Data to Support the Development of Wildlife Toxicity Reference Values for Per- and Polyfluoroalkyl Substances

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1 Summary

1.1 Purpose

Per- and polyfluoroalkyl substances (PFAS) are compounds manufactured for use in paints, cleaning agents, fire suppressants, nonstick cookware and food containers, and water-impermeable products in general. PFAS are also key ingredients in highly efficient fire suppressant agents known as Aqueous Film Forming Foams (AFFFs), which have been used by the Department of Defense (DOD) since approximately 1970 for fire-training and emergency response activities. Concerns about PFAS stem from their ubiquitous presence in the environment, widespread reports of general toxicity, and the resistance of these compounds to degradation. The primary goal of the Range Finding Experiments was to ensure bioaccumulation and sufficient body burden of the chemicals for Reproductive and Developmental Toxicity Tests. Data from the Reproductive and Developmental Toxicity Tests will be used to derive Toxicity Reference Values (TRVs), which are critical components of environmental risk assessments that help determine if the risk of environmental exposure is acceptable.

In the Range Finding Experiments, PFAS was administered orally to the white-footed mouse (*Peromyscus leucopus)* for 28 consecutive days. Blood samples was collected every 7 days via facial/submandibular venipuncture and analyzed for the concentration of PFAS. Selected tissues were weighed and processed for histopathology. This study will provide information on how various PFAS accumulate in the body with repeated, daily exposure via drinking water (i.e., bioaccumulation and body burden), how dose levels impact PFAS accumulation, and the toxic effects and possible target organs. Data will also help determine exposure levels and refine the design for upcoming reproductive/developmental toxicity studies with PFAS.

In the Reproductive and Developmental Toxicity Tests, PFAS was administered orally to the white-footed mouse for 28 consecutive days prior to establishing mating pairs. Mating pairs were orally exposed until a litter is generated. Fetuses and offspring were only exposed to the test compounds through pregnancy and maternal transfer (lactation). All animals were monitored for body weight changes and signs of toxicity. Immune function was evaluated toward the end of the dosing and observation periods through a foreign red blood cell challenge (IP injection). At the end of the study, blood was collected from anesthetized animals via decapitation and trunk blood collection. Data from this study will ultimately be used to derive TRVs.

2 Range Finding Experiments

2.1 Animals and Housing Conditions[1]

Adult male (n=198; 22.59 ± 0.237 g) and female (n=199; 20.43 ± 0.206 g) *Peromyscus leucopus* were obtained from the Peromyscus Genetic Stock Center at the University of South Carolina, Columbia, South Carolina. Animals were obtained for Experiments 1 and 2 in one shipment. Animals were obtained for Experiment 3 in a separate shipment. Animals were acclimated to the facility for at least 5 days before initiation of dosing in Experiments 1 and 3. Animals were acclimated the facility for 5 months before initiating dosing in Experiment 2. Females were between 136 and 238 days of age at the initiation of Experiment 1. Males were between 301 and 348 days of age at the initiation of Experiment 1. Females were between 304 and 406 days of age at the initiation of Experiment 2. Males were between 133 and 180 days of age at the initiation of Experiment 2. Females were between 301 and 392 days of age at the initiation of Experiment 3. Males were between 343 and 392 days of age at the initiation of Experiment 3.

Assignment to dose group and chemical was accomplished using a stratified random procedure, with animals stratified according to body mass and groups assigned by random draw. Within each experiment, body mass did not differ among groups prior to initiation of dosing. Each animal was uniquely identified by a cage card. Within each cage, animals were identified by a tail color. All animals were housed in temperature-, relative humidity-, and light-controlled rooms. The target conditions of the rooms were 68-72 degrees Fahrenheit (°F) and 30-70% humidity. An automatically controlled 12: 12 hour light: dark cycle was maintained, with the dark period beginning at 1800 hours. A certified pesticide-free rodent chow (Harlan Teklad® 2016C Certified Rodent Diet) was available *ad libitum*. Depending on the experiment, animals were provided with filtered tap water or treated filtered tap water *ad libitum*. Animals were same sex group housed in suspended 7” wide x 10.5” tall x 5” deep plastic cages on a static, stainless steel rack. Animal care and use procedures were approved by the U.S. Army Public Health Center (APHC) Institutional Animal Care and Use Committee. Animal care and use was conducted in accordance with *The Guide for the Care and Use of Laboratory Animals* (NRC 2011) and all applicable Federal and DOD regulations. The APHC Animal Care and Use Program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. This study was conducted consistent with Good Laboratory Practices (CFR 1989).

2.2 Test Substances

The six test substances were obtained from Sigma Aldrich, St. Louis, Missouri. Purity analyses for these compounds were conducted by the manufacturer. The six test substances are: perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), perfluorohexane sulfonate (PFHxS), perfluorobutane sulfonate (PFBS), tridecafluoroctane-1-sulphonic acid (6:2 FTS), and perfluorononanoic acid (PFNA).

[1] Animal use procedures were approved by the APHC Institutional Animal Care and Use Committee. Animal care and use was conducted in accordance with *The Guide for the Care and Use of Laboratory Animals* and all applicable Federal and DOD regulations. The APHC Animal Care and Use Program is fully accredited by the AAALAC International.

2.3 Dose Selection and Test Substance Preparation and Administration

Dose selection was based on the ultimate objective of the study: to establish how various PFAS accumulate in the body with repeat, daily exposure, how dose levels impact PFAS accumulation, and the toxic effects and possible target organs. The intent was not to cause or detect toxicity, if possible.

2.3.1 Experiment 1

Dosing solutions were prepared by weighing the required amount of neat PFOA, PFHxS, and PFBS, transferring to a 1000 milliliters (mL) volumetric flask, adding approximately 900 mL of water from the animal room, stirring using a magnetic stir bar and stir plate until dissolved, and adding water to 1000 mL. Three drinking water dosing solutions, 37.5, 75, and 150 milligrams per milliliter (mg/mL), were used through the experiment. Using an assumed default water consumption rate of 0.003 liters per kilogram per day (L/kg-day) and a default body weight of 0.0225 kg, dosing solutions corresponded to approximately 5, 10, and 20 milligrams per kilogram per day (mg/kg-day). Solutions were prepared weekly. Drinking water/dosing reservoirs were replaced completely every 4 days and reservoirs were changed every 2 weeks.

2.3.2 Experiment 2.1

Dosed treats were prepared by weighing the required volume of neat PFOS and adding the required amount of methanol to make a stock solution (18.5 mg/mL). Stock solution was applied to Fruit Loops with a pipette (at variable volumes) to create dosed treats. Treats were dried for 24 hours to ensure methanol evaporated off prior to being administered to animals. Three doses, 2.5, 25, and 250 mg/kg-day, were used through the experiment. Stock solution was prepared daily. Treats were prepared daily, 1 day prior to administration.

2.3.3 Experiment 2.2

Dosing suspensions were prepared by weighing the required amount of neat PFOS, transferring to a polypropylene container, measuring the appropriate volume of filtered tap water or corn oil using a graduated cylinder, and adding the tap water or corn oil to the polypropylene container. Three dosing solutions or suspensions, 0.25, 4.0, and 10.0 mg/mL, were used throughout the experiment, which corresponded to 2.5, 40, and 100 mg/kg-day. Solutions were prepared once and suspensions were prepared bi-weekly.

2.3.4 Experiment 3

Dosing solutions and suspensions were prepared by weighing the required volume of neat 6:2 FTS and PFNA, adding the required amount of methanol to make a stock solution (500 mg/mL), adding the appropriate amount of stock solution to a 50 mL volumetric flask, adding approximately 40 mL of water from the animal room, stirring using a magnetic stir bar and stir plate until dissolved, and adding water to 50 mL. Solutions were prepared bi-weekly.

Three dosing solutions, 0.25, 1.0, and 2.0 mg/mL, were prepared for 6:2 FTS, which corresponded to 2.5, 10, and 20 mg/kg-day. The 0.25 and 1.0 mg/mL 6:2 FTS dosing solutions were volumetrically adjusted such that they had the same volume of methanol as the 2.0 mg/mL 6:2 FTS dosing solution.

Two dosing solutions, 0.25 and 1.0 mg/mL, were prepared for PFNA, which corresponded to 2.5 and 10 mg/kg-day. One dosing suspension, 2.0 mg/mL, was prepared for PFNA, which corresponded to 20 mg/kg-day. The 0.25 and 1.0 mg/mL PFNA dosing solutions were volumetrically adjusted such that they had the same volume of methanol as the 2.0 mg/mL PFNA dosing suspension.

Dosing solutions were prepared by weighing the required volume of neat PFHxS, transferring to a 50 mL volumetric flask, adding approximately 40 mL of water from the animal room, stirring using a magnetic stir bar and stir plate until dissolved, and adding water to 50 mL. Two dosing solutions, 1.0 and 2.0 mg/mL, were used through the experiment, which corresponded to 10 and 20 mg/kg-day.

Because of the shared control group with 6:2 FTS and PFNA, the 1.0 and 2.0 mg/mL dosing solutions were volumetrically adjusted such that they had the same volume of methanol as the 2.0 mg/mL (6:2 FTS and PFNA) dosing solution and suspension.

2.4 Study Design

2.4.1 Experiment 1

PFOA, PFHxS, and PFBS were administered via drinking water to male and female white-footed mice for 28 consecutive days. This experiment utilized a shared control group.

2.4.2 Experiment 2.1

PFOS was administered via food treats to male and female white-footed mice for 2 and 3 days, respectively. Food treats are a well-documented exposure route for toxicity testing with rodents and even with flame retardant materials (Patisaul et al. 2009; McCaffrey et al. 2013; Hays et al. 2002).

2.4.3 Experiment 2.2

PFOS was administered via oral gavage to male and female white-footed mice for 28 consecutive days. Males and females received their first four and three doses, respectively, via filtered tap water. Animals received their remaining doses via corn oil (i.e., days 5 and 4 through 28 for males and females, respectively).

2.4.4 Experiment 3

6:2 FTS, PFNA, and PFHxS were administered via oral gavage to male and female white-footed mice for 28 consecutive days. This experiment utilized a shared control group.

2.5 Clinical Observations and Body Mass

Animals were removed from their home cages and observed daily by study personnel in conjunction with dosing for signs of toxicity, morbidity, and mortality. Body weights were taken at the start of test substance administration, at least weekly thereafter, and at termination.

2.6 Blood Collection

Blood was collected via submandibular venipuncture on days 0, 7, 14, and 21 of test substance administration. Blood was collected in weigh boats free of additives, transferred to microcentrifuge tubes, allowed to clot at room temperature for 60 to 120 minutes, and centrifuged twice for approximately 10 minutes at 600 x g. Serum was removed and frozen at -80 degrees Celsius (°C).

2.7 Necropsy, Trunk Blood Collection, and Organ Mass

After 28 days of dosing, surviving animals were anesthetized with carbon dioxide (CO_2). Animals were euthanized via decapitation and trunk blood was collected for serum analysis.

A full, detailed gross necropsy, including a careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents was performed on all experimental animals following euthanasia. At necropsy, the brain, heart, kidneys, liver, ovaries, uterus, spleen, thymus, testes, and epididymides were removed, trimmed, and weighed. Testes were weighed as pairs. Any observed lesions were retained for processing.

The brain, heart, kidneys, liver, ovaries, uterus, spleen, and thymus, were stored in 10% buffered formalin for fixation. The testes and right epididymis from each animal were placed in Davidson's fixative overnight (no longer than 24 hours). After fixation, the tissues were rinsed with deionized water and stored in 70% ethanol.

2.8 Sperm Analysis

Sperm analysis was only conducted for Experiment 3. The left epididymis from each male was removed during necropsy and submitted for sperm analysis. In preparation, 10 mL of Gibco® Medium 199 (M199) was pipetted into each well of Corning® Costar® 6-well cell culture plates and warmed on a slide warmer to approximately 37ºC. Each epididymis was weighed, placed in a well containing M199, minced using small scissors, and incubated for 5 minutes at approximately 37ºC. Then, samples were loaded on a standard count chamber slide (Leja®). Slides were immediately loaded into a Hamilton-Thorne IVOS Sperm Analysis System® and read using the IDENT® program.

2.9 Histopathology

Full histopathology was performed for all high-dose and control animals at the discretion of the pathologist and based on observed toxicity and gross pathology findings. Organs demonstrating treatment-related changes were examined in animals in the lower dose groups. Gross lesions were subjected to histopathological analysis at the discretion of the pathologist. Histopathologic findings were subjectively graded across a 6-point scale: Grade 0 (normal: <1% of tissue area affected); Grade 1 (minimal: <5% of tissue area

affected); Grade 2 (mild: 6-20% of tissue area affected); Grade 3 (moderate: 21-40% of tissue area affected); Grade 4 (marked: 41-80% of tissue area affected); and Grade 5 (severe: >80% of tissue area affected) (Schafer et al. 2018).

2.10 Data Collection and Statistical Analyses

Experimental data generated during the course of this study were recorded by band and tabulated, summarized, and/or statistically analyzed using Microsoft® Excel and Minitab®. Environmental data were automatically recorded using MetaSys® Building Management System.

Data not normally distributed was log transformed and retested for normality. Variance equality was determined by Levene's test. Analyses were conducted for males and females separately. Parameters measured multiple times (i.e., body mass) were analyzed using repeated measures one-way analysis of variance (ANOVA) and those measured at the end of the study (i.e., organ weights) were analyzed using ANOVA with dose group as the main effect. Absolute organ mass was analyzed by analysis of covariance (ANCOVA) using dose group as the main effect and body weight at necropsy as the covariate (Bailey et al. 2004). Fisher's exact test was used to determine significant differences between treated and control groups for nominal or count data (e.g., histology). Statistical significance are defined at the $p < 0.05$ level. If dose effect was significant ($p < 0.05$), appropriate post hoc analyses were performed (Tukey's multiple comparison (ANOVA) or Sidak (for ANCOVA). Interpretation of changes in absolute organ mass, organ-to-body mass ratio, and organ-to-brain mass ratio in the evaluation of compound-related effects was based on published analysis of control animal data (Bailey et al. 2004).

3 Reproductive and Developmental Toxicity Tests

3.1 Animals and Housing Conditions

For Experiment 1, adult male (n=124; 22.45 ± 0.312 g) and female (n=124; 19.80 ± 0.256 g) *Peromyscus leucopus* were obtained from the Peromyscus Genetic Stock Center at the University of South Carolina, Columbia, South Carolina. For Experiment 2, adult male (n=125; 21.23 ± 0.251 g) and female (n=125; 18.90 ± 0.233 g) *Peromyscus leucopus* were obtained from the Peromyscus Genetic Stock Center at the University of South Carolina, Columbia, SC. Animals were acclimated to the facility for at least 5 weeks before initiation of dosing in Experiments 1 and 2. Females were between 203 and 406 days of age at the initiation of Experiment 1. Males were between 150 and 412 days of age at the initiation of Experiment 1. Females were between 117 and 357 days of age at the initiation of Experiment 2. Males were between 118 and 349 days of age at the initiation of Experiment 2.

In Experiment 1, assignment to dose group was accomplished using a stratified random procedure, with animals stratified according to body mass and evenly spread among the dose groups, also taking age and known genetic background into consideration. In Experiment 1, mating pairs were randomly assigned within each dose group. In Experiment 2, assignment to dose group was accomplished using a stratified random procedure, with animals stratified according to age and evenly spread among the dose groups, also taking body mass and known genetic background into consideration. In Experiment 2, mating pairs were assigned according to age. Within each experiment, body mass did not differ among groups prior to initiation of dosing. Each animal was uniquely identified by a cage card.

Within each cage, animals were identified by a tail color. All animals were housed in temperature-, relative humidity-, and light-controlled rooms. The target conditions of the rooms were 68-72°F and 30-70% humidity. An automatically controlled 16:8 hour light: dark cycle was maintained, with the dark period beginning at 1800 hours. A certified pesticide-free rodent chow (Harlan Teklad 2016C Certified Rodent Diet) was available *ad libitum*. Animals were same sex group housed in suspended 7" wide x 10.5" tall x 5" deep plastic cages on a static, stainless steel rack. Animal care and use procedures were approved by the APHC Institutional Animal Care and Use Committee. Animal care and use was conducted in accordance with *The Guide for the Care and Use of Laboratory Animals* (NRC 2011) and all applicable Federal and DOD regulations. The APHC Animal Care and Use Program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. This study was conducted consistent with Good Laboratory Practices (CFR 1989).

3.2 Test Substances

The six test substances were obtained from Sigma Aldrich, St. Louis, Missouri. Purity analyses for these compounds were conducted by the manufacturer. The six test substances are: PFOS, PFOA, PFHxS, PFBS, 6:2 FTS, and PFNA.

3.3 Dose Selection and Test Substance Preparation and Administration

3.3.1 Experiment 1

Dosing suspensions were prepared by weighing the required amount of neat PFOS, transferring to a polypropylene container, measuring the appropriate volume of filtered tap water using a graduated cylinder, and adding the tap water to the polypropylene container. Three dosing solutions 0.02, 0.1, and 0.5 mg/mL, were used throughout the experiment, which corresponded to 0.2, 1.0, and 5.0 mg/kg-day. Solutions were prepared bi-weekly.

3.3.2 Experiment 2

Dosing solutions were prepared by weighing the required volume of neat PFNA, adding the required amount of methanol to make a stock solution (500 mg/mL), adding the appropriate amount of stock solution to a 1000 mL volumetric flask, adding approximately 600 mL of water from the animal room, stirring using a magnetic stir bar and stir plate until dissolved, and adding water to 400 mL. Four dosing solutions, 0.004, 0.02, and 0.1, and 0.5 mg/mL, were used throughout the experiment, which corresponded to 0.04, 0.2, 1.0, and 5.0 mg/kg-day. Solutions were prepared bi-weekly.

3.4 Study Design

3.4.1 Experiment 1

PFOS was administered via oral gavage drinking water to male and female white-footed mice (Parental (P) generation animals) for 28 consecutive days prior to establishing mating pairs. Pairs were orally exposed until a litter was generated or until 16 weeks of co-housing had elapsed without the generation of a litter. Fetuses

and offspring (First Filial (F1) generation animals) were only exposed to the test compounds through pregnancy and maternal transfer (lactation).

3.4.2 Experiment 2.1

PFNA was administered via oral gavage drinking water to male and female white-footed mice (Parental (P) generation animals) for 28 consecutive days prior to establishing mating pairs. Pairs were orally exposed for a total of 16 weeks, regardless of generation of a litter. Fetuses and offspring (First Filial (F1) generation animals) were only exposed to the test compounds through pregnancy and maternal transfer (lactation).

3.5 Clinical Observations and Body Mass

Animals were removed from their home cages and observed daily by study personnel in conjunction with dosing for signs of toxicity, morbidity, and mortality. Body weights were taken at the start of test substance administration, at least weekly thereafter, and at termination.

3.6 Necropsy, Trunk Blood Collection, and Organ Mass

In Experiment 1, at PND25 (or after 16 weeks of co-housing without the generation of a litter), surviving P generation animals were anesthetized with carbon dioxide (CO_2). Animals were euthanized via decapitation and trunk blood was collected for hormone analysis. In Experiment 1, at PND57-63, surviving F1 generation animals were anesthetized with carbon dioxide (CO_2). Animals were euthanized via decapitation and trunk blood was collected for hormone analysis (if possible).

In Experiment 2, surviving P generation animals will be anesthetized with carbon dioxide (CO_2) after 16 total weeks of exposure to PFNA. Animals will be euthanized via decapitation and trunk blood was collected for hormone analysis. In Experiment 2, at PND4-10, surviving F1 generation animals will be anesthetized with carbon dioxide (CO_2). Animals will be euthanized via decapitation and trunk blood was collected for hormone analysis (if possible).

A full, detailed gross necropsy, including a careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents was performed on all experimental animals following euthanasia. At necropsy, the brain, heart, kidneys, liver, ovaries, uterus, spleen, thymus, testes, and epididymides were removed, trimmed, and weighed. Testes were weighed as pairs. Any observed lesions were retained for processing.

The brain, heart, kidneys, liver, ovaries, uterus, spleen, and thymus, were stored in 10% buffered formalin for fixation. The testes and right epididymis from each animal were placed in Davidson's fixative overnight (no longer than 24 hours). After fixation, the tissues were rinsed with deionized water and stored in 70% ethanol.

3.7 Sperm Analysis

Sperm analysis was only conducted for P generation animals. The left epididymis from each male was removed during necropsy and submitted for sperm analysis. In preparation,

10 mL of Gibco Medium 199 (M199) was pipetted into each well of Corning Costar 6-well cell culture plates and warmed on a slide warmer to approximately 37ºC. Each epididymis was weighed, placed in a well containing M199, minced using small scissors, and incubated for 5 minutes at approximately 37ºC. Then, samples were loaded on a standard count chamber slide (Leja). Slides were immediately loaded into a Hamilton-Thorne IVOS Sperm Analysis System and read using the IDENT program.

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Full histopathology was performed for all high-dose and control animals at the discretion of the pathologist and based on observed toxicity and gross pathology findings. Organs demonstrating treatment-related changes were examined in animals in the lower dose groups. Gross lesions were subjected to histopathological analysis at the discretion of the pathologist. Histopathologic findings were subjectively graded across a 6-point scale: Grade 0 (normal: <1% of tissue area affected); Grade 1 (minimal: <5% of tissue area affected); Grade 2 (mild: 6-20% of tissue area affected); Grade 3 (moderate: 21-40% of tissue area affected); Grade 4 (marked: 41-80% of tissue area affected); and Grade 5 (severe: >80% of tissue area affected) (Schafer et al. 2018).

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