

SPECIAL ISSUE-LETTER

Trophic transfer of microplastics in an estuarine food chain and the effects of a sorbed legacy pollutant

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Scientific Significance Statement

Microplastics are ingested by estuarine species, yet the trophic transfer of microplastics from common single-celled prey to larval predator has never been investigated in estuarine systems. Furthermore, the influence of microplastic-associated contaminants on trophic transfer in estuarine systems is unknown. This study provides the first evidence for the trophic transfer of microplastics in a model food chain relevant to North American estuaries, as well as the potential impacts of a sorbed legacy pollutant on microplastic trophic transfer. Our study is also the first to use a unicellular microzooplankton as prey in a microplastic trophic transfer study. Microzooplankton are key microbial consumers and play an important role in estuarine food webs. We also demonstrate the negative impacts of microplastic ingestion on growth in larval fish.

Abstract

Microplastics are of increasing concern as they are readily ingested by aquatic organisms. This study investigated microplastic trophic transfer using larval inland silversides (*Menidia beryllina*) (5 d posthatch) and unicellular tintinnid (*Favella* spp.) as a model food chain relevant to U.S. estuaries. Low-density polyethylene microspheres (10–20 μm) were used to compare direct ingestion of microplastics by larval fish and trophic transfer via tintinnid prey. Dichlorodiphenyltrichloroethane (DDT)-treated microspheres were used to determine sorbed pollutant effects on microplastic ingestion. Larval fish exposed directly to microspheres ingested

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Author Contribution Statement: SNA, SDA, CAG, ART and SMB conceived the study and developed the experimental approach. ALA performed sample preparation of microplastics for analysis. SNA, SDA, CAG carried out the experiments and analysis of samples. SNA, ART and SMB analyzed data and SNA and SMB conducted statistical analysis. SNA, SMB and ART wrote the manuscript and prepared figures. SMB, ART, PS, and BM supervised the project and edited the manuscript.

Additional Supporting Information may be found in the online version of this article.

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This article is an invited paper to the Special Issue: Microplastics in marine and freshwater organisms: Presence and potential effects
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significantly fewer than those exposed via contaminated prey. Larvae ingested significantly more ciliates containing DDT-treated microspheres than ciliates containing untreated plastics but did not discriminate when exposed directly. Larvae reared for 16 d following a direct 2 h exposure had significantly lower wet weight values than unexposed controls. Our results demonstrate that trophic transfer is a significant route of microplastic exposure that can cause detrimental effects in sensitive life stages.

Microplastics (plastic particles ranging from 1 μm to 5 mm) are the most abundant type of anthropogenic debris found in marine and freshwater environments (Wright et al. 2013; Eriksen et al. 2014). Due to their proximity to land-based sources, microplastics are also well documented in estuarine systems, (Weinstein et al. 2016; Gray et al. 2018). Because estuaries are highly productive ecosystems that act as shallow nurseries for sensitive life stages (Weinstein et al. 1980; Fisher et al. 1982), understanding the impacts of microplastics is important to evaluating the environmental health of these areas.

Plastic debris is well known to have a wide variety of impacts on aquatic life, though negative effects associated with microplastics occur primarily due to ingestion (Andrady 2011). Reported effects of microplastic ingestion in different aquatic organisms vary from no acute effect (Kaposi et al. 2014; Mazurais et al. 2015; Mateos-Cárdenas et al. 2019) to reduced growth (Lo and Chan 2018), depressed energy reserves (Wright et al. 2013), and altered endocrine function (Rochman et al. 2014). Apart from the physical impacts of microplastic ingestion, there are a number of toxic persistent organic compounds associated with microplastics that have been shown to accumulate in the fatty tissues of marine organisms (Brander et al. 2011; Brander 2013; Batel et al. 2016). The legacy pesticide DDT (dichlorodiphenyltrichloroethane) and associated breakdown products are some of the most common pollutants found sorbed to the surface of plastics collected in coastal waters (Rios et al. 2010; Van et al. 2012).

Although prey misidentification and accidental ingestion of plastic debris is common, organisms can also be exposed to microplastics through the ingestion of microplastic-contaminated prey, a phenomenon known as trophic transfer (Welden et al. 2018). The trophic transfer of microplastics is an emerging concern and has been documented in both freshwater (Setälä et al. 2014; Santana et al. 2017; Tosetto et al. 2017) and marine systems (Batel et al. 2016; Mateos-Cárdenas et al. 2019). Ingested microplastics have been found in a number of important estuarine species (Alves et al. 2016; Bessa et al. 2018), yet the trophic transfer of microplastics has never been investigated in estuarine systems. Furthermore, the implication of microplastic-associated contaminants on trophic transfer in estuarine systems is unknown. Batel et al. (2016) investigated the trophic transfer of microplastics in a freshwater system as an exposure route for microplastic-associated pollutants to enter upper trophic levels. Their study found microplastics can act as a vehicle for pollutant exposure in freshwater adult fish and their prey (Batel et al.

2016). More research is needed to understand the impacts of sorbed pollutants on the trophic transfer of microplastics in estuarine systems, where chemical interaction with microplastics may differ from that of a freshwater environment (Bakir et al. 2014; Napper et al. 2015).

Relevant estuarine trophic models are needed in order to understand the potential for trophic transfer of microplastics and effects of their associated organic compounds in estuaries. One such model is the inland silverside, *Menidia beryllina*, a common euryhaline fish native to U.S. estuaries along the North and Gulf coasts and introduced to California (Gleason and Bengtson 1996), with related species distributed throughout North and South America (Strüssmann et al. 2010). These mid-trophic level fish ingest a variety of prey items, including protozoan zooplankton, and are an important food source for upper trophic level species (Middaugh and Hemmer 1992). Tintinnid ciliates, including *Favella* spp., are common in the tidal estuaries along the U.S. coast and elsewhere globally (Capriulo and Carpenter 1983; Montagnes et al. 2012).

Prior studies have demonstrated the trophic transfer of microplastics and their associated pollutants in freshwater and marine systems; however, this phenomenon in estuarine habitats is poorly understood. Furthermore, research on the impacts of microplastic ingestion in larval fish and the role of microzooplankton in the transfer of microplastics throughout marine or estuarine food webs is also lacking. Therefore, the objectives of this study were to (1) investigate the impacts of a sorbed pollutant (DDT) on the trophic transfer of microplastics in an estuarine system, (2) compare microplastic exposure via direct ingestion to that of trophic transfer, and (3) to evaluate the long-term effects of microplastic ingestion in larval fish. To do this, we used a model food chain relevant to North American estuaries consisting of larval *Menidia beryllina* and the microzooplankton prey *Favella* spp.

Methods

Animal husbandry

Complete husbandry details can be found in the Supporting Information. *Favella* spp. were maintained in 200 mL batches at 16°C with a 12h:12h day:night light cycle in 30 ppt filtered seawater supplemented with a trace metal solution (Strom et al. 2007). Ciliates were fed a mixture of phytoplankton, including *Heterocapsa triquetra*, *Isochrysis galbana*, and *Manoniella squamata*.

Adult silversides were reared to maturity in a recirculating system (22°C, 15 ppt) at the UNCW Center for Marine Science. Adult silversides were spawned according to protocols described in Brander et al. (2016) and DeCourten and Brander (2017). Hatched larvae were fed a powdered diet of B.P. American (Ocean Star International [O.S.I.], Snowville, Utah, U.S.A.) twice daily while being acclimated over 4 d to conditions favored by *Favella*. All experiments were performed in accordance with UNCW IACUC protocols 1410 and 1415-024.

Microplastic preparation

Low-density polyethylene (LDPE) microspheres (10–20 μm) were chosen for the following experiment since LDPE microspheres have been found to sorb DDT in the estuarine environment (Napper et al. 2015) and this particle size range is similar to the size of *Favella*'s natural prey (Strom et al. 2007). Preliminary trials were conducted to determine if direct ingestion and trophic transfer of microplastic particles could be tracked in the silverside larvae (*see* Supporting Information). Because microspheres could easily be detected in the digestive tract of larvae using bright-field and polarized light microscopy and due to recent work showing the transfer of fluorescent dye from microplastics into tissues (Schür et al. 2019), nonfluorescent LDPE particles were subsequently used in all feeding experiments conducted as part of the present study (Supporting Information Fig. S1).

Untreated and DDT-treated LDPE (10–20 μm) were provided as a dry powder by Dr. Anthony Andrady (North Carolina State University, Raleigh, North Carolina, U.S.A.). Full microsphere preparation details can be found in the Supporting

Information. Following a 7 d incubation period in methanol, the DDT concentration on the microspheres was determined to be $2.15 \times 10^6 \text{ ng g}^{-1}$. This concentration is higher than reported environmental concentrations on LDPE but was deemed appropriate for this proof-of-principle experiment. A preliminary validation experiment determined that no detectable DDT levels leached from the particles into the experimental medium during the 2 h exposure (*see* Supporting Information). Prior to experimentation, microspheres were added to a 0.01% Tween20 surfactant solution to ensure even dispersal then added to treatment beakers for a final concentration of approximately $5 \times 10^5 \text{ microspheres mL}^{-1}$ (*see* Supporting Information). The microplastic concentrations used in the present study were similar to those used in other experimental studies of microplastic feeding (Lo et al. 2018). These microplastic levels are still orders of magnitude higher than concentrations typically found in southeastern U.S. estuaries (Gray et al. 2018). However, there remains a great deal of uncertainty on environmental microplastic concentrations for particles in the 10–20 μm size range used in our study because field sampling devices are typically restricted to $> 333 \mu\text{m}$. For studies that have analyzed this size fraction in environmental samples, the most abundant particles typically fall into the 10–20 μm size range (Enders et al. 2015).

Favella microplastic exposure

Ciliates were starved 24 h prior to experimentation to ensure ciliates ingested microspheres instead of any residual natural food particles. Ciliates were concentrated to 100 mL via reverse filtration using a 40 μm cell strainer. Three 1 mL subsamples were preserved in Lugol's solution at 4°C for up to 48 h to determined ciliate density ($10 \text{ ciliates mL}^{-1}$).

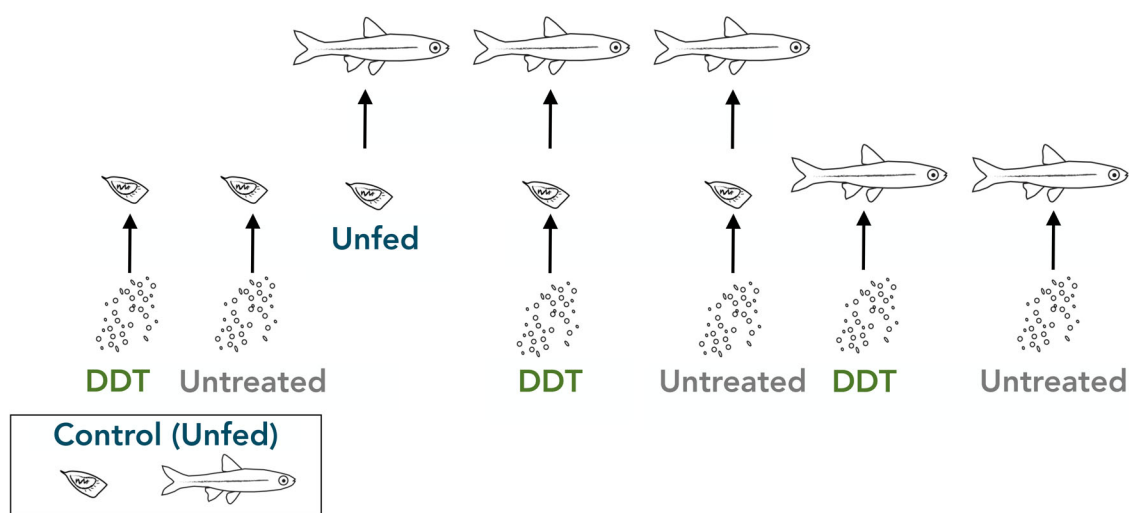


Fig. 1. An overview of different treatment and control groups from the feeding experiments. DDT-treated (green) or untreated microspheres (blue) were directly to larval silversides (*Menidia beryllina*) and tintinnid ciliates (*Favella* spp.) to investigate the effects of a sorbed pollutant on direct ingestion. Larval silversides were then added to containers containing ciliate prey that have been fed either DDT-treated or untreated microspheres to investigate trophic transfer in this estuarine model food chain. This experiment also contained unfed controls (gray) for both direct ingestion and trophic transfer feedings.

Following concentration of prey, microspheres were added to each 100-mL treatment group to achieve the final particle concentration of 5×10^5 particles mL^{-1} . One treatment group ($n = 4$) was fed DDT-treated microspheres and the other fed untreated microspheres (Fig. 1).

In order to observe LDPE ingestion by the ciliates, a subset was tethered with a suction micropipette (see Echevarria et al. 2016 for details) and exposed to the same concentration of untreated microspheres while observing with an Olympus XI 71 microscope (Waltham, Massachusetts, U.S.A.) equipped with a Fastec Inline 250 frames s^{-1} camera (Fastec Imaging, San Diego, CA, U.S.A.). Ciliates were only tethered for the purpose of filming and were allowed to swim freely in unfiled feeding experiments. An example high-speed video of LDPE microplastic ingestion by the ciliates can be found in Supporting Information Video S1.

Silverside microplastic exposure and trophic transfer

Acclimated 5 d posthatch (dph) silverside larvae were starved for 24 h prior to being gently transferred to treatment beakers using a plastic pipette. There were six treatment groups: (1) control (unfed) larvae, (2) control larvae exposed to unfed ciliate prey (unfed ciliates), (3) larvae exposed to DDT-treated plastics directly (DDT direct ingestion), (4) larvae exposed to ciliate prey containing DDT-treated plastics (DDT trophic transfer), (5) larvae exposed to untreated (UT) plastics directly (UT direct ingestion), and (6) larvae exposed to prey containing untreated plastics (UT trophic transfer). See Fig. 1 for an illustration of the experimental setup. Each direct ingestion treatment group and the unfed control groups contained four replicate beakers. Each trophic transfer treatment group contained three replicate beakers due to restrictions on the amount of *Favella* available. Each replicate beaker contained five larvae. Beakers containing *Favella* contained approximately 10 cells mL^{-1} .

Following a 2 h feeding, larvae were placed on ice, rinsed in phosphate-buffered solution (PBS), transferred to a 2.5% glutaraldehyde solution in PBS (prepared according to Yoshioko and Hirano 1988), and stored at room temperature until further analysis. Ingestion of LDPE microplastic particles by larvae was enumerated using an Olympus BX 60 compound microscope equipped with digital camera or a Zeiss Axio microscope (Goettingen, Germany) with a polarizing light filter and first-order phase plate to enable ingested particles to be visualized and counted (Fig. 2).

Microplastic egestion rate and long-term effects

In order to investigate microsphere retention time in the digestive tract of larval silversides, an additional 2 h feeding experiment was conducted. Larvae were fed either untreated or DDT-treated microspheres directly to determine the effect of a contaminant on microsphere retention. An unfed control group was also analyzed. There were four replicate beakers for each treatment, each containing five larvae. Immediately following a 2 h feeding period, larvae were sacrificed according

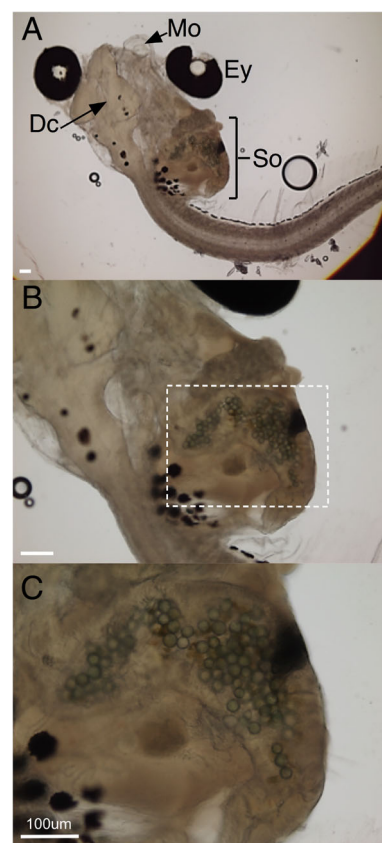


Fig. 2. Bright-field images of *Menidia beryllina* larvae with ingested microspheres. (A) Whole field view of larvae, Mo = mouth, Ey = eye, Dc = dorsal cranium, So = stomach. (B) Close up of the stomach region in (A) with white dotted square indicating location of microspheres. (C) Magnified region indicated in (B) clearly showing > 100 ingested microspheres. All scale bars indicate 100 μm .

to the methods above at 30 min intervals from 2.5 to 6 h, at 24, 48, and 72 h and stored in 2.5% glutaraldehyde until further analysis.

A separate longer-term experiment was conducted to determine effects of directly ingested microplastic and sorbed DDT on larval growth. Larvae were fed either untreated or DDT-treated microspheres directly. All replicate beakers ($n = 4$) within each treatment contained 12 larvae. After the 2 h direct feeding period, two larvae from each beaker were sacrificed and analyzed using the Olympus BX-60 microscope (Waltham, Massachusetts, U.S.A.) to confirm plastic ingestion. The remaining larvae were then maintained in clean water at 16°C and 30 ppt salinity for 16 d. Larvae were fed a powdered B.P. American diet twice daily and then switched to live *Artemia* prey after 8 dph. After 16 d, larvae were sacrificed and growth parameters (including larval wet weight and caudal length) were recorded.

Statistical analysis

A Student's *t*-test was used to compare the untreated and DDT-treated microplastic exposed ciliate groups to determine

whether *Favella* fed differentially on contaminated particles. An ANOVA and post hoc analyses (including a Tukey HSD and Scheffe's test) were used to determine the effects of sorbed DDT on the trophic transfer and direct ingestion of microplastics in this model food chain. Retention time was analyzed using a generalized linear model (GLM, Poisson distribution). An ANOVA with Tukey HSD post hoc analysis was used to compare wet weight and length between treatment groups after a 16 d grow out period. Statistical analyses were run using JMP 11.0.

Results and discussion

Favella microplastic exposure

Favella spp. readily ingested the LDPE particles and video of tethered ciliates indicated they began ingesting particles immediately upon provision (see Supporting Information Video S1; Fig. 3). Forty-four percent of *Favella* spp. contained ingested plastics after the 1 h feeding with 15% containing 10 or more particles (Fig. 3). No plastics were detected in the

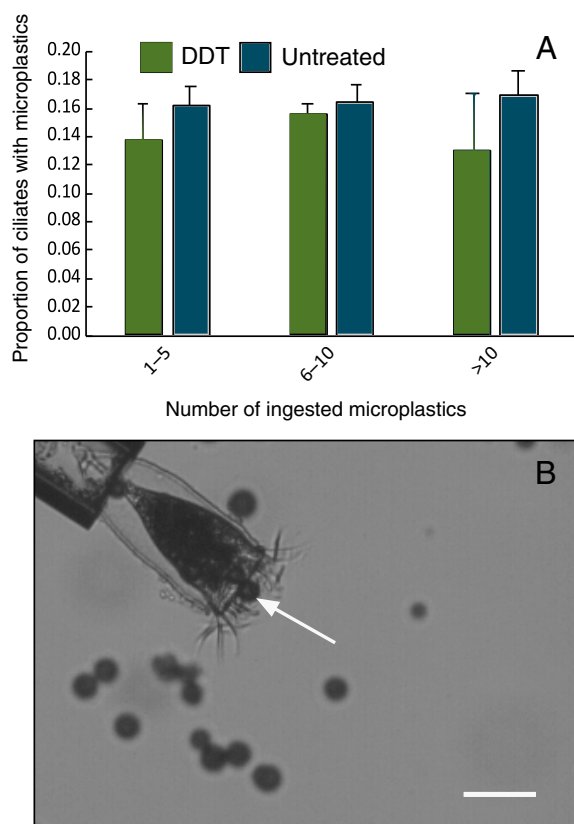


Fig. 3. *Favella* microplastic exposure (A) bars indicate proportion of ciliates with ingested microplastics (untreated = blue, DDT-treated = green). There were no significant differences in the number of ingested untreated and DDT-treated particles in ciliates (t -test, $n = 3$, $p > 0.05$). Error bars indicate standard error (SD). (B) An image of a tethered ciliate (*Favella* spp.) ingesting 10–20 μm microspheres. See Supporting Information Video S1. Arrow indicates microsphere at the cytostome about to be ingested. Scale bar = 50 μm .

controls and no significant difference in particle ingestion was detected between untreated LDPE microspheres and DDT-treated LDPE microspheres (Fig. 3, Student's t -test, $p > 0.05$). This demonstrates that ciliates did not feed differentially and that they are not able to detect or discriminate against particles with high levels of sorbed DDT. Further understanding of ciliate ingestion of plastic particles is a priority given their status as a common prey item for larval fish globally and now demonstrated potential to transfer microplastics in estuarine systems.

Silverside microplastic exposure and trophic transfer

The present study demonstrated that larval silversides will directly ingest microspheres following 24 h starvation (Supporting Information Fig. S1). Microspheres were only detected in the gut and did not appear to translocate to other tissues through the experiment. Subsequent experiments with LDPE microspheres showed that direct ingestion of DDT-treated (25.9 ± 35.2 particles larvae⁻¹) vs. untreated microspheres (37.1 ± 23.9 particles larvae⁻¹) by larval silversides was not significantly different (ANOVA, Tukey HSD, $p > 0.05$) (Fig. 4A), indicating the larvae were unable to detect sorbed DDT or discriminate between DDT-treated and untreated LDPE microspheres. None of the control larvae contained microspheres.

Silverside larvae also readily ingested microplastic-fed *Favella* spp. as water samples taken from beakers before and after the addition of larval silversides contained significantly fewer ciliates than those without larvae (ANOVA, Tukey HSD, $p = 0.02$, Fig. 4B), confirming that the larvae were actively consuming ciliate prey during the feeding sessions. Larvae in these trophic transfer experiments retained microplastics from this prey in their guts (Fig. 4A) at up to 320 particles individual⁻¹. Such a high accumulation of particles in the gut could increase predation risk due to altered swimming behavior (Lankford et al. 2000).

Interestingly, larvae from the DDT trophic transfer treatment contained significantly greater amounts of microspheres (205.4 ± 89.9 particles larva⁻¹) in their digestive tracts compared to larvae from the untreated trophic transfer treatment (81.8 ± 42.0 particles larva⁻¹, ANOVA, Tukey HSD, $p = 0.0001$) (Fig. 4A), despite not ingesting significantly more ciliates overall (Fig. 4B; ANOVA, Tukey HSD, $p > 0.05$). This suggests ciliates that had ingested DDT treated microspheres were more susceptible to predation by larvae compared to ciliates that had not ingested them, leading to an increased exposure to microspheres for the same number of ingested prey. One possible explanation is that the sorbed DDT interferes with prey behavior (i.e., swimming speed or pattern) such that the predation rate is higher in this treatment group. DDT is known to interfere with the activity of voltage gated sodium channels (Field et al. 2017) and could affect channel-mediated ciliary beating (swimming) or compromise fast action-potential mediated predator escape responses (Rios et al. 2007; Echevarria et al. 2016). More work is needed to understand the biological effects of

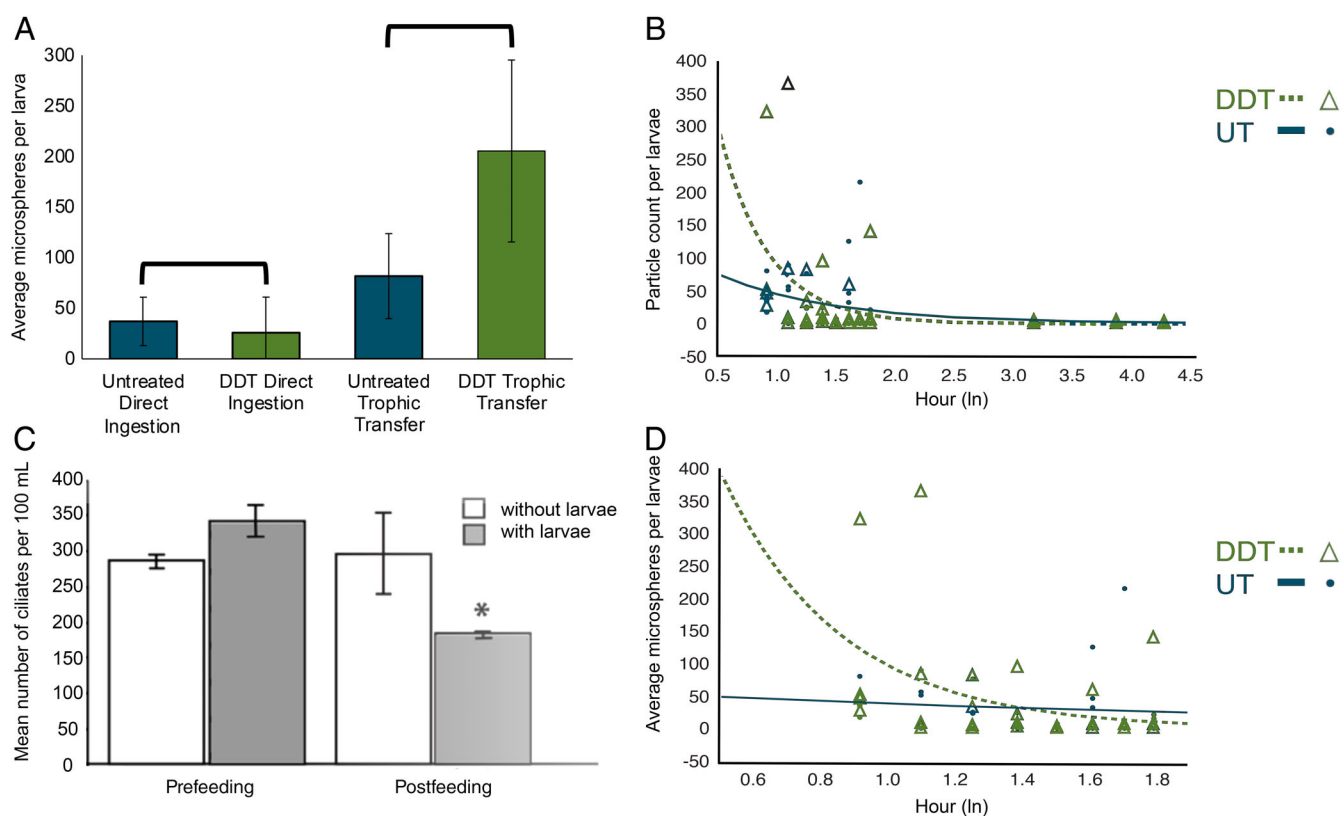


Fig. 4. (A) Each bar represents the average microspheres per larvae. Larval silversides do not feed differentially on untreated and DDT-treated microspheres when exposed to particles directly (ANOVA, Tukey HSD, $p > 0.05$, $n = 4$). Larvae did feed significantly more on prey contaminated with DDT-treated particles than those with untreated particles (ANOVA, Tukey HSD, $p = 0.0001$, $n = 3$). Larvae in direct ingestion groups ingested significantly less than those in trophic transfer groups (ANOVA, Scheffe's test, $p < 0.05$, $n = 2$). Brackets denote significant findings. Error bars indicate 1 SD. (B) Bars indicate mean number of ciliates per 100 mL. Treatments containing larval silversides (both UT and DDT treatments) had a significant reduction in the mean number of *Favella* 100 mL⁻¹ ($n = 3$, t -test, $p < 0.021$). Symbol (*) denotes significant findings. Error bars indicate 1 SD. (C) The rate of egestion integrated over the first 6 h was 0.4 particles h⁻¹. (D) The rate of egestion integrated over the entire sampling period (2.5–72 h after feeding) was 0.15 particles h⁻¹ (GLM, Poisson distribution). Solid lines and (•) represent untreated microsphere treatment, dotted lines and (Δ) represent DDT-treated microsphere treatment.

DDT-treated microplastic ingestion and the potential effects of DDT exposure on locomotion and predator escape response in *Favella*. Additionally, larvae ingested significantly more LDPE particles from microplastic-contaminated prey than by direct ingestion of particles from the water column (ANOVA, Scheffe's test, $p < 0.05$, $n = 2$). Overall, these results imply trophic transfer from microzooplankton prey can be a significant route for microplastic exposure in larval fish predators. The DDT effect on silverside-ciliate predation illustrates the potentially complex trophic impacts of microplastic-associated pollutants in aquatic systems.

Microplastic egestion rate and long-term effects

While most directly ingested microplastics were excreted within 24 h of ingestion, particles were found throughout the larval silverside digestive tract up to 72 h postfeeding. Rate of egestion for the first 6 h after ingestion was 0.4 particles h⁻¹ (Fig. 4C). The egestion rate integrated over the entire

sampling period (72 h) was 0.15 particles h⁻¹ (Fig. 4D) (GLM, Poisson distribution), with no significant differences in the rate of egestion of DDT-treated and untreated microspheres (ANOVA, Tukey HSD, $p > 0.05$).

Menidia spp. grow rapidly during their larval stage, reaching maturity within 190 dph (Lankford et al. 2000) and we therefore investigated the impacts of a brief direct microplastic ingestion event on growth in larval fish. Sixteen days post direct-ingestion of LDPE microspheres, wet weight values for both plastic treatment groups were significantly smaller than the wet weight values of the control group (ANOVA, Tukey HSD, $p = 0.0108$ [UT], $p = 0.0105$ [DDT], Fig. 5). These results suggest a short-term accumulation of microplastics in the gut negatively affects growth, possibly through obstruction of the gut. When energy demands are not met, as through the ingestion of non-nutritious material, growth can be reduced (McCauley and Bjorndal 1999). Reduced growth during early life could increase the amount of time spent in the larval stage

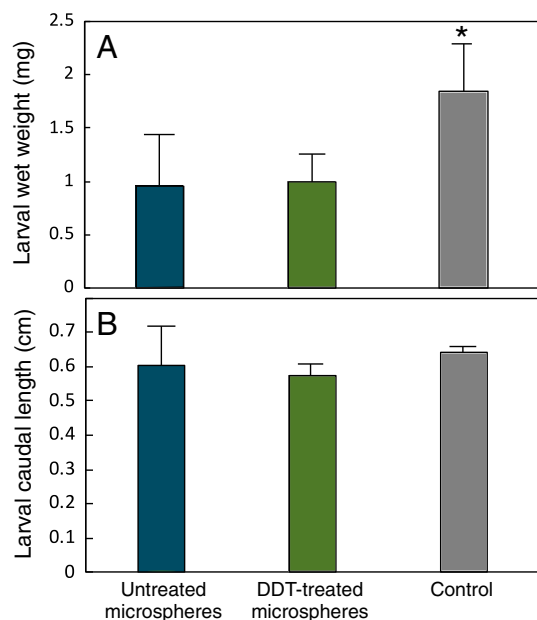


Fig. 5. (A) Larval wet weight (mg) of larvae exposed directly to microspheres for 2 h on day 1 were significantly lower than control (unfed) larvae by day 16 postfeeding (ANOVA, $n = 4$, Tukey HSD, $p = 0.0108$ [UT], $p = 0.0105$ [DDT]). (B) Caudal length (cm) was not significantly different between control and microsphere-fed treatment groups (ANOVA, Tukey HSD, $p > 0.05$). Symbol (*) denotes significant findings. Error bars indicate 1 SD.

and ultimately prolong the period of high predation risk (Skajaa et al. 2003). There was no significant difference in length for microplastic-exposed larvae after the 16 d rear out period (ANOVA, $p > 0.05$) (Fig. 5). Pepin (1995) demonstrated that weight-length relationships in larval fish are nonlinear and that wet weight is more tightly associated with larval development than length at this developmental stage and therefore a more sensitive metric of developmental effects.

In this study, we demonstrate the trophic transfer of microplastics in a model estuarine food chain, as well as the impact of the LDPE microplastics and an associated pollutant on each species at an organismal level. Reduced growth of fish larvae and apparent effects on larval-ciliate interactions were observed albeit at high LDPE particle and DDT concentrations compared to typical environmental levels. Nevertheless, our data suggest that trophic transfer may be an important route of exposure for estuarine species. Estuaries are productive ecosystems that are home to many economically valuable seafood species, as well as providing important nursery habitat for sensitive life stages across taxa. They are also one of the most highly impacted habitats when it comes to marine litter (Nixon et al. 2010; Weinstein et al. 2016). Better understanding the impacts of microplastics in estuarine food webs will be important for evaluating sources of marine pollution, establishing policies to mitigate litter, and determining the human health risks of microplastics (Santillo et al. 2017; Carbery et al. 2018). Further studies are now needed to

examine how trophic interactions can mediate microplastic exposure in a wider range of estuarine predator-prey models and under more environmentally relevant conditions.

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