PER- AND POLYFLUOROALKYL SUBSTANCES IN SURFACE WATER AND BLUEGILL AND ITS RELATIONSHIP TO SWIMMING PERFORMANCE AND HISTOLOGY

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ABSTRACT

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Per- and poly-fluorinated alkyl substances (PFAS) can be found in many household, industrial, and personal care products, including furniture, aqueous film forming foam, and sunscreen. Many recent studies have shown PFAS in surface waters and aquatic organisms around the world. PFAS levels are higher near contamination sources, such as the Former Wurtsmith Air Force Base in Oscoda, Michigan, USA. Unexpectedly, PFAS levels have not biomagnified in Clark's Marsh near Wurtsmith Air Force Base, a known PFAS source to the surrounding environment. Bluegill (*Lepomis macrochirus*) in Clark's Marsh have higher PFAS levels than their predators, however, the reasons and effects of these high levels are unknown. To investigate these effects, Bluegill were sampled in various inland lakes and rivers in Michigan with differing PFAS concentrations. Bluegill were individually tested for critical swimming speed, and liver and gill histology. The data was analyzed to determine differences between PFAS effects at different sites and with different levels of PFAS. We hypothesized higher PFAS levels would relate to higher extent of liver vacuoles, more prevalent gill alterations, and slower swimming speeds. Results show decreased and increased swimming speeds at different PFAS levels, and increased liver vacuoles and abnormal gill morphology with increased PFAS levels. While there's no consensus in the literature regarding swimming behavior after PFAS exposure, decreased swimming performance may have been caused by the histological alterations exhibited due to decreased oxygen uptake and organ dysfunction. The increased lesions in both liver and gill tissues were consistent with ecotoxicological literature, especially that of organophosphate pesticides. This research showed how individual fish were impacted by environmentally relevant PFAS concentrations which also cause widespread effects on aquatic communities.

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INTRODUCTION

Many anthropogenic chemicals, such as pharmaceuticals, personal care products, and per- and poly-fluorinated alkyl substances (PFAS), eventually enter the environment without knowing the consequences of their release to the environment beforehand. These emerging contaminants have been entering the environment for many years, many of which were developed and began mass production in the 1920s to 1960s but, only in the last few decades have they been detected in the environment (Bernot et al. 2013; Chandler 1969; Ramirez et al. 2009; Valdersnes et al. 2017). Analytical testing capabilities and accuracy to detect anthropogenic chemicals have only recently improved, allowing researchers to detect minute, parts per trillion level, concentrations in the environment. More than 25 individual pharmaceuticals and personal care products are now found in surface waters worldwide at parts per trillion levels and larger (Ebele et al. 2017). Currently, an abundance of contaminants is widespread in the environment and has been for the past several decades.

PFAS are among the anthropogenic chemicals causing increased human and environmental health concerns. PFAS have been produced since the 1940s and are still manufactured today (Clara et al. 2008; Svihlikova et al. 2015). Because they are both hydrophobic and lipophobic, PFAS are commonly used in surfactants, coatings for furniture, textiles, paper, and many household products (Lindstrom et al. 2011). PFAS are also commonly used in flame retardants, specifically aqueous film-forming foam (Hu et al. 2016). Given the widespread use of PFAS, there is a high risk of products, containing these chemicals, entering aquatic and terrestrial ecosystems.

Wastewater treatment plant effluent, biosolids, flame retardant application, and industrial businesses are some mechanisms in which PFAS enter the environment. Due to inadequate

processes at wastewater treatment plants, PFAS are found in wastewater treatment plant (WWTP) effluent, which flow into local waterways, and biosolids, which are directly applied to fields (Clara et al. 2008). Another source of contamination is the direct application to the environment, by use of flame retardants (AFFF) at emergency sites and at training sites, such as military bases (Hu et al. 2016). Landfills and their leachate also allow PFAS to enter the environment through the disposal of PFAS-containing products and PFAS leaching from the products into the landfill's runoff (Barzen-Hanson et al. 2017). Lastly, some industrial businesses, such as chrome platers and historical dry cleaners, have discharged PFAS into the soil and water (Moeller et al. 2010). There are numerous ways PFAS can enter the groundwater, surface water, air and soil, which have led to a multitude of sites being contaminated with PFAS.

Both surface waters and groundwater worldwide have been contaminated by PFAS. For reference, the water quality values for the protection of aquatic life, as opposed to other criteria which protect human health, for the two most commonly found PFAS are 140 µg/L perfluorooctane sulfonate (PFOS) and 880 µg/L perfluorooctanoic acid (PFOA) for the final chronic value (for aquatic life) (MDEQ 2018a). In Michigan, the surface water quality criteria for water not used as drinking water, human non-cancer values are 12 ppt PFOS and 12,000 ppt PFOA (MDEQ 2018a). Practice sites in the U.S. Northwest, such as fire training areas on military bases, have concentrations up to 6,900 ppt in groundwater (Backe et al. 2013). In addition, the Tennessee River had a maximum concentration of 600 ppt PFOS and 150 ppt PFOA (Hekster et al. 2003). Concentrations throughout the world vary with distance from a contamination source. Thus, surface water PFAS concentrations vary widely on county, national and global scales (Lam et al. 2014; Moeller et al. 2010). Not only are PFAS abundant in the environment, but PFAS are also very persistent, taking decades to degrade. The half-lives of

PFOS in water samples from photolysis and hydrolysis ranged from 3.7 years to 41 years, respectively, at 25º C (Giesy et al. 2010). Due to PFAS entering the environment and their persistence, many aquatic systems are now contaminated and may have been for decades.

Because of PFAS' ubiquity in nature, awareness of contamination levels found in many animals has been increasing. Animals are exposed to PFAS from many sources including water, soil, plants, and the atmosphere (Falk et al. 2012). In some sites, PFAS are diluted from the source, which create widespread low levels. Most Norwegian Cod (*Gadus morhua*) tested on the Norwegian coast were at low levels, with a maximum of 21.8 ppb PFOS, and under the environmental quality standards (Valdersnes et al. 2017). However, similar to surface water concentrations, there is a wide range of contamination levels in fish tissues, including sunfish in a Michigan marsh, adjacent to the contamination source (Bush et al. 2015). The sunfish exhibited mean PFOS concentrations of 5,498 ppb, several times more than the highest consumption advisory thresholds at 300 ppb (Bush et al. 2015). Fish tissues with the highest PFAS concentrations are commonly the blood, kidney, and liver (Martin et al. 2003), likely because PFAS are associated with protein-rich tissues (Bossi et al. 2015). Animal exposure to PFAS is influenced by many factors, including distance from a source, biotransformation, and biomagnification.

Organisms are exposed to different PFAS concentrations which vary spatially, partially based on the distance to a source discharging PFAS into the environment. The distance from a source can affect PFAS exposure by influencing the PFAS concentrations in the surface water to which the animal is directly exposed (Junttila et al. 2019). Indeed, the most contaminated surface waters with WWTP effluent were near chemical and textile industrial sites (Svihlikova et al. 2015). Animals in these 'hotspot' areas have higher levels of PFAS than in water bodies a short

distance down gradient (Bush et al. 2015). Biotransformation can also influence the PFAS compounds present at a site.

Biotransformation of PFAS occurs not only in the environment, but also in fish and other organisms (Babut et al. 2017). Polyfluoroalkyl substances are less stable and many are precursors to perfluoroalkyl substances. Zabaleta et al. (2017) found 8:2 FTCA, a fluorotelomer acid, PFOA, and other transformation products in Gilthead Bream (*Sparus aurata)* exposed to 8:2 diPAP, a polyfluoroalkyl phosphate diester. Further, 8:2 FTCA transformed into PFOA in multiple Rainbow Trout studies (Butt et al. 2013). At higher trophic levels, biotransformation is assumed to be more likely than at lower trophic levels, which can confound bioaccumulation models (Tomy et al. 2004). Indeed, Babut et al. (2017) found higher biotransformation, of perfluorooctane sulfonamide (FOSA) to PFOS and of N-methyl perfluorooctane sulfonamidoacetic acid (MeFOSAA) to FOSA, in the sediment-invertebrates-barbels food chain. Biotransformation and other PFAS characteristics create different opportunities for exposure to aquatic organisms.

Due to the characteristics of individual PFAS compounds, certain compounds partition out of the water column and into sediments, while other compounds stay in the water (Lee et al. 2020). This phenomenon creates locations within a water body where there are different opportunities for exposure. Suspended sediments and bottomland sediments had contrasting temporal trends of PFAS concentrations in the Niagara River, indicating that benthic and pelagic organisms are exposed to different PFAS compounds and at different concentrations (Myers et al. 2012). Thus, interspecies differences in PFAS concentrations occur due to feeding differences (Babut et al. 2015). Partially due to the feeding differences in aquatic organisms and locations of

specific PFAS in the environment, aquatic organisms have varying levels of bioaccumulation and biomagnification that does not always follow canonical amplification.

PFAS bioaccumulate and biomagnify at different rates in aquatic communities. PFAS bioaccumulate in Great Lakes food chains, however, the transport and fate of PFAS are poorly understood (Myers et al. 2012). PFOS accumulation occurs in association with time and concentration of exposure (Giesy et al. 2010). Some PFAS do not biomagnify or bioaccumulate, especially short chain perfluorocarboxylic acids – those with less than seven carbons in the carbon chain (Goeritz et al. 2013; Xu et al. 2014). PFOS has been frequently found to biomagnify in aquatic communities (Tomy et al. 2004; Xu et al. 2014). In some instances, however, the highest PFOS concentrations were found in the lower trophic levels potentially due to differential contamination source signatures (Bush et al. 2015; Martin et al. 2004). PFAS biomagnification is not only important to examine on the community-level, but it also informs how organisms may be affected by exposure levels.

PFAS concentrations affect individual's behavior and physiology. PFOS and PFOA have hepatotoxic, carcinogenic, developmental toxic, reproductive toxic, and neurotoxic effects in animals (Valdersnes et al. 2017). PFAS-exposed fish have exhibited abnormal physiology, anatomy, and behavior, as seen in altered body weight, spinal curvature, and swimming performance, after the exposure period ended (Hagenaars et al. 2014; Jantzen et al. 2016a, 2016b). Due to the broad range of toxic effects, PFAS contamination can also influence fish tissue health by causing increased liver lesions and presumably decreased liver function (Du et al. 2008; Giari et al. 2015). While broad effects of high level PFAS exposure are known, behavioral and physiological effects from PFAS mixtures at environmentally relevant levels have not been well studied.

The major PFAS contamination area near the Former Wurtsmith Air Force Base in Oscoda, Michigan was investigated in this research. The Base used PFAS-containing AFFF for decades before the Base closed in the early 1990's. For decades, PFAS have migrated from the Base's fire-training area to the adjacent Clark's Marsh, Au Sable River and surrounding areas. High PFOS levels in water and fish tissues in both the Marsh and River resulted in a 'do not eat fish' consumption advisory from the State of Michigan (Delaney et al. 2015). Currently, surface water PFAS concentrations, from 17.1 to 12,266 ppt Total PFAS, including 3.23 to 5,099 ppt PFOS, are known at these sites, however, their effect on fish physiology and behavior is yet to be determined. Further, if PFAS influenced fish swimming behavior then, PFAS levels and critical swimming speed would be inversely related. Also, if PFAS influenced tissue health, then PFAS levels, liver vacuoles, and gill lesions would be positively related. The objective of this research was to determine the behavioral and physiological effects, as measured by critical swimming speed and liver and gill histology, of Bluegill exposed near the AFFF-contaminated Former Wurtsmith Air Force Base. Generally, the research question was: How do Bluegill from PFAS-contaminated sites have altered behavior and physiology? These endpoints are critical for fisheries managers and natural resource decision-makers to begin identifying how fisheries population size, fitness, and growth are influenced by PFAS exposure.

METHODS

Experimental Design

To determine the behavioral and histological effects of PFAS exposure, Bluegill (*Lepomis macrochirus)* were collected from six different locations across Northern Michigan (Figure 1; Figure 2). A correlational approach was taken by using an anaerobic swimming performance assay along with gill and liver histology to investigate potential effects of PFAS on Bluegill. A total of 36 swimming trials were conducted and 28 histology samples were collected. Three to four gill and liver histology samples were collected per water body; eight to eleven swimming trials were conducted per water body (Table 1).

Study Sites

Due to the high level of contamination surrounding the Former Wurtsmith Air Force Base, two nearby sampling sites were chosen, along with a Northern site with low-level contamination. Clark's Marsh, the Lower Au Sable River, Pickerel Lake, and Susan Lake were chosen as sampling sites for their variety of PFAS concentrations. Pickerel Lake, near Petoskey, MI, served as a control due to the lack of urban and industrial land uses within its watershed and was assumed to have little to no contamination. Susan Lake, near Charlevoix, MI, has low level PFAS contamination (TOMWC 2019). Clark's Marsh and the Lower Au Sable River were adjacent to the Former Wurtsmith Air Force Base and known to be highly contaminated with PFAS (Bush et al. 2015).

PFAS Water Collection and Analysis

Surface water PFAS concentrations were used as a surrogate for fish PFAS concentrations to compare PFAS and swimming speed. Surface waters, where fish were collected, were sampled by Michigan agencies between 2013 and 2019 (Table 1, Table 2; Bush

et al. 2015). Tip of the Mitt Watershed Council sampled Susan Lake in Summer 2019 (TOMWC 2019). Au Sable River and Clark's Marsh were sampled by the Michigan Department of Environmental Quality (MDEQ) in 2013 (Bush et al. 2015). Total PFAS concentrations are the combined concentration of all analyzed PFAS compounds from a single site. Test America analyzed the MDEQ samples for 19 individual PFAS compounds with a reporting limit of 2 ng/L for all compounds (Bush et al. 2015).

Susan Lake was sampled by Tip of the Mitt Watershed Council in Summer 2019 in accordance with the Michigan Department of Environmental Quality Surface Water PFAS Sampling Guidance Document (MDEQ 2018b; TOMWC 2019). Water samples were collected at the water's surface, by hand dipping the bottle, and just above the deepest point of the lake bottom, using a 2 L stainless-steel Kemmerer bottle (TOMWC 2019). Samples were transported in an iced cooler to the University of Michigan Biological Station Analytical Chemistry Laboratory, where Freshwater Future analyzed the samples for 14 PFAS compounds (Table 2, Shoemaker et al. 2009; TOMWC 2019). Freshwater Future analyzed the samples based on EPA Method 537 rev1.1 (TOMWC 2019).

In addition to water samples for PFAS analysis, basic physical and chemical parameters were quantified at each site, within 28 days of fish sampling, by using a Eureka Amphibian 2 handheld PC (Austin, TX, USA) with a Manta 2 sub 3 Hydrolab probe or a YSI Professional Plus (Yellow Springs, OH, USA). Temperature, pH, dissolved oxygen, conductivity, and turbidity were collected at 0.79 m ± 0.04 m (mean depth ± SEM) from the surface of all the sites.

Fish Collection and Housing

Fish were collected in 2019 from Pickerel Lake, Susan Lake, Au Sable River, and Clark's Marsh (Figures 1& 2; Table 1). Adult Bluegill (14.9 cm ± 0.43 cm; total length ± SEM) were

collected using hook and line sampling under the University of Michigan IACUC Protocol: PRO00009089. Fish housing and transport were based on the MDEQ Fish Tissue PFAS Sampling Guidance Document (MDEQ 2019). Bluegill were placed into one of 3 aerated coolers, Coleman Xtreme 70 quart cooler: Wichita, KS, USA; Coleman 48 quart performance cooler: Model 5286B; or Rubbermaid 80 cooler: Atlanta, GA, USA, with water from the site and transported to the University of Michigan Biological Station Stream Research Facility, Pellston, MI (45.563973, -84.751218).

Bluegill were housed in a flow-through artificial stream for at least 2 hours at the University of Michigan Biological Station Stream Research Facility. The artificial stream, 2.6 m x 0.8 m x 0.3 m (L x W x H), was composed of cement blocks covered in 4 mil polyethylene sheeting and fed by unfiltered stream water from the East Branch of the Maple River. Flow into the stream was 0.995 L/s. Although some Bluegill were collected from slow- to no-flow environments, to standardize swimming trials for the best comparison, all Bluegill were held in the flow-through holding stream and began the swimming trial at the same flow velocity. Bluegill fed on detritus and other organic matter flowing through the artificial stream. The stream was covered with polyethylene snow fence to prevent outside predation and escape of the fish. The holding stream was located outside, thus, the fish were exposed to natural sunlight, light: dark schedule (approximately 15:9 light: dark schedule), weather conditions, and rainfall. Fish were exposed to an ambient temperature of approximately 19 ºC. There was minimal sand substrate which entered the stream through the inflow. The fish started the trial a maximum of 55 hours after being placed into the artificial stream, which is less than the depuration half-lives which range from 3 to 35 days in fish tissues, depending on the individual PFAS compounds (Martin et al. 2003).

Swimming Flume Design

A recirculating flume was used to measure the fish's swimming ability (Webb 1988). The swimming area of the flume was 55.9 cm x 17.6 cm x 24.6 cm (L x W x H). The swimming area was enclosed with hardware cloth on either end. Prior to the trials, the hardware cloth was soaked in acetone, isopropyl alcohol, and water to remove oils from the metal and prevent potential PFAS contamination. Black curtains surrounded the front of the swimming area, 46.4 cm x 24.6 cm (L x H), to prevent outside influences from affecting swimming. A Motor Master 200 (Minarik Electric Company, Los Angeles, CA) with a Blue Chip II™ Adjustable Speed Motor (180 V DC, 2400 RPM, 1 HP; Minarik Electric Company) attached to a propeller created the flume's flow (Figure 3).

A mirror was placed under the swimming area which faced both the swimming area and a handheld Panasonic High Definition Video Camera (Model No.: HDC-HS250P; Osaka, Japan) which recorded the swimming trials. A Hach flow meter (Hach FH950, Loveland, Colorado) was used to determine the flow at specific revolutions per minute (RPMs). A linear regression was used to find the RPM's needed for any desired flow rate. The RPM's were recorded at each flow rate during the trials and converted into the estimated flow rate for each step.

Swimming Performance Testing Protocol

The temperature of the flume water, 19.3 ºC ± 0.69 ºC (mean ± SEM), was measured before each trial. A bubbler ran for the entire trial to prevent oxygen limitation. Dissolved oxygen was measured in two trials and dissolved oxygen was higher after each trial. The acclimation period allowed the fish to adjust to the temperature and the slow flow rate. The acclimation period of 150 minutes at 20 cm/s caused the fish to swim in a combination of two gaits: median and paired fins swimming and body/caudal fin swimming (Gregory and Wood

1999). After the acclimation period, the flow increased quickly to determine the effects of PFAS during anaerobic swimming as opposed to common critical swimming speed tests with longer intervals which approximate aerobic swimming (Bainbridge 1962; Dussault et al. 2008). Although many of the longer-type critical swimming speed trials acclimate the fish overnight, in this study, the acclimation period was shortened to prepare for anaerobic swimming. After the acclimation period, the flow increased 4 cm/s every minute until the fish's tail rested on the back grate and its body wavered laterally, while facing upstream. If the fish was unable to stay off the back grate during the acclimation period, the fish was nudged with a 2.54 cm PVC pipe to ensure the Bluegill was exhausted. If no swimming response followed three pokes or if the fish was unable to stay off the back grate out of the acclimation period, the trial ended. The video recording verified when the trial ended at the specific time of an individual flow step to confirm the observation during the trial.

Fish Dissection and Histological Sampling

Fish were euthanized using a benzocaine solution (≥ 250 mg/L; ChemCenter; La Jolla, CA, USA) as per University of Michigan IACUC protocol: PRO00009089 (Appendix C). Once opercular movement ceased, the fish remained in benzocaine for at least 10 minutes to ensure death. Total length and weight were measured for each fish with a non-coated fish board and an O'HAUS scout scale (Model #H-5851; Parsippany, NJ, USA). Before weighing, the fish was placed in a Ziploc® bag to prevent contamination. The fish was then decapitated and dissected atop a Ziploc® bag. The liver was removed and weighed at 0.828 g ± 0.91 g (mean liver mass ± SEM). The liver sample was double bagged using Ziploc® bags, labelled, and was stored at -18 °C or colder (Lam et al. 2014; Newsted et al. 2008; Pan et al. 2017). Gill samples and additional liver samples were used to determine differences in histology (Table 1). These samples were cut

into cubes up to 2.5 cm x 2.5 cm x 2.5 cm and placed in Bouin's fixative at a 1:3 tissue to fixative volume ratio. The fixative was exchanged for fresh fixative in mid-August. The samples were stored in fixative for up to 55 days.

Equipment Handling

Equipment was screened for PFAS-containing products (MDEQ 2019). Fishing equipment and supplies were washed with Alconox® Powdered Precision Cleaner (Alconox, Inc; White Plains, NY, USA) mixed with Milli Q water in a Coleman Model 5277 cooler between sampling events to prevent cross contamination of PFAS. The coolers were also cleaned with Alconox® between sampling events. PFAS-free materials, like Ziploc® bags were used during fish weighing, dissecting, and storage. In addition, dissection scissors, scalpels, and hemostats were washed using Alconox® detergent and rinsed with Milli-Q water in Ziploc® bags between dissections to prevent cross-contamination of samples.

Lab Analysis

Fish liver and gill samples were analyzed for changes in histology. The samples stored in Bouin's fixative were embedded in paraffin, sectioned, and photographed using a light microscope (Yasser & Naser 2011). To prepare for embedding, samples were soaked in multiple reagents each for three times at 15 minutes each. Each sample was soaked in 50% ethanol, 75% ethanol, 95% ethanol, 100% ethanol, xylene, and liquid paraffin. The tissue samples were then set in a cassette and made into a block. The blocks were sectioned with a Microm HM 325 microtome (Waldorf, Germany), into 5 µm and 10 µm slices, placed on microscope slides, floated on a Boekel Scientific water bath (Feasterville-Trevose, PA, USA), and warmed on a Precision Scientific Slide Warmer (Chicago, IL, USA). The microscope slides were stained with hematoxylin and eosin techniques (Bancroft and Stevens 1990). Specifically, the microscope

slide was soaked twice for one minute each in 100% xylene, 100% ethanol, 95% ethanol, 70% ethanol, and 50% ethanol in coplin jars to remove the paraffin from the slide and rehydrate the sample. The slide was also soaked in hematoxylin for 2-5 minutes, rinsed with water for 3-5 minutes and dipped in 70% ethanol with 10-20 drops of hydrochloric acid for 10 seconds. Hematoxylin stained nucleic chromatin a blue-purple color (Feldman and Wolfe 2014). After being rinsed with water for 1 minute and soaked in 1% eosin for 10 minutes, the slide was soaked twice for one minute each of 95% and 100% ethanol, and 100% xylene. Eosin stained cytoplasms and other proteins pink (Feldman and Wolfe 2014). Finally, the slide was dried and mounted with permount and a coverslip. After at least 24 hours to allow for drying, slides were viewed under the following light microscopes with attached cameras: Nikon Eclipse E200 with Nikon Y-THF camera (Tokyo, Japan) or Leica DM750P with Leica ICC50W camera (Wetzlar, Germany). Multiple pictures of each sample were taken at magnification up to 400X for gills and 1000X for livers including the objective magnification. The photomicrographs were used to identify tissue structures and determine whether structures were healthy, an alteration due to an artifact of sacrifice, or a true alteration (Wolf et al. 2015).

Data Analysis

Critical swimming speed was calculated from the flow step and time information from each trial:

$$Ucrit = last\ successful\ flow\ step + (time\ swam\ in\ last\ step * flow\ interval)$$

(Brett 1964; Kelsch 1996). In addition, the time to exhaustion was calculated by adding the total amount of time the fish swam during the trial, including the acclimation period.

Polynomial regression models, using the lm and poly functions in R statistical software (version 3.6.1; R Core Team, 2019), determined if there were any significant differences

between PFAS levels taken from different water bodies, swimming performance, and histology. Fish whose entire swimming trial lasted less than 10 minutes were deemed as non-participating and were not included in the regression models.

Linear mixed models, using the lmer function in the lme4 package (Bates et al. 2019) in RStudio (version 1.3.776; Rstudio Inc., Boston, MA), were completed using Total PFAS and an individual PFAS compound, which had concentrations for all four sites, to determine standardized critical swimming speed values, with water temperature in the swimming flume as a random factor. Repeated linear mixed models were ran with Total PFAS and a different individual PFAS compound each time to retain the simplicity of only two factors in each model.

Histological alterations identified in the photomicrographs were analyzed for their severity (Table 6). A fish's lesions for a specific tissue were categorized for each lesion type into 4 severity scores: minimal, mild, moderate, and severe (Wolf et al. 2004, 2010). The same scoring methods were not standardized for each lesion type due to the ranges of size and severity between lesion types. A minimal score indicated the lesion type was present, but with the least change possible in the tissue. Whereas, a severe score indicated a lesion type had the most change possible in a tissue and the lesion type compromising the vast majority of the tissue sample. Some lesions, like lamellar adhesions were scored based on the area of tissue altered by this lesion type (i.e. minimal: <25%, mild: 25-50%, moderate: 50-75%, severe: 75-100%). However, other lesion types, like lamellar fusion, were categorized by their number of discrete lesions (i.e. minimal: 1-3 lesions, mild: 4-6 lesions, moderate: 7-9, severe: >9). With the scores for each lesion type present in each tissue sample, lesion type and severity trends were made in comparison to surface water PFAS concentrations and samples from the other sites.

RESULTS

Surface Water PFAS Concentrations

Surface water total PFAS concentrations, and physical and chemical water characteristics varied between sites (Table 3; Table 4). Total PFAS concentrations are the combined concentration of all the 14 or 19 analyzed PFAS analytes from a single site. Water samples from Pickerel Lake were not taken to determine PFAS concentrations, therefore, total PFAS concentrations were assumed to be less than 0 ppt based on a third-order polynomial regression with total PFAS and critical swimming speed ($R^2 = 0.205$, p-value = 0.03) of the remaining sites. The range of analyzed PFAS concentrations throughout the sampling sites was from 13 ppt total PFAS (Susan Lake) to 12,266 ppt total PFAS (Clark's Marsh; Figure 4).

Fish Behavior

Of the thirty-six swimming trials started, twenty-nine trials were deemed successful. Pickerel Lake and Clark's Marsh had the highest mean critical swimming speeds when swimming speed was standardized with the fish's total length (Clark's Marsh: 2.93 ± 0.4 body lengths travelled per second (BL/s), mean swim speed ± SEM; Pickerel Lake: 3.52 ± 0.3 BL/s, mean swim speed ± SEM; Figure 5).

Linear and polynomial regression analysis were completed to determine the relationships between individual PFAS concentrations and swimming performance. Linear regression analysis resulted in a significant inverse relationship between standardized critical swimming speed and PFHxDA concentrations ($R^2 = 0.246$; p-value = 0.006; Figure 6). Polynomial regression analysis resulted in significant relationships between standardized U_{crit} and the PFAS compounds: Total PFAS, PFPeA, PFOSA, PFHxDA, PFBA, PFHpS, and PFBS (Table 5). Using a second-order polynomial regression, critical swimming speed was slower at moderate surface water total

PFAS concentrations and was faster at both non-detect and very high contamination levels (adjusted $R^2 = 0.347$; p-value < 0.002; Figure 7).

Linear mixed models were used to examine the contributions of individual PFAS compounds to critical swimming speed. Linear mixed models showed perfluorocarboxylic acids (PFCAs) with carbon chain length 6-8, including PFOA, and total PFAS significantly described critical swimming speed (p-value < 0.05), when the random factor was water temperature in the swimming flume for each swimming trial.

Fish Histology

Numerous gill lesions were exhibited by samples from all sites (Tables 6 and 7). Lamellar adhesions generally became more consistent and severe with an increase in total PFAS (Figure 8). Lamellar atrophies generally decreased with an increase in total PFAS. There was an increase in variation in epithelial cell hyperplasia and lamellar disorganization as total PFAS increased. Nearly all samples had severe lamellar clubbing and at least minimal telangiectasis, however, these lesions were likely artifacts of handling or sacrifice. Thus, since the lesions were likely not due to toxicosis, therefore likely not present during the swimming trials, the lesions were excluded from further analysis. Although these samples were analyzed for lesion severity, a margin of error exists due to excessive section thickness.

Liver lesion presence and severity varied between sites. The most common lesion in Pickerel Lake liver tissues was glycogen vacuolation (Table 6). There were no lesions frequently found in Susan Lake liver tissues. Au Sable River liver lesions most commonly found were cytoplasmic hypertrophy, nuclear degeneration, cytoplasmic degeneration, and glycogen vacuolation (Table 7; Figure 9). The most common lesions in Clark's Marsh liver tissues were glycogen vacuolation, cytoplasmic hypertrophy, irregularly shaped nuclei, cytoplasmic

degeneration, and nuclear degeneration (Table 7). Liver lesions were not classified with severity (extent) scores as gill lesions were due to lacking quality. While there is uncertainty of specific amounts of the lesions, this presence-absence data is helpful to determine liver lesions from PFAS.

DISCUSSION

The presence of PFAS in surface waters was related to significantly altered critical swimming speed of Bluegill (*Lepomis macrochirus)* collected from those water bodies. Overall, these results clearly show that PFAS exposure modified Bluegill's behavior and caused damage to both liver and gill tissue. The effects of PFAS exposure were most pronounced when examining the behavioral data (i.e. critical swimming speed). Critical swimming speeds had mixed effects, decreasing at low PFAS levels and increasing at high PFAS levels. The increasing swimming speeds at high PFAS levels may have been influenced by the difference in oxygen levels between the swimming flume (i.e. approximately 8 mg/L) and Clark's Marsh (i.e. approximately 1.5 mg/L). This increase in available oxygen may have allowed Clark's Marsh fish to swim faster than their normal ability. However, there is no consensus in the literature as to how PFAS exposure should affect swimming speed in fish. Indeed, exposed larval Zebrafish studies have shown increased and decreased swimming speed in separate studies (Hagenaars et al. 2014; Khezri et al. 2017). Goldfish (*Carassius auratus*) exposed to PFOS had an insignificant downward trend in critical swimming performance with increasing PFOS concentration in the parts per million range (Xia et al. 2013). Topmouth Gudgeon (*Pseudorasbora parva*) exposed at parts per million levels had decreased critical swimming speed (Xia et al. 2014). While there's little consensus among other studies, in this study, Bluegill critical swimming speed decreased as PFAS increased at low level contamination and critical swimming speed increased with PFAS concentration at high level contamination.

Histological analysis of liver tissues demonstrated two main findings. First, glycogen vacuolation increased with surface water PFAS concentrations. Second, cytoplasmic and nuclear hypertrophy and degeneration also increased as surface water PFAS concentrations increased.

Increased hepatic glycogen vacuolation is a common response to toxins such as PFAS and organophosphate pesticides (Giari et al. 2015; Singh 2012). For example, wild-caught European Eels (*Anguilla anguilla*) exposed to parts per billion-range PFOA exhibited increased glycogen vacuolation (Giari et al. 2015). Similarly, Common Carp (*Cyprinus carpio*) exposed to dimethoate, an organophosphate pesticide, exhibited increased glycogen vacuolization, in addition to, irregular arrangement of liver cells, rupture of vessels, and necrosis (Singh 2012). While many liver histopathological studies showed an increase in lipid vacuolation, there was no evidence of this lesion type in any of the sampled Bluegill tissues (Cheng et al. 2016). Yet, larval Zebrafish exposed to PFOS at 0.250 ppm and 1 ppm showed an increase in lipid vacuolation (Cheng et al. 2016; Du et al. 2008). Bluegill exhibited many lesion types in both liver and gill tissues, similar to other ecotoxicological research findings.

Additionally, gill tissues demonstrated three main histological findings. First, lamellar adhesions increased with surface water PFAS concentrations (Table 6, Table 7). Second, lamellar atrophies decreased with increased surface water PFAS concentrations (Table 6, Table 7). Third, the variation of severity increased for epithelial cell hyperplasia and lamellar disorganization with increasing PFAS concentrations (Table 6, Table 7). Similar results for gill histology are found in other ecotoxicology studies, including those identifying effects of pesticides (Sakr et al. 2002). For example, Common Carp exposed to heavily contaminated river water, in which pesticides, trace metals, and hydrocarbons were found, exhibited epithelial hyperplasia, epithelial lifting, and lamellar atrophy, among other abnormal morphology (Yasser and Naser 2011). These histological alterations are nonspecific effects, which are present due to toxin exposure, not specifically to PFAS exposure (Cengiz 2006; Velmurugan et al. 2007). While in most cases, gills can regrow once the contamination has been removed, the damage could

greatly alter fish metabolism and behavior by potentially limiting oxygen intake and blood detoxification (Cerqueira and Fernandes 2002).

These physiological and histological changes could explain some of the findings regarding swimming speed. Gill damage in the form of epithelial lifting and lamellar fusion, can decrease oxygen uptake (Peuranen et al. 1994; Skidmore 1970; Skidmore and Tovell 1972). This decreased oxygen uptake, in turn, can influence the fish's metabolism and behavior. Rainbow Trout (*Oncorhynchus mykiss*) exposed to lethal levels of zinc sulfate, which caused severe epithelial lifting, altered blood flow patterns, and increased granulocytes (white blood cells), had increased ventilation volume, opercular rate, and coughing rate to maintain adequate oxygen uptake (Skidmore 1970, Skidmore and Tovell 1972). Thus, more effort was required to achieve the same oxygen uptake rates. Brown Trout (*Salmo trutta*) exposed to iron and humic acids showed lamellar fusion and epithelial cell hypertrophy, which along with iron precipitate on the gills, led to an increased diffusion distance. This increased distance led to decreased oxygen uptake and impaired ion regulation (Peuranen et al. 1994). With decreased oxygen uptake and ion regulation, there are limited options for behaviors a fish can enact to maintain homeostasis. For example, maximum aerobic demand is dependent on the total functional gill area (Duthie and Hughes 1987). A reduction in functional gill area via cautery led to decreased oxygen consumption and decreased critical swimming speeds at normoxic and hyperoxic conditions (Duthie and Hughes 1987). Thus, a reduction in functional gill area due to damage by toxins may lead to decreased critical swimming speeds as well. Gill damage, as was found in the Bluegill, can reduce oxygen uptake and the behaviors a fish can maintain. Gill and liver damage alter internal physiology, which is ultimately displayed in abnormal behavior and long-term fish health.

Damaged liver tissues, including those with increased glycogen vacuolation and hepatocyte apoptosis, can alter metabolism (de la Torre et al. 2000; Krajnovic-Ozretic and Ozretic 1987; Roy and Bhattacharya 2006). Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) are key enzymes in metabolism, and an increase in their levels can be onset by chemical exposure and in association with the histological lesions exhibited in this study (Chavan and Muley 2014; Krajnovic-Ozretic and Ozretic 1987; Roy and Bhattacharya 2006). Ultimately, the increased levels of GPT and GOT show both hepatic and myocardial toxicity, thereby indicating organ dysfunction, altered metabolic functions or structural tissue damage (Chavan and Muley 2014, Krajnovic-Ozretic and Ozretic 1987). So, liver damage, leading to organ malfunction and alterations in metabolism, can influence homeostasis and cause abnormal fish behavior.

In combination, both liver and gill deformities related to PFAS could modify fish swimming behavior. Similar to fish in hypoxic environments, fish with decreased oxygen uptake and metabolism would exhibit decreased speed and duration of aerobic swimming, such as during cruising, habitat exploration, and foraging; and anaerobic swimming, such as during predator evasion (Domenici et al. 2013). Due to limitations in metabolic scope and maximum metabolic rate, there is a limited ability to keep red “slow-twitch” oxidative muscles, responsible for aerobic swimming, oxygenated which reduces swimming performance results (Domenici et al. 2013). Decreased metabolism and the effective hypoxia would increase the recovery time after anaerobic swimming when a fish is recovering from its ‘oxygen debt’, or post-exercise oxygen consumption, thereby making a fish vulnerable to predators during this extended recovery period (Domenici et al. 2013; Jain et al. 1998). For example, Sockeye Salmon (*Oncorhynchus nerka*) exposed to dehydroabietic acid (DHA), a toxin in pulp mill effluent, had

decreased critical swimming speeds in both of the repeated trials, increased oxygen consumption during two repeated swimming trials and the recovery period in between, and increased plasma lactate concentration during the three time periods (Jain et al. 1998). Epithelial hyperplasia, which is also found in our study's Bluegill, may be partially attributed to the increased oxygen consumption (Jain et al. 1998). Thus, the decreased swimming speed of the exposed fish may be attributed to respiratory limitations (Jain et al. 1998).

Altered swimming speeds likely lead to modifications of ecological aspects, such as foraging, predator evasion, and mating. The increased critical swimming speeds may aid in the survival of contaminated fish, due to the increased chance of success in predator evasion, increased chance of securing a mate, and increased opportunity for foraging (Plaut 2001). Indeed, critical swimming speed is positively correlated with standard metabolic rate and routine activity, which applies to foraging, migration, and predator avoidance (Plaut 2001). On the other hand, fish from low PFAS contaminated sites with decreased swimming speed, are subject to decreased survival due to less success in predator evasion, securing a mate and foraging (Plaut 2001). With altered swimming speed and survival, contaminants, such as PFAS, may alter population dynamics.

Due to the presence of multiple PFAS in the environment, populations are inevitably exposed to multiple stressors. Despite the frequency of studies focusing on organismal effects of individual PFAS, there are a limited number of studies researching organismal effects of the more relevant PFAS mixtures and whether wild populations of aquatic organisms may have additive or synergistic effects from the multiple PFAS to which they are exposed. Khezri et al. (2017) appears to be the only study which focuses on behavioral responses to PFAS-containing mixtures, which found increased swimming speed in PFAS mixture- and PFOS-only exposed

fish and is similar to responses to persistent organic pollutant mixtures. Giari et al. (2015) researched multiple PFAS and histology in wild-caught European Eels to find that liver vacuoles increased with PFOA concentrations, similar to this study. While multiple stressors studies are more environmentally relevant, currently, there is a gap in knowledge in this area that this study begins to fill.

Altered aspects of population and community ecology due to contaminant exposure is clearly possible from changes in metabolic processes, altered oxygen uptake, organ dysfunction, and altered critical swimming speed. Although many studies focus on the organismal effects of PFAS and other contaminants, there is a gap in knowledge regarding effects at larger ecological scales. Ecological aspects which could be altered include foraging, mating, and predation. For example, Abalone (*Haliotis iris*) exposed to an algal stimulant exhibit increased feeding activity (Allen et al. 2000). Increased foraging may lead to a trophic cascade, where some trophic levels are overabundant and adjacent trophic levels are scarce, which could result in increased conspecific competition and potential population decline. At low level contamination, more severe population decline is possible, with decreased foraging, mating, and chance of success in predator evasion. To exacerbate the situation, PFAS contaminated individuals may produce offspring with lethal deformities, leading to a decline in population recruitment (Chen et al. 2013). However, over time, fish may become more resistant to the contaminant, like when Mummichog (*Fundulus heteroclitus*) were found in elevated concentrations of mercury (Weis and Khan 1990). While large-scale responses to contaminants vary, there is potential for fish population decline in contaminated waters.

Since PFAS occur in the environment, estimating biomagnification potential will aid in public health and fisheries management decision making. Kannan et al. (2005) suggest that

PFOS biomagnifies via diet in Great Lakes food chains by over 5 orders of magnitude. However, there are mixed results in the literature, suggesting biomagnification does not occur at all sites (Bush et al. 2015). Thus, the factors that determine the specific biomagnification potential for different trophic levels and locations are not widely known. Known factors that contribute to biomagnification in aquatic organisms are diet, the tendency of the compound to stay in the water column or attach to the sediment, the organism's location in the environment (i.e. benthic, pelagic, etc.), and the tissue type to which the contaminant binds (Ahrens et al. 2015; Kannan et al. 2005). In addition, the increased chance of success in predator evasion may decrease the biomagnification potential in highly contaminated sites. For example, PFAS requires new models than many common contaminants, like DDT, because PFAS bind to proteins instead of lipids (Kannan et al. 2005). Some studies have indicated that aquatic organisms are exposed to PFAS and other contaminants both by gill uptake and diet (Ahrens et al. 2015, Kannan et al. 2005). As toxin concentrations increase in higher trophic levels, more ecological aspects may be altered.

Exposure to PFAS and other anthropogenic contaminants likely cause wide reaching effects extending passed the physiology and behavior of individual organisms to community- and ecosystem-level scales. This research described organism-level behavioral and physiological changes at different environmentally relevant PFAS contamination levels. While the gap in knowledge of organismal effects of PFAS has begun filling, there's still little known about PFAS effects on broader temporal and ecological scales. For example, next steps may include identifying the PFAS concentrations and effects on fish after varying length of exposure. Another route may include using population surveys, which measure fish condition, growth, age,

PFAS concentrations, and population size, for multiple exposed fish populations. These projects would help determine the broader effects of PFAS on fish populations over time.

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APPENDIX A. TABLES

Table 1. Locations of Bluegill collection in July-September 2019. Swimming sample size is the number of fish per site that completed the swimming trial. The histology sample size indicates the number of liver and gill samples each per site, unless otherwise stated.

Site Name	**Latitude**	**Longitude**	**Swimming Sample Size**	**Histology Sample Size**
Clark's Marsh – Site 2	44.438076	-83.390103	5	2 gill; 1 liver
Clark's Marsh – Site 3	44.437707	-83.389940	5	1 gill; 2 liver
Au Sable River – Site 1	44.418956	-83.337097	1	1
Au Sable River – Site 2	44.43560	-83.439190	6	2
Susan Lake	45.329179	-85.179604	8	3
Pickerel Lake	45.390555	-84.754775	11	4

Table 2. The PFAS compounds analyzed at Freshwater Future, in samples taken by Tip of the Mitt Watershed Council (TOMWC), and PFAS compounds analyzed at Test America and taken by the Michigan Department of Environmental Quality (MDEQ) (Bush et al. 2015; TOMWC 2019). Chain length indicates the number of carbons in the carbon chain in the individual PFAS compound.

Name of Compound	**Acronym**	**Chain Length**	**TOMWC**	**MDEQ**
Perfluorobutanoic acid	PFBA	4		X
Perfluorobutane sulfonate	PFBS	4	X	X
Perfluoropentanoic Acid	PFPeA	5		X
Perfluorohexanoic acid	PFHxA	6	X	X
Perfluorohexane sulfonate	PFHxS	6	X	X
Perfluoroheptanoic acid	PFHpA	7	X	X
Perfluoroheptane sulfonic acid	PFHpS	7		X
Perfluorooctanesulfonamide	PFOSA	8		X
N-Methyl perfluorooctane sulfonamidoacetic acid	N-MeFOSAA	8	X	
N-Ethyl perfluorooctane sulfonamidoacetic acid	N-EtFOSAA	8	X	
Perfluorooctanoic acid	PFOA	8	X	X
Perfluorooctane sulfonate	PFOS	8	X	X
Perfluorononanoic acid	PFNA	9	X	X
Perfluorodecanoic acid	PFDA	10	X	X
Perfluorodecane sulfonic acid	PFDS	10		X
Perfluoroundecanoic acid	PFUdA	11	X	X
Perfluorododecanoic acid	PFDoA	12	X	X
Perfluorotridecanoic acid	PFTrDA	13	X	X
Perfluorotetradecanoic acid	PFTeDA	14	X	X
Perfluorohexadecanoic acid	PFHxDA	16		X
Perfluorooctadecanoic acid	PFODA	18		X

Table 3. Physical and chemical characteristics of the surface water at fish sampling sites.

Site Name	**Temperature (C)**	**pH**	**Dissolved Oxygen (mg/L)**	**Conductivity (µS/cm)**	**Turbidity (NTU)**
Clark's Marsh – Site 2	21.4	6.88	1.51	231.8	-
Clark's Marsh – Site 3	21.5	6.91	1.51	240.6	-
Au Sable River – Site 1	23.1	7.74	5.43	274.8	-
Au Sable River – Site 2	20.22	8.33	8.21	306.3	3.12
Susan Lake	27.48	8.82	11.86	247.1	3.18
Pickerel Lake	26.56	8.64	9.19	302.5	5.89

Table 4. PFAS concentrations in each sampled water body (Bush et al. 2015; TOMWC 2019). BRL indicates PFAS concentrations below the reporting limit; <1 ppt for Susan Lake and <2 ppt for the Au Sable River and Clark's Marsh. NA indicates the sample was not tested for a specific analyte. All PFAS analytes are displayed in ppt or ng/L. Total PFAS concentrations are the combined concentration of all 14 or 19 analyzed PFAS analytes from a single site.

PFAS compound	**Au Sable River**	**Clark's Marsh**	**Susan Lake**
Total PFAS	17.1	12266.0	13.0
PFBA	2.8	116.0	NA
PFBS	BRL	104.0	13.0
PFHxA	1.6	922.0	BRL
PFHxS	4.8	3756.0	BRL
PFHpA	0.9	173.0	BRL
PFHpS	BRL	171.0	NA
PFOA	1.9	1309.0	BRL
PFOS	3.2	5099.0	BRL
PFNA	BRL	24.0	BRL
PFDA	BRL	2.5	BRL
PFDS	BRL	BRL	NA
PFUdA	BRL	BRL	BRL
PFDoA	BRL	BRL	BRL
PFTrDA	BRL	BRL	BRL
PFTeDA	0.2	BRL	BRL
PFHxDA	0.5	BRL	NA
PFODA	BRL	BRL	NA
PFOSA	BRL	172.0	NA
PFPeA	1.3	418.0	NA
N-MeFOSAA	NA	NA	BRL
N-EtFOSAA	NA	NA	BRL

Table 5. Characteristics of the significant polynomial regressions between individual PFAS compounds and standardized critical swimming speed. All PFAS concentrations are natural log transformed except PFHxDA.

PFAS compound	**Type of polynomial regression**	**Equation**	**F-statistic**	**R^2**	**p-value**
Total PFAS	2nd order	$4.7111x^2 + 0.4862x + 2.7272$	8.445	0.3938	0.001
Total PFAS	3rd order	$-1.3239x^3 + 4.6571x^2 + 0.4880x + 2.7322$	6.020	0.4192	0.003
PFBS	2nd order	$4.1041^2 – 0.5876x + 2.8172$	5.073	0.2807	0.014
PFHpS	2nd order	$3.8432x^2 + 1.5556x + 2.8172$	4.073	0.2807	0.014
PFBA	2nd order	$4.4118x^2 + 0.8685x + 2.7377$	6.874	0.3459	0.004
PFBA	3rd order	$-2.2113^3 + 4.2288x^2 + 0.9520x + 2.7322$	6.020	0.4194	0.003
PFHxDA	2nd order	$3.4227x^2 – 3.4320x + 2.7364$	9.055	0.4106	0.001
PFOSA	2nd order	$3.8432x^2 + 1.5556x + 2.8172$	5.073	0.2807	0.014
PEPeA	2nd order	$4.2183x^2 + 1.4308x + 2.7398$	6.645	0.3383	0.005
PFPeA	3rd order	$-2.3195x^3 + 4.0113x^2 + 1.4861x + 2.7322$	6.020	0.4194	0.003

Table 6. Descriptions of liver and gill lesions found (Wolf et al. 2015). Lesions which were likely an artifact of handling and sacrifice are denoted by an asterisk.

Type of Lesion	**Affected Organ**	**Lesion Description**
Epithelial Cell Hyperplasia	Gill	A proliferation of epithelial cells on the filament
Epithelial Lifting	Gill	A gap is presence between lamellae pavement cells and capillaries
Filament Branching	Gill	A branch in the distal region of a filament
Lamellar Adhesion	Gill	Lamellae attach to each other at one or more points
Lamellar Atrophy	Gill	Absence of lamellae for all or part of their length
Lamellar Clubbing*	Gill	Enlargements of the lamellae's distal end
Lamellar Disorganization	Gill	Lamellae are abnormally angled away from filament and within lamellae
Lamellar Epithelial Hyperplasia	Gill	A proliferation of epithelial cells on the lamellae
Lamellar Fusion	Gill	Occurs when proliferating pavement cells fill interlamellar sulci partially or completely
Telangiectasis*	Gill	Oval-shaped, blood-filled enlargements of lamellar capillaries
Cytoplasmic Degeneration	Liver	Break down of the cytoplasm in which the nucleus and organelles may not be present
Cytoplasmic Hypertrophy	Liver	Enlargement of the cytoplasm
Increased Glycogen Vacuolation	Liver	Large, light-colored vacuoles without distinct edges
Irregularly Shaped Nucleus	Liver	Nucleus that does not exhibit a round shape
Nuclear Degeneration	Liver	Break down of the nucleus in which the nucleus may have a different texture or shape

Table 7. Lesions found in gill tissues. Lesions likely to be artifacts of sacrifice or handling are excluded. Total PFAS concentrations increase in sites from left to right. An increase in the number of +'s indicates a higher degree of severity: lesion absent = 0; minimal = +; mild = ++; moderate = +++; and severe = ++++. See Table 6 for lesion descriptions.

Lesion Type	**Pickerel Lake**				**Susan Lake**		**Au Sable River**			**Clark's Marsh**		
Individual Sample	**1**	**2**	**3**	**4**	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**3**
Epithelial Cell Hyperplasia	+	+	+	+	+	0	+	0	0	+	+++	+
Epithelial Lifting	++	0	0	0	0	0	0	+++	0	0	0	0
Filament Branching	0	0	0	0	0	0	0	+	0	0	0	0
Lamellar adhesion	+	0	+	+	0	+	+	+	+	+	++	+
Lamellar atrophy	+	+	+	+	0	+	0	0	+	0	0	0
Lamellar Disorganization	++	+++	++	+	0	++	++	+	++	++++	0	++
Lamellar Epithelial Hyperplasia	0	0	0	0	0	0	0	0	0	0	0	+
Lamellar fusion	+	+	+	+	0	+	+	0	0	+	+	+

APPENDIX B. FIGURES

Figure 1. The fish sampling sites in Northwest Michigan near Petoskey and Charlevoix. PIL indicates Pickerel Lake and SUL indicates Susan Lake.

Figure 2. The fish sampling sites in Northeast Michigan near Oscoda. ASR indicates the Au Sable River and CLM indicates Clark's Marsh. The Former Wurtsmith Air Force Base, heavily contaminated with PFAS, is shown North of the marsh and river.

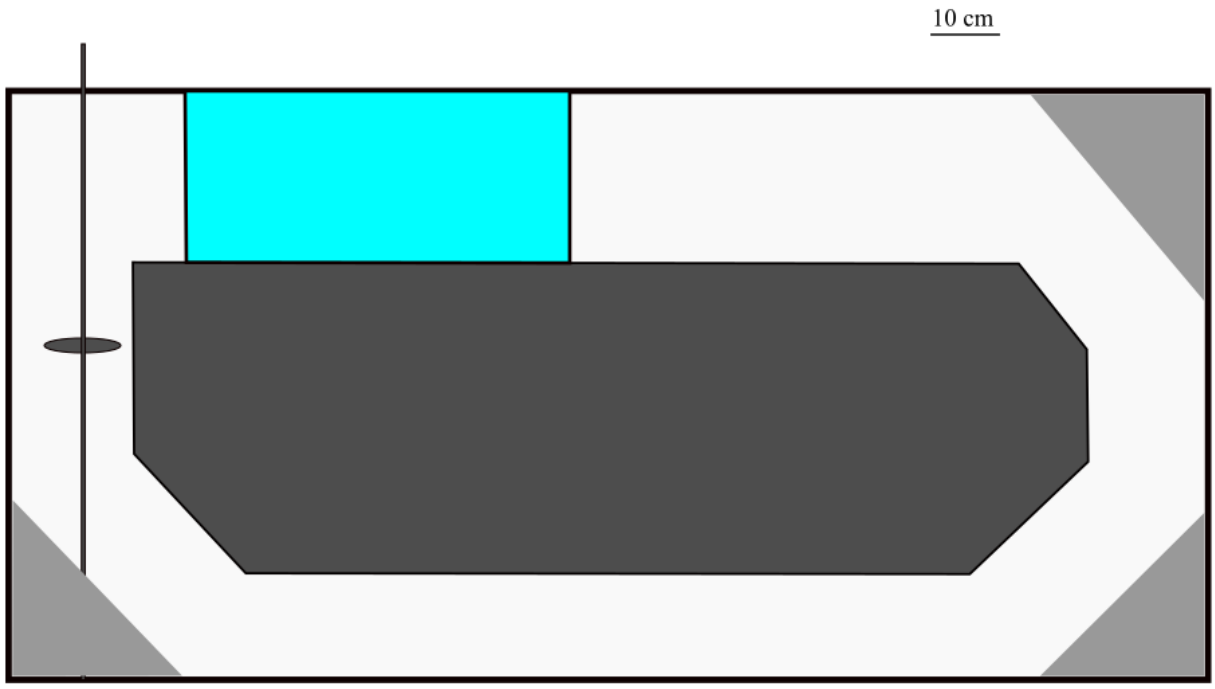

Figure 3. A diagram of the recirculating flume used for swimming trials. The fish were in the blue box during the swimming trial, which has metal grates on either side. Water flowed to the top left direction and around the flume in the white and blue areas.

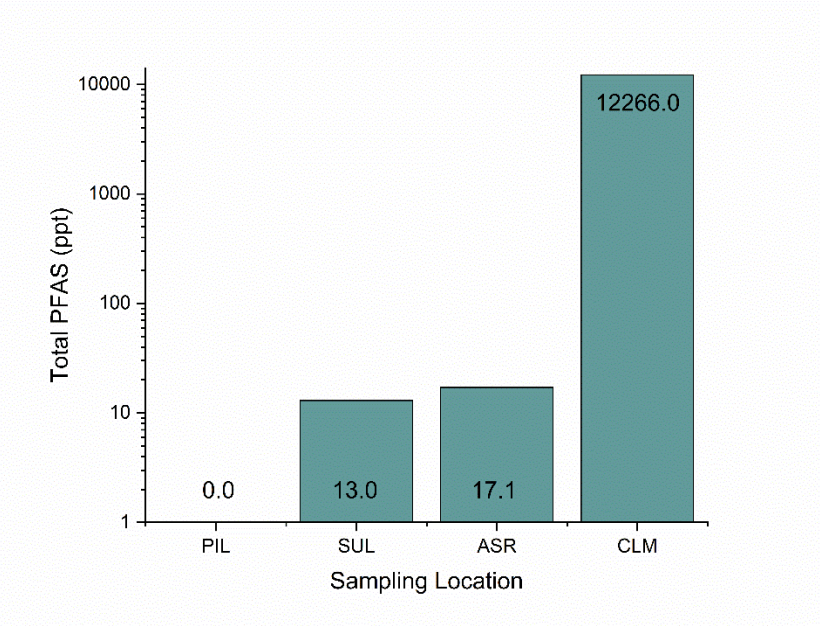

Figure 4. Surface water concentrations of total PFAS from the four sampling sites. PIL indicates Pickerel Lake, SUL is Susan Lake, ASR is Au Sable River, and CLM indicates Clark's Marsh. These concentrations are the combination of 14 individual PFAS compounds for Susan Lake and 19 individual PFAS compounds for Au Sable River and Clark's Marsh.

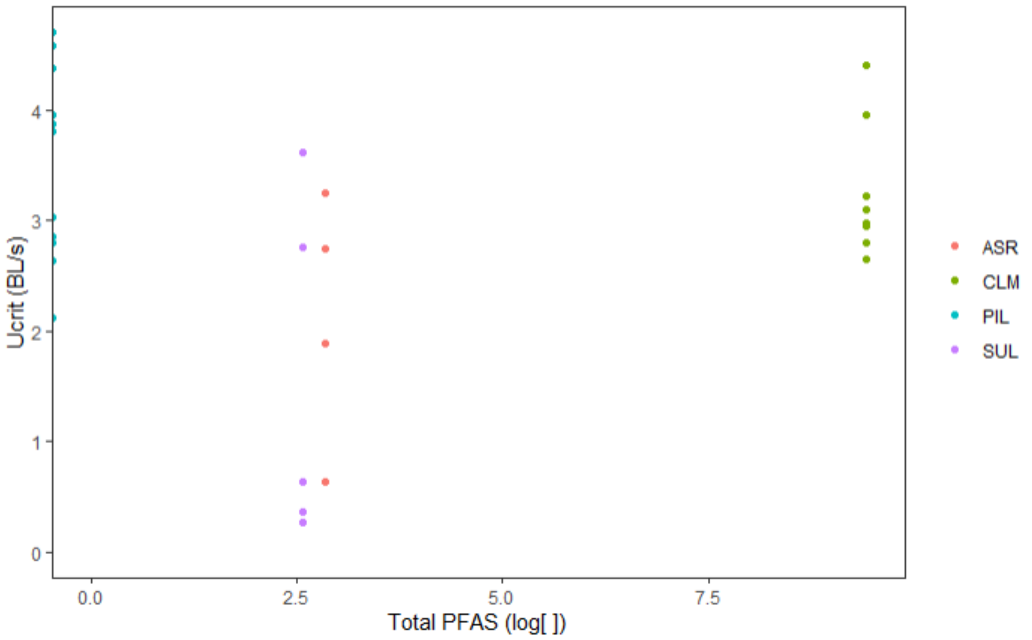

Figure 5. Scatter plot of critical swimming speed (U_{crit}) based on Total PFAS concentrations at the sampling site. Successful swimming trials are included in this plot. PIL indicates Pickerel Lake, SUL is Susan Lake, ASR is Au Sable River, and CLM indicates Clark's Marsh.

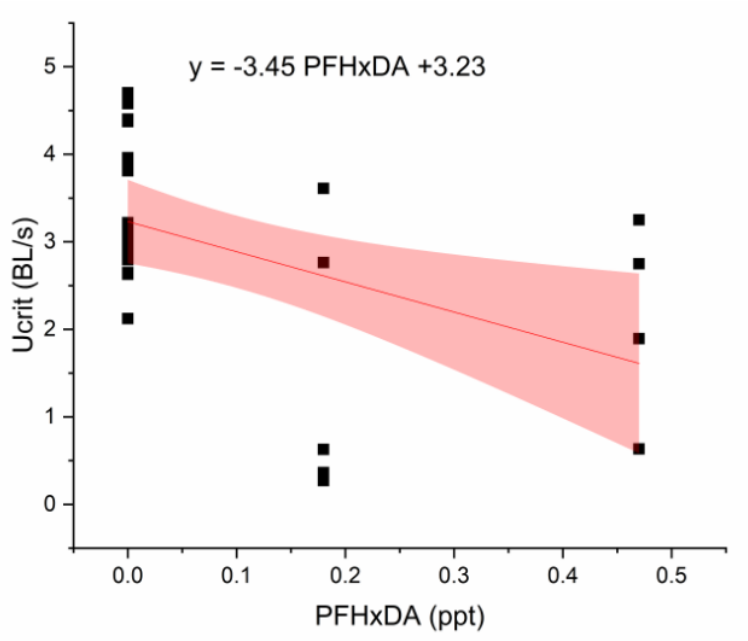

Figure 6. A significant linear regression between PFHxDA and standardized critical swimming speed demonstrating higher levels of PFHxDA relate to slower critical swimming speeds.

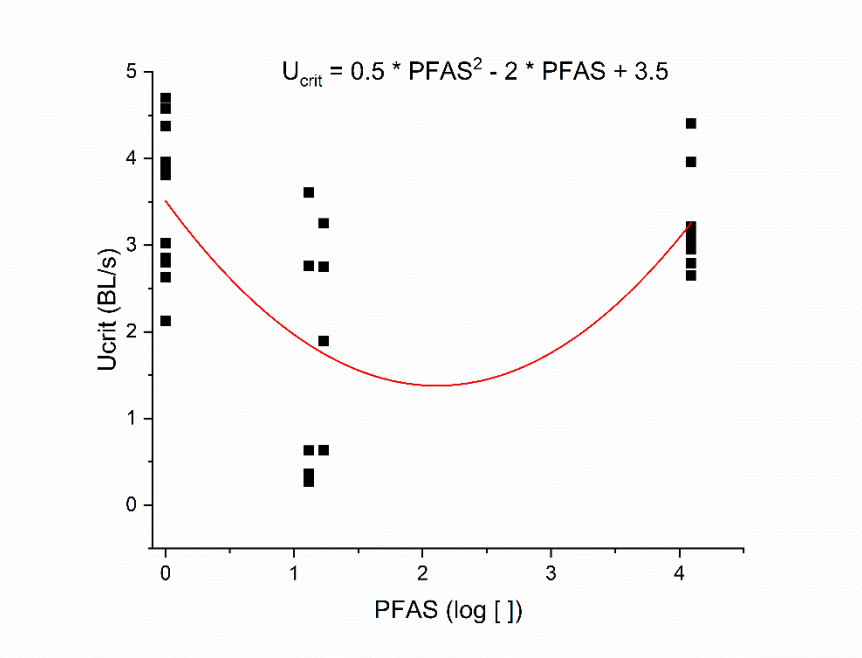

Figure 7. The second-order polynomial regression demonstrates slower critical swimming speeds at moderate total PFAS concentrations (13.0-17.1 ppt total PFAS).

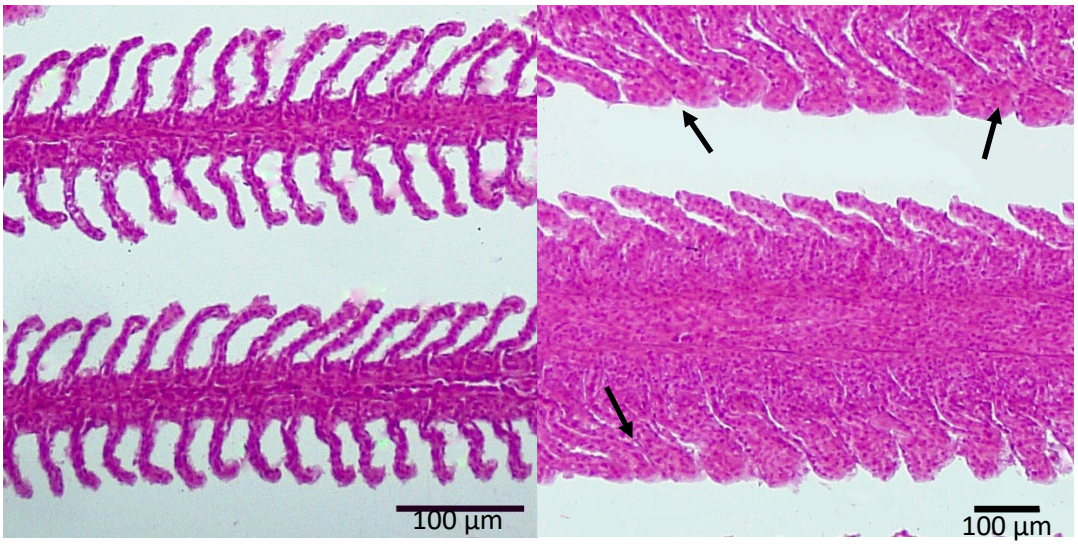

Figure 8. Gill tissues from the control site (Pickerel Lake; left panel) and gill tissues from Clark's Marsh (right panel). Clark's Marsh tissue exhibits lamellar adhesions (black arrows).

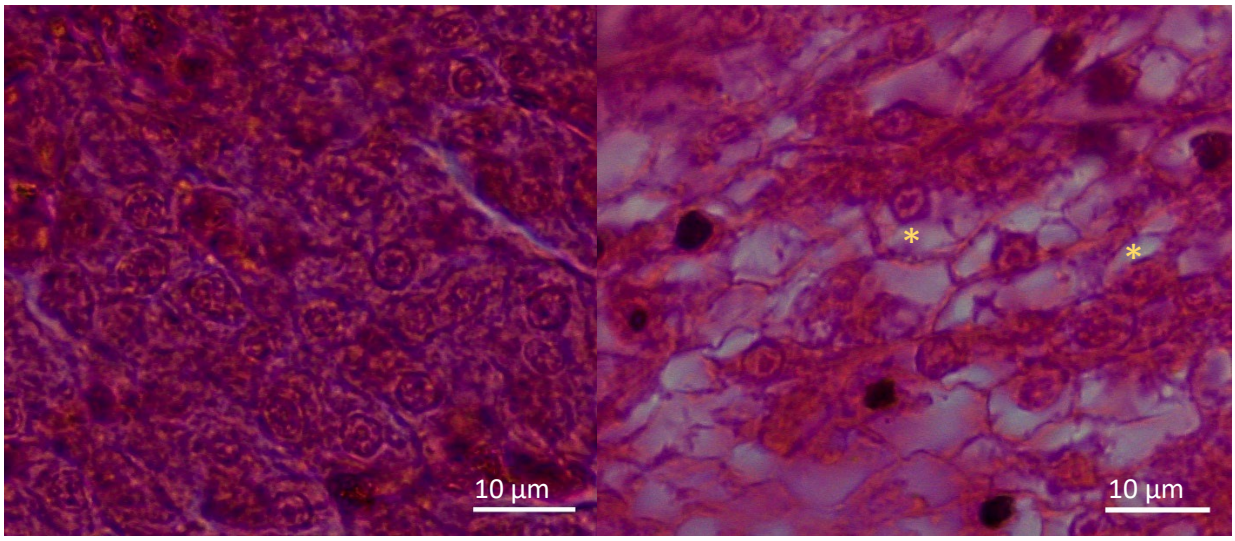

Figure 9. Liver samples from the Pickerel Lake control site (left panel) and Au Sable River (right panel). Au Sable River tissue exhibits abundant cytoplasmic vacuolation (*).

APPENDIX C. IACUC LETTER

ANIMAL CARE & USE OFFICE
UNIVERSITY OF MICHIGAN

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5/16/2019

Paul Moore
Biological Station
710 Dennison
Ann Arbor
48109-1090

Dear Dr. Moore:

The Institutional Animal Care & Use Committee (IACUC) has reviewed the animal use application referenced below. The proposed animal use procedures are in compliance with University guidelines, State and Federal regulations and the standards of the "Guide for the Care and Use of Laboratory Animals. This project has been approved.

There may be additional issues that need to be addressed prior to initiation of the associated research. It is your responsibility, as Principal Investigator, to secure all the necessary requirements and recommendations and notify funding agencies of changes made to the study.

Committee approval must be obtained prior to making changes from what is originally stated in the protocol. An amendment must be approved prior to the implementation of the change. Contact the ACU Office for further information.

The United States Department of Agriculture (USDA), Department of Defense (DOD), and University policy require an annual administrative review of any animal use protocols funded by the DOD or approved to use USDA-covered species. Your continued animal use approval is contingent upon the completion of this online form. Additionally, your renewal application must be submitted, reviewed and approved in a timely manner prior to the expiration date of the current protocol. You will receive notification prior to the deadlines for both the annual review and protocol expiration.

The University's Animal Welfare Assurance Number on file with the NIH Office of Laboratory Animal Welfare (OLAW) is A3114-01, and most recent date of accreditation by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Intl.) is November 22, 2017.

If you receive news media inquiries concerning any aspect of animal use or care in this project, please contact James Erickson, News and Information Services, (734) 647-1842. If you have a security concern regarding the animals or animal facilities, or if you need emergency veterinary medical care, contact Joseph Piersante, Chief Operations Officer, at (734) 763-3434. UMPD will contact the appropriate veterinarian.

Sincerely,

Dr. Daniel Myers,
D.V.M., MPH
Professor of
Surgery
Chairperson, Institutional Animal Care and Use Committee

Protocol ID: PRO00009089
Protocol Title: Swimming performance and tissue contamination of blue gills from PFAS contaminated habitats Approval Period: 5/16/2019 - 5/16/2022

External Funding Sources:

PAF ID	PAF Title	PAF PI	PAF Sponsor

Internal Funding Sources:

Project Title	Department
PFAS in northern Michigan water fish and deer	LSA Biological Station