOPU-UHFFFAOYSA-N

★ CAS registry number for the corresponding potassium salt
✪ CAS registry number for the corresponding sodium salt
✧ CAS registry number for the corresponding ammonium salt

Table B2: Tandem mass spectrometry (MS/MS) parameters. Transitions (*m/z* for precursor and product ions) for quantifying PFAS in water or serum samples. Analyses were performed with ESI in negative ion mode, using the multiple reaction monitoring (MRM) mode.

Analyte	Transition	Mass-Labeled Internal Standard†	Transition
PFBA	213.0 > 168.9	$^{13}C_4$-PFBA or MPFBA	217.0 > 172.0
PFPeA*	263.0 > 218.9	$^{13}C_2$-PFHxA or MPFHxA	315.0 > 269.9
PFHxA	313.0 > 268.9	$^{13}C_2$-PFHxA or MPFHxA	315.0 > 269.9
PFHpA	363.0 > 318.9	$^{13}C_4$-PFOA, MPFOA, or M4-PFOA	417.0 > 371.9
PFOA	413.0 > 368.9	$^{13}C_4$-PFOA, MPFOA, or M4-PFOA	417.0 > 371.9
PFNA	463.0 > 418.9	$^{13}C_5$-PFNA or MPFNA	468.0 > 422.9
PFDA	513.0 > 468.9	$^{13}C_2$-PFDA or MPFDA	515.0 > 470.0
PFBS**	298.9 > 80.0 298.9 > 98.9	$^{13}C_3$-PFBS or M3-PFBS $^{18}O_2$-PFHxS, MPFHxS, or M4-PFHxS	301.9 > 80.0 402.9 > 83.9
PFHxS**	398.9 > 80.0 398.9 > 98.9	$^{18}O_2$-PFHxS, MPFHxS, or M4-PFHxS	402.9 > 83.9
PFOS**	498.9 > 79.9 498.9 > 98.9	$^{13}C_4$-PFOS or MPFOS	502.9 > 80.0
GenX (or HFPO-DA)	284.9 > 168.9	$^{13}C_3$-HFPO-DA ($^{13}C_3$-GenX) or M3-HFPO-DA (M3-GenX)	286.9 > 168.9
4:2 FTS**	327.0 > 307.0	$^{13}C_2$-4:2 FTS or M2-4:2 FTS $^{13}C_2$-6:2 FTS or M2-6:2 FTS	328.9 > 308.9 429.0 > 409.0
6:2 FTS	427.0 > 407.0	$^{13}C_2$-6:2 FTS or M2-6:2 FTS	429.0 > 409.0
		$^{13}C_2$-PFOA or M2-PFOA	414.8 > 369.9

†all analytical standards sourced from Wellington Laboratories
*PFPeA is not reported in blood due to analytical issues.
**For some analytes, blood and serum had different internal standards or transitions. Information relevant to only blood is formatted in red text, and information relevant to only water is formatted in blue text.
Blood serum was spiked with 10 ng each of MPFAC-MXA, M3-GenX, M3-PFBS, M2-4:2 FTS, and M2-6:2 FTS, and with 10 ng of M2-PFOA.
Water was spiked with 20 ng each of MPFAC-MXA, M3-GenX, and M2-6:2 FTS.

Table B3: LC and source parameters

LC Parameters	**Value**
Injection Volume	20 μL
Flow Rate	0.4 mL/min
Column Oven Temperature	45 °C
Source Parameters	**Value**
Gas Temp	250 °C
Gas Flow	8 L/min
Nebulizer	25 psi
Sheath Gas Heater Temp	350 °C
Sheath Gas Flow	12 L/min
Capillary – Negative	3,000 V
V Charging	0

Table B4: Average concentrations (ng/g) in NIST Standard Reference Material (SRM) 1958 fortified human blood serum and relative standard deviation (RSD) along with reference values and method detection limits (MDL). Data are reported in separate batches as (T1, T2) with n=3 per batch and total n=6.

Analyte	MDL (ng/g)	SRM 1958 (ng/g)	SRM 1958 RSD (%)	SRM 1958 Reference Values†	Percent of NIST Value (%)
PFOS	0.13, 0.15	19.7, 19.8	5.3%, 3.5%	16.6 ± 0.9† 17.0 ± 0.3**	118%, 119%
PFOA	0.06, 0.51	4.0, 3.7	2.6%, 4.3%	4.11 ± 0.17† 4.03 ± 0.21**	88%, 88%
PFNA	0.14, 0.33	0.6, 0.9	7.0%, 3.3%	0.66 ± 0.13† 0.553 ± 0.259**	82%, 132%
PFHxS	0.02, 0.01	2.7, 2.6	2.3%, 3.5%	2.66 ± 0.07† 2.63 ± 0.05**	101%, 98%
PFHxA	0.45, 0.36	0.41, 0.71	2.5%, 15.7%	<0.388**	N/A
PFHpA	0.43, 0.09	0.38, ND	5.6%, ND	0.180 ± 0.044**	N/A
PFDA	0.25, 0.29	0.37, 0.43	5.9%, 6.0%	0.353 ± 0.141**	N/A

†reference and information values as reported on the NIST Certificate of Analysis for SRM 1958 (NIST, 2018a)
**values as reported in Keller *et al.* (2010)
N/A: not applicable; values are not reported in the NIST Certificate of Analysis
ND: not detected

Table B5: Percent recoveries of mass-labeled internal standards

Internal Standard	Average Percent Recovery			
	Serum Samples, T1 (n=48)	Serum Samples, T2 (n=44)	Water Samples, T1 (n=48)	Water Samples, T2 (n=44)
$^{13}C_4$-PFBA	51.9%	75.6%	64.9%	985.7%
$^{13}C_2$-PFHxA	63.6%	94.8%	89.7%	555.0%
$^{13}C_4$ -PFOA	66.6%	87.4%	96.6%	134.5%
$^{13}C_5$-PFNA	45.6%	51.4%	84.1%	110.5%
$^{13}C_2$-PFDA	31.4%	36.1%	59.3%	74.0%
$^{13}C_3$-PFBS	114.2%	107.0%	not used	not used
$^{18}O_2$ -PFHxS	107.0%	82.5%	91.0%	98.9%
$^{13}C_4$-PFOS	39.9%	32.3%	69.1%	36.8%
$^{13}C_3$-HFPO-DA ($^{13}C_3$-GenX)	218.2%	168.9%	129.0%	8576.8%
$^{13}C_2$-4:2 FTS	163.9%	153.6%	not used	not used
$^{13}C_2$-6:2 FTS	156.8%	114.4%	126.0%	187.9%

Table B6: Regression analysis of serum PFAS predictors of health measures in blood serum. Models were adjusted for age and sex. Beta-coefficients (β), 95% confidence intervals (95% CI), and adjusted p-values (p) are reported. Significant results are formatted in bolded or red text for convenience. *p<0.05

		Serum PFAS																	
		PFHxA			PFHxS			PFOA			PFOS			PFNA			PFDA		
Outcome	**Units**	**β [1]**	**95% CI**	**p**	**β [1]**	**95% CI**	**p**	**β [1]**	**95% CI**	**p**	**β [1]**	**95% CI**	**p**	**β [1]**	**95% CI**	**p**	**β [1]**	**95% CI**	**p**
Total Cholesterol	mg/dL	−0.06	(−0.25, 0.12)	0.50	**0.41**	**(0.04, 0.77)**	***0.03**	**0.37**	**(0.004, 0.73)**	***0.048**	−0.001	(−0.48, 0.47)	0.99	0.04	(−0.38, 0.46)	0.84	0.32	(−0.35, 0.99)	0.35
HDL Cholesterol	mg/dL	−0.01	(−0.04, 0.03)	0.75	−0.04	(−0.15, 0.07)	0.48	−0.01	(−0.13, 0.11)	0.8915	−0.01	(−0.14, 0.11)	0.85	0.02	(−0.10, 0.14)	0.73	0.01	(−0.19, 0.21)	0.92
non-HDL Cholesterol	mg/dL	−0.05	(−0.23, 0.13)	0.55	**0.47**	**(0.09, 0.84)**	***0.02**	**0.39**	**(0.01, 0.76)**	***0.04**	0.01	(−0.47, 0.49)	0.97	0.04	(−0.38, 0.47)	0.84	0.36	(−0.33, 1.04)	0.30
ALP	IU/L	**−0.07**	**(−0.13, −0.02)**	***0.008**	0.12	(−0.03, 0.28)	0.12	0.08	(−0.08, 0.24)	0.32	0.18	(−0.002, 0.35)	0.05	0.04	(−0.12, 0.21)	0.61	−0.02	(−0.29, 0.26)	0.91
Total Protein	g/dL	−0.0003	(−0.002, 0.002)	0.75	−0.001	(−0.005, 0.002)	0.39	0.001	(−0.002, 0.004)	0.68	−0.004	(−0.01, 0.0003)	0.07	0.001	(−0.003, 0.004)	0.76	−0.002	(−0.01, 0.004)	0.49
Globulin	g/dL	−0.001	(−0.002, 0.001)	0.48	−0.001	(−0.003, 0.002)	0.71	0.001	(−0.002, 0.004)	0.40	−0.001	(−0.005, 0.002)	0.40	0.001	(−0.002, 0.004)	0.62	0.0002	(−0.005, 0.01)	0.93
BUN	mg/dL	0.002	(−0.02, 0.02)	0.80	−0.001	(−0.04, 0.04)	0.97	0.002	(−0.04, 0.04)	0.91	0.03	(−0.01, 0.08)	0.17	−0.004	(−0.05, 0.04)	0.87	0.04	(−0.04, 0.11)	0.34
BUN:Cre	unitless ratio	0.01	(−0.02, 0.03)	0.61	−0.02	(−0.06, 0.03)	0.43	−0.01	(−0.05, 0.03)	0.60	**0.06**	**(0.004, 0.11)**	***0.04**	−0.0002	(−0.05, 0.05)	0.99	0.06	(−0.01, 0.13)	0.11
eGFR	mL/min / 1.73	−0.01	(−0.05, 0.04)	0.83	−0.03	(−0.13, 0.06)	0.47	−0.03	(−0.12, 0.06)	0.48	0.05	(−0.06, 0.17)	0.36	−0.00003	(−0.1, 0.1)	0.99	0.11	(−0.06, 0.27)	0.20
Sodium	mmol/L	**−0.02**	**(−0.04, −0.01)**	***0.0005**	0.0003	(−0.02, 0.02)	0.98	−0.01	(−0.03, 0.01)	0.36	0.02	(−0.01, 0.05)	0.12	0.0001	(−0.02, 0.02)	0.99	−0.02	(−0.06, 0.01)	0.24
Potassium	mmol/L	−0.001	(−0.003, 0.001)	0.34	−0.001	(−0.004, 0.003)	0.67	−0.0005	(−0.004, 0.003)	0.77	−0.001	(−0.01, 0.003)	0.64	0.00005	(−0.004, 0.004)	0.98	0.003	(−0.003, 0.01)	0.35
Total Carbon Dioxide	mmol/L	**−0.01**	**(−0.02, −0.0002)**	***0.04**	0.004	(−0.01, 0.02)	0.46	0.002	(−0.01, 0.01)	0.70	0.01	(−0.01, 0.02)	0.42	0.0004	(−0.01, 0.01)	0.96	−0.001	(−0.02, 0.02)	0.90
Chloride	mmol/L	**−0.01**	**(−0.03, −0.001)**	***0.03**	−0.01	(−0.03, 0.01)	0.44	−0.01	(−0.03, 0.01)	0.25	0.02	(−0.01, 0.04)	0.31	−0.01	(−0.03, 0.02)	0.50	−0.02	(−0.05, 0.02)	0.42

		PFHxA			PFHxS			PFOA			**PFOS**			PFNA			PFDA		
Outcome	**Units**	**β [2]**	**95% CI**	**p**	**β [2]**	**95% CI**	**p**	**β [2]**	**95% CI**	**p**	**β [2]**	**95% CI**	**p**	**β [2]**	**95% CI**	**p**	**β [2]**	**95% CI**	**p**
Bilirubin	% change	0.10	(−0.03, 0.22)	0.12	0.008	(−0.22, 0.23)	0.94	−0.02	(−0.24, 0.20)	0.89	−0.10	(−0.39, 0.20)	0.51	0.10	(−0.16, 0.36)	0.44	−0.08	(−0.49, 0.32)	0.68
Glucose	% change	0.02	(−0.04, 0.08)	0.50	0.02	(−0.04, 0.09)	0.52	0.001	(−0.06, 0.07)	0.97	**0.10**	**(0.02, 0.19)**	***0.02**	0.05	(−0.03, 0.13)	0.19	0.05	(−0.07, 0.17)	0.42
Calcium	% change	−0.003	(−0.01, 0.01)	0.56	−0.001	(−0.02, 0.01)	0.85	0.0002	(−0.01, 0.02)	0.98	0.005	(−0.01, 0.03)	0.59	0.005	(−0.01, 0.02)	0.60	0.0005	(−0.03, 0.03)	0.97
A:G Ratio	% change	0.02	(−0.01, 0.04)	0.21	0.0003	(−0.05, 0.05)	0.99	−0.03	(−0.08, 0.03)	0.33	0.02	(−0.05, 0.08)	0.60	−0.0008	(−0.06, 0.06)	0.98	−0.002	(−0.10, 0.09)	0.96
AST	% change	**−0.08**	**(−0.15, −0.001)**	***0.047**	0.02	(−0.08, 0.12)	0.73	0.02	(−0.08, 0.12)	0.70	−0.04	(−0.18, 0.09)	0.52	−0.02	(−0.14, 0.10)	0.73	−0.004	(−0.19, 0.18)	0.96
ALT	% change	−0.02	(−0.10, 0.05)	0.56	0.02	(−0.13, 0.18)	0.75	0.01	(−0.14, 0.16)	0.86	−0.03	(−0.22, 0.17)	0.79	0.05	(−0.12, 0.22)	0.56	0.07	(−0.21, 0.34)	0.63
Creatinine	% change	−0.001	(−0.02, 0.02)	0.92	0.02	(−0.03, 0.07)	0.42	0.02	(−0.03, 0.07)	0.46	−0.03	(−0.09, 0.03)	0.33	−0.003	(−0.06, 0.05)	0.91	−0.06	(−0.14, 0.03)	0.20
Albumin	% change	−0.001	(−0.01, 0.01)	0.92	−0.004	(−0.02, 0.01)	0.65	−0.001	(−0.02, 0.02)	0.87	−0.02	(−0.05, 0.0005)	0.06	−0.00004	(−0.02, 0.02)	0.997	−0.02	(−0.05, 0.01)	0.20

[1] Beta-coefficients represent the unit change in serum concentration for each 1% increase in serum PFAS

[2] Beta-coefficients represent the % change in serum concentration for each 1% increase in serum PFAS

95% CI: 95 percent confidence interval

Table B7: Regression analysis of two univariate predictors, age and sex, of health measures in blood serum. Beta-coefficients (β), 95% confidence intervals (95% CI), and p-values (p) are reported. Significant results are formatted in bolded or red text for convenience. *p<0.05

		Sex[1]			Age[2]		
Outcome	**Units**	**β [3]**	**95% CI**	**p**	**β [3]**	**95% CI**	**p**
Total Cholesterol	mg/dL	**13.77**	**(1.5, 26.1)**	***0.03**	0.38	(−0.5, 1.2)	0.37
HDL Cholesterol	mg/dL	**7.50**	**(3.2, 11.8)**	***0.001**	0.29	(−0.01, 0.6)	0.06
Non-HDL Cholesterol	mg/dL	6.31	(−6.3, 19.0)	0.32	0.09	(−0.7, 0.9)	0.83
ALP	IU/L	1.16	(−4.3, 6.7)	0.67	0.16	(−0.2, 0.5)	0.38
Total Protein	g/dL	−0.03	(−0.14, 0.07)	0.54	**−0.007**	**(−0.01, −0.0009)**	***0.03**
Globulin	g/dL	0.04	(−0.05, 0.1)	0.39	−0.005	(−0.01, 0.001)	0.11
BUN	mg/dL	−0.58	(−2.1, 0.9)	0.44	**0.14**	**(0.05, 0.2)**	***0.003**
BUN:Cre	unitless ratio	1.30	(−0.1, 2.7)	0.07	**0.10**	**(0.01, 0.2)**	***0.03**
eGFR	mL/min / 1.73	−1.18	(−6.1, 3.7)	0.62	**−0.85**	**(−1.1, −0.7)**	***<0.0001**
Sodium	mmol/L	−0.29	(−0.9, 0.31)	0.34	0.01	(−0.03, 0.05)	0.58
Chloride	mmol/L	−0.28	(−1.0, 0.4)	0.41	0.01	(−0.03, 0.06)	0.62
Potassium	mmol/L	−0.09	(−0.19, 0.02)	0.10	0.003	(−0.003, 0.01)	0.33
Total Carbon Dioxide	mmol/L	0.05	(−0.3, 0.4)	0.80	0.001	(−0.02, 0.03)	0.91
		Sex[1]			**Age[2]**		
Outcome	**Units**	**β [4]**	**95% CI**	**p**	**β [4]**	**95% CI**	**p**
Glucose	% change	2.03	(−2.7, 7.0)	0.40	0.07	(−0.2, 0.4)	0.65
Albumin	% change	**−1.56**	**(−2.8, −0.3)**	***0.02**	−0.06	(−0.15, 0.03)	0.17
Creatinine	% change	**−10.49**	**(−14.1, −6.7)**	***<0.0001**	0.29	(−0.04, 0.6)	0.09
A:G Ratio	% change	−3.48	(−7.4, 0.6)	0.09	0.14	(−0.1, 0.4)	0.32
AST	% change	**−10.17**	**(−16.6, −3.3)**	***0.01**	0.23	(−0.3, 0.8)	0.37
ALT	% change	**−13.75**	**(−23.1, −3.3)**	***0.01**	−0.41	(−1.2, 0.4)	0.30
Calcium	% change	0.23	(−0.9, 1.4)	0.68	0.02	(−0.05, 0.1)	0.56
Bilirubin	% change	**−16.86**	**(−29.8, −1.5)**	***0.03**	0.69	(−0.5, 1.8)	0.23
Serum PFHxA	% change	**−12.10**	**(−21.0, −2.2)**	***0.02**	0.11	(−0.6, 0.8)	0.76
Serum PFHxS	% change	−14.62	(−31.1, 5.8)	0.14	0.86	(−0.6, 2.3)	0.23
Serum PFOA	% change	−7.29	(−26.4, 16.8)	0.51	**1.50**	**(0.05, 3.0)**	***0.04**
Serum PFOS	% change	−11.34	(−24.2, 3.7)	0.13	**1.22**	**(0.2, 2.2)**	***0.02**
Serum PFNA	% change	−6.14	(−22.3, 13.4)	0.50	**1.49**	**(0.3, 2.7)**	***0.01**

Serum PFDA	% change	−0.78	(−11.8, 11.6)	0.89	0.53	(−0.2, 1.3)	0.16
Serum Σ(6)PFAS	% change	−9.90	(−22.9, 5.2)	0.18	**1.14**	**(0.3, 2.1)**	***0.02**

[1] Referent group is males; participant sex is defined as categorical variable (male vs. female)
[2] Age is defined as a continuous variable (in years)
[3] Beta-coefficients represent the unit change in serum concentration relative to the reference group of males for sex, or per 1-year increase for age
[4] Exponentiated beta-coefficients represent the % change in serum concentration relative to the reference group of males for sex, or per 1-year increase for age

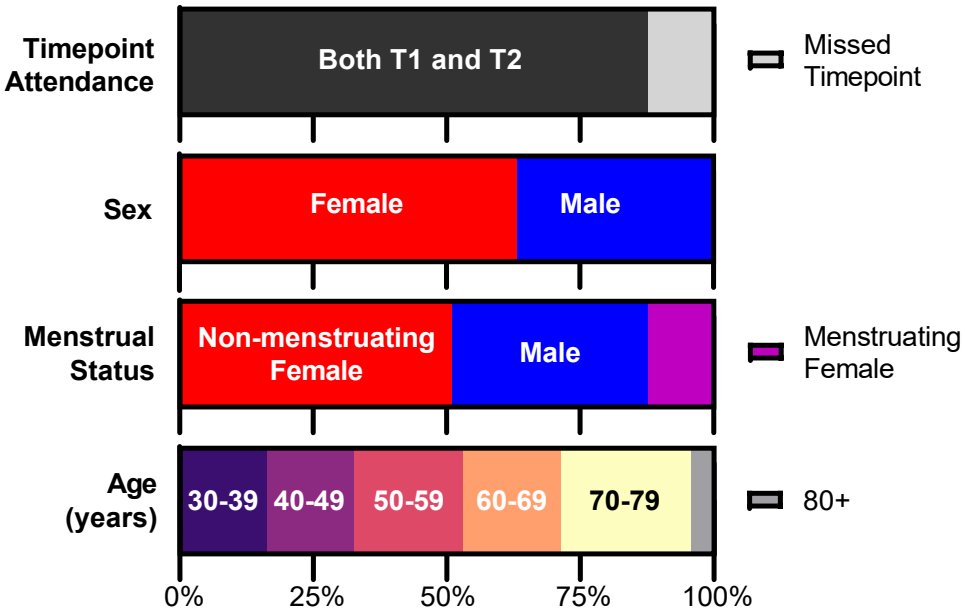


Figure B1: Demographic characteristics of Pittsboro cohort.

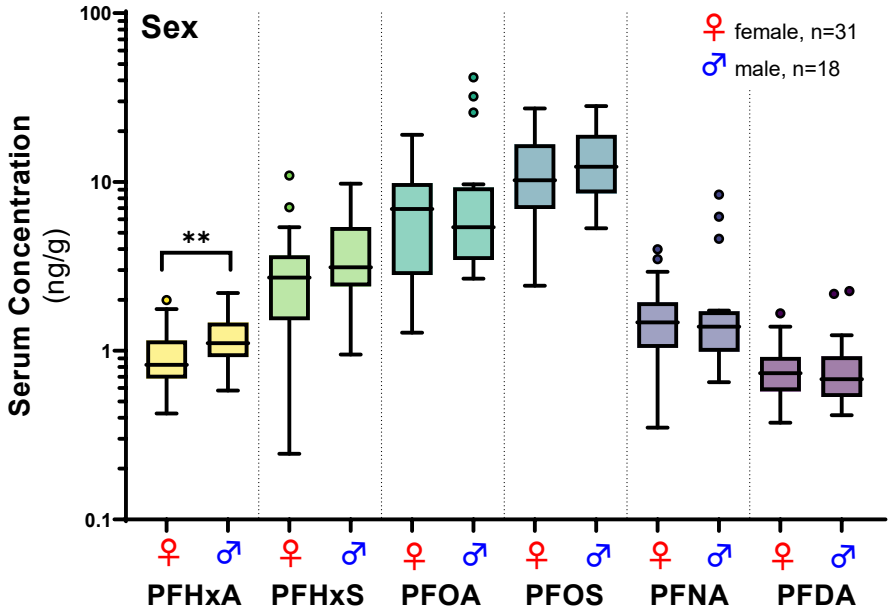


Figure B2: Pittsboro blood serum PFAS concentrations by participant sex. Females had significantly lower PFHxA blood concentrations than males (Mann-Whitney, two-tailed). Concentrations are reported on log scale with Tukey boxplots. **p<0.01

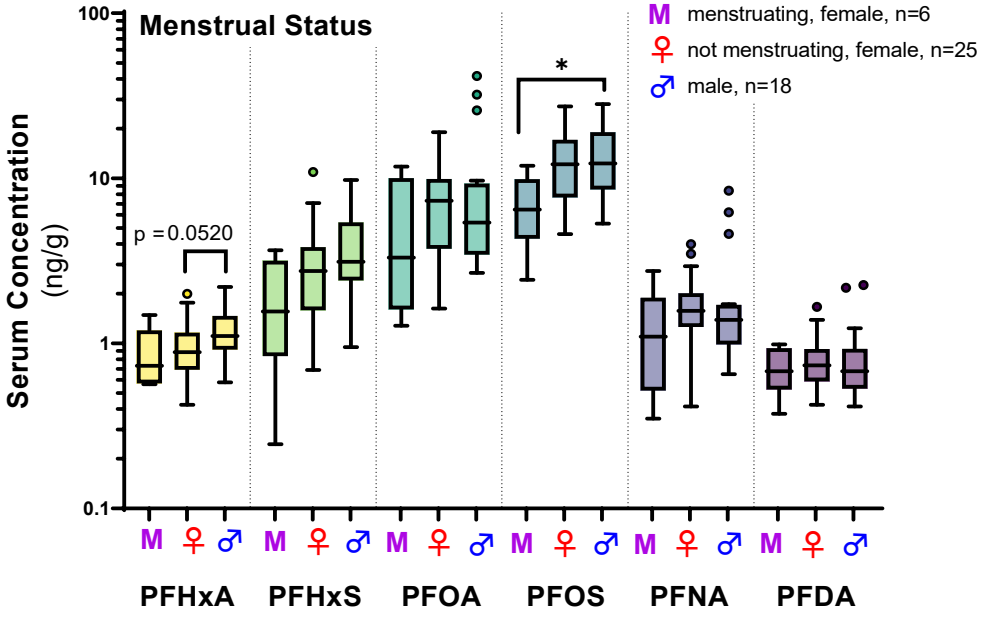


Figure B3: Pittsboro blood serum PFAS concentrations by participant menstrual status. Menstruating females (i.e., participants who reported that they still regularly menstruated) had significantly lower blood concentrations of PFOS than males (Kruskal-Wallis, followed by Dunn's multiple comparisons test). Concentrations are reported on log scale with Tukey boxplots. *p<0.05

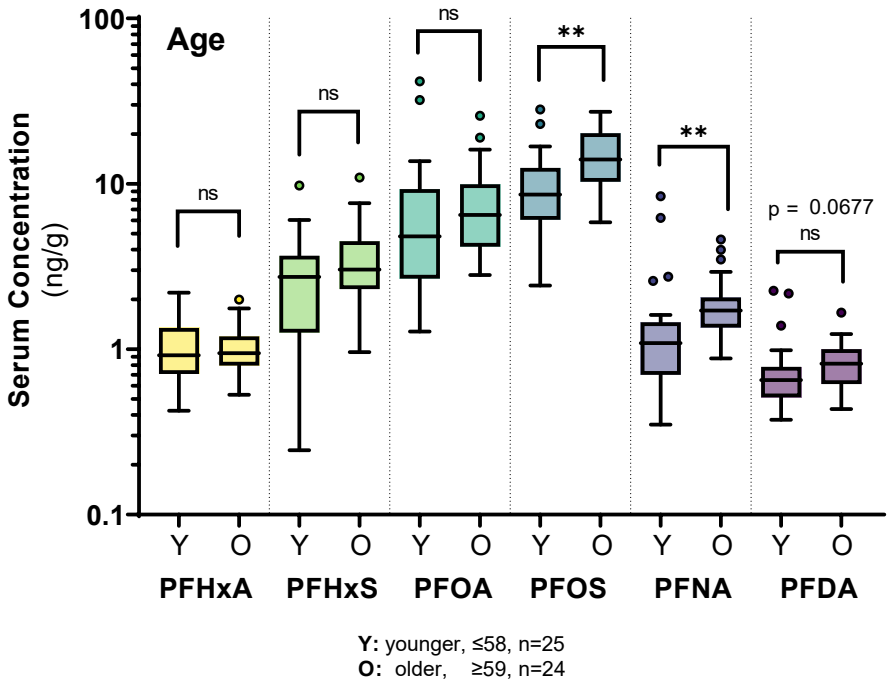


Figure B4: Pittsboro blood serum PFAS concentrations by participant age. Data were dichotomized at the median age of 58 years old. Participants who were 59 or older had significantly higher blood concentrations of PFOS and PFNA than younger participants (Mann-Whitney, two-tailed). Concentrations are reported on log scale with Tukey boxplots. **p<0.01, ns: not significant

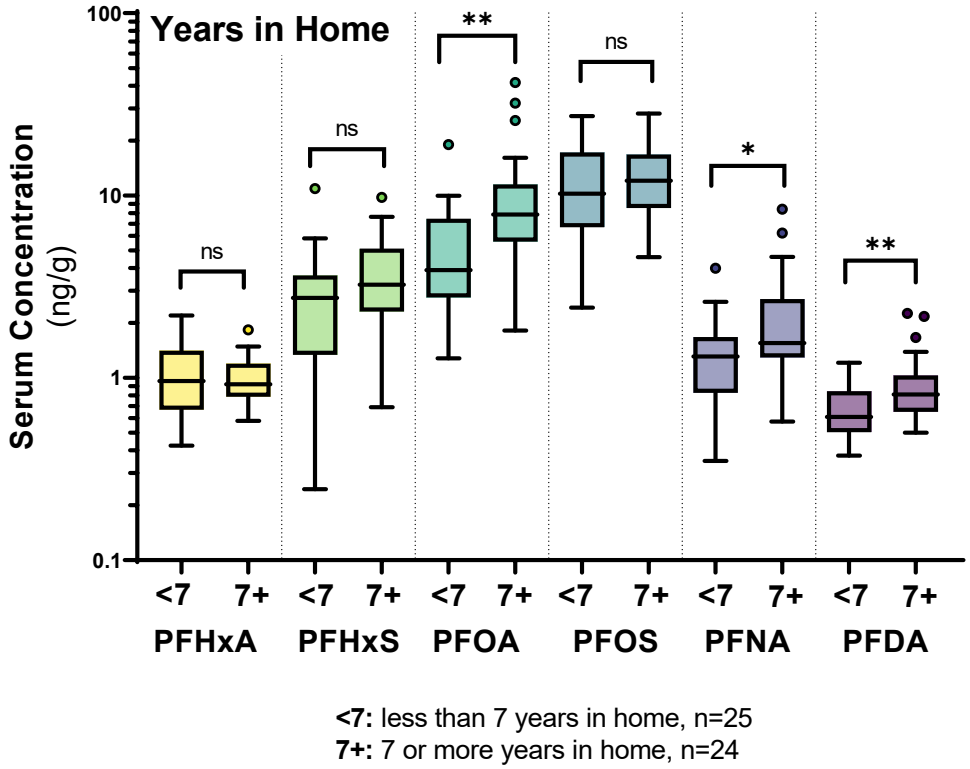


Figure B5: Pittsboro blood serum PFAS concentrations by years lived in homes. Participants who lived in their homes for seven or more years had significantly higher blood levels of PFOA, PFNA, and PFDA than participants who had lived in their homes for less than seven years (Mann-Whitney, two-tailed). Concentrations are reported on log scale with Tukey boxplots. *p<0.05, **p<0.01, ns: not significant

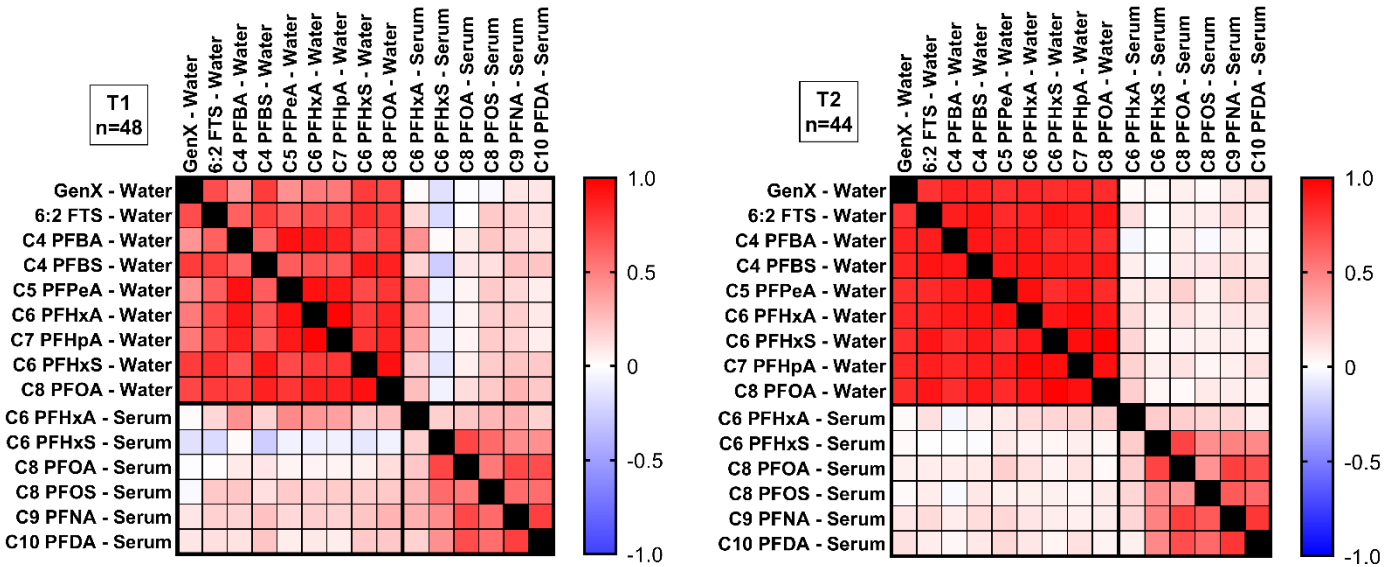


Figure B6: Spearman correlation coefficients for PFAS frequently detected in serum and drinking water. Color indicates strength and directionality of correlation.

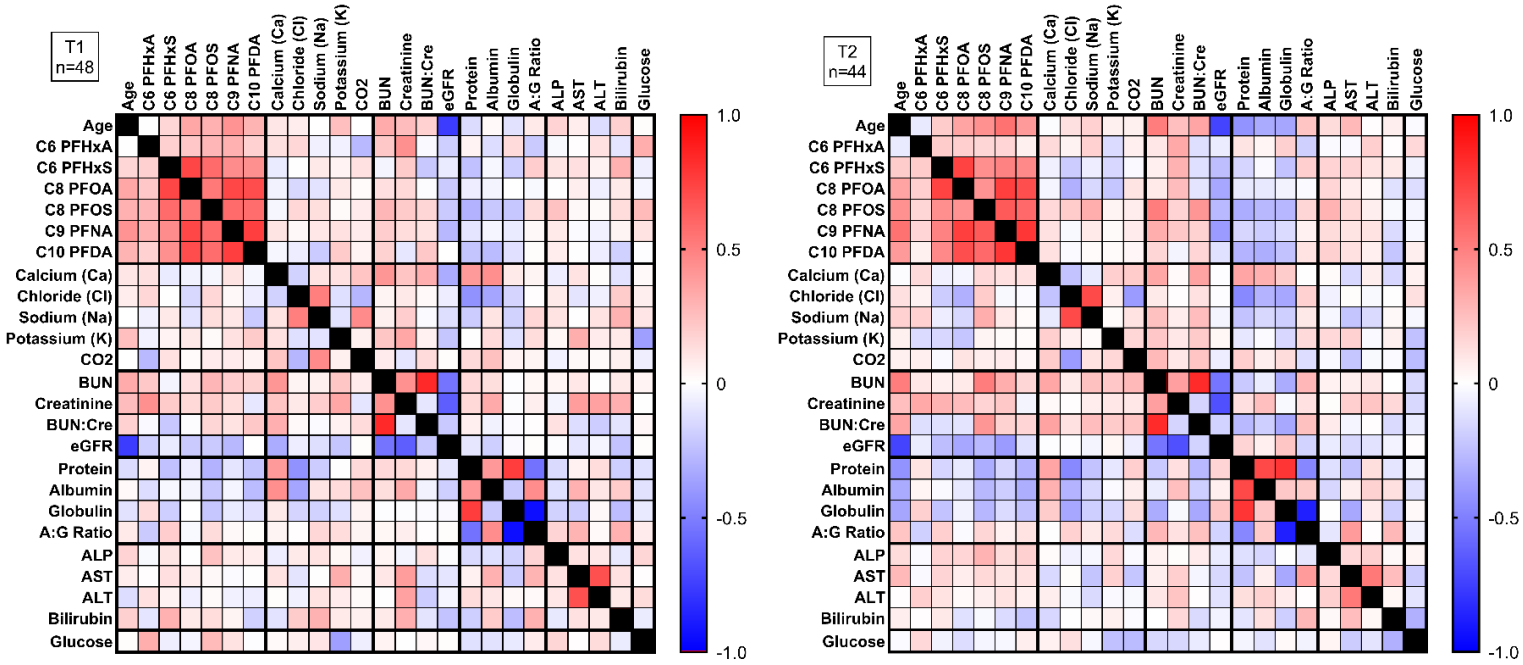


Figure B7: Spearman correlations for serum PFAS and serum CMP measures.

Appendix C

Appendix C contains supporting information for Chapter 4.

Table C1: Chemical information for PFAS drinking water mixture

Analyte	CAS Number	Lot or Batch Number	Chemical Formula	Molecular Weight (g/mol)	Water Concentration (ng/L)
PFBA	375-22-4	#00017623	C4HF7O2	214.04	96.1
PFPeA	2706-90-3	#00015707	C5HF9O2	264.05	158.4
PFHxA	307-24-4	#00017040	C6HF11O2	314.05	261.4
PFHpA	375-85-9	#00017038	C7HF13O2	364.06	144.7
PFOA	335-67-1	#00017912	C8HF15O2	414.07	48.2
PFNA	375-95-1	#00010188	C9HF17O2	464.08	7.9
PFDA	335-76-2	#00018431	C10HF19O2	514.08	9.0
PFBS*	29420-49-3	#00019005	C4F9KO3S	338.19	7.9*
PFHxS*	3871-99-6	BCCD2559	C6F13KO3S	438.20	9.6*
PFOS*	2795-39-3	BCCC4690	C8F17KO3S	538.22	15.4*

*Sulfonic acids were purchased as potassium salts, not free acids; water concentration reflects concentration of chemical present as anion, not salt

Table C2: Average concentrations (ng/g) in NIST Standard Reference Material (SRM) 1947 Lake Michigan fish tissue and relative standard deviation (RSD) along with reference values and method detection limit (MDL). These data were used as QA/QC for rabbit liver PFAS concentrations.

Analyte	MDL (ng/g)	SRM 1947 (n=2) (ng/g)	SRM 1947 RSD (%)	SRM 1947 Reference Values†	Percent of NIST Value (%)
PFOS	0.04	6.5	3.2%	5.90 ± 0.39†	110%
PFNA	0.22	0.3	2.5%	0.20† 0.18 ± 0.01**	150%
PFDA	0.06	0.2	2.3%	0.26† 0.28 ± 0.06**	77%
PFOA	0.09	0.2	22.9%	<0.297** <0.676**	N/A

†reference and information values as reported on the NIST Certificate of Analysis for SRM 1947 (NIST, 2017)
**values as reported in Reiner *et al.* (2012)
N/A: values are not reported in the NIST Certificate of Analysis

Table C3: Average concentrations (ng/g) in NIST Standard Reference Material (SRM) 1947 Lake Michigan fish tissue and relative standard deviation (RSD) along with reference values and method detection limit (MDL). These data were used as QA/QC for rabbit feces PFAS concentrations.

Analyte	MDL (ng/g)	SRM 1947 (n=3) (ng/g)	SRM 1947 RSD (%)	SRM 1947 Reference Values†	Percent of NIST Value (%)
PFOS	0.02	5.7	10.5%	5.90 ± 0.39†	97%
PFNA	0.1	0.43	16.1%	0.20† 0.18 ± 0.01**	215%
PFDA	0.1	0.19	12.5%	0.26† 0.28 ± 0.06**	73%
PFOA	0.1	0.7	12.9%	<0.297** <0.676**	N/A

†reference and information values as reported on the NIST Certificate of Analysis for SRM 1947 (NIST, 2017)
**values as reported in Reiner *et al.* (2012)
N/A: values are not reported in the NIST Certificate of Analysis

Table C4: Average concentrations (ng/mL) in NIST Standard Reference Material (SRM) 1958 fortified human blood serum and relative standard deviation (RSD) along with reference values and method detection limits (MDL). These data were used as QA/QC for rabbit urine PFAS concentrations. Rabbit urine samples were extracted for PFAS using the same extraction method as typically used for serum.

Analyte	**MDL** (ng/mL)	**SRM 1958** (n=3) (ng/mL)	**SRM 1958 RSD** (%)	**SRM 1958 Reference Values**†	**Percent of NIST Value** (%)
PFOS	0.01	17.0	2.7%	16.6 ± 0.9† 17.0 ± 0.3**	102%
PFOA	0.16	3.4	1.0%	4.11 ± 0.17† 4.03 ± 0.21**	83%
PFNA	0.17	0.6	5.4%	0.66 ± 0.13† 0.553 ± 0.259**	91%
PFHxS	0.01	2.3	2.8%	2.66 ± 0.07† 2.63 ± 0.05**	87%
PFHxA	0.38	0.13	11.0%	<0.388**	N/A
PFHpA	0.21	0.3	8.1%	0.180 ± 0.044**	N/A
PFDA	0.07	0.13	3.0%	0.353 ± 0.141**	N/A

†reference and information values as reported on the NIST Certificate of Analysis for SRM 1958 (NIST, 2018a)
**values as reported in Keller *et al.* (2010)
N/A: values are not reported in the NIST Certificate of Analysis

Table C5: Tandem mass spectrometry (MS/MS) parameters. Transitions (*m/z* for precursor and product ions) for quantifying PFAS in rabbit tissue samples. Analyses were performed with ESI in negative ion mode, using the multiple reaction monitoring (MRM) mode.

Analyte	Transition	Mass-Labeled Internal Standard†	Transition
PFBA	213.0 > 168.9	$^{13}C_4$-PFBA or MPFBA	217.0 > 172.0
PFPeA	263.0 > 218.9	$^{13}C_5$-PFPeA or M5-PFPeA	268.0 > 223.0
PFHxA	313.0 > 268.9	$^{13}C_2$-PFHxA or MPFHxA	315.0 > 269.9
PFHpA	363.0 > 318.9	$^{13}C_4$-PFHpA or M4-PFHpA	367.0 > 322.0
PFOA	413.0 > 368.9	$^{13}C_4$-PFOA, MPFOA, or M4-PFOA	417.0 > 371.9
PFNA	463.0 > 418.9	$^{13}C_5$-PFNA or MPFNA	468.0 > 422.9
PFDA	513.0 > 468.9	$^{13}C_2$-PFDA or MPFDA	515.0 > 470.0
PFBS	298.9 > 80.0	$^{13}C_3$-PFBS or M3-PFBS	301.9 > 80.0
PFHxS	398.9 > 80.0	$^{18}O_2$-PFHxS, MPFHxS, or M4-PFHxS	402.9 > 83.9
PFOS	498.9 > 79.9 498.9 > 98.9*	$^{13}C_4$-PFOS or MPFOS	502.9 > 80.0
		$^{13}C_2$-PFOA or M2-PFOA	414.8 > 369.9
		$^{13}C_8$-PFOS or M8-PFOS*	506.9 > 80.0

†all analytical standards sourced from Wellington Laboratories
*Rabbit serum was analyzed with a slightly different acquisition method; those differences are formatted in red text.
Rabbit liver and wastes were spiked with 20 ng each of MPFAC-MXA, M3-PFBS, M4-PFHpA, and M5-PFPeA, and with 20 ng of M2-PFOA.
Rabbit serum was spiked with 12.8 ng of MPFAC-MXA, M3-PFBS, M4-PFHpA, and M5-PFPeA, and with 10 ng each of M2-PFOA and M8-PFOS.

Table C6: Average percent recoveries (%) of mass-labeled internal standards for rabbit samples. Internal standards were added immediately prior to start of extraction, and a recovery standard was added at the very end of the extraction prior to the LC-MS/MS run. M2-PFOA was used as the sole recovery standard for all mass-labeled internal standards, except for the blood serum. In blood serum, M8-PFOS was also added and used as the recovery standard for the mass-labeled sulfonates. Perfect recovery is 100%, generally acceptable recovery is between 50-150% for QA/QC; recoveries outside that range are formatted in red text. All analytical standards were sourced from Wellington Laboratories.

Internal Standard	Serum	Liver		Feces		Urine	
	Sample Recovery	Sample Recovery	SRM Recovery	Sample Recovery	SRM Recovery	Sample Recovery	SRM Recovery
MPFBA	79%	73%	74%	322%	73%	5%	72%
M5-PFPeA	63%	112%	72%	98%	84%	17%	70%
MPFHxA	66%	70%	61%	66%	76%	37%	80%
M4-PFHpA	80%	93%	69%	84%	76%	82%	81%
MPFOA	62%	54%	60%	66%	67%	92%	84%
MPFNA	51%	68%	63%	111%	76%	116%	91%
MPFDA	32%	7%	36%	104%	62%	117%	92%
M3-PFBS	75%	225%	83%	728%	99%	212%	102%
MPFHxS	76%	180%	65%	700%	96%	279%	91%
MPFOS	60%	65%	18%	487%	57%	203%	80%

Table C7: Comparison of PFAS concentrations in NIST standard reference materials (SRMs).

Analyte	**Lake Michigan Fish Tissue (1947)** (ng/g)	**Fortified Human Serum (1958)** (ng/mL)	**House Dust (2585)** (ng/g)	**Other Human Blood SRM (Serum & Plasma)** (ng/mL)	**Other SRM** (ng/g)
PFBA	--	--	**230**	--	--
PFPeA	--	--	--	--	--
PFHxA	--	--	**260**	--	**13✧**
PFHpA	--	--	**249**	**0.305✪**	**7.96✧**
PFOA	--	**4.11**	--	**3.21★, 5.00✪**	**28.5✧**
PFNA	0.2	**0.66**	**99.4**	**0.705★, 0.878✪**	--
PFDA	0.26	--	--	**0.315★, 0.39✪**	--
PFBS	--	--	--	--	--
PFHxS	--	**2.66**	**1440**	**3.19★, 4.00✪**	**9.39✧**
PFOS	5.9	**16.6**	**2310**	**10.43★, 21.1✪**	**225✧, 3.41▼, 0.778▲, 2.19Δ**
Comparison Matrix	Tissue (Placenta, Liver), Feces	Serum, Urine	House Dust	--	--

Bolded values are reference values; other values are for information only, per NIST. Certified values are the best and most reliable values, but no PFAS concentration is certified for any NIST SRM. Full information is available at the NIST website: www-s.nist.gov/srmors/viewTableH.cfm?tableid=247
★ Human Plasma (1950); ✪ Non-Fortified Human Serum (1957);
✧ Domestic Sludge (2781); ▼ Soil (2586); ▲ Great Lakes Sediment (1936); Δ Lake Superior Fish Tissue (1946)

Appendix D

Appendix D contains supporting information for Chapter 5, published under the title "Per- and Polyfluoroalkyl Substances in Dust Collected from Residential Homes and Fire Stations in North America" in *Environmental Science & Technology* in 2020. It is reprinted with permission from Hall, S. M.; Patton, S.; Petreas, M.; Hoffman, K.; Zhang, S.; Phillips, A.L.; Stapleton, H. M. Per- and Polyfluoroalkyl Substances in Dust Collected from Residential Homes and Fire Stations in North America. *Environ. Sci. Technol.* 2020, *54* (22), 14558–14567. DOI: 10.1021/acs.est.0c04869. Copyright 2020 American Chemical Society.

Additional Methods

Dust Sample Preparation and Extraction

Dust samples were prepared to extract the 17 PFAS analytes listed in Table D1 and are grouped into classes following previously described nomenclature (Buck *et al.*, 2011; Wang *et al.*, 2017). In Hall *et al.* (2020) analytes are split into two large subclasses: legacy and precursor PFAAs. Legacy PFAAs refers to the PFAAs (PFCAs and PFSAs) which are more persistent in the environment. PFAA precursors refers to the shorter-lived PFAS that are potentially capable of transforming into PFAAs.

Dust extraction methods have previously been described in Phillips *et al.* (2018). In brief, dust samples from fire station samples (n=49) and residential homes (n=184) were sieved to <150 μm or <500 μm and extracted with a total 30 mL of 1:1

dicholoromethane:hexane (v/v) using sonication. Extracts were concentrated to approximately 1 mL using a SpeedVac vacuum concentrator (ThermoFisher Scientific). Extracts were fractionated using Florisil solid-phase extraction cartridges (Supelclean™, ENVI-Florisil®, 6 mL, 500 mg; Supelco®), eluting three fractions with 6 mL hexane, 10 mL ethyl acetate, and 6 mL methanol. The F2 fraction eluted using 10 mL ethyl acetate was used for analysis of FTOHs and FOSEs, and the F3 fraction eluted using 6 mL methanol was used for analysis of PFAAs and diPAPs (the F1 and F2 fractions were prioritized for analysis of other compounds in other studies). The F2 fractions were concentrated to approximately 1 mL using a SpeedVac concentrator and were reconstituted in hexane prior to GC-MS analysis. Samples were spiked with mass-labeled internal standards prior to extraction for fire station samples and prior to cleanup for house dust samples.

HPLC-MS/MS Analysis

Twelve PFAS compounds were analyzed using an Agilent 1260 Infinity II high-performance liquid chromatograph (HPLC) instrument coupled to an Agilent 6460A triple quadrupole mass spectrometer. The mass spectrometer was operated in negative electrospray ionization mode (HPLC-ESI-MS/MS). Separation of analytes by LC was performed using a 4.6 mm (I.D.) x 50 mm Agilent ZORBAX Eclipse XDB-C18 reversed-phase HPLC column (1.8 μm particle size) preceded by a 4.6 mm x 5 mm XDB-C18 guard cartridge.

Mobile phases were 2 mM ammonium acetate in water (mobile phase A) and 2 mM ammonium acetate in methanol (mobile phase B) using a flow rate of 0.4 mL/min. Gradient conditions for chromatographic separation were as follows: initial condition (30% B) was increased to 60% B over 1.5 minutes; then increased to 95% B over 2 minutes and held for 5.5 minutes; then increased to 100% B over 3 minutes, returned to initial condition (30% B) over 0.5 minutes, and held for 5.5 minutes. The column temperature was 45 °C and the injection volume was 20 μL. Data were acquired under multiple reaction monitoring (MRM) transitions using optimized parameters. Additional methods information, including transitions, is included in Table D2.

GC-MS Analysis

Five PFAS compounds were analyzed using gas chromatography (GC) with an Agilent 7890A gas chromatograph instrument coupled to an Agilent 5975C mass spectrometer operated in electron impact mode (GC-EI-MS). Methods were previously described in Hammel *et al.* (2019). In brief, pressurized temperature vaporization (PTV) injection was employed in the inlet, and a 0.25 mm (I.D.) x 30 m fused silica capillary column coated with 5% phenyl methylpolysiloxane (J&W Scientific, 0.25 μm film thickness) was used in the GC for separation of analytes. Helium was used as carrier gas at a constant flow rate of 1.3 mL/min. The inlet was set to a temperature of 80 °C for 0.3 minutes, and a 600 °C/min ramp was employed to increase the inlet temperature to 300 °C in order to efficiently transfer the samples to the head of the GC column. The GC

oven was held at 80 °C for 2 minutes followed by a temperature ramp of 20 °C/min to 250 °C, a ramp of 1.5 °C/min to 260 °C, and a ramp of 25 °C/min to 300 °C, and the ion source was held at 230 °C. Additional methods information, including ions used for quantification, is available in Table D2.

Table D1: List of PFAS analytes measured in dust

Legacy PFAAs			PFAA Precursors		
PFCAs	**Perfluoroalkyl carboxylic acids**	**CASRN**	**diPAPs**	**Fluorotelomer phosphate diesters**	**CASRN**
PFBA	Perfluorobutanoic acid	375-22-4	6:2 diPAP	6:2 fluorotelomer phosphate diester or sodium bis(1H,1H,2H,2H-perfluorooctyl) phosphate	57677-95-9
PFPeA	Perfluoropentanoic acid	2706-90-3	8:2 diPAP	8:2 fluorotelomer phosphate diester or sodium bis(1H,1H,2H,2H-perfluorodecyl) phosphate	678-41-1
PFHxA	Perfluorohexanoic acid	307-24-4	**FTOHs**	**Fluorotelomer alcohols**	**CASRN**
PFHpA	Perfluoroheptanoic acid	375-85-9	6:2 FTOH	6:2 fluorotelomer alcohol or 2-Perfluorohexyl ethanol (FHET)	647-42-7
PFOA	Perfluorooctanoic acid	335-67-1	8:2 FTOH	8:2 fluorotelomer alcohol or 2-Perfluorooctyl ethanol (FOET)	678-39-7
PFNA	Perfluorononanoic acid	375-95-1	10:2 FTOH	10:2 fluorotelomer alcohol or 2-Perfluorodecyl ethanol (FDET)	865-86-1
PFDA	Perfluorodecanoic acid	335-76-2			
PFSAs	**Perfluoroalkyl sulfonic acids**	**CASRN**	**PASF-based substances**	**Perfluoroalkane sulfonamido ethanols**	**CASRN**
PFBS	Perfluorobutane sulfonic acid	375-73-5	MeFOSE	*N*-methyl perfluorooctane sulfonamido ethanol (N-MeFOSE)	24448-09-7
PFHxS	Perfluorohexane sulfonic acid	355-46-4	EtFOSE	*N*-ethyl perfluorooctane sulfonamido ethanol (N-EtFOSE)	1691-99-2
PFOS	Perfluorooctane sulfonic acid	1763-23-1			

Table D2: MS conditions for quantifying PFAS analytes in dust samples. Transitions (*m*/*z* for precursor and product ions) are included for compounds measured with LC-MS/MS, and quantifier and qualifier ions are included for compounds measured with GC-MS.

LC Compounds	Transition	Internal Standard	Transition
6:2 diPAP	789.0 > 96.9	$^{13}C_4$-6:2 diPAP	793.0 > 96.9
8:2 diPAP	989.0 > 96.9	$^{13}C_4$-8:2 diPAP	992.9 > 96.9
PFBA	213.0 > 168.9	$^{13}C_4$-PFBA	217.0 > 172.0
PFBS	298.9 > 98.9	$^{13}C_4$-PFHxS	402.9 > 83.9
PFPeA	263.0 > 218.9	$^{13}C_2$-PFHxA	315.0 > 269.9
PFHxA	313.0 > 268.9	$^{13}C_2$-PFHxA	315.0 > 269.9
PFHxS	398.9 > 79.9	$^{18}O_2$-PFHxS	402.9 > 83.9
PFHpA	363.0 > 318.9	$^{13}C_4$-PFOA	417.0 > 371.9
PFOA	413.0 > 368.9	$^{13}C_4$-PFOA	417.0 > 371.9
PFOS	498.9 > 79.9	$^{13}C_4$-PFOS	502.9 > 80.0
PFNA	463.0 > 418.9	$^{13}C_5$-PFNA	468.0 > 422.9
PFDA	513.0 > 468.9	$^{13}C_2$-PFDA	515.0 > 470.0
GC Compounds	**Quant / Qual Ions**	**Internal Standard**	**Quant / Qual Ions**
6:2 FTOH	365 / 327	^{13}C 6:2 FTOH	367 / 329
8:2 FTOH	465 / 427	^{13}C 8:2 FTOH	467 / 429
10:2 FTOH	565 / 527	^{13}C 8:2 FTOH	467 / 429
MeFOSE	558 / 540	d_7-N-MeFOSE	547 / 565
EtFOSE	572 / 554	d_9-N-EtFOSE	581 / 563

Table D3: Average dust levels (ng/g dust) in house dust SRM 2585 and relative standard deviation (RSD).

Compound	House Dust			Fire Station Dust			SRM 2585 Reference Values†
	MDL (ng/g)	SRM 2585 (n=5)	SRM 2585 RSD (%)	MDL (ng/g)	SRM 2585 (n=5)	SRM 2585 RSD (%)	
6:2 diPAP	0.48	299	25.6	2.54	698	2.6	421 ± 83* 675 ± 28.3**
8:2 diPAP	10.63	321	27.4	9.63	748	5.2	868 ± 30* 227 ± 27.3**
PFBA	1.72	54.7	31.4	1.03	104	9.7	230 ± 16† 249 ± 25* 229 ± 25.2**
PFBS	22.28	23.8	14.8	8.56	13.8	20.9	18.8 – 130* 21.3 ± 2.87**
PFPeA	0.14	67.1	27.2	1.2	139	14.5	226 ± 31* 235 ± 24.4**
PFHxA	0.42	185	18.5	10.30	238	2.5	260 ± 25† 279* 349 ± 33.2**
PFHxS	0.25	1630	13.2	0.97	1300	7.4	1440 ± 250† 1400 ± 170** 1420 ± 191**
PFHpA	0.51	205	31.5	6.77	310	9.9	249 ± 32† 259* 360 ± 26.6**
PFOA	0.26	480	6.9	1.60	551	2.2	561* 747 ± 191**
PFOS	0.20	2420	8.8	1.44	2190	7.5	2310 ± 420† 2280 ± 200** 1860 ± 256**
PFNA	0.15	76.7	10.1	0.47	65.4	31.7	99.4 ± 4.9† 101 ± 5** 90.1 ± 12**
PFDA	0.06	55.6	10.8	1.49	51.4	5.4	34.6 ± 4.5† 38.6* 66.9 ± 10.7**
6:2 FTOH	6.44	4490	7.4	48.90	3450	101.8	4560 ± 868**
8:2 FTOH	15.80	5580	13.5	7.23	6940	57.3	5220 ± 850**
10:2 FTOH	N/A	N/A	N/A	5.00	3630	48.0	3730 ± 687**
MeFOSE	13.17	2250	23.8	5.00	2490	17.2	2790 ± 180**
EtFOSE	15.45	4500	8.5	5.00	3530	11.4	4200 ± 338**

†reference values as reported on the NIST Certificate of Analysis for SRM 2585 (NIST, 2018b)
*reference and information values as reported in Reiner *et al.* (2015)
**reference values as reported in Winkens *et al.* (2018)
N/A: not applicable as this compound was not measured in house dust

Table D4: Fire station dust concentrations stratified by country*

Compound	United States Fire Stations (2015, n=25)		Canadian Fire Stations (2018, n=24)	
	Median Concentration (ng/g dust)	**Maximum Concentration** (ng/g dust)	**Median Concentration** (ng/g dust)	**Maximum Concentration** (ng/g dust)
6:2 diPAP	291	5,770	243	6,270
8:2 diPAP	95.9	1,460	105	2,250
MeFOSE	32.0	627	28.5	1,110
EtFOSE	19.8	17,540	<MDL	213
6:2 FTOH	1,430	86,060	544	22,620
8:2 FTOH	445	65,170	175	17,480
10:2 FTOH	268	183,700	46.0	8,020
PFBA	5.0	213	3.5	33.6
PFOA	26.4	791	9.5	305
PFNA	7.1	181	6.3	203
PFDA	7.0	88.7	<MDL	137
PFHxS	10.3	8,280	2.4	204
PFOS	104	74,370	13.2	1,190

*only analytes detected in >50% of all 49 fire stations are reported here
<MDL: below method detection limit

Table D5: Characteristics of 49 fire stations included in Hall et al., 2020*

Characteristic	Sample Size
Country	
United States	25
Canada	24
Year of fire station construction	
Pre-1970	22
Post-1970	23
Square footage	
Less than 3,500 square feet	21
3,500 or greater square feet	19
Carpeting	
Greater than 20% carpeting	20
20% or less carpeting	19

*sample size may not always sum to total due to incomplete survey information

Table D6: Characteristics of 184 residential homes included in Hall et al., 2020

Characteristic	Sample Size
Year of home construction	
Pre-1970	34
Post-1970	86
No data	64
Square footage	
Less than 1,720 square feet	67
1,720 or greater square feet	67
No data	50
Carpeting	
Carpeted (any %)	124
Not carpeted	60

Table D7: Summary of median PFAS dust concentrations (ng/g dust) in U.S. house dust reported in the published literature and Hall *et al.* (2020).

Reference	Year of Sample Collection	Sample Size (n)	PFOS	PFOA	PFHxS	PFHxA	PFBS	PFPeA	PFHpA	PFNA	PFDA
House dust SRM 2585 [NIST (2018b) and Reiner *et al.* (2015)]	1993-1994	Unknown	2,310	561	1,440	260	19–130	226	249	99	35
Strynar and Lindstrom (2008)	2000-2001	102 homes	201	142	46	54	9	-	50	8	7
Kato *et al.* (2009)[†]	2004	10 homes	~95	~95	~100	-	-	-	-	-	-
Knobeloch *et al.* (2012)	2008	39 homes	47	44	16	0	2	5	17	12	6
Fraser *et al.* (2013)*	2009	30 homes	27	24	-	9	-	-	12	11	-
Karaskova *et al.* (2016)	2013	14 homes	14	9	9	6.5	1	3	4	4	1.8
Hall *et al.* (2020)	2014-2016	184 homes	4	8	2	9	-	-	9	3	6

[†]medians for U.S. homes estimated from reference

*geometric mean concentrations reported in lieu of median values

Blank values were either not reported or <MDL

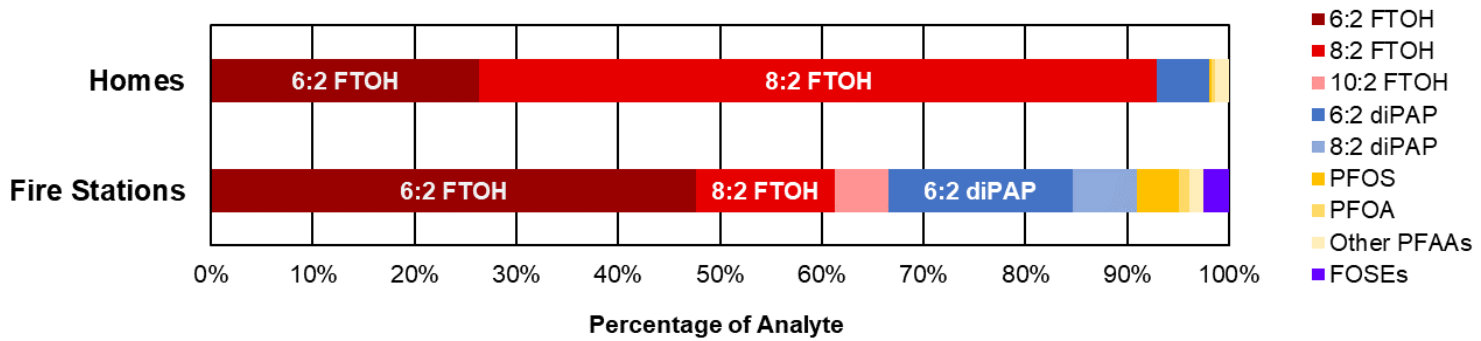


Figure D1: PFAS profiles in dust from homes and fire stations. Relative amounts of individual PFAS in dust from homes (n=184) and fire stations (n=49) based on the median concentrations and reported as percentage of dust composition. For homes, 10:2 FTOH was not measured.

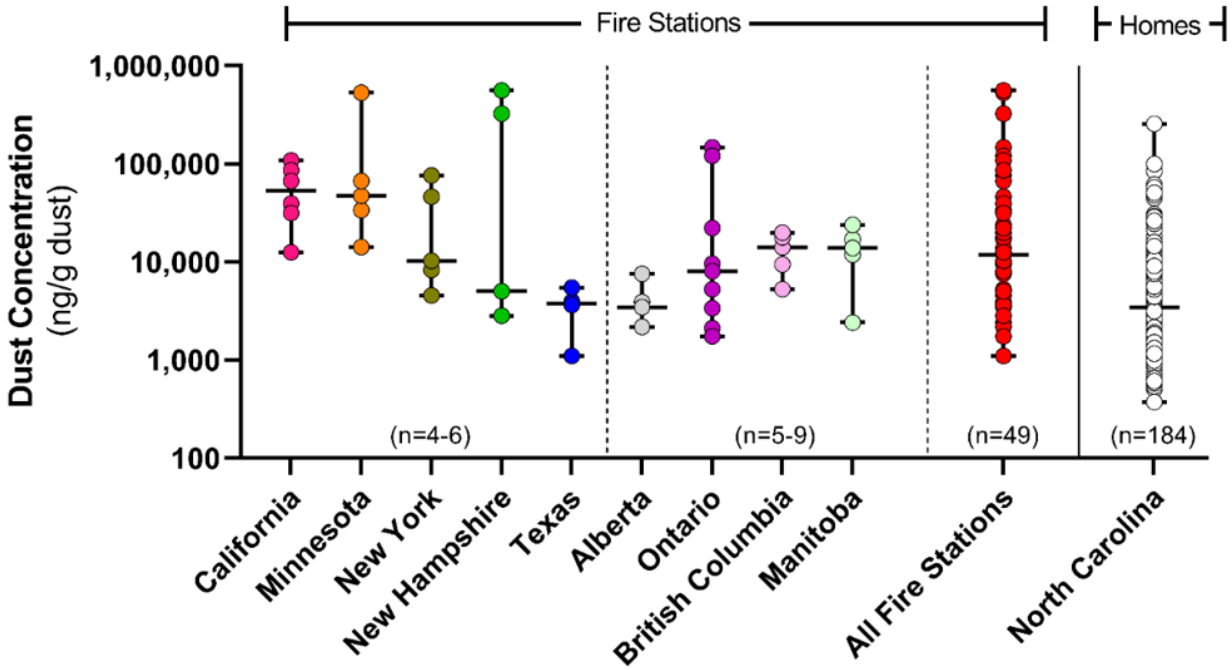


Figure D2: ΣPFAS concentrations in dust samples by region. ΣPFAS in dust samples stratified by region and represented by scatterplots depicting individual samples, with lines at the minimum, median, and maximum concentrations. ΣPFAS refers to the sum of all PFAS analytes measured in the dust. In fire station dust, 17 analytes were measured, and 16 analytes were measured in house dust (10:2 FTOH was not measured in house dust). Fire station dust samples were collected from across the United States and Canada (n=4-9 per region, 49 total) while dust from residential homes was collected in North Carolina (n=184). Concentrations are reported on a log scale. Due to small sample sizes, no statistical tests were performed. Note: British Columbia fire stations collected dust from living quarters and fire truck interiors.

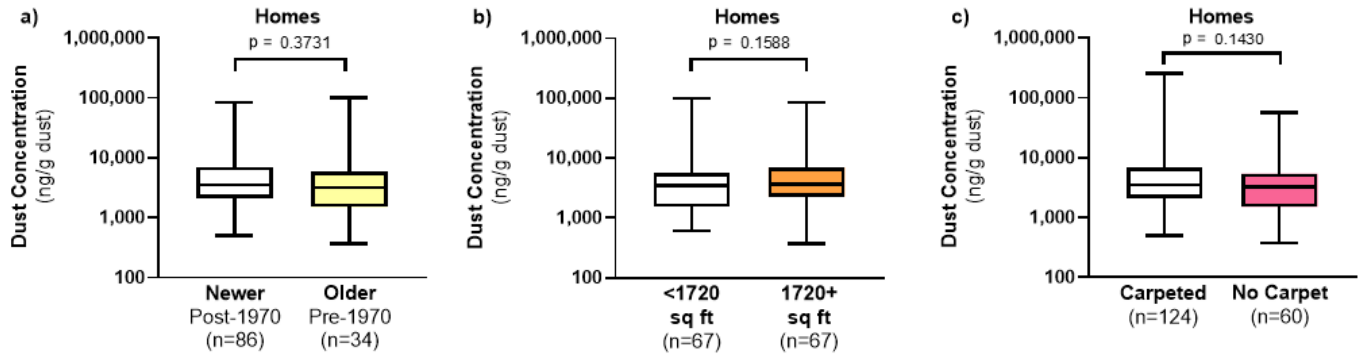


Figure D3: ΣPFAS dust concentration and survey responses for residential homes. Differences in dust ΣPFAS concentrations in homes due to: a) year of construction, b) square footage of home, and c) carpeting. No significant differences in house dust ΣPFAS concentrations were seen based on year of construction, square footage, or carpeting. Significance determined by two-tailed Mann-Whitney test. Sample sizes are variable due to missing survey data. Boxplots represent the minimum, 25th percentile, median, 75th percentile, and maximum concentrations. Concentrations are reported on a log scale. The ΣPFAS concentration represents the sum of all 16 PFAS analytes measured in a house dust sample.

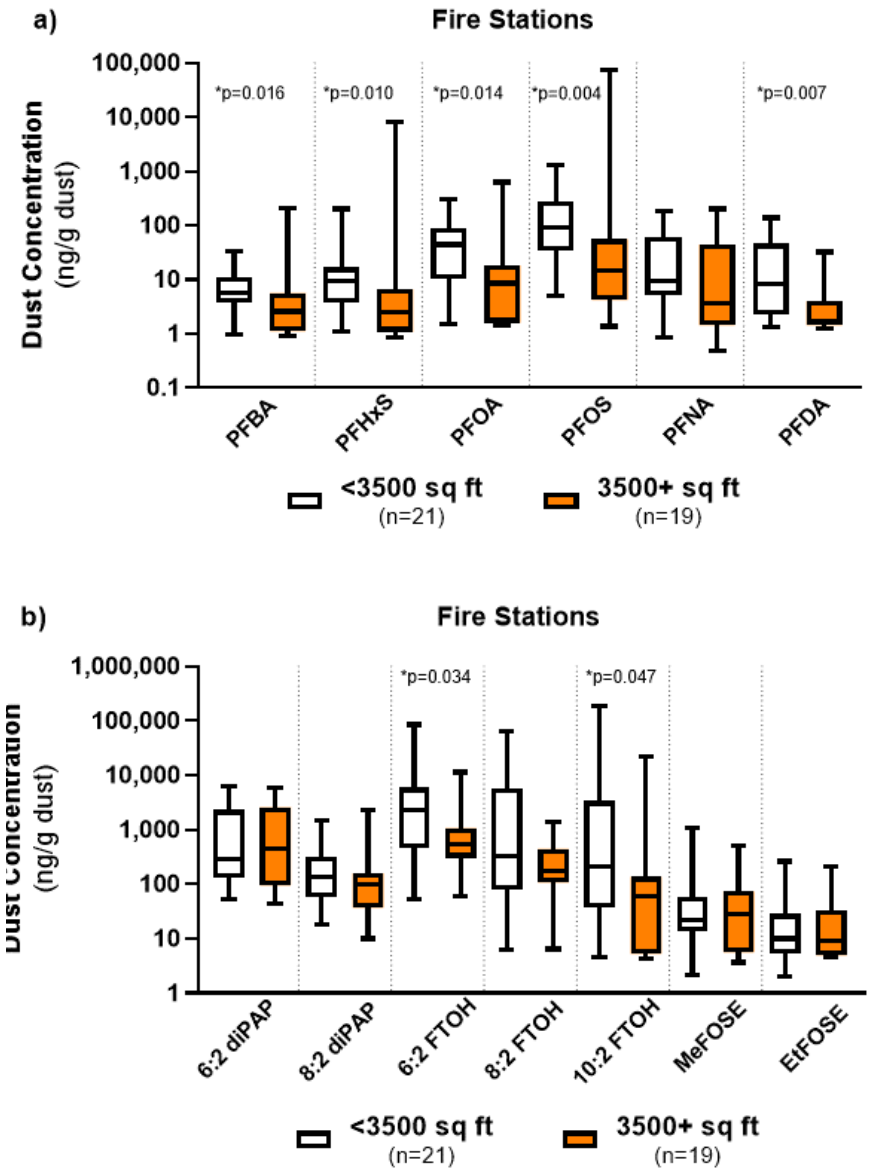


Figure D4: Square footage area of fire station living quarters and PFAS dust concentrations. Dust concentrations by individual analyte for a) legacy PFAAs and b) PFAA precursors. Significance determined by the two-tailed Mann-Whitney test. Boxplots represent the minimum, 25th percentile, median, 75th percentile, and maximum concentrations. Concentrations are reported on a log scale.

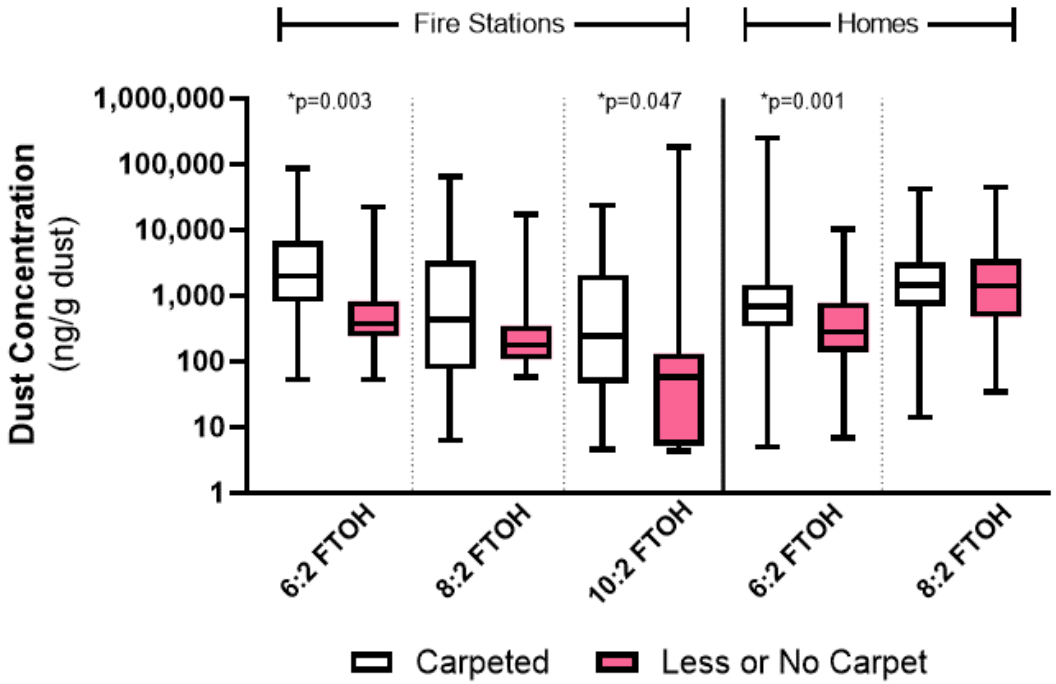


Figure D5: Carpeting and FTOH dust concentrations in fire stations and homes. Dust concentrations by individual analyte for the FTOHs. Significance determined by the two-tailed Mann-Whitney test. Boxplots represent the minimum, 25th percentile, median, 75th percentile, and maximum concentrations. Concentrations are reported on a log scale. For homes, 10:2 FTOH was not measured. Fire stations were stratified by greater than 20% carpeting (n=20) and 20% or less carpeting (n=19). Homes were stratified by carpeted (n=124) or not carpeted (n=60).

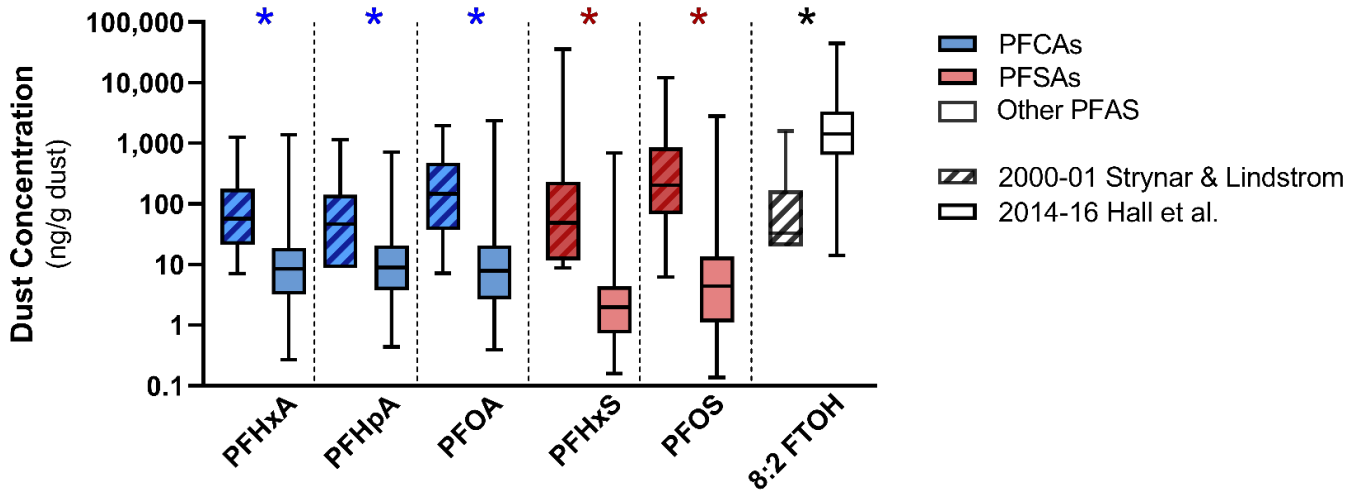


Figure D6: PFAS concentrations in house dust in 2000-2001 and 2014-2016. Significance determined by the two-tailed Mann-Whitney test (p-values are all *p<0.0001). Boxplots represent the minimum, 25th percentile, median, 75th percentile, and maximum concentrations. Concentrations are reported on a log scale. All analytes plotted were detected in >50% of dust samples in both studies. Data are from Strynar & Lindstrom, 2008 (n=102) and Hall et al., 2020 (n=184). While 6:2 FTOH was detected in <50% of dust samples in Strynar & Lindstrom, 6:2 FTOH concentrations were low in 2000-01 and high in 2014-16, similar to 8:2 FTOH. For Hall et al., 10:2 FTOH was not measured in house dust.

Table D8: Average percent recoveries (%) of mass-labeled internal standards used for dust measurements. All house dust data included in this table are adapted from the supplemental information of Kassotis *et al.* (2021), which also reports on PFAS concentrations in the same house dust samples. Perfect recovery is 100%, generally acceptable recovery is between 50-150%; recoveries outside that range are formatted in red text. Note: this Table D8 is an addition to the original publication; it was not originally published with the supplemental information of Hall *et al.* (2020).

Mass-Labeled Internal Standard (ISTD)	House Dust			Fire Station Dust		
	Amount Spiked (ng)	Average Percent Recovery	Unlabeled Analytes Quantified with ISTD	Amount Spiked (ng)	Average Percent Recovery	Unlabeled Analytes Quantified with ISTD
^{13}C 6:2 diPAP	172.4	43%	6:2 diPAP	125	61%	6:2 diPAP
^{13}C 8:2 diPAP	172.4	101%	8:2 diPAP	125	161%	8:2 diPAP
^{13}C PFBA	86.2	50%	PFBA	10	227%	PFBA
^{13}C PFHxA	86.2	49%	PFHxA, PFPeA	10	65%	PFHxA, PFPeA
^{13}C PFOA	86.2	48%	PFOA, PFHpA	10	64%	PFOA, PFHpA
^{13}C PFNA	86.2	52%	PFNA	10	52%	PFNA
^{13}C PFDA	86.2	47%	PFDA	10	55%	PFDA
^{18}O PFHxS	86.2	49%	PFHxS, PFBS	10	90%	PFHxS, PFBS
^{13}C PFOS	86.2	48%	PFOS	10	66%	PFOS
^{13}C 6:2 FTOH	100	32%	6:2 FTOH	NR	NR	6:2 FTOH
^{13}C 8:2 FTOH	100	42%	8:2 FTOH, 10:2 FTOH	NR	NR	8:2 FTOH
Deuterated MeFOSE	100	106%	MeFOSE	NR	NR	MeFOSE
Deuterated EtFOSE	100	84%	EtFOSE	NR	NR	EtFOSE

NR: not reported

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Biography

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