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CHARACTERIZATION AND ENUMERATION OF MICROPLASTIC POLLUTION IN THREE FISH SPECIES OF THE UPPER MISSISSIPPI RIVER

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CHARACTERIZATION AND ENUMERATION OF MICROPLASTIC POLLUTION IN THREE FISH SPECIES OF THE UPPER MISSISSIPPI RIVER

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Biology Aquatic Science Concentration.

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ABSTRACT

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Microplastics have become a widespread pollutant in terrestrial and aquatic ecosystems over the past 50 years. Microplastics can cause a variety of negative health effects in the organisms that consume them, from changes in feeding habits to increased exposure to toxic chemicals. The majority of recent research has focused on marine microplastics, so the extent that microplastics are impacting freshwater ecosystems is less resolved. In this project, we assessed microplastic pollution in three fish species collected in 2019 from Pools 4 and 8 of the Upper Mississippi River (UMR). Digestive tracts of emerald shiners (*Notropis atherinoides*) (n=89), yellow perch (*Perca flavescens*) (n=97), and shorthead redhorse (*Moxostoma macrolepidotum*) (n=95) were removed for microplastic analysis. Tissue and contents were digested, density separated and filtered for microplastic enumeration. Microplastics were counted and identified, and subsamples were verified via Raman Spectroscopy. In total, 891 microplastic particles were found among the 281 fish individuals and ranged from 0-22 particles per fish. The most prevalent type of microplastic found across species was fibers. Common colors included blue, black, red and clear. Within the size range of microplastics collected (250µm-5mm), microplastic particle prevalence decreased as size of particle increased across all species. Within each species, there were no significant differences in microplastic content when comparing fish from Pool 4 versus Pool 8 ($p>0.05$). In addition, habitat strata (e.g., backwater, main-channel, side-channel, etc.) did not have a significant effect on microplastic content in any species ($p>0.05$). Microplastic content of fish decreased as fish length (mm) increased ($p<0.05$). In addition, smaller fish tended to contain proportionately more microplastics than larger fish (microplastics per mm fish length) ($p<0.05$). Between the three species, emerald shiner contained significantly more microplastics per mm fish length than both yellow perch and shorthead redhorse ($p<0.05$). Raman verification was conducted on 115 randomly selected particles and revealed the most common microplastic polymers as styrene-isoprene, polyester (PES), and acrylonitrile butadiene styrene (ABS). This research confirms microplastic ingestion by UMR fish and highlights the need for further monitoring of microplastic pollution in the UMR system.

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INTRODUCTION

Since the early 1950s, plastic pollution has been an increasing problem and has become a significant threat to natural systems including marine and freshwater ecosystems (Ivar do Sul and Costa 2014, Geyer et al. 2017, Li et al. 2020). Microplastics, broadly defined as plastic particles <5mm in size, are created either through primary manufacturing processes or through secondary degradation of larger plastic items (Frias and Nash 2019). Microplastics are commonly composed of high-density polyethylene (HDPE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polycarbonate (PC) or polyurethane (PU) (Lagarde et al. 2016, Digka et al. 2017, Wang et al. 2017). Size, shape, and condition of microplastic pollution can vary greatly, and are generally dependent on a variety of abiotic factors including chemical and physical weathering. While much of the previous research has focused on large areas of plastic pollution in marine environments, microplastics are emerging as a significant threat to freshwater biodiversity (Reid et al. 2019, Hu et al. 2019). Further research is needed to identify microplastic occurrence in freshwater ecosystems and their potential impacts on these important ecosystems.

Research over the past 10-15 years has confirmed the presence of microplastics in a variety of freshwater ecosystems, including lakes, rivers, wetlands, and groundwater aquifers (Mani et al. 2015, Chia et al. 2021, Kumar et al. 2021, Yang et al. 2022). However, the source of microplastic pollution in these systems varies widely and is dependent on several factors. Terrestrial runoff from rainfall and snowmelt can introduce microplastics into aquatic environments (Pinon-Colin et al. 2019, Vogelsang et al. 2018). Due to this runoff, microplastic concentrations are generally higher in lakes and rivers in urban areas (McCormick et al. 2014, Yonkos et al. 2014, Luo et al. 2019). However, agricultural runoff can also contribute microplastics to nearby aquatic ecosystems due to the use of synthetic fabrics and plastic equipment/machinery. (Grbic et al. 2020). Waste-water treatment effluent has been shown to contain high levels of microplastics, serving as a point source for these pollutants (McCormick et al. 2014, Sun et al. 2019). While waste-water treatment plants remove some microplastics during treatment, the plants release microplastics into the environment due to the high concentrations of microplastics found in influent, the inability to screen micro-sized particles, and the recalcitrant nature of plastic (Murphy et al. 2016). Shedding of synthetic fibers from textiles during manufacturing and washing has been shown to be a contributor to environmental microplastic pollution (Hernandez et al. 2017, Almroth et al. 2018). Research conducted in 2011 on washing machine discharge found that a single piece of clothing could produce more than 1900 microfibers per wash cycle (Browne et al. 2011). In addition, airborne microplastics are prevalent in the atmosphere, therefore the deposition of airborne microplastics into aquatic environments is likely (Chen et al. 2020).

Once in river systems, emerging evidence suggests that a large proportion of microplastics in rivers are retained for extended periods, rather than flowing through the system (van Emmerik et al. 2022). The amount of microplastics retained and the duration of retention in river systems are dependent on weather conditions, flow conditions, distribution of vegetation, and the presence of infrastructure (van Emmerik et al. 2022). Microplastics tend to accumulate in vegetation, along riverbanks, in sediments, at dams, and in other areas where water velocity is low (Watkins et al. 2019). Microplastic retention is also dependent on particle density and shape (Nizzetto et al. 2016, Hoellein et al. 2019, Drummond et al. 2020). Because of these factors, microplastic retention times can vary significantly; however under certain conditions microplastics can be retained for over a decade (Tramoy et al. 2019, van Emmerik et al. 2022). The release and export of retained microplastics through rivers is influenced by flow conditions, as periods of high flow or flooding conditions due to rainfall/snowmelt can release microplastics and cause them to flow out of the system (Hurley et al. 2018, Hitchcock 2020, Ockelford et al. 2020). Research outlining microplastic retention in rivers is of particular concern, since retention rates will likely be linked to effects on organisms that inhabit these ecosystems.

Microplastics can be ingested by aquatic organisms in several ways. Recent research which analyzed microplastics in museum fish samples found that consumption of microplastics has been steadily increasing since the 1950s (Hou et al. 2021). For predatory fish, the most frequent source of microplastics are prey organisms (Guilherme et al. 2019). Microplastic ingestion by zooplankton has been observed in both environmental samples and in laboratory experiments (Cole et al. 2013, Botterell et al. 2019). Ingestion of microplastics has also been observed in both terrestrial and aquatic invertebrates (Wright et al. 2013, Akindele et al. 2020, Ribeiro-Brasil et al. 2022). Due to the large volume of water they filter, mussels can contain significant concentrations of microplastic particles (Li et al. 2016, Qu et al. 2018, Wardlaw and Prosser 2020). Furthermore, microplastic ingestion by larval fish has been observed (Steer et al. 2017). For planktivorous fish, microplastic ingestion may occur by accidental feeding as microplastic particles can be mistaken for prey if particles are of a similar size to prey organisms (Lopes et al. 2020). Since microplastics are ubiquitous in the prey organisms, further research is needed to explore the extent of microplastic pollution to which predatory fish are being exposed. In addition, there is a lack of consensus around the role that functional feeding groups play in influencing microplastic ingestion across fish species. Some research has found links between fish feeding traits and types/quantities of microplastics ingested, whereas other studies have found no significant relationship between fish feeding functional groups and microplastic ingestion (Vendel et al. 2017, McNeish et al. 2018, Hurt et al. 2020, Merga et al. 2020). Additionally, mechanisms of feeding, rather than prey type, might contribute to differences in microplastic ingestion across species (Li et al. 2021). While several unanswered questions remain regarding the relationship between species traits and microplastic ingestion, research investigating the health impacts of microplastic pollution on fish has drastically increased over the past 10 years.

Microplastics may pass through the digestive tracts of fish within ~24 hours, however some evidence suggests that microplastics may be harmful during that time (Hou et al. 2023). Microplastics may cause blockages in the digestive tracts of fish, causing internal tissue damage and preventing proper feeding (Jovanovic et al. 2017, Wang et al. 2018). Once ingested, microplastics may move beyond the digestive tract via translocation to other organs and tissues (Sharifinia et al. 2020, McIlwraith et al. 2021). Ingestion and bioaccumulation of microplastics can also cause changes in the immune response of fish (Kim et al. 2021). Along with the direct physical harm caused by microplastics, they may also release chemicals that can cause negative health effects (Wang et al. 2020a). The primary group of chemicals of concern are Endocrine Disrupting Chemicals (EDCs), which may be present in microplastics due to the manufacturing process or adhere to the surface of plastics and leach into the organisms that consume them (Chen et al. 2019, Naqash et al. 2020, Wu et al. 2021). Microplastic particles, particularly those exposed to extended periods of UV radiation, can also adsorb heavy metals (Wang et al. 2020b). To evaluate the health impacts that microplastic ingestion has on freshwater fish, further research needs to be conducted to describe the extent that fish species are exposed to microplastic pollution in their natural habitats.

RESEARCH OBJECTIVE AND HYPOTHESES

The primary objective of this work was to quantify microplastic abundance, size (between 250µm-5mm), color, and shape in gut samples from three species of Upper Mississippi River (UMR) fish: emerald shiner (*Notropis atherinoides*), shorthead redhorse (*Moxostoma macrolepidotum*) and yellow perch (*Perca flavescens*). These measurements will allow us to better assess how fish in the UMR are being exposed to microplastic pollution and determine if microplastic patterns are related to fish species, size, UMR Pool (proximity to urban areas) and sub-habitat locations within pools. The main hypotheses are summarized below:

1. Emerald shiner and yellow perch will contain higher proportions of microplastic fibers than shorthead redhorse, as fibers are more likely to be suspended in the water column
2. Adult yellow perch will contain higher concentrations of microplastic particles than adult emerald shiner and adult shorthead redhorse, as they will accumulate microplastics from prey
3. Microplastic concentrations will be positively correlated with fish size, and larger fish will contain more microplastics than smaller fish when comparing within species

We hypothesized that microplastic content would differ in the gut samples among the different species. This prediction is due to different feeding patterns between species and different distribution of species within the water column. We would expect differences in microplastic particle counts as well as microplastic types (e.g., material, color, size) between the three species. We predicted a higher proportion of microplastic fibers relative to other microplastic types in emerald shiners and yellow perch, compared to shorthead redhorse because of differences in feeding habits (Sparks and Immelman 2020). We hypothesized that yellow perch will contain more microplastic particles than shorthead redhorse and emerald shiner, because adult yellow perch are more piscivorous and could accumulate microplastic particles from prey. In addition, we hypothesized microplastic gut concentrations to be positively correlated with fish size, as larger fish have larger gut tissues and likely have more contents within their digestive tracts. Since gut contents are dependent on when the fish most recently fed before being collected, standardizing microplastic data across gut masses will normalize microplastic concentrations across fish sizes. We did not expect to see a significant relationship between microplastic particles per gram gut mass and fish size (length, mass). Proportional to their body mass, smaller fish generally eat more compared to larger fish of the same species. However, we hypothesized that because of the type of prey that smaller fish feed on, much of the microplastic they ingest will be below the size range that will be analyzed in this project (250µm-5mm).

METHODS

Study Species

In this study, three UMR fish species occupying different feeding guilds were evaluated for microplastic pollution: yellow perch (*Perca flavescens*), shorthead redhorse (*Moxostoma macrolepidotum*), and emerald shiner (*Notropis atherinoides*). Yellow perch are a popular sport fish in the state of Wisconsin, are tolerant to low oxygen conditions, and are often harvested and eaten by anglers. Adults generally feed on aquatic invertebrates and small fish near the bottom of the water column (Mecozzi 2008), while juveniles are planktivorous. Adult yellow perch reach 20-25cm in length. Shorthead redhorse are a benthic fish that are common throughout the UMR and feed primarily on larval aquatic invertebrates (Paulson and Hatch 2002, Fuller 2021). Adults generally reach 40-50cm in length. Emerald shiner are the smallest of the three species, with adults growing to 13cm (Nico 2023). They are common throughout the entirety of the Mississippi River and feed primarily on zooplankton and small insects (Hartman et al. 1992). They serve as an important food source for larger game fish, such as walleye (*Sander vitreus*) and northern pike (*Esox Lucius*) (Nico 2023). Previous research has found microplastics in the gut contents, liver, and muscle tissue of wild yellow perch (McIlwraith et al. 2021, Munno et al. 2022). There has also been research assessing microplastics in the GI tracts of emerald shiners (Campbell et al. 2017, Munno et al. 2022). Currently there is no published research studying microplastic pollution in wild-caught shorthead redhorse. Research into the prevalence of microplastic pollution in fish of the UMR is very limited (but see Gad et al. 2023). Most of the previous research on microplastic in the Mississippi River has focused on particles present in the sediment and water column (Hasenmueller et al. 2017, Scircle et al. 2020).

Upper Mississippi River

The UMR system extends from Pool 1 at St. Anthony Falls, Minneapolis, Minnesota to the mouth of the Ohio River near Cairo, Illinois (Figure 1). The river system consists of 26 pools segmented by lock and dams which help control flow and allow for navigation between pools. The lock and dam system maintains much of the historical assortment of aquatic habitat, including the main channel, side channels, and backwater lakes. In addition, impounded habitats located directly upriver of each of the dams have introduced reservoir-like areas throughout the system. Pool 4 runs from Lock and Dam 3 near Hager City, WI to Lock and Dam 4 near Alma, WI. Pool 8 runs from Lock and Dam 7 near Dresbach, MN to Lock and Dam 8 near Genoa, WI. Habitats vary significantly between the two pools (Figure 1). Pool 4 contains Lake Pepin, a 34-km-long lake with a maximum depth of 18 meters. At the northwest and southeast ends of Pool 4 there are large areas of backwater habitats and terrestrial floodplains. In addition, Pool 4 is closer to Pool 1 which collects runoff from the urban areas of Minneapolis, MN and St. Paul, MN. Pool 8 has large sections of side channel habitats especially in the northern half of the pool (Carhart and De Jager 2018). Pool 8 also contains large areas of shallow floodplain and contiguous impounded aquatic habitat (Figure 1).

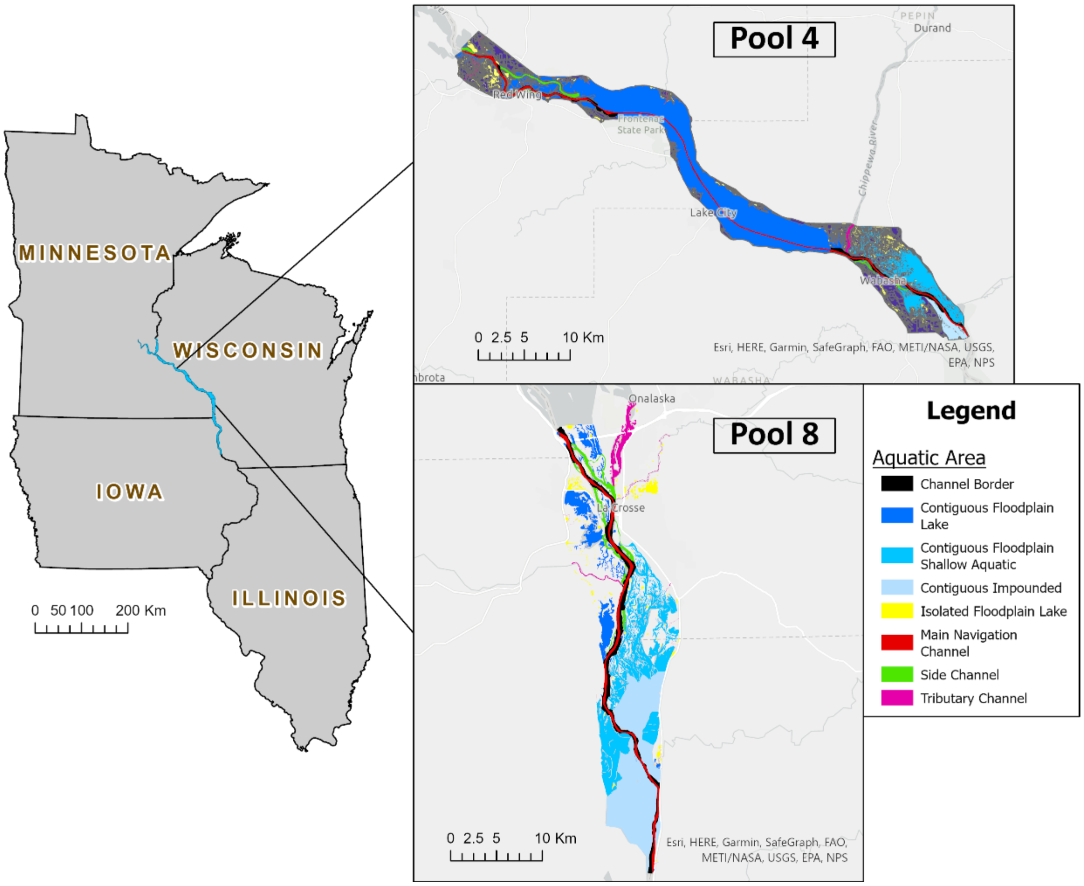

Figure 1. Northern section of Upper Mississippi River (UMR) system and aquatic areas in Pools 4 and 8. Data from UMRR HNA-II 2010/11 Aquatic Areas, sciencebase.gov.

Fish Samples

All fish were collected in the Upper Mississippi River (Pools 4 and 8) between June 17, 2019, and October 28, 2019 by the Upper Mississippi River Restoration Program Long Term Resource Monitoring (UMRR LTRM) group. The UMRR is a federally mandated program consisting of federal and state partners focused on understanding and maintaining the UMR system. The Missouri Department of Conservation and biologists at Missouri State University (MSU) coordinated the collection of fish for a study examining fish distribution and condition. Fish were frozen and donated to the University of Wisconsin- La Crosse. Accompanying data obtained included fish species, total length (mm), and mass (g). In addition, UMR pool, date of collection, GPS coordinates and sub-habitat strata (Table 1) were collected for each fish. Sub-habitat strata describe areas within pools where each fish was collected. These include side channel border (SCB), main channel border (MCB), backwater contiguous shoreline (BWC-S), and impounded shoreline (IMP-S).

Table 1. Summary information on fish samples included in study (total n=281).

Species	Pool 4	Pool 8	Dates Collected	Mass (g)	Length (mm)
Shorthead Redhorse	48	47	06/17/2019-10/28/2019	<1-1292	57-506
Yellow Perch	48	49	06/17/2019-10/25/2019	<1-486	55-314
Emerald Shiner	19	70	06/17/2019-10/04/2019	<1-8	34-105

Dissection

Fish were removed from the freezer and allowed to thaw for 12-24 hours in a cooler prior to dissection. Once thawed, fish were placed on fiberglass dissection trays and moved to a fume hood for dissection. Preliminary studies showed that dissecting fish in the fume hood minimized microplastic contamination (Wesch et al. 2017). Using scalpels and scissors, the entire intact digestive tract was removed and placed in a 125mL glass Erlenmeyer flask and the digestive tract was weighed to the nearest 0.01g. In addition, throughout each set of dissections (n=10 samples), a filtered DI water wetted filter was set in a petri dish adjacent to the dissecting tray to assess any airborne microplastic contamination (Kutralam-Muniasamy et al. 2021). All tools used during dissections were cleaned and rinsed with filtered deionized (DI) water between sample dissections. Following dissection, samples immediately underwent chemical digestion.

Chemical Digestion

Digestive tract samples were digested in approximately 100mL of 15% potassium hydroxide (KOH) solution to remove organic matter (Dawson et al. 2020, Garces-Ordonez et al. 2020). The volume of KOH used was dependent on the size of the sample being digested, with approx. four times the volume of the fish digestive tract added in KOH. Flasks were covered with aluminum foil and digested at 50°C in a Thermo Scientific gravity convection drying oven (Munno et al. 2018). Digestion was complete when the solution was transparent, and no organic material was visible. Samples with visible fat content required additional treatment with an Alcojet (a low-foaming, nonionic detergent) solution to remove fats (Naesheim 2020). Following KOH digestion, fatty samples were rinsed through stacked sieves (5.6mm, 250µm) and suspended in a 4% Alcojet solution. Samples were left for 1-2 days to allow the detergent to break down the fat. Following Alcojet treatment, samples were rinsed through stacked sieves with DI water to remove the Alcojet solution. Samples were then resuspended in 15% KOH solution and subjected to a second digestion at 50º C for 1-2 days. All solutions (KOH, Alcojet, and DI water) were filtered (Whatman 934-AH) prior to use.

Density Separation

Following digestion, a density separation procedure was performed to remove heavier, inorganic non-plastic material from samples (Karlsson et al. 2017). Digested samples were rinsed with filtered DI water through 250µm sieves to remove KOH solution. Samples were then suspended in a 1.4 kg/L filtered (934-AH) $CaCl_2$ solution and placed in the density separator apparatus (Figure 2, Samandra et al. 2022). Most microplastic polymers have densities <1.4 kg/L and will float to the surface of this solution (Syakti

2017). Heavier materials such as small rocks, sand, and shells settle to the bottom of the separator. The solution was allowed to separate for 2-3 hours undisturbed. Following the first separation, the bottom two-thirds of the solution was drained from the separator and discarded. Samples were then resuspended in a fresh $CaCl_2$ solution and allowed to undergo a second separation for 2-3 hours. The second separation was necessary to remove heavy, inorganic material from samples and release any microplastics which may have adhered to heavier inorganic materials. Following the second density separation, the bottom two-thirds of the solution was drained and discarded. The remaining solution in the density separator was drained into a 250mL glass beaker. The funnel and watch glass were rinsed thoroughly with filtered water to collect any microplastics which may have adhered to the separator.

Figure 2. Density separation apparatus. Apparatus is composed of glass funnel attached to rubber tubing, with a plastic pinch clip to control flow. Apparatus is attached to ring stand and topped with glass watch glass. Photo by Sam Munk.

Enumeration

Samples were vacuum filtered through 0.45µm mixed cellulose ester gridded membrane filters (47mm Cytiva Whatman No. 1040670) to isolate microplastics for enumeration. Contaminate-free filters were prepared by visually inspecting the filters using a dissection microscope and removing all particles with forceps or compressed air prior to filtration. Filters were placed in covered 50 mm glass petri dishes and allowed to dry completely. Throughout the filtration process, the filters and sample solutions were covered to prevent airborne microplastic contamination.

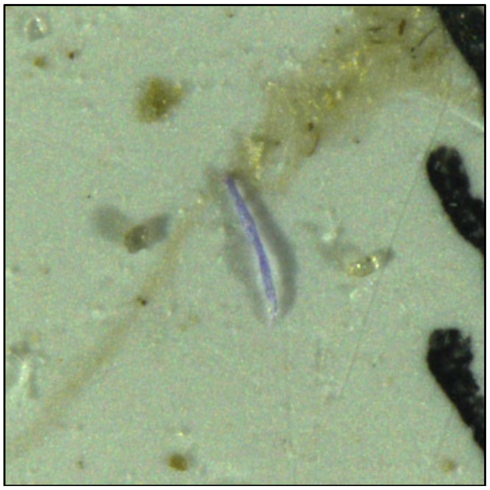

Figure 3. Blue fiber particle on filter paper. Photo by Veronica Sannes, 2021

Microplastics were visually identified via microscopy using a Nikon SMZ18 dissecting scope equipped with a Nikon DS-Ri2 camera. Standard microplastic identification techniques outlined in the Marine and Environmental Research Institute's Guide to Microplastic Identification were used (MERI 2012, Watkins et al. 2019). Important characteristics for consideration when identifying potential microplastics were the presence of cellular structures, shape, color/transparency, and texture. Photos were taken of all particles identified as microplastics (e.g., Figure 3). Fish sample ID number, color, shape, and length along longest axis were recorded for each microplastic particle.

Raman Analysis

Using a random number generator, 115 particles (12.9% of total MP particles) were selected for Raman verification (Lenz et al. 2015, Arujo et al. 2018, Cabernard et al. 2018, Thiele et al. 2021). To reduce background interference from chitinous invertebrate exoskeletons and other debris on filters, each microplastic particle was manually isolated from samples. Each sample filter was viewed under the dissecting scope, referencing previously taken pictures to locate microplastic particles. Using fine tip foreceps, particles were individually removed from sample filters and placed in a 10mL glass vial of filtered ethanol. The vial contents were then filtered through a clean gridded filter, and the vial was thoroughly rinsed with filtered DI water. This process produced a pristine filter containing only microplastics previously identified via visual microscopy. Filters were mounted to an aluminum foil covered cardboard filter holder mounted on a glass slide (Figure 4).

Raman spectroscopy verification was conducted using a HORIBA XploRA PLUS Raman Spectrometer with LabSpec6 Software (532nm excitation wavelength, 100µm slit and 300µm hole) (Pitroff et al. 2021). A 100-2000 cm^{-1} spectral range with a 1200T diffraction grating was used. A 50x objective was used throughout, and the filter was varied from 0.1-100% to produce the best spectra while preventing particle damage (burning).

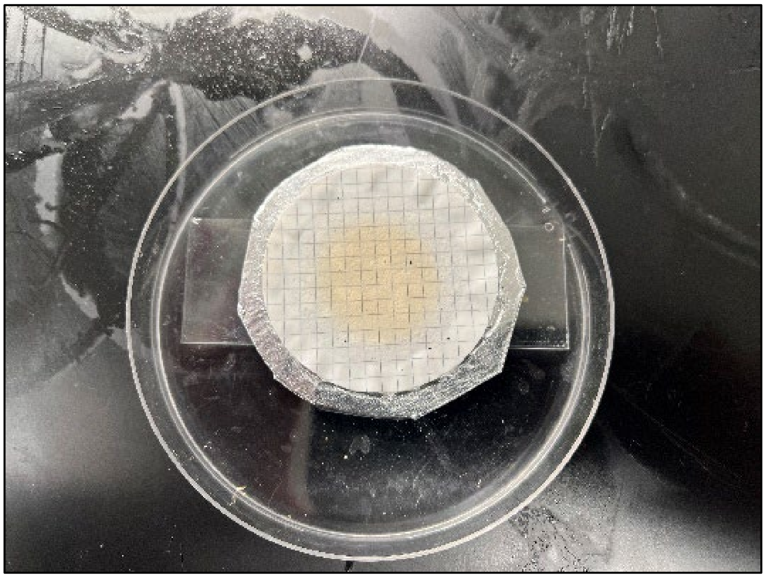

Figure 4. Filter mounted to slide for Raman analysis. Photo by Sam Munk

The "Autofocus" function (-20 to 20) was used throughout the process to help with focusing on irregular surfaces which commonly occur with microplastic fibers. The instrument was calibrated using a silicon sample prior to use. One to three spectra were obtained from each particle at different locations, and the clearest spectra was stored. The "FLAT" baseline correction was performed on each spectra obtained. The spectra obtained from each microplastic particle was compared to the published Spectral Library of Plastic Particles (SLoPP-E) containing spectra from environmentally weathered polymers using SpectraGryph software (v1.2.16.1) (Munno et al. 2020, Ragusa et al. 2021, Stefánsson et al. 2021). The SLoPP-E library contains 113 spectra from microplastic particles collected from a variety of environmental matrices. Due to harsh processing (e.g., KOH digestion) and physical wear, a match rating >60% was considered a successful match for this study (Thiele et al. 2021).

Quality Control

Blanks and spikes were used throughout laboratory processing procedures to assess and account for contamination and particle recovery. Blanks were incorporated every ten samples and were used to assess microplastic contamination during processing. Following dissection, the “blank” Erlenmeyer flask was filled with 100 mL of KOH solution and allowed to incubate along with sample set. This blank solution was run through the same procedure as the samples, from digestion to enumeration. Since some samples required additional processing steps (e.g., Alcojet washing), samples were organized so that blanks underwent identical treatment to the ten samples with which they were associated. The blank was used to determine if any of the processing steps introduced microplastic contamination into the samples. Spikes were used every twenty samples to assess microplastic recovery (Santana et al. 2021, Yuan et al. 2022). A sample was randomly designated as the spike using a random number generator. Twenty blue polyethylene microspheres (250-300 µm diameter, Cospheric) were added to spike sample prior to KOH addition (Connors et al. 2017). Spiked samples were treated as a normal sample and were run through laboratory processing steps. During enumeration, the number of spike microspheres captured on a filter was counted, and a spike recovery percentage was calculated. Due to their bright blue color, microsphere spike particles were easily distinguishable from environmental microplastics in samples.

Microplastic data from samples were corrected using blank contamination and spike recovery data. First the spike recovery rate was calculated as a percentage of the 20 spike particles recovered during enumeration. These recovery percentages were converted to a factor, which was applied to each blank and environmental sample within its associated batch. The environmental samples were first corrected using the spike recovery factor, then corrected using the data from the blank which it was associated with. The 281 environmental samples were grouped into 15 batches (20 samples each), with two sets within each batch (30 sets).

Example: A fish sample was found to contain 10 microplastic particles. The blank associated with the sample contained 2 microplastic particles, and the spike recovery associated with the sample was 90% (18/20 spike particles recovered). We therefore assume 10% of particles were lost during processing. A 10% loss means our spike recovery factor is 1.1, and each sample must be multiplied by 1.1 to correct for this loss. This spike correction factor is applied to the blanks and spikes in the sample, resulting in the fish sample containing 11 particles and the blank containing 2.2 particles. The blank concentration is then subtracted from the fish sample (11-2.2=8.8), and our resulting fish sample concentration is 8.8 microplastic particles.

Contamination

Limiting contamination was an essential part of assessing microplastics in environmental samples. Airborne microplastic contamination was of particular concern, since sample processing was conducted in an enclosed laboratory. Synthetic fibers from clothing often become airborne and settle on samples throughout processing, inflating microplastic counts. As previously outlined, wetted filters were used to assess airborne contamination during fish dissection. Dissections were conducted in fume hoods, which has been shown to limit airborne contamination (Wesch et al. 2017). During sample processing, purple cotton shirts were worn to prevent shedding of synthetic fibers (Wang and Wang 2018). Purple is an uncommon color for environmental microplastics, so any purple cotton fibers found on filters were not counted during enumeration. Additionally, all laboratory spaces were equipped with HEPA air filters to remove airborne microplastics which may contaminate samples (Brander et al. 2020).

To ensure reagents and chemicals used during processing were not contaminating samples, all solutions and water were filtered through glass fiber filters (Whatman 934-AH) prior to use. Glass laboratory equipment was used throughout processing to prevent microplastic contamination from interfering with sample analysis. Following use, glassware was rinsed thoroughly with deionized water and placed in an acid bath for 12 hours to remove any organic material. Glassware was then rinsed three times with filtered water and allowed to dry. All glassware was covered when in storage to prevent settling of airborne microplastics.

Data Analysis

Data were analyzed using R software (R 4.1.1) and Microsoft Excel. Levene's Test for Equality of Variances was used to test for homogeneity of variance. A Shapiro-Wilks Test was used to assess whether data were normally distributed (when data violated parametric assumptions, non-parametric tests were used). All microplastic concentration data were determined to be non-parametric, therefore a Kruskal-Wallis Test (*kruskal.test*) and Dunn post-hoc test (*dunnTest*) were used to determine differences among treatment groups (fish species, UMR Pool, sub-habitat strata). Linear regression was used to quantify relationships between microplastics and fish size (across and within fish species). Significance level (α) of 0.05 was used to determine statistical significance for all tests performed.

RESULTS

Prior to data analysis, all fish sample data were corrected with blank and spike data (see “Methods: Quality Control”). Our analysis quantified a total of 891 microplastic particles from the 281 fish samples. We estimated a total of 594 microplastic particles were present in 164 (58%) of the fish examined. We did not find any microplastic particles in the remaining 42% of fish samples. Analysis was first performed to determine effects of UMR Pool and sub-habitat strata on microplastic concentrations (See: “Results: Pool and Habitat Strata”). It was determined that microplastic concentrations did not significantly differ across UMR Pool or sub-habitat strata (within species), therefore fish data were combined for comparisons across species.

Species Comparisons

Emerald shiner had a higher percentage of individuals that contained microplastics (~76%) than shorthead redhorse (~51%) and yellow perch (~49%) (Figure 5A). Data did not meet parametric assumptions so non-parametric tests were performed (Kruskal-Wallis, Dunn post-hoc). The Kruskal-Wallis test revealed that microplastic content (particles per fish) was significantly different between emerald shiner, shorthead redhorse, and yellow perch ($H(2)=16.028$, $p<0.001$) (Figure 5B). The Dunn post-hoc test revealed that emerald shiner contained significantly higher concentrations of microplastics (particles per fish) than both shorthead redhorse ($p<0.001$) and yellow perch ($p<0.001$). Similarly, the Kruskal-Wallis and Dunn post-hoc revealed mean microplastic content per mm fish length was significantly higher in emerald shiner compared to shorthead redhorse ($p<0.001$) and yellow perch ($p<0.001$) (Kruskal-Wallis: $H(2)=4.889$, $p<0.001$) (Figure 5C). For both mean microplastics per fish and mean microplastics per mm fish length, shorthead redhorse and yellow perch were not significantly different (p>0.05).

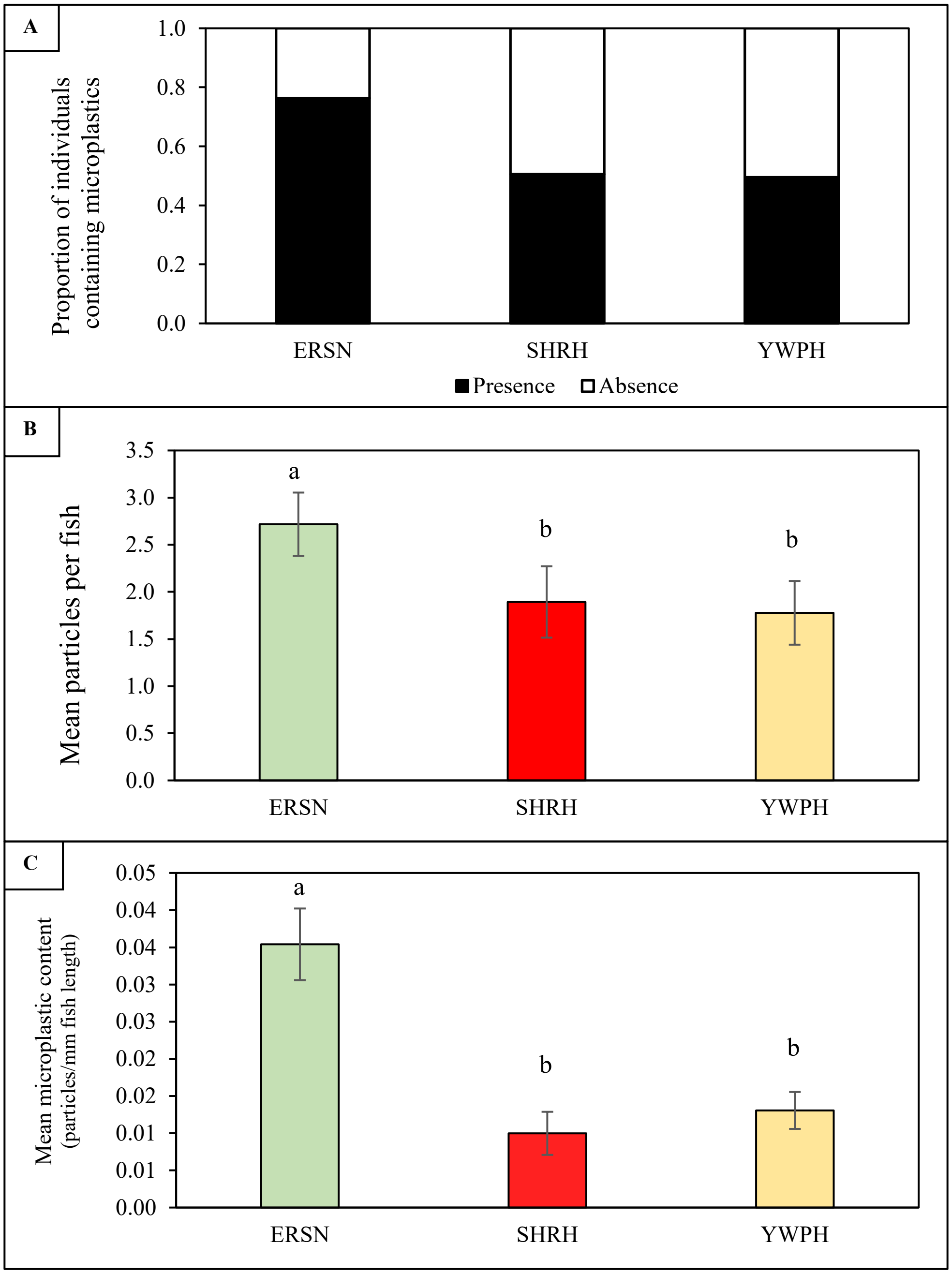

Figure 5. (**A**) Proportion of fish containing microplastic particles, (**B**) mean particles per fish, and (**C**) mean microplastic particles per mm fish length (±1 Std. Error) across three fish species, emerald shiner (ERSN), shorthead redhorse (SHRH) and yellow perch (YWPH) Different letters reported above means indicate statistically significant values ($p<0.05$, Kruskal-Wallis with Dunn post-hoc).

Pool and Habitat Strata

Within emerald shiner, neither UMR Pool (H(1)=0.2642, p=.607) nor habitat strata (H(3)=1.297, p=0.730) had a significant effect on mean microplastics per mm fish length (Kruskal-Wallis). Within shorthead redhorse, neither UMR Pool (H(1)=0.834, p=0.361) nor habitat strata (H(2)=4.504, p=0.105) had a significant effect on mean microplastics per mm fish length (Kruskal-Wallis). The same was found within yellow perch, with neither UMR Pool (H(1)=0.017,p=0.895) nor habitat strata (H(3)=1.055, p=0.788) having a significant effect on mean microplastics per mm fish length (Kruskal-Wallis).

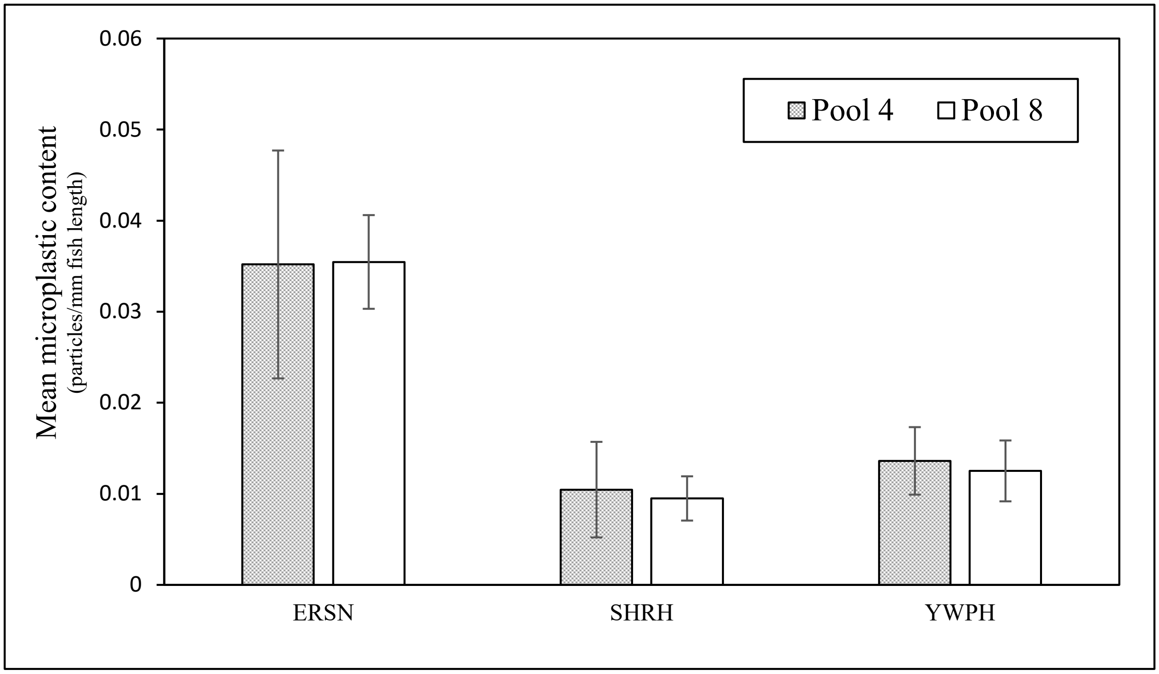

Figure 6. Mean microplastic content per mm fish length (±1 Std. Error) for emerald shiner (ERSN), shorthead redhorse (SHRH) and yellow perch (YWPH) across Pools 4 and 8 of the Upper Mississippi River.

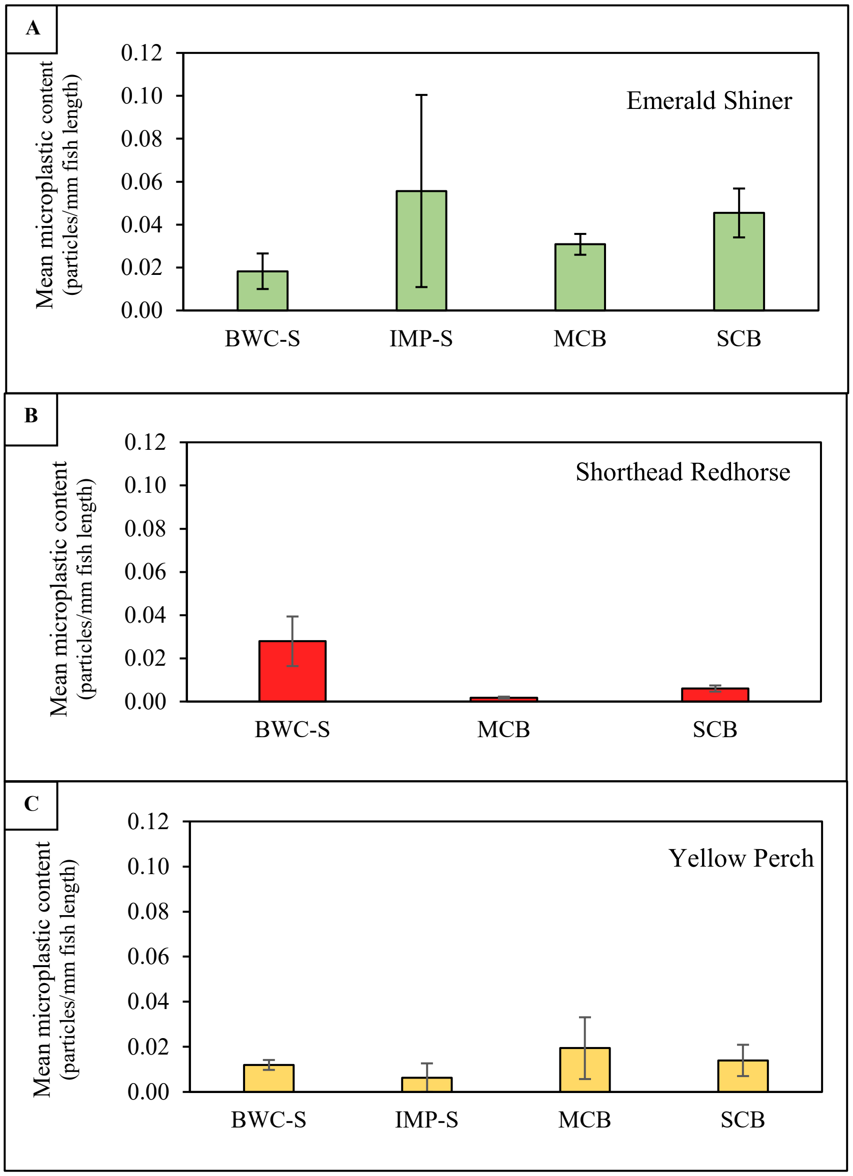

Figure 7. Mean microplastic content per mm fish length (±1 Std. Error) for (**A**) emerald shiner, (**B**) shorthead redhorse and (**C**) yellow perch across habitat strata of the Upper Mississippi River. Habitat strata include backwater contiguous shoreline (BWC-S), impounded shoreline (IMP-S), main channel boundary (MCB) and side channel boundary (SCB).

Fish Size

Fish were not measured <1g so no fish mass data is present for the smallest 76 of the 281 total fish included in the study. Gut mass (g), which consisted of both the mass of the tissue as well as the gut contents, was recorded immediately following dissection. However, some fish were dissected prior to the onset of this study, and gut masses could not be measured. Comparisons were made between microplastic particles per fish and gut mass, as well as particles and fish mass. Both comparisons yielded relationships very similar to those between particles per fish and fish length (mm). Due to fish length being the only measure of size that was complete for all 281 fish, we focused on fish length as the main variable representing fish size.

For all fish combined, fish length (mm) was a weak, but significant predictor of microplastic content (R^2=0.0148, p<0.05; Figure 8). However, within the individual species, no significant relationship between fish length and microplastic content was found (ERSN: R^2=0.002, p=0.379 | SHRH: R^2=0.028, p=0.0584 | YWPH: R^2=0.005, p=0.474). When accounting for fish size, it appears that smaller fish tend to contain more microplastics per mm fish length (Figure 9). Fish length was a significant predictor of length-corrected microplastic concentration when all fish are combined (particles per mm fish length) (R^2=0.09258, p<0.001; Figure 9A). A negative exponential fit was applied to the data but did not provide a better match than the simple linear regression. This relationship is significant within the emerald shiner (R^2=0.07383, p<0.05; Figure 9B) and shorthead redhorse (R^2=0.1121, p<0.001; Figure 9C) species as well. However, fish length was not a significant predictor of microplastics per mm fish length within the yellow perch species data (p>0.05, Figure 9D).

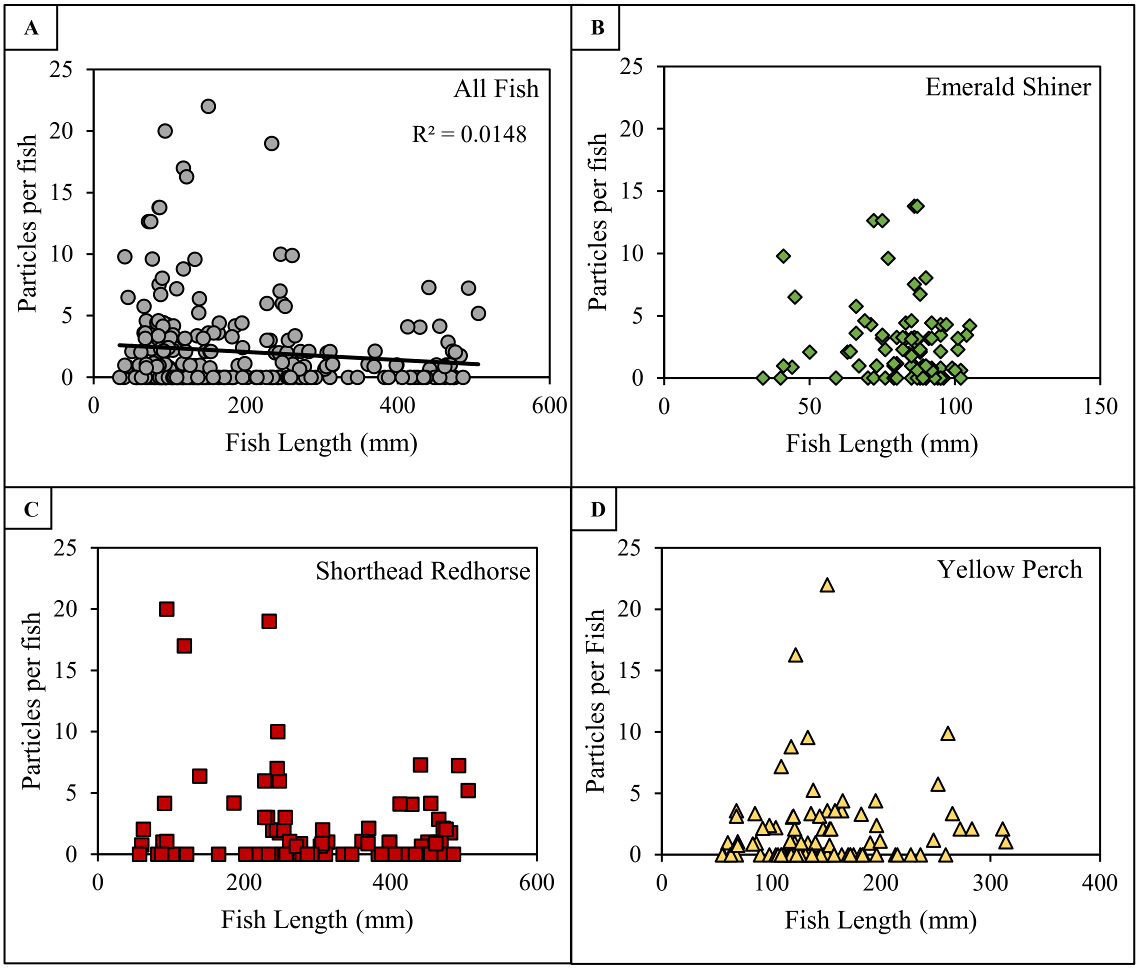

Figure 8. Microplastic particles per fish versus fish length (mm) for (**A**) all fish (n=281), (**B**) emerald shiner (n=89), **(C)** shorthead redhorse (n=95) and (**D**) yellow perch (n=97).

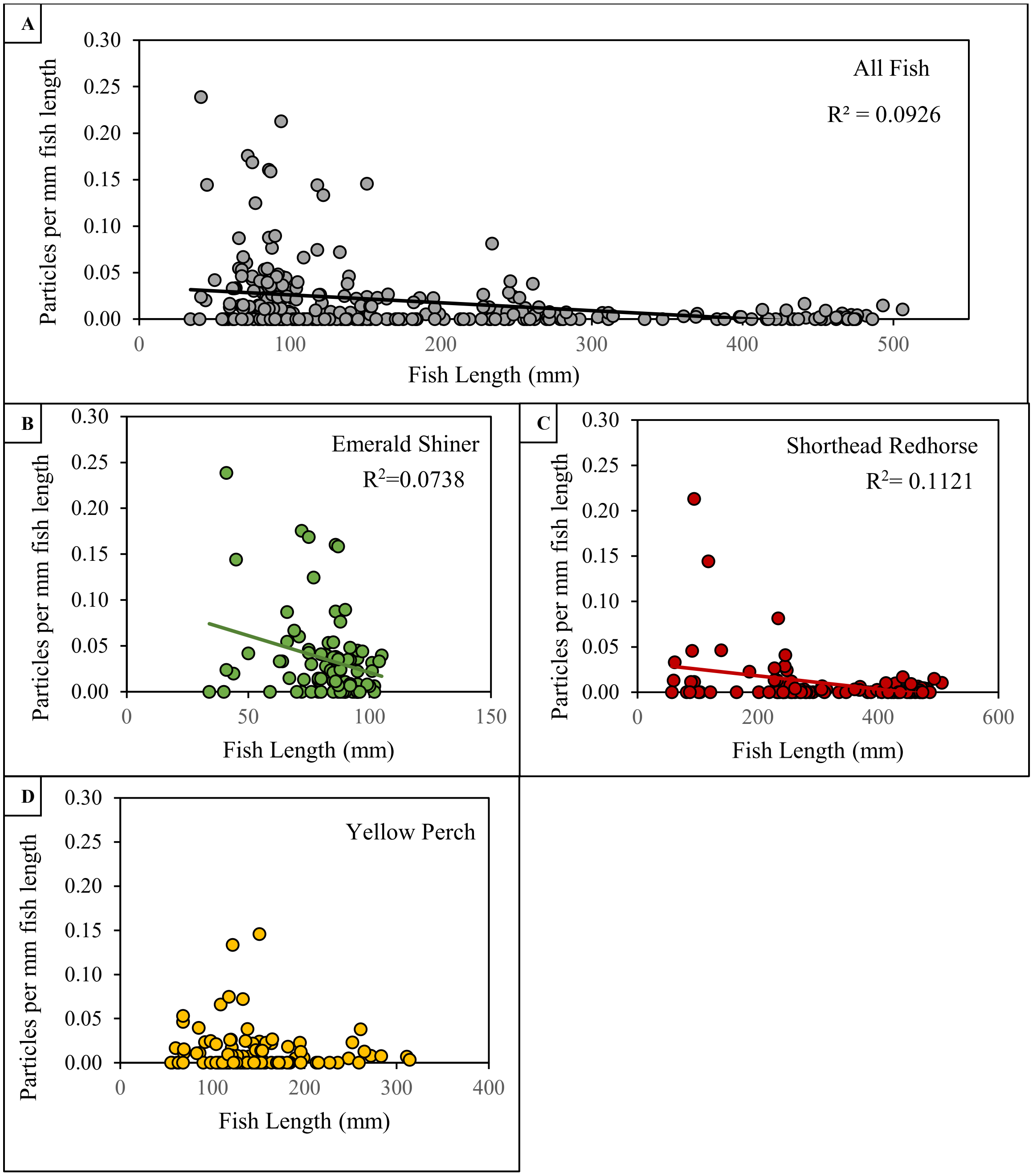

Figure 9. Microplastic particles per mm fish length versus fish length (mm) for (**A**) all fish (n=281), (**B**) emerald shiner (n=89), **(C)** shorthead redhorse (n=95) and (**D**) yellow perch (n=97).

Particle Characteristics

Color characteristics of microplastic particles were relatively consistent between the three species analyzed (Figure 10B). In emerald shiner (n=89) and shorthead redhorse (n=95), blue particles comprised 41.6% and 51.7% of all the non-purple particles collected, respectively. Black particles comprised 28.4% and 28.5% of all particles collected, respectively. In yellow perch (n=97), blue particles comprised 34.7% and black particles comprised 39.1%. In all three species, the remaining particles were red, clear, green, white, yellow or brown. Particle types were grouped based on physical characteristics, and included fibers, films, foams and fragments. Proportion of microplastic particle type were consistent across the three species (Figure 10A), with fibers being the most common type. Fibers comprised >92% of the total particles collected for each of the three species, while fragments comprised 6-7%. Only three film particles and one foam particle were collected during the study. Particle sizes were grouped into 250µm size classes, ranging from 250-5000µm. Across the three species, as particle size decreased the number of particles increased (Figure 11). Across the three species, particles between 250-500µm comprised 34-39% of the total particles. There were only minor differences among the three species.

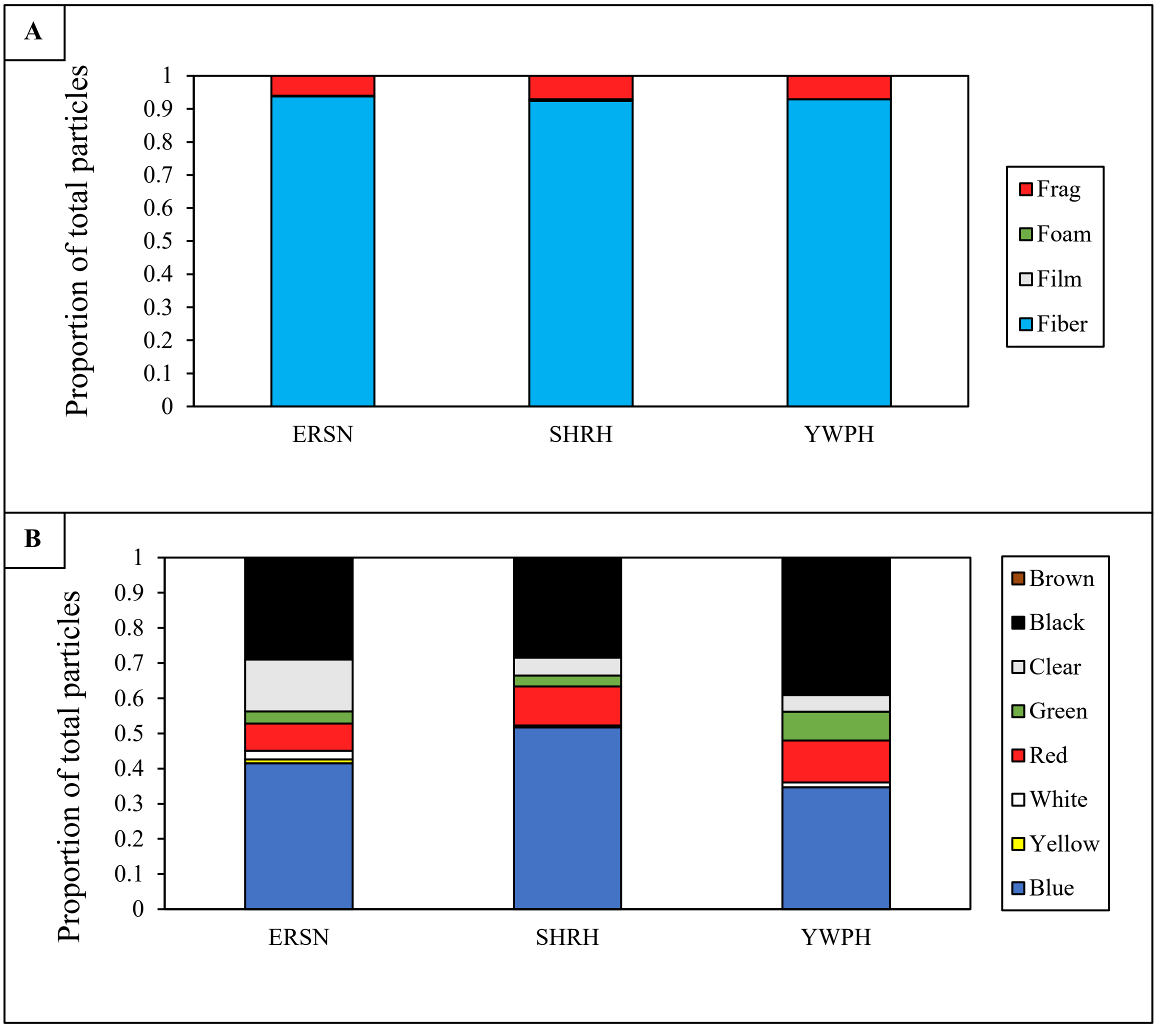

Figure 10. Microplastic particle counts (total number of particles) by color (**A**), type (**B**) for emerald shiner (ERSN), shorthead redhorse (SHRH) and yellow perch (YWPH). Purple fiber particles are excluded from figure B. “Frag” refers to microplastic fragments.

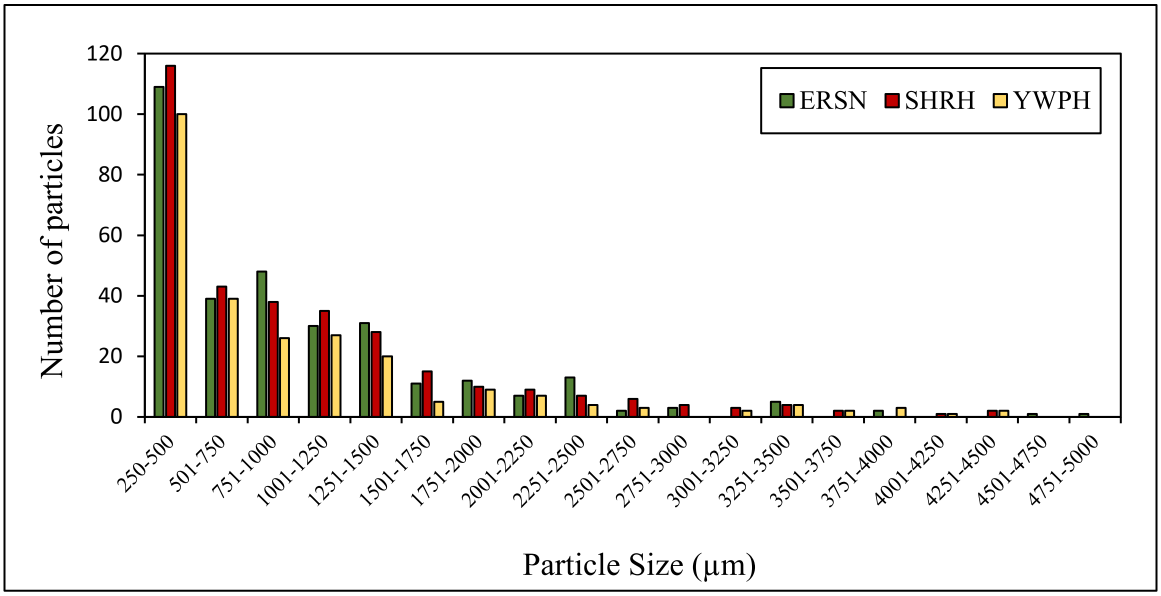

Figure 11. Microplastic particle counts (total number of particles) by size range for emerald shiner (ERSN), shorthead redhorse (SHRH) and yellow perch (YWPH). Purple fiber particles are excluded from figure.

Raman Verification

A total of 115 particles were verified via Raman spectroscopy with a mean (± Std. Dev.) match percentage of 53.3%±19.4%. The match ratings ranged from 9.8-82.3% (Figure 12). Of the 115 total particles analyzed, 74 (64%) had a match percentage >50%. Only 55 (48%) particles met the 60% match requirement used for this study (Table 2). Of the 55 particles within the acceptable range, two were fragments and the remaining were fibers. Colors included blue, black, red, and clear. The most common material identified was styrene-isoprene, which matched with 23 (41.8%) particles, which were a mix of black, blue, clear, and red fibers. Polyester (PES) matched with 11 (20%) particles which were a mix of black, blue and red fibers. Acrylonitrile Butadiene Styrene (ABS) matched with nine particles (16.4%) which were a mix of black, blue, clear, and red fibers. Polyamide (PA) was the next most common material, matching with seven particles (12.7%) which were a mix of black and blue fibers. The next most common was polyethylene (PE), which matched with the two fragment particles. Since the particles were light blue fragments, it is worth noting that these particles may have been fragments from spike particles since they matched in color, basic shape, and material. The least common materials identified were acrylic (PMMA), polypropylene (PP) and polysulfone (PSU), each with one particle match. Based on these results, there was not a correlation between the color of the particle and the material it was identified as. Low match quality was likely due to physical and chemical weathering of particles (Dong et al. 2020).

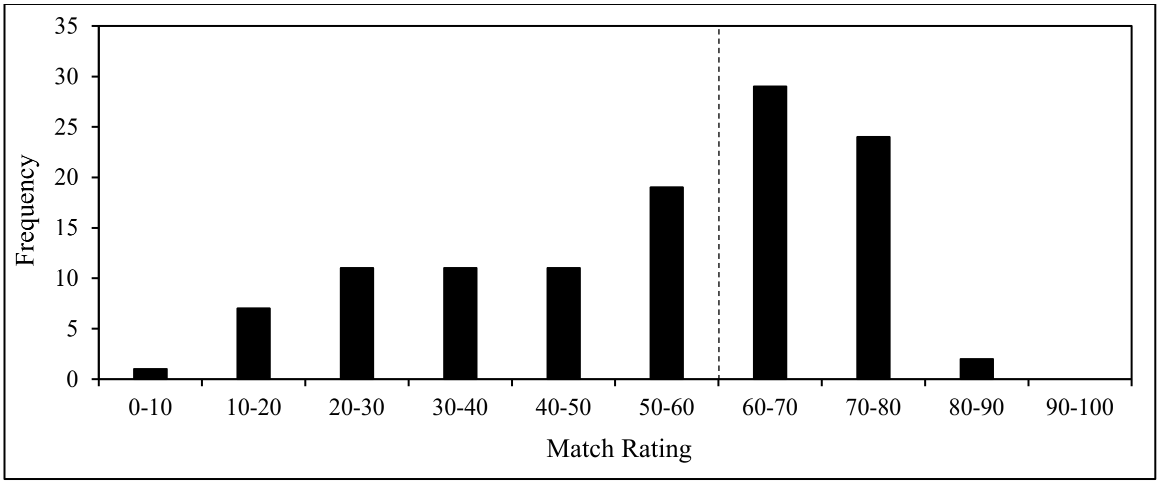

Figure 12. Match ratings for 115 particles analyzed via Raman spectroscopy. Match rating is based on top match to SLoPP-E spectral database using Spectragryph v1.2.16.1.

Table 2. Results of Raman analysis using SLoPP-E spectral library. Highest spectral match from samples with match rating>60% included

Material	Count
Acrylic	1
Acrylonitrile Butadiene Styrene	9
Polyamide	7
Polyester	11
Polyethylene	2
Polypropylene	1
Polysulfone	1
Styrene-Isoprene	23
Total	55

Controls and Contamination

Microplastic particles per blank filter and spike recovery percentage stayed relatively consistent throughout the study. While blank contamination and spike recovery varied between fish sample sets and batches, there were no trends over time (Figure 13). I assume that lab protocols were sufficient to limit contamination and ensure microplastic particles were not lost during processing. Full presentation of quality assurance (QA) and quality control (QC) is shown in the Appendix.

DISCUSSION

Species Comparisons

A recent study analyzed microplastics in the digestive tracts of four fish species collected from the Mississippi River System (including Pool 8) found that 12.66% of all fish analyzed contained microplastic particles (Gad et al., 2023). This is much lower than the 58.8% of fish found in this study. An important factor in microplastic research is the processing methods used and the contamination control methods. Since no universal standard methods exist for analyzing microplastics in the digestive tracts of fish, comparing results of different studies can be difficult. In addition, factors such as the time period when fish were collected, habitat type, and aquatic system may influence microplastic ingestion in fish. This means that comparing microplastic concentrations in certain species across studies should be done carefully.

Emerald shiner contained significantly higher concentrations of microplastics (particles/fish and particles/mm fish length) than both shorthead redhorse and yellow perch. In addition, emerald shiner had a higher proportion of individuals which contained microplastics, compared to yellow perch and shorthead redhorse. In this study, the proportion of emerald shiners containing microplastics (~76%) was nearly identical to a study analyzing emerald shiner near Regina, Saskatchewan, Canada (Campbell et al., 2017). However, the mean microplastic particles per emerald shiner in this study (~2.7) was much lower than emerald shiner collected from Lake Michigan tributaries in 2017 (~13 particles per fish) (McNeish et al., 2018). This was also lower than emerald shiners collected from Humber Bay and Toronto Bay in Lake Ontario (19 particles per fish) (Munno et al., 2021). The ~1.9 particles per yellow perch found in this study was lower than studies analyzing fish taken from Lake Ontario (39 particles per fish) and Lake Superior (15 particles per fish) (Munno et al., 2021). The microplastic concentrations found in this study were slightly lower than those found in Hou et al. (2021), however there was no overlap in fish species or sampling sites between the two studies (Hou et al. 2021). There are no published studies which have analyzed microplastic pollution in the digestive tracts of shorthead redhorse.

The higher concentrations of microplastics in emerald shiner compared to yellow perch and shorthead redhorse is likely due to two factors. First, emerald shiners generally feed on zooplankton within the size range of microplastics analyzed in this study (Pothoven et al., 2009). There has been research in marine ecosystems establishing that planktivorous fish may consume microplastics due to the overlap in size range between microplastic particles and their prey (Ozturk and Altinok 2020).Therefore, it is reasonable that emerald shiners could eat microplastics particles mistaking them for prey. Second, the relationship between fish length and microplastic ingestion may be the dominant factor. Within this study, the average length of emerald shiners was 82mm, compared to 310mm for shorthead redhorse and 147mm for yellow perch. Across all fish combined, smaller fish tended to contain more microplastics than larger fish both in particles per fish and particles per mm fish length. Therefore, it is possible that emerald shiner contained more microplastics simply because they were smaller. The higher concentrations of microplastics in smaller fish may be due to the narrower digestive tract, which could allow microplastic particles to become stuck more easily. To distinguish between these factors, a study would need to be conducted on several species within a certain size range. Since fish were collected prior to this study, we did not have enough small yellow perch or shorthead redhorse to conduct this analysis. The time period in which the fish were collected was also assessed, and microplastic concentrations were consistent within each species across the sampling period from June-October 2019.

Pools and Habitat Strata

Neither UMR pool nor habitat strata had a significant relationship with microplastic content across the three fish species. Within each of the three species, there also was not a significant difference in the amount of microplastics in the digestive tracts of fish from UMR Pool 4 versus Pool 8. While microplastics have been shown to accumulate in areas of low flow and along structure/vegetation, it is difficult to confirm by collecting fish via electrofishing (van Emmerik et al., 2022). While fish may have been collected from a certain habitat stratum (e.g., backwaters), there is no way to determine where the fish last fed before it was collected. In addition, fish may be moving between habitats. A fish collected in the main channel may have recently fed on prey from a side channel. To better assess the effects of habitat strata on microplastic ingestion in fish, researchers would need to confirm that fish were confined to certain strata and would likely need to assess microplastics in other matrices within that stratum (e.g., sediment, water column, along vegetation, etc.).

Originally it was hypothesized that fish from Pool 4 may contain more microplastics, since this pool is closer to Minneapolis, MN, a large urban area. Stormwater runoff from urban areas has been shown to contain high concentrations (1.1-24.6 particles/L) of microplastics, so it would follow that river systems receiving this runoff would also contain higher concentrations of microplastics (Werbowski et al., 2021). The lack of differences between fish from Pools 4 and 8 may be due to a few reasons. Microplastics may be moving downstream through Pools and creating a more even distribution of microplastic pollution throughout the Mississippi River system. This would is supported by research on microplastics in river shore sediment in the Rhin-Main area of Germany, in which microplastic concentrations were not related to population density of the surrounding area (Klein et al., 2015). It is possible that microplastic concentrations in Pool 4 are higher than Pool 8, but the difference is not large enough to be reflected in the ingestion of fish within the pools. As noted by Scircle et al., 2020 and Gad et al., 2023, a historically high spring flooding event occurred in the Mississippi River in 2019. It is possible that this event mobilized microplastic particles which had been stored in the Pools, causing a more homogenous distribution through the UMR system (Scircle et al., 2020, Gad et al., 2023). This may have also effectively diluted the Pools, preventing differences in microplastic concentrations between Pools 4 and 8 from being reflected in the digestive tract of the fish (Gad et al., 2023). In addition, microplastic loads in the Mississippi River have been shown to increase as move down river (Cizdziel 2020). This relationship may explain the similar concentrations in fish from Pools 4 and 8 in this study, however the same study also found higher microplastic concentrations along population centers (Cizdziel 2020). More research is needed to assess how urban runoff influences microplastic pollution in large river systems. In particular, large river systems such as the Mississippi River which receives direct inputs from urban runoff, tributaries, and wastewater treatment facilities.

Fish Size

The general trend identified in this study was that smaller fish contained more microplastic particles than large fish. This trend was significant when pooling all fish, but not when looking within the individual species. However, when accounting for fish size, the relationship was more pronounced. For all fish combined, emerald shiner, and shorthead redhorse, there was a significant inverse linear relationship between microplastic particles per mm fish length and fish size. There are two main processes which may be influencing this trend. First, smaller fish feed on smaller prey and are therefore more likely to consume microplastics that are within this prey size range. Zooplankton in the mesoplankton and macroplankton size ranges would be similar lengths to the sizes of microplastics observed in this study (Burdis and Hoxmeier 2011). Second, metabolic rates are higher for smaller fish. When researchers harvested the fish used in this study, it would be more likely that a small fish had recently fed and had a full digestive tract, compared to a larger fish. A smaller fish that needs to feed frequently would be more likely to consume microplastic particles since they need to take the risk and are willing to possibly consume non-prey items. Larger fish generally do not feed as frequently and are more selective with food choice.

Marine Systems

Research on microplastic pollution in marine ecosystems is more expansive and predates its freshwater counterpart (Cole et al., 2011, Wright et al., 2013, Ugwu et al., 2021). The sources of microplastics in marine environments are similar to freshwater environments, with wastewater, terrestrial runoff and the breakdown of large plastic pieces being the main contributors (Auta et al., 2017). A key difference between freshwater and marine systems is that the distribution of microplastics in marine systems is more strongly influenced by wave and current patterns (Wichmann et al., 2019). To compare the results from this study to marine research, fish feeding guild can be used as a common characteristic. Although ontogenetic shift occurs as these species age, we can consider emerald shiner to planktivorous, yellow perch to be piscivorous and shorthead redhorse to be benthic omnivores/insectivores. Studies on microplastics in the digestive tracts of marine planktivorous fish have found highly variable results. A study conducted with 292 planktivorous fish from the southeast Pacific found only 2.1% of fish sampled contained microplastics (Ory et al., 2018). Fish sampled from Tokyo Bay were found to contain an average of 2.3 microplastic particles per fish, with 77% of all fish sampled containing microplastics (Tanaka and Takada 2016). Our results are similar to the study of fish in Tokyo Bay, as emerald shiner in our study contained ~2.7 particles per fish (~76% contained microplastics). Spotted Seatrout (piscivorous) collected from Charleston Harbor, SC were found to contain ~83 microplastic particles per fish (Parker et al., 2020). Two benthic marine fishes (*Callionymus lyra* and *Mullus surmuletus*) collected from the northwestern Iberian continental shelf and 60-79% of individuals were found to contain microplastics (Filgueiras et al., 2020). As with freshwater microplastic research, the concentration of microplastics in the digestive tracts of marine fish seems to be highly dependent on species and location.

Particle Characteristics

Microplastic fibers were the most common shape collected across the three species analyzed in this study. This result was expected and consistent with the results of many other studies on microplastics in freshwater systems. Several studies analyzing microplastics in river sediments have found fibers to be the dominant shape (>50% of particles), (Peng et al., 2017, Alam et al., 2019, Jiang et al., 2019). The prevalence of microplastic fibers suspended in the water column of river systems has also been established. A study conducted on the Milwaukee River Basin found fibers to be the most common shape of microplastic found in both surface and sub-surface water samples (Lenaker et al., 2019). In the digestive tracts of fish, fibers are generally the most common shape of microplastics found, although this can be dependent on region, water body, and surrounding land use (Zazouli et al., 2022).

The color of environmental microplastics may provide insight into their source and may influence their interaction with aquatic biota. Blue and black were the most common color of microplastics found in the digestive tracts of fish in this study. Several other studies have found similar results, but in many cases, researchers have not established a connection between the characteristics of microplastics found in the environment versus those ingested by fish (Khan et al., 2020, Kusmierek and Popiolek 2020). A study conducted off northeast Greenland compared microplastics in the water column to microplastics in the digestive tracts of two Arctic fish species (*Triglops nybelini* and *Boreogadus saida*) (Morgana et al., 2018). Compared to water column samples, fish digestive tracts contained larger proportions of blue and black microplastics, and smaller proportions of white and transparent microplastics (Morgana et al., 2018). In order to draw conclusions about the selective feeding on certain microplastics based on color by the fish in this study, microplastic samples collected from the water column and sediment would need to be analyzed. However, given that blue and black microplastics were so prevalent in the digestive tracts of fish in this study, as well as many other studies, there may be some selective feeding behavior influencing this trend. Perhaps the blue and black color most closely mimic prey items in the water column, while white and transparent microplastics are more difficult for fish to detect. This could be supported by dosing experiments in which fish were found to be more likely to consume microplastics that mimicked their natural prey color than other colored microplastics (Roch et al., 2020).

The inverse relationship between microplastic particle size and its prevalence in the environment is a well-documented pattern (Erni-Cassola et al., 2017, Cai et al., 2018, Zhu et al., 2018). The physical and chemical breakdown of plastic particles in riverine systems is continuous, and therefore there are a greater number of smaller particles (<1mm) than larger particles (1-5mm). In addition, experiments with microplastic dosing have showed that microplastic particles size is a significant factor in the uptake of microplastic particles in aquatic organisms (Lehtiniemi et al., 2018). Since all three species exhibit similar trends in sizes of microplastics ingested, it seems likely that microplastic ingestion is due to a combination of factors such as selective feeding, accidental ingestion, and bioaccumulation.

Raman Verification

As the environmental microplastic research field grows, the need to identify the composition of microplastic particles efficiently and accurately has become more vital. While visual identification allows for faster sample processing, microplastic particles can be difficult to distinguish from natural particles or non-plastic anthropogenic particles of similar size (Ivleva et al., 2016). Raman Spectroscopy has quickly become one of the most popular methods to determine the polymer composition of microplastic particles

(Arujo et al., 2018). The 47.8% success in match rating (>60 match rating) across the 115 particles analyzed in this study is relatively low compared to non-environmental samples, though expected based on limitations in equipment and sample characteristics. It is inconclusive whether a low match rating means that a particle was not plastic, or that the spectra obtained was not clear enough. There are a variety of factors that may prevent clear spectra from being obtained, including chemical additives, particle morphology, and coloring agents (Araujo et al., 2018). A larger spectral library including environmental microplastic samples may have allowed for greater match ratings. These limitations highlight the need for standardized methods for processing and identifying microplastics. Development of larger open-source spectral libraries would be an asset for researchers and would help guide how polymer identification fits into environmental microplastic research.

Conclusion

The results of this study have confirmed that emerald shiner, shorthead redhorse and yellow perch in Pools 4 and 8 of the UMR are ingesting microplastics. In addition, it has identified that planktivorous fish may be consuming higher concentrations of microplastics than piscivorous or omnivorous fish. Within the UMR system, these three fish species seem to consume primarily microplastic fibers, with little variation between species. This study has also helped develop more accessible methods for analyzing microplastics in the digestive tracts of fish. Future research should focus on creating a standard method for analyzing microplastics in fish samples. In addition, further advancements are needed to chemically analyze microplastic samples so visual identification is less relied upon. These improvements would allow for higher quality microplastic research and make it easier to compare between studies. This study, as well as many previous studies, have confirmed that microplastics are prevalent in freshwater ecosystems. As plastic production continues to increase, research and monitoring is needed to assess how microplastic pollution will impact freshwater biodiversity in the future.

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APPENDIX

Controls and Contamination (QA/QC)

Spike recovery percentage ranged from 80-100%, with a mean (± Std. Dev.) of 89.7%±5.5% (Figure A-13A). Spike recovery stayed relatively consistent throughout time and was deemed acceptable for this study. Examination during lab processing revealed that spike loss tended to occur during two processing steps: the sieving of samples following chemical digestion and during the final filtration step. Spike particles occasionally became lodged to the sieve mesh or inside of the glass filtration funnel. Significant rinsing of the sieve and funnel with DI water increased spike recoveries. Some spike particles were damaged (irregular shape or fractured) upon receival from the manufacturer, so it was important to inspect each particle prior to using it for spiking samples.

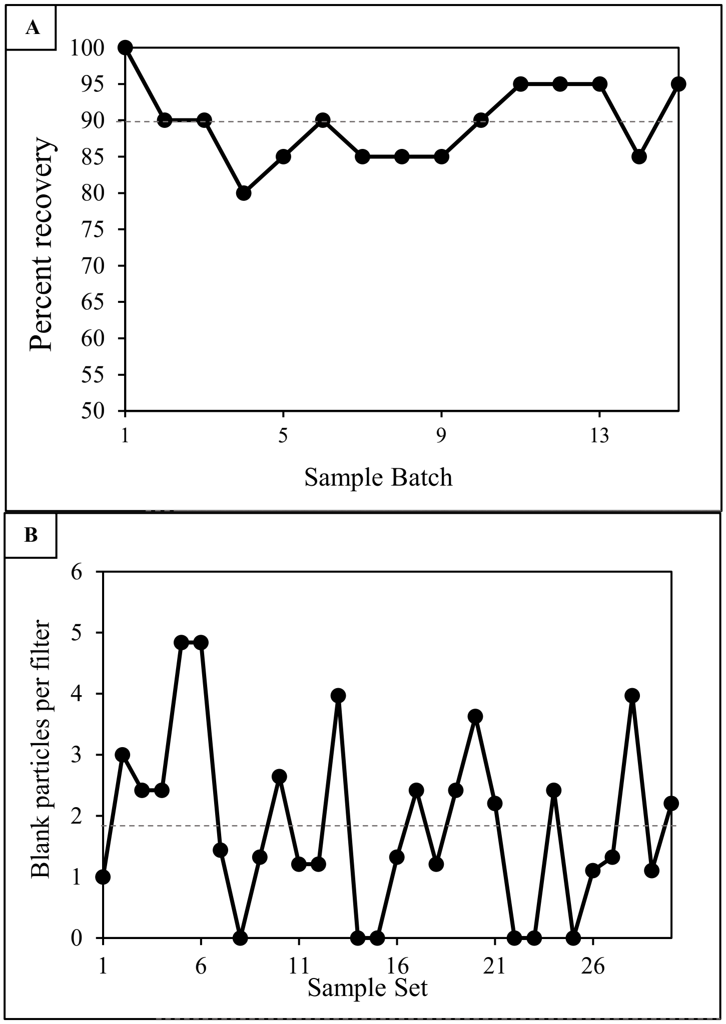

Figure A13. Spike recovery percentage (n=15) and blank concentration (particles per blank filter) (n=30) for sample batches and sets. Lab processing occurred from February- June 2022. Dotted line represents mean spike recovery (**A**) or mean blank concentration (**B**). Blank concentrations are corrected with spike recovery data.

Blanks were assessed by counting the number of non-purple microplastic particles on the filter after filtration. Blank concentrations were corrected using spike concentrations prior to use in correcting fish samples. Blank concentrations stayed relatively consistent throughout the study, ranging from 0- 4.8 particles with a mean (± Std. Dev.) of 1.85 particles ±1.4 (after adjustment with spike recovery) (Figure A-13B).

Sample sets which required Alcojet washing (Sets:1,2,3,4,7) had a mean blank concentration of 2.0 particles per filter and did not show an increase in blank contamination compared to standard sets. Microplastic contamination on blank filters did not show any unique patterns compared to fish samples, and purple fibers were the most common type of particle identified (purple fibers were not included in blank/spike/sample data). It was not possible to determine which steps contributed most to contamination of blank samples. Filters used to assess airborne contamination during dissection (wet filter placed in open petri dish) did not reveal any airborne contamination.

Controls and contamination procedures have been a limiting factor in producing high quality microplastic research. While environmental microplastic research is a rapidly growing field full of new advancements and discoveries, it is particularly vulnerable to poor study design. This is due to the ubiquitous nature of microplastics, as well as the lack of standardized methods and procedures. The use of blanks, spikes, and various procedures to limit microplastic contamination are vital to producing high quality research (Rochman et al., 2019). In this study, the blank contamination and spike recovery stayed relatively consistent throughout sample processing. The blanks were likely contaminated due to incomplete washing of glassware or settling of airborne microplastics on glassware. It is nearly impossible to limit all contamination since microplastics are present throughout the laboratory and in the chemicals used during sample processing. Spike recovery was satisfactory given the limitations for this study. For future studies, using fibers as spike particles would likely be a better measure of microplastic recovery since fibers were the most common type of particle found in environmental samples. While the blank and spike recovery factors did effect sample data (including dropping sample concentrations <0 particles/fish), there is no way to prevent this aside from limiting contamination and improving microplastic spike recovery. Incomplete control measures would undermine the legitimacy of any results found in the study. Interestingly, airborne contamination during dissection did not play a role in assessing microplastic contamination. Throughout all dissections, the open filter in the fume hood did not contain a single microplastic. This is likely due to the regular cleaning of the fume hood and keeping the hood partially closed during dissection. The most shocking contamination result was the high concentrations of purple fibers found in samples and blanks. These were due to purple cotton t-shirts being worn throughout all sample processing steps (including enumeration). This result reenforces that microplastic contamination from clothing is a significant factor that needs to be properly accounted for when performing microplastic laboratory research. This would likely also apply to any field sample collection or field processing. It is possible that some purple fibers found in samples were environmental, which would mean that microplastic concentrations are slightly underreported.