Microplastics in fish relative to point-source proximity and trophic level in an urban river

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Abstract: Microplastics are widespread in the environment, including in the bodies of freshwater fish, with their concentrations influenced by factors such as proximity to point sources, such as wastewater treatment plants (WWTPs), and trophic level. However, few studies have simultaneously assessed the combined effects of these factors on microplastic abundance in urban stream fish. To do so, we measured microplastics and assessed trophic level via N stable isotope ($\delta^{15}N$) content in 6 species of small-bodied fishes (species = *Lepomis macrochirus* Rafinesque, 1819, *Neogobius melanostomus* [Pallas, 1814], *Fundulus notatus* [Rafinesque, 1820], *Pimephales notatus* [Rafinesque, 1820], *Notemigonus crysoleucas* [Mitchill, 1814], and *Dorosoma cepedianum* [Lesueur, 1818]) collected upstream, at, and downstream of a large WWTP in Chicago, Illinois, USA. Additionally, we analyzed stomach contents for 2 of these species (*L. macrochirus* and *N. melanostomus*). Four of the six species exhibited $\delta^{15}N$ enrichment at and downstream of the WWTP, indicating prolonged residence times at the study sites (i.e., several weeks). Stomach contents of the 2 species measured supported this pattern, showing high chironomid consumption at the WWTP and variable stomach contents elsewhere. For microplastics, 1 species had higher concentrations near the WWTP, but microplastic concentrations did not differ among locations in the other 5 species. We found no evidence of a relationship between $\delta^{15}N$ enrichment and microplastic concentration. Overall, the stable isotope and stomach content results suggest a strong relationship of WWTP effluent with fish diet but not with microplastic concentrations in fish. The results suggest that microplastic concentrations in fish are shaped by interacting, species-specific factors including behavior (i.e., movement and foraging) and physiology (i.e., egestion rates and feeding mechanisms), in addition to proximity to point sources. Our study highlights the complexity of microplastic infiltration into food webs

and underscores the need for further research to disentangle the drivers of microplastic accumulation in aquatic organisms.

Key words: urban ecosystems, aquatic food webs, plastic pollution, wastewater, stable isotopes, gut contents

INTRODUCTION

Microplastics (particles <5 mm) are pervasive in aquatic environments globally (Li et al. 2020, Du et al. 2021, Wright et al. 2021). In freshwater ecosystems, sources of microplastics to the environment include stormwater, agricultural runoff, wastewater treatment plants (WWTPs), littering, and atmospheric deposition (Hoellein and Rochman 2021). Urban landscapes have high densities of point and nonpoint sources of microplastics, and microplastic concentrations in rivers are positively related to urban land use (Grbić et al. 2020, Kunz et al. 2023, Li et al. 2023).

Fish consume microplastics in urban freshwater ecosystems (Scherer et al. 2017, Hou et al. 2021). Organisms may consume microplastics intentionally if mistaken for food or unintentionally via ingestion of plastics within prey (Foley et al. 2018, Wang et al. 2020). Previous studies on the biological effects of microplastic ingestion by freshwater fish identified a range of potential impacts, consisting of neutral and negative effects on consumption, growth, reproduction, and survival that are variable across taxa and trophic levels (as reviewed by Foley et al. 2018, Galafassi et al. 2021, Wootton et al. 2021). Understating the sources, fate, and biological interactions of microplastics in fish is an important step in their protection and conservation.

WWTPs are a point source of microplastic pollution in urban rivers (Edo et al. 2020). Microplastics are in wastewater because of fragmentation of plastic textiles in washing machines, microplastics in personal care products, and plastic fragments in sewers (Hamidian et al. 2021, Yaseen et al. 2022). Although WWTPs can remove up to 99% of microplastics from raw sewage (Carr et al. 2016), the high volume of treated effluent released can enrich microplastic concentrations at discharge locations (McCormick et al. 2016). Organisms near effluent release sites are exposed to higher microplastic concentrations from WWTP effluent and

can have more microplastics in their digestive tracts (Ziajahromi et al. 2016). For example, Park et al. (2020) found higher microplastic concentrations in fish downstream (mean = 12.4 particles/fish) relative to upstream (7.3 particles/fish) of a WWTP.

In addition to environmental exposure, the microplastic body burden of fish may be a result of their trophic position. Every trophic level has the potential for direct microplastic ingestion into the food web, which can lead to biomagnification of plastics in top predators (Krause et al. 2021). Preliminary evidence of positive links between trophic level and microplastic abundance in freshwater fish are mixed. Some studies showed higher microplastic abundance in predators (McNeish et al. 2018, da Costa et al. 2023), whereas others suggest omnivorous fish have more microplastics (Garcia et al. 2020), and some research showed no relationship between trophic level and microplastics (Pei et al. 2024).

Fish trophic level and diet are assessed by several different techniques. Common approaches include using database records for species trophic levels (Froese and Pauly 2017), analyses of stomach or feces contents, and analysis of accrued chemicals like fatty acids and stable isotopes (Iverson 2009, Layman et al. 2012). Database records represent a broad average for the species across its range. In contrast, stomach contents consider a snapshot of an individual organism's foodweb interactions at single moment (Garcia et al. 2021). The abundance of stable isotopes of N ($\delta^{15}N$) indicates trophic level (Alp and Cucherousset 2022) and reflects an individual's diet over several weeks to months (Hette-Tronquart 2019). Stable isotope analyses are individual-based assessments and are valuable for quantifying the variability in trophic levels and foraging habits that can occur within a species, even across a small geographic range. WWTPs are also point sources of elevated $\delta^{15}N$ from human waste, and organisms near WWTP effluent are enriched in $\delta^{15}N$ (Kendall 1998, Hoffman et al. 2012). To

date, few studies assess how microplastic abundance in freshwater fish are related to point-source proximity, trophic levels estimated via stable isotopes, and stomach content composition, despite the benefits of including multiple methods for estimating trophic level and diets (Garcia et al. 2021, Pei et al. 2024).

The objective of this study was to quantify the relationships of point-source proximity and foodweb position with the abundance of microplastics in stream fish. Our research questions were 1) Does microplastic concentration in stream fish vary by distance from a WWTP point source? 2) Is microplastic concentration in stream fish higher at higher trophic levels? We predicted that 1) fish would have higher microplastic concentrations and $\delta^{15}N$ immediately downstream of a WWTP-effluent location and at a location several kilometers downstream compared with an upstream location, and 2) microplastic abundance would be positively related to $\delta^{15}N$ because of trophic transfer and WWTP influence.

METHODS

Study site

We measured microplastic concentrations, estimated trophic position via $\delta^{15}N$, and assessed stomach contents for fish of varying functional groups at multiple sites in a heavily modified urban river to better understand factors driving microplastic infiltration into food webs. The Chicago River (Illinois, USA) is part of the Chicago area waterway system, which is a network of waterbodies engineered to manage storm- and wastewater (Fig. 1). From 1889 to 1910, a system of locks and dams was constructed to regulate water movement from Lake Michigan into the Chicago River. The North Shore Channel (NSC) was created to help drain Chicago's northern communities as well as to move Lake Michigan water into the Chicago River

to increase its flow. The Terrence J. O'Brien water reclamation plant processes about 966 million liters of water per day (McCormick et al. 2014), and treated effluent is released into the NSC near Howard Street in Evanston, Illinois, ~7.4 km downstream of Lake Michigan. The T. J. O'Brien WWTP is a point source of microplastics to the NSC (McCormick et al. 2014, 2016, Hoellein et al. 2017).

For our study we chose 3 locations surrounding the T. J. O'Brien WWTP: upstream (Up), at the effluent site (WWTP), and downstream (Down) (Fig. 1). Our upstream site is ~2 km upstream from the effluent release site. Water at this location is largely from Lake Michigan and has low concentrations of microplastics (2 particles/L) and nutrients (dissolved inorganic N = 181 µg/L) (McCormick et al. 2014, 2016, Hoellein et al. 2017). The WWTP location is 115 m downstream of the WWTP-effluent discharge point and has higher concentrations of microplastics (18 particles/L) and nutrients (dissolved inorganic N = 7405 µg/L) (McCormick et al. 2014, 2016) than the upstream site. Finally, the downstream location is ~10 km downstream of the WWTP. This site has a combination of water from Lake Michigan, WWTP effluent, and water from the North Branch Chicago River watershed (Vincent and Hoellein 2021), but >70% of the water is discharge from the WWTP (Duncker and Johnson 2015). Baseline concentrations were not available for this location.

Fish specimen collection

Fish specimens were collected during an annual monitoring program conducted by the Metropolitan Water Reclamation District of Greater Chicago. In 2019, fishes were captured from multiple locations throughout the Chicago area waterway system via pulsed-direct current electrofishing surveys (120 pulses/s targeting 12–14 amps for 400 m). To facilitate identification

and measurement, smaller (<10 cm) individuals were euthanized (MS-222; Tricaine-S: 0.26 g/L) and preserved in a propylene gycol solution (Carosafe®; Carolina Biological Supply, Burlington, North Carolina). Collection and handling protocols were consistent with accepted methods from the American Fisheries Society (Midway et al. 2022) and the American Veterinary Medical Association (Leary et al. 2013). From these stored samples of euthanized individuals we selected 6 species: Bluegill *Lepomis macrochirus* Rafinesque, 1819, Round Goby *Neogobius melanostomus* (Pallas, 1814), Blackstripe Topminnow *Fundulus notatus* (Rafinesque, 1820), Bluntnose Minnow *Pimephales notatus* (Rafinesque, 1820), Golden Shiner *Notemigonus crysoleucas* (Mitchill, 1814), and Gizzard Shad *Dorosoma cepedianum* (Lesueur, 1818). We selected these species because they had large enough sample sizes across our 3 sites of interest and spanned a gradient of trophic levels and feeding guilds (Table 1). Our target was $n = 5$ individuals per species per site; however, in some cases there were not 5 individuals collected, as reported in Table 1.

Microplastics analysis

Sampling and identification We examined microplastics in fish digestive tracts following similar procedures in previous studies (McNeish et al. 2018, Hou et al. 2021, 2023). First, we rinsed each preserved fish and materials used for dissection (i.e., enamel tray, scalpels, dissecting scissors, forceps, and ruler) with deionized water that was prefiltered through a 363-µm mesh (hereafter DI water). We measured fish wet mass (to nearest 0.01 g) and standard length (to the nearest mm; i.e., from the tip of the snout to the caudal fin). We used a scalpel or dissecting scissors to cut from the mouth to the urogenital opening along the ventral side of the fish, exposing the entire digestive tract. We removed the digestive tract and placed it in a precleaned

glass mason jar and immediately covered it with a metal lid. We recorded the amount of time elapsed during the dissection (i.e., from when the fish was removed from its container to the time the digestive tract was placed in the glass jar) to conduct timed accounts for contamination (see below). Between dissections we rinsed scalpels, forceps, dissecting scissors, and tray with DI water, and we changed gloves to prevent contamination (McNeish et al. 2018, Hou et al. 2021, 2023).

After dissections, fish digestive tracts were dried, oxidized, and filtered (Hou et al. 2021). We covered the glass jars containing the samples with aluminum foil and placed them in a drying oven at 40°C overnight or until the sample was dry (1320 Economy Oven; VMR, Radnor, Pennsylvania). After cooling to room temperature, we added 20 mL of 30% hydrogen peroxide and 20 mL of 0.05 Fe (II) solution (0.05 mol/L $FeSO_4$ + 3 mL H_2SO_4) and placed the jars on a rotation table for at least 24 h at room temperature to oxidize organic material without affecting the microplastics (Lusher et al. 2017, Munno et al. 2018). Next, we added 20 mL of H_2O_2 and placed samples in the drying oven at 40°C for 24 h. We allowed the solution to return to room temperature and filtered it with a vacuum onto a gridded cellulose-fiber filter (0.45-µm pore size; Whatman®, Buckinghamshire, UK). We transferred the filter onto a 20-mL aluminum pan (Thermo Fisher Scientific®, Inc., Waltham, Massachusetts) and covered it with aluminum foil (Hoellein et al. 2021, Hou et al. 2021). We placed the pan and filter in the drying oven at 40°C overnight, then stored the samples at room temperature until further analysis. Using a dissecting microscope (25–30× magnification; model ASZ30L3; Bausch & Lomb, Rochester, New York), we counted all suspected microplastics and categorized them by shape (fiber, fragment, film, and foam) and color, and we measured the length and width of each particle. Samples were assessed by 2 independent researchers. If counts did not agree (by ±3 microplastics), a 3rd independent

researcher counted the sample, and we recorded the lowest value out of the 3 researchers' assessments (Hou et al. 2021).

We prepared microplastics for micro-Fourier transform infrared (μFT-IR) identification. We placed a thin layer of Skin Tac® (Torbot® Group, Inc., Cranston, Rhode Island), a rosin-based adhesive, on a precleaned glass microscope slide (Thaysen et al. 2020). We individually selected the first 3 particles of each color–shape combination from each slide (e.g., the first 3 clear fibers, the first 3 black fibers, and so on) and used forceps to place them on the rosin. If a filter did not have 3 particles of a particular color–shape combination, then we placed all microplastics of that color–shape combination on the slide. We used a fine-tip marker to draw circles around each particle, and we left the slides covered with a box while the rosin dried. Then we placed glass cover slips over the particles, secured the edges with tape, and stored slides securely until polymer identification. For polymer identification we used a micro-FT-IR spectrometer (model Spotlight 200i; PerkinElmer®, Shelton, Connecticut) in attenuated total reflectance (ATR) mode via a 100-μm-diameter germanium crystal. Spectrum results from 16 scans were saved under micro-ATR mode across wavelengths from 650 to 4000/cm. We compared the results with a reference library and known standards with Spectrum 10 Software (PerkinElmer), with a target match between samples and standards at 0.53 to 0.95 (Hoellein et al. 2024). We took background scans before each analysis scan of a particle.

Reducing and accounting for contamination We used multiple approaches to reduce contamination. We wiped all laboratory surfaces with a cellulose sponge (model nCratch10; JINCLEAN®, Republic of Korea) soaked in DI water. All researchers wore yellow polypropylene-coated smocks (Kleenguard® A70, Ansell Healthcare Products, LLC, Iselin, New

Jersey) to reduce clothing contamination. We chose yellow because it is rarely found in fish and water samples from this site (McNeish et al. 2018, Hoellein et al. 2024). We washed and rinsed all glassware with DI water prior to use and stored it with aluminum foil covers.

We measured contamination by running laboratory controls across a gradient of timed exposures. Contamination can occur as dust settles onto the enamel tray during dissection, and fish dissection times were variable depending on size and species. Interspersed among the dissections we conducted a series of contamination measurements by allowing our clean enamel tray to sit for a defined amount of time: 3, 5, or 7 min. After the allotted time, we rinsed the tray with filtered DI water and poured it into a glass mason jar (n = 18). Control samples underwent the same drying, digestion, filtering, counting, and polymer identification procedures as described above for our fish samples. We calculated microplastic contamination in units of no. cm^{-2} min^{-1} (enamel tray area = 778 cm^2). We considered the area of potential contamination of the fish during dissection to be the area accounted for by fish size and placement of tools. For Gizzard Shad, Golden Shiner, Bluntnose Minnow, and Blackstripe Topminnow, this area was 120 cm^2. For Bluegill and Round Goby, the potential contamination area was larger (198.5 cm^2) to account for the workspace needed for stomach content analysis (see below). We used the time for each dissection and the estimated area of the fish to determine the potential contamination value. We rounded up to the nearest integer to generate conservative estimates for particle subtraction. We did not detect any microplastic shapes other than fibers in laboratory controls. Based on the contamination results, we subtracted 2 microplastic fibers from the sample total to determine final counts for Gizzard Shad, Golden Shiner, Bluntnose Minnow, and Blackstripe Topminnow. For Bluegill and Round Goby samples, we subtracted 3 microplastic fibers to determine final counts.

Spectroscopy correction We used the spectroscopy results to determine the number of particles that were microplastics and those that were anthropogenic particles (Hoellein et al. 2024). First, we classified each of the identified particles as plastic, anthropogenic cellulose, or natural cellulose. We considered all items identified as plastic polymers to be microplastics. For cellulose we differentiated natural and anthropogenic items by color. We considered cellulose particles that were white, clear, gray, black, green, yellow, or brown to be natural. We considered any other color of cellulose to be anthropogenic. We determined the number of anthropogenic particles as the sum of microplastics and anthropogenic cellulose. This approach is conservative because some of the naturally colored cellulose may have been anthropogenically modified. We considered particles with poor matches to be unknown and removed them from analysis.

We used the proportions of particles identified as microplastics and anthropogenic particles to complete the final calculation. The number of identified polymers was highest at the site scale (n = 142 upstream, n = 140 at the WWTP, and n = 69 downstream; Table S1), rather than at the individual species scale (n = 30–80 across the 6 species; Table S1). Thus, we did the same calculation for all fish collected at each site. For example, 39.4% of the identified particles at the upstream site were plastic, so we multiplied each of the control-corrected particle counts for all upstream fish by 0.394 to obtain the estimated number of microplastics. We repeated this process for anthropogenic particles (Hoellein et al. 2024).

Particle differences among locations and species We compared microplastics and anthropogenic particles among locations and species by constructing general linear mixed-effects models (GLMMs) with microplastic or anthropogenic particle concentration as the dependent

variable (negative binomial regression for these concentration data, rounded to whole numbers) and species and location as fixed effects. We used the *glmmTMB* package (version 1.1.11; Brooks et al. 2017, McGillycuddy et al. 2025) in R statistical software (version 4.4.2; R Project for Statistical Computing, Vienna, Austria) in the RStudio® Integrated Development Environment (version 2025.00.1+401; Posit® Software, PBC, Boston, Massachusetts). The interaction between species and locations was of primary interest, so we included a species × location interaction term in both models. To account for differences in microplastic and anthropogenic particle concentrations related to the size of individual fish, we included fish mass as a random covariate. We used the *DHARMa* package (version 0.4.7; Hartig 2024) in R to simulate residuals and quantile–quantile plots, confirming adequate fit and no major deviations from distributional assumptions. We used the *emmeans* package (version 1.11.1; Lenth 2025) to conduct post hoc comparisons of predicted means among locations within each species with a Tukey's adjustment and to estimate 95% CIs, which we then displayed with *ggplot2* (version 3.5.2; Wickham 2016). Finally, we characterized particles by polymer type and color, and we compared the relative abundances across these categories among the 3 study locations and the control samples.

Stable isotopes

δ^{15}N measurements We measured $\delta^{15}N$ from fish tissue taken from the nape of each fish, except for Blackstripe Topminnow, where the peduncle was used because the fish were smaller. We rinsed the fish with DI water and removed the tissue using a cleaned scalpel. We rinsed the tissue samples with DI water and individually stored the samples in microcentrifuge tubes with 95% denatured ethanol until later processing. Tissue samples were dried at 60°C for 24 to 48 h,

until mass of the samples did not vary over ~2 h of drying. We used a mortar and pestle to grind the samples to a fine powder, rinsing the mortar and pestle with DI water between samples. Stable isotopes were analyzed at the Boston University Stable Isotope Laboratory (Boston, Massachusetts) with a GV Instruments IsoPrime isotope ratio mass spectrometer (Elementar®, Langenselbold, Germany) interfaced through a GV Instruments Diluter and Ref Gas box to a vario ISOTOPE cube elemental analyzer (Elementar). We then corrected the $\delta^{15}N$ data to the international standard (atmospheric N_2).

$\delta^{15}N$ differences among locations and species We compared $\delta^{15}N$ among locations and species by constructing general linear mixed-effects models (GLMMs) with the *glmmTMB* package as above, with $\delta^{15}N$ as the dependent variable (normal linear [Gaussian] regression) and with location, species, and a species × location interaction as fixed effects and mass as a random effect. Finally, we examined any potential connection between microplastics and $\delta^{15}N$ using similar model framing, but with microplastic abundance as the response variable (negative binomial regression model for count data) and $\delta^{15}N$, species, location, and a species × location interaction as fixed effects.

Stomach contents

Identification We examined stomach contents to quantify the diet of collected specimens of Bluegill and Round Goby. Of the 6 study species, stomach contents for these 2 were a priori deemed most amenable to taxonomic identification via dissecting scope, whereas the other 4 taxa were either too small or the stomach contents were not expected to be visually enumerable. For the analysis, we dissected the fish's digestive tract from mouth to anus. We separated the

stomach and placed it in a clean enamel tray, and we placed the remaining digestive tract into a precleaned glass jar. We cut open the stomach, removed the contents, and visually identified organisms to the lowest taxonomic group possible under a dissecting microscope. We classified 5 prey taxa in Round Goby and Bluegill stomachs: *Bosmina*, chydorids, chironomids, copepods, amphipods, and other/unknown. After identification, we added the stomach contents to the glass jar containing the rest of the digestive tract for microplastics analysis (described above). We recorded the time elapsed from dissection to completion of stomach content analysis to account for contamination as above.

Composition differences among locations and species To assess if stomach content composition (percentage of sample total) differed by location or species, or if there was a location × species interaction effect, we used the adonis2 function (*vegan* package, version 2.7.2; Oksanen et al. 2025) to conduct permutational multivariate analysis of variance (PERMANOVA). When we found a difference in stomach content composition (determined as a p-value <0.05), we then used the betadisper function to determine whether the difference could be attributed to differences in variability of the composition data (i.e., dispersion) or differences in stomach content (i.e., locations of data centroids). We visually assessed compositional data via constrained analysis of principal coordinates (CAP) plots of Bray–Curtis similarities among samples with the capscale function in the *vegan* package.

RESULTS

Microplastics and stable isotopes among locations and species

We examined microplastics, anthropogenic particles, and stable isotopes in 76 fish from 3 locations: upstream (n = 28), WWTP (n = 27), and downstream (n = 21) (Table 1). All individuals contained microplastics in their digestive tracts. Microplastic concentrations in fish differed among locations (χ^2 = 26.83, df = 2, p < 0.001) and among species (χ^2 = 45.79, df = 5, p < 0.001) (Table 2). There was also a species × location interaction (χ^2 = 19.44, df = 10, p = 0.04). This interaction was explained by trends for Bluegill, which had the highest microplastic concentrations at the WWTP-effluent location, whereas other species did not differ in microplastic concentration among locations (Fig. 2). The same patterns occurred with anthropogenic particles (Table S2, Fig. S1).

Stable isotope analysis identified differences in δ^{15}N among species (χ^2 = 79.21, df = 4, p < 0.001), locations (χ^2 = 198.84, df = 2, p < 0.001), and an interaction between species and location (χ^2 = 65.47, df = 8, p < 0.001) (Table 2). For Gizzard Shad, δ^{15}N did not differ among the 3 locations (pairwise Tukey-adjusted t-tests p > 0.9; Fig. 3). However, Golden Shiner and Bluntnose Minnow both differed among locations, with lower δ^{15}N values upstream than downstream of the WWTP (pairwise Tukey-adjusted t-tests p < 0.001 for each species; Fig. 3). Bluegill and Round Goby differed among all 3 locations, with downstream fish the most enriched in ^{15}N and upstream fish the least enriched (all pairwise Tukey-adjusted t-tests $p \leq 0.06$; Fig. 3). The sample size of Blackstripe Topminnow was too low to be included in δ^{15}N analysis.

Generalized linear models demonstrated no relationship between δ^{15}N and either microplastic or anthropogenic particle concentrations in stomachs (Tables 2, S2). Results suggest independence between δ^{15}N and both microplastic and anthropogenic particles across the environmental gradients included in this study.

Characterization of microplastics in environmental samples and controls

Microplastic shapes, colors, and polymers were similar across the 3 locations. By shape, 99.5% of all microplastics were fibers, whereas only 5 fragments were found out of 1070 total particles counted. Of the particles for which we completed polymer identification (n = 464, including from controls), 32.1% (n = 149) were plastic polymers, 31.0% (n = 144) were anthropogenic cellulose, and 36.9% (n = 171) were natural cellulose (Table S1). The most common plastic polymers were polyester, polybutylene, polyethylene, and acrylic (Fig. S2). For anthropogenic particles, cellulose and rayon were the most common materials, representing ~53% of all particles (Table S1, Fig. S3). For both microplastic and anthropogenic particles, the most common colors were clear, blue, black, and red (Figs S4, S5). The control particles had a greater abundance of polyester and cellulose relative to the particles isolated from fish (Figs S2, S3) but similar color distributions (Figs S4, S5).

Stomach contents

Stomach contents differed in diet between Bluegill and Round Goby and among the 3 study locations (PERMANOVA location × species interaction $F_{2,28}$ = 2.22, p = 0.05; Table S3, Fig. 4A, B). Our primary interest was to assess variation in stomach contents among sites, so we analyzed each species separately. Stomach contents of Bluegill differed by location (PERMANOVA $F_{2,13}$ = 3.23, p = 0.01; Table S3), but betadisper indicated much lower variability in diet composition at the WWTP location compared with both upstream and downstream locations ($F_{2,11}$ = 5.82, p = 0.012 Fig. 4A). The final CAP ordination included 2 constrained and 8 unconstrained axes, with CAP1 explaining 59% and CAP2 explaining 41% of the constrained variation (Fig. S6). Stomach contents of Round Goby differed by location

(PERMANOVA $F_{2,14} = 5.72$, $p = 0.005$; Table S3, Fig. 4B), with no difference in diet composition variability among locations (betadisper $F_{2,12} = 0.21$, $p = 0.8$). Pairwise PERMANOVA tests indicated that stomach contents of Round Goby were different between WWTP and both upstream ($F_{1,2} = 8.96$, $p = 0.05$) and downstream ($F_{1,2} = 8.63$, $p = 0.02$; Fig. 4B) sites. The final CAP ordination contained 2 constrained and 6 unconstrained axes, with CAP1 explaining 87% and CAP2 explaining 13% of the constrained variation (Fig. S7). Round Goby at the WWTP consumed more chironomids and chydorids, whereas amphipods characterized diets at the other 2 locations (Fig. 4B). No amphipods were found in any fish stomachs at the WWTP.

DISCUSSION

Microplastic infiltration into stream food webs has widespread consequences for freshwater ecosystems, but the combined effects of WWTP effluent and fish trophic level on the abundance of microplastic particles in the bodies of fish are not well understood. In this study, our objective was to quantify the relationships of point-source proximity and foodweb position with microplastic concentrations in stream fish from a heavily modified urban environment. Microplastic concentrations were highly variable and not consistently related to WWTP proximity. In contrast, $\delta^{15}N$ values of fish tissues, along with stomach content analyses for 2 species, clearly reflected WWTP influence. Contrary to our expectations, $\delta^{15}N$ content of fish did not predict microplastic abundance, indicating that factors other than WWTP proximity were likely stronger drivers of the patterns in microplastics that we observed. Given that the study area is relatively small (12 river km) and all locations were in an urban area, the role of atmospheric deposition and proximity to nonpoint sources were likely the same among locations. Thus, other

potential driving factors include species-specific differences in fish movement, behavior, egestion rates, and particle characteristics.

Microplastics in fish: Variation among locations and species

Our results suggest that fish movement relative to the WWTP did not contribute to differences in fish microplastic concentration. Stomach content and stable isotope analyses provided insight into which fish likely remained stationary relative to the WWTP-effluent location in the weeks to months before collection. Golden Shiner, Bluntnose Minnow, Round Goby, and Bluegill had lower $\delta^{15}N$ values upstream of the WWTP, suggesting limited exposure to $\delta^{15}N$-enriched effluent and, thus, limited movement into waters downstream of the WWTP. Round Goby and Bluegill stomach contents also showed differences among locations, suggesting some location fidelity in the 24 to 48 h prior to their collection. It was therefore surprising that similar patterns were not seen in the data on consumed microplastics. Species-specific factors that we did not measure in this study (e.g., behavior, physiology) thus likely played a role in microplastic dynamics.

There were 2 species, Gizzard Shad and Bluegill, with patterns in $\delta^{15}N$ and microplastics that were especially noteworthy, and some details about their life history could be useful for interpreting the results. Unlike the other species, Gizzard Shad, a highly mobile species (Drenner et al. 1984), showed no spatial differences in $\delta^{15}N$ or microplastic accumulation. We infer that individuals of this species were more mobile than the other fish species, moving among the study locations, and therefore did not demonstrate any signature of WWTP influence on $\delta^{15}N$ or microplastic. In contrast, Bluegill was the only species to show the predicted patterns for both $\delta^{15}N$ and microplastics, with the lowest values for both metrics upstream of the WWTP and

higher values downstream, which we speculate may be related to Bluegill life history. Bluegill are territorial benthic feeders and can have relatively small home ranges in streams (Gerking 1953). Previous analyses of microplastic distribution among benthic habitats in the Chicago River showed that areas rich in fine sediments, as well as coarse benthic organic matter (e.g., leaf litter), were hot spots of microplastic abundance relative to other surfaces like gravel (Vincent and Hoellein 2021). Thus, the association of Bluegills with benthic habitats rich in organic matter, combined with limited movement, as evidenced by differences in $\delta^{15}N$, may explain the increased abundance of microplastics in Bluegill individuals downstream from the WWTP.

Particle egestion rates may have contributed to the microplastic patterns we observed. Microplastic measurements in the digestive tract represent a snapshot of an individual's recent ingestion and egestion patterns (Farrell and Nelson 2013). For example, Hou et al (2023) found that acrylic microplastic fibers had a mean residence time of 24 h in the digestive tracts of Round Goby from the Chicago area, suggesting that microplastics found in their digestive tracts were consumed within the previous day. Moreover, egestion rates can differ among fish species and across microplastic sizes. For instance, Roch et al (2021) fed Rainbow Trout (*Oncorhynchus mykiss* [Walbaum, 1972]) and Common Carp (*Cyprinus carpio* Linnaeus, 1758) microplastics ranging from 0.02 to 1.00 mm and found that the trout preferentially egested larger microplastics, whereas the carp showed no size-based differences in microplastic egestion. These species-specific egestion dynamics may have contributed to the lack of spatial variation in microplastics for most species in our study. To account for these effects, future studies could collect multiple specimens over an extended time period to better capture the influence of ingestion and egestion rates.

Previous research has shown mixed evidence for a correlation between microplastic abundances within freshwater organisms and proximity to WWTP effluent or other microplastic sources. Park et al. (2020) measured higher microplastics in fish collected downstream of a WWTP relative to upstream. Microplastics in Brown Trout (*Salmo trutta* Linnaeus, 1758) and Brook Trout (*Salvelinus fontinalis* [Mitchill, 1814]) were also higher within and downstream of the city of River Falls (Wisconsin, USA) relative to rural upstream locations (Simmerman and Coleman Wasik 2020). However, in the Milwaukee River, Wisconsin, Hoellein et al. (2021) found high variation in microplastics of *Dreissena* spp. mussels, with no difference between those at a WWTP-effluent location and those at other locations in the same waterway. These inconsistencies suggest that the impacts of discrete point sources on microplastic abundance in aquatic organisms may vary by location and species. Understanding the factors that mediate this relationship remains a key area for future research.

Fish $\delta^{15}N$ values: Variation among locations and trophic levels

The difference in $\delta^{15}N$ values of fish collected upstream vs downstream of the WWTP was likely due to the effluent itself rather than changes in diet. The results followed expected patterns of WWTP-effluent enrichment of $\delta^{15}N$ values in aquatic food webs (Kendall 1998), including in fish near WWTP effluent (Hoffman et al. 2012, Morrissey et al. 2013, Loomer et al. 2015). For example, fish found near effluent loading locations had substantially enriched $\delta^{15}N$ values and overall poorer health than those found outside the impacted area in the Maroochy Estuary (Queensland, Australia) (Schlacher et al. 2007). In a study across urban rivers in South Wales (UK), macroinvertebrates showed elevated $\delta^{15}N$ values in wastewater-affected locations (Morrissey et al. 2013). Our data illustrate that WWTP effects can generate substantial

differences across small scales (e.g., $\delta^{15}N$ in fish 2 km upstream relative to those at the WWTP), which were sustained for at least 10 km downstream of the effluent location.

We expected that isotopic fractionation would generate higher $\delta^{15}N$ in fish predators relative to omnivores or fish that consume detritus or primary producers. Within individual locations, our findings followed this expected pattern. Bluegill and Round Goby, both invertebrate predators, generally had higher $\delta^{15}N$ values compared with Golden Shiner and Bluntnose Minnow, which consume detritus, biofilms, and zooplankton. However, $\delta^{15}N$ values were most similar among fish species at the WWTP, which was likely because of the influence of wastewater. One exception to this trend was Gizzard Shad, which did not always have the lowest $\delta^{15}N$ values among fish species at each location, even though they are typically considered detritivores (Yako et al. 1996, De Brabandere et al. 2009). At the downstream location, Gizzard Shad $\delta^{15}N$ values were lower than Bluegill and Round Goby, as expected, but at the upstream location, Gizzard Shad $\delta^{15}N$ values were the same or higher than Bluegill and Round Goby. Given that Gizzard Shad $\delta^{15}N$ and microplastic abundances were similar across all locations and that this species can swim long distances to forage or filter feed (Drenner et al. 1984), it is possible that Gizzard Shad movement among locations was high enough that $\delta^{15}N$ values did not stabilize to reflect any one location's influence, in contrast with the other species.

Fish microplastics and $\delta^{15}N$ were unrelated

We predicted a positive relationship between $\delta^{15}N$ and microplastics in fish because 1) microplastics may transfer up trophic levels, and 2) WWTP effluent enriches $\delta^{15}N$ in aquatic organisms and is a point source for microplastics (Setälä et al. 2014, Krause et al. 2021). However, we found no evidence to support greater microplastic abundance with elevated $\delta^{15}N$.

The lack of correlation between $\delta^{15}N$ and microplastics could be attributed to differences in the temporal duration of their influence. Isotopic fractionation allows for inferences about food webs (e.g., benthic vs pelagic, or trophic level comparisons) to be drawn from a period of recent weeks (Busst and Britton 2018, Winter et al. 2019). In contrast, the microplastics measured in this study were likely not subject to long-term retention in the digestive tract (Hou et al. 2023) and are indicative of the feeding of the most recent 1 to 2 d. In addition, $\delta^{15}N$ measurements can have limited capacity for differentiating narrow changes in diet (e.g., a switch between prey with similar niches) or over brief time scales. This rationale has been employed by other researchers when using multiple trophic tracers to quantify food webs (Happel et al. 2015, 2018). The lack of trends in microplastic composition demonstrated here suggests microplastics may not be useful as indicators of trophic level, at least under the highly dynamic conditions typical of urban rivers. It is possible that collection of microplastics in digestive tracts over longer time periods may better match trophic dynamics, as shown by $\delta^{15}N$, although longer-term collections have not yet been attempted.

Other studies have examined stable isotopes, foodweb dynamics, and microplastics in fish and have found variable results when assessing relationships among these factors (Au et al. 2017). For example, trophic position (as indicated by $\delta^{15}N$) was not related to microplastic concentration in fish collected in the Garonne River, France (Garcia et al. 2021), a variety of deep-sea fish species collected from Monterey Bay canyon (California, USA; Hamilton et al. 2021), or fish in the Three Gorges Reservoir (China; Pei et al. 2024). In contrast, Andolina et al (2022) found that stable isotope values were positively related to microplastic ingestion for fish collected in the Mediterranean Sea, Italy. Finally, analyses of $\delta^{15}N$ and microplastics in marine fish suggest that the diversity of microplastic particles may increase with trophic level, even with

no differences in the abundance of particles (Valente et al. 2023, Gao et al. 2024). More research is needed, including studies that span a range of species, exposures, fish tissues, and data collection periods to better identify the mechanisms that explain how microplastics move through individual fish and freshwater food webs.

Polymer composition: Anthropogenic cellulose and plastic microparticles

We confirmed polymer composition on a representative subset of particles and adjusted the visual counts based on the proportion of particles identified as anthropogenic cellulosic and plastic. Both categories of material include an array of different compounds and chemical additives, are derived from similar sources, and have similarities in their toxicological impacts and rates of environmental degradation (Rochman et al. 2019, Earn et al. 2021). In this study, both anthropogenic particles and microplastics showed the same patterns relative to locations and fish species. Some previous studies report the categories together as microplastics, whereas others have separated them. For example, recent policy changes in the state of California include anthropogenic cellulose as microplastics when measuring contamination in drinking water (Coffin 2023), given their shared toxicological properties. However, separating the categories can be useful for placing microplastic abundance and composition in the broader context of plastic or C budgets for individual ecosystems. For example, Hoellein et al. (2024) measured macroplastics, microplastics, and anthropogenic cellulose microparticles in floating organic matter rafts in the Chicago River, adjacent to the downstream location from this study (Fig. 1). The authors measured plastic polymers separately, given their objectives to quantify the total abundance of plastic (g/m^2) across particle size classes and to convert plastic mass to mass of C. The percentage of particles that were visually suspected to be microplastics but confirmed as

plastic polymers was 42.1%, and the remainder were cellulosic (Hoellein et al. 2024). The percentage in this study was similar, although slightly lower, averaging 32.1% for all fish (Table S1). Because plastics were reported separately, these data can contribute to a long-term goal of uniting this dataset with other plastic pollution studies in this watershed, thereby generating an ecosystem-scale budget of plastic pools and fluxes.

Broader implications and future work

Our aim was to quantify the spatial and ecological dynamics of microplastics, $\delta^{15}N$, and stomach content composition in multiple freshwater fish species to better understand mechanisms that influence microplastic abundance in food webs. Our results indicated that a combination of exposure, physiology, and behavior likely affect microplastic abundance in the digestive tracts of freshwater fish and that their relative influences vary among species. The lack of relationship between stable isotopes and microplastics suggests that, at least in the urban rivers we studied, trophic position may not be a good indicator of microplastic abundance in fish. And although our stomach content analysis indicated differences in diet among the locations, microplastic concentration in fish was not different among locations (except for 1 species), so change in diet did not consistently explain microplastic concentrations in fish. We suggest that future studies could benefit by quantifying smaller particles in fish digestive tracts (i.e., <100 μm), assessing particle degradation during fish digestion, and measuring microplastic abundance across the whole aquatic food web (e.g., plankton, macroinvertebrates, fish, and birds) and habitats. Urban waterways are valuable study sites for examining foodweb assemblages because they can offer distinct environmental conditions across a relatively small spatial scale. We suggest that further investigation is needed into the drivers of microplastic infiltration into

aquatic food webs, including studies that take advantage of preserved specimens to extend the temporal scope of analysis and leverage the sharp environmental gradients typical of urban freshwater ecosystems.

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FIGURE CAPTIONS

Fig. 1. The location of Illinois (in black) within the United States (A). The location of Chicago (black star) within the state of Illinois (B). The 3 sampling locations in this study were upstream (Up), at (At), and downstream (Down) of the T. J. O'Brien wastewater treatment plant (WWTP) (C). Satellite image showing where the effluent from the WWTP enters the North Shore Channel between Oakton and Howard streets (white arrow) (D).

Alt Text: At the top is a map of the United States showing the location of Illinois and a map of Illinois showing the location of Chicago. At the bottom is a map of the North Shore Channel and the North Branch Chicago River in Chicago, along with the location of a wastewater treatment plant and the sampling locations used in this study. The final image is a satellite picture of wastewater effluent entering the North Shore Channel.

Fig. 2. Microplastic in fish taxa (ordered by increasing trophic level from left to right: Gizzard Shad [*Dorosoma cepedianum*], Golden Shiner [*Notemigonus crysoleucas*], Bluntnose Minnow [*Pimephales notatus*], Blackstripe Topminnow [*Fundulus notatus*], Bluegill [*Lepomis macrochirus*], and Round Goby [*Neogobius melanostomus*]), collected in 2019 from locations upstream (Up) of the T. J. O'Brien wastewater treatment plant in Chicago, Illinois, USA, at the effluent release location (WWTP), and downstream (Down) of the WWTP. Vertical lines indicate 95% CIs, red boxes indicate the estimated marginal mean number of microplastics of a given species at a location. Raw data are displayed with filled circles. Brackets indicate pairwise differences in stable isotope values between locations as identified via the *emmeans* package used to conduct post hoc comparisons of

predicted means among locations within each species with a Tukey's adjustment. Brackets are only shown for values where $p \leq 0.05$.

Alt Text: A box and whisker plot of microplastic concentration in 6 species of fish, all measured upstream of a wastewater treatment plant, at a wastewater treatment plant, or downstream. Only 1 of the 6 species, Bluegill, shows elevated microplastics at the wastewater treatment plant.

Fig. 3. Stable isotopes in fish taxa (ordered by increasing trophic level from left to right: Gizzard Shad [*Dorosoma cepedianum*], Golden Shiner [*Notemigonus crysoleucas*], Bluntnose Minnow [*Pimephales notatus*], Blackstripe Topminnow [*Fundulus notatus*], Bluegill [*Lepomis macrochirus*], and Round Goby [*Neogobius melanostomus*]). Fish were collected in 2019 from locations upstream (Up) of the T. J. O'Brien wastewater treatment plant in Chicago, Illinois, USA, at the effluent release location (WWTP), and downstream (Down) of the wastewater treatment plant. Vertical lines indicate 95% CIs, red boxes indicate the estimated marginal mean $\delta^{15}N$ of a given species at a location. Raw data are displayed with filled circles. Brackets indicate pairwise differences in stable isotope values between locations as identified via the *emmeans* package used to conduct post hoc comparisons of predicted means among locations within each species with a Tukey's adjustment. Brackets are only shown for values where $p \leq 0.05$

Alt Text: A box and whisker plot of ^{15}N content in 6 species of fish, all measured upstream of a wastewater treatment plant, at a wastewater treatment plant, or downstream. Four of the species show a low ^{15}N upstream of the wastewater treatment plant. Gizzard Shad shows no difference among species, and Blackstripe Topminnow had too few individuals to quantify a pattern.

Fig. 4. Canonical analysis of principal coordinates (CAP) plot of stomach content composition (percentages of counts, Bray–Curtis similarities) for Bluegill (*Lepomis macrochirus*) (A) and Round Goby (*Neogobius melanostomus*) (B) collected in 2019 from locations upstream (Up) of the T. J. O'Brien wastewater treatment plant in Chicago, Illinois, USA, at the effluent release location (WWTP), and downstream (Down) of the wastewater treatment plant.

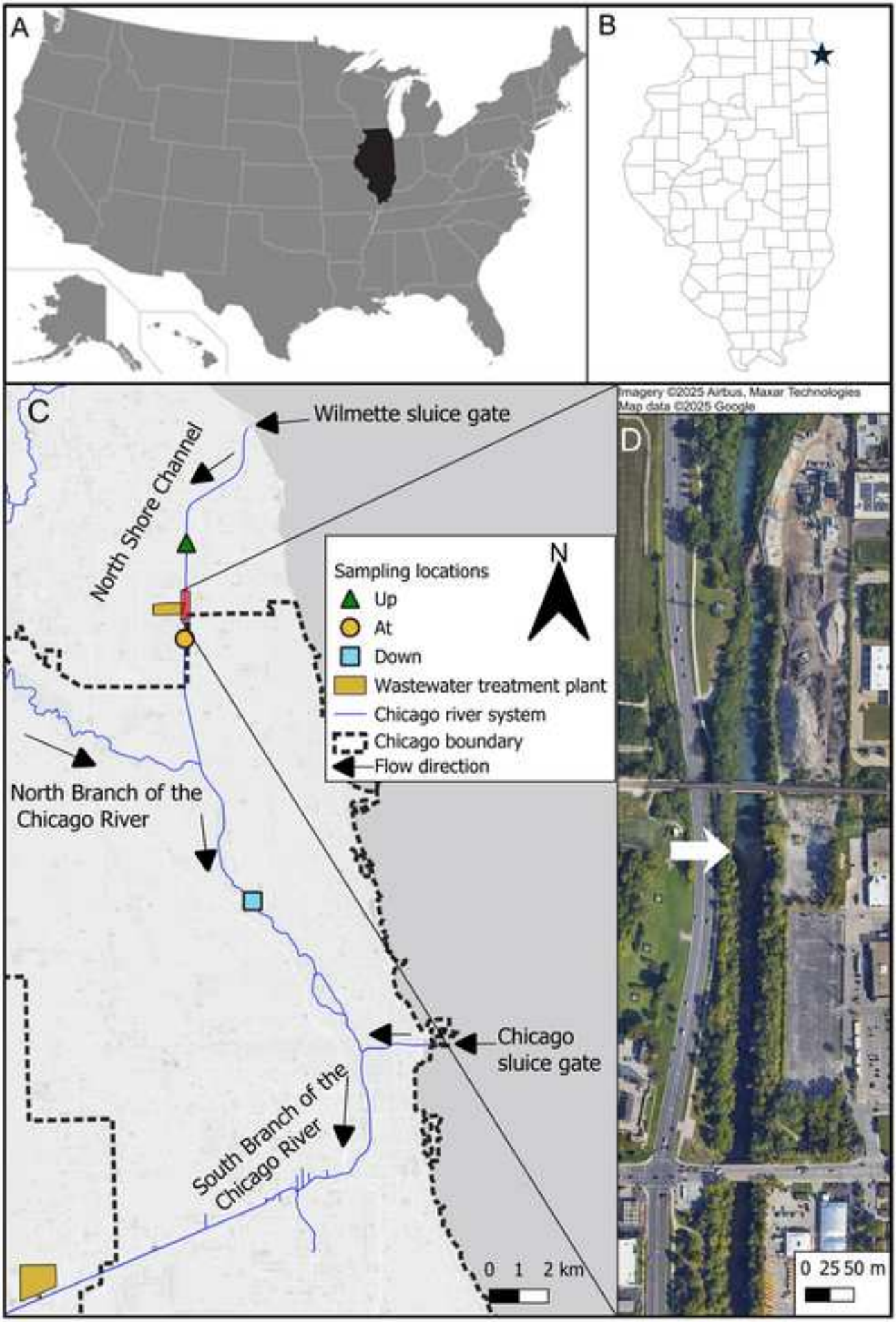
Alt Text: A 2-panel figure showing an ordination of the community of organisms found in the guts of Bluegill and Round Goby, each collected upstream of a wastewater treatment plant, at a wastewater treatment plant, or downstream. The composition of diet for both fish is least diverse at the wastewater treatment plant.

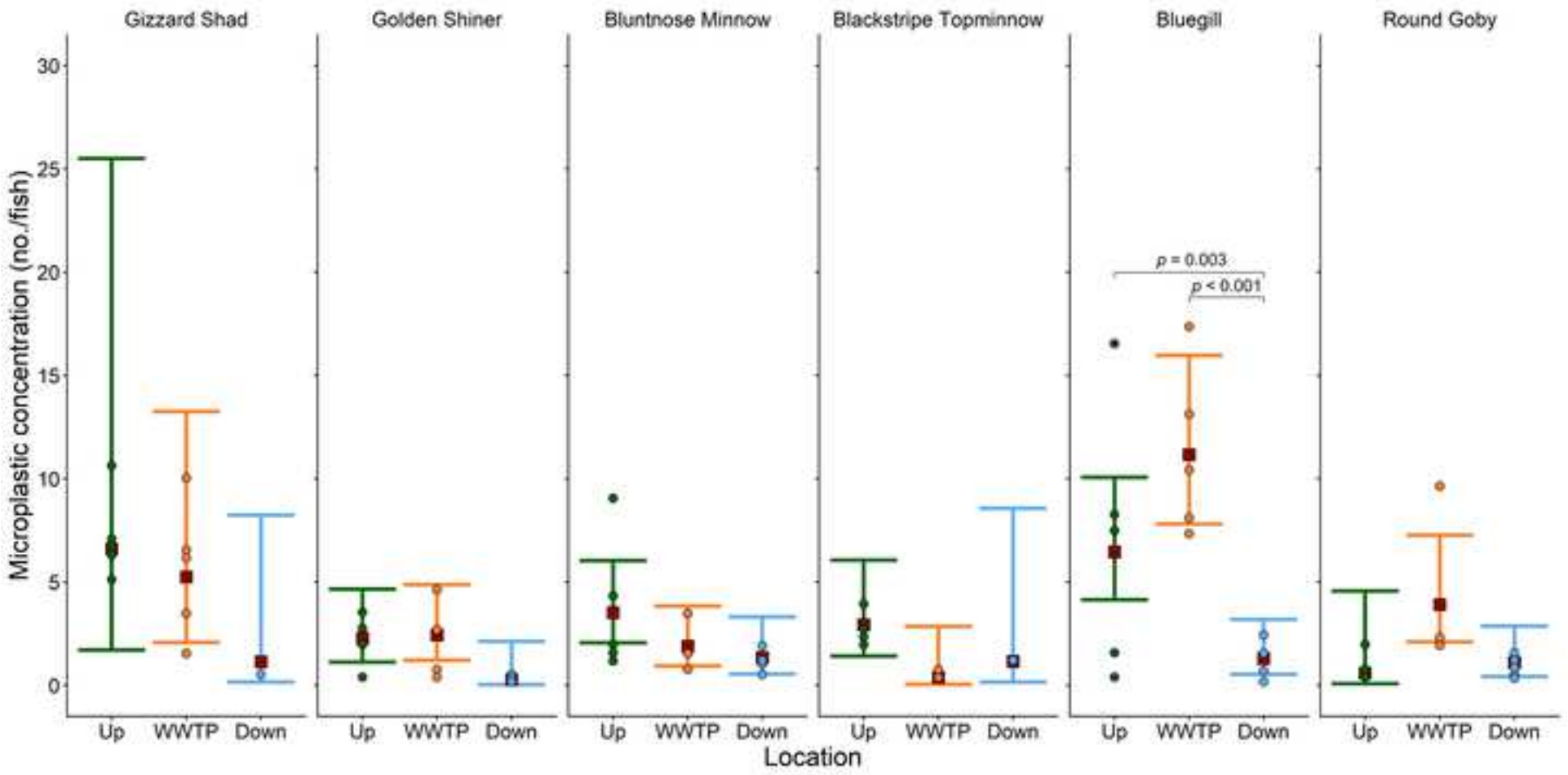
Table 1. Summary of all fishes studied for microplastic concentration, including species characteristics, the number of individuals at each location (*n*), and size range (minimum to maximum). Up = upstream, Down = downstream, and WWTP = at the effluent release site of the wastewater treatment plant. Fish were collected in 2019 by the Metropolitan Water Reclamation District of Greater Chicago from sites throughout the Chicago area waterway system, Illinois, USA.

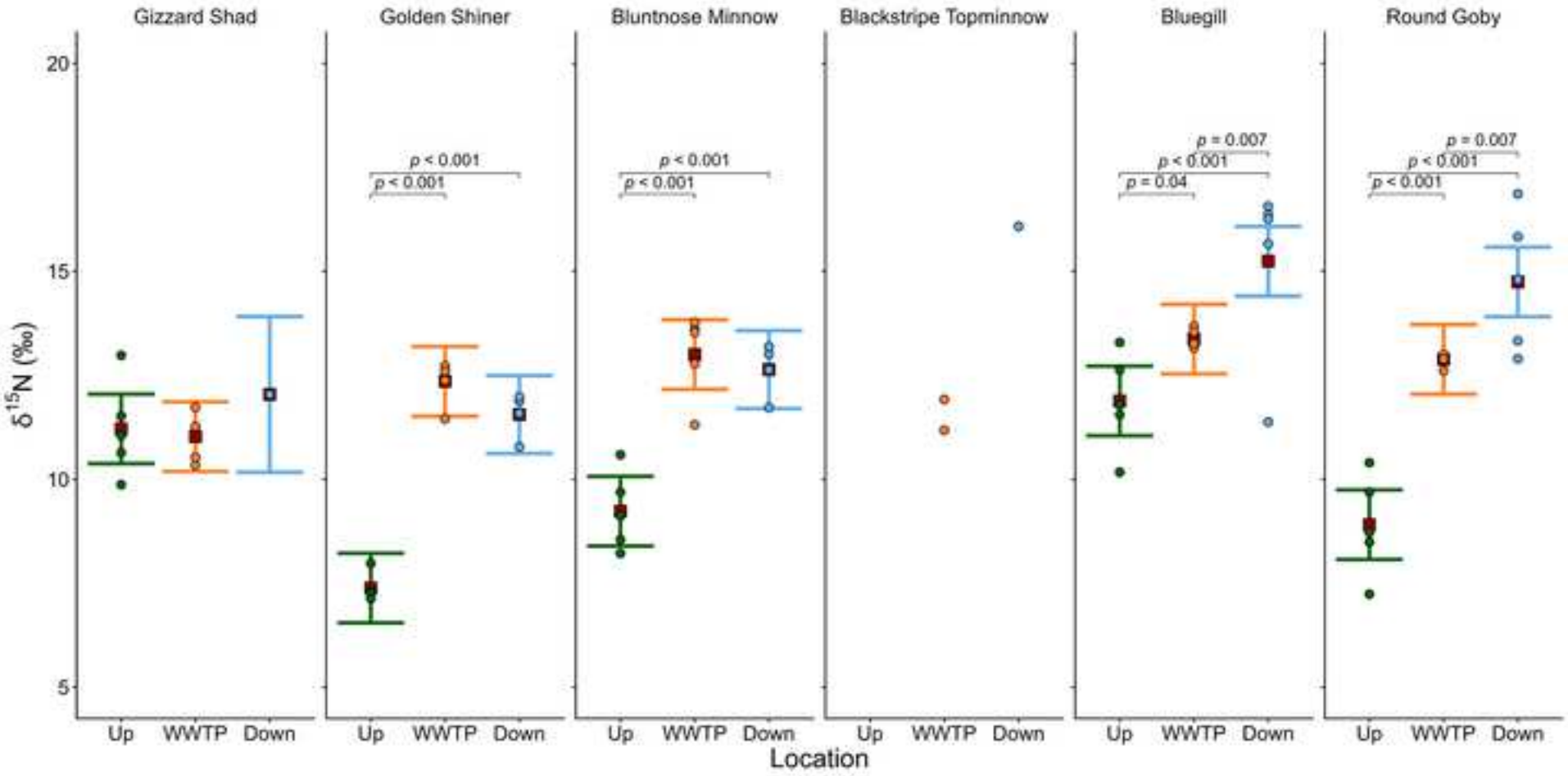
Taxa	Common name	Functional feeding group	Habitat	Trophic fraction	Location	*n*	Wet mass (g)
Dorosoma cepedianum	Gizzard Shad	Detritivore/planktivore	Pelagic	2.4	Up	5	7.00–13.70
					WWTP	5	7.60–8.90
					Down	1	1.43
Notemigonus crysoleucas	Golden Shiner	Invertivore	Demersal	2.7	Up	5	0.32–0.64
					WWTP	5	0.46–0.71
					Down	4	0.12–0.22
Pimephales notatus	Bluntnose Minnow	Omnivore	Demersal	2.7	Up	5	1.50–2.10
					WWTP	5	2.30–3.10
					Down	5	0.10–0.30
Fundulus notatus	Blackstripe Topminnow	Invertivore	Benthopelagic	2.9	Up	4	0.01–0.19
					WWTP	3	0.04–0.13
					Down	1	0.04
Lepomis macrochirus	Bluegill	Invertivore	Benthopelagic	3.2	Up	5	1.10–1.50
					WWTP	5	0.90–1.40
					Down	5	0.50–0.66
Neogobius melanostomus	Round Goby	Zoobenthivore	Benthic	3.3	Up	4	8.60–13.90
					WWTP	4	0.27–1.70
					Down	5	0.16–1.49

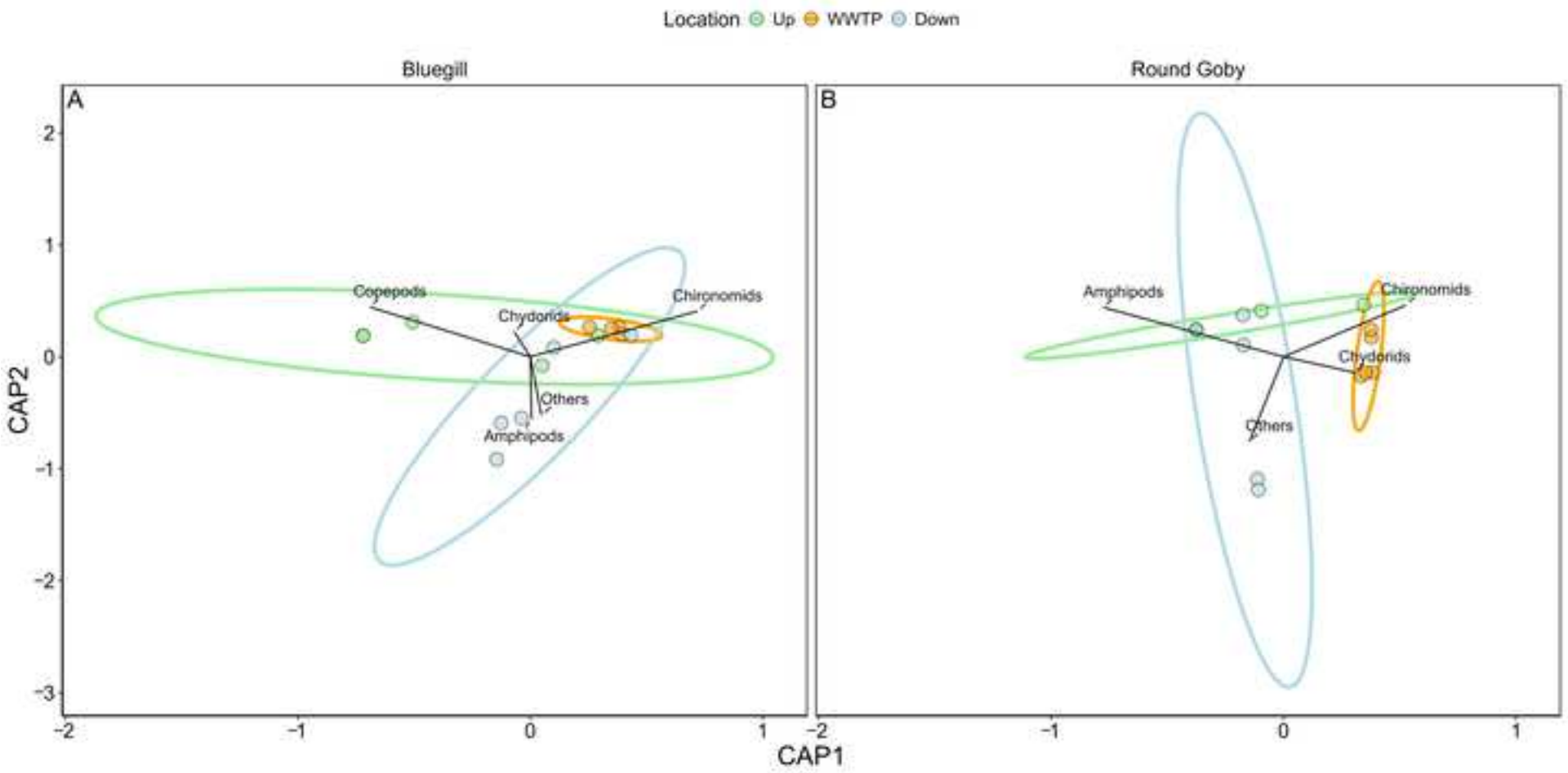
Table 2. Type II analysis of deviance table of best-fitting models to explain microplastic concentrations and $\delta^{15}N$ values in fish with fixed effects of species, location, and a species × location interaction, and to explain microplastic concentrations in fish with fixed effects of $\delta^{15}N$ values, species, location, and species × location. All models include a random effect of mass.

Model	Predictor	χ^2	df	*p*
Microplastics	Mass	0.02	1	0.9
	Species	45.79	5	<0.001
	Location	26.83	2	<0.001
	Species × location	19.44	10	0.04
$\delta^{15}N$	Species	79.21	4	<0.001
	Location	198.84	2	<0.001
	Species × location	65.47	8	<0.001
Microplastics	$\delta^{15}N$	0.24	1	0.6
	Species	29.67	5	<0.001
	Location	22.75	2	<0.001
	Species × location	16.64	9	0.06









Supplemental Materials

Microplastics in fish relative to point source proximity and trophic level in an urban river

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Table S1. Polymer identification (count of each type and percentage of the total) for all specimens and fish collected by site and species. (Species = *Lepomis macrochirus* Rafinesque, 1819, *Neogobius melanostomus* [Pallas, 1814], *Fundulus notatus* [Rafinesque, 1820], *Pimephales notatus* [Rafinesque, 1820], *Notemigonus crysoleucas* [Mitchill, 1814], and *Dorosoma cepedianum* [Lesueur, 1818]). Anthro=anthropogenic, WWTP = wastewater treatment plant. Fish were collected in 2019 by the Metropolitan Water Reclamation District of Greater Chicago from sites throughout the Chicago area waterway system, Illinois, USA.

	Plastic (no.)	Anthro. cellulose (no.)	Natural cellulose (no.)	Total anthro. (no.)	Total identified (no.)
All fish	149 32.10%	144 31.00%	171 36.90%	293 63.10%	464
Upstream	56 39.40%	37 26.10%	49 34.50%	93 65.50%	142
WWTP	54 38.60%	41 29.30%	45 32.10%	95 67.90%	140
Downstream	12 17.40%	21 30.40%	36 52.20%	33 47.80%	69
Blackstripe Topminnow	6 20.00%	11 36.70%	13 43.30%	17 56.70%	30
Bluegill	34 42.50%	16 20.00%	30 37.50%	50 62.50%	80
Bluntnose Minnow	14 25.90%	20 37.00%	20 37.00%	34 63.00%	54
Gizzard Shad	44 62.90%	11 15.70%	15 21.40%	55 78.60%	70
Golden Shiner	9 17.60%	16 31.40%	26 51.00%	25 49.00%	51
Round Goby	15 22.70%	25 37.90%	26 39.40%	40 60.60%	66
Control	27 23.90%	45 39.80%	41 36.30%	72 63.70%	113

Table S2. Type II analysis of deviance table of best fitting models to explain anthropogenic particles values in 6 species of fish with fixed effects of either mass or $\delta^{15}N$, as well as fixed effects of species, location, and a species × location interaction, and a random effect of mass. Fish were collected in 2019 from locations upstream of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA, at the effluent release location, and downstream of the WWTP.

	Chi Square	df	*p*
Anthropogenic Particles			
Mass	0.06	1	0.8
Species	53.29	5	<0.001
Location	21.54	2	<0.001
Species × Location	26.14	10	0.004
Anthropogenic Particles			
$\delta^{15}N$	0.07	1	0.8
Species	36.40	5	<0.001
Location	16.77	2	<0.001
Species × Location	24.71	9	0.003

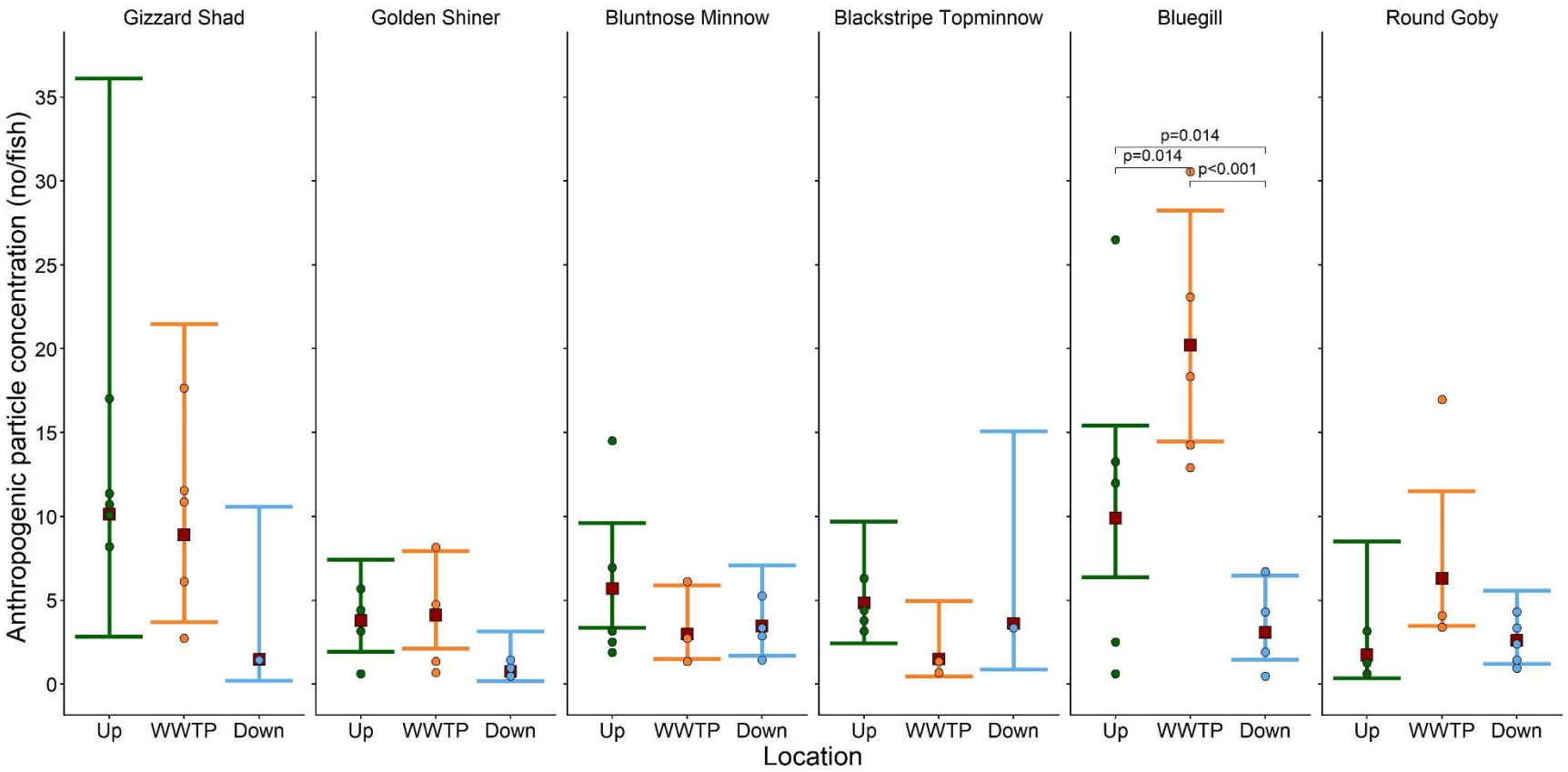

Figure S1. Anthropogenic particles in fish taxa (ordered by increasing trophic level from left to right), collected upstream (Up), downstream (Down), and at the effluent release site of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA. Vertical lines indicate 95% confidence intervals, red boxes indicate the estimated marginal mean number of microplastics of a given species at a location. Raw data is displayed with filled circles. Brackets indicate pairwise differences in stable isotope values between locations as identified via the *emmeans* package used to conduct post hoc comparisons of predicted means among locations within each species with a Tukey's adjustment. Brackets are only shown for values where $p \leq 0.05$.

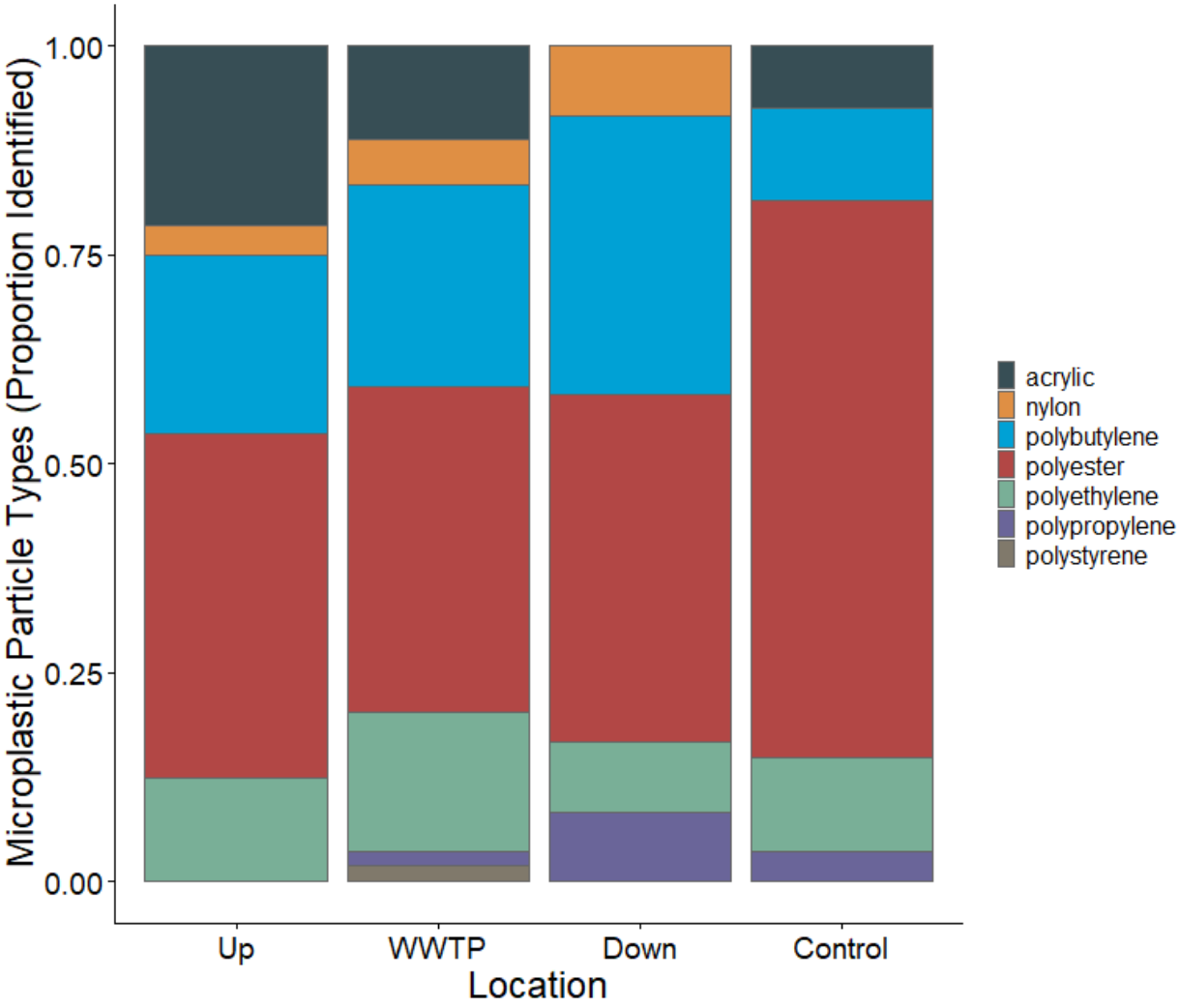

Figure S2. Composition of microplastics by polymer type from fish collected in 2019 upstream (Up), downstream (Down), and at the effluent release site of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA, and laboratory controls (Control).

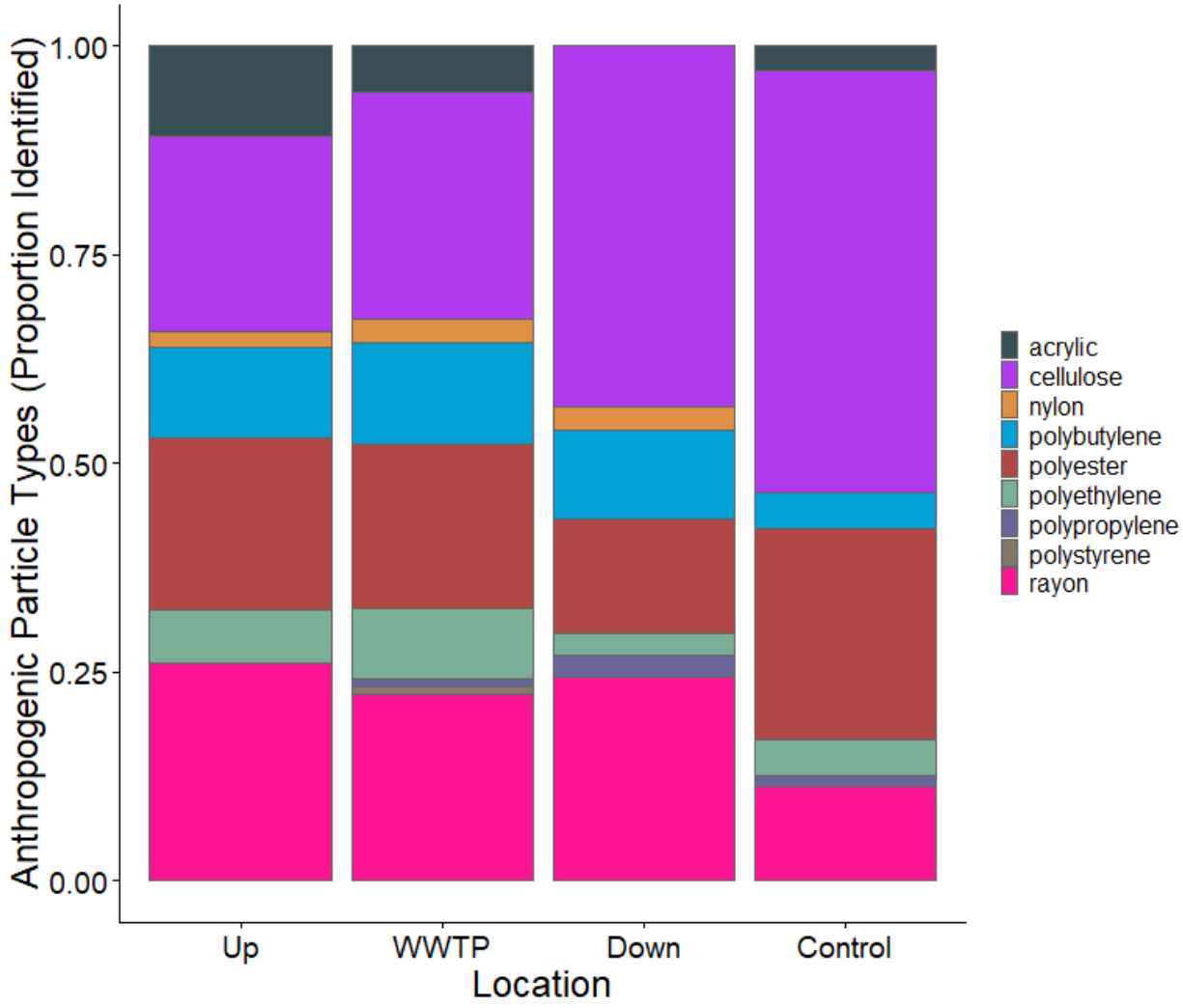

Figure S3. Composition of anthropogenic particles by polymer type from fish collected in 2019 upstream (Up), downstream (Down), and at the effluent release site of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA, and laboratory controls (Control).

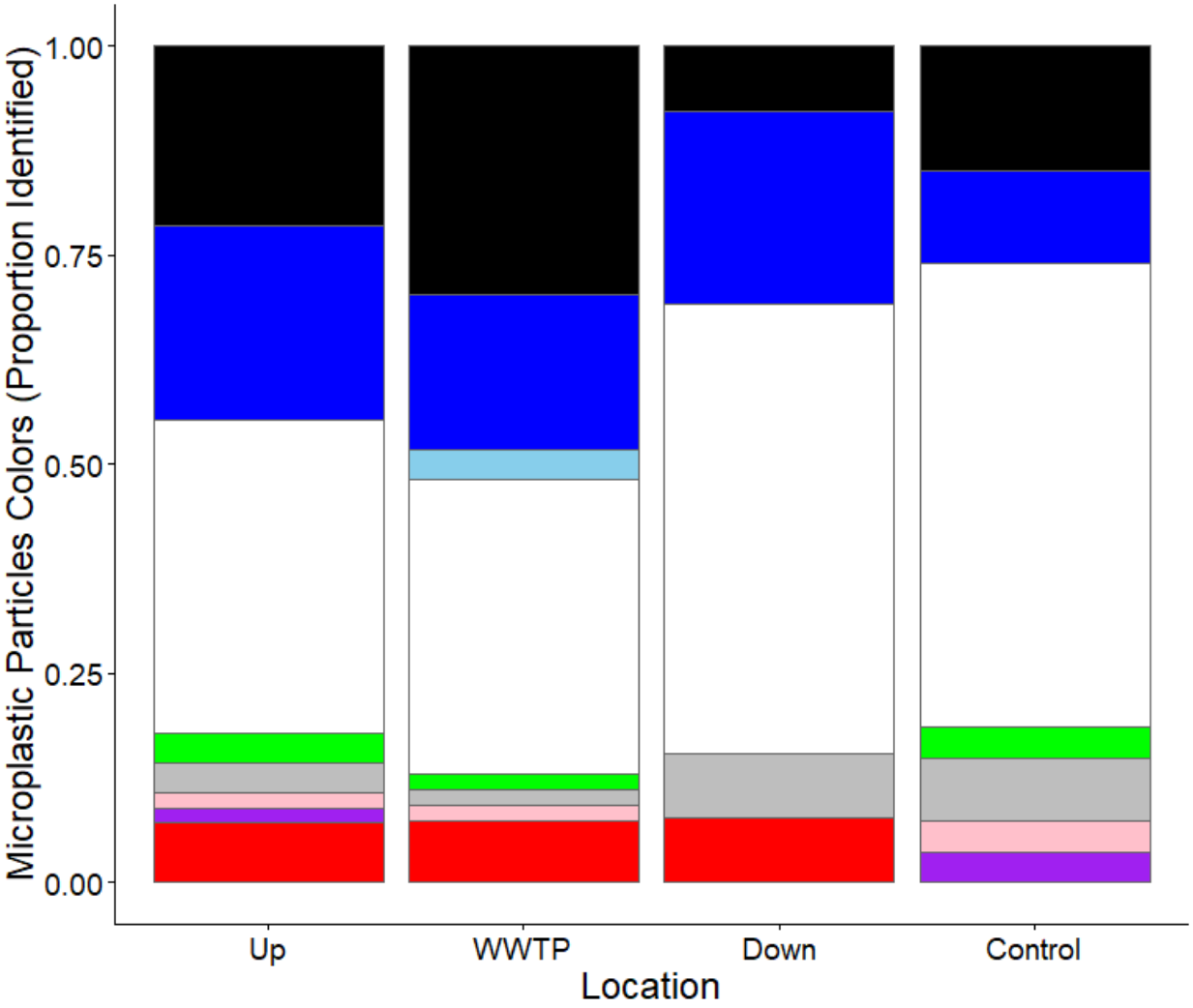

Figure S4. Composition of microplastic colors from fish collected in 2019 upstream (Up), downstream (Down), and at the effluent release site of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA, and laboratory controls (Control). Colors are shown as appeared, except white = clear.

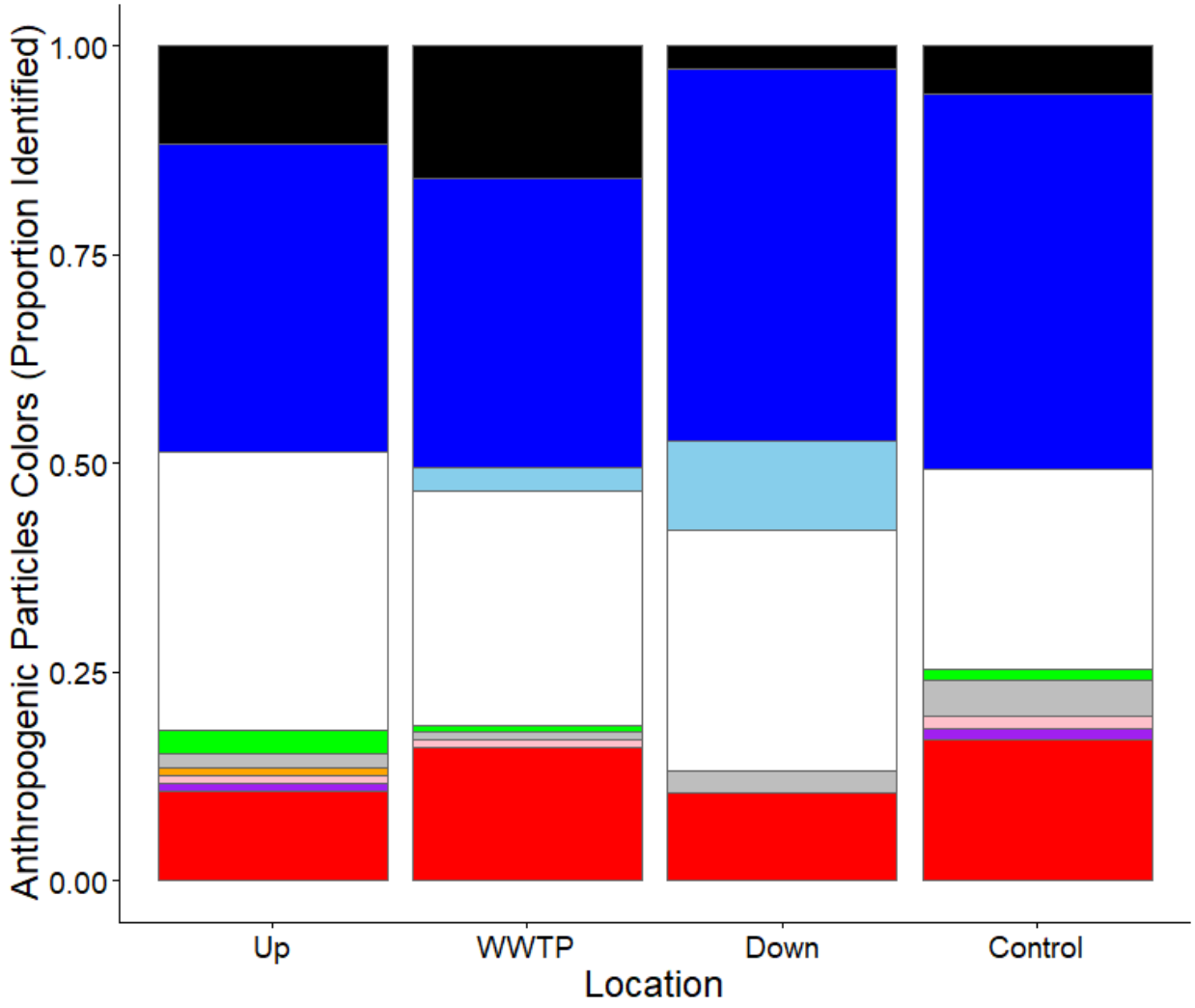

Figure S5. Composition of anthropogenic particle colors from fish collected in 2019 upstream (Up), downstream (Down), and at the effluent release site of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA, and laboratory controls (Control). Colors are shown as appeared, except white = clear.

Table S3. Permutational analysis of variance (PERMANOVA) testing the effect of location on stomach contents of Bluegill and Round Goby, considered together and individually. SS = sum of squares. Fish were collected in 2019 from locations upstream of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA, at the effluent release location, and downstream of the WWTP.

Source	df	SS	R^2	F	p
Both taxa together					
Location	2	2.28	0.29	6.30	<0.001
Species	1	0.74	0.09	4.11	0.01
Location × species	2	0.80	0.10	2.22	0.05
Residual	23	4.16	0.52	0.52	
Total	28	7.98	1.00	1.00	
Bluegill alone					
Location	2	1.28	0.37	3.23	0.01
Residual	11	2.19	0.63		
Total	13	3.47	1.00		
Round Goby alone					
Location	2	1.88	0.49	5.72	0.005
Residual	12	1.97	0.51		
Total	14	3.85	1.00		

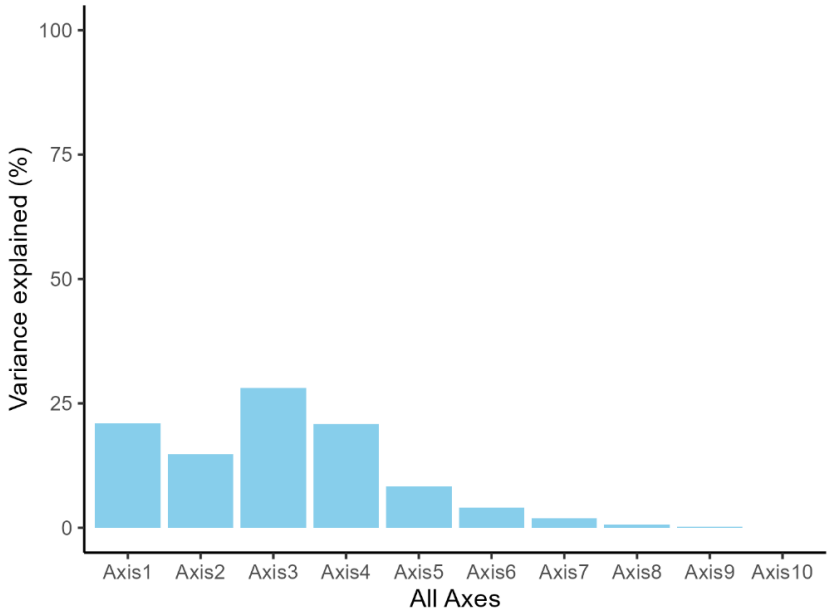

Figure S6. Scree plot showing the proportion of constrained variation in Bluegill stomach contents collected in 2019 upstream (Up), downstream (Down), and at the effluent release site of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA, explained by each axis of the species-specific Canonical Analysis of Principal Coordinates (CAP) ordination.

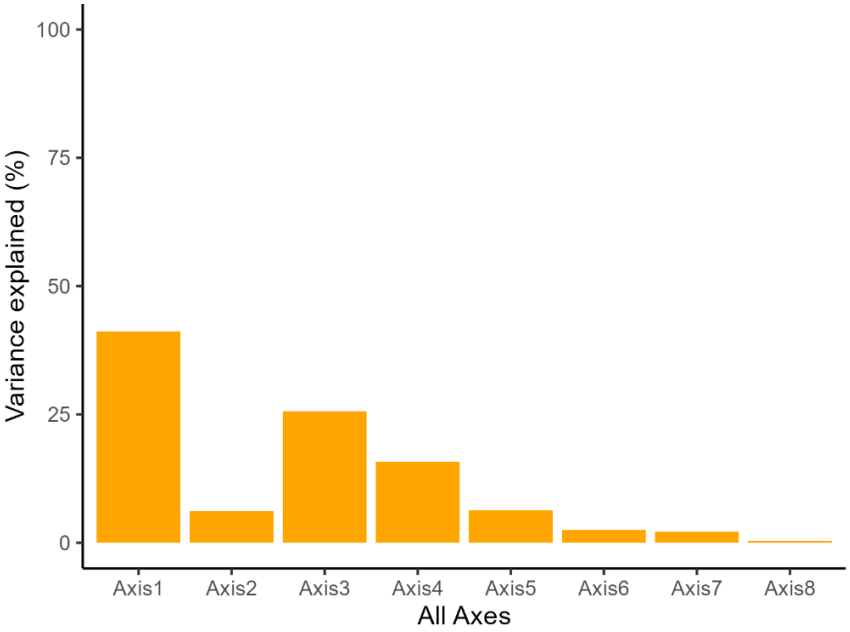

Figure S7. Scree plot showing the proportion of constrained variation in Round Goby stomach contents collected in 2019 upstream (Up), downstream (Down), and at the effluent release site of the T. J. O'Brien wastewater treatment plant (WWTP) in Chicago, Illinois, USA, explained by each axis of the species-specific Canonical Analysis of Principal Coordinates (CAP) ordination.