Domestic Dogs and Horses as Sentinels of Per- and Polyfluoroalkyl Substance (PFAS) Exposure and Associated Health Biomarkers in Gray's Creek North Carolina

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Supplemental Methods

Materials for PFAS analysis

Calibration solutions were prepared from neat standards (Wellington, PFAC-24PAR, fluoroether standards provided by the Chemours company) and combined in charcoal stripped fetal bovine serum (FBS; Life Technologies, Grand Island, NY; cat #10437, Lot #1754113; total protein 3.7 g/dL). Acetonitrile (ACN; Optima®), methanol (Optima®), ammonium formate (99%), formic acid (99.5%,), and isopropyl alcohol (99%,) used for extractions and UPLC mobile phase were purchased from Fisher Scientific (Hampton, NC). Laboratory glassware was rinsed with isopropyl alcohol, methanol, and acetonitrile prior to use. Deionized water (diH_2O) was used for laboratory blanks and were analyzed for contamination prior to use. National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1957 was utilized as an external validation to ensure accuracy and comparability of the method utilized to measure PFAS within serum.

Mass spectrometry

The PFAS analyzed were chosen based on compounds measured by Strynar et al. (2015), Sun et al. (2016), Kotlarz et al. (2020) and Guillette et al. (2020), and the availability of authentic standards [1, 2, 3, 4, 5]. At the time of analysis, analytical standards were available for 33 PFAS of interest (**Supplemental Table S2**). Standard curves were prepared in charcoal stripped fetal bovine serum and the % error was acceptable with +/- 30% accuracy for all points. There were three method blanks utilized for this analysis, a method blank containing fetal bovine serum, a method blank containing fetal bovine serum plus internal standard (12.5 ng), and a diH_2O only double blank. These were used to assess background PFAS contamination from several potential sources, including internal standard addition, solvents used in the analysis, and instrument background.

The mass spectrometry conditions and UPLC gradient were similar to Guillette et al. 2020 [1,2]. Briefly, PFAS were detected using a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray ionization (HESI) source operated in negative mode. Data were collected in data dependent mode for compound validation, with a preferred ion list consisting of the quantitated PFAS standards. Quantitation was based on an eight-point calibration curve (with two injections per concentration) of the internal standard normalized integrated peak area of the extracted ion chromatogram of the [M-H]-ion with a 3 to 5-ppm mass tolerance. The r^2 values of all calibration curves used for analysis were > 0.98. Branched and linear isomers of PFOS and PFOA were summed and reported as total PFOS and total PFOA. The mobile phase gradient and composition, source conditions, and quality assurance documents of this method (spike recovery of 10 ng/mL for all compounds monitored) were identical to our previous studies [3].

Quality controls

Within each batch analysis (three in total), four homogenous mixtures of dog and horse serum (10 µl taken from each experimental sample) were analyzed throughout the batch. Replicates of SRM 1957 (n = 6) were compared to the values on the Certificate of Analysis for the NIST SRM 1957 standards and were within 15.41% of expected values (**Table 1**). Charcoal stripped FBS used for the matrix-matched preparation of the standard curves was run as a method blank with internal standard and without.

Table 1: SRM 1957 Analysis

PFAS Abbreviation	SRM 1957 (ng/ml)	Certified Value (ng/mL)	% Difference
PFHpA	0.258 ± 0.025	0.305 ± 0.051	15.41
PFOA	4.995 ± 0.025	5.000 ± 0.440	0.10
PFNA	0.799 ± 0.067	0.878 ± 0.077	9.00
PFDA	0.407 ± 0.017	0.390 ± 0.120	4.36
PFOS	21.928 ± 1.728	21.100 ± 1.300	3.92
PFHxS	4.037 ± 0.067	4.000 ± 0.830	0.92
PFUnDA	0.194 ± 0.044	0.172 ± 0.036	12.79
NIST Standard Reference Material 1957, ran in triplicate within each batch of samples (n = 6)			

References

1. Sun, M. *et al.* Legacy and Emerging Perfluoroalkyl Substances Are Important Drinking Water Contaminants in the Cape Fear River Watershed of North Carolina. *Environ. Sci. Technol. Lett.* **3**, 415–419 (2016).
2. Guillette, T. C. *et al.* Elevated levels of per- and polyfluoroalkyl substances in Cape Fear River Striped Bass (Morone saxatilis) are associated with biomarkers of altered immune and liver function. *Environ. Int.* **136**, 105358 (2020).
3. Guillette, T. C., Jackson, T. W., Guillette, M. P., McCord, J. P. & Belcher, S. M. *Blood Concentrations of Per- and Polyfluoroalkyl Substances are Associated with Autoimmunity-like Effects in the American Alligator*. http://biorxiv.org/lookup/doi/10.1101/2022.02.15.480575 (2022) doi:10.1101/2022.02.15.480575.
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5. Strynar, M. *et al.* Identification of Novel Perfluoroalkyl Ether Carboxylic Acids (PFECAs) and Sulfonic Acids (PFESAs) in Natural Waters Using Accurate Mass Time-of-Flight Mass Spectrometry (TOFMS). *Environ. Sci. Technol.* **49**, 11622–11630 (2015).

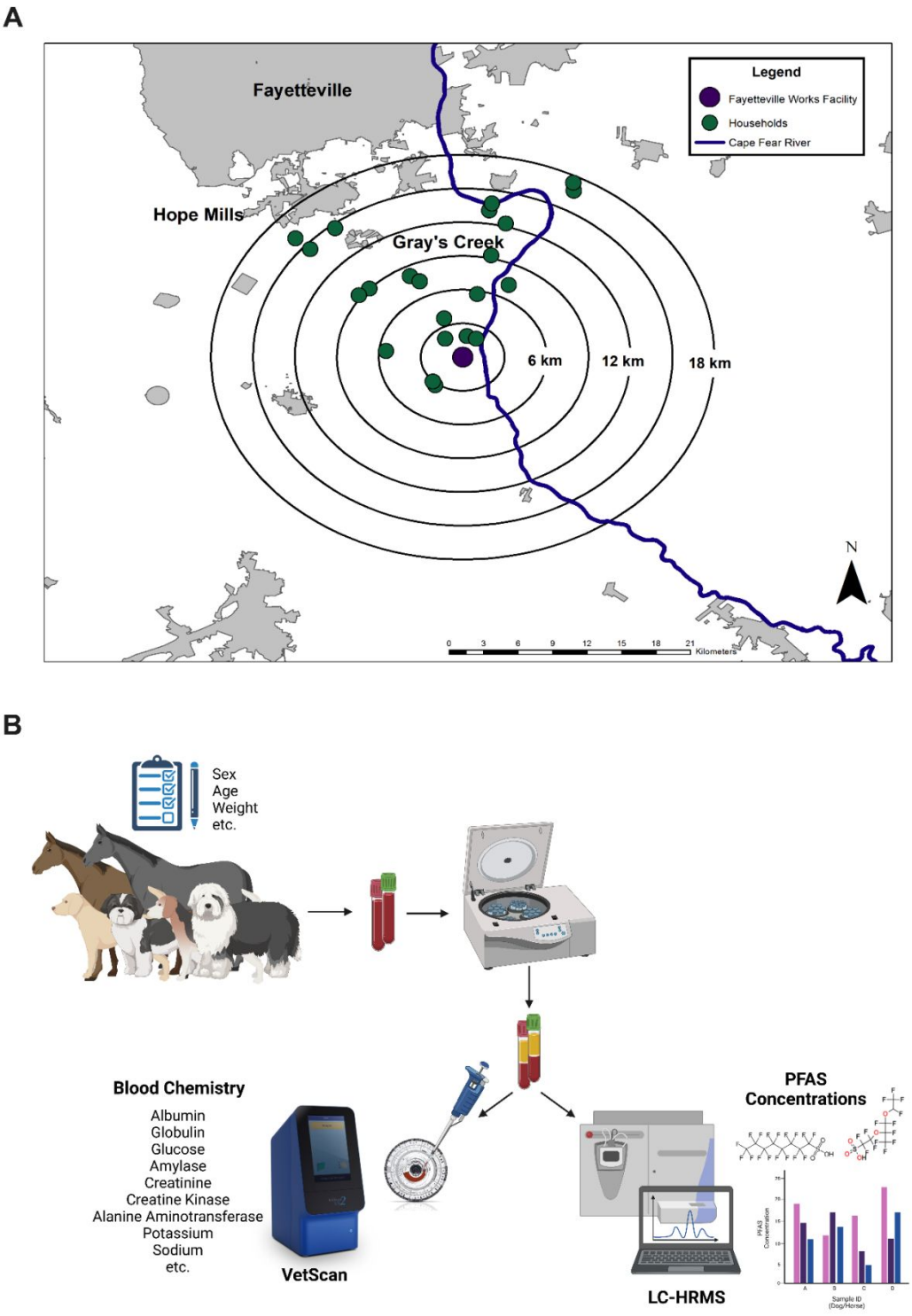

Figure S1. Study location and design. A) Distribution of households and their approximate distance from a nearby fluorochemical production facility. There was a total of 22 households, 31 dogs, and 35 horses enrolled in the study. **B)** Diagram of study design, created with BioRender.com.

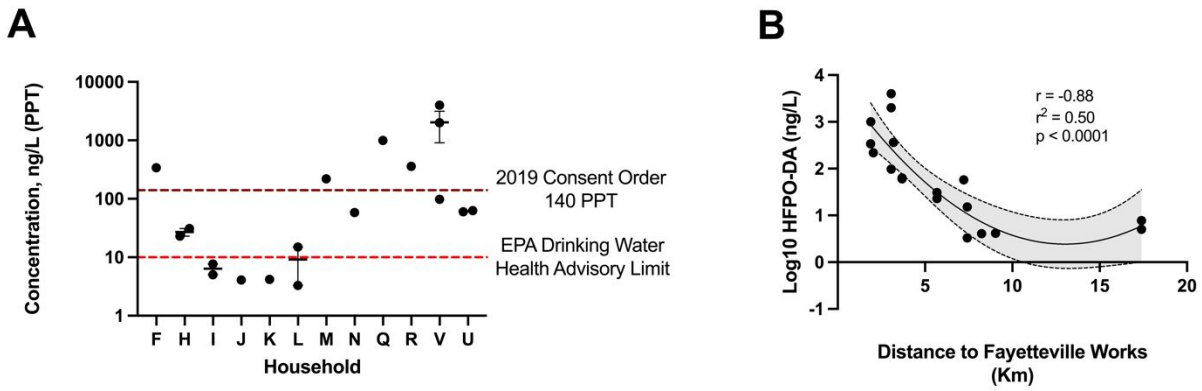

Figure S2. Concentrations of GenX in well water. A) Concentrations of GenX (HFPO-DA) in well water samples from households that participated in this study. **B)** Scatterplot of non-linear regression analysis with 95%CI, and Spearman rank correlation, for Log_{10} HFPO-DA concentration in well water and household distance from Fayetteville Works. Details regarding the company that analyzed the sample and date of analysis can be found in **Supplemental Table S1.**

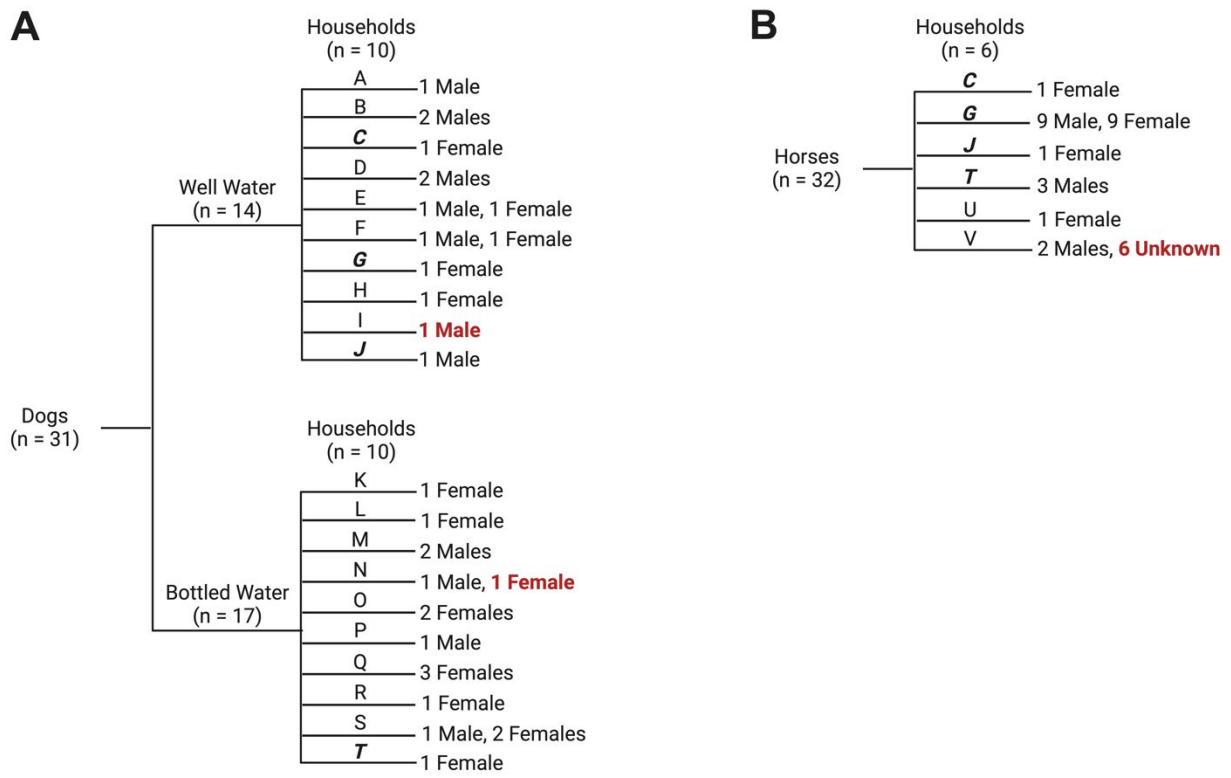

Figure S3. Flow diagram of dog and horse samples stratified by water source, household, and sex. A) Dogs (n = 31) in this study were provided with well water (n = 14) or bottled water (n = 17) as their primary source of drinking water. Households (n = 10) that provided their dogs with well water include A – J. Details on the number and sex of the animals from each household are provided with the total for well water dogs equaling 9 males and 5 females. Households (n = 10) that provided their dogs with bottled water include K – T, with a total of 5 males and 12 females. **B)** Horses (n = 32) in this study came from 6 different households with a total of 14 males and 12 females. Metadata, including sex, age, and weight were not obtained for 6 animals at household V. Serum samples from all animals shown in **A** and **B** were analyzed for PFAS (dogs n = 31, horses n = 32). However, samples labeled in red text were not used for diagnostic clinical chemistry assays due to lack of sufficient volume (dogs: household I, 1 male and household N, 1 female) or lack of metadata needed for statistical analysis (horses: household V, 6 unknown), making the final sample sizes for VetScan analysis 29 dogs and 26 horses. Bold, italicized letters (***C, G, J, T***) indicate households that enrolled both dogs and horses in the study.