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Direct ingestion, trophic transfer, and physiological effects of microplastics in the early life stages of *Centropristis striata,* a commercially and recreationally valuable fishery species☆

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ABSTRACT

Microplastics are ubiquitous in marine and estuarine ecosystems, and thus there is increasing concern regarding exposure and potential effects in commercial species. To address this knowledge gap, we investigated the effects of microplastics on larval and early juvenile life stages of the Black Sea Bass (*Centropristis striata*), a North American fishery. Larvae (13–14 days post hatch, dph) were exposed to 1.0×10^4, 1.0×10^5, and 1.0×10^6 particles L^{-1} of low-density polyethylene (LDPE) microspheres (10–20 μm) directly in seawater and via trophic transfer from microzooplankton prey (tintinnid ciliates, *Favella* spp.). We also compared the ingestion of virgin and chemically-treated microspheres incubated with either phenanthrene, a polycyclic aromatic hydrocarbon, or 2,4-di-tert-butylphenol (2,4-DTBP), a plastic additive. Larval fish did not discriminate between virgin or chemically-treated microspheres. However, larvae did ingest higher numbers of microspheres through ingestion of microzooplankton prey than directly from the seawater. Early juveniles (50–60 dph) were directly exposed to the virgin and chemically-treated LDPE microspheres, as well as virgin LDPE microfibers for 96 h to determine physiological effects (i.e., oxygen consumption and immune response). There was a significant positive relationship between oxygen consumption and increasing microfiber concentration, as well as a significant negative relationship between immune response and increasing virgin microsphere concentration. This first assessment of microplastic pollution effects in the early life stages of a commercial finfish species demonstrates that trophic transfer from microzooplankton can be a significant route of microplastic exposure to larval stages of *C. striata*, and that multi-day exposure to some microplastics in early juveniles can result in physiological stress.

1. Introduction

The demand for plastic has steadily increased over the last half century, driving the current global annual plastic production to 335 million metric tons (Geyer et al., 2017; PlasticsEurope, 2017), and with developed nations such as the United States leading in the production of plastic waste (Borelle et al., 2020). Globally, plastic ingestion has been documented in over 220 species of marine organisms, including finfish (Lusher et al., 2017, Savoca et al., 2020).

Microplastics (synthetic particles ranging between 1 μm–5 mm in size; Brander et al., 2020) of both primary and secondary sources are ubiquitous and persistent in the aquatic environment (Barnes et al., 2009; Eriksen et al., 2014). The effect of microplastics on commercial fisheries is of growing concern due to the potential impact of exposure on populations, as well as possible human health risks of consuming microplastic-contaminated seafood (Santillo et al., 2017; Karami et al., 2018). There is limited information about the effects in commercial fish species, particularly those native to North America (Baechler et al., 2020; Granek et al., 2020). Field studies involving commercial fisheries primarily report presence or absence of microplastics (Foekema et al.,

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2013; Lusher et al., 2013; Bessa et al., 2018; Liboiron et al., 2018) and laboratory studies often use the same few non-commercial freshwater species, e.g. Zebrafish, Fathead Minnows, Japanese Medaka (reviewed in Jacob et al., 2020), and species sensitivity can vary widely (e.g. Besseling et al., 2019). Thus, it is important to gather data on responses to emerging persistent contaminants, such as microplastics, across a wider range of species (Granek et al., 2020). To provide a greater understanding of how species outside of these typical models, such as commercial finfish, may be affected by microplastic pollution, we used the Black Sea Bass (*Centropristis striata*) as the focal species for our experiments.

C. striata, a commercially and recreationally valuable fishery along the Atlantic coast of North America, is a widely distributed temperate reef fish with a range from the Gulf of Maine to the Gulf of Mexico (Able and Hales, 1997). This species feeds opportunistically upon a variety of prey items and thus accidental ingestion of microplastics from the water column as the fish mistakes plastic for prey is a potential concern (Sedberry, 1988; Devriese et al., 2015). *C. striata* utilize nursery habitats in estuaries and coastal waters that are notably impacted by anthropogenic activities, during their early life stages (Beck et al., 2001; Rabalais, 2015; Vendel et al., 2017). Interspecific variation in microplastics ingestion is likely due to the species-specific feeding strategies and abundance of plastics in their surrounding environment (Lusher et al., 2013; de Ruijter et al., 2020). Also of importance, microplastics prevalence is pronounced in coastal zones due to their proximity to terrestrial inputs and tidal processes that cause accumulation and fragmentation (Weinstein et al., 2016; Gray et al., 2018).

The potential risks of direct microplastic ingestion during early life stages of fishes likely arise from a combination of physical stress and chemical exposure (Jacob et al., 2020, Pannetier et al., 2020). An additional exposure route includes ingestion of microplastics and associated pollutants via trophic transfer from contaminated prey items (Nelms et al., 2018), documented in both natural systems and in artificial laboratory food webs (Carbery et al., 2018; Welden et al., 2018). Notably, both Athey et al. (2020) and Hasegawa and Nakoaka (2021) demonstrated that fish obtain more microplastics from prey (ciliates and mysid shrimp, respectively) than they do directly from the water. To what degree commercial finfish are affected by the trophic transfer of microplastics and associated pollutants remains unknown and the mechanisms poorly understood, particularly under environmentally relevant conditions.

Additionally, due to their ubiquity and high surface area to volume ratio, microplastics have the potential to serve as transport vectors not only for plastic additives but also for hydrophobic persistent organic pollutants (Rios et al., 2007; Bakir et al., 2014; Gallo et al., 2018). Chemicals commonly associated with microplastics are adsorbed hydrophobic aqueous pollutants (DDT, PAHs, PCBs) (Ziccardi et al., 2016). It has been suggested that the transfer of chemicals adsorbed to microplastics from the environment is not a significant means of exposure when compared to other exposure pathways (e.g., through the environment or prey) (Koelmans et al., 2016). However, plastic additives, added at high concentrations during manufacturing, may be a greater concern because of their potential for endocrine disruption at low concentrations (Brander, 2013; Brander et al., 2016; Franzellitti et al., 2019; Bucci et al., 2021).

Given these knowledge gaps, we sought to address the impacts of microplastics of different morphologies with and without associated chemicals in early life stages (larval, juvenile) of an estuarine commercial fishery species. Our objectives were 1. To assess ingestion directly from the water compared to trophic transfer in larvae, and 2. To investigate whether physiological responses were perturbed by microplastic exposure in young juveniles, by measuring respiration and immunity. To accomplish the first objective, we used a model food chain with single-celled microzooplankton (tintinnid ciliates; *Favella* spp.) and larval *C. striata*, and exposed larvae to microspheres with and without associated chemicals. Ciliates are important food sources for larval fish, including *C. striata*, in marine and freshwater habitats (Zingel et al., 2019) and may serve as significant vectors of microplastics to enter food webs via trophic transfer (Athey et al., 2020). For the second objective, we conducted exposures to microplastics of two morphologies (sphere and fiber) with and without associated chemicals in early juvenile stage *C. striata* and assessed two physiological endpoints: oxygen consumption and gross immune response. Three microplastic concentrations were used for both objectives to provide the type of dose-concentration data necessary for risk assessment. Microplastic-associated chemicals used were the common environmental pollutant phenanthrene, and a frequently used UV stabilizer - 2,4-di-tert-butylphenol (2,4-DTBP) (Black et al., 1983; Samanta et al., 2002; Rani et al., 2015; McConville et al., 2018; Coffin et al., 2019). To the best of our knowledge, this is the first set of laboratory microplastic and microfiber exposures conducted with early life stages of an estuarine commercial finfish species native to North America.

2. Methods

2.1. Contamination mitigation

All glassware used in the laboratory feeding experiments was rinsed with deionized (DI) water, soaked in a nitric acid solution (10% v/v) for 24 h prior, and soaked in DI water for 24 h prior to experimentation. The glassware was then baked at 450 °C for 4 h and rinsed with either dichloromethane (DCM) or acetone (ultrapure grade) to prevent additional contamination. Equipment (e.g., glass pipettes, dip nets, etc.) was designated to specific treatment groups to ensure no cross-contamination between virgin, phenanthrene-treated, and 2,4-DTBP-treated microspheres. Beakers were covered with foil (larvae) or lids (juveniles) during exposures to prevent contamination from plastics in the air.

2.2. Microsphere and microfiber stock preparation

Given polyolefins such as polyethylene are frequently documented in the water column due to their extensive use in fishing gear and single-use plastic products (Jambeck et al., 2015; Reisser et al., 2015; Conkle et al., 2018; Pozo et al., 2019), we selected low-density polyethylene (LDPE) microspheres for both the larval and juvenile exposures, and PE microfibers for use only in the juvenile experiments. LDPE microspheres (10–20 μm in diameter; Grant Industries, NJ, USA) were used for the larval and early juvenile laboratory feeding experiments. Microspheres were rinsed with methanol for 6 d and then dried in a fume hood at ambient temperature. To ensure proper dispersion of the microspheres in aqueous media, a 0.01% (v/v) solution of the non-ionic surfactant Tween20 (Fisher Scientific, Pittsburgh, PA, USA) was prepared in 100 mL Milli-Q ultrapure (MQ) water, stirred at ambient temperature for 30 min, and heated to 100 °C for 5 min in a water bath (Athey et al., 2020). The methanol-rinsed LDPE microspheres were resuspended in 0.01% Tween20 solution and vortexed in a glass bottle. Stock LDPE microsphere solutions were prepared by adding MQ water to the Tween20-microsphere mixture, to yield stocks of 1.0×10^4, 1.0×10^5, 1.0×10^6 particles per L^{-1}. A hemocytometer was used to confirm microsphere concentrations. PE microfibers (700 μm in length, 10–15 μm diameter; MiniFIBERS, Inc, Johnson City, TN, USA) were resuspended in 0.01% Tween20 solution at 30 mg in 15 mL (Cole, 2016). The stock solution was created by adding MQ water (85 mL) to the Tween20-microfiber mixture and vortexed in a glass bottle to break up fiber clumps. The microfibers were not solvent rinsed and small clumps were visible, making it difficult to validate the exact number of microfibers mg^{-1}. As a result, the microfiber stock solutions and experimental concentrations are expressed in mass of microplastics L^{-1} rather than fiber count L^{-1}.

2.3. Phenanthrene and 2,4-di-tert-buytlphenol (2,4-DTBP) loading

LDPE microspheres were stirred in a mixture of toluene: hexane (1:1 v/v) containing phenanthrene (>99.5% purity) or methanol containing 2,4-DTBP (>99% purity) (Sigma-Aldrich, St. Louis, MO, USA) for 6 d at ambient temperature. The resulting slurry was filtered through a glass fiber filter (Whatman #1820–021, retention: 1.6 μm) (Sigma-Aldrich, St. Louis, MO, USA) before being washed four times with hexane and dried at ambient temperature for 24 h. The concentrations of phenanthrene (1.9 μg g^{-1}) and 2,4-DTBP (12 μg g^{-1}) sorbed on the microspheres were selected to reflect environmental or additive concentrations, respectively, of these compounds (Rani et al., 2015; Peng et al., 2019). Sorption was confirmed by extraction and subsequent gas chromatography and flame-ionization detection (GC/FID) analysis (see Supplemental 1 for details). Fibers were not treated with chemicals.

3. Larval exposures

3.1. Larval C. striata maintenance

C. striata broodstock were maintained at the UNC-Wilmington Aquaculture Facility, Wrightsville Beach, NC according to the methodology described by Watanabe (2011) and Watanabe et al. (2021) and in accordance with UNCW IACUC Protocol #A1819-009. Approximately 1000 *C. striata* larvae were obtained at 12 dph (days post hatch) and stocked in 18 L rearing containers of artificial seawater (ASW, 30 ppt) at a density of 30 larvae L^{-1} in a temperature-controlled room (16 °C). ASW was prepared using Instant Ocean (Middleton, Wisconsin, USA) and DI water until the appropriate salinity was reached. Larvae were fed nutritionally enriched rotifers (10 rotifers mL^{-1}) twice daily during the acclimation period. Salinity (29.40 ± 1.96 ppt), temperature (17.25 ± 0.14 °C), dissolved oxygen (8.29 ± 0.93 mg L^{-1}), ammonia (0.00 ± 0.00 ppm), and pH (7.35 ± 0.04) were monitored daily during the acclimation and experimental periods (see Supplemental 2).

3.2. Culturing of tintinnid ciliates

Tintinnid ciliates (*Favella* spp.) were cultured based on previous methodology described in Athey et al. (2020). Ciliate cultures were maintained in 200 mL batches of filtered seawater in a temperature-controlled incubator (14–16 °C, 30 ppt) and sub-cultured every 3–4 d. The ciliates were fed phytoplankton (*Heterocapsa triquetra, Isochrysis galbana, and Mantoniella squamata*) every 3–4 d. The phytoplankton cultures were maintained in 40–1000 mL batches of filtered seawater supplemented with f/2 media and Guillard's vitamins. The phytoplankton were maintained in an illuminated incubator with 50–100 μmol photons m^{-2} s^{-1} on a 14:10 day:night cycle at 14–16 °C and were sub-cultured every 1–2 wks.

3.3. Experimental design of larval exposures

The purpose of this feeding experiment was to assess microplastic ingestion in cultured *C. striata* larvae (13–14 dph) exposed to virgin and chemically-treated LDPE microspheres through direct ingestion and trophic transfer. For the direct ingestion and trophic transfer feeding experiments we used virgin, phenanthrene-treated, and 2,4-DTBP-treated LDPE microspheres (10–20 μm) at three concentrations (1.0×10^4, 1.0×10^5, and 1.0×10^6 particles L^{-1}). The lowest concentration was 10,000 particles L^{-1}, or 10 particles mL^{-1}, an approximation of an environmentally relevant level of small microplastic particles recently recommended for use in experiments by Bucci et al. (2019). It is difficult at this time to verify how accurate this approximation is, as many field surveys do not account for plastics smaller than 300 μm (Brander et al., 2020). For the trophic transfer groups, *C. striata* larvae were exposed to rinsed ciliates that were previously exposed to virgin or chemically treated microspheres at the three concentrations (see below). In total, there were 18 experimental groups (exposed to microplastics directly and via trophic transfer) and 3 control groups (not exposed to microplastics) with 4 replicates for each group

3.4. Larval direct exposure

Immediately prior to the feeding experiment, glass treatment beakers were filled with 250 mL ASW (16 °C, 30 ppt) into which virgin and chemically microspheres (1.5×10^5 beads mL^{-1} stock) were added volumetrically: 16.7 μL, 167 μL and 1.67 mL to achieve the low, medium, and high concentration replicates of 1.0×10^4, 1.0×10^5, and 1.0×10^6 particles L^{-1} respectively. These concentrations are on the low end of those typically used in exposures with larval and juvenile fish (reviewed in Jacob et al., 2020). Given that most field measurements focus on larger plastic size fractions (Brander et al., 2020), an accurate estimate of 10–20 μm LDPE found in estuarine waters was not available at the onset of our experiments. A glass pipette was used to gently stir each replicate to disperse the microspheres evenly. No microspheres were added to the control group. Black sea bass larvae were starved 3 h prior to experimentation before transferring 10 individuals into each experimental replicate. After the 2 h microplastics exposure in foil-covered beakers, 3 larvae from each of the direct ingestion replicates were sampled to obtain microsphere ingestion counts. The larvae were rinsed in MQ water to remove any microspheres adhered to the skin, sacrificed on ice, rinsed in phosphate buffer saline (PBS), and preserved in glutaraldehyde (2.5% v/v) to prevent degradation until microscopic analysis (Oozeki and Hirano, 1988).

3.5. Larval trophic transfer exposure

Ciliate cultures were starved 24 h prior to experimentation, pooled into a 2 L glass container, gently reverse filtered using a 40 μm nylon mesh cell strainer, and reconstituted to 2 L with ASW. This washing process was repeated several times to remove algal prey cells and culture debris. Three 1 mL subsamples of the final ciliate pool were counted using a Sedgewick-Rafter counting chamber to determine ciliates mL^{-1}.

For each trophic transfer replicate, washed ciliates were volumetrically added from the pooled container to a glass beaker to achieve a concentration of 15 ciliates mL^{-1} in 100 mL ASW (16 °C, 30 ppt). Three 1 mL samples were collected and preserved in Lugol's iodine (20 μL) and glutaraldehyde (20 μL, 2.5% v/v) and stored at 4 °C for later counting to confirm the starting ciliate density. Then, chemically treated or virgin microspheres were added to these beakers to achieve the high, medium, and low concentration replicates as described above before stirring with a glass pipette to disperse the microspheres and ciliates evenly. Ciliates were allowed to feed on microspheres for 1 h. One set of ciliate controls were not fed microplastics and were not fed to C. *striata* larvae. The other set of ciliate controls were not fed microplastics but *were* fed to *C. striata* larvae for trophic transfer experiments.

Following 1 h exposure, ciliates in each beaker were reverse filtered through a 40 μm nylon mesh cell strainer from 100 mL to 20 mL and reconstituted to 100 mL with ASW. This was repeated twice to remove any extraneous microspheres. Three 1 mL ciliate samples were taken to enumerate the number of ingested microspheres per ciliate and the number of ciliates per mL following the 1 h exposure. The final volume of each beaker was increased from 100 mL to 250 mL before transferring 10 *C. striata* larvae that were allowed to feed on ciliates for 2 h, after which 3 larvae from each replicate, including ciliate control, were sampled to obtain microsphere ingestion counts. Larvae were sacrificed and preserved as described above in the previous section.

3.6. Microsphere quantification

Each of the 1 mL samples collected after the 1 h ciliate feeding period were centrifuged for 15 s and pipetted into a glass depression slide, and viewed using a polarized light microscope (ZEISS Axioskop,

Oberkochen, Germany) to quantify the total number of ciliates in 1 mL and determine microspheres ingested per ciliate. The preserved *C. striata* larvae were whole mounted on a microscope slide and also analyzed using polarized light microscopy (ZEISS Axioskop, Oberkochen, Germany). A first-order phase plate was used to provide additional contrast between the microspheres and the soft tissues of the gut. The number of microspheres within the gut of each larva were obtained to determine total microplastic consumption across all treatment groups.

4. Juvenile exposures

4.1. Juvenile C. striata maintenance

C. striata juveniles were maintained at the UNC-Wilmington Aquaculture Facility in Wrightsville Beach, NC according to the methodology described by Watanabe (2011) and Watanabe et al. (2021) in accordance with IACUC Protocol #A1819-009. Approximately 1000 *C. striata* juveniles (50–60 dph, each approx. 0.75 g) were temporarily stocked in aerated 10 L glass aquaria with full-strength high-quality seawater (HQSW, 20–22 °C, 30–34 ppt). HQSW was obtained from the Center of Marine Science's Seawater Systems: raw seawater from the Intracoastal Waterway is processed through a series of filters (60 μm, 10 μm, 1 μm). Juveniles were fed a commercially prepared diet (Otohime, Reed Mariculture Inc, Campbell, CA) twice daily *ad libitum*. Salinity (ppt), temperature (°C), dissolved oxygen (mg L^{-1}), ammonia (ppm), and pH were monitored daily, and 50% water changes in the holding tanks were conducted daily (Supplemental 3).

4.2. Experimental design of juvenile exposures

The purpose of this experiment was to measure the rates of oxygen consumption and immune response in early juvenile *C. striata* following a 4-d direct exposure to virgin microspheres, chemically-treated microspheres (phenanthrene or 2,4-DTBP), and virgin microfibers. We used virgin, phenanthrene-treated, and 2,4-DTBP-treated LDPE microspheres (10–20 μm) and virgin LDPE microfibers (700 μm in length) at three concentrations (1.0×10^4, 1.0×10^5, and 1.0×10^6 microplastic particles L^{-1}). In total, there were 12 experimental groups and 1 control group each with 4 replicates.

4.3. Juvenile direct exposure

For each treatment, 8 *C. striata* juveniles were removed from the stock tanks and placed in 3 L glass containers filled with aerated HQSW (20–22 °C, 30–34 ppt). The 8 juveniles in each experimental unit were of similar sizes (~0.75 g) to avoid cannibalism which has been observed during nursery rearing (Watanabe, 2011). The fish were initially starved 24 h prior to the first addition of microspheres. Virgin and chemically-treated microspheres were added to each replicate volumetrically: 5.0 mL, 0.50 mL, and 0.05 mL of microspheres were added from the 100 mL stock solutions (6×10^5 mL^{-1} stock) to achieve the low (10,000 particles L^{-1}), medium (100,000 particles L^{-1}), and high (1,000,000 particles L^{-1}) microplastic treatments, respectively. These are the same concentrations used for larval *C. striata*. Complete water changes of the *C. striata* juvenile tanks were conducted after each 24 h period, followed by microplastic addition to maintain the same level of exposure. Subsets of juveniles were randomly selected for endpoint analyses (immune response assay and respiration analysis) following the 4-d exposure to virgin and chemically treated microspheres.

4.4. Respiration analysis

Using methodology adapted from Watts et al. (2014), closed-system respiration chambers (RC400 Respiration Cell, Strathkelvin Instruments, Motherwell, Scotland, UK) were used in conjunction with oxygen electrodes and a six-channel oxygen meter (SI130 Microcathode Oxygen Electrode; SI929 6-Channel Oxygen Meter, Strathkelvin Instruments, Motherwell, Scotland, UK) to measure oxygen concentration. The respiration analysis was designed to measure rates of oxygen consumption in a subset of juvenile *C. striata* after the 4-d microplastic exposure.

Oxygen electrodes were calibrated daily in both oxygen-saturated water and oxygen-free water (by addition of sodium sulfite). Each respiration chamber was fitted with a stir bar below a grated bottom to insure mixing, filled with fully saturated HQSW, and spatially arranged to prevent any interaction between fish that could affect the respiration rates. The temperature (°C) and salinity (ppt) of the HQSW along with the atmospheric pressure (mmHg), were measured to calculate the oxygen saturation of the water. Oxygen concentration data were collected for a minimum of 30 min prior to experimentation to determine background oxygen concentration.

Two fish per replicate were placed in each chamber and oxygen consumption recorded continuously for a total of 20 min (10 min of acclimation to the chambers and 10 min of recording to be used in analysis). Following the data collection period, fish were removed from the chambers and euthanized via lethal concentration of MS-222 (described below in *Immune Response Assay*). The water was discarded, the chamber was rinsed, and refilled with fully saturated HQSW prior to every subsequent respiration trial.

Oxygen concentration (μmol L^{-1}) was analyzed via Strathkelvin SI929 Software (Strathkelvin Instruments, Motherwell, Scotland, UK). The background O_2 levels were recorded in chambers with no fish and then subtracted from the measured O_2 concentrations for each experimental replicate. The rate of oxygen consumption (μmol hr^{-1}) of *C. striata* juveniles from exposed and control treatments was calculated over the 10 min period after acclimation for each replicate. Oxygen consumption calculations were normalized to the body mass of the fish (approximately 0.75 g per individual).

4.5. Immune response assay

The immune response assay, a proxy for stress, was measured at the end of the 4-d microplastic exposure experiment using 3 juvenile *C. striata* per replicate. The assay was performed as described by DeCourten et al. (2020) and adapted from Breckels and Neff (2013). Phytohemagglutinin (PHA) is a novel antigen known to induce a cell-mediated response of T-cell proliferation and localized swelling at the site of injection (Ardia and Clotfelter, 2006). As a result, injection of PHA can provide an assessment of immune function though a localized swelling response. The caudal peduncle of *C. striata* was selected as the injection site because it is a measurable location with limited variability (Ardia and Clotfelter, 2006; Clotfelter et al., 2007). Two fish from each experimental and control replicate were randomly assigned to receive a subcutaneous injection of 2 μg PHA (Sigma-Aldrich, St. Louis, Missouri, USA) in 1 μL of phosphate buffered saline (PBS) using a 10 μl 26-gauge syringe with a beveled tip (Hamilton Company, Reno, NV, USA). The third fish of the same replicate was assigned to receive a control injection of only 2 μl of PBS. Juveniles were first anaesthetized with a sublethal dosage of tricaine methanesulfonate (MS-222, 0.25 g L^{-1}) (Sigma-Aldrich, St. Louis, Missouri, USA) for approximately 90 s. The caudal peduncle width was measured three times with a manual caliper before a subcutaneous injection of either PHA or PBS was administered to that site for each fish. The post-injection fish were placed in isolation chambers (20–22 °C, 30–34 ppt) to recover for 24 h without food, after which they were euthanized with a lethal concentration of MS-222 (1.25 g L^{-1}). The average of three caudal peduncle measurements was taken and the immune response of each juvenile was determined as the difference in swelling between pre-injection and post-injection caudal peduncle widths.

4.6. Hurricane Florence impact statement

As a result of severe building damage caused by Hurricane Florence at UNC-Wilmington in September 2018 all frozen samples from these experiments were lost when the back-up generator failed due to severe flooding. Therefore, we were unable to confirm ingestion/quantify the number of microspheres and microfibers within juvenile gut or gill tissues. Ingestion was however confirmed in larvae. Due to funding constraints and our use of a non-model fish species, we could not spawn more fish to repeat these experiments within the timeframe of the project. These results therefore provide a baseline study for understanding of how juvenile *C. striata* may be physiologically impaired after direct exposure to microplastics.

4.7. Statistical analyses

A generalized linear model (GLM + Poisson distribution) was used to analyze the average number of microspheres ingested per ciliate across virgin, phenanthrene-treated, or 2,4-DTBP-treated microspheres. The same approach was also used to analyze the number of microspheres ingested per *C. striata* larva, and to compare the number of microspheres ingested directly from the water or via trophic transfer from prey. A GLM (+normal distribution) was used to compare the effects of virgin microfibers and virgin, phenanthrene-treated, and 2,4-DTBP-treated microspheres on juvenile *C. striata* oxygen consumption. Immune response measurements were analyzed in a similar manner to compare caudal peduncle measurements across treatment groups. In the case of both respiration and immune response, treatment responses were normalized by subtracting the mean control responses. We represent the range of control data as a shaded area in each graph. To estimate the potential effect of low replication within the GLM prior to line-fitting, a leave-one-out analysis was conducted to determine the marginal effect of having even fewer data points. In all cases, the average effect on the slope of the line was <1%, indicating that the data were sufficient to fit the regression (Simberloff, 1978). Regressions were also fit to determine the relationship between increasing concentration of microplastics and either immune response or respiration. We calculated the 95% confidence interval around the regression line, using the point at which the lower bound of the confidence interval is>0 to be the point of departure, or the point at which the effect is greater than zero (Montgomery et al., 2021). All statistical analyses were performed in JMP Pro 14. Regressions were fit in lieu of using categorical comparisons (e.g. ANOVA with post-hoc comparison) based on recommendations from Cottingham et al. (2005) and implementing curve-fitting approaches similar to those published in Brander et al. (2016), Goff et al. (2017), and Mundy et al. (2020). All model parameters are reported in Supplemental tables 4A-4E.

5. Results and discussion

5.1. Ciliate LDPE microsphere ingestion

Ciliates (*Favella* spp.) ingested virgin, phenanthrene-treated, and 2,4-DTBP-treated LDPE microspheres at the three microplastic densities (1.0×10^4, 1.0×10^5, and 1.0×10^6 microspheres L^{-1}) following a 1 h direct exposure (Table 1). As might be expected, ciliate ingestion of microspheres increased with microsphere concentration (Fig. 1, GLM (Poisson), microsphere concentration effect: $P < 0.0001$). However, there was no effect of chemical treatment on the average number of microspheres ingested per ciliate (Fig. 1, GLM + Poisson distribution, chemical effect: $P < 0.9999$). No microspheres were detected in the unfed control ciliates.

The data show that *Favella* spp. readily ingested the LDPE microspheres but did not ingest a greater number of virgin or chemically treated microspheres. Similar results were reported by Athey et al. (2020) in which *Favella* spp. did not differentiate between virgin and DDT-treated microspheres, even though the amount of DDT (2.15×10 ng^6 g^{-1}) sorbed onto the microspheres exceeded environmentally relevant concentrations. Tintinnid ciliates have a preferred prey size range of 5–25 μm, indicating the organisms will reliably ingest objects – natural or synthetic – within the appropriate size range (Echevarria et al., 2014). Although microzooplankton are selective feeders that can use chemical as well as physical cues to feed upon prey (Griniene et al., 2016), the *Favella* spp. used in this study did not demonstrate a difference in ingestion of virgin vs. contaminated microspheres.

5.2. Larval C. striata: direct ingestion and trophic transfer of LDPE microspheres

Black sea bass larvae ingested virgin, phenanthrene-treated, and 2,4-

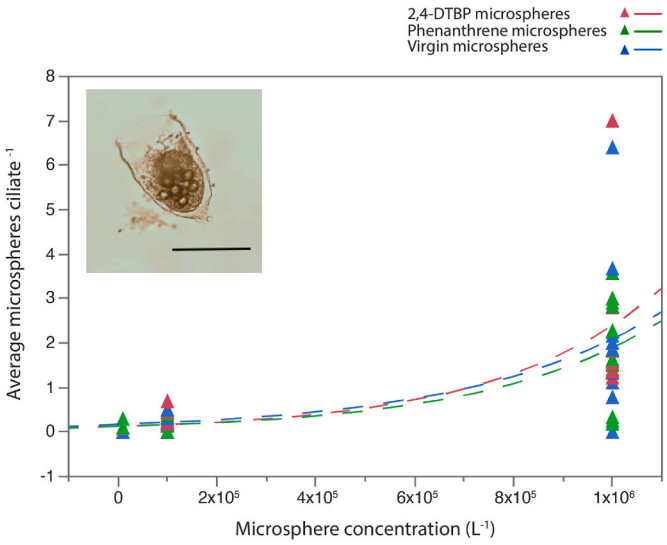

Fig. 1. Average number of microspheres internalized per ciliate, the red line and red (▲) represent the 2,4-DTBP treatment, green line and green (▲) represent the phenanthrene microsphere treatment, and blue line and (▲) represent the virgin microsphere treatment. The micrograph scale bar is 100 μm. Solid lines are a significant fit, dotted lines are not significant. GLM (Poisson), $\alpha = 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Average number (±SEM) of virgin (untreated), phenanthrene-treated, and 2,4-DTBP-treated microspheres ingested by ciliates and *C. striata* larvae across three microplastic densities.[a]

	Low	Virgin			Phenanthrene			2,4-DTBP	
		Medium	High	Low	Medium	High	Low	Medium	High
Ciliate - direct ingestion	0	0.25 ± 0.06	2.04 ± 0.06	0.03 ± 0.02	0.21 ± 0.05	1.84 ± 0.33	0.01 ± 0.01	0.24 ± 0.06	2.34 ± 0.46
Larvae – direct ingestion	0.08 ± 0.08	0	0.91 ± 0.56	0	0.25 ± 0.25	0.33 ± 0.33	0	0	0.32 ± 0.22
Larvae – trophic transfer	0	0.42 ± 0.19	2.18 ± 1.70	0	0	2.17 ± 0.87	0	0.42 ± 0.26	2.08 ± 1.28

[a] Ciliate data reflects the average number of microspheres that were ingested by ciliates in three 1 mL samples for each of the 4 replicates. The larval data refers to the average number of microspheres ingested by 3 individual larvae for each of the 4 replicates – either directly from the water or via trophic transfer from prey.

DTBP-treated LDPE microspheres of three microplastic densities (1.0×10^4, 1.0×10^5, and 1.0×10^6 microspheres L^{-1}) following a 2 h exposure to microspheres directly in the water and via trophic transfer from prey (Table 1). Larvae that fed upon microplastic-containing ciliates ingested significantly more microspheres than larvae directly exposed to microplastics in the water (Fig. 2, A, GLM (Poisson), direct ingestion vs. trophic transfer effect: $P = 0.0168$). There was no effect of chemical treatment on the total number of microspheres ingested by *C. striata* larvae via trophic transfer from prey (Fig. 2, B, GLM (Poisson), chemical effect: $P = 0.3722$). *C. striata* initially appeared to ingest a greater number of virgin microspheres directly from the water at the highest concentration (Fig. 2, C, GLM (Poisson), microsphere effect: $P = 0.6824$), but this result was not significant. Significantly more microspheres across all treatments were ingested at the highest microplastic density (Fig. 2, A-C, GLM (Poisson), microsphere effect: $P < 0.0001$). No microspheres were detected in the control larvae and limited ingestion occurred at the low and medium microplastic concentrations (Table 1).

Larval *C. striata* did not ingest a greater number of the virgin microspheres compared to either of the chemically treated microspheres when directly available in the water or through the ciliate prey. The olfactory system is important for discriminating odors that mediate feeding and social behaviors in larval fish (Firestein, 2001), but the sensitivity of olfaction is not well established for many species of marine finfish larvae (Lara, 2008). The olfactory system becomes more developed as fishes transition into juvenile and adult life stages, so it is plausible that *C. striata* larvae were unable to discriminate against or are indifferent to the chemically treated microspheres via olfaction.

Larval fish are visual predators (Voesenek et al., 2018), which is consistent with our finding that *C. striata* larvae potentially ingest more microspheres via contaminated prey items (i.e., tintinnid ciliates) than directly from the water. For the highest concentration of microspheres, ciliates ingested an average of 2 microspheres per individual (Table 1), but larval fish exposed to high concentrations of microspheres (direct ingestion) contained less than 1 microsphere per individual across all concentrations. However, slightly greater than 2 microspheres per individual fish were observed in the highest trophic transfer concentration treatments. At 15 ciliates mL^{-1}, each trophic transfer treatment beaker had a microplastic exposure of approximately 3×10^3 microspheres L^{-1}, which is an order of magnitude lower than the lowest direct ingestion exposure treatment (1×10^4 microspheres L^{-1}) in which only one microsphere was ingested among 12 specimens (Table 1). Microplastic-containing zooplankton in the natural environmental may pose significant risk of exposure to juvenile salmon (Desforges et al., 2015), indicating that trophic transfer of plastics is an important consideration for estuarine and coastal food webs. Athey et al. (2020) recently demonstrated increased ingestion of microplastics through microzooplankton prey by larvae of the estuarine model species *Menidia beryllina* and here we show that common microzooplankton such as ciliates also have the potential to serve as significant vectors of microplastics in commercially valuable fishes.

5.3. Early juvenile C. striata: physiological responses following LDPE microsphere and microfiber exposures

Only juvenile *C. striata* exposed to virgin microfibers exhibited a significant increase in oxygen consumption with increasing plastic concentration (Fig. 3, GLM (Normal), $P = 0.0352$), indicating the microfibers had a more pronounced effect on the respiratory system in comparison to microspheres. Based on the 95% CI we estimate that juveniles began to respond to microfibers at a concentration of 2.7×10^5 per L^{-1}. Respiratory distress (measured in terms of increased oxygen consumption) is a likely physiological response to a microplastic exposure, considering the potential for microsphere and microfiber uptake via the gills (Watts et al., 2016). Recently, increased mucus production in the gills was observed in maturing *O. latipes* following a 10-week dietary exposure to 10 μm polystyrene microplastics (Zhu et al.,

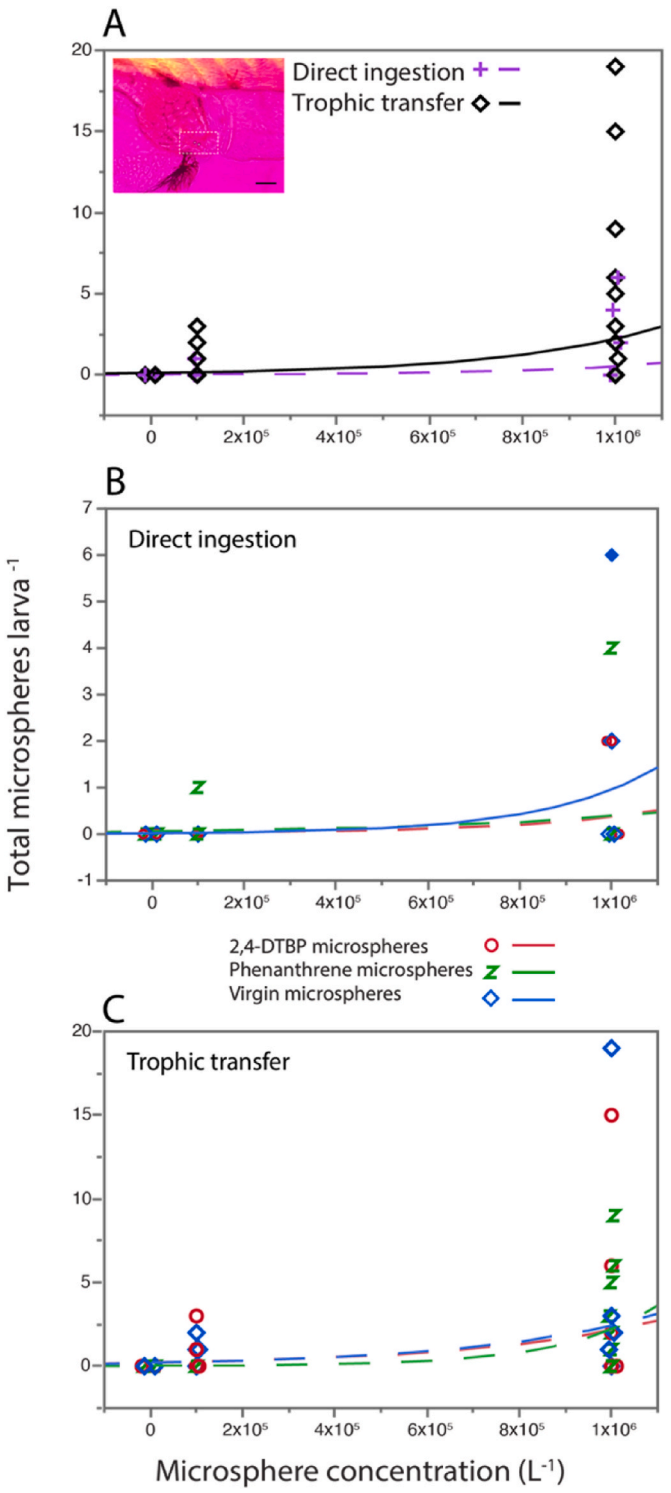

Fig. 2. (A) Trophic transfer and direct microsphere ingestion by C. striata larvae, the purple line and purple (+) represent the direct ingestion treatments and black line and black (⋄) represent the trophic transfer treatments. The micrograph scale bar is 100 μm. (B) Microspheres ingested by C. striata larvae via trophic transfer from prey, the red line and red (○) represent the 2,4-DTBP treatment, green line and green (z) represent the phenanthrene microsphere treatment, and blue line and (⋄) represent the virgin microsphere treatment. (C) Microsphere ingestion by C. striata larvae directly from the water, same colors and symbols as (B). Solid lines are a significant fit, dotted lines are not significant. All analyses used GLM (Poisson), $\alpha = 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

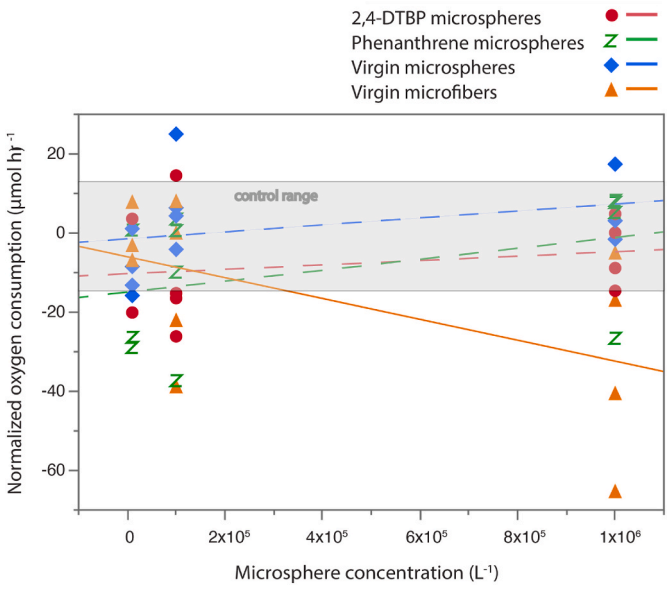

Fig. 3. Oxygen depletion in juvenile *C. striata* following a direct 96-h exposure to microplastics, the red line and red (●) represent the 2,4-DTBP treatment, green line and green (z) represent the phenanthrene microsphere treatment, blue line and (◆) represent the virgin microsphere treatment, and the orange line and orange (▲) represent the virgin microfiber treatment. Data from exposure treatments were standardized by subtracting the mean oxygen depletion in the control treatment (not exposed to microplastics), hence control data are not included in the regression. The shaded box (centered on zero) represents the range of control values. GLM (Poisson), $\alpha = 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

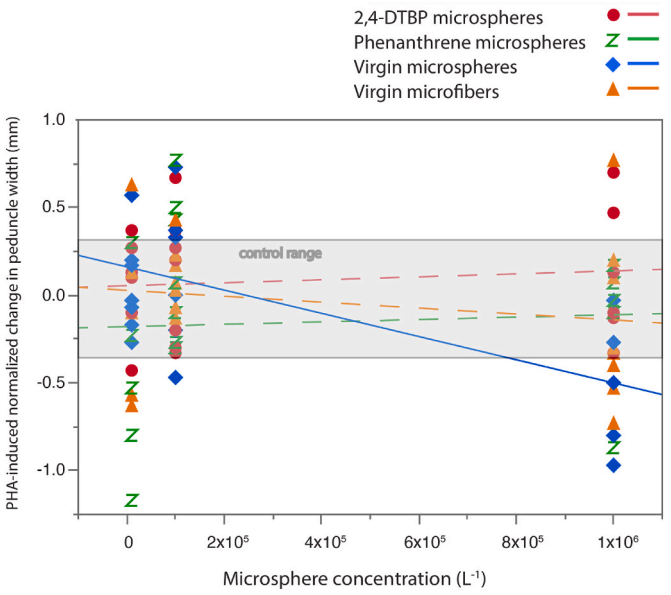

Fig. 4. PHA-induced change, normalized by saline-injected control, in peduncle width (proxy for immune response) in juvenile *C. striata* following a direct 96-h exposure to microplastics, the red line and red (●) represent the 2,4-DTBP treatment, green line and green (z) represent the phenanthrene microsphere treatment, blue line and (◆) represent the virgin microsphere treatment, and the orange line and orange (▲) represent the virgin microfiber treatment. Data from exposure treatments were standardized by subtracting the mean response in the control treatment (not exposed to microplastics), hence control data are not included in the regression. The shaded box (centered on zero) represents the range of caudal peduncle swelling for the control animals. Solid lines are a significant fit, dotted lines are not significant. GLM (Poisson), $\alpha = 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2020). Given that the gills are extremely sensitive to toxicants and the presence of foreign substances (Wang et al., 2013), respiratory distress and increased oxygen consumption may occur when a foreign substance (i.e., microplastics) interferes with normal gill function (Van Cauwenberghe et al., 2015). It is possible that this toxicity is dependent on the shape of the microplastic, and that microfibers may have become entrapped in the gills of the exposed juvenile *C. striata,* although it is important to mention that another recent study in finfish found little impact on fish gills from microplastic exposure (Batel et al., 2018).

Only juveniles exposed to increasing concentrations of virgin microspheres for 96-h had a significant decrease in normalized caudal peduncle widths (Fig. 4, GLM (Normal), $P = 0.0049$), with no effect observed with chemically treated microspheres. The adaptive immune response (T cell-mediated) works to identify foreign substances, proliferate in the infected area, and remove the substance (Janeway, 2001). A smaller caudal peduncle indicates less T cell proliferation and a potentially suppressed immune response. This relationship is most evident at higher concentrations of microspheres, and calculations based on the 95% CI estimate that an effect was measurable at a concentration of 3.23×10^5 per L^{-1} and above. The presence of ingested or inhaled microplastics alone may be enough to elicit an inflammatory response within the organism (Wright and Kelly, 2017). The apparent lack of response to the other treatments is difficult to explain because potentially toxic additives and monomers are used to manufacture plastics (Avio et al., 2017), however, it is possible that unlike the larval *C. striata*, juveniles (50–60 dph) were able to differentiate between virgin and chemically treated microspheres, hence avoiding the latter. This was not possible to determine following exposures due to hurricane-related sample loss, as explained in the Methods.

The effects of microplastics on finfish are diverse and variability in experimental design can make it difficult to compare across studies. Laboratory studies investigating the trophic transfer of virgin and chemically treated microspheres from prey to finfish report different physiological endpoints, some with significant latent impacts at high concentrations (e.g., reduced growth two weeks post-exposure in larval Inland Silversides (*Menidia beryllina*; Athey et al., 2020) and others showing no effect. A study in Zebrafish indicated that microplastic-associated pollutants ingested from prey (*Artemia* nauplii) potentially desorb in fish intestines (Batel et al., 2016). However, no altered behavior was observed in Krefft's Frillgobies (*Bathygobius kreffii*) exposed via trophic transfer (Tosetto et al., 2017) and no effect on hepatic CYP1A levels was found in Zebrafish exposed to microplastics with sorbed benzo(*k*)fluoranthene trophically via *Daphnia magna* and *Chironomus riparius* (Hanslik et al., 2020).

The physiological effects of microplastics in non-commercial finfish include decreased lipid metabolism and oxidative and hepatic stress in adult Zebrafish (*D. rerio)* (Lu et al., 2016), decreased growth and body condition of juvenile forage fish (*Acanthochromis polyacanthus*) (Critchell and Hoogenboom, 2018), decreased body length and mass in juvenile Glassfish (*Ambassis dussumieri*) (Naidoo and Glassom, 2019), reduced predatory performance in juvenile Common Goby (*Pomatoschistus microps*) (de Sa et al., 2015), and endocrine disruption in adult Japanese Medaka (*Oryzias latipes*) (Rochman et al., 2014). It is therefore apparent that concern is warranted and additional research is necessary, especially in commercial species. Even in the limited studies on commercial species, microplastic exposure can result in weakened feeding behaviors and reduced energy reserves in juvenile Korean Rockfish (*Sebastes schlegelii*) (Yin et al., 2018) and pathological alterations to intestinal epithelium in juvenile European Sea Bass (*Dicentrarchus labrax*) (Peda et al., 2016), although minimal effects were observed in European Sea Bass larvae (*D. labrax)* (Mazurais et al., 2015) and juvenile Gilt-head Seabream (*Sparus aurata*) (Jovanovic et al., 2018). Additional

experiments are needed to resolve the interaction of microplastics across different morphologies and polymer types, with a focus on frequently detected fibers (Ross et al., 2021), as well as there being a need for a better understanding of the role of olfaction and particle selection across early life stages in fishes.

6. Conclusions

This study provides the first assessment of the effects of microplastic exposure in early life stages of a commercially and recreationally important fish species (*C. striata*). We found that direct ingestion of LDPE microspheres by larval *C. striata* was only detected at high levels of exposure with no difference between virgin and chemically treated microspheres. Importantly, *C. striata* larvae ingested significantly more microspheres via trophic transfer from microzooplankton (*Favella* spp.), indicating that ingestion via prey should be further evaluated in future assessments. Juvenile *C. striata* are susceptible to physiological impairment (i.e., increased oxygen consumption and altered immune response) following 96-h exposure to some but not all microplastic treatments, additional research in this area is clearly needed.

In the present study, chemically treating microspheres with a plastic additive and a PAH did not have a significant effect on ingestion, oxygen consumption, or immune response of early juvenile *C. striata*. However, the presence of microfibers resulted in significantly increased oxygen consumption in early juvenile *C. striata* compared to the presence of microspheres (virgin or chemically treated). This information is important considering the growing body of literature suggesting that microfibers are the most prevalent type of microplastic ingested by wild-caught marine organisms and may present the greatest risk to the respiratory system in aquatic animals (Lusher et al., 2013; Mishra et al., 2019).

This study aimed to address several critical knowledge gaps, particularly through using a commercial marine finfish species at early life stages, evaluating relatively low microplastic concentrations in a concentration-response design, and plastic additive-treated microplastics. Data such as those produced here can be used to inform future risk assessments, especially considering that studies measuring responses across microplastic concentrations are currently limited in commercial fishery species (Granek et al., 2020). Future research is necessary to fully understand how commercial finfish will be affected by microplastics across shapes, sizes, and polymer types (e.g. Rochman et al., 2019; Cunningham et al. in review) and the role of microplastics as one of a suite of multiple stressors (e.g., overharvest, ocean warming, and hypoxia; Baechler et al., 2019), but there are unique challenges associated with using commercial finfish in the laboratory (e.g., complex life histories, feeding strategies, nutrient requirements, and intensive husbandry; Watanabe et al., 2019). With over 88% of global fisheries production and aquaculture being utilized for human consumption (FAO, 2018), it is imperative to determine if the trophic transfer of microplastics and associated pollutants and additives present a potential risk of exposure to humans by way of seafood consumption.

Author Statement

We have uploaded a corrected Highlights document.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117653.

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