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Responses of the symbiotic sea anemone Anthopleura elegantissima to microplastics

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Responses of the symbiotic sea anemone *Anthopleura elegantissima* to microplastics

By

Robert Beck

Offered in Partial Completion
of the Requirements for the Degree
Master of Science

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Master's Thesis

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Robert Ihaia Beck

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A Thesis
Presented to
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In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Robert Ihaia Beck
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Abstract

The Salish Sea, a large and complex fjord estuary, receives waters impacted by a watershed that includes 8 million people aggregated in several large urban and industrial centers. Microplastics, defined as plastic particles less than 5 mm in their largest dimension, are transported from this watershed into the Salish Sea where they are easily ingested by filter feeders, herbivores and predators. To measure effects of microplastics on one common and important intertidal species, we exposed the sea anemone *Anthopleura elegantissima* to polyester microfibers at concentrations of 0, 0.01, or 0.1 g/L in the laboratory and measured the responses of the anemones throughout a 38-day exposure period. Because *A. elegantissima* hosts photosymbionts, we hypothesized that microplastics could affect the host, the symbiont, or both and took measurements to evaluate performance of both the hosts and the symbionts. We used linear mixed model analyses to evaluate changes in anemone size via oral disc diameter, digestive efficiency, and respiration rate, and to measure effects to symbionts we measured photosynthetic efficiency (*Fv/Fm*), symbiont density, and symbiont mitotic index. We interpret the effects of the microplastic exposure against the background effect of the environmental changes experienced through our experiment and found that smaller anemones had lowered digestive efficiencies in high microplastic concentrations, and that photosymbionts seem to play a minor role in the success of the anemone in the presence of microplastics when looking at *Fv/Fm* over time and anemone size over time. The results suggest that, under the experimental conditions we used, the short-term effects of microplastic exposure on *A. elegantissima* are not large. However, microplastics could have more lasting impacts over time that could affect the success of this species living in seas already impacted by other environmental stressors including rising temperatures, acidification, and chemical pollutants.

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Introduction

Petroleum based polymers collectively known as "plastic" dominate in packaging, consumer goods, and manufacturing with an estimated total production of 8.3 billion metric tons of virgin plastic produced worldwide since it was first invented in the early 20th Century (Geyer et al. 2017). Due to insufficient management of highly durable plastic waste, plastic debris is now a global phenomenon with a reach that continues to expand into terrestrial, freshwater, and marine habitats, including previously assumed untouched regions of the deep sea (Taylor et al. 2016, Jamieson et al. 2019), Arctic ice (Obbard et al. 2014), Antarctic marine sediments (Munari et al. 2017, Dawson et al. 2018), and even circulating in the atmosphere (Zhang et al. 2020). Human consumption of plastic is estimated to be in the millions of particles per year (Cox et al. 2019). Plastic debris is now so common that geologists have characterized a new type of rock, plastiglomerate, and propose its inclusion in future records as an anthropogenic marker for our current epoch (Corcoran et al. 2014).

An estimated 5 trillion pieces of plastic debris weighing over 250,000 tons is estimated to be floating in oceanic surface waters as of 2013, with 4.9 trillion particles less than 5 millimeters in diameter, weighing over 35,000 tons, concentrated either in subtropical gyres or along coastlines (Eriksen et al. 2014). Efforts to quantify the extent of plastic pollution in subtropical gyres like the "Great Pacific Garbage Patch" are ongoing, but it is clear that each basin and body of water has unique characteristics that determine the nature, extent, and impact of plastic contamination.

The Salish Sea, a protected, flooded fjord estuary, stretches along the U.S. – Canada Border from the southern reaches of the Puget Sound northwards to Desolation Sound including the Straights of Georgia and Juan de Fuca. The Salish Sea is impacted by an estimated 8 million people concentrated in several large urban centers and distributed across many smaller coastal communities. Residents rely on this vital shared ocean resource for commercial and recreational fishing, international shipping, oil refining, and transportation, and as an essential attribute of identity and culture. The watershed of the Salish Sea extends inland towards the Cascade Mountain range on the east and Olympic and Vancouver Island ranges on the west. Water circulation is primarily driven by tidal currents flushing between the various inlets and passages, but this flow is complicated by freshwater inputs and winds, resulting in a highly dynamic system supporting a rich and unique biodiversity affected by a range of human activity including agriculture, dairy farming, silviculture, mining, and urbanization. While few published studies have quantified local plastic contamination in the Salish Sea or identified the factors that contribute to this problem, it has been estimated that a one-meter-wide band of beach wrack along the shores of the Salish Sea contains 5.8 metric tons of plastic (Davis and Murphy 2015). Local circulation and differences in supply, however, lead to inequalities in distribution with areas of particularly heavy accumulations (Sutherland et al. 2011, Desforges et al. 2014) that can put local fauna at particular risk.

Plastic debris kills or otherwise negatively affects seabirds (reviewed in GESAMP 2015), marine mammals (Williams et al. 2011, Denuncio et al. 2017), sea turtles (Carr 1987) and fish (Murphy 2018b, Pazos et al. 2017, Savoca et al. 2016) through entanglement and ingestion. Plastics may also become a vector for introduction of persistent organic pollutants (POPs; Fisner et al. 2017), heavy metals (Brennecke et al. 2016) and microbes (Teuten et al. 2009). Because of

the non-biodegradable nature of plastic (Andrady 2011), it persists in the environment. When weathering breaks large plastic debris into smaller particles, the plastic does not simply disappear, but instead becomes even more pervasive with higher concentrations of particles.

Microplastics, defined as plastic particles less than 5 mm in the longest dimension (U.S. EPA 2016, Lusher et al. 2017), increase the probability of encounter and ingestion by marine organisms because they are in the same size range as food sources to lower trophic levels and could, consequently, accumulate through the food web (Farrell and Nelson 2013, Nelms et al. 2018). Even primary producers are at risk from plastics, with measured effects including elevated production of reactive oxygen species (ROS) and decreasing photosynthetic efficiency (Bhattacharya et al. 2010, Yokota et al. 2017). Zooplankton and larval fish can ingest microplastics by direct consumption or by feeding on other organisms that have ingested the particles. Both processes can decrease assimilation efficiency and produce reproductive changes in both zooplankton and fish (Cole et al. 2013, Kaposi et al. 2014, Lee et al. 2013, Dedman 2014, Jeong et al. 2016). Commercially important shellfish species including clams (Baechler et al. 2020), mussels (Wegner et al. 2012, Santana et al. 2016, Mathalon and Hill 2014) and oysters (Sussarellu et al. 2016, Murphy 2018a, Martinelli et al. 2020) also ingest microplastics.

As indiscriminate filter feeders, bivalves are of special concern as they can bioaccumulate dispersed plastic particles. However, less obvious benthic detritivores like the lugworm *Arenicola* sp. also ingest microplastic particles, capturing debris that sinks from the water column, and have been shown to translocate toxins from the plastics to their bodies (Teuten et al. 2007, 2009). Reef-forming scleractinian corals, already suffering unprecedented losses due to rising temperatures and bleaching, may be further threatened directly by ingestion of microplastic particles (Hall et al. 2015, Allen et al. 2017) and indirectly by potential impacts

on their symbiotic algae. With the possibility of microplastics affecting both the heterotrophic feeding efficiency of the coral host and the photosynthetic capacity of the photosymbionts, corals and other symbiotic cnidarians are prime candidates for studying the impacts of microplastic pollution on marine species.

The cloning, aggregating, and photosynthetically symbiotic sea anemone, *Anthopleura elegantissima*, a temperate-water analog to tropical corals, is a common intertidal species along the west coast of North America. Like tropical corals, *A. elegantissima* forms symbioses with marine algae, deriving photosynthetically produced nutrition to supplement heterotrophic feeding. *A. elegantissima* contribute significantly to the energetics of the communities where they occur; Fitt (1982) suggested that the productivity of *A. elegantissima* rivals that of macroalgae in some intertidal communities. Unlike the obligate cnidarian/dinoflagellate symbiont relationships in tropical corals, *A. elegantissima* forms flexible endosymbiotic relationships with one or a combination of two photosymbionts: green chlorophytes (*Eliptochloris marina*) called zoochlorellae and brown dinoflagellates (*Breviolum muscatinei*) (previously known as *Symbiodinium muscatinei;* LaJeunesse et al. 2018) called zooxanthellae. As a means of studying pathways and mechanisms of stress in cnidarians, *A. elegantissima* has increased our understanding of the effects of temperature stress (Bingham et al. 2011), light (Dimond et al. 2012), and ocean acidification (Coleman 2021) on photosymbiosis. While there are no published data showing that microplastics damage *A. elegantissima*, the dependence of this species on opportunistic capture of prey items ranging from micro- to mesoscales and their presence in intertidal habitats subjected to daily tidal exchanges suggest they may be vulnerable to microplastics, both through direct impacts to the anemone and through impacts on their photosymbiotic partners.

The goal of our work was to determine how *A. elegantissima* and its symbionts respond to microplastics by exposing the anemones to different concentrations of microplastics and measuring multiple responses of the holobiont, including growth and respiration of the anemone and growth and photosynthetic efficiency of the symbionts.

Methods

Anemone collection and identification

Anthopleura elegantissima were collected from the intertidal zone at Lawrence Point, Orcas Island, Washington (48° 39' 43" N, 122° 44' 34" W) on June 27, 2018. The anemones were collected via snorkeling at tidal heights ranging from +6.5 to +7.28 ft MLLW (NOAA Tides and Currents). We collected 70 anemones hoping to get individuals in different symbiotic conditions. Symbiotic state can be estimated by the coloration of the anemone oral surface and tentacles with brown coloration indicating symbiosis with *B. muscatinei* and green coloration indicating symbiosis with *E. marina* (Figure 1) though this method is not precise. The anemones were carefully pried from a variety of aspects and angles of attachment to the rock substratum with a small spatula, immediately placed in seawater, and transported to the Shannon Point Marine Center where they were placed in a flow-through seawater table. To confirm symbiotic state, a single tentacle was clipped from each anemone, squashed, and examined under the microscope where the identity of the symbionts could be determined by color and size. Based on the results, each anemone was defined as brown (hosting primarily *B. muscatinei*) or green (hosting primarily *E. marina*). Each anemone was then placed in a numbered watch glass, lined with traction tape on the inside lip to discourage escape, and held in a flow-through sea table. The anemones were acclimated this way for 8 weeks prior to microplastic exposure.

To run our microplastic exposure experiment, it was necessary to hold the anemones in flow-through seawater while maintaining contact with microplastic particles. To accomplish this, we glued a clear, flat plexiglass cap on the bottom of 5-cm-tall sections of clear, round plexiglass tubing (7.6 cm diameter). We then drilled a 4.5 cm circular hole in the upper half of the tube wall and hot-glued a 60 μm Nitex mesh screen over the hole. The containers were placed in a flow-through sea table with the water level rising above the Nitex but below the open top of the tube. This allowed seawater to flow across the Nitex while preventing loss of the microplastics. We also prefiltered the seawater source with a 60 μm filter to minimize particulate matter entering the experimental chambers.

Microfiber preparation

Plastic microfibers were generated from a red (for ease of visual identification), 100% polyester t-shirt by cutting the fabric into small pieces then running the pieces through a kitchen blender at high speed to break up and mechanically wear the fabric strands. We then used a consumer grade hair clipper to "massage" the material, further separating and wearing the microfibers.

To constrain the sizes of our fibers, we next mixed them in tap water and used Nitex sieves to remove fibers below 63 μm and above 1000 μm. Finally, the microfibers were collected in aluminum foil dishes, dried for 24 hours at 60 degrees Celsius, then stored in Ziplock baggies until they could be weighed and added to the anemone treatment containers.

To express our microfiber concentration in both particles per ml and g per ml (permitting comparison with other studies), a 1 x 1 cm piece of the original fabric was weighed. Under the microscope, the structure of the fabric was observed, the number fibers within each thread

counted, and the total length of fibers in that piece of fabric calculated (Figure 2). This allowed us to calculate a weight by length relationship of the microfibers. Next, a sample of the shredded fabric was weighed then examined under the microscope where we used Image J software (Rasband 1997-2018) to measure the lengths of 50 arbitrarily chosen fibers in each sample. Using the average length of those fibers, we calculated the number of microfibers per unit weight of the sample. These values allowed us to express the total amount of microplastic we added to the experimental chambers in both grams and number of particles.

Experiment setup

To start the experiment, a single *A. elegantissima*, categorized as green or brown was placed in each plexiglass container and microfibers were added at concentrations of 0, 0.01, or 0.1 g/l of seawater. Ten anemones (7 green, 3 brown) received 0 microfibers, 30 anemones (18 green, 12 brown) received 0.01 g/l, and 17 anemones (17 green, 12 brown) received 0.1 g/l. The anemones had been sorted by size and were distributed systematically through the experiment so that each treatment received a diversity of anemone sizes. The treatments were systematically arranged in a single seawater table and subjected to ambient lighting and seawater temperature.

The experiment was started with addition of the microplastics to the containers on August 31, 2018. The containers were drained, cleaned, and fresh measures of the microfibers added at 7–10-day intervals afterward until October 8 when final measurements were taken with a total of 38 days of exposure to microplastic treatments.

Measuring Anemone Responses

To measure impacts of microplastic exposure on anemone size, oral disk diameters were calculated using the average of two perpendicular measurements across the oral surface intersecting at the mouth. Measurements were made using Image J software (Rasband 1997-2018) with digital photographs of each anemone taken on August 13 before the experiment started and on October 8 at the end of the experiment.

One possible impact of microplastics could be interference with feeding and digestive efficiency of the anemones. To test that possibility, we fed each anemone pre-weighed pellets of squid mantle that were approximately 5% of the estimated anemone wet weight as calculated from measured oral disc diameters (see Hiebert and Bingham 2012 for details of those calculations).

The anemones were first fed on August 17 then at 7-10-day intervals thereafter approximately 48 hours prior to cleaning of the plexiglass chambers and replacement of the microplastics. Pellets were cut from thawed squid mantle with a cork borer before being weighed on a scale.

Twenty-four hours after each feeding, we collected any egested material from the chamber and froze it at -80° C until it could be processed. Processing involved drying each pellet at 60° C for 24 hours, carefully picking out salt crystals left behind, and weighing what was left. While there were sometimes microplastic fibers adhering to the egested pellet surface, it was not possible to remove them without losing food material, so we assumed their contribution to weight of the egested pellet was negligible.

To calculated digestive efficiency, we converted the initial pellet wet weights to dry weights by weighing 10 control squid mantle pellets of different sizes before and after drying.

Using linear regression, we created an equation that allowed us to calculate dry weight from wet weight:

$$\text{dry weight} = 0.2147 * \text{wet weight} + 3.48 \times 10^{-4}$$

Digestive efficiency was then calculated as the percent difference in mass of the pre-ingestion squid pellet and mass of the egested material we collected from the container 24 hours later.

We used respiration rate as another potential indicator of anemone stress and measured respiration of each anemone on September 7, 14, 22, and October 2. The measurements were made at the same time the water and microplastics in the plexiglass container were changed as part of the renewal process 48 hours after the anemones had been fed. To make the measurement, the anemones were transferred to a 1-liter glass jar with a PreSens O_2 dot attached inside. The jars were completely filled with filtered seawater, capped to make an air-tight seal, and wrapped with aluminum foil to block out light that could stimulate photosynthesis of the anemones' symbionts. Anemones were given 30 minutes to acclimate to the dark before initial readings were taken. O_2 readings in each jar were made with the PreSens Precision Sensing Fibox 4 Fiber optic oxygen transmitter, which collects an O_2 reading by shining a laser onto the dot inside the jar and interpreting the reflectance. The meter also has a temperature probe and internal barometer that corrects for temperature and pressure. A salinity correction, based on measurements from the Shannon Point Marine Center seawater system, was manually entered. Prior to O_2 measurements, the dots were calibrated using a 2-point calibration of oxygen-depleted and 100% saturated seawater blanks. Initial O_2 in the jar was measured prior to the introduction of the anemones. A second measurement was taken 20 – 60 minutes after the anemone was introduced. Each week, prior to respiration measurements with anemones, blank

test measurements were taken to account for bacterial respiration. The bottles were held in an insulated container to maintain constant temperature during the measurement period. Respiration rates were calculated as the change in dissolved O_2 over time per anemone biomass with biomass calculated as a function of average measured oral disc diameter using the following equation from Dimond et al. (2011) where oral diameter is measured in cm and protein biomass in mg.

$$\text{protein}=45.55e^{(0.650*\text{diameter})}$$

Measuring responses of the symbionts

The density of symbionts in each anemone was quantified 22 days before the start of the experiment by cutting three individual tentacles from each individual, combining them in a microcentrifuge tube with 1 ml of filtered seawater, then grinding the tissue with an automatic mechanical stirrer. The homogenized samples were stored at -80° C until they could be later counted. To count the symbionts, the homogenates were thawed then vortexed well before a sample was pipetted onto a hemacytometer for microscopic examination. Replicate 1 mm^2 square sections of the hemocytometer were examined until at least 800 symbiont cells had been counted. The number of cells per ml of tentacle homogenate could then be calculated based on the number of cells counted and the number of hemacytometer sections examined.

Because the two symbionts (*B. muscatinei* and *E. marina*) are different in both color and size, we could easily distinguish and count each under microscopic examination (Figure 3). While examining the samples, we also determined mitotic index (the percent of the cells that were actively dividing) by identifying and counting symbionts with a clearly defined cleavage

furrow. Mitotic index has been used as an index of stress with rates of division decreasing with increasing stress (Verde and McCloskey 2007).

To determine symbiont densities from counts it was necessary to determine protein content (µg/ml) of the tentacle homogenates. This allowed us to convert counts (symbionts per ml) to symbionts per µg protein. We used a Thermo Scientific Pierce™ BCA Protein assay kit to prepare samples for microplate reading on a Biotek Synergy LX microplate reader, following the assay kit microplate procedures with a working range of 20-2000 µg/ml. We used two replicate samples of each anemone homogenate and ran each microplate three times since the absorbance values can change over time due to the dynamic nature of the dye. The three readings were averaged to give a single value for each anemone and the reading was converted to protein concentration by comparison to a protein standard curve.

Thinking that microplastics could potentially affect photosynthesis of the *A. elegantissima* symbionts, we used a pulse-amplitude modulated fluorometer (Diving-PAM II, Walz, Germany) to measure the photochemical efficiency of photosystem II of the photosymbionts within the tissues of their host anemones before and during the microplastic exposure. Measurements were taken on September 9, 22, and 27 by holding anemones in a dark room for 30 minutes to completely relax photosystem II. We then submerged the waterproof fiber optic tip of the PAM fluorometer, holding it approximately 5 mm above the exposed oral surface of the anemone. The instrument sent out a weak pulse of light to measure minimum fluorescence (F_o) of the dark-adapted photosystems before using a saturating pulse to overwhelm photosystem II, yielding a measurement of maximum fluorescence (Fm). The difference between the minimum (F_o) and maximum (Fm) measured values gave the variable fluorescence (Fv) used to calculate the maximum quantum efficiency of photosystem II as the ratio Fv/Fm

(Kitajima and Butler 1975). Symbionts that were stressed in some way are predicted to show lower Fv/Fm ratios.

Statistical analysis:

We used the open-source statistical software, R (R core team, 2020, R studio team, 2021) and a mixed effects model analysis using the R Package *nlme* (Pinheiro et al. 2021). We visualized the data using the R package *ggplot2* (Wickham 2016). We examined the effects of time (days of exposure to microplastic), microfiber concentration, and symbiotic state (hosting *B. muscatinei* or *E. marina*), and all two and three-way interactions of those factors on anemone size, digestive efficiency, and anemone respiration rate, symbiont density, symbiont mitotic index, and Fv/Fm. The mixed effects model allowed for repeated measurements taken from each anemone over the course of the experiment, and we were able to fit random intercepts for each anemone and random slopes for each anemone over time where appropriate to account for the non-independence of the measurements and to improve the model fits. Our model fitting process included the following steps:

1. We fit a saturated fixed-effects model with all main effects and interactions of the microplastic concentration treatment, the symbiont algal species associated with the anemone, and time of the measurements. (i.e., Days, Concentration, Symbiont, Days:Concentration, Days:Symbiont, Concentration:Symbiont, and Days:Concentration:Symbiont). We added an additional effect of anemone size and all possible interactions to our model for digestive efficiency. Since our measurements of symbiont density and mitotic index were done independently for *E. marina* and *B.*

muscatinei, we ran separate models for each thus removing the need for a symbiont factor and related interactions in those analyses.

2. We tested the effects of adding random intercepts and random slopes for each anemone to the model using REML (restricted maximum likelihood) to fit the model which was assessed with AIC values to determine the value of those random effects. If inclusion of the random terms lowered the AIC value, they were retained in the model.
3. We refined the fixed effects using ML (maximum likelihood) fitting, by removing factors that appeared to have little predictive value (P-values > 0.1) and checking for changes in AIC. If the AIC dropped with removal of a term, that term was left out of a model. If a higher-order interaction was significant, the main effects contributing to that interaction were retained regardless of their individual significance values.
4. The final reduce model was fit with REML to get model coefficients and accurate significance values for each predictor and interaction.

Separate mixed model analyses were run for each of our outcome measurements and marginal r^2 (the percentage of variance explained by fixed effects) and conditional r^2 (the percentage of variance explained by both fixed and random effects) were calculated using the R package *r.squaredglmm* (Jaeger 2017), which employs the methods of Nakagawa et al. (2017).

Results

Through our processing of the polyester t-shirt, we produced microfibers ranging from 50- 2000 µm in length with an average of 450 µm (± 303 µm, SD). Using this average length, we

calculated the concentrations of particles in our microplastic treatments (0, 0.01, or 0.10 g/L) to be equivalent to 0, 5.04 x 10^4, or 5.04 x 10^5 particles/L.

Over the 38 days of our experiment, the anemones were generally open and responsive. Seawater supplied to the sea tables were consistent with 30.5 ($\pm$0.1s.d.) ppt salinity and water temperature of 11.9 ($\pm$0.1s.d.) degrees Celsius. Most wandered within their containers, but none escaped. However, ten of the smaller anemones died before the experiment ended and had to be removed. It did not appear that the mortality was caused by the microplastics since the deaths were scattered among the treatments with four occurring in the "high" and six in the "low" microplastic treatments and ranged in the time of death from the first week to the last.

Over time in the containers, the microplastic fibers tended to gather at the water's surface or at the bottom though weekly cleaning of the containers and replenishment of the microplastics resuspended the fibers. Through their movement and active feeding behaviors, the anemones regularly contacted the fibers, and the red threads could be seen adhering to the tentacles, oral disk, and body column of the anemones.

To determine whether microplastic exposure affected anemone growth, we measured oral disc diameter 18 days prior to the experiment and again after 38 days of continuous microplastic exposure. The results (Figure 4) show a general decrease in size of all anemones over the period of the experiment. Green anemones (those hosting *E. marina*) decreased in size at a similar rate in all the treatment conditions. Brown anemones (those hosting *B. muscatinei*) lost size at similar rates in the two microplastic conditions, but the slope of the loss looked much greater in the control (no microplastic) conditions. However, the 95% confidence intervals for the regression are broad making it difficult to draw strong conclusions based solely on the figure, but our model

did suggest a significant three-way interaction between microplastic concentrations, symbiont type, and day.

The final mixed model for anemone size over time included random intercepts for each anemone and all the fixed factors and interactions of day, symbiont, and microplastic concentration (Table 1). AIC values indicated that the concentration:symbiont:day term should be retained, so all lower-order interactions and factors were also kept in the model. The results indicated that day was the strongest predictor of oral disk diameter, pointing to the overall decrease in size over time in all treatments with the coefficient indicating an average drop of 0.012 cm/day. However, the concentration:symbiont:day interaction term suggests that the effect of the different microplastic concentrations over time was different for green and brown anemones, presumably pointing to the more rapid drop in size of brown anemones in the no-microplastics condition. This model, incorporating both the random variability attributable to individual anemones and the fixed factors explained 60% of the pattern in anemone oral disk diameters over the course of the experiment.

Because digestive efficiency could be influenced by size of the anemone, with larger anemones having larger and more complex gastrovascular cavities, we included anemone size as a factor in our analysis of anemone digestive efficiency. The full model results, which included random intercepts for the anemones, showed no particularly strong predictors when all main effects and interactions were included (Table 2).

Using AIC values to guide refinement of the full model, we found that the most parsimonious final model included the interaction effects of day:size and size:concentration and the contributing factors (Table 3). This reduced model, again including random intercepts for each anemone, accounted for 38% of the pattern in digestive efficiency.

Figure 5 shows the size:concentration interaction. Smaller anemones had lower digestive efficiencies in all treatments, though this effect was less obvious in the anemones without microplastics. The effect was increasingly pronounced as the concentration of microplastics increased; smaller anemones in the higher microplastic treatments had a harder time digesting the food. This difference however, got smaller as the anemones got larger. The same experiment plotted over time (Figure 6) shows that anemones in all treatments decreased in their digestive efficiency over the duration of the experiment, and Figure 7 shows the day:size interaction on anemone digestive efficiency but with no effect of microplastics. Smaller anemones decreased more in digestive efficiency over time than did large anemones.

Respiration rates of the anemones varied little over time or between treatments, though anemones hosting the symbiont *B. muscatinei* had slightly lower respiration rates on average than anemones hosting *E. marina* (Figure 8). There was no obvious indication that microplastic concentration had any effect on respiration. The full LMM analysis, which included random intercepts for each anemone and an adjusted covariance structure across days to improve the model fit, showed few patterns beyond a possible decrease in respiration over time (Table 4). AIC-guided refinement of the full model produced a final model that included day, symbiont, microplastic concentration and a two-way interaction between day and symbiont as predictors despite relatively high p-values in some cases (Table 5). However, the coefficients for these terms were very small, and the final model accounted for only 2% of the variability in *A. elegantissima* respiration suggesting little pattern in the data.

The algal symbionts of *A. elegantissima* photosynthesize in the light and contribute to the metabolic requirements of their host. We measured photosynthetic efficiency (*Fv/Fm*) of the symbionts within their hosts to determine whether the ability of the symbionts to photosynthesize

was affected by the host anemone's exposure to microplastics. The data showed distinctly different patterns for the two symbionts with the photosynthetic efficiency of *B. muscatinei* increasing and that of *E. marina* decreasing over time in the experiment. The respective slopes of each symbiont type seem relatively constant between treatments, but the intersection point between the symbiont types advanced in time with increasing microplastic concentrations (Figure 9). Our full LMM model included random intercepts for each anemone (Table 6). The final reduced model, with an optimized AIC score, included the day:symbiont and symbiont:concentration interactions (Table 7) suggesting that the pattern of *Fv/Fm* over time differed for the two symbionts and that the microplastic treatments affected the two symbionts differently. Figure 9 suggests that the overall *Fv/Fm* of *E. marina* increased with increasing microplastic concentration but the *Fv/Fm* of *B. muscatinei* fell. The final model accounts for 44% of the variability in *Fv/Fm* of each anemone and their symbionts.

Initial and final symbiont density counts were made for each anemone and patterns were analyzed as a function of time and microplastic concentration. Because the two symbionts have distinct natural histories, and we knew they might respond differently to being moved to laboratory conditions, we analyzed anemones in each symbiotic state independently to better asses the effect of the microplastic treatments. The results for *E. marina* show a similar decrease in density in all treatments over time (Figure 10). In contrast, *B. muscatinei* showed little change over time in the control and low microplastic treatments but a dramatic decline in the high microplastic concentration (Figure 11).

The LMM analyses of these data included random intercepts for the anemones, and we changed the variance structure allowing for different variances on each day of measurement. The full model for *E. marina* symbiont densities (Table 8) showed no particularly strong predictors,

and AIC-guided refinement produced a final model with only day as a predictor. This final model explained only 6% of the pattern in the data (Table 9).

In contrast, the final model for *B. muscatinei* symbiont densities, which was the same as the full model, included both day and concentration and a day:concentration interaction. The interaction confirms the much stronger negative response of the symbionts in the high microplastic treatment. The final model accounted for 35% of the pattern in the data (Table 10).

Similar to anemone symbiont density counts, the symbiont mitotic index ratios were taken initially and at the end of the experiment for samples from each anemone, and separate analyses were run for each symbiont. The data for *E. marina* showed a slightly positive trend for the control and low microplastic treatments and a slightly negative slope in the high microplastic treatment. However, the variability in these responses, as indicated by the 90% confidence intervals on the regression slopes, was quite large (Figure 12).

The LMM analysis for these data included random intercepts for anemones and a covariance structure that allowed for different variances on each day of measurement (Table 11). Model refinement again produced a final model that included only day. The coefficient for day was extremely low and the final model explained only 0.3 % of the overall pattern (Table 12), suggesting the absence of any real pattern.

The *B. muscatinei*, however, showed a different pattern in mitotic index with a clear negative slope in all treatments (Figure 13). The full model, which again included random intercepts for anemones and a covariance structure that allowed for different variances on each day of measurement suggested a strong effect over time (Table 13). The final model, confirmed that the only significant factor was day, indicating that microplastic concentration had little

impact on mitotic index of the *B. muscatinei* symbionts. This final model explained 24% of the pattern in the mitotic index (Table 14).

Discussion

Our experimental goal was to see if we could measure any change in the anemone *A. elegantissima* and its symbionts after exposing them to microplastics for 38 days by measuring a variety of features that reflect performance of the anemones and of their symbionts. All our results show changes in our measured feature as a function of time. The effects of microplastics, however, were complex. When microplastic concentration created an effect, that effect was generally tied to at least one other factor in an interactive way (Table 15). Therefore, to understand the microplastic effects, we must first understand the underlying patterns of the anemones and their symbionts over time.

The temporal changes observed in the anemones and in their photosymbiont partners indicate an effect of environmental variables. Previous studies have established that *A. elegantissima* vary in size over an annual cycle (Sebens 1982b). They grow in the spring and summer but then began shrinking in late summer and fall as they switch their metabolism from food freshly acquired by heterotrophic feeding or through translocation from their photosymbionts to a metabolism based on lipids stored in body tissues when food and light become less available (Fitt and Pardy 1981, Ponce-McDermott 2012). Our experiment coincided with a seasonal transition as summer turned into fall and as the anemones presumably began to make this switch. This alone might explain our observed decrease in anemone size, but it is hard to separate that from any additional stress related to collection and movement to laboratory conditions. Photosynthetic activity of the symbionts undoubtedly dropped as the anemones were

moved from outside sunlight to an indoor sea table with indirect light filtered through north-facing windows. At the same time, opportunities for natural heterotrophic feeding disappeared as the anemones were held in filtered seawater. We attempted to compensate by regularly feeding the anemones pellets of squid mantle, but the drop in anemone size suggests that they were not feeding as much or receiving as much fixed carbon from their symbionts as they might have under field conditions.

While size decreased in all anemones, our model results (Table 1) suggest that there was a significant difference in the rate of decrease over time between microplastic concentrations and symbiont type. While anemone's hosting *E. marina* showed a similar drop in size across microplastic treatments, anemone hosting *B. muscatinei* lost the most size in the no-plastic control group with progressively less loss as microplastic concentrations increased (Figure 4). The relatively large change in size for brown anemones in the control group may be related to low sample size and high variability. We have no other explanation for this counterintuitive result (e.g., that anemones hosting *B. muscatinei* shrunk less when more microplastic were present) and can only conclude that something about the energetics of hosting different symbionts affected the way the anemones responded to the microplastics.

Anemone body size is a crucial metric of anemone fitness because a larger feeding surface area increases the potential for prey capture and because a larger anemone has more surface area for digestion and can process larger prey (Sebens 1982c). For this reason, we included oral disc diameter as a potential factor in our model testing for effects of microplastic exposure on the anemones' digestive efficiency.

As was seen in our other analyses, digestive efficiency changed over time probably reflecting the effects of a changed environment on anemone physiology. There was also some

evidence in the interaction of time with anemone size that the digestive efficiency of small anemones dropped more over time than did that of larger individuals (Figure 7, Table 3). The analysis also showed an overall decline in anemone digestive efficiency over time. We believe this was a result of the anemones being moved to laboratory conditions, though the late season alone may have also contributed to a change in feeding behavior. Our analysis showed no relationship of digestive efficiency to symbiont type. This was not surprising since *A. elegantissima* is facultatively symbiotic and not reliant on fixed carbon provided by the symbionts as is the case in symbiotic corals. Instead, these anemones rely primarily on heterotrophic feeding that is merely supplemented by photosynthetically derived carbon (Sebens 1981, 1982a, Fitt 1982, Hiebert and Bingham 2012).

Our analysis also showed that the effect of microplastic concentration on digestion varied with anemone oral disk diameter. With no microplastics present, smaller anemones digested less of the food we gave them than did larger individuals (Figure 5). That difference, however, became more pronounced as the amount of microplastic present increased such that the smallest anemones in the highest microplastic concentrations performed the worst (Figure 5). Since the surface area of the gastrovascular cavity is dependent on the size of the anemone, it makes sense that smaller anemones might be less effective digestors. It seems that microfibers disproportionally interfered with the ability of these smaller anemones to ingest or digest their food and could explain the anemone body size by microplastic concentration interaction.

Microplastics are notable for their tendency to be ingested by lower trophic levels (Andrady 2011) and subsequently biomagnified in the bodies of larger predators (Lusher et al. 2017). As the non-digestible materials fill the digestive system, the organisms are wasting energy and digestive space to capture, consume, and process items that have no caloric value. There is

also potential for damage from mechanical blockage of the digestive tract, further limiting the ability of the animal to efficiently consume and digest food in a negative feedback cycle.

Hierl et al. (2021) reported that coral species with larger polyps may be able to better control uptake and egestion of microplastics than species with smaller polyps. The symbiotic *A. elegantissima* we tested are single large polyps with large mouths and digestive cavities. We would expect, therefore, that they might be better able to control ingestion of microplastics. To investigate possible clogging of the *A. elegantissima* gastrovascular cavities, we preserved several of our anemones at the end of the experiment and dissected them to see if we could locate microplastics. Despite the fact that red microplastic fibers were readily observed adhering to the body column, tentacles, and oral disk of the anemones, careful examination with a dissecting microscope revealed no fibers within the gastrovascular cavity.

We believe that the absence of ingested fibers is a combination of two processes. First, feeding behavior of the *A. elegantissima* is not stimulated by plastic microfibers. Though the fibers may be present across the tentacles and oral disk, there is no chemical or mechanical stimulus to induce a feeding behavior. During our experiment it was common to see fully expanded individuals with microfibers scattered across the oral disk and tentacles making no effort to ingest them. Second, when food was offered, the anemones captured and moved the pellets across the oral disk and into the mouth, picking up microfibers along the way. We believe those adherent microfibers were ingested since microparticles were evident within the bolus of mucus and waste the anemones egested a short time later. We also believe any microplastic particles that had been ingested were effectively egested prior to our final collection and preservation. *A. elegantissima* seem to be efficient at removing indigestible materials. The microplastic were simply not retained in the gastrovascular system for any significant period or

in any significant volume. Our experiment focused on the difference between anemones hosting two distinct symbionts, but we did not test asymbiotic anemones. Interestingly, De Orte et al. (2019) reported that symbiotic state (i.e., symbiotic or asymbiotic) of the facultatively symbiotic anemone *Aiptasia pallida* affects their ability to reject microplastics. We saw no such distinction between *A. elegantissima* hosting green or brown symbionts in our study.

A. elegantissima often inhabit and thrive in wave-washed intertidal zones where they are exposed to and ingest all manner of debris including shell bits, pebbles, and sand (Littler et al. 1983, Pineda and Escofet 1989, Hossfeld et al. 2020). An animal in this niche would have to be highly efficient in cleaning out its gastrovascular cavity, and we believe that was the case with the anemones in our study. If *A. elegantissima* in the field capture larger microplastic particles those could harbor diverse assemblages of microbial life and fouling invertebrates, and the anemone could potentially receive some nutrition by retaining the plastic, digesting the biofoul, and egesting the cleaned plastic (Zettler et al. 2013, Martinez-Campos et al. 2022). This, however, would not be the case with microfibers, which lack the surface area to accumulate much carbon. Furthermore, the space required to hold non-digestible plastic, if competing with more appropriate food items, could offset any potential benefits of the energy acquired from the digestion of fouling organisms. More importantly, a variety of toxic materials, including hydrocarbons and heavy metals, can be adsorbed to plastics (Teuten et al. 2009, US EPA 2016, Wang et al. 2018), and these could certainly have negative effects on the feeding anemones. We expect, therefore, that non-digestible materials are egested quickly by *A. elegantissima.*

The plastic microfibers were replaced weekly in our experiment and were unlikely to have formed any significant microbial biomass useful as a resource for the anemones. At the same time, putting in fresh material would have replaced any chemicals leached from the red

polyester microfibers and could, over time, have led to negative effects. We did not investigate that, but it is known that chemical additives present in plastics, including those in color dyes, can have strong negative effects on some invertebrate species. For example, Aminot et al. (2020) found that polystyrene fragments leached significant amounts of flame retardants like hexabromocyclododecane (HBCDD) that bioaccumulated in coral tissues. Leachates from the polystyrene consistently caused polyp retraction, suggesting that the corals were avoiding ingestion of the material. In contrast, Rotjan et al. (2019) found that the temperate, facultatively symbiotic coral *Astrangia poculata* preferentially ingested microplastic compared to brine shrimp eggs. However, polyps fed biofouled microplastics died within four weeks, suggesting the accumulation of toxic materials. Rocha et al. (2020) found that the cnidarian zoanthid *Zoanthus sociatus* react differentially to different microplastic polymer types with polyvinyl chloride (PVC) microplastics being the most detrimental. A recent study found that environmentally aged microfiber leachate was much less toxic than fresh microfiber leachate to mysid shrimp (*Americamysis bahia*) with fresh red polyacrylonitrile fibers being the most toxic (Johnson 2021). It is possible that our red polyester microfiber treatments had some nonlethal toxic effect on the anemones, but we did not test that. It is a possibility that should be pursued.

Verde and McCloskey (1996) reported that respiration rates track anemone size and digestive efficiency in decreasing from summer months into the winter. The respiration rates of our anemones dropped slightly over the course of our experiment (Figure 8) with that pattern most noticeable in the low and high microplastic treatments. It is interesting to note that the trends in respiration rates appear to diverge depending on symbiont association with anemones hosting *B. muscatinei* being more strongly affected than those hosting *E. marina*.

As intertidal invertebrates, *A. elegantissima* are common occupants of mid-intertidal pools where they can be left in stagnant water for hours at a time, illustrating their ability to tolerate stressful conditions (Sebens 1982a, Jensen and Muller-Parker 1994). Recent observations confirm the ability of *A. elegantissima* to even persist while being completely buried in sand for up to 9 months (Hossfeld et al. 2020). This resilience in the face of changing environmental conditions probably accounts for the lack of obvious respiratory responses to the addition of microplastics in our experiment.

Environmental conditions including seasonal trends in light and temperature affect not only *A. elegantissima*, but also their symbionts. The two photosymbionts of *A. elegantissima* are dramatically different from each other with each carrying its unique evolutionary history and symbiotic strategy that can even dictate basic life history strategies of the host anemone (Verde and McCloskey 1996, 2007, Dimond et al. 2011, Bingham et al. 2014). In our experiment, we found that measures of symbiont performance (*Fv/Fm*, symbiont density, symbiont mitotic index) changed over time, but the pattern of change was distinct to each symbiont species, with limited evidence of microplastic effects.

Fv/Fm measures photosynthetic efficiency and is used as a common indicator of stress to the photosystems (Maxwell and Johnson 2000, Baker 2008). In our experiment, the *Fv/Fm* of *E. marina* decreased over time while that of *B. muscatinei* increased (Figure 9). Previous research established that *B. muscatinei* is the more tolerant photosymbiont, outperforming *E. marina* in most conditions but particularly with increased temperatures and irradiance, which it can survive without producing as many destructive reactive oxidation species (Dimond et al. 2017). This higher tolerance allows *A. elegantissima* hosting *B. muscatinei* to dominate in the higher intertidal and, if climatic warming continues, we may eventually see it replace *E. marina* as the

sole form of *A. elegantissima* (Dimond et al. 2011). However, while *E. marina* is more sensitive to environmental conditions, it seemed unaffected by the microplastic treatments. In contrast, as microplastic concentrations increased, *B. muscatinei Fv/Fm* declined (Figure 9). Our final model highlights this in the interaction between symbiont type and microplastic concentration (Table 7) and suggests that *E. marina* may be the more robust of the two symbionts under conditions of elevated microplastics. We do not know by what mechanism the microfibers affect the symbiont's *Fv/Fm*.

Each symbiont-host relationship responds to seasonal changes differently as *A. elegantissima* can expel symbionts to lower symbiont densities, while the symbionts, under favorable conditions, can divide more frequently, increasing their density. This pull-push dynamic is a function of elevated productivity of the symbiont, which can contribute to the anemone's energy reserves, but also potentially exposes it to photosynthetically produced oxidative chemicals that can damage its tissues (Dimond et al. 2017). To index the effects of microplastics on these two processes, we measured both symbiont density within the host tissues and symbiont growth rate as estimated through measurements of the proportion of actively dividing symbiont cells (mitotic index).

Symbiont densities and mitotic indices were analyzed independently for each symbiont type allowing us to compare relative trends and assess what was happening between the symbionts and the host during our experiment. Since we measured these parameters before and after 10 weeks of microplastic exposure, we hoped to see changes related to the effects of the microplastics. However, the patterns we observed must still be interpreted within the context of environmental changes to which our experiment exposed the host anemones and the limitation of

having only two measurement points over time (which was done to reduce the impact of cutting tentacles from the anemones).

We found that *E. marina* cell densities were approximately twice as high as those of *B. muscatinei* as described in previous studies (Verde and McCloskey 1996, Dimond et al. 2011). Because *E. marina* is smaller, it can exist in higher densities within the host cells (Verde and McCloskey 1996). *E. marina* in our experiment had a much higher mitotic index than *B. muscatinei* as has also been reported previously (Verde and McCloskey 2007). Interestingly, the trends over time were distinct for the two symbionts with *E. marina* mitotic index increasing over time (Figure 12) while the *B. muscatinei* mitotic index decreased (Figure 13). Mitotic indices vary by season with *E. marina;* in general, division rates increase in the spring and summer and decrease in fall and winter (Verde and McCloskey 2007, Dimond et al. 2011).

Previous studies describe stable symbiont densities throughout the year for both algal species in non-laboratory settings (Bergschneider and Muller-Parker 2008, Dimond et al 2011). However, *B. muscatinei* showed a large decrease in density over time in the high microplastic treatment while symbiont densities in the control and low microplastic treatments were relatively stable (Figure 11). This was reflected in the microplastic concentration by time interaction in the *B. muscatinei* model (Table 10). This result suggests again that the symbiotic relationship of *B. muscatinei* to its anemone host was somehow affected by our microplastic treatments.

Since symbiont densities in the host are the result of the balance of symbiont growth and anemone expulsion, we can explore whether the effects we saw resulted from a symbiont response or from an anemone response by also examining the symbiont mitotic index. We found that the mitotic indices of *E. marina* stayed relatively constant throughout our experiment in all treatments (Figure 12), suggesting that the symbiont growth rates were unaffected by

microplastic exposure. The drop in *E. marina* density in their *A. elegantissima* hosts (Figure 10), therefore, appears to have resulted from an increase in expulsion by the anemones, with no difference among the microplastic treatments, again suggesting that the change in symbiont density was unrelated to the microplastics.

In contrast, the mitotic index of *B. muscatinei* decreased over time in all the microplastic treatments (Figure 13), suggesting that movement from the field to the laboratory was responsible for the decreased symbiont growth rates. However, while overall symbiont densities stayed relatively constant in the no microplastic and low microplastic treatments, we saw a significantly greater decrease in symbiont density among anemones hosting *B. muscatinei* in the high microplastic treatment (Figure 11). Together these results suggest that the symbionts did not react to the microplastics, but that the host responded to the high microplastic treatment by expelling more symbionts.

In our experiment we wanted to see if anemones and their symbiotic relationship could be affected by exposure to plastic microfibers. With a few notable exceptions (e.g., Hierl et al., 2021, Jiang et al., 2021) many studies of microplastics last from a few hours to a few days at most (e.g., Axworthy and Padilla-Gamino 2019, De Orte et al. 2019). Because *A. elegantissima* has such distinct seasonal patterns, longer-term exposure seems very important. Though we followed the anemones through a full month of exposure, more work is needed to fully understand impacts, particularly considering changes in feeding, metabolism, and symbiotic relationships during the different seasons.

We found an environmental background effect that we assume to be related to the seasonality of the anemones and their symbionts and to their movement from the field to the laboratory. On top of seasonal changes, we found that microplastic treatments affected digestive

efficiency and anemone size but not anemone respiration. Anemone size decreased over time with anemones hosting *B. muscatinei* losing less as microplastic concentrations increased. However, anemones with *B. muscatinei* decreased in photosynthetic efficiency as microplastic concentrations increased and *B. muscatinei* density dropped significantly in the high concentration treatment of microplastic. Anemones hosting *E. marina* on the other hand decreased in overall performance but seemed to be unaffected by microplastic exposure. Our results seem to show that anemones hosting *B. muscatinei* may be affected negatively by microplastic exposure, but overall maintain a higher tolerance in conditions that result in anemones hosting *E. marina* to decline. While we were able to see some effects of microplastic exposure to anemone and symbionts, these effects seem to be minor compared to the external environmental factors that affected all measurements.

Environmental levels of microplastics are typically defined by either total mass or particles per volume of sampled water. The most recent reports record levels of up to 9.2 $x10^3$ particles/m^3 of seawater in the Queen Charlotte Sound of British Columbia (Desforges et al. 2014), a geometric mean in the inside passage of 0.092 ug/L, up to 0.08 mg/L in the Puget Sound surface waters (J. Masura, pers. comms.), and an average sum of 0.39 g/m^2 of microdebris along the wrack line of Puget Sound shorelines (Davis and Murphy 2015). Our concentrations of microplastic by weight, 0.1g/L and 0.01g/L were substantially higher than these levels. However, our densities of 51 and 514 particles per mL are in the range described in recent studies with the scleractinian coral *Stylophora pistillata* (Lanctot et al. 2020).

One of the major challenges to quantifying plastic pollution in general is the immense variability and heterogeneity of plastic debris (Fisner et al. 2017). Microplastic pollution along the west coast of North America is highly variable as debris is transported by dynamic and

shifting winds and currents. Microplastics that affect this stretch of coast may originate locally or could be remnants of the North Pacific gyre. Atmospheric transport to the ocean is also a highly likely source of small microfibers similar to those we use in our experiment (Zang et al. 2020).

One of the main issues in understanding microplastics in the environment is the wide variety in characteristics and properties that microplastics can have. Quantifying microplastic concentration for this reason must be a multidimensional endeavor that includes not only volume or number of particles and size ranges of those particles, but also polymer type, color, and shape. Many experiments in the published literature have used plastic microspheres, or microbeads purchased directly from plastics manufacturers for laboratory exposure experiments (Hall et al. 2015, Allen et al. 2017, Axworthy and Padilla Gamino 2019, Lanctot et al. 2020, Okubo et al. 2020, Hierl et al. 2021). Microfibers, however, are the dominant form of microplastic debris found in local waters (Desforges et al. 2014, Davis and Murphy 2015). polymer type, color, and shape.

Our experimental setup included only polyester microfibers, which is a gross oversimplification of what a microplastic exposure could entail in the natural environment. However, we believe using them in our experimental assay is not an unreasonable approach to testing potential impacts on the anemones. Our decision to use red microfibers was simply based on a desire to use a color that would be most make the fibers obvious in the experimental treatments. The different dyes used in plastics, however, have been shown to elicit differential feeding behaviors (Allen et al. 2017, Rotjan et al. 2019) and may show different levels of toxicity (Johnson et al 2021), *A. elegantissima* has not yet been studied in this regard but it is an interesting avenue for future work.

Anthopleura elegantissima is an incredibly robust marine invertebrate with a life history and suite of strategies that position it as a dominant species in the intertidal zone of the North American west coast. It can tolerate rapid changes in temperature and the stresses associated with repeated aerial exposure as tides rise and fall. And, despite living in a swirling soup of sand and other undigestible materials, it can capture the prey it needs while benefitting from additional carbon passed to it by symbiotic partners living in its tissues. Microplastics could disrupt capture, ingestion, and digestion of prey, act as a vector for disease, or accumulate and transport toxic chemicals to the anemones. The magnitude of effects we saw were relatively small: primarily reduced digestive efficiency in smaller anemones and differential impacts on symbiosis with *E. marina* and *B. muscatinei.* While it is unlikely that *Anthopleura elegantissima* will experience the levels of microplastics we exposed them to anytime soon, they will be dealing with increasing loads and microplastic complexity, with both repeated and chronic exposure as plastic manufacturing continues to increase (Lebreton and Andrady 2019) without adequate resources for diverting waste streams that contribute to the accumulating pollution in the marine environment.

Despite their resilience, these important animals, like those in all our world's oceans, are facing an ever-increasing rate of change related to a changing climate, ocean acidification, and pollution, and even minor effects like those we observed, could be sufficient to harm animals that may be increasingly pushed toward the limits of survival.

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Table 1. *A. elegantissima* oral disk diameter (full and final model results were the same). LMM analysis of microplastic effects over a 38-day exposure period. The full model included random intercepts for each anemone. The marginal and conditional r^2 values were 0.28 and 0.60 respectively

Factor	Coefficient	SE	df	T	p
Intercept	1.53	0.110	65	13.92	< 0.01
Day	-0.012	0.003	54	-0.20	< 0.01
Symbiont	-0.03	0.138	65	-0.22	0.82
Concentration	-0.002	0.009	65	-0.21	0.84
Concentration:Symbiont	0.008	0.010	65	0.80	0.42
Concentration:Day	3.0E-4	1.8E-4	54	1.64	0.11
Symbiont:Day	4.6E-3	3.2E-3	54	1.44	0.15
Concentration:Symbiont:Day	-4.5E-4	2.4E-4	54	-1.89	0.06

Table 2. *A. elegantissima* digestive efficiency (full model results). LMM analysis of microplastic effects over a 38-day exposure period. The full model included random intercepts for each anemone.

Factor	Coefficient	SE	df	T	p
Intercept	0.796	0.110	226	7.23	< 0.01
Day	-0.008	0.0057	226	-1.33	0.18
Symbiont	0.055	0.169	96	0.33	0.75
Size	0.090	0.063	96	1.44	0.15
Concentration	-0.011	0.009	96	-1.34	0.18
Day:Symbiont	-5.4E-4	9.1E-3	226	-0.59	0.55
Day:Size	1.7E-3	3.2E-3	226	0.53	0.60
Day:Concentration	6.0E-5	4.6E-4	226	0.13	0.90
Symbiont:Size	-0.047	0.099	96	-0.47	0.64
Symbiont:Concentration	0.009	0.014	96	0.70	0.49
Size:Concentration:	5.9E-3	5.2E-3	96	1.14	0.26
Day:Symbiont:Size	4.5E-3	5.3E-3	226	0.85	0.40
Day:Symbiont:Concentration	-3.9E-4	7.1E-4	226	-0.55	0.59
Day:Size:Concentration	1.2E-6	2.7E-4	226	0.00	0.99
Symbiont:Size:Concentration	-3.7E-3	7.9E-3	96	-0.47	0.64
Day:Symbiont:Size:	1.1E-4	4.1E-4	226	0.27	0.78
Concentration					

Table 3. *A. elegantissima* digestive efficiency (final reduced model results). LMM analysis of microplastic effects over a 38-day exposure period. The final model, based on the minimized AIC value, included random intercepts for each anemone. The marginal and conditional r^2 values were 0.21 and 0.38 respectively.

Factor	Coefficient	SE	df	T	p
Intercept	0.82	0.071	232	11.62	< 0.01
Day	-0.01	0.003	232	-3.27	< 0.01
Size	0.06	0.042	100	1.61	0.11
Concentration	-9.6E-3	5.0E-3	100	-1.90	0.06
Day:Size	3.7E-3	1.8E-3	232	2.06	0.04
Size:Concentration	5.7E-3	2.9E-3	100	1.82	0.07

Table 4. *A. elegantissima* respiration rate (full model results). LMM analysis of microplastic effects over a 38-day exposure period. The full model included random intercepts and slopes for each anemone.

Factor	Coefficient	SE	df	T	p
Intercept	0.025	0.009	191	2.87	< 0.01
Day	-6.34E-4	3.9E-4	191	-1.61	0.11
Symbiont	-5.74E-3	1.1E-2	65	-0.51	0.61
Concentration	-1.28E-4	6.5E-4	65	-0.20	0.85
Day:Symbiont	7.05E-4	5.0E-4	191	1.41	0.16
Day:Concentration	3.77E-6	3.0E-5	191	0.13	0.90
Symbiont:Concentration	8.00E-5	8.4E-4	65	0.09	0.92
Day:Symbiont:Concentration	-2.00E-5	3.7E-5	191	-0.54	0.59

Table 5. *A. elegantissima* respiration rate (reduced model results). LMM analysis of microplastic effects over a 38-day exposure period. The model included random intercepts and an adjusted covariate structure for day of measurement. The marginal and conditional r^2 values were 0.02 and 0.02 respectively.

Factor	Coefficient	SE	df	T	p
Intercept	2.70E-2	6.3E-3	193	4.26	< 0.01
Day	-5.94E-4	2.7E-4	193	-2.22	0.03
Symbiont	-5.03E-3	7.7E-3	66	-0.65	0.52
Concentration	-2.49E-4	1.8E-4	66	-4.26	0.17
Day:Symbiont	5.14E-4	3.4E-4	193	1.49	0.14

Table 6. *A. elegantissima Fv/Fm* (full model results). LMM analysis of microplastic effects over a 38-day exposure period. The full model included random intercepts for each anemone.

Factor	Coefficient	SE	df	T	p
Intercept	0.665	0.023	126	29.60	< 0.01
Day	0.002	0.001	126	2.02	0.04
Symbiont	0.090	0.029	65	3.15	< 0.01
Concentration	-0.001	0.002	65	-0.73	0.47
Day:Symbiont	-3.8E-3	1.2E-3	126	-2.98	< 0.01
Day:Concentration	9.1E-6	7.4E-5	126	-0.12	0.90
Symbiont:Concentration	0.003	0.002	65	1.59	0.12
Day:Symbiont:Concentration	-5.7E-5	9.5E-5	126	-0.60	0.55

Table 7. *A. elegantissima Fv/Fm* (reduced model results). LMM analysis of microplastic effects over a 38-day exposure period. The final model included random intercepts for each anemone. The marginal and conditional r^2 values were 0.19, and 0.44 respectively.

Factor	Coefficient	SE	df	T	p
Intercept	0.667	0.018	128	29.60	< 0.01
Day	0.002	6.7E-4	128	2.87	< 0.01
Symbiont	0.099	0.023	65	4.28	< 0.01
Concentration	-0.001	0.001	65	-1.37	0.17
Day:Symbiont	-4.3E-3	8.7E-4	128	-4.97	< 0.01
Symbiont:Concentration	0.002	0.001	65	1.82	0.07

Table 8. Density of *E. marina* symbionts in *A. elegantissima* (full model results). LMM analysis of microplastic effects over a 38-day exposure period including random intercepts for individual anemones and an adjusted covariance structure for days.

Factor	Coefficient	SE	df	T	p
Intercept	2764.60	234.03	31	11.55	< 0.01
Day	-11.35	6.86	31	-1.65	0.11
Concentration	-14.24	18.33	31	-0.78	0.44
Day:Concentration	-0.13	0.52	31	-0.26	0.80

Table 9. Density of *E. marina* symbionts in *A. elegantissima* (reduced final model results). LMM analysis of microplastic effects over a 38-day exposure period including random intercepts for individual anemones and an adjusted covariance structure for days. The marginal and conditional r^2 values were 0.06 and 0.06 respectively.

Factor	Coefficient	SE	df	T	p
Intercept	2632.52	167.5	32	15.72	< 0.01
Day	-12.61	4.8	32	-2.62	0.01

Table 10. *A. elegantissima* density of *B. muscatinei* symbionts (full and final model results). LMM analysis of microplastic effects over a 38-day exposure period including random intercepts for individual anemones and a covariate structure for days. The marginal and conditional r^2 values were 0.18 and 0.35 respectively.

Factor	Coefficient	SE	Df	T	p
Intercept	1486.25	218.63	19	6.80	< 0.01
Day	-0.30	4.86	19	-0.06	0.95
Concentration	28.51	16.61	19	1.72	0.10
Day:Concentration	-0.97	0.36	19	-2.63	0.02

Table 11. Mitotic index of *E. marina* symbionts in *A. elegantissima* (full model results). LMM analysis of microplastic effects over a 38-day exposure period including random intercepts for individual anemones.

Factor	Coefficient	SE	df	T	p
Intercept	0.1571	0.0156	32	10.04	< 0.01
Day	0.0005	0.0003	32	1.39	0.17
Concentration	-0.0005	0.0014	32	-0.42	0.68
Day:Concentration	-3.3E-5	2.5E-5	32	-1.34	0.19

Table 12. *A. elegantissima* mitotic index of *E. marina* symbionts (final reduced model results). LMM analysis of microplastic effects over a 38-day exposure period. This model included random intercepts for individual anemones. The marginal and conditional r^2 values were 0.003 and 0.003 respectively.

Factor	Coefficient	SE	df	T	p
Intercept	0.1523	0.011	33	14.12	< 0.01
Day	1.4E-4	2.3E-4	33	0.60	0.55

Table 13. *A. elegantissima* mitotic index of *B. muscatinei* symbionts (full model results). LMM analysis of microplastic effects over a 38-day exposure period including random intercepts for individual anemones and a covariate structure for days.

Factor	Coefficient	SE	df	T	p
Intercept	0.046	0.006	19	7.55	< 0.01
Day	-0.0006	0.0002	19	-3.98	< 0.01
Concentration	-0.0006	0.0005	19	-1.40	0.18
Day:Concentration	1.1E-5	1.3E-5	19	0.86	0.40

Table 14. *A. elegantissima* mitotic index of *B. muscatinei* symbionts (final reduced model results). LMM analysis of microplastic effects over a 38-day exposure period including random intercepts for individual anemones and a covariate structure for days. The marginal and conditional r^2 values were 0.24 and 0.24 respectively.

Factor	Coefficient	SE	df	T	p
Intercept	0.039	0.004	20	9.27	< 0.01
Day	-5.6E-4	1.2E-4	20	-4.77	< 0.01

Table 15. Summary of anemone and symbiont response to 38 days of exposure to microplastics.

Response	**Predictors retained in the final model**	Conditional r^2
Oral disk diameter	Day, Concentration:Symbiont:Day	0.60
Digestive efficiency	Day, Size, Concentration, Day:Size, Concentration:Size	0.38
Respiration	Day	0.02
Fv/Fm	Day, Symbiont, Day:Symbiont, Symbiont:Concentration	0.44
***E. marina* density**	Day	0.06
***B. muscatinei* density**	Day, Concentration, Concentration:Day	0.35
***E. marina* mitotic index**	Day	0.003
***B. muscatinei* mitotic index**	Day	0.24

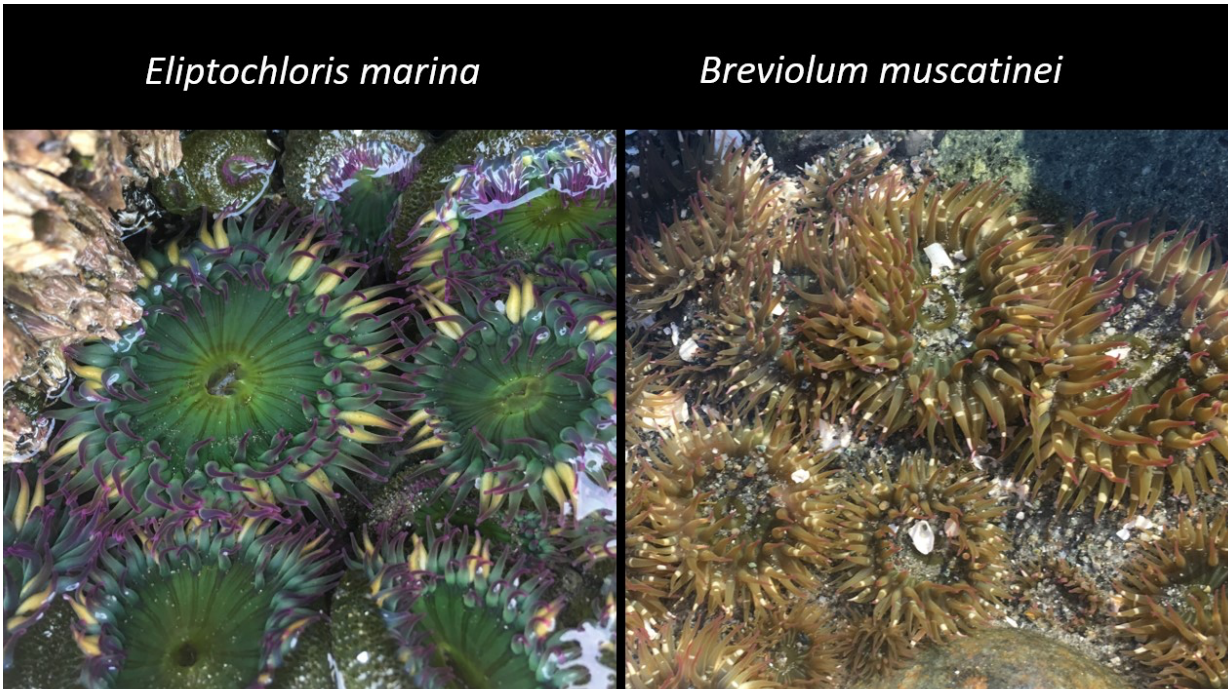

Figure 1. Photographs of the aggregating anemone *A. elegantissima* in the environment hosting the green, *Elliptochloris marina* (left) and the brown *Breviolum muscatinei* (right).

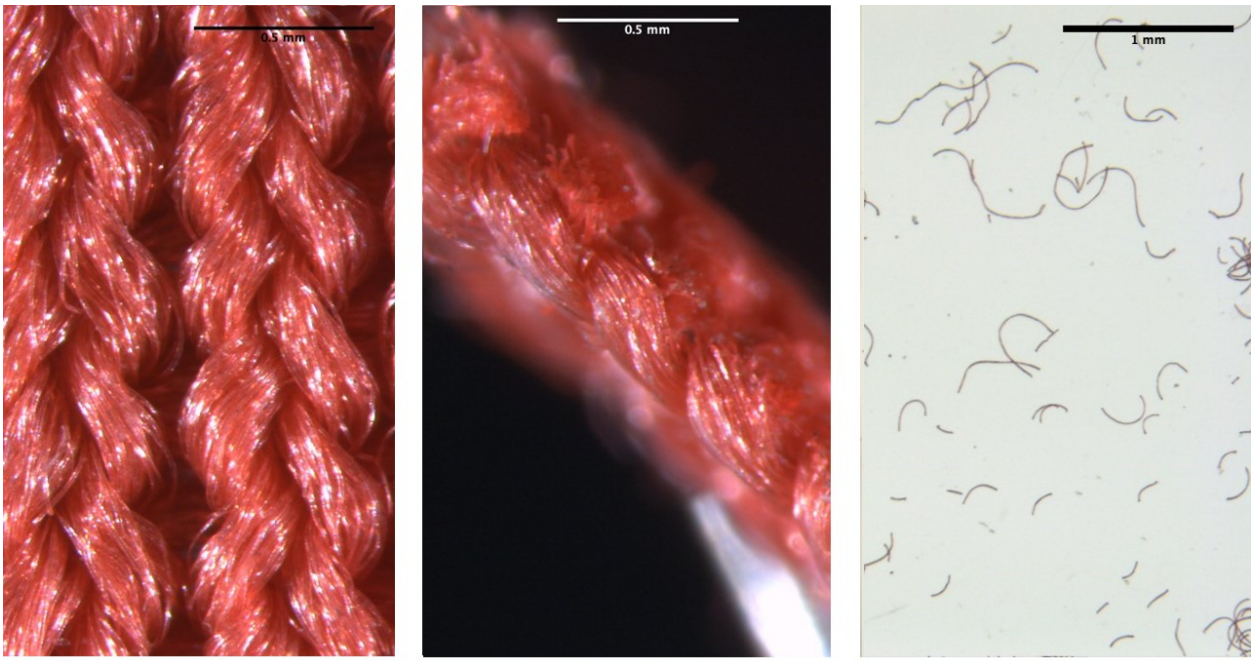

Figure 2. A photograph of (A) red polyester cloth weave from top view showing four “threads” each composed of multiple “fibers” wrapped in bundles, (B) side view, (C) and of individual microfibers after preparation.

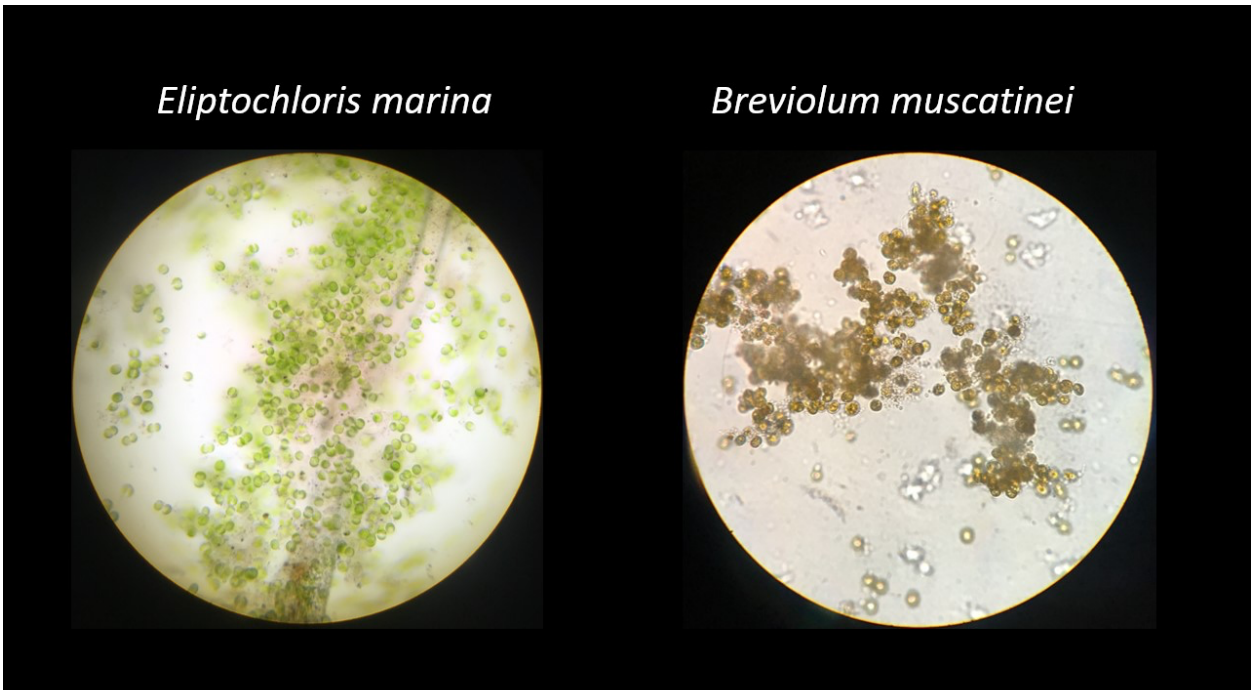

Figure 3. Photograph of *E. marina* (left) and *B. muscatinei* (right) isolated from *A. elegantissima* under 40X magnification.

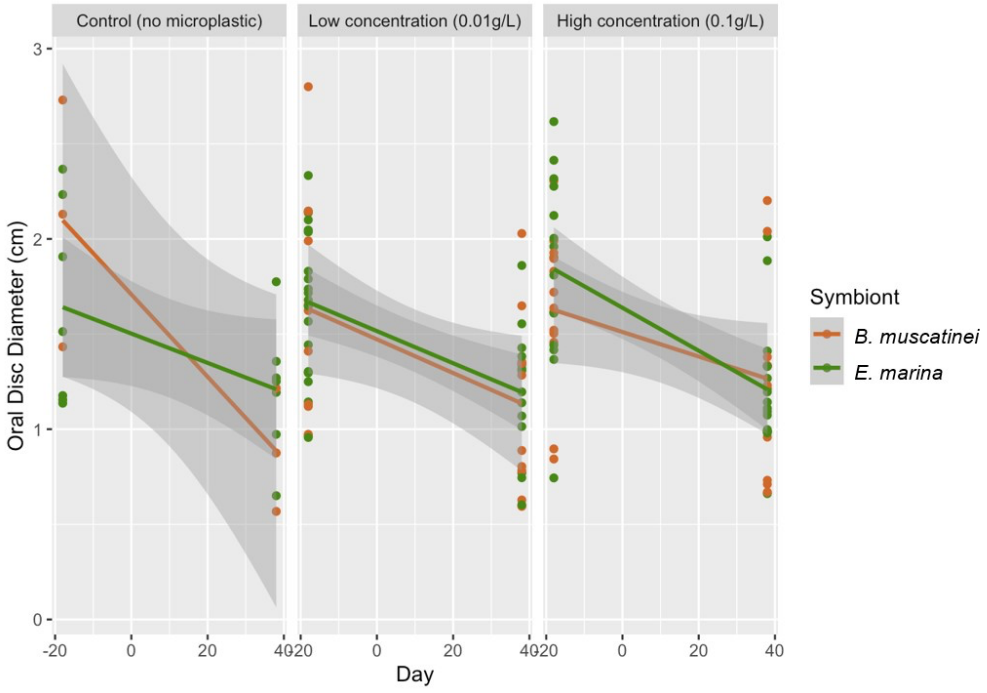

Figure 4. Oral disc diameters of *A. elegantissima* by symbiont (indicated by different colors) and microplastic concentration (three panels) over time. Overall regression lines with 95% confidence intervals are shown for individuals in the two symbiotic states.

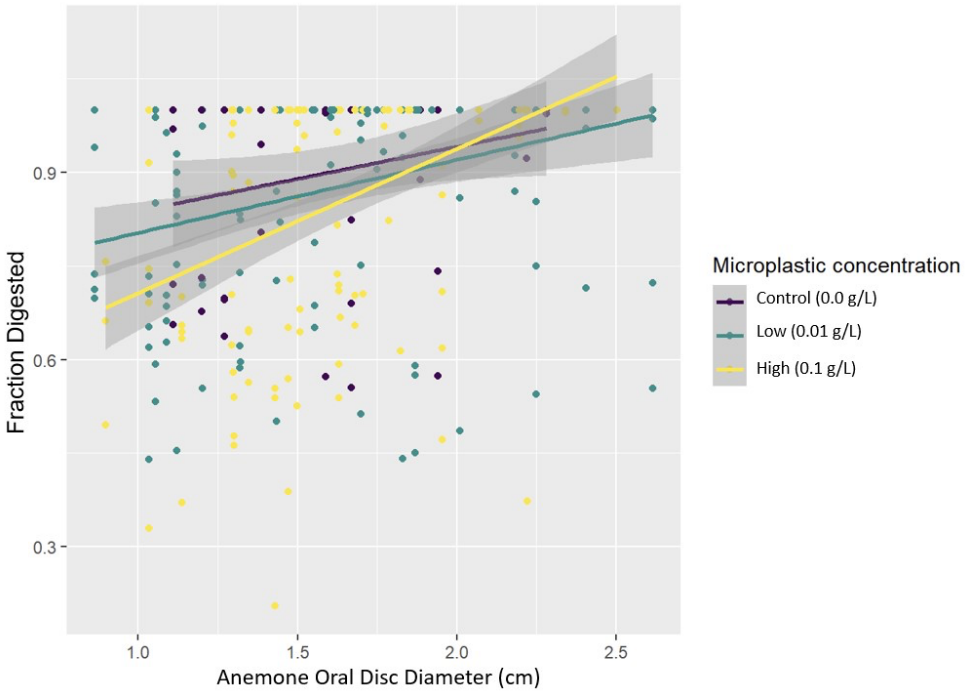

Figure 5. Anemone digestive efficiency for each of three microplastic concentrations (colors) as a function of anemone oral disc diameter. Smaller anemones in general show lower digestive efficiencies, and smaller anemones exposed to the highest concentrations of microplastics (yellow) showed the lowest digestive efficiencies.

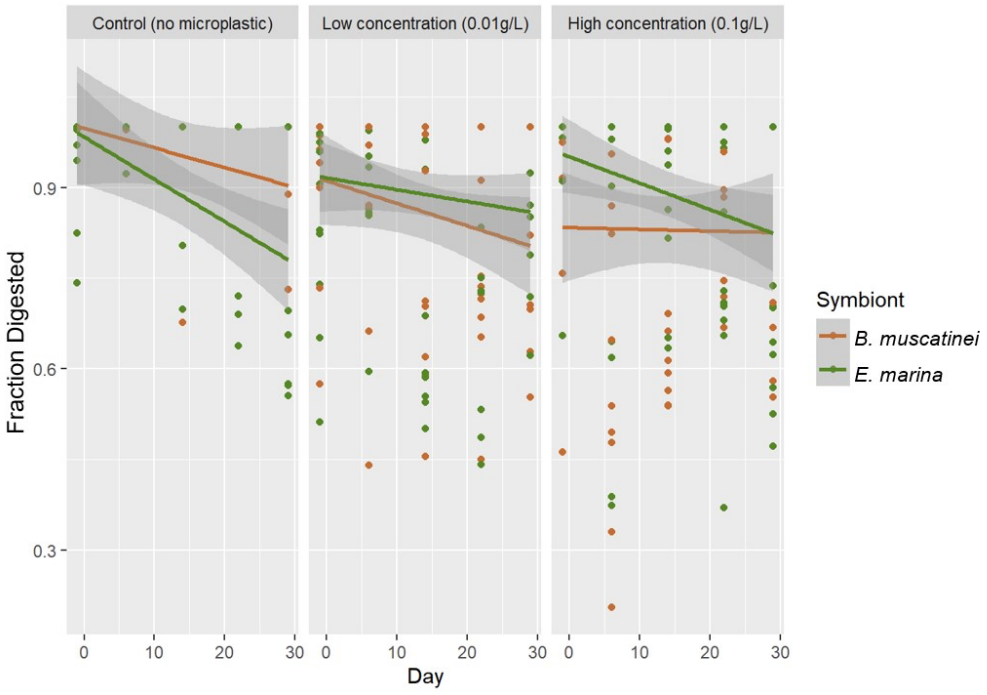

Figure 6. Digestive efficiency of anemones with different symbiont assemblages (color) in three microplastic concentration treatments (panels) over time.

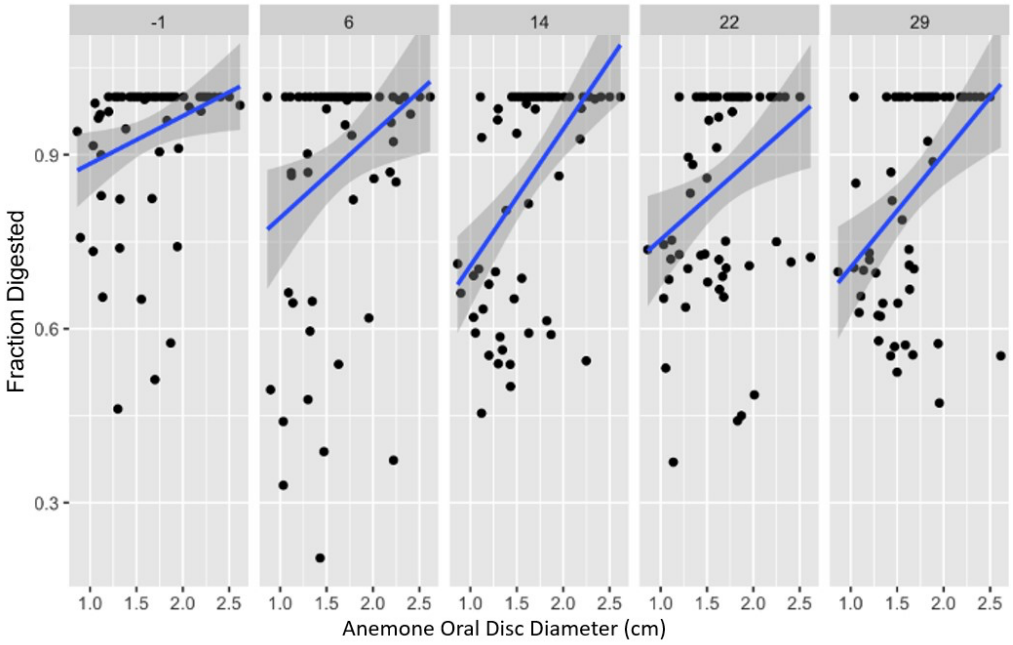

Figure 7. Digestive efficiency of anemones by average oral disc diameter by day of measurement in the experiment. Each panel shows a different day of the experiment relative to the first day of microplastic exposure where day –1 is the day before microplastics were introduced.

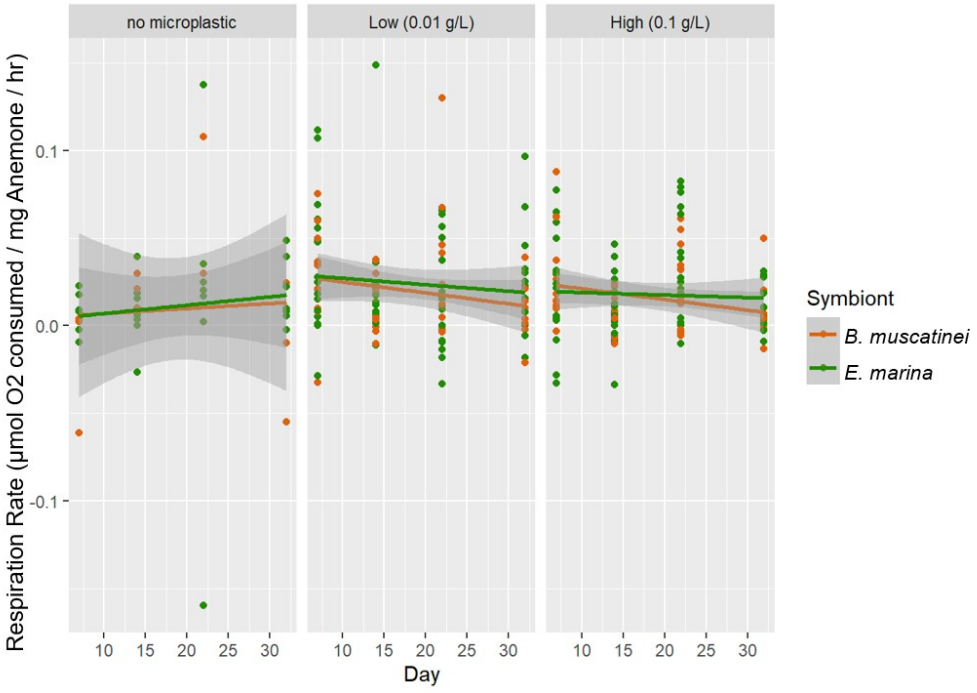

Figure 8. Respiration rates of *A. elegantissima* as a function of microplastic concentration (panels) and by symbiont type (color). Anemones with *B. muscatinei* symbionts appear to have slightly lower respiration rates compared to anemones with *E marina* with the difference growing over time.

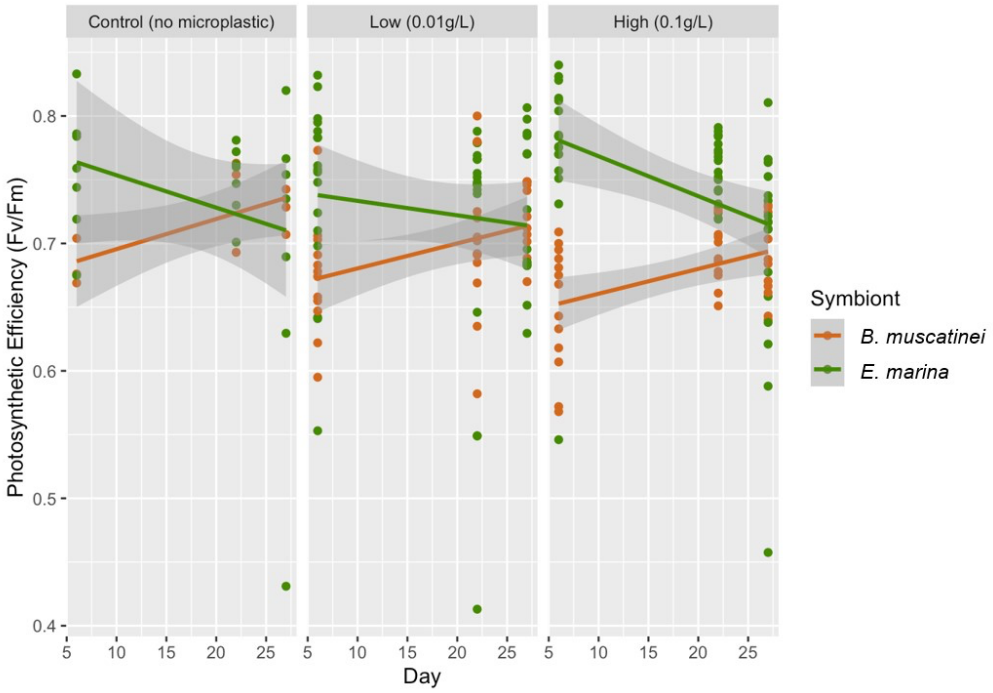

Figure 9. Change in photosynthetic efficiency over time as a function of microplastic concentration (panels) and symbiont type (color). While anemones with different symbiotic algae had significantly different responses over time, and their respective slope appear relatively constant across microplastic treatments, the intersection between the two symbiont trajectories tend to happen later in the experiment with increasing microplastic concentration.

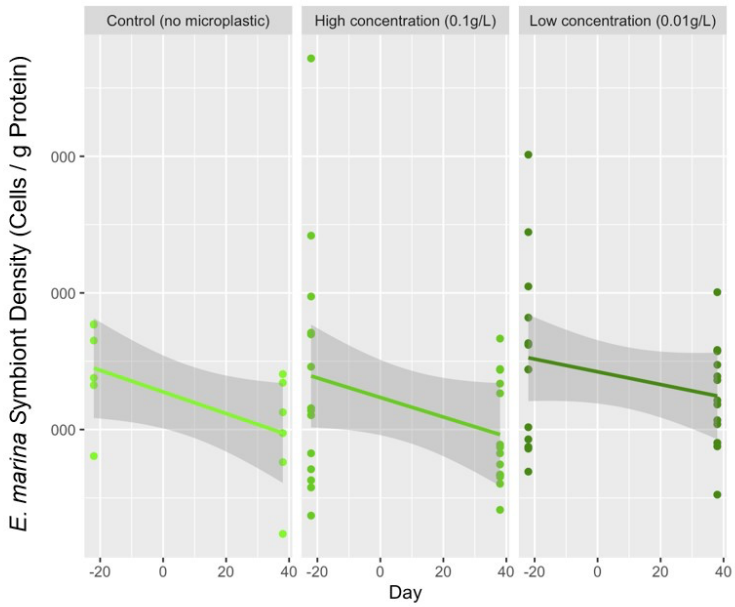

Figure 10. *E. marina* density over time in the experiment. There was a consistent negative slope in symbiont density over time regardless of microplastic concentration (panels). Day zero is defined as the beginning of microplastic exposure.

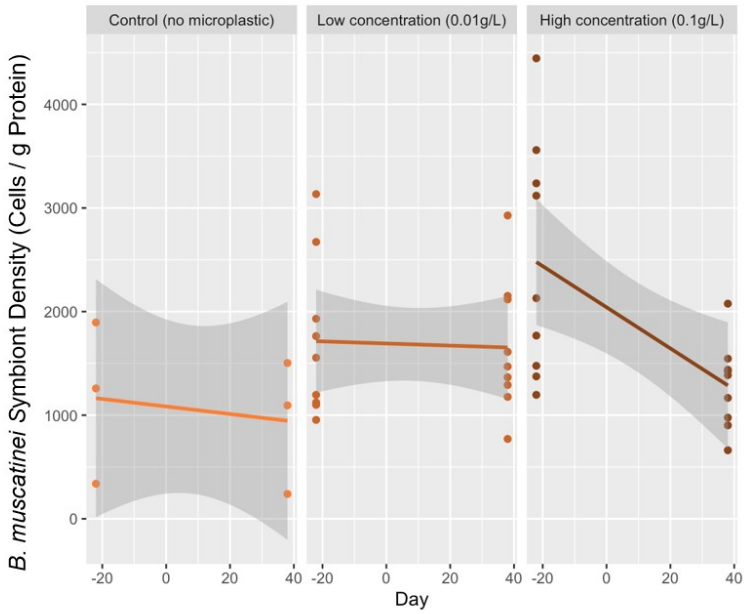

Figure 11. *B. muscatinei* densities over time in the experiment. There was little change over time in the control and low microplastic treatments, but a strong decline in the high microplastic treatment. Day zero is defined as the beginning of microplastic exposure.

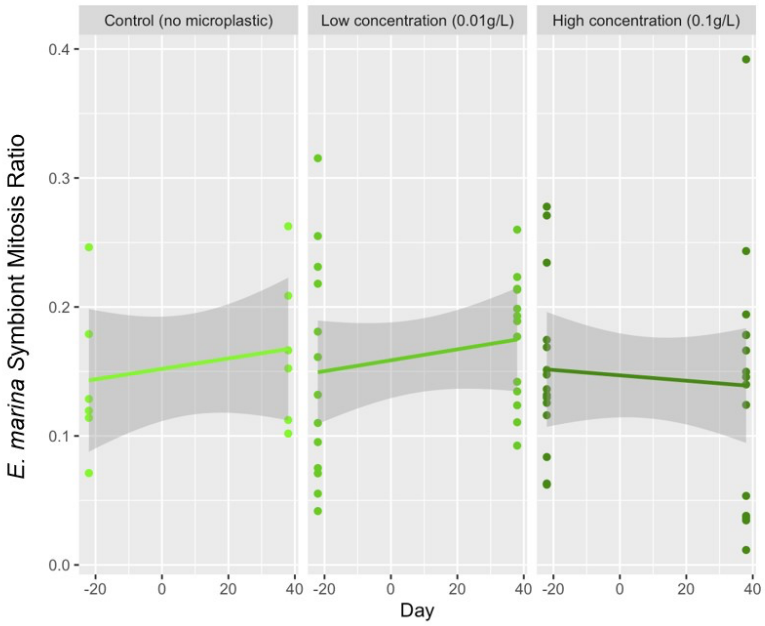

Figure 12. Mitotic index of *E. marina* symbionts during the experiment. Day zero is defined as the beginning of microplastic exposure.

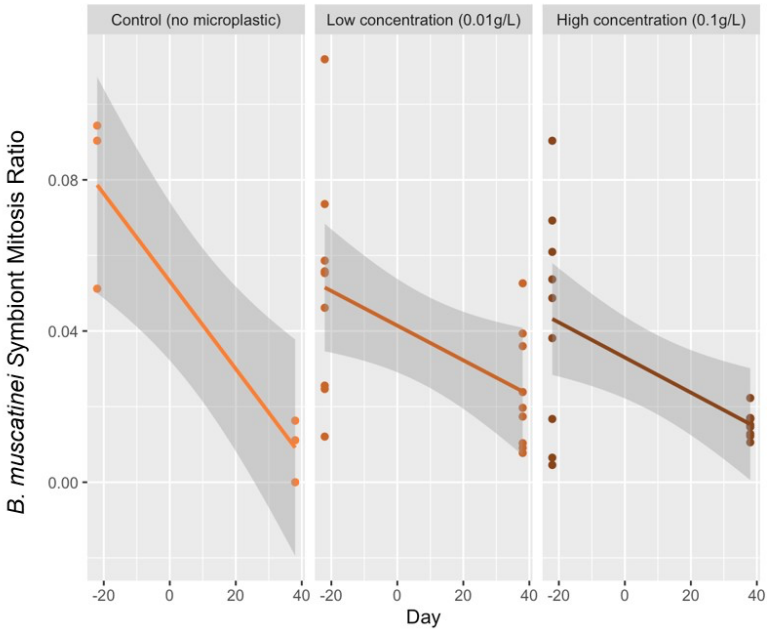

Figure 13. Mitotic index of *B. muscatinei* during the experiment. Day zero is defined as the beginning of microplastic exposure.