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Nylon microfibers develop a distinct plastisphere but have no apparent effects on the gut microbiome or gut tissue status in the blue mussel, *Mytilus edulis*

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Abstract

Ingestion of microplastics (MP) by suspension-feeding bivalves has been well-documented. However, it is unclear whether exposure to MP could damage the stomach and digestive gland (gut) of these animals, causing ramifications for organism and ecosystem health. Here, we show no apparent effects of nylon microfiber (MF) ingestion on the gut microbiome or digestive tissues of the blue mussel, *Mytilus edulis*. We exposed mussels to two low concentrations (50 and 100 particles/L) of either nylon MF or *Spartina* spp. particles (dried, ground marsh grass), ca. 250–500 μm in length, or a no particle control laboratory treatment for 21 days. Results showed that nylon MF, when aged in coarsely filtered seawater, developed a different microbial community than *Spartina* spp. particles and seawater, however, even after exposure to this different community, mussel gut microbial communities resisted disturbance from nylon MF. The microbial communities of experimental mussels clustered together in ordination and were similar in taxonomic composition and measures of alpha diversity. Additionally, there was no evidence of damage to gut tissues after ingestion of nylon MF or *Spartina* spp. Post-ingestive particle processing likely mediated a short gut retention time of these relatively large particles, contributing to the negligible treatment effects.

INTRODUCTION

Elucidating the effects of plastic particles is important because of the emergence of microplastics (MP, <5 mm along the longest axis) as a contaminant in marine, terrestrial, and freshwater environments. MP can be directly manufactured for consumer products (primary MP) or produced by the degradation of macroplastics (secondary MP; Andrady, 2011; Cole et al., 2011). MP of various shapes (spherical, fibres) and polymer types (e.g., polystyrene, polypropylene, nylon, etc.) have been documented across all marine compartments (Andrady, 2011; Cole et al., 2011). Increasing MP pollution has led to concerns about the effects that these abiotic particles have on marine organisms. MP occupy the same size fraction as natural particles such as sand/silt and planktonic organisms, and, as a result, plastic particles can be ingested by organisms that feed on suspended and deposited materials (Wright et al., 2013). In particular, suspension-feeding bivalves, such as the blue mussel, *Mytilus edulis*, are exposed to MP as a result of their feeding strategy (Wright et al., 2013). Blue mussels are common members of the rocky intertidal and are considered ecosystem engineers, playing important roles in habitat formation, nutrient cycling, and benthic-pelagic coupling (Officer et al., 1982; Prins et al., 1998; Smaal & Prins, 1993; Stewart & Myers, 1980). They are widely distributed along the coasts of the North Atlantic. In these animals, lateral cilia on the gill filaments create a current of water that is drawn through the inhalant aperture, into the infrabranchial cavity, and towards the frontal gill surface (Ward & Shumway, 2004). Particles contained in this flow (microalgal cells, MP, etc.) can be

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captured on the gill filaments, transported to the labial palps, and in many cases, ingested through the mouth. Therefore, blue mussels have been productive model organisms for research on the uptake and potential effects of MP on benthic animals.

In some laboratory experiments, ingestion of MP by suspension-feeding bivalves has induced negative physiological or cellular outcomes. For example, MP were found to affect the reproduction of eastern oysters (*Crassostrea virginica*) by shifting energy allocation from reproduction to maintenance (Sussarellu et al., 2016), or inducing a heightened immune response in mussels (*Mytilus* sp.) through hyalinocyte-granulocyte ratios and increased superoxide dismutase activity (Cole et al., 2020). Another avenue by which MP may impact bivalves is through interactions with the consortia of microbiota that inhabit digestive tissues. Host-associated microbial communities have been shown to aid in digestion, mediate stressors, and affect host immunity in many animal species (Parfrey et al., 2018). They may, therefore, perform similar functions in bivalves. It has been hypothesised that MP may alter the gut microbiome by acting as a vector for anthropogenic chemicals or foreign/pathogenic microbial species, or by causing direct mechanical damage to gut tissues (Fackelmann & Sommer, 2019). Experiments with well-studied model animals such as zebrafish, mice, and earthworms have produced evidence that MP exposures can cause changes in the bacterial taxonomic composition of the gut, as well as other disorders associated with organismal health (Jin et al., 2019; Lu et al., 2018; Qiao et al., 2019; Wan et al., 2019). However, these studies all utilise virgin polystyrene microspheres, which are not commonly found in the marine environment (Phuong et al., 2016). Additionally, concentrations of MP used in the above studies (1816 MP/mL, 114–186 MP/mL, 1456,000-14,560,000 MP/mL, 730–7300 MP/mL) were orders of magnitude higher than environmentally relevant concentrations, which are typically <1/L in the open ocean (Enders et al., 2015; Lusher et al., 2014) and <1–2 particles/L in coastal environments (Mladinich et al., 2023; Zhao et al., 2018). Several studies using lower MP concentrations have reported absent or minimal effects on bivalve physiology (Fabra et al., 2021; Gonçalves et al., 2019; Hamm & Lenz, 2021; Harris & Carrington, 2020; Joyce & Falkenberg, 2022; Opitz et al., 2021; Revel et al., 2020; van Cauwenberghe et al., 2015). Therefore, there is lingering uncertainty regarding the effects of prolonged exposure to low concentrations of MP on the gut microbiome of many species.

There is also evidence suggesting that MP may cause abrasion, translocation, or trigger cellular defence mechanisms within the digestive systems of invertebrates. Polystyrene microspheres (3–10 μm) were shown to translocate to the circulatory system of blue mussels by entering the hemolymph within 3 days of exposure and persisting for 48 h (Browne et al., 2008). Furthermore, high-density polyethylene (HDPE) fragments have been found to accumulate in lysosomes of *M. edulis* and cause histological changes and inflammatory responses, which were effects that increased in magnitude with exposure time, albeit at high particle concentrations (von Moos et al., 2012). There is, however, evidence that MP do not cause tissue alteration at environmentally relevant concentrations in *Crassostrea gigas* (Revel et al., 2020). Thus, there is a need to evaluate the effect of nylon microfibers on digestive tissues.

Given the importance of suspension-feeding bivalves to coastal environments, providing a full examination of the effect of MP on the gut microbiome and digestive tissues of bivalves such as *M. edulis* is important for understanding the ramifications of MP pollution in the marine environment. Negative effects of MP on bivalve digestive tissues and gut microbial communities may have far-reaching implications for both the health of the individual and the health of ecosystems as a whole. This study investigated the effects of exposure to nylon microfibers (MF; an abundant type of MP) and *Spartina* spp. particles (dried, ground marsh grass, used as a particle control) on the gut microbiome and tissue integrity of *M. edulis*.

EXPERIMENTAL PROCEDURES

Animal collection and acclimation

Two experiments were conducted in succession. For each experiment, mussels (*M. edulis*, shell length 30–40 mm) were collected from natural populations in Long Island Sound and scrubbed clean of epibionts. Temperature and salinity at the time of collection were approximately 30 ppt and 19–21°C. One strip of Velcro was secured to the left valve of each animal using a two-part marine epoxy. Fifty-five mussels were then placed in a 76-L aquarium filled with 30–40 L of filtered seawater (cartridge, nominal pore size 0.22 μm) in a temperature-controlled environmental chamber (20°C, 12:12 h light/dark cycle) to acclimate for 2 days. Animals were fed a mixture of Shellfish Diet® (aquarium concentration = 5000 cells/mL, size range 4–20 μm) and *Tetraselmis* sp. (aquarium concentration = 15,000 cells/mL, approximately 7 μm in size) four times daily. For each experiment, 16 additional mussels were collected at the start of each exposure and used as environmental, or in situ, controls. In situ mussels were allowed to depurate for 16 h prior to sterile gut dissection (Supporting Information).

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Microcosms and feeding

Each of the two exposures lasted 21 days and was conducted in an environmental chamber (20°C, 12:12 h light/dark cycle). Mussels were held in 1 L glass containers (microcosms) that were filled with 800 mL cartridge-filtered seawater (nominal pore size 0.22 μm) and were supplied air that passed through 0.22 μm Whatman in-line air filters. Two mussels were housed in each microcosm, one to be used for microbial analysis and the other for histology. Mussels were attached by Velcro to craft sticks that were then attached to the rim of the container using a wooden clip. All microcosms were outfitted with a magnetic stir bar and sat atop magnetic stir plates. Stir plates were set at 130 rpm and bars stirred for 30 s once every 15 min to resuspend any settled particles or food throughout the day. All components of the microcosm system were sterilised prior to experimentation. Microcosm jars were autoclaved, and lids and air tubing were dipped in 95% ethanol.

For the duration of the experiments, all mussels were fed a microalgal diet consisting of Shellfish Diet™ (microcosm concentration = 5000 cells/mL, size range 4–20 μm) and cultured *Tetraselmis* spp. microalgae (microcosm concentration = 15,000 cells/mL, approximately 7 μm in size). Food was delivered in discrete batches concurrently with particle treatments four times per day with 2.5 h in between each feeding/dosing. Seawater in microcosms was replaced daily with freshly filtered seawater to prevent a cumulative increase in particle concentration over time. Water changes were conducted with sterile procedures, including cotton lab coats and gloves to minimise both microbial and plastic contamination. Mussel feeding activity was determined both visually and by verifying a decrease in particle concentration over time using an electronic particle counter (Coulter Multisizer).

Fibre and natural particle preparation

Nylon MF (500-μm-length, 30-μm-diameter) was chosen for this study because of their prevalence in the marine environment and previous research demonstrating that such MF can be captured and ingested by the blue mussel (Claessens et al., 2011; van Cauwenberghe et al., 2015; Ward et al., 2019). Additionally, the density of these fibres (1.15 g/mL) is higher than the density of seawater (1.03 g/mL), therefore, particles will sink to the benthos and suspension-feeding bivalves will be exposed to these particles and their corresponding bacterial community. Nylon thread (Coats & Clark® Extra Strong Nylon Upholstery Thread) was purchased and cut to the appropriate size (average length 556 μm, median 525 μm) under a dissecting microscope with a razor blade (Ward et al., 2019). The polymer composition of fibres was previously verified using Raman microspectroscopy (Renishaw System 2000, Renishaw plc), with spectra compared against commercial Raman spectral libraries (KnowItAll Software, Bio-Rad Laboratories, Inc.) (Ward et al., 2019). Cut fibres were placed into 50 mL tubes filled with 50 mL of 95% ethanol and dispersed by vortexing. Stock particle concentrations were determined by transferring 1 mL onto a Sedgwick rafter cell and counting individual filaments. Ethanol was then decanted from the tubes. Fibres were washed three times by suspending them in 5 mL of Type 1 laboratory water (Milli-Q Integral A10 system; MQ), centrifuging for 5 min at 1500 rcf, followed by decanting. After the washing procedure, fibres were suspended in approximately 40 mL of MQ water.

A non-plastic refractory particle was included as a treatment in the experiments to distinguish the effects of MF specifically from the effects of an indigestible particle of similar size and shape. Particles produced from ground *Spartina* spp. stems and leaves were chosen for the non-plastic control because *Spartina* spp. particles are refractory and, although they can be captured and ingested by bivalves (Milke & Ward, 2003), they are minimally digested and a poor source of carbon (Newell & Langdon, 1986). Dried stems and leaves of *Spartina* spp. were collected from a coastal saltmarsh in Stonington, CT and cut into small pieces with scissors before being ground in a blender with natural seawater. The suspension of ground *Spartina* spp. was washed through a 500 μm sieve with 0.22 μm filtered seawater onto a 210 μm sieve (Ward et al., 2019). Particles in the 210–500 μm size range, which was roughly equivalent to the size of MF, were collected for use in the experiment. Stock suspensions of *Spartina* spp. were created by suspending particles in approximately 40 mL of MQ water and were held at 4°C until use. Separate working suspensions of MF and *Spartina* spp. particles were created for each day of the exposure period by diluting an appropriate volume with filtered seawater (GF/C, pore diameter 1.2 μm) in a separate container. Working suspensions were created 3 days in advance of use and aged at room temperature to allow particles to develop a biofilm.

Exposure treatments

During each experiment, mussels were exposed to one of three treatments: nylon MF ($n = 16$, designated 'plastic mussels'), *Spartina* spp. ($n = 16$, designated '*Spartina*' mussels) or no particles ($n = 14$, designated 'control mussels'). Half the mussels in each treatment were processed for microbiome analysis and the other half processed for histology (Figure S1). Mussels in the exposure control group received a volume of coarsely filtered seawater equivalent to the volume of suspended particles received by mussels in the other treatments.

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Bivalves in the natural environment are exposed to a steady supply of particles at a given concentration. In a static experimental system, however, particle concentration decreases over time, limiting exposure. Therefore, several doses of particles were delivered to microcosms over the day to approximate a more continuous exposure concentration over time. Particle treatment concentrations were selected by taking into account the average filtering capacity of a 45–55 mm shell length mussel (approximately 1 g dry weight, ca. 0.8 L/h; Cranford & Ward, 2011), the volume of water in each microcosm (800 mL), and the number of doses given each day (4). For the first experiment, a target concentration of 50 particles/L/h/mussel was used (low particle-concentration experiment, hereafter referred to as experiment 1), which equates to 240 particles per mussel per dose. Under these conditions, each mussel was exposed to approximately 960 particles per day. For the second experiment, the target exposure concentration was doubled to 100 particles/L/h/mussel (high particle-concentration experiment, hereafter referred to as experiment 2), which equates to 480 particles per mussel per dose. Under these heightened conditions, each mussel was exposed to approximately 1920 particles per day. Typical verified concentrations of MP in seawater are <1 to 2 particles/L (Enders et al., 2015; Lusher et al., 2014; Mladinich et al., 2023; Phuong et al., 2016; Zhao et al., 2018). Therefore, in the natural environment mussels could be exposed to <33–504 particles per day assuming 12 h of active feeding (Zhao et al., 2018). Although higher than in the environment, the concentrations of MP used in the present study were far lower than those used in most other exposure studies with bivalves, for example, 1×10^5–1×10^6 MP/L (Cole et al., 2020; Jin et al., 2019; Sussarellu et al., 2016). Hence, the target concentrations were chosen to be well under those used in previous studies but slightly higher than environmental concentrations to examine potential effects.

Settling containers (without mussels) were used to account for particle settling and separate bottles with seawater were spiked with aliquots of either MF or *Spartina* spp. particles at each dosing to verify particle concentrations delivered to mussels (see Supporting Information). On each day, faeces from a subset of animals in each treatment (two from MF, two from *Spartina* spp., and one from control) were collected and examined using microscopy to verify visually that the particles were incorporated into feces and thus ingested (Figure S2A, B, see Supporting Information; Ward et al., 2019). Feces were then digested using 1 N NaOH and constituent particles counted to quantify the approximate number of nylon MF and *Spartina* spp. fragments ingested by the mussels (see Supporting Information). Particles of both types were quantified in all feces sampled.

Microbial community sampling

Following 21 days of exposure in both experiments, mussels were removed from individual microcosms and sacrificed following aseptic techniques outlined by Greenberg and Hunt (Greenberg & Hunt, 1985). Half of all mussels (including in situ mussels) were processed for microbial analysis and the other half were processed for histology (see below). Mussels remained in their microcosms until immediately prior to dissection, at which point their shells were washed briefly with 95% ethanol (EtOH) to remove contaminating microbes. Each mussel was opened by cutting the posterior adductor muscle, and the pallial cavity rinsed with 10 mL of 3.0% sterile sodium chloride. The stomach and digestive diverticula complex (gut) was isolated using sterile instruments over a cold surface to minimise nucleic acid degradation. Isolated tissues were placed in 1.5 mL microcentrifuge tubes, suspended in 400–600 μL of ZymoBiomics DNA/RNA Shield (Zymo Research Corp., Orange County, CA), and homogenised into a slurry with a sterile pestle. Homogenised gut samples were stored at -80°C until DNA extraction.

Samples of the coarsely filtered seawater used to age particles (hereafter referred to as 'ageing seawater') and each particle type (nylon MF and *Spartina* spp. particles) were taken from stock suspensions at 5–6 time points throughout the experiments (Figure S1). Approximately 2–3 mL of ageing seawater and particle stock suspensions were vacuum-filtered under sterile conditions. Ageing seawater was filtered through a 0.22 μm nitrocellulose filter (Millipore) to collect planktonic bacteria. Particle stocks were filtered through a 5 μm nitrocellulose filter (Millipore) to capture MF and *Spartina* spp. particles but exclude planktonic bacteria. All filters were placed in 1.5 mL microcentrifuge tubes with 400–600 μL of ZymoBiomics DNA/RNA Shield (Zymo Research Corp., Orange County, CA) and stored at -80°C until DNA extraction.

The ZymoBiomics DNA Miniprep Kit (Zymo Research Corp., Orange County, CA) was used to extract total genomic DNA from the gut, filtered ageing seawater, and filtered particle samples. Extractions were performed following the manufacturer's instructions, with the addition of a Proteinase K (20 mg/mL) digestion step. After DNA extraction, 16S rRNA gene fragments (V4 hypervariable region) were amplified by PCR using modified primers from the Earth Microbiome Project (Apprill et al., 2015) and indices supplied by the University of Connecticut Microbial Analysis, Resources, and Services Center (MARS, for full details see Supporting Information). Amplicons were subjected to amplicon sequencing on an Illumina MiSeq platform (see Supporting Information).

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Microbiome data analysis

Bioinformatic amplicon read analysis was conducted in R (version 4.1.0) (R Core Team, 2022), and amplicon reads were analysed with the DADA2 pipeline (version 1.20.0, see Supporting Information; Callahan et al., 2016). Further data manipulation and analyses were conducted with the phyloseq package (version 2.20.0; Schliep, 2010; Wright, 2015). Both Bray–Curtis dissimilarities and UniFrac distance metrics were computed and compared (Lozupone et al., 2011). DESeq2 was also used to examine the differential representation of ASVs between treatment groups. Differences in multivariate community composition were tested statistically when possible with permutational analysis of variance (PERMANOVA; Anderson, 2014). Homogeneity of variance was evaluated with the betadisper function, which uses a multivariate analog of Levene's test to compare category dispersions. Comparisons of alpha-diversity between treatment groups were conducted with ANOVA. Homoscedasticity was verified using Levene's test and the normality of distributions was examined with Shapiro–Wilk tests. Data for relative taxon abundance of mussel gut microbial communities were compared statistically using ANOVA. These data were log and square-root transformed when necessary to meet the assumptions of ANOVA. Data for relative taxon abundance of particle and water microbial communities failed to meet the assumptions of ANOVA even after transformation, therefore relative abundances were compared statistically using a nonparametric Kruskal–Wallis test. Pairwise comparisons were conducted using a Dunn test for multiple comparisons of groups.

Histology

The remaining half of the mussels (including in situ mussels) were processed for histological analysis (for full sectioning and staining procedures see Supporting Information). Briefly, gut tissues were fixed in 10% formalin and stored at room temperature until sectioning. Prior to sectioning tissue samples were rinsed in 1X phosphate-buffered saline (PBS) and rehydrated with a 30% w/v sucrose in PBS solution. Gut tissue sections were created using cryogenic microtome techniques and stained with Haemotoxylin and Eosin. Twenty-four sections were created for each mussel.

Histological data analysis

Histopathological surveys were used to identify pathogens and cellular defence mechanisms indicative of a stress response in gut tissues (stomach and digestive gland, Figure S3). Stress responses prioritised for assessment included digestive gland atrophy (DGA), diffuse and focal hemocytic infiltration (HI), necrosis, and presence/encapsulation of particles. The occurrence of digenetic trematode parasitization in mussels was common, so this pathology was assessed as well. Six of the 24 sections created for each animal were selected to quantify stress responses and pathologies. Sections were selected based on quality and the amount of tissue visible. Necrosis and presence/encapsulation of particles were scored as either present or absent. HI and DGA were quantified for prevalence and then rated semi-quantitatively for intensity on a 0- to 4-point severity scale following the methods of Apeti et al. and Jones et al. (see Supporting Information; Apeti et al., 2014; Jones et al., 2021). Prevalence and intensity were also calculated for digenetic trematode parasitization, using formulas from Apeti et al. (2014). Prevalence is defined as the proportion of mussels within the population with a certain pathology or stress response. Intensity only considers infected individuals.

For DGA and HI analyses, the average prevalence for each group was calculated (see Supporting Information). For each experiment, prevalence and intensity values were compared statistically in R (version 4.1.0) using a nonparametric Kruskal–Wallis test. Pairwise comparisons were conducted using a Dunn test for multiple comparisons of groups, when necessary. The intensity of trematode parasitization did not satisfy the assumptions of ANOVA; therefore, counts were also compared using a nonparametric Kruskal–Wallis test and pairwise Dunn test.

RESULTS

During experiment 1 a single mussel from the control treatment died on day 6. On the 14th day of the experiment, a mussel from the *Spartina* spp. treatment died and was frozen immediately for subsequent dissection and histological analysis. There were no mortalities observed during experiment 2. Nylon MF and *Spartina* spp. particles were observed within the fecal matrix throughout the experiment, confirming that both particles were ingested by the mussels (Figure S2A, B). In experiment 1 on average 73 nylon MF (standard deviation 84, range 5–411) and 81 *Spartina* spp. particles (standard deviation 77, range 3–441) were recovered from feces. In experiment 2 on average 112 nylon MF (standard deviation 147, range 9–922) and 173 *Spartina* spp. particles (standard deviation 151, range 0–791) were recovered from feces. Previous studies have demonstrated that >90% of anthropogenic particles are egested by bivalves within 48 h (Doyle et al., 2015; Kach & Ward, 2008; Ward et al., 2019), therefore these numbers likely indicate the number of particles ingested during the previous 1–2 days.

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Microbial community composition

16S rRNA gene amplicon sequencing

A total of 2904,036 raw reads from the 96 samples (median 18,150 reads) were loaded into the DADA2 pipeline. After quality control (i.e., read filtering and removal of erroneous taxonomic assignments), 2866 unique ASVs were obtained. Rarefaction analysis revealed that the ASV richness of all target communities was sufficiently sampled by the sequencing effort, regardless of variability in filtered read depth (Figure S4).

Beta-diversity analysis

Upon ordination of the multivariate ASV count data, there was a distinct clustering of microbial communities for all samples by treatment. Non-metric multidimensional scaling on Bray–Curtis dissimilarities for both experiment 1 (50 particles/L/h) and 2 (100 particles/L/h) identified three distinct clusters (Figure 1). The microbial communities from in situ mussels clustered together, as did those from control-, plastic-, and *Spartina* spp.-exposed gut communities. The microbial communities on nylon and *Spartina* spp. particles and in ageing seawater clustered together (Figure 1). When the data were separated by experiment, the ordinations displayed the same patterns.

On a finer scale, differences in grouping among ageing seawater and particle-associated communities were apparent when those communities were considered apart from gut samples. Specifically, microbial communities on nylon MF grouped more tightly together than those in water samples or on *Spartina* spp. particles. Ordination also indicated relatively more similarity between ASV composition on *Spartina* spp. particles and in water than between those of nylon MF and either *Spartina* spp. or water (Figure S5). PERMANOVA on Bray–Curtis dissimilarities confirmed the significant difference in multivariate ASV composition between sample types (pseudo-$F_{(5,\ 32)} = 3.891$, $r^2 = 0.419$, $p < 0.01$).

Alpha-diversity analysis

Comparisons of Inverse Simpson index values between mussels in both experiments 1 and 2 found no significant differences between microbial communities (Table S1, Figure 2A, ANOVA $F_{(7,\ 53)} = 2.063$,

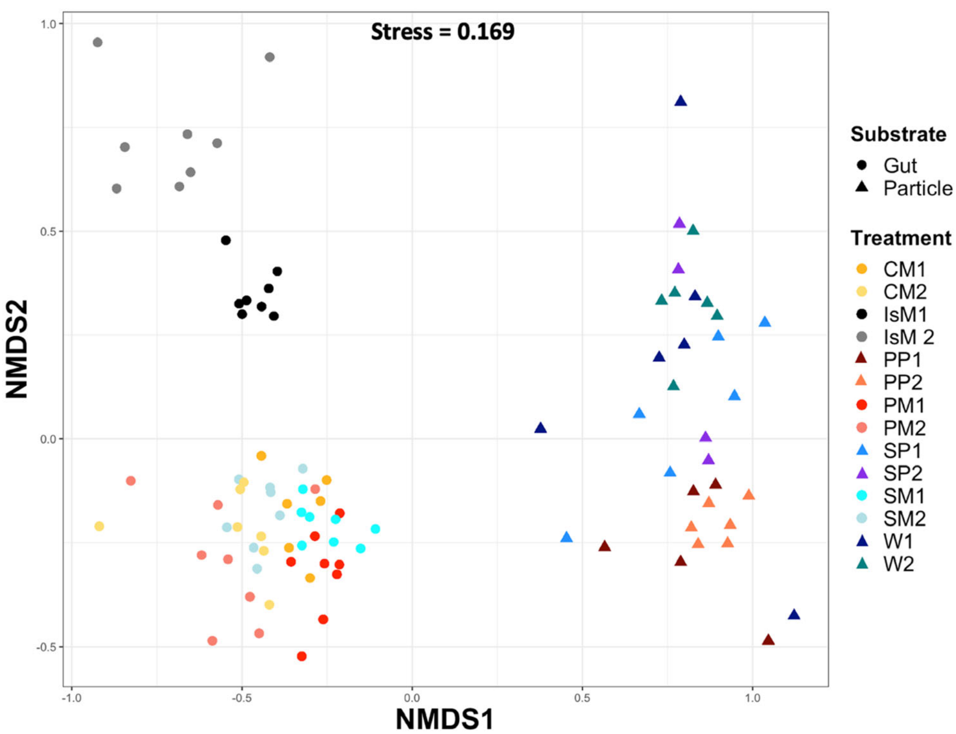

FIGURE 1 Ordination plot displaying non-metric multidimensional scaling on Bray–Curtis dissimilarities from all microbial communities for both experiments. The stress value is indicated on the plot. In the legend, sample abbreviations are as follows: 'CM' for Control Mussel, 'IsM' for in situ Mussel, 'PP' for Plastic Particle, 'PM' for Plastic Mussel, 'SP' for *Spartina* Particle, 'SM' for *Spartina* Mussel, and 'W' for ageing seawater. Samples are further distinguished by '1' or '2' for experiment 1 (50 particles/L/h) and 2 (100 particles/L/h), respectively. Microbial communities are coloured according to treatment group and experiment number and are shaped according to substrate type (mussel gut, or particle and ageing seawater).

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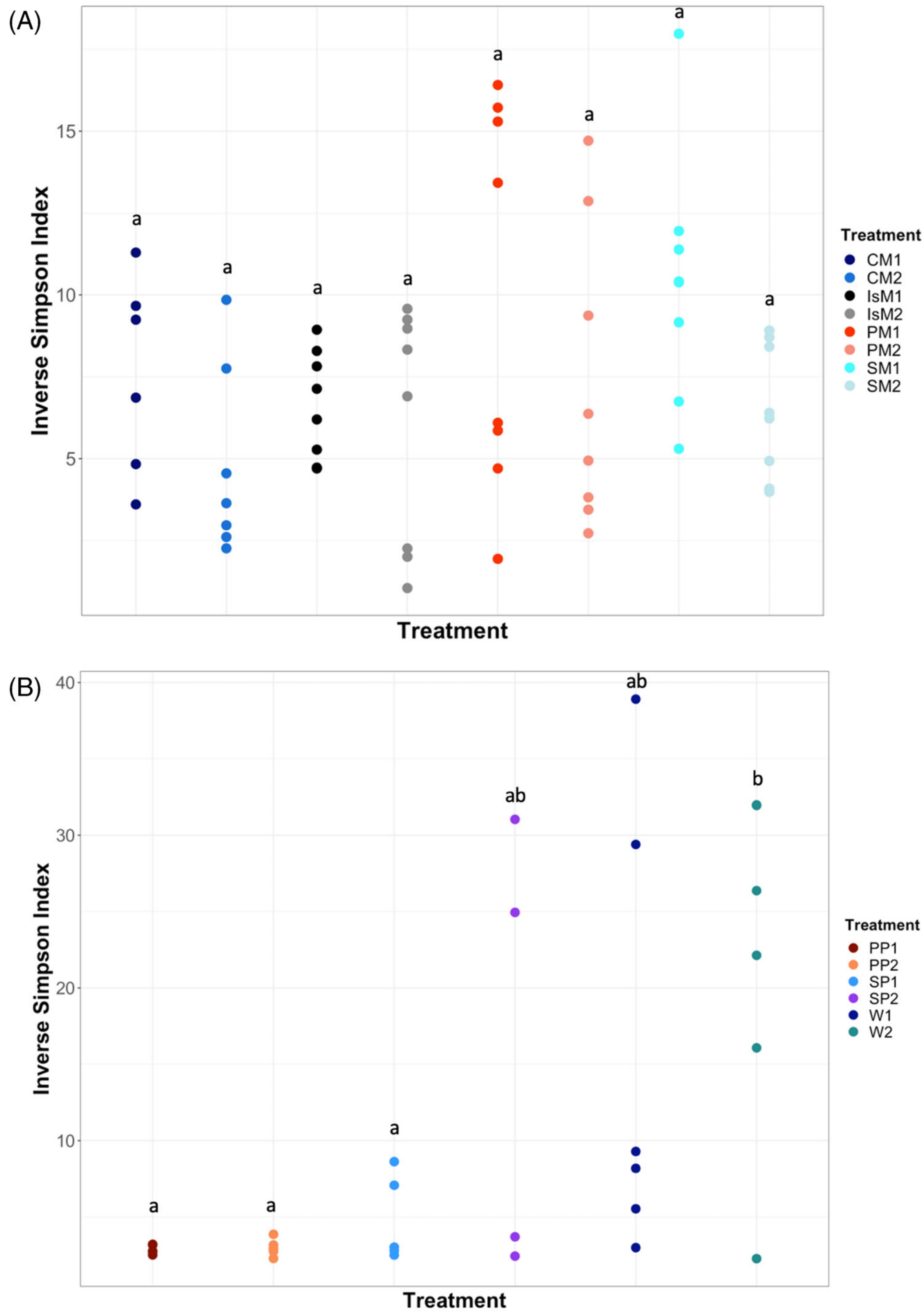

FIGURE 2 A comparison of Inverse Simpson index values for (A) mussel gut and (B) particle and water samples from both experiments. In the legend, sample abbreviations are as follows: 'CM' for Control Mussel, 'IsM' for in situ Mussel, 'PP' for Plastic Particle, 'PM' for Plastic Mussel, 'SP' for *Spartina* Particle, 'SM' for *Spartina* Mussel, and 'W' for ageing seawater. Samples are further distinguished by '1' or '2' for experiment 1 (50 particles/L/h) and 2 (100 particles/L/h), respectively. Microbial communities are coloured according to the treatment group and experiment number. Compact letter display indicates significant and nonsignificant differences between means of treatment groups (Tukey posthoc tests, $p < 0.05$).

$p = 0.06$). Mussel gut communities showed similarities in species richness and evenness among all treatment groups. However, there were differences in species richness and evenness between nylon MF and ageing seawater samples (ANOVA $F_{(5, 27)} = 4.208$, $p = 0.006$). The microbial communities on nylon MF showed a lower Inverse Simpson index (decreased species richness and evenness) than those of ageing seawater samples from the corresponding experiment (Table S1 and Figure 2B). In experiment 2 (100 particles/L/h), this difference was significant (Tukey posthoc test experiment 1, $p = 0.065$; experiment

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2, $p = 0.02$). While the difference in the Inverse Simpson index was not always significant, the microbial community on *Spartina* spp. particles had a mean Inverse Simpson index of 8.92 (SD = 10.36) which was approximately 3× higher than the mean Inverse Simpson index of nylon MF (2.95, SD = 0.42). Differences in diversity between nylon MF and ageing seawater samples were also evident at higher taxonomic levels (e.g., class level, Figure 4).

Taxonomic composition

In experiment 1, the taxonomic composition of gut communities of control, plastic, and *Spartina* spp. mussels were comparable with respect to relative abundance at the class level (Figure 3). Alphaproteobacteria, Bacteroidia, and Gammaproteobacteria were the most dominant classes in all gut samples. The microbial communities of in situ mussels showed a decreased relative abundance of Alphaproteobacteria compared to the gut communities of experimental mussels (ANOVA $F_{(3,\ 26)} = 15.82$, $p < 0.001$; Tukey posthoc $p < 0.01$). Results from experiment 2 showed a slightly different pattern (Figure S6). At the class level, plastic mussels had a significantly lower relative abundance of Bacteroidia than control mussels (ANOVA $F_{(3,\ 26)} = 5.816$, $p = 0.003$; Tukey posthoc $p = 0.002$), and a marginally significant lower relative abundance compared to *Spartina* spp. and in situ mussels (ANOVA $F_{(3,\ 26)} = 5.816$, $p = 0.003$; Tukey posthoc $p = 0.055$ and $p = 0.06$). It was not uncommon to find samples that had taxa that could not be classified with the SILVA database or by manual BLAST (Altschul et al., 1990). In these situations, communities were dominated by one to two unidentifiable bacterial ASVs. In some cases, the relative abundance of unidentifiable bacteria was as high as 97% (Figure S6).

In both experiments, 1 and 2, the microbial communities on plastic particles showed a relatively low richness of classes. Samples of nylon MF had a significantly lower relative abundance of Alphaproteobacteria compared to both *Spartina* spp. and ageing seawater (Kruskal–Wallis test $p = 0.0003$, Dunn's test $p = 0.02$ and $p = 0.0003$). In addition, nylon MF showed a significantly higher relative abundance of Bacteroidia than both *Spartina* spp. and ageing seawater (Kruskal–Wallis test $p < 0.0001$, Dunn's test $p = 0.0002$ and $p = 0.0009$). The microbial communities on *Spartina* spp. particles and in ageing seawater also had a higher richness of class-level taxonomic groups, with Planctomycetes, Campylobacteria, Bdellovibrionia, and Acidimicrobiia among those present, albeit at lower relative abundances. Differential abundance analysis with DESeq2 revealed that six ASVs were significantly more abundant in the microbial communities on nylon MF than on *Spartina* spp. particles (Figure S7, $p_{adjusted} < 0.01$). Of these ASVs, four were from class Gammaproteobacteria, one was from class Alphaproteobacteria, and one belonged to class Bacilli. A member of the genus *Psychromonas* (class Gammaproteobacteria) was specifically more highly abundant in the communities on nylon MF than on *Spartina* spp. particles. Differential abundance analysis also revealed 10 ASVs that were significantly more abundant in the communities on nylon MF than in ageing seawater

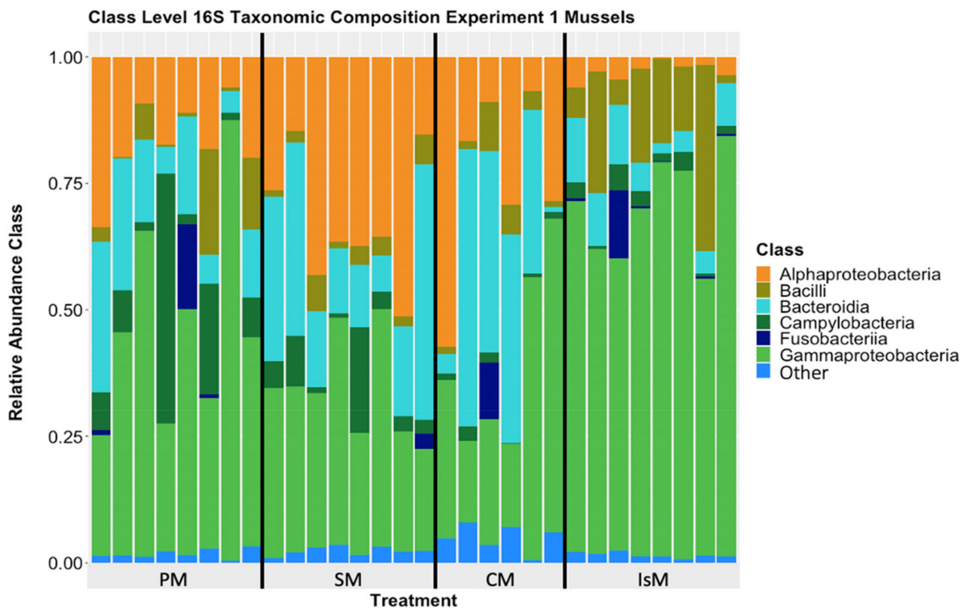

FIGURE 3 Relative abundance of ASVs grouped at the Class level for mussel gut microbial communities in experiment 1. On the *x*-axis sample abbreviations are as follows: 'PM' for Plastic Mussel, 'SM' for *Spartina* Mussel, 'CM' for Control Mussel, and 'IsM' for in situ Mussel.

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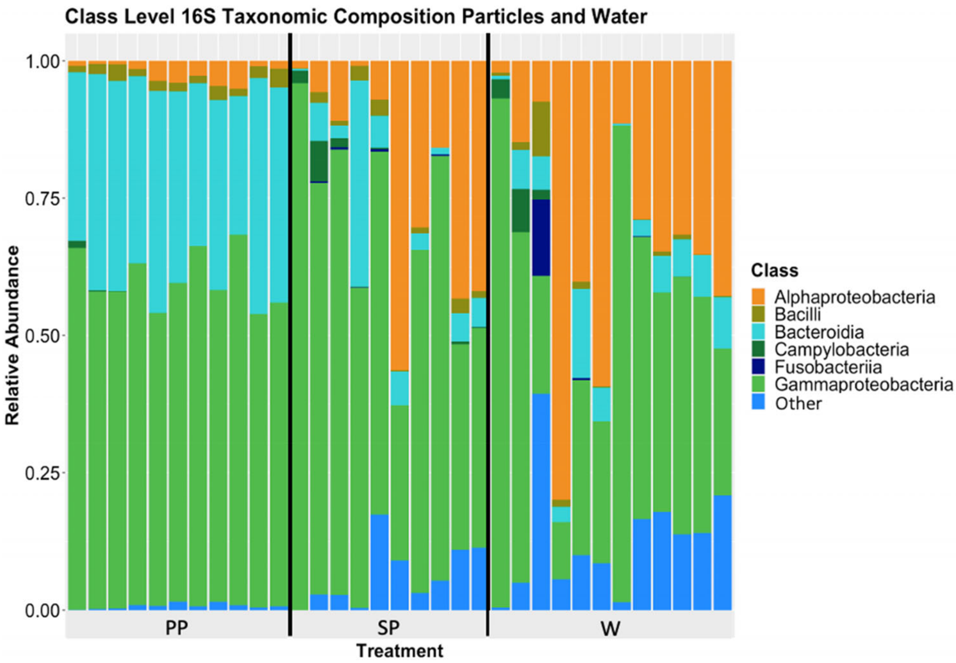

FIGURE 4 Relative abundance of ASVs grouped at the Class level for particles and ageing seawater for both experiments. On the *x*-axis, sample abbreviations are as follows: 'PP' for Plastic Particle, 'SP' for *Spartina* Particle, and 'W' for ageing seawater.

(Figure S8, $p_{adjusted}$ <0.01). Of these ASVs, seven belonged to class Gammaproteobacteria, one to class Bacilli, one to Alphaproteobacteria, and one to Bacteroidia. In particular, a member of the genus *Psychrobacter* (class Gammaproteobacteria) was more highly abundant in the microbial communities on nylon MF than in ageing seawater. When examining both comparisons (nylon MF vs. *Spartina* spp. and nylon MF vs ageing seawater) it was apparent that the species *Umboniibacter marinipuniceus* was more highly represented in the microbial communities on nylon MF than in *Spartina* spp. particles or in ageing seawater.

Histopathology

There was no occurrence of necrosis or of nylon MF penetrating tissues. Additionally, no nylon MF was encapsulated by hemocytes in the tissue sections. There were no significant differences in the prevalence or intensity of DGA in mussels in either experiment (Kruskal–Wallis test $p > 0.05$; Table S2). Furthermore, no differences were observed in the prevalence of focal HI for the same comparisons (Kruskal–Wallis test $p > 0.05$), or the prevalence of diffuse HI in experiment 1 (Kruskal–Wallis test $p > 0.05$). There was a significant difference in the prevalence of diffuse HI in experiment 2. A pairwise posthoc Dunn test with Bonferroni adjustments revealed that in situ mussels had a higher prevalence of diffuse HI than control mussels ($p = 0.039$, epsilon-squared $= 0.282$). No significant differences in the intensity of diffuse HI were observed for either experiment (Kruskal–Wallis test $p > 0.05$). The parasitic sporocysts observed in this study were identified as the digenean *Proctoecus maculatus* (Looss 1901) based on the location of sporocysts within tissues and the geographic range of this species (Stunkard & Uzmann, 1959; Sunila et al., 2004; Uzmann, 1953). Trematode parasites were common in experiment 1, conducted in July, but there were no significant differences in the prevalence or intensity between treatments (Kruskal–Wallis test $p > 0.05$). The low prevalence of trematode parasitization in experiment 2 (only two out of 30 mussels were infected), conducted in late August and early September, precluded statistical analysis. Overall, mussels showed a relatively consistent low intensity of both DGA and diffuse HI, with occasional occurrences of focal HI (Figure 5A–D).

DISCUSSION

The presence of MP in the marine environment necessitates the investigation of whether these synthetic particles negatively impact the physiology of marine animals. Results from this study showed no apparent effects of nylon MF on the gut microbiome or digestive tissues of the blue mussel. Gut microbial communities of mussels exposed to nylon MF did not differ from those of mussels exposed to *Spartina* spp. particles or the control mussels (Figure 1). Importantly, mussel gut microbiomes showed resistance to disturbance, even after 21 days of exposure to nylon MF. Resistance, defined as the absence of a change in microbiome

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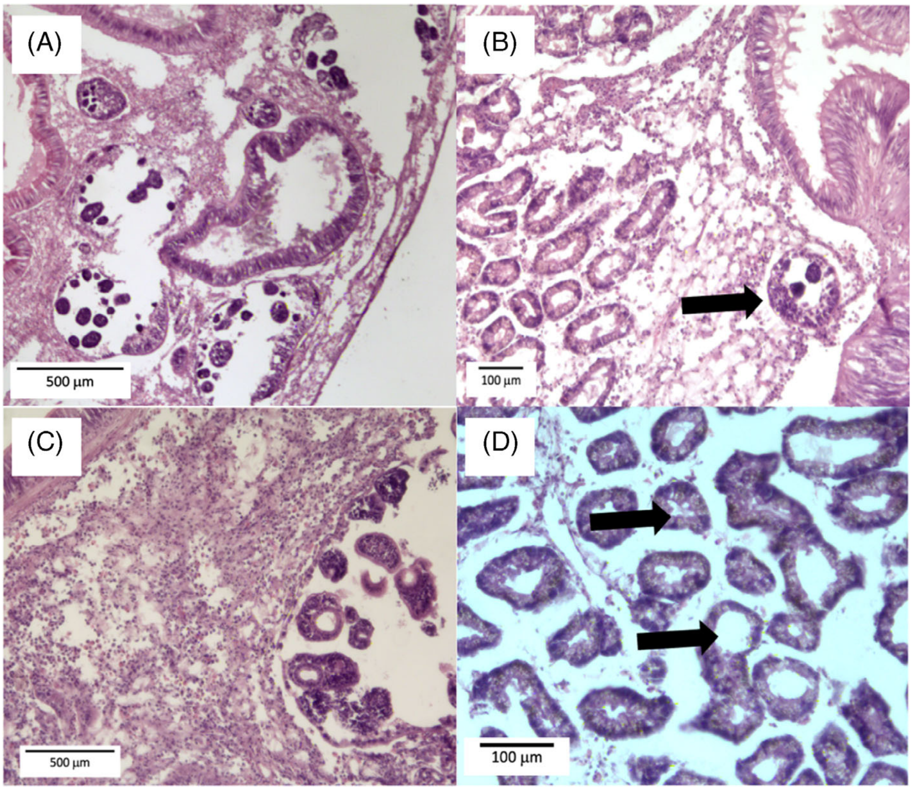

FIGURE 5 Examples of four pathologies prioritised for histological assessment. (A) Heavy digenetic trematode sporocyst parasitization, 40× magnification. (B) Occurrence of focal HI surrounding a small trematode sporocyst (black arrow), 100× magnification. (C) Diffuse HI adjacent to a trematode sporocyst containing cercaria, 40× magnification. (D) Black arrows pointing to a 'normal' tubule with thick epithelial cells and a quadriradiate lumen (top), and a digestive tubule showing atrophy with thinned epithelial walls and round, enlarged lumen (bottom), 100× magnification.

composition after a given disturbance (Moya & Ferrer, 2016), was evident in this study. Mussels were exposed to a microbial community on nylon microfibers that differed from microbiota on *Spartina* spp. particles and in seawater; however, even after exposure to this disturbance, there was no corresponding change in the gut microbiome of mussels.

Results from this study support prior research on the presence of a 'plastisphere', or a distinct microbial community that develops on macro- and MP in the marine environment (Amaral-Zettler et al., 2020; Zettler et al., 2013). The microbial community on nylon MF in this work differed from the communities on comparably sized natural particles (*Spartina* spp.) and coarsely filtered seawater in terms of relative abundance of ASVs grouped at the class level. In particular, Alphaproteobacteria was significantly underrepresented and Bacteroidia significantly overrepresented in the microbial communities on nylon MF, compared to those on *Spartina* spp. particles and in ageing seawater. It is possible that these shifts were due to the physicochemical properties of the nylon MF in comparison to the *Spartina* spp. particles. Microbial communities on nylon MF also showed a much lower Inverse Simpson index of alpha diversity than those on *Spartina* spp. particles and in ageing seawater. In comparison, the microbial communities on *Spartina* spp. particles and in coarsely filtered seawater showed a much more variable taxonomic community, with many more classes represented at varying relative abundances. DESeq comparisons showed that in particular, an ASV assigned to *Umboniibacter marinipuniceus*, a member of class Gammaproteobacteria, was more highly abundant in the microbial communities on nylon MF compared to those on *Spartina* spp. particles and in ageing seawater. Furthermore, two separate ASVs assigned to the genus *Psychrobacter* (class Gammaproteobacteria) were more highly abundant in the microbial communities on nylon MF, one in comparison to ageing seawater and one to *Spartina* spp. particles.

The effects of plastic particles on the gut microbiota of bivalves require further investigation, however, results from the present study are not congruent with some previous findings. Li et al. found that fine-scale changes occurred in the gut microbiomes of *M. edulis* after exposure to HDPE microspheres (4–6 and 20–25 μm) at high concentrations (ca. 1170 and 117,000 MP/mL; Li et al., 2020). Although these authors used MP with different sizes, shapes, polymer types, and concentrations than the current work, they also did not

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provide adequate food to the animals during their experiments and did not include a particle control. Additionally, their design included pseudoreplication and the pooling of mussel guts which masks individual variability. These shortcomings along with the use of high concentrations of MP likely accounted for the results they obtained.

Results from the present study are more in line with those of Yang et al. who revealed no significant effect of plastic particles on the gut microbial community of marine mussels (Yang et al., 2021). Yang et al. examined the effect of polystyrene microbeads (diameter = 10 μm) in tandem with ocean acidification on *Mytilus coruscus* by exposing animals to 2 pH levels (7.3 and 8.1) and either no MP or 1000 MP/L for 21 days (Yang et al., 2021). Although their study used virgin MP, which are not commonly found in the marine environment, concentrations were closer to in situ observations (Lusher et al., 2014). Yang et al. found no change in mussel gut microbial communities after exposure to both decreased pH and MP (Yang et al., 2021), which matches the result of resistance demonstrated by the gut microbial communities in the present study.

The most marked shifts in gut microbial composition observed in this study occurred between the in situ mussels and all of the mussels brought into the laboratory for both experiments (Figures 1, 3, S5). These changes in microbiome composition are unsurprising and can be attributed to a combination of factors associated with housing mussels in the laboratory, which include removing the animals from their natural environment, isolating pairs into individual static microcosms with filtered seawater, and delivering them a controlled microalgal diet. Notably, these laboratory-associated shifts in microbiomes occurred even though all mussels, in situ and experimental, underwent depuration prior to being sacrificed. All in situ mussels were left undisturbed in isolated microcosms for 16 h to allow them to void transient microbiota associated with feces prior to dissection. Furthermore, in both experiments, mussels from the plastic, *Spartina*, and control groups were dissected 15–16 h after the time of their final feeding, allowing these animals a comparable amount of time to void transient microbes associated with feces. As a consequence, the resulting gut tissues were occupied primarily by resident communities of microbes upon dissection (Griffin et al., 2021). Therefore, the laboratory-associated shift in gut microbial composition in mussels is driven by changes in the resident community.

Ingestion of MP and MF by animals has been hypothesised to cause intestinal damage, blockages, or cellular defence responses (Wright et al., 2013). Results from this study indicate that nylon MF approximately 500 μm in length at low concentrations do not block the digestive system nor cause tissue necrosis or excessive stress responses (Table S2). Mussels from all treatment groups showed a low level of DGA, a response that can be indicative of stressors of many kinds (Winstead, 1995). DGA analysis in bivalves has been used in conjunction with other biomarkers to evaluate oil-spill effects and recvery, and has even been linked to climate variation (Garmendia et al., 2011; Kim & Powell, 2009). DGA is a common pathology in bivalves, and prevalence levels from this study do not exceed those typically seen in the Northeastern USA (Apeti et al., 2014). Similarly, in the current study, HI was present at low levels in most of the examined mussels, which is common for mussels in coastal waters of the Northeastern USA (Apeti et al., 2014).Trematode sporocysts were common parasites present in the digestive gland of mussels from all treatment groups during experiment 1. Infection of *M. edulis* by *P. maculatus* is common in Long Island Sound, with a high prevalence up to 60% reported in some cases (Sunila et al., 2004). MP and *Spartina* spp. were confirmed to have been ingested and egested (therefore traversing the digestive organs), yet their presence did not cause any evidence of abrasion, blockage, or cellular defence responses.

The histopathological data presented here disagree with some previously published results. Teng et al. exposed *Crassostrea gigas* to irregularly sized fragments of PE (mean diameter = 36.72 μm) and PET (mean diameter = 31.11 μm) at two concentrations (10 μg/L and 1000 μg/L), equivalent to ca. to 3.0×10^3 MP/L and 3.0×10^6 MP/L (Teng et al., 2021). These authors used MP concentrations orders of magnitude higher than those found in the natural environment (Everaert et al., 2018; Phuong et al., 2016), and did not use a particle control, which could explain the discrepancies between the current study and their work. von Moos et al. exposed *M. edulis* to HDPE spheres between <1 and 80 μm in diameter for 96 h (von Moos et al., 2012). After the exposure, they observed MP in the primary and secondary ducts of the digestive gland, the digestive tubules, and an inflammatory response of granulocytomas in the connective tissue combined with lysosomal membrane destabilisation. The discrepancy between the results of von Moos et al. and the current study could be a consequence, in part, of the size and shape of MP used (von Moos et al., 2012). The small, (<80 μm) spherical particles used by von Moos et al. could have penetrated the ducts of the digestive gland more easily than the long fibres used in the current study. Both the length and shape of the long nylon fibres could have prevented them from entering the ducts, while shorter and/or more spherical particles may enter more easily. However, it is also possible that the high concentration of particles used (2.5 g of HDPE/L; equivalent to ca. to 7.8×10^7 MP/L) contributed to their result. This point is particularly important because it is well known that exposing bivalves to high

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concentrations of natural refractory particles (between 0.001–2.5 g/L) can cause stress and negatively affect feeding and digestive processes (Benitez-Polo & Velasco, 2020; Bricelj & Malouf, 1984; Cranford & Gordon Jr., 1992; Goldsmith et al., 2021; Loosanoff, 1962; Robinson et al., 1984; Shumway et al., 2003).

The histopathological data presented here are in agreement with results published previously in several other papers. Revel et al. exposed *M. edulis* to PE (diameter range = 0.4–720 μm, mean = 287 μm) and PET (diameter range = 0.4–950 μm, mean = 204 μm) fragments at 0.008 μg/L, 10 μg/L, or 100 μg/L (equivalent to ca. 9, 1.1×10^4 and 1.1×10^5 MP/L, respectively) for 10 days, followed by a 10-day depuration period (Revel et al., 2019). The authors found no significant differences in a variety of histological parameters, including DGA and hemocytic infiltration, between mussels exposed to plastic and the unexposed controls. Gonçalves et al. exposed *M. galloprovincialis* to 2 and 10 μm polystyrene microspheres at 1.0×10^4 MP/L and 1.0×10^6 MP/L in both short-term (minutes) and 48-h exposures (Gonçalves et al., 2019). These authors identified MP in the bivalve stomach cavity, but not the digestive gland, and found no evidence of histological damage. In conjunction with the current study, the evidence presented suggests that low concentrations (i.e., 10^1–10^3 MP/L), and with some types of MP even high concentrations (i.e., 10^4–10^6 MP/L) cause little if any abrasive damage, blockage, or other negative effects on the gut tissues of suspension-feeding bivalves.

It is likely that the nylon MF used in this experiment passed quickly through the mussel digestive system without triggering the stress responses assayed. Postingestive particle selection occurs in the bivalve gut based on size, shape, and chemical properties (Ward & Shumway, 2004). As described comprehensively by both Morton (1960) and Owen (1966), ingested particles travel down the esophagus to the anterior chamber of the complex stomach. Within the stomach, particle selection occurs on sorting areas comprised of ciliated ridges and grooves. Larger, denser particles are typically directed toward the intestine, where they are incorporated with other waste products into fecal pellets for egestion (Morton, 1960). Finer, organic particles and particle fragments are typically kept in circulation and are eventually transported to tubules in the digestive diverticula, where they are phagocytosed by digestive cells (Owen, 1966). Postingestive selection within the bivalve stomach has been documented to affect the gut retention time of particles (Bricelj et al., 1984; Brillant & MacDonald, 2000; Brillant & MacDonald, 2002; Brillant & MacDonald, 2003; Gagnon & Fisher, 1997; Shumway et al., 1985). Accordingly, the relatively large (500-μm-length) fibres used in the current study likely had a short gut retention time compared to other ingested particles; MP with a longer gut retention time may increase the potential for gut colonisation by foreign microbes or trigger histological stress responses. Evidence for preferential postingestive selection of organic particles and particles with an organic coating further necessitates the use of aged plastic particles in exposure studies, rather than virgin MP.

Inconsistencies also persist regarding the histopathological effects of MP of varying shapes and sizes. Future experiments involving chronic exposures that utilise appropriate particle controls and comprehensive assemblages of MP sizes, shapes, and polymer types found at environmentally relevant concentrations should be a priority for the field. Such experiments would provide a fuller picture of how MP may affect the physiology of suspension-feeding bivalves. Regardless, data from the present study indicate that, although the prokaryotic community on nylon MF differs from the community on *Spartina* spp. particles and in seawater, exposure to nylon MF did not impact the gut microbiome of the blue mussel. Furthermore, no significant physiological stress responses were observed in the gut tissues of mussels following ingestion of MF. These novel results represent a biological reality that has positive implications for the health of this important coastal species.

AUTHOR CONTRIBUTIONS

Hannah Collins: Conceptualization (equal); formal analysis (lead); funding acquisition (supporting); investigation (lead); writing – original draft (lead); writing – review and editing (lead). **Tyler Griffin:** Formal analysis (supporting); investigation (supporting); writing – review and editing (supporting). **Bridget A. Holohan:** Investigation (supporting); supervision (supporting); writing – review and editing (supporting). **J. Evan Ward:** Conceptualization (equal); funding acquisition (lead); supervision (lead); writing – review and editing (supporting).

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the NCBI Short Read Archive (SRA) under BioProject ID: PRJNA823368.

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