Determination of Ten Perfluorinated Compounds in Bluegill Sunfish (*Lepomis macrochirus)* Fillets

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Abstract

A rigorous solid phase extraction/liquid chromatography/tandem mass spectrometry method for the measurement of 10 perfluorinated compounds (PFCs) in fish fillets is described and applied to fillets of bluegill sunfish (*Lepomis macrochirus)* collected from selected areas of Minnesota and North Carolina. The 4 PFC analytes routinely detected in bluegill fillets were perfluorooctane sulfonate (PFOS), perfluorodecanoic acid (C10), perfluoroundecanoic acid (C11), and perflurododecanoic acid (C12). Measures of method accuracy and precision for these compounds showed that calculated concentrations of PFCs in spiked samples differed by less than 20% from their theoretical values and that the %RSD for repeated measurements was less than 20%. Minnesota samples were collected from areas of the Mississippi River near historical PFC sources, from the St. Croix River as a background site, and from Lake Calhoun, which has no documented PFC sources. PFOS was the most prevalent PFC found in the Minnesota samples, with median concentrations of 47.0 – 102 ng/g at locations along the Mississippi River, 2.08 ng/g in the St. Croix River, and 275 ng/g in Lake Calhoun. North Carolina samples were collected from two rivers with no known historical PFC sources. PFOS was the predominant analyte in fish taken from the Haw and Deep Rivers, with median concentrations of 30.3 and 62.2 ng/g, respectively. Concentrations of C10, C11, and C12 in NC samples were among the highest reported in the literature, with respective median values of 9.08, 23.9, and 6.60 ng/g in fish from the Haw River and 2.90, 9.15, and 3.46 ng/g in fish from the Deep River. These results suggest that PFC contamination in freshwater fish may not be limited to areas with known historical PFC inputs.
Keywords: LC/MS/MS; PFOS; PFOA; fish; SPE; perfluorinated compounds

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Human and Animal Studies

No human subject samples were used in this study. All fish in this study were deceased before arrival at the U.S. EPA laboratories.

Introduction

Over the past 50 years, perfluorinated compounds (PFCs) have been used in many industrial and consumer products, including water repellants, paper coatings, cosmetics, and fire fighting foams (Prevedouros et al., 2006). As a result of their widespread use and resistance to degradation, PFCs are now globally distributed in the environment, and some are routinely measured at the ng/mL level in the blood of people living in industrialized nations (Kannan et al., 2004; Calafat et al., 2007). Human exposure to PFCs is of concern because the two most well-known and most commonly detected PFCs in human blood, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), have long half-lives in humans and both compounds exhibit toxicity in laboratory animals (Seacat et al., 2003; Kennedy et al., 2004; Lau et al., 2007).

Many of the PFCs are soluble in water, and a number of studies have indicated that PFCs are dispersed in the environment through normal hydrological processes (Simcik and Dorweiler; 2005; Scott et al., 2006). Many studies have found that PFOS and other PFCs are present in fish, with some of the compounds having bioconcentration factors of 4000 or more (Martin et al., 2003; Taniyasu et al., 2003; Sinclair et al., 2006; Furdui et al., 2007). Most of these investigations have focused on the analysis of whole fish homogenates and fish livers (Taniyasu et al., 2003; Martin et al., 2004; Hoff et al., 2005; Houde et al., 2006a), but a smaller number have focused on fish fillets (Giesy and Kannan, 2001; Hoff et al., 2003; Kannan et al., 2005; McCann et al., 2007). From a human exposure standpoint, the analysis of fish fillets should present a more accurate assessment of potential human dietary exposure to PFCs, because fillets of fish are commonly consumed by humans.

The bluegill sunfish (*Lepomis macrochirus*) is a widely distributed fish species that is commonly caught and consumed by humans, making it potentially useful for evaluating human exposures to aquatic contaminants. This species is native to much of the eastern United States, southern Canada and northern Mexico and has been introduced to many areas throughout the world including Asia, Africa, Central America, South America and islands in the Caribbean (Page and Burr, 1991). PFCs have been shown to accumulate in bluegill at concentrations similar to those found in some higher trophic level predators (McCann et al., 2007), suggesting that bluegills should be sensitive indicators of PFCs in the environment. Moreover, individual bluegills have a home range of less than 30 square meters (Parr, 2002). Therefore, PFCs accumulated in bluegill fillet should be representative of the immediate environment, making this fish species a sensitive indicator of local contaminant conditions. Because bluegills are prolific, easy to catch, and have favorable culinary attributes, this commonly eaten fish may be a good sentinel species for determining potential human dietary exposures to PFCs. This species should also be an excellent sentinel species for determining the geographic distributions of PFCs in the environment.

The analysis of perfluorinated compounds has been historically difficult due to a variety of issues, including contamination of analytical reagents and instrumentation, a lack of high quality native and internal standards, and insufficient cleanup of biological and environmental extracts (van Leeuwen et al., 2006). While contamination continues to be a challenge, researchers better understand this issue and have been able to take steps to monitor and minimize potential problems associated with background contamination of laboratory equipment, supplies, and solvents. Over the past several years, there has

been a dramatic increase in the number of commercially available perfluorinated standards and particularly labeled perfluorinated internal standards, which greatly aids with the development of reliable analytical methods. Additionally, recent widespread use of improved extraction and clean up techniques, such as alkaline digestion and solid phase extraction (SPE), has led to more consistent results in inter-laboratory studies (Taniyasu et al., 2005; So et al., 2006) when compared with earlier studies that used ion-paring extraction techniques. These improvements in PFC analysis have allowed researchers to more reliably perform analysis on a wide variety of analytical matrices, including blood serum, tissue homogenates, house dust, and breast milk (Longnecker et al., 2008; Reiner et al., 2008; Strynar and Lindstrom, 2008; Tao et al., 2008). While analytical methods have been steadily improving over the past several years, it remains important for analysts to continue to strive for improvement. An essential part of assessing potential method improvement and performance is to continuously evaluate a method in terms of its precision, accuracy, specificity, and recovery.

The objectives of this study were to rigorously characterize the performance of a solid phase extraction (SPE) liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the determination of 10 target PFCs in bluegill fillets and to demonstrate the applicability of the assay to fish fillet samples. The 10 PFC analytes include 3 perfluorinated sulfonates (PFBS, PFHS, PFOS) and 7 perfluorinated carboxylic acids (C6 – C12). Method performance was thoroughly evaluated by determining precision, accuracy, recovery, and matrix effects. Bluegill fillets from several bodies of water in Minnesota (MN), including the St. Croix River, Lake Calhoun, and the Mississippi River were analyzed and compared with bluegill taken from parts of the Cape

Fear River Basin in North Carolina (NC) that are known to contain PFCs (Nakayama et al., 2007). With method performance parameters established, the resulting data were used to help assess PFC distributions in these river systems and provide information concerning potential human exposures.

Materials and Methods

Chemicals and Reagents. Potassium salts of perfluorobutane sulfonate (PFBS, 98% purity) and perfluorohexane sulfonate (PFHS, 93%) were provided by 3M Company (St. Paul, MN). The potassium salt of perfluorooctane sulfonate (PFOS, 98%) was purchased from Fluka (Buchs, Switzerland). Perfluorohexanoic acid (C6, 97%), perfluoroheptanoic acid (C7, 99%), perfluorooctanoic acid (PFOA, 96%), perfluorononanoic acid (C9, 97%), and perfluorodecanoic acid (C10, 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Perfluoroundecanoic acid (C11, 96%) and perfluorododecanoic acid (C12, 96%) were purchased from Oakwood Products (West Columbia, SC). Two mass labeled internal standards were used for the quantitation of PFCs. $^{18}O_2$-Ammonium perfluorooctane sulfonate ($^{18}O_2$-PFOS) was purchased from RTI International (Research Triangle Park, NC). 1,2-$^{13}C_2$-labeled PFOA ($^{13}C_2$-PFOA) was purchased from Perkin-Elmer Life and Analytical Sciences, Inc. (Waltham, MA). HPLC-grade methanol (MeOH) was purchased from Burdick-Jackson (Muskegon, MI) and contained no measurable PFCs. Deionized (DI) water was obtained from a Barnstead EASYpure UV/UF compact reagent grade water system (Dubuque, IA) and had no detectable amounts of PFCs. Sodium hydroxide (NaOH), sodium acetate, glacial acetic acid,

ammonium acetate and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich (St. Louis, MO).

Fish Collection. Bluegill from Minnesota (n = 30) were collected by the Minnesota Department of Natural Resources and the Minnesota Pollution Control Agency. Fish were collected by electroshocking and netting in November 2006 and included 5 fish each from the St. Croix River, Lake Calhoun, and 4 locations (Pools 3, 4, 5, and 5a) on the Mississippi River (Figure 1). Sampling locations on the Mississippi River were chosen due to their downstream proximity to known historical sources of PFCs (McCann et al., 2007). The St. Croix River is named as a National Scenic Riverway by the National Park Service and was chosen as a potential background sampling site because it has minimal known industrial inputs. Samples from Lake Calhoun were collected and included in this study because a previous study found that samples from this location contained elevated levels of PFOS (McCann et al., 2007). Samples were kept on ice at the time of collection and then stored in a laboratory freezer until they were filleted skin-on and ground using a meat grinder in a preparatory lab. Aliquots of the ground fillets were stored at -20ºC until they were shipped to the analytical laboratory on dry ice, after which they were stored at -20ºC until analysis.

Bluegill collected in North Carolina (NC) were caught in the Haw (n = 31) and Deep Rivers (n = 30) in May 2007 by angling and electroshocking (Figure 2). These rivers are located in north central NC and represent the headwaters of the Cape Fear River Basin, the largest river basin in NC (NCDENR, 2004). Samples were taken approximately 3 miles upstream of the confluence of the Haw and Deep Rivers, which join to form the Cape Fear River (Figure 2). These areas were chosen for sampling

because a previous study indicated that PFCs were present in the river water at these locations in the tens of ng/L and because water from the two rivers had different proportions of various PFCs in spite of their proximity to one another (Nakayama et al., 2007). All fish were stored on ice until they were filleted. Fillets from each fish were cut into smaller chunks and were stored at -20ºC until sample analysis.

Sample Homogenization and Preparation. For each fish, an aliquot of the ground fillets (MN) or the entire unground fillet (NC) was homogenized. Deionized water was added to fillets at a ratio of 3 mL for every gram of fish tissue prior to homogenization using either a Polytron PT 10/35 homogenizer (Brinkmann Instruments, Westbury, NY) or a Waring blender (Waring Laboratory Science, Torrington, CT). Fish fillet homogenate was stored in 50 mL Falcon tubes at -20ºC until analysis. Fillets from several species of fish were tested for use as blank matrix in sample analysis. Tilapia (*Tilapia aurea)* fillets purchased from a local market (Grand Asia Market, Cary, NC) had the lowest PFC concentrations of all species tested. Homogenates of tilapia fillet tissue (3:1 DI water:fillet tissue) were used for matrix blanks and matrix matched calibration curves. Tissue from each tilapia was tested to ensure that all PFCs were below the limit of quantitation (LOQ) before use. All fish fillet homogenate samples, matrix blanks, standards, and quality controls (QCs, discussed below) were thawed and re-homogenized using a Polytron homogenizer immediately before sample preparation.

Each analytical batch consisted of approximately 20 bluegill samples, 1 method blank (deionized water), 2 matrix blanks (tilapia fillet homogenate), 8 calibration curve standards (spiked tilapia homogenate), and 4 QCs (unspiked bluegill homogenate with naturally occurring PFCs). All samples, blanks, standards, and QCs were subjected to the

same sample preparation, which was a modification of a previously described method that has been used for fish fillet and whole fish analysis (Taniyasu et al., 2005; Ye et al., 2008a; Ye et al., 2008b). Briefly, 2 mL of bluegill homogenate, tilapia blank homogenate, or deionized water (for method blanks) were placed into pre-weighed 15 mL Falcon tubes (Becton Dickinson, Franklin Lakes, NJ). The net weight of fish fillet homogenate was determined by reweighing the tubes containing fillet homogenate. Appropriate amounts of all 10 PFC analytes were spiked into blank tilapia fillet samples to make 8 matrix matched calibration curve standards. Eight mL of a 0.01 N NaOH in MeOH solution containing both $^{18}O_2$-PFOS and $^{13}C_2$-PFOA (15 ng each per 8 mL of solution) were added to each sample tube.

Samples were vortexed and placed on a Lab-Line Lab Rotator (Melrose Park, IL) with gentle shaking action at room temperature overnight (16 hours). Vigorous shaking was then performed for 30 minutes using a Lab-Line Orbit Environ-Shaker (Melrose Park, IL). Each sample was centrifuged at 2000 *g* for 5 minutes. An aliquot of the supernatant (3 mL) was added to 27 mL of deionized water and vortexed. SPE was performed on the diluted supernatant using Waters Oasis 3 cc WAX cartridges (60 mg sorbent, 60 µm particle size) and a Waters vacuum manifold. Cartridges were conditioned with 4 mL of 0.03% NH_4OH in MeOH followed by 4 mL of MeOH and 4 mL of deionized water. The entire 30 mL sample was then loaded onto the cartridge. The cartridges were washed with 4 mL of acetate buffer (25 mM, pH = 4) followed by 4 mL of MeOH. PFCs were eluted from the SPE cartridge using 4 mL of 0.03% NH_4OH in MeOH. The eluate was captured in 15 mL Falcon tubes and evaporated under nitrogen gas to 0.5 mL in a Zymark TurboVap concentrator (Caliper Life Sciences, Mountain

View, CA). After vortexing, a 280 µL aliquot of the concentrated methanolic eluate was placed in an autosampler vial with 120 µL of 2 mM ammonium acetate buffer (final solvent composition of 70% MeOH, 30% aqueous buffer).

Instrumental Analysis. Sample analysis was performed using an LC/MS/MS system consisting of an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) interfaced with a Sciex 3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The HPLC method consisted of a 10 minute isocratic run using a mobile phase of 75% MeOH and 25% 2 mM ammonium acetate. The flow rate was 200 µL/min and the column used was a Phenomenex Luna C18 (2) (3 mm x 50 mm) with an injection volume of 10 µL. Electrospray negative ionization was used in the mass spectrometer source, which was maintained at 350ºC. An ionspray voltage of -1500 V was used for all compounds. Mass transitions for each analyte and internal standard were monitored using multiple reaction monitoring (MRM) and analyte-specific mass spectrometer parameters were optimized for each compound (Table 1). A mass spectrometer method monitoring a quantitation ion for each analyte was used for sample analysis. The potential for incorrect quantitation of PFOS and PFHS in biological samples has been reported (Benskin et al., 2007). Therefore, a subset of the samples were analyzed using a mass spectrometer method containing quantitation and confirmation ion transitions for each analyte (Table 1) in order to ensure proper analyte identification and quantitaiton.

Quantitation. Matrix-matched calibration curves, which were run at the beginning and end of each sample batch analysis, consisted of 8 points that ranged in concentration from 1 – 600 ng/g fish (wet weight) for PFOS and from 0.4 – 50 ng/g fish (wet weight) for the

remaining 9 PFCs. Analyst software (version 1.4.2, Applied Biosystems/MDS Sciex, Foster City, CA) was used for quantitation of all compounds. $^{18}O_2$-PFOS was the internal standard for PFOS, PFHS, and PFBS, whereas $^{13}C_2$-PFOA was the internal standard for the C6 – C12 acids. Ratios of analyte peak area to internal standard peak area were used for quantitation. Linear calibration curves were used with a weighting of 1/x. Calibration curves were required to have correlation coefficients of 0.99 or greater and calibration points were required to be within ± 30% of the theoretical value for the lowest calibration curve point and ± 20% of the theoretical value for all other points in order to be used for the quantitation of unknown samples.

Quality Control. Two pools of quality control samples (QCs) were made from unspiked bluegill fillet homogenate that contained naturally occurring levels of PFCs. Homogenates used in the QC pools consisted of 3 mL of deionized water for each gram of bluegill fillet. Aliquots of each QC pool (approximately 2 mL) were placed into pre-weighed Falcon tubes and stored at -20ºC until analysis. Each QC pool was characterized by the mean and standard deviation of QC samples that were analyzed on multiple days. QC samples and analytical batches were considered acceptable if the mean of duplicate QC samples were within 2 standard deviations of their characterized mean.

Method Performance. Inter-day and intra-day precision and accuracy were determined by spiking blank tilapia matrix with 3 different known PFC concentration levels on multiple days. Accuracy measurements were calculated as the absolute value of the percent difference between theoretical and calculated concentrations, whereas precision was calculated as percent relative standard deviation (%RSD). Recovery of the method was determined by spiking one set of blank tilapia homogenate with PFCs prior to SPE

(Set A), spiking a different set of blank tilapia homogenate extracts with PFCs after SPE (Set B), and comparing the ratios of calculated concentrations between the two sample groups (Set A/Set B).

Matrix effects were assessed by comparing the ratios of tilapia blank homogenate extracts spiked with PFCs after SPE elution to a solvent standard of equal concentration. Values of 100% indicate no ion suppression or enhancement, values above 100% indicate ion enhancement, and values below 100% indicate ion suppression. An additional matrix effect experiment was performed by spiking a known amount of PFCs into previously analyzed bluegill samples and measuring the accuracy of the concentration increase in order to assess whether similar instrument responses were obtained for spiked bluegill homogenate as compared to spiked tilapia homogenate.

The limit of detection (LOD) and limit of quantitation (LOQ) for each PFC was determined by calculating the standard deviation of the calculated concentrations for each of the lowest 4 calibration standards from several calibration curves that were each run on separate days. The standard deviations were plotted against the theoretical concentrations for each calibration point. A linear regression analysis was performed and the y-intercept was equal to S_0, the standard deviation as the concentration approaches zero. The LOD was calculated as $3S_0$ and the LOQ was determined as $10S_0$ (Taylor, 1987).

Statistical Analysis. Statistical analysis of the data was performed using R version 2.6.0 software (Vienna University of Economics and Business Administration, Vienna, Austria). Wilcoxon rank sum tests were applied for assessing differences in measurements between the Haw and Deep Rivers. Additional statistical analysis of North Carolina bluegill data was performed using Spearman's correlation tests for non-

parametric analysis with a significance level (p) of 0.05. The Spearman's correlation coefficient (ρ), given to show the strength of correlations, and the significance level (p) are given for each comparison. Samples below the LOQ were assigned a value of one half of the LOQ for determination of statistical differences, averages, medians, standard deviations, and ranges.

Results and Discussion

Method Performance. The LOQ values were calculated using analytical values measured for the lowest four calibration curve standards from several calibration curves on separate days and are as follows: 0.52 ng/g for PFOS; 5.21 ng/g for C7; and a range from 0.01 – 1.89 ng/g for the remaining eight analytes (Table 2). Inter-day and intra-day precision and accuracy values in spiked tilapia matrix had less than 10% RSD and were less than 10% different from theoretical values for PFOS and were less than 20% different and had less than 20% RSD for all other analytes with the exception of C6 and C7 (Table 2). All PFC concentrations quantitated in QC samples were within 2 standard deviations of the characterized average PFC concentrations in the QC samples. Recoveries in spiked tilapia were 100% ± 10% for PFOS and ranged from 76 to 133% for all other analytes (Table 2). Matrix effects were 100% ± 10% for PFOS and ranged between 61 and 111% for the remaining analytes (Table 2). Addition of PFCs to bluegill samples indicated that in most cases matrix effects were minimal because the accuracy of these samples was 100% ± 20% for 22 out of 30 measurements made in 3 fish fillet samples (Table 3). The fact that good accuracies were obtained for both tilapia and bluegill suggests that this method may also work well for fillets from additional fish

species. Calculated concentrations for PFOS were consistently higher for the confirmation ion than for the quantitation ion. However, for each individual sample for which quantitation and confirmation ions were monitored, calculated concentrations were within 25% of each other for all detectable analytes, indicating that all analytes were properly identified and quantitated. Taken together, these method performance parameters represent a more thorough method characterization than has been previously reported and indicate that the method is reliable and reproducible for the analysis of the most commonly identified PFCs in bluegill homogenate.

Concentrations of PFCs in Minnesota Bluegill. Mean concentrations, standard deviations, medians, geometric means, and concentration ranges for Minnesota bluegills can be found in Table 4. Only PFOS, C10, C11, and C12 were detected in these samples, while the remaining 6 analytes were below the LOQ for all samples. PFOS was the most prevalent PFC, as it was detected in all samples and represented greater than 82% of total PFCs in all 30 samples. Fish collected from the St. Croix River, an area with minimal known industrial activity, had low but measureable PFOS concentrations, with median levels of 2.08 ng/g. Samples from the Mississippi River were collected from areas that are downstream from known historical PFC sources in the Minneapolis-St. Paul metropolitan area, with Pool 3 located closest to the known potential PFC sources and Pool 5a located the most distant downstream (Figure 1). Median PFOS concentrations were 89.5 ng/g (Pool 3), 102 ng/g (Pool 4), 47.0 ng/g (Pool 5), and 47.0 ng/g (Pool 5a), suggesting an overall reduction in PFOS accumulation as the distance from the historical sources increased. Bluegill from Lake Calhoun had the highest PFOS levels measured in this study, with a median concentration of 275 ng/g. Lake Calhoun is located within the

Minneapolis – St. Paul metropolitan area (Figure 1), but there are no documented sources of PFCs in this lake. An investigation is underway to evaluate potential PFC sources in this area (Minnesota Pollution Control Agency, 2008).

The C10, C11, and C12 acids were detected in 67%, 37%, and 37% respectively, of all MN samples, with each acid individually accounting for less than 10% of the total PFC contribution in all 30 samples. These compounds exhibited trends that were similar to PFOS in that the highest concentrations were noted in Lake Calhoun, there was a general pattern of decreasing concentrations progressing from Mississippi River Pools 3 to 5a, and the lowest concentrations of perfluorinated acids were found in the St. Croix River. Median C10, C11, and C12 levels in Lake Calhoun were 6.09 ng/g, 4.50 ng/g, and 5.91 ng/g, respectively. The highest median levels in the Mississippi River occurred in Pool 3, with C10, C11, and C12 concentrations of 2.84 ng/g, 1.33 ng/g, and 1.31 ng/g, respectively.

Concentrations of PFCs in NC Bluegills. Mean concentrations, standard deviations, medians, geometric means, concentration ranges, and 95% confidence intervals associated with the median for North Carolina bluegills are reported in Table 4. As was the case with the MN fish, only PFOS and the C10 – C12 acids were detected in bluegill fillets from NC (Figure 2), with the remaining 6 analytes remaining below the LOQ for all samples. However, PFOS represented a lower percentage of the total PFCs in NC fish compared to MN fish due to higher concentrations of the C10 – C12 acids. PFOS was detected in all 61 North Carolina bluegill samples, with concentrations ranging from 15.9 ng/g to 136 ng/g. PFOS was the predominant analyte measured in fish from both rivers, accounting for 43% of all PFCs in Haw River fish and 80% of all PFCs in Deep River

fish (Figure 3), with median levels at 30.3 ng/g and 62.2 ng/g PFOS for the Haw and Deep Rivers, respectively. Although there are no known sources of PFCs in the NC sampling locations, PFOS concentrations were similar to those found in areas of the Mississippi River that are downstream of known historical PFC sources.

C11 was also detected in all 61 NC samples, with concentrations ranging from 1.31 to 50.5 ng/g (Table 4). Median C11 levels were 23.9 ng/g on the Haw River and 9.15 ng/g on the Deep River. C11 accounted for 34% of total PFCs in fish from the Haw River and 12% of total PFCs in fish from the Deep River (Figure 3). C10 and C12 were, respectively, detected in 87% and 93% of samples from the Deep River and in 100% of samples from fish on the Haw River. C10 ranged from below the LOQ of 1.11 ng/g to 22.8 ng/g, with median concentrations of 9.08 ng/g in Haw River fish, and 2.90 ng/g in Deep River fish. C12 was found in concentrations from below the LOQ of 0.72 ng/g to 24.3 ng/g, with median levels of 6.60 ng/g on the Haw River and 3.46 ng/g on the Deep River. Although there are no known sources of PFCs on the Haw or Deep Rivers, C10 – C12 acid concentrations on these rivers were higher than those found in the Mississippi River.

Statistically significant differences were observed for concentrations of PFOS, C10, C11, and C12 between fish collected from the Haw and Deep Rivers ($p < 0.001$, Figure 4). PFOS concentrations in Haw River fish were significantly correlated with C11 concentrations only ($p < 0.05$, $\rho = 0.3899$), while PFOS concentrations in Deep River fish were not significantly correlated with C10, C11, or C12 acid concentrations in fish ($p > 0.05$, $\rho = 0.0501$ - 0.0866). C10, C11, and C12 acid concentrations were all significantly correlated with one another both in Haw River fish ($p < 0.001$, $\rho = 0.6753 – 0.8515$) and

in Deep River fish ($p < 0.001$, $\rho = 0.9387 – 0.9881$). Taken together, these findings suggest different PFC input sources on the Haw and Deep Rivers.

Comparisons of PFOS Concentrations in Fish. To date, very few studies have focused upon the analysis of PFCs in fish fillets. All of these investigations, as well as the current study, found that PFOS was the predominant PFC detected in fish muscle (Giesy and Kannan, 2001; Hoff et al., 2003; Kannan et al., 2005; McCann et al., 2007; Ye et al., 2008b). One of these studies also measured PFC concentrations in whole fish and found that PFOS was the predominant compound found in whole fish samples as well (Kannan et al., 2005). PFOS concentrations in the fillets of several fish species from Michigan waters ranged from 2 – 300 ng/g (Giesy and Kannan, 2001, Kannan et al., 2005). Bib and plaice in the North Sea had PFOS concentrations of <10 – 111 ng/g (Hoff et al., 2003). PFOS in the fillets of bluegill, carp, channel catfish, smallmouth bass, largemouth bass, smallmouth buffalo, walleye, whitebass, and northern pike from Minnesota ranged from <0.5 ng/g to about 600 ng/g in Pools 2 to 5a of the Mississippi River (McCann et al., 2007). More specifically, bluegills in the same study (n = 28) had PFOS fillet concentrations of approximately 75 ng/g to 250 ng/g in Mississippi River Pools 2 to 5a and an average concentration higher than 300 ng/g in Lake Calhoun. Together, these studies show that PFOS is found in freshwater fish fillets from various locations and indicate that continued monitoring will help to provide a better understanding of the geographical distributions of PFCs and the environmental factors which influence these distributions.

Comparison to Previously Reported C10 – C12 Concentrations in Fish. Previous analysis of fish fillet samples has generally shown that PFOA is detected above the LOQ

in very few samples (Kannan et al., 2005; McCann et al., 2007). This is consistent with the findings of the current study, where PFOA was never detected above the LOQ. One study measured the remaining C6 – C12 perfluorinated acids in the fillets of fish collected from Pools 2 to 5a of the Mississippi River in Minnesota and found that all samples from several species of fish in Minnesota had no detectable C6, C7, or C9 (McCann et al., 2007), a finding that is also consistent with the current study. The same study found that all species of fish had C10 levels below 10 ng/g (wet weight) and maximum concentrations of C11 and C12 in all species of fish were 5.79 and 14.6 ng/g wet weight, respectively (McCann et al., 2007).

Median concentrations of the C10 – C12 acids found in North Carolina bluegill were among the highest reported in the literature, as the median concentrations of the C11 acid in NC bluegill from both rivers (23.9 ng/g on Haw River , 9.15 ng/g on Deep River) were greater than previously reported maximum C11 concentrations in fish fillets (McCann et al., 2007) and are more than double the highest median concentration of Minnesota bluegill analyzed in the current study (Lake Calhoun, 4.50 ng/g, see Table 4). Median concentrations of the C10 and C12 acids found in Minnesota bluegill in the current study agreed well with previously reported values (McCann et al., 2007). Lake Calhoun also has no known PFC inputs, yet fish from this lake had higher C10 – C12 concentrations than fish from Pools 3 – 5a of the Mississippi River. These data indicate that, similar to PFOS, C10 – C12 acids are found in some U.S. freshwater fish and further investigations will be useful in determining the geographical distributions of these PFCs.

Concentrations of the C10 – C12 acids in fillets from the current study were generally greater than or equal to most previously reported concentrations of C10 – C12

acids found in fish plasma, liver, and whole fish homogenates, which range from <0.8 to 31.6 ng/g wet weight (Martin et al., 2004; Houde et al., 2006a; Houde et al., 2006b; Hart et al., 2008). However, PFC concentrations are typically higher in fish plasma, liver, and whole fish than in fillet (Houde et al., 2006b). Only one known study has reported higher levels of the C10 and C11 acids, but these were fish were living in or near a creek in Canada where fire fighting foams containing PFCs had been released (Moody et al., 2002). Elevated C10 – C12 acid concentrations found in NC and Lake Calhoun bluegills indicate that there were inputs of PFCs into these bodies of water. Further investigation would be useful to assess potential sources of perfluorinated acids in these areas.

Dietary Exposure. While the factors that influence human exposure to the PFCs are still very poorly understood, a recent modeling study estimated that daily human exposure to PFOS for a 60 kg adult through all exposure routes is an average of 1.6 ng/kg bodyweight, with an upper daily level of exposure of 8.8 ng/kg bodyweight (Fromme et al., 2009). These values are not tolerable daily intake concentrations, but instead provide an estimate of human exposures to PFCs. The authors of that study concluded that PFOS exposures are dominated by dietary intake.

Results from the current study suggest that individuals who consume fish caught in some areas of MN and NC may be exposed to higher levels of PFCs than is suggested in the previous modeling study. For example, on a given day, if a 60 kg person consumes one meal (195 g) of fish caught in most of the locations sampled in MN and NC, that individual would greatly exceed the modeled upper daily exposure estimate of 8.8 ng/kg on that particular day. The amount of PFOS ingested in a 195 g meal of bluegill fillet from MN or NC would be as follows (based on median concentrations): Lake Calhoun

(894 ng/kg), Pool 4 (332 ng/kg), Pool 3 (291 ng/kg), Deep River (202 ng/kg), Pool 5 (153 ng/kg), Pool 5a (153 ng/kg), Haw River (98.5 ng/kg), and St. Croix River (6.76 ng/kg). This indicates that it would be beneficial for future modeling studies to consider incorporating freshwater fish consumption data when determining daily estimates of PFOS exposure. It also underscores the fact that very little data are available for these modeling efforts, and that additional measurement work would be helpful in order to obtain a more complete understanding of potential exposure routes.

The Minnesota Department of Health (MDH) has issued guidelines on fish consumption based on levels of PFOS measured in fish fillets. The MDH advises eating no more than one meal per week of fish if PFOS concentrations in fish fillets exceed 40 ng/g and eating no more than one meal per month of fish if fillet concentrations exceed 200 ng/g (Minnesota Department of Health, 2008). Following this advice keeps exposures below 80 ng/kg/day. Mean concentrations of PFOS measured in bluegill from the Deep River and Pools 3, 4, 5, and 5a of the Mississippi River exceeded 40 ng/g and the mean concentration of PFOS in Lake Calhoun bluegill exceeded 200 ng/g.

Amounts of C11, the acid that was measured in the highest concentrations in NC fish, that a 60 kg person would ingest in a 195 g meal would be as follows based on median concentrations: Haw River (77.7 ng/kg), Deep River (29.7 ng/kg), Lake Calhoun (14.6 ng/kg), Pool 3 (4.32 ng/kg), Pool 4 (3.93 ng/kg), Pool 5 (not determined), Pool 5a (not determined), and St. Croix River (not determined). To our knowledge, consumption advisories for compounds other than PFOS have not been issued by any entity. But considering that the C11 concentrations at some of the NC locations are in the same range as PFOS, and that a previous study concluded that toxicity increases as carbon

chain length increases with the perfluorinated acids (Kudo et al., 2006), further investigations regarding PFCs other than PFOS in fish would be useful.

In summary, a method for the measurement of PFCs in bluegill sunfish fillets has been thoroughly described, comprehensively evaluated, and applied to samples collected from areas both with and without known historical inputs of PFC contamination. The use of this method with bluegill fillets has been determined to be accurate and precise for PFOS and other PFCs that are often detected in fish tissues. As described above, the method is relatively easy to apply, and its performance characteristics indicate that it is useful for the analysis of PFCs. Additionally, bluegill appear to be a good sentinel species for monitoring environmental distributions of PFCs and human dietary exposures to PFCs for the following reasons: bluegills have widespread distributions, live in a small area for prolonged periods, have an apparent ability to accumulate PFCs, are easy to catch, and are often eaten by humans. The current method could be applied to samples of a sentinel species such as bluegill to evaluate geographical and historical trends in PFC distributions in the environment, examine the impact of potential PFC sources, and compare potential risks from consumption of fish from different water sources.

In the current study, the method has been successfully applied to the MN samples to demonstrate distinct differences in regional contamination in areas with known but uncertain historical sources of PFC contamination. Waters with fish that are above the current MN fish consumption advisory limit (40 ng PFOS/g fish fillet) are clearly distinguishable from nearby areas that show little evidence of contamination. In NC, the method has been used to document the existence of fish contamination that is apparently similar in magnitude to some areas of MN. Moreover, the occurrence of C10 – C12

carboxylates at higher concentrations than most previous studies and distinctive contamination patterns in fish from adjoining NC rivers suggests that a variety of PFC sources can contribute to PFCs present in freshwater fish.

The similarity of the data from MN and NC suggests that U.S. freshwater fish in various locations contain PFCs and that contamination may not be limited to a small number of known historical sources. Further investigation would be useful in order to more fully evaluate the actual extent of this situation on a national and international basis. With the information provided in this work, this method can be applied to help provide comparable measurements that would be useful to perform a more accurate and complete description regarding the nature of this emerging concern.

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Figure Legends

Figure 1. Minnesota sampling sites

Figure 2. North Carolina sampling sites

Figure 3. PFOS and C10-C12 acid concentrations in fish fillet samples as a percentage of total PFCs based on median concentrations

Figure 4. Box plots of PFOS and C10-C12 acid concentrations in the fillets of bluegills collected from the Haw and Deep Rivers

Table 1. MRM Transitions and Analyte-Specific Mass Spectrometer Parameters

Compound	Molecular Weight	Quantitation Ion MRM Transition (m/z)	Confirmation Ion MRM Transition (m/z)[a]	DP[b]	FP[b]	CE[b]	CXP[b]
PFBS	299	299→80	299→99	-51	-200	-64	-15
PFHxS	399	399→80	399→99	-66	-200	-80	-15
PFOS	499	499 →80	499 →99	-76	-200	-94	-15
$^{18}O_2$-PFOS	521	503→84	503→103	-76	-200	-94	-15
$^{13}C_2$-PFOA	416	415 →370	none monitored	-21	-80	-14	-23
PFHxA	314	313 →269	313 →119	-16	-60	-14	-15
PFHpA	364	363 →319	363 →169	-21	-80	-14	-19
PFOA	414	413→369	413→169	-21	-80	-14	-23
PFNA	464	463 →419	463 →219	-26	-90	-14	-15
PFDA	514	513 →469	513 →219	-26	-90	-16	-15
PFUnA	564	563 →519	563 →269	-31	-110	-16	-15
PFDoA	614	613 →569	613 →169	-31	-120	-18	-15

[a]Quantitation ion listed first and confirmation ion listed second for all analytes; approximately 30% of samples were verified with mass spectrometer method containing quantitation and confirmation ions

[b]Parameters specific to Sciex mass spectrometers, DP = declustering potential, FP = focusing potential, CE = collision energy, CXP = collision cell exit potential

Table 2. Method Performance Parameters Determined Using Spiked Tilapia Samples

Compound	Concentration[a]	Intra-day[b] Precision	Inter-day[b] Precision	Intra-day[c] Accuracy	Inter-day[c] Accuracy	Recovery[d]	Matrix[e] Effects	LOQ[f,g]
PFOS	4	3.38	7.39	6.52	5.52	94.9	107	0.52
	100	3.07	7.15	6.33	4.98	97.4	110	
	400	3.36	7.58	5.74	5.29	95.3	99.1	
C6	0.4	9.71	23.5	29.8	20.3	<LOQ	<LOQ	1.89
	4	8.46	20.9	19.3	4.84	85.5	72.7	
	20	6.53	11.6	8.47	2.50	109	60.6	
C7	0.4	10.7	31.1	37.3	36.4	<LOQ	<LOQ	5.21
	4	6.39	16.2	18.6	20.3	<LOQ	<LOQ	
	20	5.90	11.7	7.52	1.17	133	62.3	
C8	0.4	10.2	18.5	18.9	18.8	<LOQ	<LOQ	0.77
	4	3.16	9.45	8.65	8.90	88.9	79.9	
	20	4.09	8.40	7.14	7.91	122	65.8	
C9	0.4	4.49	10.1	8.26	9.47	<LOQ	<LOQ	1.88
	4	10.3	19.5	9.15	5.69	92.5	98.8	
	20	6.48	17.2	6.69	1.36	125	73.1	
C10	0.4	5.78	14.5	12.3	5.81	<LOQ	<LOQ	1.11
	4	10.5	15.4	6.63	0.52	101	71.9	
	20	7.24	9.77	5.53	4.37	116	75.8	
C11	0.4	12.8	17.8	12.7	2.99	<LOQ	<LOQ	1.05
	4	10.4	13.5	6.95	7.65	97.2	111	
	20	8.73	9.91	7.08	8.40	122	69.0	

C12	0.4	8.82	12.3	9.74	12.3	<LOQ	<LOQ	0.72
	4	9.49	15.1	6.02	7.55	88.0	100	
	20	6.36	10.3	10.8	10.6	112	73.0	
PFHS	0.4	5.76	6.76	4.52	14.3	106	96.1	0.01
	4	3.88	10.1	2.92	12.8	85.1	100	
	20	4.98	8.20	6.06	9.00	111	88.6	
PFBS	0.4	12.1	16.7	10.2	8.66	<LOQ	<LOQ	0.48
	4	3.81	6.18	2.95	10.3	76.2	88.7	
	20	4.34	8.85	3.85	4.65	97.0	77.9	

[a]Concentrations reported as ng/g fish, wet weight. Concentrations are approximated in this table for clarity. Actual concentrations for each analyte were slightly different due to weighing out different amounts of each compound when making original stock solutions. Accuracy was determined using actual concentrations for each analyte.

[b]Precision values expressed as %RSD.

[c]Accuracy values reported as absolute value of % difference from theoretical value.

[d]Recovery reported as a percentage.

[e]Matrix effects expressed as percentages, values of 100 indicate no suppression, values above 100 indicate ion enhancement, values below 100 indicate ion suppression.

[f]LOQ reported as ng/g fish. Determinations of precision and accuracy at 0.4 ng/g were made before the LOQ was determined.

[g]LOQ values determined for PFHS (0.01 ng/g) and PFOS (0.52 ng/g) were less than the lowest calibration point and therefore for analysis of unknowns, the lowest calibration points for PFHS (0.40 ng/g) and PFOS (1 ng/g) are the LOQ for PFHS and PFOS.

Table 3. Accuracy of Spiked Bluegill Samples[a]

Bluegill	Unspiked[b] PFOS Concentration	PFOS	C6	C7	C8	C9	C10	C11	C12	PFHS	PFBS
1	15.2	102	76.9	102	101	120	125	103	110	104	83.8
2	45.7	95.5	74.9	93.9	108	124	132	115	136	110	88.5
3	77.4	80.7	121	101	97.1	118	116	95.8	116	123	113

[a]Accuracy is expressed as a percentage. All 3 unspiked bluegill fillet homogenates contained PFOS and Bluegill 1 also contained 1.44 ng/g of C12. Aliquots of each bluegill fillet homogenate were spiked with 20 ng/g PFOS and 4 ng/g of the remaining 9 PFCs. Accuracy is expressed as a percentage based on the ratio of observed PFC concentration increase between unspiked and spiked samples compared to the theoretical PFC concentration increase (n = 2 for both spiked and unspiked samples).

[b]Bluegill samples used in accuracy assessment were chosen based on PFOS concentrations determined by previous analysis.

Table 4. Statistics for Minnesota and North Carolina Samples, Including Sample Concentration Means, Standard Deviations, Medians, and Ranges (ng/g fish, wet weight) for PFOS, C10, C11, and C12

Sample Site[a]	State	n	Value	PFOS	C10	C11[b]	C12
Miss. River Pool 3	MN	5	Mean (St. Dev.)	156 (152)	3.66 (2.34)	2.11 (1.87)[c]	2.95 (4.02)[c]
			Geometric Mean	121	3.12	1.44[c]	1.36[c]
			Median	89.5	2.84	1.33[c]	1.31[c]
			Range	77.0-428	1.62-7.41	0.53-4.44[c]	0.36-9.92[c]
Miss. River Pool 4	MN	5	Mean (St. Dev.)	84.8 (42.0)	1.63 (0.85)[c]	1.45 (1.03)[c]	1.46 (1.23)[c]
			Geometric Mean	74.7	1.42[c]	1.16[c]	1.01[c]
			Median	102	1.73[c]	1.21[c]	1.07[c]
			Range	32.8-130	0.56-2.78[c]	0.53-2.70	0.36-3.03[c]
Miss. River Pool 5	MN	5	Mean (St. Dev.)	45.0 (9.03)	1.29 (0.51)[c]	<LOQ[d]	<LOQ[d]
			Geometric Mean	44.2	1.19[c]	<LOQ[d]	<LOQ[d]
			Median	47.0	1.28[c]	<LOQ[d]	<LOQ[d]
			Range	32.3-55.4	0.56-1.97[c]	<LOQ[d]	<LOQ[d]
Miss. River Pool 5a	MN	5	Mean (St. Dev.)	48.4 (24.9)	0.97 (0.39)[c]	<LOQ[d]	<LOQ[d]
			Geometric Mean	43.0	0.90[c]	<LOQ[d]	<LOQ[d]
			Median	47.0	1.13[c]	<LOQ[d]	<LOQ[d]
			Range	23.5-78.7	0.56-1.39[c]	<LOQ[d]	<LOQ[d]
St. Croix River	MN	5	Mean (St. Dev.)	2.87 (2.47)	<LOQ[d]	<LOQ[d]	<LOQ[d]
			Geometric Mean	2.27	<LOQ[d]	<LOQ[d]	<LOQ[d]
			Median	2.08	<LOQ[d]	<LOQ[d]	<LOQ[d]
			Range	1.22-7.17	<LOQ[d]	<LOQ[d]	<LOQ[d]
Lake Calhoun	MN	5	Mean (St. Dev.)	272 (63.2)	5.82 (1.44)	4.18 (1.62)	4.72 (1.75)
			Geometric Mean	266	5.64	3.90	4.42

			Median	275	6.09	4.50	5.91
			Range	205-339	3.40-7.05	2.14-6.02	2.70-6.08
Haw River	NC	31	Mean (St. Dev.)	29.8 (6.08)	10.3 (3.85)	26.9 (8.05)	7.25 (2.63)
			Geometric Mean	29.2	9.74	25.8	6.87
			Median (95% C.I.)[e]	30.3 (27.5-31.5)	9.08 (8.11-11.1)	23.9 (21.3-31.4)	6.60 (5.46-7.85)
			Range	15.9-47.5	6.07-22.8	14.3-42.2	4.16-16.1
Deep River	NC	30	Mean (St. Dev.)	66.3 (29.6)	5.25 (5.32)[c]	14.2 (12.6)	4.66 (4.68)[c]
			Geometric Mean	59.8	3.19[c]	9.16	3.13[c]
			Median (95% C.I.)[e]	62.2 (50.9-76.8)	2.90[c] (1.70-6.21)	9.15 (5.97-16.7)	3.46[c] (2.23-5.22)
			Range	21.4-136	0.56-22.7[c]	1.31-50.5	0.36-24.3[c]

[a]Miss. River = Mississippi River

[b]Samples above the linear range for C11 were diluted with blank tilapia homogenate and rerun.

[c]Samples below the LOQ were assigned a value equal to half the LOQ. These values were used in the determination of means, standard deviations, medians, and ranges.

[d]Value not determined because all 5 samples were below the LOQ.

[e]95% confidence interval associated with the median included for NC samples; MN sample size too small to determine 95% confidence interval

Figure 1

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Figure 2

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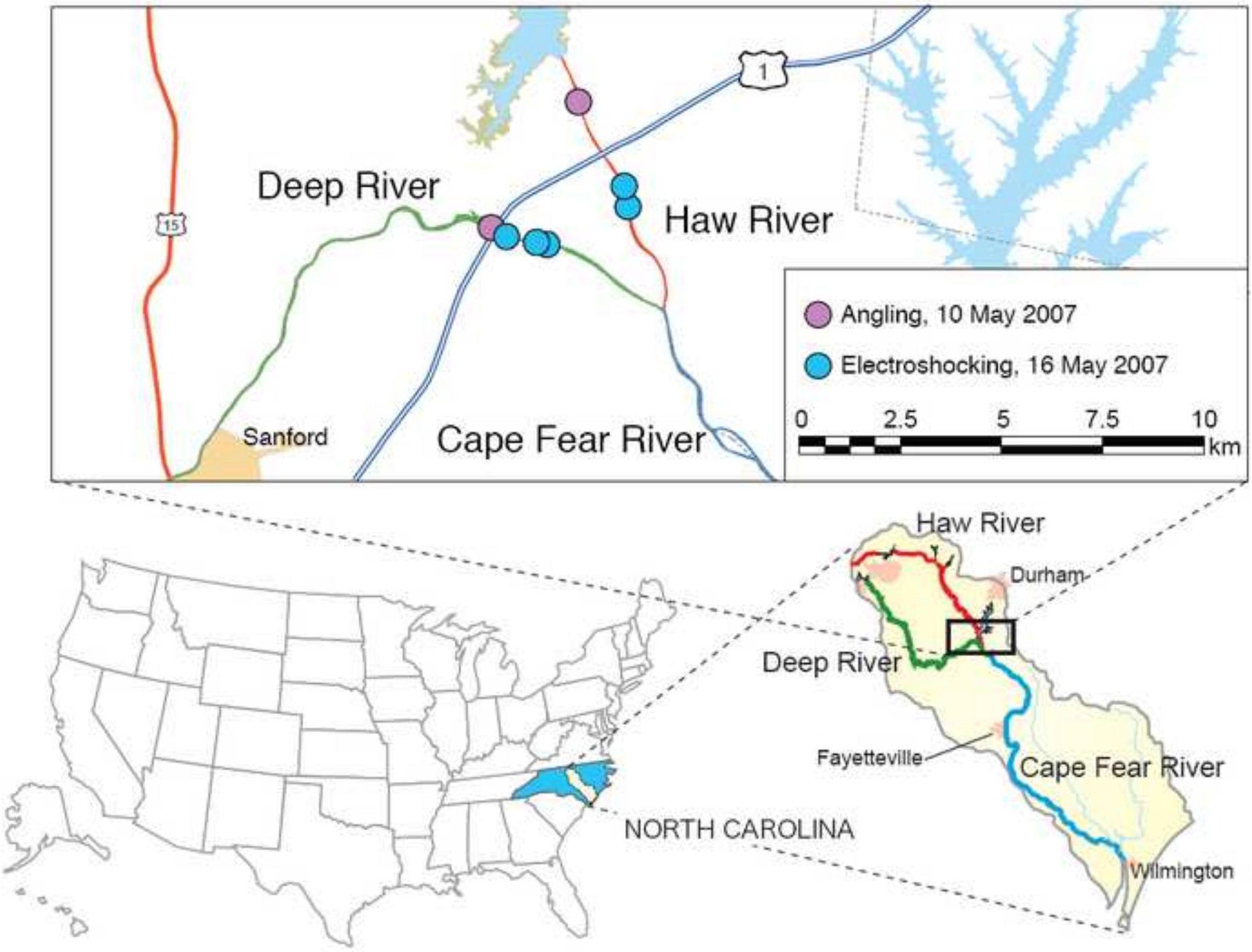

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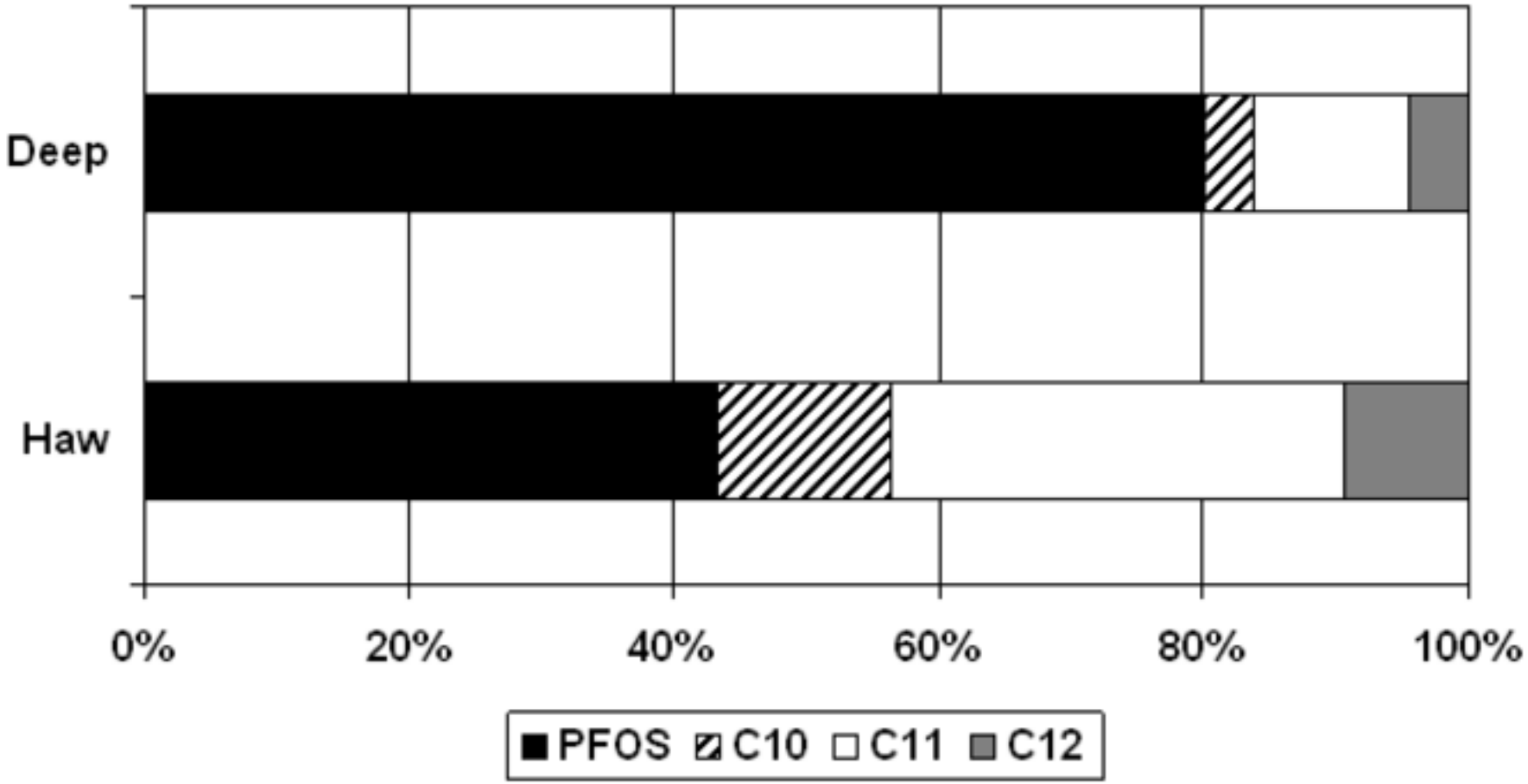

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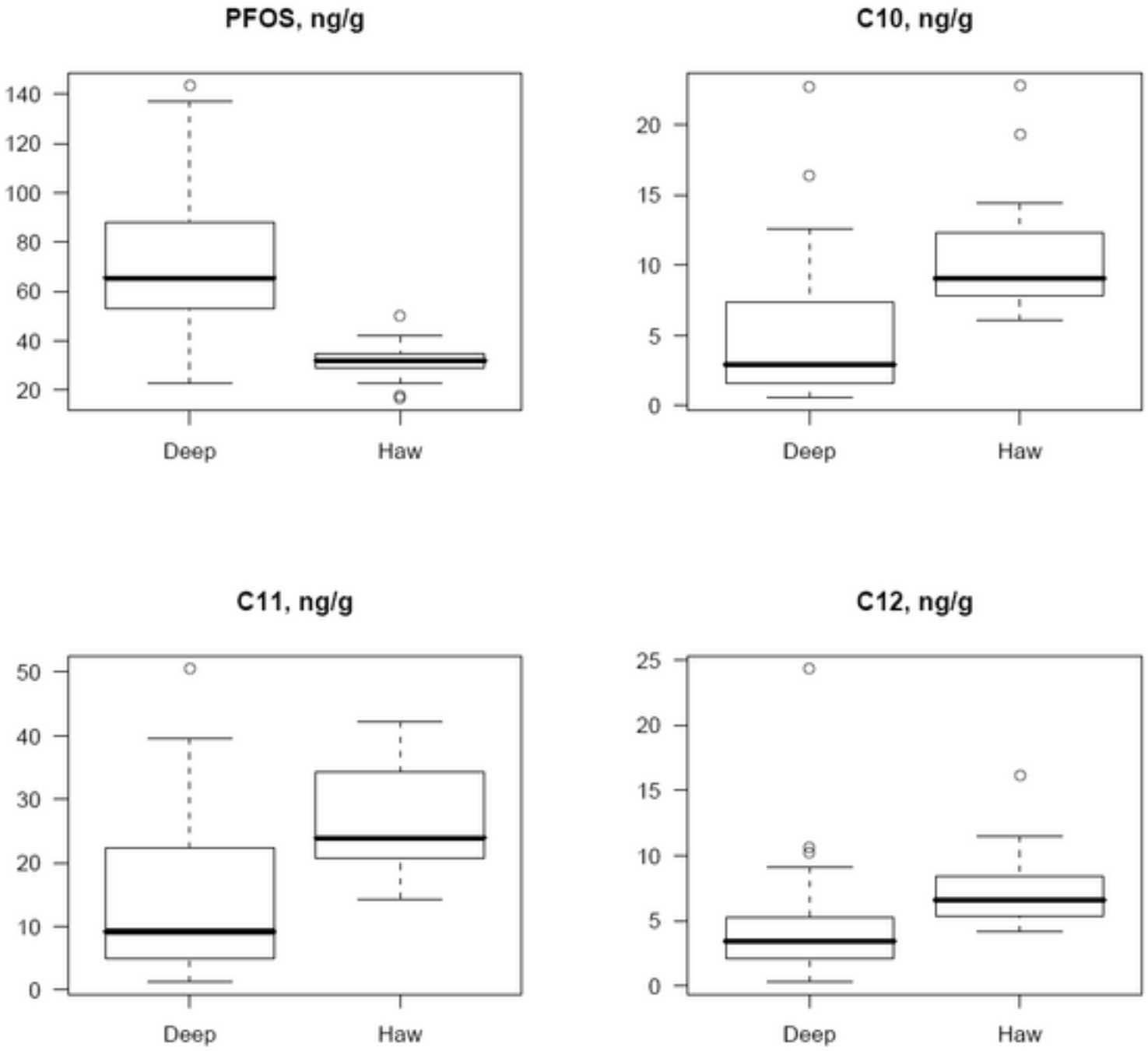

Figure 1 Black and White

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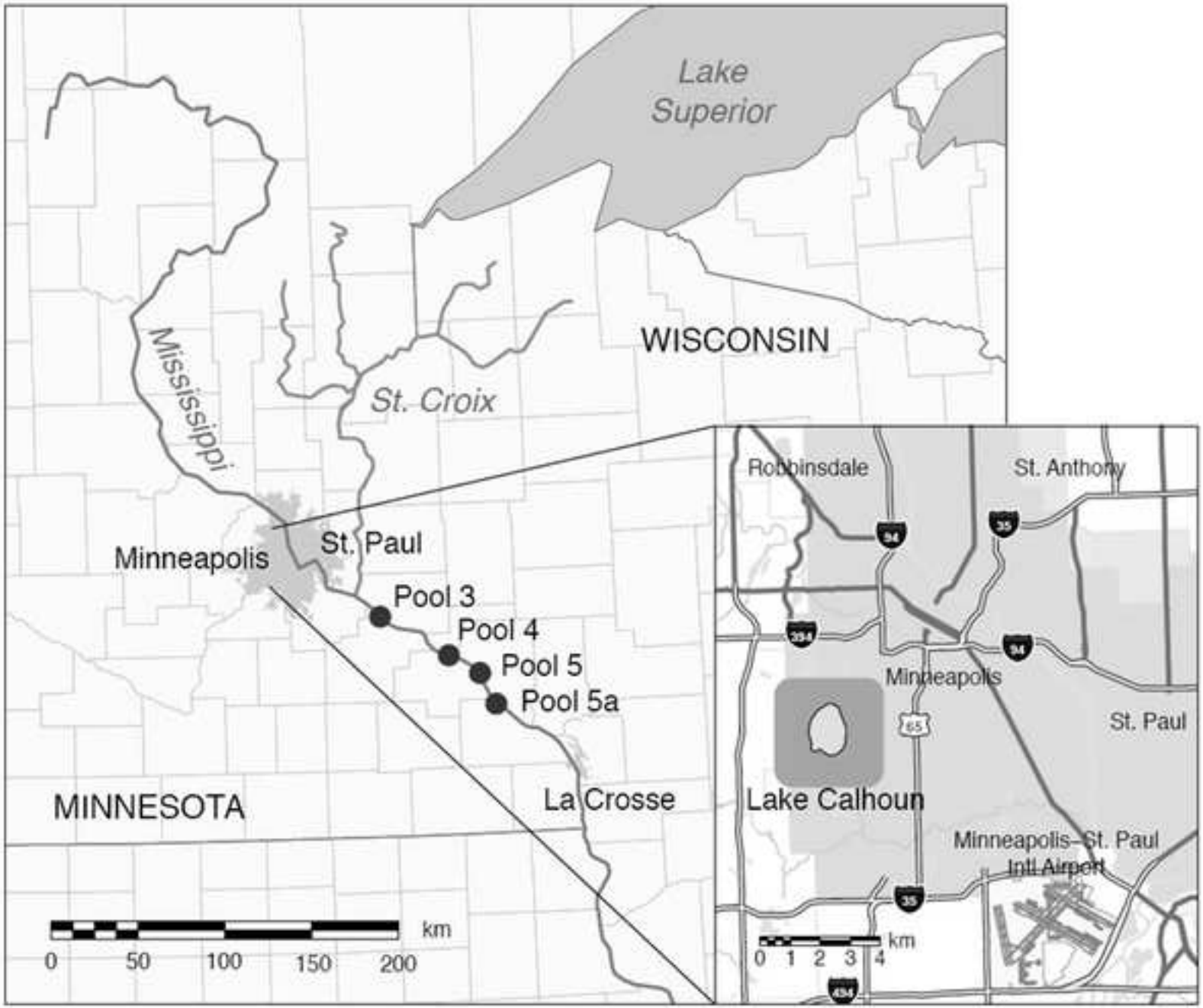

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