

Petroleum-based and biodegradable microplastics alter tissue structure and fecundity in the eastern mudsnail (*Ilyanassa obsoleta*)

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Abstract

Microplastics are hazardous to aquatic life. Most experiments focus on the effects of a single type of microbead, while in the environment, organisms are exposed to irregularly shaped fragments belonging to several chemical groups. The effects of biodegradable plastics are unknown. We tested the effects of mixed-source (MS) petroleum-based and biodegradable (polylactic acid, PLA) microplastics on the intertidal eastern mudsnail, *Ilyanassa obsoleta* (Say, 1822), a benthic grazer. MS plastics were collected from local coastal areas (polystyrene, polyethylene, polypropylene, polyvinyl chloride and polyethylene terephthalate, combined) and were tested at three exposures, including one similar to concentrations found locally (2250 particles/kg sediment). Plastics were milled to be similar in size to the biofilm–sediment mix provided to the snails as food (32.94 μm^2 for sediment, 137.99 μm^2 for MS, and 31.16 μm^2 for PLA). Locally relevant exposures of MS microplastics disrupted digestive gland histology, while extreme exposures additionally increased the number of hemocytes and reduced fecundity. Effects of PLA were similar to those of MS microplastics, at the extreme exposure tested here. These results indicate that both petroleum-based and biodegradable microplastics disrupt the structure of the digestive gland and that environmentally relevant exposures induce “hidden” tissue-level changes that are invisible without specialized techniques.

Key words: microplastics, polylactic acid, benthic grazer, estuarine snail, histopathology, tissue structure, intertidal snail, *Ilyanassa obsoleta*

Résumé

Les microplastiques constituent un danger pour la vie aquatique. La plupart des expériences s'intéressent aux effets d'un seul type de microbilles, alors que, dans le milieu, les organismes sont exposés à des fragments de forme irrégulière appartenant à différents groupes de produits chimiques. Les effets des plastiques biodégradables ne sont pas connus. Nous vérifions les effets de microplastiques de sources mixtes (SM) à base de pétrole et biodégradables (acide polylactique, APL) sur la nasse (*Ilyanassa obsoleta* (Say, 1822)), un brouteur benthique. Des plastiques SM ont été recueillis de zones littorales locales (polystyrène, polyéthylène, polypropylène, polychlorure de vinyle et polyéthylène téréphtalate, combinés) et testés à trois niveaux d'exposition, dont un semblable aux concentrations observées localement (2250 particules/kg sédiments). Les plastiques ont été broyés jusqu'à des tailles semblables à celles du mélange de biofilm et sédiments fourni aux nasses comme nourriture (32,94 μm^2 pour les sédiments, 137,99 μm^2 pour les SM et 31,16 μm^2 pour l'APL). Des niveaux d'exposition aux microplastiques SM pertinents pour la région perturbent l'histologie de l'hépatopancréas, alors que des expositions extrêmes rehaussent le nombre d'hémocytes et réduisent la fécondité. Les effets de l'APL sont semblables à ceux des microplastiques SM au niveau d'exposition extrême testé. Ces résultats indiquent que les microplastiques tant à base de pétrole et que biodégradables perturbent la structure de l'hépatopancréas et que des niveaux d'exposition environnementalement pertinents induisent des changements « cachés » dans les tissus qui sont invisibles sans l'utilisation de méthodes spécialisées. [Traduit par la Rédaction]

Mots-clés : microplastiques, acide polylactique, brouteur benthique, escargot estuarien, histopathologie, structure des tissus, nasse, *Ilyanassa obsoleta*

Introduction

Rapid increase in plastic production since the 1950s has led to roughly 8 million tons of plastic waste entering the

world's oceans each year (Jambeck et al. 2015; Ostle et al. 2019). Microplastics (<5 mm in diameter) and nanoplastics (<100 nm) are of concern as their small size means they are

easily overlooked, they are difficult to quantify in the field, and they overlap in size with food particles for many aquatic organisms. To combat global accumulations of plastic waste that can remain in the environment for hundreds of years, biodegradable plastics, such as polylactic acid (PLA), are being developed for a broad range of applications. Biodegradable plastics pose their own risk to aquatic organisms as breakdown rates vary widely under different conditions. PLA, for example, completely degrades but only in specific industrial composting and anaerobic digestion conditions, is estimated to take over three decades to completely degrade in soil (Narancic and O'Connor 2019) and shows no appreciable breakdown in seawater after 400 d (Bagheri et al. 2017). The ecotoxicological effects of microplastics on aquatic organisms is a highly active area of research (Browne et al. 2008; von Moos et al. 2012; Cole et al. 2015;) with most studies focusing on a single source of microplastics, and the effects of bioplastics are largely unknown.

Our objective is to investigate the effects of mixed-source (MS) microplastics and biodegradable microplastics on the eastern mudsnail (*Ilyanassa obsoleta* (Say, 1822)), a common benthic grazer on tidal flats of the North Atlantic. Marine invertebrates take up microplastics by selective targeting or accidental ingestion (Lusher 2015; Lusher et al. 2017) and once ingested, microplastics induce sublethal effects including pathological stress, stunted growth rates, altered fecundity, and oxidative stress (Prinz and Korez 2020). For example, in the deposit-feeding marine lugworm (*Arenicola marina* (Linnaeus, 1758)), ingested polyvinyl chloride (PVC) microplastics caused inflammation and a compromised feeding apparatus (Wright et al. 2013), while ingested polystyrene (PS) led to polychlorinated biphenyl (PCBs) build up in tissues, weight loss, and reduced feeding activity (Besseling et al. 2013). In the filter-feeding blue mussel (*Mytilus edulis* Linnaeus, 1758), PS that accumulated in the gut cavity and digestive tubules was translocated to the hemolymph, reducing phagocytic activity (Browne et al. 2008), while the uptake of PS by the oyster (*Crassostrea gigas* (Thunberg, 1793)) increased feeding rates and decreased fecundity (Sussarellu et al. 2016). While few studies have investigated the effects of PLA on marine invertebrates, Green et al. (2017) found that the effects of PLA exposure were species specific, decreasing filtration rates in *M. edulis* while increasing filtration in *Crassostrea gigas*, effects that were similar to those induced by high-density polyethylene (HDPE).

Microplastics have been discovered in field-collected marine invertebrates (e.g., Karlsson et al. 2017) and some studies indicate high levels of uptake by benthic grazers (Sfriso et al. 2020). Benthic grazing invertebrates are an abundant, taxonomically diverse group at high risk of ingesting pollutants as they graze small particles from a variety of surfaces. They also are active in the sediment–water interface, where microplastics undergo repeated cycles of deposition and resuspension (Xia et al. 2021). As our goal was to expose *I. obsoleta* to microplastics that are similar to what the snails would encounter in local coastal ecosystems (e.g., in terms of particle size and chemical composition), we collected and combined plastics from nearby beaches and milled them to be similar in size to particles in a biofilm–sediment mix (see Results) that was provided to the snails as food. The washed-up plastic debris

included PS, polyethylene (PE), polypropylene (PP), PVC, and polyethylene terephthalate (PET), the most widely produced types of plastic and thus the most likely to be encountered in marine environments by aquatic taxa (Stock et al. 2019). The milled plastic particles varied in size and shape and were therefore similar to secondary microplastics created by breakdown of larger plastics in the field. We exposed snails to a range of MS concentrations to determine whether effects were dose dependent; importantly, our middle exposure (i.e., high MS) falls within the range of microplastics detected in coastal ecosystems near our study site for both sediments (Halifax Harbour, 1500 particles/kg; Mathalon and Hill 2014) and seawater (Gulf of Maine, 7600 particles/m; Barrows et al. 2018).

We tested the following hypotheses: (i) that MS petroleum-based microplastics cause tissue level changes in a digestive organ (hepatopancreas) at high risk of exposure in the benthic grazing snail, *I. obsoleta*; and (ii) that biodegradable plastics (PLA) elicit changes that are similar to those induced by MS microplastics at similar concentrations. We focused on the effects of microplastic exposure on the hepatopancreas, hemocytes (immune cells) in interstitial tissues, and fecundity. As we exposed snails to MS microplastics of irregular shapes and sizes, results provide insights into how invertebrates respond to the diversity of microplastics found in coastal ecosystems.

Materials and methods

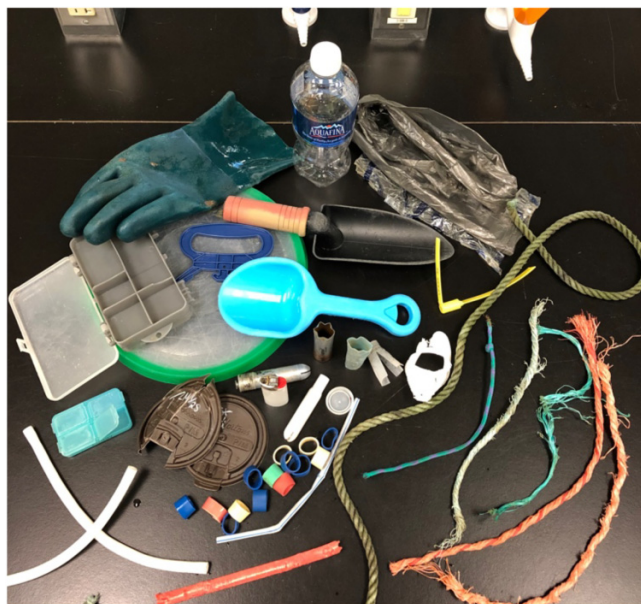
Collection and culture

Ilyanassa obsoleta were collected from tidal flats at Avonport Beach, Nova Scotia, Canada (45.1158°N, 64.2472°W), on 3 July 2019. There were no significant differences in shell length in the different treatment groups (range of 15–20 mm shell length). Egg capsules were collected by gently removing them from the substrate (e.g., *Spartina*, *Fucus* spp.). A mix of biofilm and surface sediment was collected to use as food for the snails by using the edge of a trowel to skim the top 1–2 mm of surface sediment from a 1 m² region of the tidal flat, in the same region of the tidal flat where snails were collected. The biofilm–sediment mix was stored at –20 °C until needed.

Ilyanassa obsoleta were cultured on an ambient photoperiod in 37 L aquaria in a glass house at the K.C. Irving Environmental Centre at Acadia University. Seawater (30 ppt, pH of 8.0) was obtained at Halls Harbour, Nova Scotia, Canada, and stored at the Acadia University Animal Care Facility in an Enviro Monitron Recirculating Aquaculture System (Aquabiotek) which provided filtration. Water temperature was maintained at 17–20 °C.

After a 1-week acclimation period, snails were exposed to microplastics by mixing aliquots of MS or PLA microplastics (described above) with the biofilm–sediment mix and feeding the mixture to the snails every day for 1 month. Before the biofilm–microplastic mix was added, aquaria were cleaned by siphoning out old seawater, biofilm, sediment, feces, etc. Each aquarium received 20 g (wet weight) of the biofilm–sediment mix daily. Seawater was added to a depth of 10 cm/aquarium for an approximate volume of seawater

Fig. 1. Plastic debris collected from Lawrencetown and Conrad's Beaches, Nova Scotia, Canada, that was processed into microplastic dust and used in the mixed-source (MS) plastic exposure groups. Lobster bands (rubber) were not included in the microplastic dust.



of 0.013 m³ and gently aerated. Aquaria also contained two small rocks and small pieces of seaweed (*Fucus* spp.) to provide a substrate for females to lay their egg capsules. No snails died during the experiment.

Microplastic production

For the MS microplastics, we collected plastic debris that was washed up on Lawrencetown Beach (44.6438°N, 63.3409°W) and Conrad's Beach (44.6443°N, 63.3743°W), Nova Scotia, Canada. The beach-collected debris included: PET plastic bottles, PS coffee lids, PS plastic utensils, PP drinking straws, PP and nylon fishing rope, low-density polyethylene plastic bags, and PVC food packaging (Fig. 1). Small pieces were cut or broken from each type of plastic for a total of 10 g per chemical group. These were combined in a small electric kitchen grinder and milled into fine powder (20 min; see Results) until no large pieces (i.e., visible by eye) remained. The powder was rinsed out of the grinder using 100% ethanol and vacuum filtered with coarse paper filter (Fisherbrand P8 Grade) to select fine particles (see Results); larger particles retained by the filter paper were discarded. Ethanol was evaporated from the filtrate with a rotary evaporator (Buchi Rotavapor R-210; 65 °C) and the dry microplastic powder stored in 20 mL glass scintillation vials.

PLA microplastics were milled from 3D printer ink (Verbatim 3D printer filament). The ink was weighed and cut into 10 g pieces based on ink colour and processed into a fine powder as described above for MS microplastics.

Microplastic exposures

Stock suspensions of MS and PLA microplastics were approximately 1.0×10^6 particles/mL 0.01% Tween 20 in reverse

osmosis (RO) water. Tween 20 was used to reduce clumping of the microplastics in the stock solution. The concentration of microplastics in the stock suspensions was determined by counting particles in replicate 100 µL samples with a hemocytometer. To count the particles, micrographs of the stock solutions were taken with an AmScope MU500 camera on a Nikon Alphaphot2 YS-2 compound microscope (400× magnification) and adjusted with ImageJ with the following parameters: 8 bit, image threshold (black and white), measure: area, FFT (complex Fourier transform), analyse particles: outlines, size: 5 µm Infinity, circularity: 0.00–1.00. After adjustment, particles were counted with Hemocytometer Sidekick app for Apple iOS 13.3.1. At the same time, we compared the size distribution of the milled plastics to particles in the snail's food (i.e., biofilm–sediment mix) to ensure that they overlapped in size. Five micrographs (at 400× magnification) of each type of particulate (biofilm–sediment mix, MS microplastic, and PLA microplastic) were taken with an AmScope MU500 camera on a Nikon Alphaphot2 YS-2 compound microscope, analysed as described above, and size distribution compared in SPSS version 26.

Once the concentration of the stock suspension was determined, 100 µL of the stock suspension was added to 1 L of 0.01% Tween 20 in RO water yielding a working solution of roughly 100 plastic particles/mL. Different exposures of microplastics were generated by adding aliquots of the working solution to a final volume of 15 mL 0.01% Tween 20, and vigorously shaking the microplastic suspension into 20 g (wet weight) of the biofilm–sediment mix to ensure a homogeneous distribution. Given the small size of the microplastics (median particle size of 50 µm; see Results), it is likely that some of the particles became suspended in the seawater of the aquaria (seawater depth of 10 cm) during the experiment. Therefore, we provide estimates of exposure with all particles retained in the biofilm–sediment mix (as number of particles/kg sediment) and with all particles in suspension (as number of particles/m³ seawater). For the controls, 15 mL of 0.01% Tween 20 was added to the biofilm–sediment mix (i.e., no microplastics). The low MS exposure was 75 particles/kg biofilm–sediment mix (or, up to 116 particles/m³ seawater), the high MS exposure was 2250 particles/kg biofilm–sediment mix (or up to 3500 particles/m³ seawater), and the extreme MS and PLA exposures were 75 000 particles/kg biofilm–sediment mix (or up to 100 000 particles/m³ seawater). In the low and high MS treatment, it is likely that most individual snails did not encounter microplastics daily, given how few particles were added to each aquarium (i.e., ~1.5 particles or ~50 particles added to the aquaria daily, in the low and high MS groups, respectively). Also, as the original microplastic dust was highly diluted in the aquaria, it is likely that the snails were exposed to different relative concentrations of the chemical groups in their daily feedings, but this would presumably “average out” over the experiment.

Histology and histochemistry

At the end of the 1-month exposure period, snails were transferred to clean seawater for 24 h to allow depuration of sand from the digestive system before tissue processing.

Snails were immobilized (4 °C; 5 min), shells removed and the visceral mass fixed in Bouin's solution (5% acetic acid, 9% formaldehyde, and 0.9% picric acid; Sigma-Aldrich) for 24 h at room temperature. Tissues were dehydrated in ethanol, cleared in toluene (1 h each), infiltrated in three 20 min changes of paraffin wax in a vacuum oven (Fisher Scientific Isotemp 280A; 58 °C, 10 bar), and embedded in Paraplast (58 °C). Tissues were sectioned at 6 µm with an America Optical Company 820 Spencer rotary microtome and sections placed on Poly-L lysine dipped slides. Sections were stained with Periodic acid – Schiff – Alcian blue (Presnell and Schreiber 1997). Brightfield micrographs were taken with a Zeiss Axioplan 2 microscope and SPOT camera. Images were manipulated using the Axioplan software but only by using the white balance and histogram functions to balance colours so that they were similar to those seen on the slides. Plates were generated using Microsoft PowerPoint version 16.33.

Scanning electron microscopy (SEM)

SEM was used to examine tissue sections using backscattered electron shadow mode, following Sawaguchi et al. (2018). The hepatopancreas of snails from the control and extreme MS groups were sectioned at 30 µm using a rotary microtome, dried, deparaffinized in xylene, and rehydrated in an ascending ethanol series. Tissues were stained in 1.0% uranyl acetate in 70% methanol (5 min), rinsed in RO water, then stained with Reynold's lead citrate (3 min). Slides were then well rinsed with RO water and left to dry overnight in the fume-hood before examination with SEM.

Data analysis

We analysed the histology of the anterior hepatopancreas for the height of the epithelium of the hepatopancreas tubules, the number of intercellular gaps in a 100 µm region of the hepatopancreas tubules, and the distribution of granules in the cytoplasm of the digestive cells (400× magnification). For the distribution of granules, a score of 0.25 indicates the granules were located in the apical quarter of the cell, 0.5 the apical half of the cell, 0.75 the apical three-quarters of the cell, and 1 meaning the granules were evenly distributed from the apical to basal surface. We examined potential effects of microplastic exposure on the immune system by counting the numbers of mature (granular) hemocytes in the connective tissue surrounding the hepatopancreatic tubules (0.16 mm² area; 400× magnification). Each of these was determined in five regions of histological sections of the hepatopancreas for each snail.

Histological data were compared among groups with a generalized linear mixed (GLM) model in SPSS version 26. Subjects were individual snails and the five measurements taken per subject analysed as repeated measures. A Poisson distribution was used for count data. As we analysed the slides, we found many snails (~40%) were parasitized by trematodes. Fixed effects were treatment group (control, low MS, high MS, extreme MS, and PLA) and parasite load (absent/present), with the interaction term "group × parasite load". Significant differences among groups were estimated with a post hoc pairwise comparison.

Fecundity was estimated as the number of viable embryos per egg capsule and analysed in SPSS version 26 with an independent-samples Kruskal-Wallis test. The field, low MS, extreme MS, and PLA groups were analysed as they were the only ones that produced egg capsules.

Results

Size distribution of microplastics

The size distribution of the biodegradable microplastics was similar to the size of the particles in the field-collected biofilm-sediment mix that was provided to the snails as food, while the MS microplastics were slightly larger (Fig. 2A). Note that we were not able to quantify particles less than 1 µm (e.g., nanoparticles) with this technique and that particles >500 µm were either excluded from the analysis (for the biofilm-sediment mix) or from the microplastic treatments during preparation (e.g., filtration) of the stock solutions. Both the MS and biodegradable microplastics were irregular in shape and contained both smooth and jagged fragments (Fig. 2B).

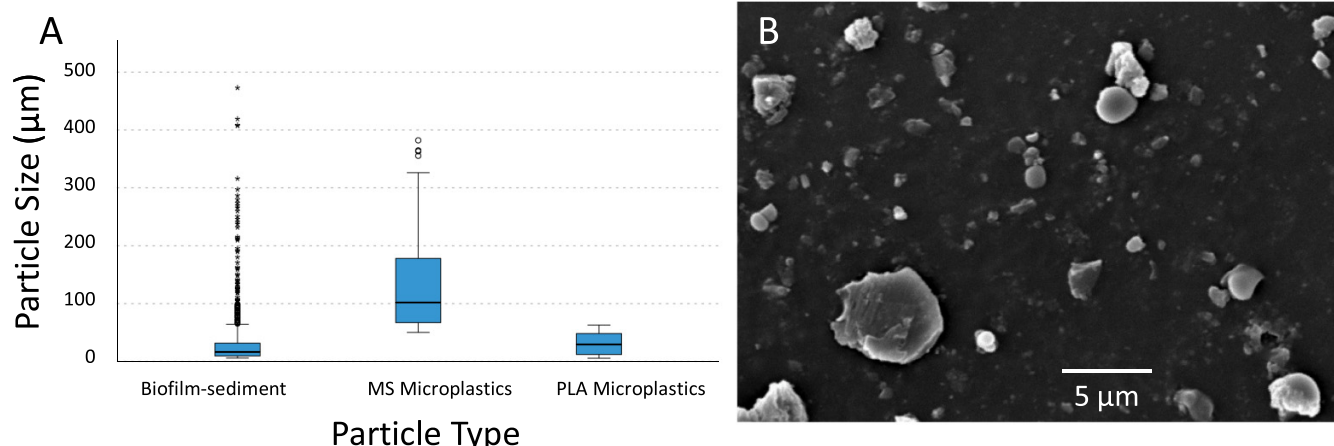
Histology of the hepatopancreas

The hepatopancreas is a large, extensively branched organ with a tubular-alveolar structure (Brown 1969). The most abundant cells are digestive cells (secretory and absorptive cells with glycogen granules and pinocytic vacuoles), while pyramidal cells (characterized by calcareous and small secretory granules) and vacuolated cells (with lipid vacuoles, function in storage) are also present (Reader 1976).

In our control group, the epithelium of the tubules formed a contiguous layer of digestive cells, with PAS-positive granules concentrated in the apical part of the cell, and scattered vacuolated cells (Figs. 3A–3C). In contrast, the epithelium in the microplastic treatment groups appeared disrupted. For example, in the extreme MS group, the tubule epithelium contained intercellular gaps that extended to the basement membrane, and the cytoplasmic granules of the digestive cells were distributed from the apical to basal parts of the cells (Figs. 3D and 3E). The epithelium of the hepatopancreas of the PLA group also had intercellular gaps and granules that were distributed throughout the digestive cells (Fig. 3F). In all groups, the loose connective tissue surrounding the tubules contained hemocytes, often clustered near small arterioles (Figs. 3B, 3D, and 4). The hemocytes also contained PAS-positive granules (Fig. 4A) and with SEM, were seen to have small filopodia with which they contacted connective tissue fibers (Fig. 4B). In both the PLA and extreme MS groups, the hemocytes were often observed clustered on the outer surface of the basement membrane of the tubules (Figs. 4A, 4C, and 4D). Parasites were detected in the gonads of approximately 40% of the snails examined (Fig. 3A).

GLM revealed a significant increase in the number of intercellular gaps between epithelial cells (counted in a 100 µm length of tubule) in response to plastic treatments but not in response to the presence of parasites (Fig. 5A and Table 1a, corrected model). A post hoc comparison revealed significant

Fig. 2. Comparison of the milled microplastics and particles in the biofilm–sediment mix used as a source of food for the snails. (A) Boxplot of particle size for the biofilm–sediment mix ($n = 860$), MS microplastics ($n = 166$), and biodegradable microplastics ($n = 315$). (B) Scanning electron micrograph of the MS microplastics.



differences in all microplastic exposures, even the low MS exposure, relative to the controls (Table 1b).

Microplastic exposure also influenced the distribution of PAS-positive granules within the digestive cells (Table 2a, GLM). In the controls, the granules were primarily distributed in the apical part of the cell while the extreme MS and PLA groups had granules dispersed throughout much of the digestive cell (Table 2b and Fig. 5B, note that an apical concentration of granules is noted by 0.5 or less, while a distribution of granules throughout the height of the cell is indicated by 1). The presence of parasites also influenced the distribution of cytoplasmic granules (Tables 2a and 2c) but the effect of parasites was small, with the ratio of distribution of granules across the cell to overall cell height of 0.635 ± 0.023 (mean $\pm$ SE) if parasites were present, and of 0.532 ± 0.020 if parasites were not detected. There was no significant interaction between microplastic exposure and presence of parasites (Table 2a).

Height of the tubule epithelium also differed among groups in response to plastic treatments, but effects of parasites was not significant (Fig. 5C and Table 3a, GLM corrected model). A post hoc comparison revealed a significant decrease in cell height in the high and extreme MS plastics groups relative to controls, but no significant difference between the low MS and control groups (Table 3b). The bioplastics (PLA) group appeared to have lower columnar cells than the controls (Fig. 5C), but this was not significant ($p = 0.08$; Table 3b).

Among treatment groups, there were also significant differences in the number of mature hemocytes in the interstitial tissue surrounding the hepatopancreas tubules (counted in a 0.16 mm^2 in area) (Fig. 5D and Table 4a, GLM corrected model). Although there was no effect of the presence of parasites on the number of hemocytes, there was a significant interaction between the plastic exposures and parasites (Table 4a, group $\times$ parasite load), suggesting that the immune response of parasitized snails differed in response to microplastics than nonparasitized snails. The predicted results of the GLM showed an overall increase in the number of

mature hemocytes in the extreme and biodegradable plastic groups, relative to controls, but no differences were evident in the low or high MS groups (Table 4b and Fig. 5D).

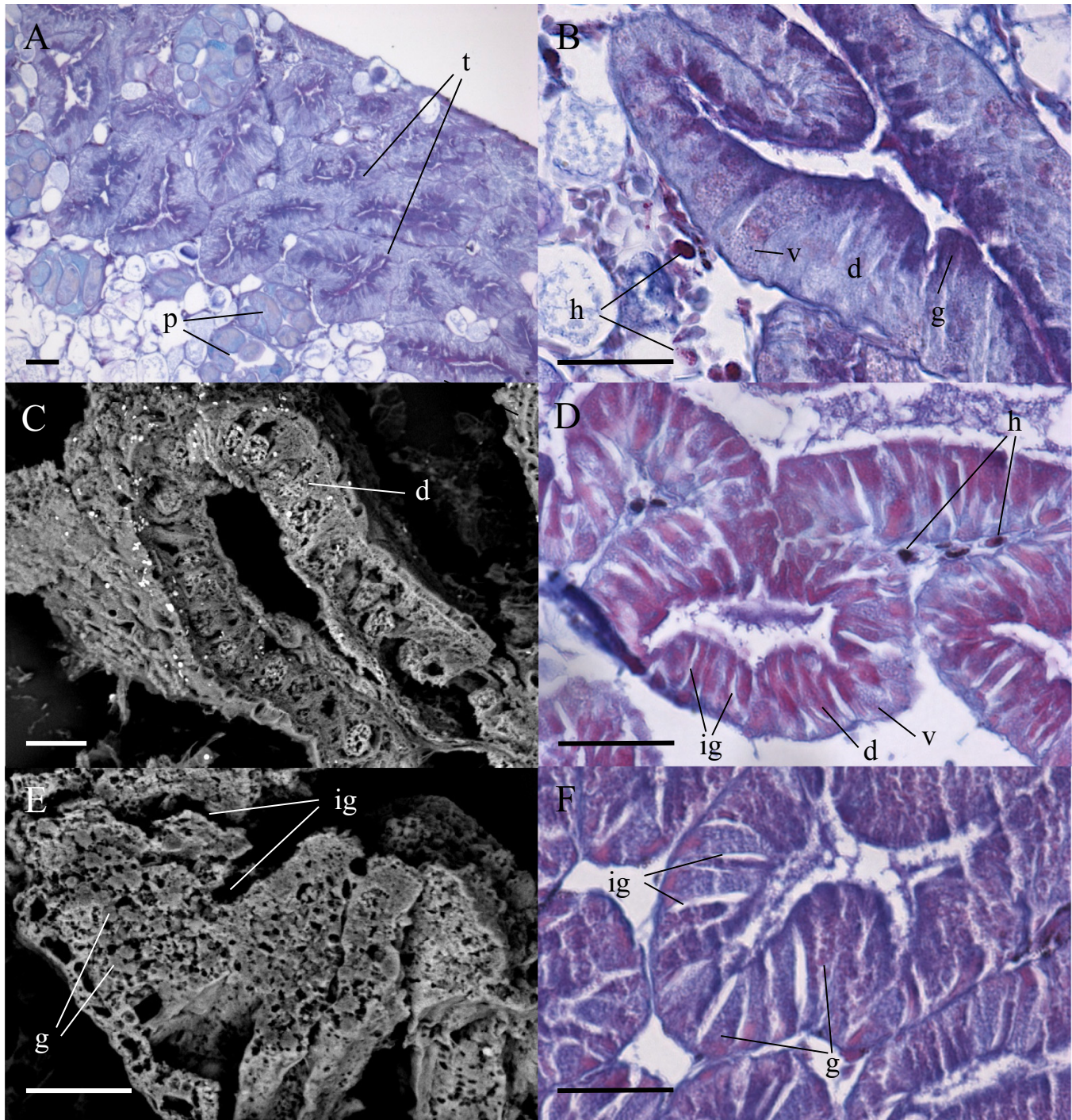
Effects of microplastics on fecundity

Egg capsules from the field contained 48.80 ± 9.08 embryos per egg capsule (mean $\pm$ SD, $n = 10$; Fig. 6). The number of embryos per egg capsule was significantly different among groups (independent samples Kruskal–Wallis test, total $n = 40$, test statistic = 7.841, $df = 3$, $p = 0.049$; Fig. 6). Data are not shown for the control group as those snails did not spawn during the experiment. Post hoc pairwise comparisons indicated a significant decrease in the number of embryos per egg capsule in the extreme MS group relative to the field collected egg capsules ($p = 0.048$) and also, a significant decrease in embryo number in the extreme MS ($p = 0.031$) and PLA ($p = 0.048$) relative to the low MS group (Fig. 6).

Discussion

We tested the hypotheses (i) that MS, petroleum-based microplastics induce tissue level changes in a digestive organ (hepatopancreas) at high risk of exposure in the benthic grazing snail, *I. obsoleta*; and (ii) that biodegradable plastics elicit changes that are similar to those induced by MS microplastics at similar concentrations. Results support both hypotheses. Response to the MS microplastics was dose dependent and even the lowest exposure (~ 75 particles/kg sediment or $116 \text{ particles/m}^3$ seawater) induced intercellular gaps to form in the digestive tubules. Exposure to microplastics at concentrations found in coastal ecosystems near our study site (i.e., our high MS; similar to reports for sediment from Halifax Harbour and seawater from the Gulf of Maine; Mathalon and Hill 2014; Barrows et al. 2018) induced the formation of intercellular gaps and decreased the height of the tubule epithelium, suggesting a loss of function and a decreased ability of the epithelium to maintain a barrier. The biodegradable microplastic (PLA) induced effects that were similar to

Fig. 3. Micrographs of the hepatopancreas of eastern mudsnails (*Ilyanassa obsoleta*) with bright field (A, B, D, F) and scanning electron microscopy (C, E, backscatter mode). The tubules from the control group (A–C) had contiguous epithelium and digestive cells with PAS-positive granules in the apical cytoplasm. In the extreme MS group (D, E), the epithelium contained gaps between the cells, and the granules were distributed throughout the digestive cell. The hepatopancreas from the PLA group (F) was similar to that of the extreme MS group. d, digestive cell; g, granules; h, hemocyte; ig, intercellular gap; p, parasite; t, tubule of the hepatopancreas; v, vacuolated cell. Scale bars = 50 μ m (A, B, D, F) and 10 μ m (C, E).



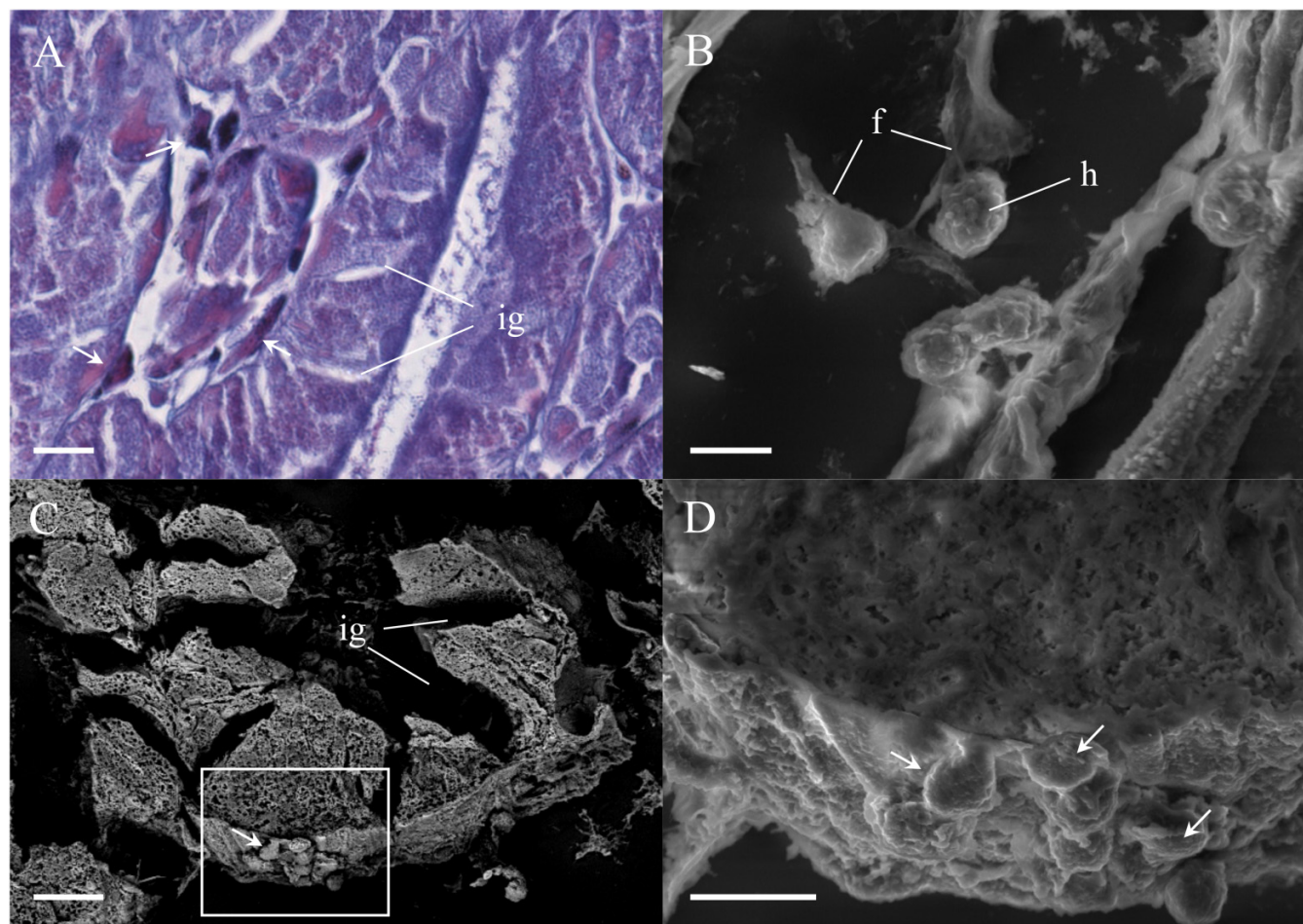
MS microplastics at the extreme concentration used in this preliminary study.

Effects of microplastics on gut structure

Most previous studies on the effects of microplastics on aquatic organisms used manufactured, uniform micro-

spheres of one polymer type in their experiments (Cole et al. 2011; Cole 2016; de Sá et al. 2018) while in contrast, field-collected invertebrates contain chemically diverse microplastics of different sizes (Mathalon and Hill 2014; Karlsson et al. 2017; de Sá et al. 2018). Lei et al. (2018) demonstrated histopathological changes and intestinal disruption in the ze-

Fig. 4. Micrographs of hemocytes in the interstitial tissues of the hepatopancreas of eastern mudsnails (*Ilyanassa obsoleta*). (A) Bright field micrograph of the hepatopancreas from the PLA group showing hemocytes in the interstitial tissue and clustered under the basement membrane of the tubules (arrows). Note the intercellular gaps and basal distribution of granules of the digestive cells, both characteristic of the epithelium of the PLA group. (B) SEM (secondary electron mode) of the interstitial tissue of the hepatopancreas from the control group showing granular hemocytes (h) with filipodia (f) attached to connective tissue fibers. (C) SEM (backscatter mode) of the hepatopancreas of the extreme MS group showing a cluster of hemocytes (arrows) near the basement membrane. The box in C shows a cluster of hemocytes on the outside of the tubule, enlarged in D. (D) SEM (secondary electron mode) of hemocytes. f, filipodia; h, hemocyte; ig, intercellular gap. Scale bars = 5 μ m (B), 10 μ m (A, D), and 20 μ m (C).

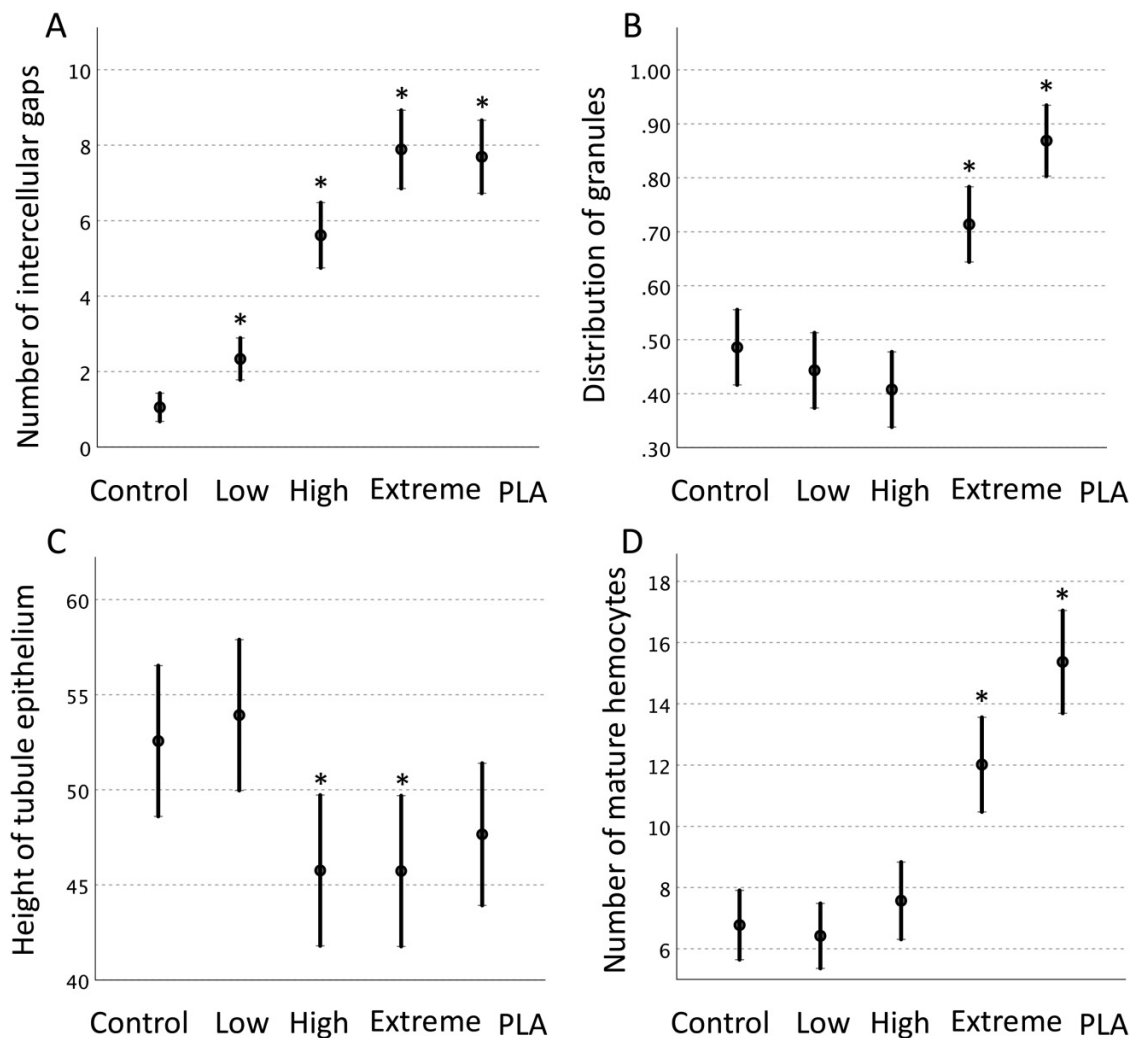


brafish (*Danio rerio* (Hamilton, 1822)) following exposure to several types of microplastics in separate treatment groups (PA, PS, PP, PE, and PVC). Similarly, Wang et al. (2019) discovered that PS microplastics at concentrations higher than our extreme exposure (100 particles/mL) decreased the size and number of microvilli, increased mitochondria, and increased autophagosome formation in epithelial cells lining the digestive tract in brine shrimp (*Artemia parthenogenetica* Bowen and Sterling, 1978). While few experimental studies have exposed invertebrates to MS microplastics, Revel et al. (2019) found that exposing the filter feeding mussel *M. edulis* to a mix of PP and PE microplastics had dose-dependent effects on enzyme activity of the digestive gland and gills, but no significant histopathological effects on the digestive gland. In a similar study, Revel et al. (2020) exposed the omnivorous ragworm (*Hediste diversicolor* (O.F. Müller, 1776)) to a mixture of PP and

PE and found a decrease in coelomocyte viability. Thus, exposure to MS microplastic effects the digestive and immune systems, as has also been shown in response to exposure to a single chemical type of microbead.

We found that disruption of the epithelium of the hepatopancreas tubules was dose dependent, with an increase in the number of intercellular gaps with exposure levels. Epithelia function as a sheet and gaps between the digestive cells would decrease the capacity of the tissue to generate a barrier, diminish control over transport and reduce cell-cell communication. Exposure to PS nanoparticles increased gut permeability in the nematode *Caenorhabditis elegans* (Maupas, 1900) which increased susceptibility to environmental toxins (Zhao et al. 2017). Intercellular gaps could arise through loss of cell junctions, change in cell structure, formation of residual bodies, or loss of specific cell types.

Fig. 5. The effects of microplastic exposure on the histology of the hepatopancreas of eastern mudsnail (*Ilyanassa obsoleta*), showing (A) disruption of the tubule epithelium as the number of intercellular gaps in a 100 μm region of the tubule, (B) distribution of cytoplasmic granules in the digestive cells, (C) height (in μm) of the epithelium of the digestive tubules, and (D) number of mature hemocytes in a 0.16 mm^2 area of the hepatopancreas. For (B), the distribution of granules was estimated as 0.25 if granules were concentrated in the apical quarter of the cell, 0.5 if granules were in the apical half of the cell, 0.75 if granules were present in the top three-quarters of the cell, and 1 if granules were present throughout the cell. Data are the estimated means and 95% confidence intervals from a GLM with microplastic exposure and presence of parasites as fixed effects. Count data were analysed with a Poisson distribution. The effect of parasites was either not significant (A, C, and D) or very small (B), so data shown are for the main model and include snails both with and without parasites. An asterisk (*) indicates significant differences from the control group ($p \leq 0.05$, post hoc pairwise comparison). $n = 10$ snails/group. MS, mixed-source microplastics; PLA, biodegradable (polylactic acid) microplastics.



In snails, for example, Marigómez et al. (1990) found exposure to cadmium caused a loss of digestive cells in the periwinkle (*Littorina littorea* (Linnaeus, 1758)), while Snyman et al. (2005) found that exposure to the fungicide copper oxychloride induced a loss of calcium cells in the digestive glands of the brown gardensnail (*Helix aspersa* O.F. Müller, 1774; now known as *Cornu aspersum* (O.F. Müller, 1774)). Digestive cells in snails are known to accumulate ingested contaminants (Snyman et al. 2005; Browne et al. 2008), and this coupled within a decreased ability to maintain a barrier could increase translocation of contaminants into nearby tissues. The dose-dependent changes in the tissue structure in *Ilyanassa*

also included redistribution of cytoplasmic granules in the digestive cells and an overall thinning of the epithelium. Redistribution of granules may be related to the release of residual bodies, a common response of molluscan digestive cells that have sequestered contaminants (Lobo-da-Cunha et al. 2018). A dose-dependent thinning of the digestive gland epithelia also occurs in response to copper in the terrestrial snail, *Helix aspersa* (now *Cornu aspersum*) (Snyman et al. 2005). Collectively these histological changes may reduce the capacity of *Ilyanassa* to digest and assimilate nutrients, and also, increase the risk of ingested contaminants entering the tissues.

Table 1. Effects of microplastic exposure on the number of intercellular gaps (per 100 μm length of tubule) in the hepatopancreas epithelium of eastern mudsnail (*Ilyanassa obsoleta*).

(a) GLM fixed effects.							
Source	F	df	Significance				
Corrected model	38.53	9, 140	0.000				
Group	81.05	4, 140	0.000				
Parasite load	0.003	1, 140	0.957				
Group $\times$ Parasite load	0.668	4, 140	0.615				

(b) Results of the post hoc pairwise comparison of the number of intercellular gaps in the hepatopancreatic epithelium; an alpha of $p = 0.05$ or less was considered significant.							
Treatment group	Contrast estimate	SE	t	df	Adjusted significance	95% CI	
						Lower	Upper
Control vs. Low	-1.281	0.338	-3.795	140	0.000	-1.948	-0.614
Control vs. High	-4.559	0.476	-9.570	140	0.000	-5.500	-3.617
Control vs. Extreme	-6.835	0.524	-12.266	140	0.000	-7.936	-5.733
Control vs. Bioplastics	-6.638	0.524	-12.667	140	0.000	-7.674	-5.602

Note: Results of a generalized linear mixed model (GLM) of the number of gaps (Poisson distribution) in which subjects were snails, and the intercellular gaps were counted in five tubules/section and treated as repeated measures. Fixed effects were microplastic exposure (= group), presence of parasites, and the interaction between the two (group $\times$ parasite load). Groups are based on microplastic exposure (laboratory control, low MS, high MS, extreme MS, and PLA). $n = 10$ snails/group.

Table 2. Effects of microplastic exposure on the distribution of cytoplasmic granules in the epithelium of the hepatopancreatic tubules of eastern mudsnail (*Ilyanassa obsoleta*).

(a) GLM fixed effects.							
Source	F	df	Significance				
Corrected model	17.61	9, 140	0.000				
Group	33.91	4, 140	0.000				
Parasite load	10.89	1, 140	0.001				
Group $\times$ Parasite load	0.917	4, 140	0.456				

(b) Results of the post hoc pairwise comparison of the distribution of cytoplasmic granules in the hepatopancreatic digestive cells of <i>I. obsoleta</i> ; an alpha of $p = 0.05$ or less was considered significant.							
Treatment group	Contrast estimate	SE	t	df	Adjusted significance	95% CI	
						Lower	Upper
Control vs. Low	0.043	0.050	0.856	140	0.393	-0.056	0.141
Control vs. High	0.078	0.050	1.569	140	0.119	-0.020	0.177
Control vs. Extreme	-0.228	0.050	-4.574	140	0.000	-0.326	-0.129
Control vs. Bioplastics	-0.383	0.048	-7.908	140	0.000	-0.479	-0.287

(c) Results of the post hoc pairwise comparison of the distribution of cytoplasmic granules in the hepatopancreatic digestive tubules of <i>I. obsoleta</i> relative to the presence or absence of parasites; an alpha of $p = 0.05$ or less was considered significant.							
Treatment group	Contrast estimate	SE	t	df	Adjusted significance	95% CI	
						Lower	Upper
Present vs. Absent	0.103	0.031	3.300	140	0.001	0.041	0.164
Absent vs. Present	-0.103	0.031	-3.300	140	0.001	-0.164	-0.041

Note: Results of a generalized linear mixed model (GLM) of granule distribution in which subjects were snails, and the distribution of granules was estimated in five tubules/section and treated as repeated measures. Fixed effects were microplastic exposure (= group), presence of parasites, and the interaction between the two (group $\times$ parasite load). Groups are based on microplastic exposure (laboratory control, low MS, high MS, extreme MS, and PLA). $n = 10$ snails/group.

PLA is widely considered to be biocompatible with low toxicity (Gao et al. 2020) and while PLA nanoparticles are taken up by mammalian cells in culture, the physiological effects are considered minimal (da Luz et al. 2017). Only a few studies have compared the effects of biodegradable and petroleum-based microplastics in invertebrates. Green et al. (2016) discovered that the effects of PLA were similar to those of HDPE and PVC in a study of feeding behaviour in the deposit-feeding lugworm, *A. marina*. Green et al. (2017) also

found that both PLA and HDPE affected filtration rates in the bivalves *M. edulis* and European flat oyster (*Ostrea edulis* Linnaeus, 1758). Straub et al. (2017) compared the effects of another biodegradable plastic, polyhydroxybutyrate with the petroleum-based polymethylmethacrylate on feeding in the amphipod *Gammarus* and found that both types of microplastics decreased wet weight relative to a control group exposed to silica particles. In our preliminary study, we used exposures of PLA (100 000 particles/ m^3) that were higher than

Table 3. Effects of microplastic exposure on the height of digestive cells in the hepatopancreatic tubules of eastern mudsnail (*Ilyanassa obsoleta*).

(a) GLM fixed effects.			
Source	F	df	Significance
Corrected model	2.165	9, 140	0.028
Group	3.741	4, 140	0.006
Parasite load	0.223	1, 140	0.638
Group × Parasite load	0.656	4, 140	0.624

(b) Results of the post hoc pairwise comparison of cell height in the hepatopancreatic epithelium; an alpha of $p = 0.05$ or less was considered significant.

Treatment group	Contrast estimate	SE	t	df	Adjusted significance	95% CI	
						Lower	Upper
Control vs. Low	-1.364	2.836	-0.481	140	0.631	-6.970	4.242
Control vs. High	6.805	2.836	2.400	140	0.018	1.199	12.412
Control vs. Extreme	6.833	2.836	2.410	140	0.017	1.227	12.439
Control vs. Bioplastics	4.905	2.756	1.780	140	0.077	-0.543	10.353

Note: Results of a generalized linear mixed model (GLM) of cell height in which subjects were snails, and cell height was measured in five tubules/individual and treated as repeated measures. Fixed effects were microplastic exposure (=group), presence of parasites, and the interaction between the two (group × parasite load). Groups are based on microplastic exposure (laboratory control, low MS, high MS, extreme MS, and PLA). $n = 10$ snails/group.

Table 4. Effects of microplastic exposure on the number of hemocytes in the interstitial tissue near the hepatopancreas tubules (counted in a 0.16 mm² area) of eastern mudsnail (*Ilyanassa obsoleta*).

(a) GLM fixed effects.			
Source	F	df	Significance
Corrected model	18.652	9, 140	0.000
Group	28.008	4, 140	0.000
Parasite load	2.961	1, 140	0.088
Group × Parasite load	13.273	4, 140	0.000

(b) Results of the post hoc pairwise comparison of the number of mature hemocytes around the hepatopancreatic digestive tubules; an alpha of $p \leq 0.05$ was considered significant.

Treatment group	Contrast estimate	SE	t	df	Adjusted significance	95% CI	
						Lower	Upper
Control vs. Low	0.353	0.784	0.450	140	0.653	-1.197	1.903
Control vs. High	-0.794	0.856	-0.928	140	0.355	-2.488	0.889
Control vs. Extreme	-5.239	0.968	-5.411	140	0.001	-7.154	-3.325
Control vs. Bioplastics	-8.593	1.023	1.023	140	0.001	-10.616	-6.571

Note: Results of a generalized linear mixed model (GLM) of the number of hemocytes (Poisson distribution) in which subjects were snails and cells were counted in five fields of view per section and treated as repeated measures. Fixed effects were microplastic exposure (=group), presence of parasites, and the interaction between the two (group × parasite load). Groups are based on microplastic exposure (laboratory control, low MS, high MS, extreme MS, and PLA). $n = 10$ snails/group.

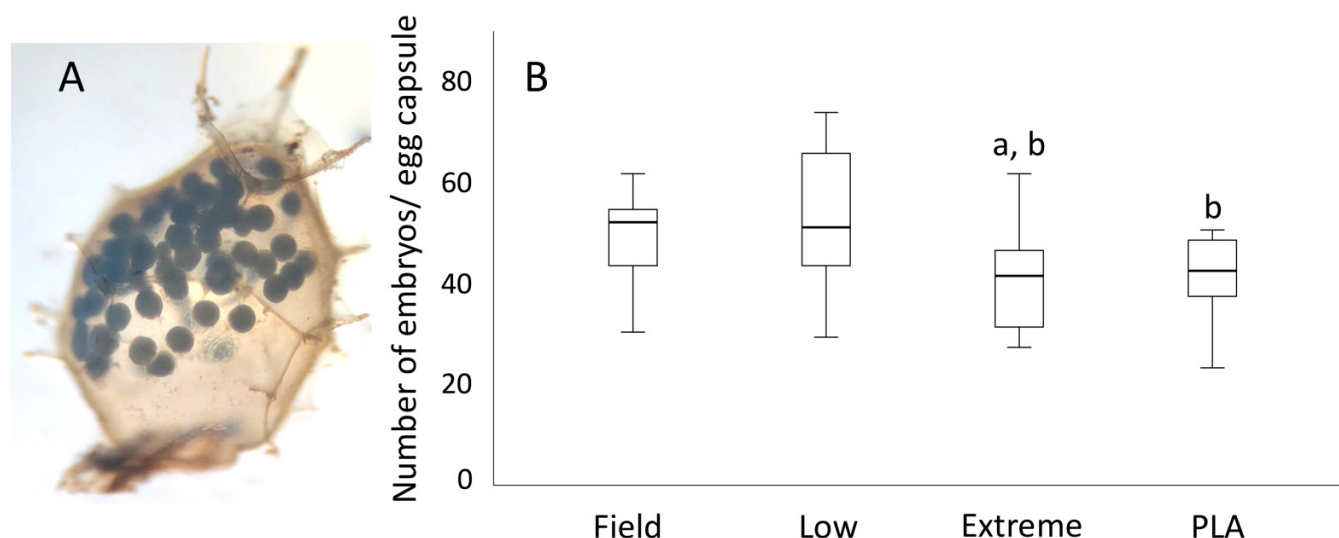
concentrations of petroleum-based microplastics found in coastal ecosystems, and found effects were similar to MS microplastics at the same exposure level. Collectively, these studies emphasize the need for further ecotoxicological research on biodegradable plastics given the rapid increase in their manufacturing as we pursue safe alternatives to traditional plastics, and their negative effects on marine invertebrates relative to controls exposed to inert particles (i.e., silica, [Straub et al. 2017](#); sediment in the biofilm mix fed to the control group in the current study).

Effects of microplastics on the number of hemocytes

We found that the number of hemocytes in interstitial tissues surrounding the hepatopancreas doubled with exposure to extreme levels of both MS and PLA microplastics, relative

to controls, but an increase was not detected in our high MS exposure, suggesting that levels of contamination that have been detected locally would have no or minimal effects. In molluscs, hemocytes are an actively circulating lineage of innate immune cells involved in defence ([Coyne 2011](#); [Pila et al. 2016](#)). Cultured hemocytes of the Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) have a dose-dependent response to PS microbeads, with the lowest tested exposure (1 µg/mL seawater) inducing an increase in extracellular reactive oxygen species and high exposures (50 µg/mL), inducing a decrease in phagocytosis and increase in apoptosis ([Canesi et al. 2015](#)); note these exposures are much higher than those used in the current study. Our results are consistent with those of [von Moos et al. \(2012\)](#) who documented a decrease in hemocyte number in the haemolymph of *M. edulis* following a 48 h exposure to HDPE particles (0–80 µm in diameter),

Fig. 6. Effects of microplastics on fecundity. (A) Egg capsule of an eastern mudsnail (*Ilyanassa obsoleta*) from the extreme MS group. Box plot of the number of embryos per egg capsule from field collected and laboratory spawned egg capsules, showing the median, quartiles, minimum and maximum values. Data are missing for the laboratory control group, as they did not spawn during the experiment. “a” indicates significant difference from the field collected egg capsules, and “b” indicates significant difference from the low MS laboratory group (independent samples Kruskal–Wallis test, $n = 40$, $p < 0.05$). $n = 10$ egg capsules/group. MS, mixed-source microplastics; PLA, biodegradable (polylactic acid) microplastics. [Colour online.]



which the authors attribute to migration of hemocytes from hemolymph to local tissues. We also found a significant interaction between the presence of parasites and microplastic exposure on the number of hemocytes, indicating that parasitized snails had an increased immune response to microplastics.

Effects of microplastics on fecundity

We found a small but significant decrease in the number of embryos per egg capsule in the extreme MS microplastic group relative to field-spawned egg capsules, and in the extreme MS and PLA groups relative to the low MS exposure. To the best of our knowledge, this is the first study to show an effect of PLA on fecundity in a marine invertebrate. Petroleum-based microplastics have dose-dependent and species-specific effects on development, and negative impacts detected in some species are not shown by other taxa exposed to the same type of microplastic (reviewed in Haegerbaeumer et al. 2019). In invertebrates, microplastics have been shown to effect egg production in the copepod *Parvocalanus* (Heindler et al. 2017), the Pacific oyster (*Crassostrea gigas*) (Sussarellu et al. 2016), and *Caenorhabditis elegans* (Zhao et al. 2017). Taliec et al. (2018) found that PS microbeads increased gamete mortality and decreased fertilization in the oyster *Crassostrea gigas*, but only if the microplastics contained a $-NH_2$ functional group, suggesting that the toxic effects are impacted by biofouling. Thus, the effects of microplastics on gametogenesis and early embryogenesis must be more fully investigated, as effects vary with type of microplastics, species, and perhaps ageing in oceanic conditions.

Microplastics

The milled microplastics were similar in size to the biofilm–sediment mix that was provided to the snails as food,

and were a mixture of smooth, irregular, and jagged particles. Almost all studies on the tissue-level effects of microplastics have used commercially available, smooth microbeads. In the oceans, however, microplastic particles are irregular in shape, with the majority being fibers, fragments, and foamed microplastics (Shim et al. 2018). While both fragments and fibers have been found to have been ingested by copepods in the field (Desforges et al. 2015), the effects of particle shape on tissue structure are unknown. Results similar to those of Desforges et al. (2015) were found by Coppock et al. (2019), who showed in laboratory experiments that *Calanus helgolandicus* (Claus, 1863) ingested microplastics provided as fibers, fragments and microbeads, and additionally, showed size-specific preferences for microplastics of different chemical composition (e.g., PE and PET). While we tried to replicate the variety of shapes of microplastics that might be found in sediments or in suspension, the particles we used were freshly milled and lacked microbial films. Biofouling is known to enhance ingestion in zooplankton (Vroom et al. 2017) and also alter vertical transport of the particles through the water (Kooi et al. 2017), making the effects of biofouling important to consider in follow-up studies.

Conclusions

The effects of microplastics on marine invertebrates are a very active area of research, yet relatively little is known about the effects of exposure to mixed chemical groups of plastics, which better reflect microplastics found in aquatic ecosystems, and almost nothing is known about the effects of biodegradable microplastics. We found that mixed microplastics affected the histology of the digestive gland in a dose-dependent manner. Specifically, environmentally relevant concentrations of microplastics, such as those found in

the general area of our study, disrupted the digestive gland tissues indicating a decrease in both digestive function and the ability of the gut to maintain a barrier between the environment and inner tissues. PLA and MS microplastics at extreme exposures produced similar disruptions to the histology of the hepatopancreas, and also, a small reduction in fecundity. The similarity in response to MS and PLA suggests that some of the deleterious effects of microplastics arise through contact with particles, rather than a response to a specific chemical contaminant, but also, that the response is specific to foreign particles and not simply inert particles, such as sediment or silica, that occur naturally in the snails' diet.

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Data availability

Data generated or analysed during this study are available from the corresponding author upon reasonable request.

Author information

Author contributions

BH conceived the study. BH and GG planned and conducted the experiment. BH did the histology. Both authors collected and analysed the data, and participated equally in writing the manuscript.

Competing interests

The authors have declared that no competing interests exist.

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