Journal of Experimental Marine Biology and Ecology 567 (2023) 151929

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Optimization of experimental conditions for exposure of larval mussels (*Mytilus californianus*) to microplastic particles

Brittany Cunningham [a,*], Matt Hawkyard [b], Stacey Harper [a,c], Chris Langdon [d]

[a] Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR, United States
[b] Aquaculture Research Institute, University of Maine, Orono, ME, United States
[c] School of Chemical, Biological and Environmental Engineering, Oregon State University, Corvallis, OR, United States
[d] Coastal Oregon Marine Experiment Station, Department of Fisheries, Wildlife, and Conservation Sciences, Oregon State University, Newport, OR, United States

ARTICLE INFO

Keywords:
Microplastics
Larvae
Bivalve mollusk
Mussel
Growth
Survival

ABSTRACT

Inputs of plastic pollution in marine environments continue to increase, making it essential to understand potential impacts on commercially important marine and estuarine species. Bivalve larvae have been shown to take up micro- and nano-sized plastics; however, exposures detailed in the literature often do not account for particle dynamics, such as eco-corona formation, agglomeration, or settlement. There is clearly a need to define and maintain suspended particle concentrations during exposure periods, while using culture systems that support the growth and survival of the test organism. Therefore, experiments were designed to optimize the components of an exposure system for both particle suspension, as well as larval mussel growth and survival. The suspension and clumping of 2 μm ultra-pure latex beads were assessed to determine the influence of flask shape, agitation, and dispersant type. These results were paired with an assessment of the effects of antibiotics, flask shape, flask rotation, and dispersant type on mussel larvae growth and survival. The combination of these experiments revealed that the optimum system for exposure of bivalve larvae to micro-sized plastics was in rotating, dimple-bottom flasks with additions of 2.5 mg.L^{-1} methyl cellulose and 2 mg.L^{-1} chloramphenicol in natural, 1 μm filtered seawater. This optimized system was used to assess the impacts of 2 μm ultra-pure latex beads on the survival and growth of *Mytilus californianus* larvae. First, a dose-response experiment was carried out to assess the impacts of a two-week exposure of larval mussels to microplastics concentrations that were 0%, 20%, 33.3%, 42.9%, and 50% of total cell volume of the algal ration. For these experiments the algal rations were all kept at 100%. Subsequently, a second experiment was carried out to assess impacts of a two-week exposure of larval mussels to microplastics where both the percent by volume of the algal (0–100%) ration and beads (0–100%) varied so that the total particle volume in the exposures remained constant (100% total). When algal food rations were held constant, a dose-dependent decrease in growth was observed with increasing concentrations of beads. When algal rations decreased, growth and survival decreased to a greater extent when microplastic beads were present. These results indicate that both concentrations of microplastic particles and volumetric ratios of algae rations to microplastic particles determine responses of mussel larvae, with microplastics having a greater effect on larval survival when added to low algal rations.

1. Introduction

Shellfish are both ecologically and economically important. Ecologically, bivalve shellfish contribute to habitat stabilization and improvements in water quality, as well as holding an important trophic position in food webs by linking planktonic and benthic communities (Rullens et al., 2019). Economically, global bivalve shellfish aquaculture for human consumption is worth an estimated $23.9 billion (van der Schatte Olivier et al., 2020). Bivalve populations are threatened by a combination of anthropogenically driven stressors (Costa et al., 2020; Serra-Compte et al., 2018). One such emerging stressor is plastic pollution. Studies have documented plastic pollutants in the guts and gills of clams, mussels, and oysters (Ward et al., 2003; Watts et al., 2016).

Microplastics are well documented in marine environments (Eriksen et al., 2014) and production is expected to increase in the near future.

* Corresponding author.
E-mail address: cunningb@oregonstate.edu (B. Cunningham).

https://doi.org/10.1016/j.jembe.2023.151929
Received 24 October 2022; Received in revised form 3 July 2023; Accepted 5 July 2023

Annual production of plastics, synthetic organic polymers that are resistant to degradation, had grown to approximately 368 million tons in 2019 (PlasticsEurope and EuPR, 2020) and is projected to continue increasing along current trajectories through to 2030 (Borrelle et al., 2020; Lau et al., 2020). It is estimated that annual global emissions of plastic pollution into the environment, often ending up in waterways and ultimately the ocean, ranges from 9 to 23 million metric tons (MacLeod et al., 2021). Microplastics, generally classified as plastic pieces between 1 μm and 5 mm, can result from the breakdown of macroplastic debris; however, there are also sources of plastics intentionally created in this size range (Azimi et al., 2016; Fendall and Sewell, 2009). Microplastics are ubiquitous (Barnes et al., 2009) and estimates in ocean surface water alone vary widely with reported values ranging from 7 thousand to 236 thousand metric tons (Cózar et al., 2014; Eriksen et al., 2014; Van Sebille et al., 2015). The negative effects of ingestion of microplastics, and to a lesser-extent of inhalation, are documented for a variety of terrestrial and aquatic organisms (Athey et al., 2020; Baechler et al., 2020b; Batel et al., 2018; Browne et al., 2008; Carr et al., 2012; Davidson and Dudas, 2016; Deng et al., 2017; Jeyavani et al., 2022; Salimi et al., 2022; Watts et al., 2016; Wright et al., 2013; Zhao et al., 2021). Though microplastic pollution is frequently discussed as a single entity, the category of plastics actually encompasses a diversity of chemical compositions, colors, additives, morphologies, and sizes (Rochman et al., 2019).

Microplastics not only persist and accumulate in the environment (Barnes et al., 2009), but have also been documented to be ingested by many marine organisms (Miller et al., 2020), including filter-feeding bivalves (Baechler et al., 2020a; Covernton et al., 2019; Liu et al., 2021; Mankin and Huvard, 2020; Van Cauwenberghe and Janssen, 2014). In fact, mussels have even been suggested as sentinel species (Li et al., 2019) and have been shown to interact with microplastics through both ingestion and adherence with their byssal threads (Ward et al., 2003; Watts et al., 2016). Microplastic internalization rate and accumulation by juvenile and adult mussels has been thoroughly investigated (Fernández and Albentosa, 2019; Rist et al., 2019b; Van Cauwenberghe et al., 2015). Exposure to microplastics have been shown to cause a variety of biochemical responses in mussels; for example, exposure of adult *Mytilus* to polystyrene (PS) microplastics has been shown to induce oxidative stress and impact immune responses (Cole et al., 2020; Paul-Pont et al., 2016). Microplastics have also been shown to impact bivalve feeding behavior and reproductive output (Sussarellu et al., 2016). Though microplastics have been shown to have negative impacts on mussels, these assessments have mainly focused on adults (Cole et al., 2020; Paul-Pont et al., 2016). While, much less is known about the rate or impacts of microplastic ingestion on larval mussels, they have been shown to ingest small (100 nm and 2 μm) plastic particles (Rist et al., 2019a).

Bivalve larvae are a sensitive life stage and their responses to microplastics are underrepresented in the literature (Table S1). Most larval bivalve exposures have been conducted over 48 h or less (Beiras et al., 2018; Bringer et al., 2020a; Bringer et al., 2020b; Capolupo et al., 2018; Franzellitti et al., 2019; Tallec et al., 2018). Rist et al. (2019a) carried out one of the few studies that investigated the impacts of longer-term exposures to microplastics on larval bivalves. They found that the effects they observed over a two-week period were different from those observed over shorter exposures to similar plastic concentrations and they emphasized the need for studies with longer exposures. Additionally, Bringer et al. (2021) found that a week-long exposure of pediveliger larval oysters to microplastics lowered settlement success.

To fully investigate the impact of microplastics on larval bivalves there is a need to develop an optimized exposure system in order to obtain accurate dose-response data. Some researchers have acknowledged settlement of microplastics during exposure, and attempted to keep particles suspended using aeration (Cole and Galloway, 2015), rotation (Beiras et al., 2018), or manual stirring (Weber et al., 2020). Rotational movement of cultures has the potential to concentrate the microplastics into the center of the vessel, making it necessary to devise methods that minimize this effect. Thus this study assessed the impact that differentially-shaped flasks had on minimizing vortexing effects on particle concentrations.

Ward et al. (2019) discuss the importance of particle size, agglomeration rate and surface coating on particle uptake by adult bivalves. The surface characteristics of particles, as well as their incorporation into agglomerates, impacts microplastic particle capture and selection; however, no studies with larval bivalves could be found that addressed microplastic clumping and agglomeration over the exposure period. This is important because larval bivalves typically ingest particles between 0.5 and 12 μm in diameter (Baldwin and Newell, 1995; Newell and Langdon, 1996). As plastic particles in a laboratory setting have been shown to readily agglomerate in seawater (Shupe et al., 2021; Summers et al., 2018), they can become less available to bivalve larvae and other filter-feeding organisms as particle size increases due to clumping over the experimental period. Overall, clumping and settlement of microplastic particles can alter the exposure dose for organisms during laboratory experiments if measures are not taken to keep particles in suspension. In this study, we investigated culture conditions that would best maintain concentrations of suspended microplastic particles.

In the environment, plastic particles become covered by an eco-corona or biofilm composed of bacteria and organic matter. This biofilm can aid in stabilization of microplastic suspensions (Rummel et al., 2017). Consequently, it would be an advantage to coat particles with a material of known composition that mimics this feature of particle stabilization in exposure experiments with microplastics. For this reason, dispersants were investigated to coat the particles. Development of a biofilm around the plastic particles should be prevented during these experiments as it could provide an unintended addition of nutrients to larvae, potentially masking the effects of the microplastic particles themselves. The addition of antibiotics should reduce the potential role of microplastic particles in delivering bacteria to the larvae (Fabra et al., 2021) by reducing bacterial colonization (Pepi and Focardi, 2021). Overall, addition of antibiotics to the system should both improve rearing conditions for larva and minimize confounding variables.

In this study, we conducted longer-term (two weeks) microplastic exposures with larval mussels (*Mytilus californianus*) and measured their responses under controlled conditions, taking into account microplastic particle size, coating, and agglomeration over time. Mussels are popular research organisms that are readily available and can be induced to reproduce throughout the year. We designed experiments with the purpose of optimizing conditions for exposure of mussel larvae to suspended microplastics. Ultra-pure latex spheres were used to ensure that no detergents were present which may have confounded interpretation of the impacts of the plastic particles on larvae.

Our goals were (1) to identify the type and agitation of flasks that optimized conditions for larval growth and particle suspension and (2) to determine the optimal dispersant type and concentration to maintain particle suspensions with no significant observable effect on the mussel larvae. Finally, we tested our optimized exposure conditions by assessing the impacts of microplastics on the growth and survival of larval *M. californianus* over a period of 14 days. We hypothesized that exposure to microplastics would impact bivalve larva survival and growth in a dose-dependent manner. First, all larvae were fed full algal rations and the concentration of microplastic beads were varied among treatments. Then, both the algal ration and the concentration of microplastic beads were varied while maintaining a constant total volume of suspended particles. This second experimental approach provided results on the effects of ration size on the impacts of microplastic additions, while avoiding the confounding effects of varying total volumetric particle concentration.

2. Materials and methods

2.1. General methodology

2.1.1. Mussel Larvae

Adult *Mytilus californianus* were collected in February 2021 from the intertidal zone at Seal Rock, Oregon, USA (44.4972° N, 124.0827° W). All filtered seawater (FSW) used in these experiments was pumped from Yaquina estuary, filtered to 1 μm, and aerated for 24 h with CO_2-striped air to raise the pH to between 8.2 and 8.6. In this way we attempted to remove both the effects of acidified sea water and undetermined particulate and nutrients as confounding variables in our experiments. The mussels were induced to spawn by heat shock in FSW at 24 °C. Following verification of egg fertilization, developing embryos were transferred to 30 L containers at a density of 50 larvae per mL, at 20 °C. At 48 h post fertilization (hpf) subsamples were taken to determine concentrations of D-hinge larvae. Larvae were rinsed on a 45 μm screen and then transferred, at a final density of 2 larvae per mL, to either 10 L plastic containers or 125 mL flasks filled with FSW at a salinity of 28–30 ppt and 20 °C. Larvae were fed *Isochrysis galbana* (clone C-Iso; CISO) at concentrations appropriate to the assigned experimental treatment (see Sections 2.4–2.6).

2.1.2. Microplastics, antibiotics, and dispersants

Stock suspensions of 8% *w/v*, 2 μm ultra-pure sulfate latex beads (Thermo Fisher Scientific Waltham, MA, USA) were diluted in seawater immediately before use. A stock solution of chloramphenicol [≥98% (HPLC); Sigma Aldrich, St. Louis, MO, USA] was prepared in distilled water at 2 $mg.mL^{-1}$. Stock solutions of the dispersants lignin sulfonate (mol. wt. 52,000; BeanTown Chemical, Hudson, NH, USA) and gum arabic (Sigma Aldrich, St. Louis, MO, USA; mol. wt. of approx. 250,000) were prepared in distilled water at 0.5% w/v. Stock solutions of the dispersant high-viscosity methyl cellulose (1500 cP; Sigma Aldrich, St. Louis, MO, USA), were prepared in distilled water at 0.5% w/v by stirring on a magnetic stirrer at 70 °C for an hour, 35 °C for an hour, and then overnight at room temperature. All stock solutions were stored in the refrigerator at 2 °C until use.

2.2. Effect of dispersants on particle clumping

Three different dispersants were assessed to determine which was most effective in (1) reducing clumping of 2 μm latex beads, and (2) maintaining the beads in suspension over a period of 48 h. Artificial seawater (ASW) at a salinity of 35 ppt was prepared in 0.8 μm filtered distilled water by additions of Red Sea Coral Pro Salt™. In addition to an ASW control, dispersant treatment levels included two concentrations (2.5 and 5 $mg.L^{-1}$) of lignin sulfonate, gum arabic, and methyl cellulose. Stock suspensions of beads, chloramphenicol, and dispersant were sonicated for 1 min before the initial concentration of beads was determined using a hemocytometer. Aliquots of 50 mL of each bead suspension were added to 125 mL Erlenmeyer flasks and placed on an orbital shaker at 60 rpm (rpm) (Digital Orbital Shaker Lab Oscillator Adjustable Variable Speed Rotator Oscillator, MXBAOHENGus Instrument Co.). Treatments were set up in triplicate ($n = 3$). At 24 and 48 h after the start of the experiment, 20 μl samples were taken from the interior and perimeter of each flask. The number of beads in each sample was counted with a hemocytometer and photos were taken to record particle clumping.

Samples were placed on a slide with a coverslip, viewed using a Leica DM1000 compound microscope, and photographed with a Leica DFC 400 camera (Leica Microsystems CMS GmbH Mannheim, Germany). Agglomerate sizes were determined as the number of beads along the longest portion of the clump. Although this method did not provide a highly accurate quantification of the clump sizes, it did allow use to comparatively assess the performance of the dispersants in relation to one another. Agglomerates were counted as a single particle when describing concentrations of suspended particles over time. This approach was adopted because larvae encounter the clump as a single particle while feeding. Bead concentrations were expressed as percentages of initial concentrations.

2.3. Acute toxicity assay: particle dispersants

As lignin sulfonate and methyl cellulose performed well in the particle dispersant experiments, both were assessed for their toxicities with mussel larvae. These experiments were structured after the EPA protocol for the Bivalve Acute Toxicity Test (USEPA, 2016). Seven concentrations of solutions of lignin sulfonate (5–400 $mg.L^{-1}$) and methyl cellulose (0.1–100 $mg.L^{-1}$) were prepared in 30 mL shell vials, with five replicate vials per dispersant concentration ($n = 5$). One hundred mussel embryos, 4 h post fertilization (hpf), were added to each vial at a density of 10 embryos per mL. These were allowed to develop until 48 hpf. Larvae in the control vials were confirmed to have reached the D-hinge development stage, and then larvae in all vials were preserved with the addition of 100 μl of 10% buffered (pH 8.6) formalin. The number of normal larvae in each vial was counted. Dose-response curves were constructed and, when possible, EC_{50} values calculated for each dispersant.

2.4. Effect of flask type and agitation conditions on particle suspension and larval performance

Four types of flasks [dimple-bottom (DB), flat-bottom (FB), custom-bottom (CB), baffled-bottom (BB)] were used for the bead suspension and larval growth experiments. A detailed description of the flasks can be found in the supplemental information. The flasks were evaluated under either stationary (flasks placed on benchtop) or agitated (rotated on an orbital shaker at 60 rpm) conditions to determine if flask agitation was needed to maintain bead suspensions and which type of flask resulted in greatest larval growth and survival (see Sections 2.4.1 and 2.4.2).

2.4.1. Effect of culture conditions on particle suspension

Flask type and agitation were assessed to identify conditions that best maintained beads in suspension. Red Sea Coral Pro Salt™ was added to distilled water to produce ASW at a salinity of 35 ppt. A stock bead suspension was prepared with final concentrations of chloramphenicol at 2 $mg.L^{-1}$, lignin sulfonate at 100 $mg.L^{-1}$, and beads at 5.8×10^6 $particles.mL^{-1}$. Lignin sulfonate was used to reduce clumping of the ultraclean latex beads in this experiment but in later experiments it was replaced by methyl cellulose as the latter was non-toxic and more effective as a dispersant. The stock was sonicated with a Vibracell™ wand-style sonicator (Sonics & Materials Inc., Danbury, CT, USA) for one minute, then the beads in a 20 μl subsample were counted using a hemocytometer to obtain the initial particle concentration. Next, 50 mL of the stock bead suspension in ASW was added to each flask type (DB, FB, CB, and BB). The stock suspension was covered and inverted at least two times prior to adding 50 mL to each flask to ensure homogeneity of the suspension. Half of the flasks of each type were not agitated (stationary) and half were agitated by rotation on an orbital shaker (60 rpm). Each treatment combination (flask ± agitation) was replicated in triplicate ($n = 3$). Samples were taken and evaluated as described in Section 2.2.

2.4.2. Effect of flask culture conditions on larval growth and survival

D-hinge mussel larvae (approx. 48 hpf) were added to either 10 L containers filled with 5 L of seawater to emulate established culture practices for mussels, or to one of four types of 125 mL flask (DB, FB, CB, and BB) (final volume 50 mL). All vessels were stocked at a density of 2 larvae per mL. Larvae were reared in 1 μm filtered seawater (FSW) obtained from Yaquina bay (Newport, OR) with 2 $mg.L^{-1}$ chloramphenicol. Half of the flasks were not agitated (stationary) and half were

agitated on an orbital shaker at 60 rpm (agitation). Each treatment combination of vessel and agitation type was set up in triplicate (n = 3). In an additional study, flat bottom flasks (n = 3 stationary and n = 3 agitated on an orbital shaker) were used to assess the utility of the addition of antibiotic, 2 mg.L^{-1} chloramphenicol, to the culture system. Larvae were fed algae daily, starting at 30,000 CISO cells per mL (i.e. 15,000 cells per larvae per day) and increasing to 50,000 cells per mL on day 6, and culture water was changed every other day. Flasks were loosely covered with foil to prevent contamination from plastic particles in the air. At 12 dpf larvae were collected on a 45 μm screen, transferred to 30 mL shell vials and preserved with 10% buffered formalin (100 μl.mL^{-1}). Larval survival in each replicate was estimated by counting live and dead individuals.

For all larval growth analyses (Sections 2.4.2, 2.5 and 2.6), the number of larvae in each vial was counted using a Leica DM1000 compound microscope (Leica Microsystems CMS GmbH Mannheim, Germany) and digital images of the larvae were taken using a Leica DFC 400 camera (Leica Microsystems CMS GmbH Mannheim, Germany). Image J software (National Institute of Health, Bethesda, MD) was used to measure the lengths and widths of haphazardly chosen larvae (30 larvae per replicate) from digital images (Schneider et al., 2012). Definitions of larval bivalve shell length and width are given by Loosanoff et al. (1966) as length being the longest distance along the anterior-posterior line of the shell, and width being the distance from the tip of the umbo to the ventral margin of the shell. In instances where the number of surviving larvae was <30, the sample size for the growth measurements was reduced.

2.5. Effects of bead concentration on mussel larval growth and survival: dose response

First, a dose-response experiment was carried out to assess if increasing bead concentrations negatively affected larval mussel growth and survival when added to a constant daily ration (cell concentration) of algae across bead treatments. All larval mussels were fed 30,000 cells per mL for 5 days and then increased to 50,000 cells per mL for the remaining experimental period. Additions of beads for these experiments were expressed as a percent of the total cell volume of the algal ration. The average volume of CISO cells is approximately 80 μm^3 per cell (Ishiwata et al., 2013). This value was used to calculate the approximate number of 2 μm latex beads (~4.189 μm^3 per bead) that would result in an equivalent volume to that of the algal ration. Approximately 19 of the 2 μm beads had the same total volume as one cell of CISO.

All larvae were grown in 125 mL DB flasks filled with FSW with the addition of 2 mg.L^{-1} chloramphenicol and 2.5 mg.L^{-1} methyl cellulose, resulting in a final volume of 50 mL. These flasks and dispersant were chosen based on the results of the trials described in Section 2.4. Larvae were added to the flasks at the D-hinge stage (approx. 48 hpf) and stocked at 2 larvae per mL, resulting in a total of 100 larvae per flask in the 50 mL total culture volume. Flasks were continuously agitated at 60 rpm. Controls of a 100% algal ration alone were set up in triplicate. The bead concentrations representing 0%, 20%, 33.3%, 42.9%, and 50% of total algal cell volume and each was each tested in duplicate. Table 1 details the amount of algae and beads added to each flask as both numbers and volumetric concentrations per unit volume. The experiment lasted until 14 dpf, at which point larvae were collected on a 45 μm screen and then washed into 30 mL shell vials and fixed with 10% buffered formalin (100 μl.mL^{-1}). The number of larvae in each vial was counted and shell lengths and widths were measured as described in Section 2.4.2.

2.6. Effects of bead concentration on mussel larval growth and survival: constant total particulate volume

A second experiment was carried out when keeping the total volume of algal cells and/or beads constant for all treatments. Larval bivalve gut processes are determined by volumetric rates of ingested particles (Gray et al., 2015); therefore, it was important to test bead microplastic concentrations under constant volumetric particle conditions to reduce the effects of different total particle volumes on feeding and digestive processes. The full (100%) algal ration used in these experiments was 30,000 cells per mL for the first 5 days and 50,000 cells per mL over the remaining experimental period to 14 dpf.

Experimental conditions (larval age and densities, flask type, antibiotics and dispersant) were the same as those described in Section 2.5 and modified as needed for experimental treatments. Two control groups that were fed the 100% algal ration (Table 2 row 1) were included: 1) those with the addition of 2.5 mg.L^{-1} methyl cellulose 2) those without methyl cellulose. These controls were included to determine if methyl cellulose had an impact on larval survival or growth. Partial algal rations of 0%, 25%, 50%, 75% by total cell volume were each set up in duplicate. Each of these algal ration treatments included additions of 2.5 mg.L^{-1} methyl cellulose. One set of flasks received partial algal rations with no microplastic bead additions (Table 2 rows 2–5, designated as “No beads additions”) and the other set received the same partial algal rations with the remaining percent suspended particulate volume of the 100% algal ration made up by bead additions (Table 2 rows 6–9, designated as “Beads additions”).

The experiment was terminated and larvae counted and measured as described in Section 2.5. Image J software (National Institute of Health, Bethesda, MD) was used to measure the surface area of each larva and the surface area of beads associated with each larva. The bead counts within each mussel shell cavity were divided into two categories, 1) those accumulated outside of the body tissues (non-ingested) that had accumulated within the shell cavity near the mouth, and 2) those that had been ingested and were present within the bodies of larvae (Fig. S3).

2.7. Data analysis

Statistical analyses were performed using R Studio (RStudio Team, 2020) and JMP software (v15.1.0; SAS Institute Inc.). Levene’s test and Shapiro-Wilk’s test were carried out to assess homogeneity of variance and normality of distributions, respectively. The data that met these assumptions included: bead concentration data and larval survival data. For these data, one- and two-way ANOVAs were applied. These were used to compare the effects of exposure conditions for both particle (factors: movement & flask type and dispersant/concentration combination) and larval optimization (factors: antibiotics & movement and flask type & movement) experiments as well as to compare fitted linear

Table 1
Experimental treatments for the dose-response larval experiment. Bead concentrations were based on the volume of the 100% daily algal ration of 30,000 cells per mL. Bead concentrations increased proportionally when the 100% algae ration was increased to 50,000 cells per mL per day on day 6.

Percent Algal Ration	Beads as percent algal volume	Algal cells per mL	Volume of algae per mL (μm^3)	Beads per mL	Volume of beads per mL (μm^3)	Total particle volume per mL (μm^3)
100	0	30,000	2,400,000	0	0	2,400,000
100	20	30,000	2,400,000	114,592	480,000	2,880,000
100	33.3	30,000	2,400,000	190,795	799,200	3,199,200
100	42.9	30,000	2,400,000	245,799	1,029,600	3,429,600
100	50	30,000	2,400,000	286,479	1,200,000	3,600,000

Table 2
Experimental treatments for the constant volume larval experiment. Bead concentrations were based on the starting 100% algal ration of 30,000 cells per mL. Bead concentrations increased proportionally when the 100% algal ration was increased to 50,000 cells per mL on day 6.

Microplastic beads	Percent Algal Ration	Beads as percent algal volume	Algal cells per mL	Volume of algae per mL (μm^3)	Beads per mL	Volume of beads per mL (μm^3)	Total particle volume per mL (μm^3)
No bead additions	100	0	30,000	2,400,000	0	0	2,400,000
	75	0	22,500	1,800,000	0	0	1,800,000
	50	0	15,000	1,200,000	0	0	1,200,000
	25	0	7500	600,000	0	0	600,000
	0	0	0	0	0	0	0
Bead additions	75	25	22,500	1,800,000	143,239	600,000	2,400,000
	50	50	15,000	1,200,000	286,479	1,200,000	2,400,000
	25	75	7500	600,000	429,718	1,800,000	2,400,000
	0	100	0	0	572,958	2,400,000	2,400,000

models. The factors here were movement (stationary and rotated), flask type (DB, FB, CB, and BB), antibiotics (with and without), dispersant type (lignin sulfonate, gum arabic, and methyl cellulose), and dispersant concentration (2.5 $mg.L^{-1}$ and 5 $mg.L^{-1}$). For analysis of the dispersants on particle suspension a one-way ANOVA was used and each combination of dispersant concentration and type were combined into a single factor because though the dispersant and concentration design was fully crossed, the addition of the control (i.e. 0 $mg.L^{-1}$) made the design unbalanced. Following identification of significant differences with ANOVAs, Tukey HSD post hoc tests were used to identify significant differences among treatment groups. Generalized linear models with Gaussian distributions were fit to assess impacts on growth and survival. A Multivariate Analysis of Variance (MANOVA) was performed to evaluate the effects of flask type and agitation conditions on larval growth and survival, as well as particle suspension. Data normality and homogeneity of variance were not met for bead agglomerate data, all larval growth measurements, and dose response survival values; therefore, the non-parametric Kruskall–Wallis and Dunn tests were used to compare means of these data. Concentration-response curves were constructed and EC_{50} values were calculated using the DRC (Analysis of Dose-Response Curves) package, fitted with the log-logistic model 4 (Ritz et al., 2015). The significance level for all statistical tests was set at $p < 0.05$.

3. Results

3.1. Particle suspension and agglomeration

Four types of 125 mL flasks (flat, dimple, baffled, and custom) were compared for their ability to maintain ultraclean latex beads in suspension. The initial concentration of beads in the experiment with different flask types was 5.8×10^6 $beads.mL^{-1}$. Rotated flasks maintained significantly higher concentrations of suspended beads than non-rotated flasks ($p \leq 0.001$, Table S2, Fig. S4). It was found that the different flasks performed similarly in maintaining particle concentrations over the 48 h experimental period; however, at the 24 h time point, perimeter concentrations in the dimple-bottom flasks were significantly higher than those for the other flask types ($p \leq 0.05$, Table S3). The interaction between 'flask type and movement' was not significant ($p > 0.05$, Table S3).

The initial concentrations of beads in the flasks with different dispersants ranged from 9.8×10^6–1.2×10^7 $beads.mL^{-1}$ (Table S4). Differences in maintaining concentrations of particle suspensions were evident for the different dispersant types (Fig. S5A). At 24 h, addition of methyl cellulose at the high (5 $mg.L^{-1}$) concentration resulted in significantly more of the initial concentration of beads remaining in suspension compared with the control ($p \leq 0.01$, Table S5). At 48 h, high concentration of methyl cellulose (5 $mg.L^{-1}$) was significantly better at maintaining the beads in suspension in the perimeter of the flasks compared to the control and all other dispersant treatments ($p \leq 0.01$, Table S6). At 48 h, both the low (2.5 $mg.L^{-1}$) and high (5 $mg.L^{-1}$) concentrations of methyl cellulose resulted in significantly higher concentrations of beads in the interior remaining in suspension than for the control and other dispersants, except for lignin sulfonate at 5 $mg.L^{-1}$ ($p \leq 0.01$, Table S7).

Beads agglomerated over the 48 h experimental period but there were clear differences in the ability of the different dispersants in reducing agglomeration. Both low (2.5 $mg.L^{-1}$) and high (5 $mg.L^{-1}$) concentrations of methyl cellulose, as well as the high (5 $mg.L^{-1}$) concentration of lignin sulfonate were significantly more effective in reducing agglomeration than the other dispersants and concentrations ($p \leq 0.05$, Table S8, Fig.S5B).

3.2. Toxicity of dispersants

Of the dispersants tested, methyl cellulose and lignin sulfonate were found to be the most effective in maintaining beads in suspension, as well as preventing agglomeration over a 48 h period; therefore, a 48 h toxicity test was carried out with both of these dispersants. Lignin sulfonate was found to be toxic for developing larval mussels at concentrations above 10 $mg.L^{-1}$ (Fig. S6A). Quantification of the abnormal larvae resulted in an EC_{50} value of 11.25 $mg.L^{-1}$ (Fig. S6A). Methyl cellulose was not toxic at concentrations as high as 100 $mg.L^{-1}$; therefore, no EC_{50} value could be calculated for the range of tested concentrations (Fig. S6B). The superior performance of methyl cellulose in maintaining microplastic particle suspensions, as well as lack of evidence of toxicity for developing mussel larvae, made it the optimal dispersant for use in the microplastic particle assays with mussel larvae.

3.3. Antibiotics

The impact of additions of 2 $mg.mL^{-1}$ of the antibiotic chloramphenicol was assessed for mussel larvae grown in both 10 L containers and flat-bottom flasks. The addition of this antibiotic significantly increased survival of larvae reared in 10 L containers ($p \leq 0.05$, Table S9, Fig. S7). Larval growth was not significantly impacted, either negatively or positively, by the presence of chloramphenicol when cultured in 10 L containers (Fig. S8). When cultured in rotating flat-bottom flasks, larvae were larger in terms of shell lengths and widths when antibiotics were added, compared to those without added antibiotics. When cultured under stationary conditions, larval growth was not significantly different between those cultured with and without antibiotics (Table S10, Fig. S8).

3.4. Flask selection and agitation

To assess the optimum flask and agitation type for mussel larval growth and survival, larvae were grown in four different flask-bottom types that were either placed on an orbital shaker or kept stationary. The performance of the larvae reared in these containers was then compared with that of larvae reared in 10 L containers. There were no significant differences in survival between larvae reared in the different

flask bottom types except the non-rotated baffled flasks, in which there was a significantly lower survival than in the other flask types ($p \leq 0.05$, Table S11, Fig. S7). Neither were there any significant differences in larval growth among the different flask bottom types (Fig. S8). In contrast, both survival (Table S12) and growth (Table S13) of larvae in the flasks were significantly greater than those reared in the 10 L containers (Fig. S8). Overall, the optimum conditions for both (1) mussel larvae survival and growth, and (2) particle suspension was in rotated, dimple-bottom flasks with additions of 2 mg.L^{-1} chloramphenicol and 2.5 mg.L^{-1} methyl cellulose (Table S14).

3.5. Growth trial with beads: dose response

The optimized exposure conditions — rotated DB flasks containing methyl cellulose and chloramphenicol (Table S14) — were used in experiments to determine the effects of bead concentration on larval mussel survival and growth. A dose-response experiment was conducted to evaluate the effects of increasing concentrations of microplastic beads when provided to mussels fed a full daily ration of microalgae. Although mussel larval survival appeared to decline with increasing concentrations of beads (Fig. 1) this trend was not significant. In contrast, a generalized linear model for growth (final shell length and width) showed an inverse correlation between bead concentration and larval growth parameters (glm, $p < 0.05$; Fig. 2). It was found that larvae exposed to a bead concentration volumetrically equivalent to a 50% algal ration were significantly smaller, in both shell length and width, than those exposed to beads at either 0% or 20% algal volumetric equivalents of beads ($p \leq 0.05$, Table S15 and S16). EC_{50} values could not be calculated because none of the treatments resulted in <75% survival, or <80% of the control's growth.

3.6. Growth trial with beads: constant total particulate volume

In this experiment, the effects on larval growth and survival of increasing concentrations of ultraclean latex beads, when provided as volumetric replacements for partial algal rations, were compared with the performance of larvae fed on the same partial algal rations alone. There were no significant differences in larval growth and survival between control flasks with and without the addition of methyl cellulose (data not shown); therefore the methyl cellulose-containing control flasks were used as the controls for statistical comparison with all other treatments in this experiment. A generalized linear model showed that a decrease in algal ration was correlated with a decrease in larval survival with and without added beads (glm, $p \leq 0.0001$; $R^2 = 0.82$ algae only, $R^2 = 0.91$ algae and beads; Fig. 3); furthermore, the addition of beads was shown to be a significant variable in the model (glm, $p = 0.00343$). The slopes of the lines in response to algal ration with and without bead additions were significantly different (glm interaction term $p = 0.0233$, Fig. 3) with additions of beads further reducing larval survival with decreasing algal rations.

Overall, both length and width of the larvae increased with increasing ration (Fig. 4). The impact of the beads on larval growth was less pronounced than their impact on survival. Only at a 0% algal ration was there a difference in length and width of larvae with and without beads, with beads having a significant negative effect on final shell dimensions ($p \leq 0.05$, Table S17 and S18).

Mussel larvae were found to ingest 2 μm latex beads (Fig. 5). The area of ingested beads increased with the volumetric percentage of beads in the ration ($p \leq 0.05$, Table S19). The area of non-ingested beads observed within the shell cavity near the mouth was significantly higher in larvae exposed to the 100% bead ration than for those exposed to the 25% bead ration ($p \leq 0.05$, Table S20).

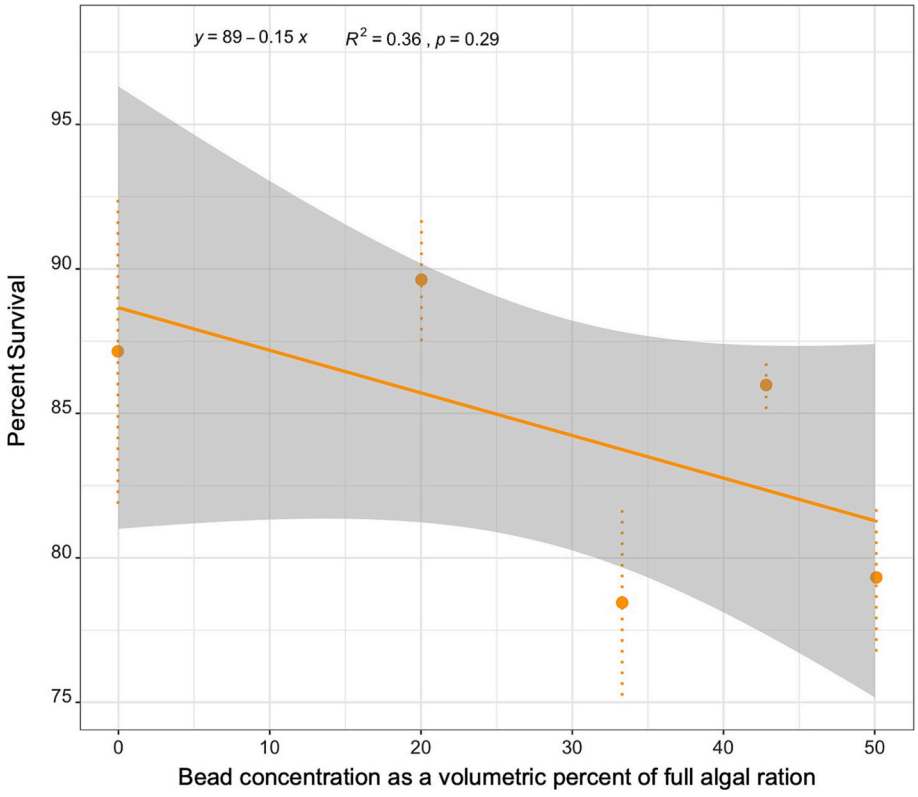

Fig. 1. Percent larval survival with increasing volumetric concentrations of beads fitted with a generalized linear model. Equation for survival: y = 88.658–0.147×, $R^2 = 0.36$, $p = 0.287$. The solid line is the fitted glm. The gray zone around the line represents the 95% confidence interval of the fitted model. The solid points are the average of the replicates, and the dashed vertical lines on either side of the points show the standard error.

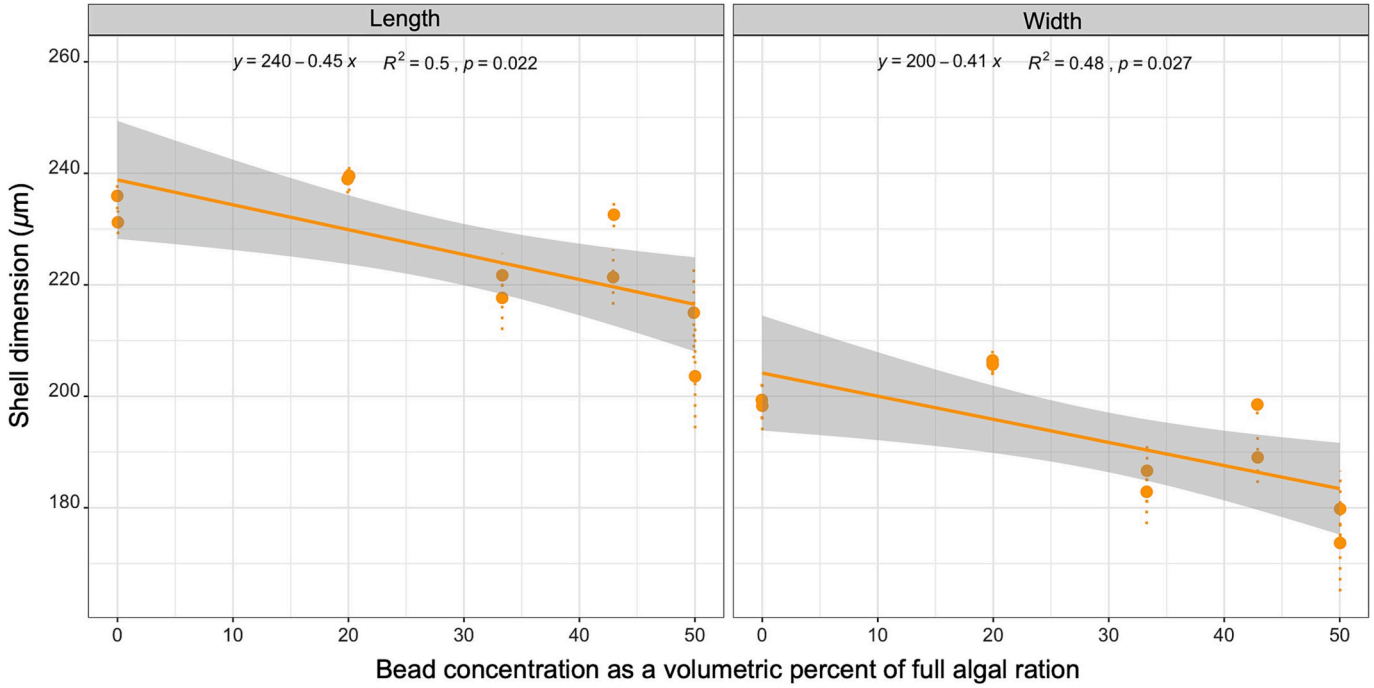

Fig. 2. Larval growth (final shell length and width) with increasing volumetric concentrations of beads fitted with a generalized linear model. Equation for shell length: y = 239.819–0.446×, $R^2 = 0.50$, $p = 0.0221$; equation for shell width: y = 200.168–0.414×, $R^2 = 0.48$, $p = 0.0271$. The solid line is the fitted glm. The gray zone around the line represents the 95% confidence interval of the fitted model. The solid points are the average of the measured larvae within the replicate, and the dashed vertical lines on either side of the points show the standard error.

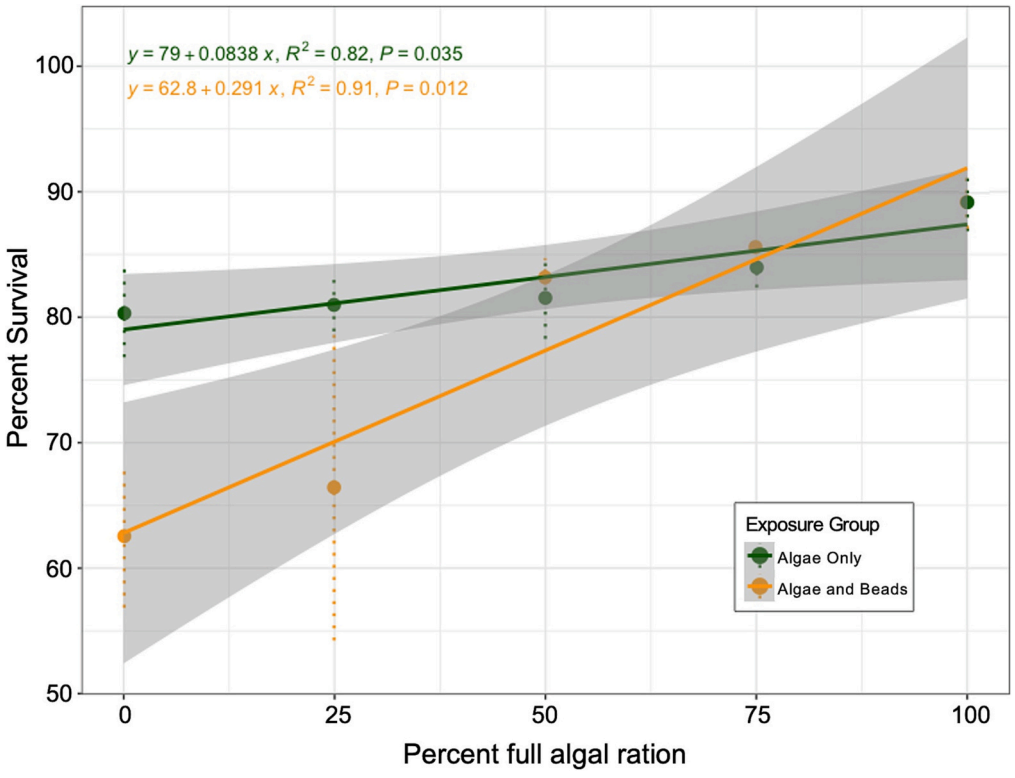

Fig. 3. Percent survival of mussel larvae fed on different rations of algae with and without the addition of microplastic beads. Lines show fitted generalized linear models. Algae-only equation: y = 79–0.0838×, $R^2 = 0.82$, $p = 0.035$; algae and beads equation: y = 62.8–0.291×, $R^2 = 0.91$, $p = 0.012$. The solid lines are the fitted glm. The gray zones around the lines represent the 95% confidence intervals of the fitted models. The solid points are the average values for the replicates, and the dashed vertical lines on either side of the points show the standard errors.

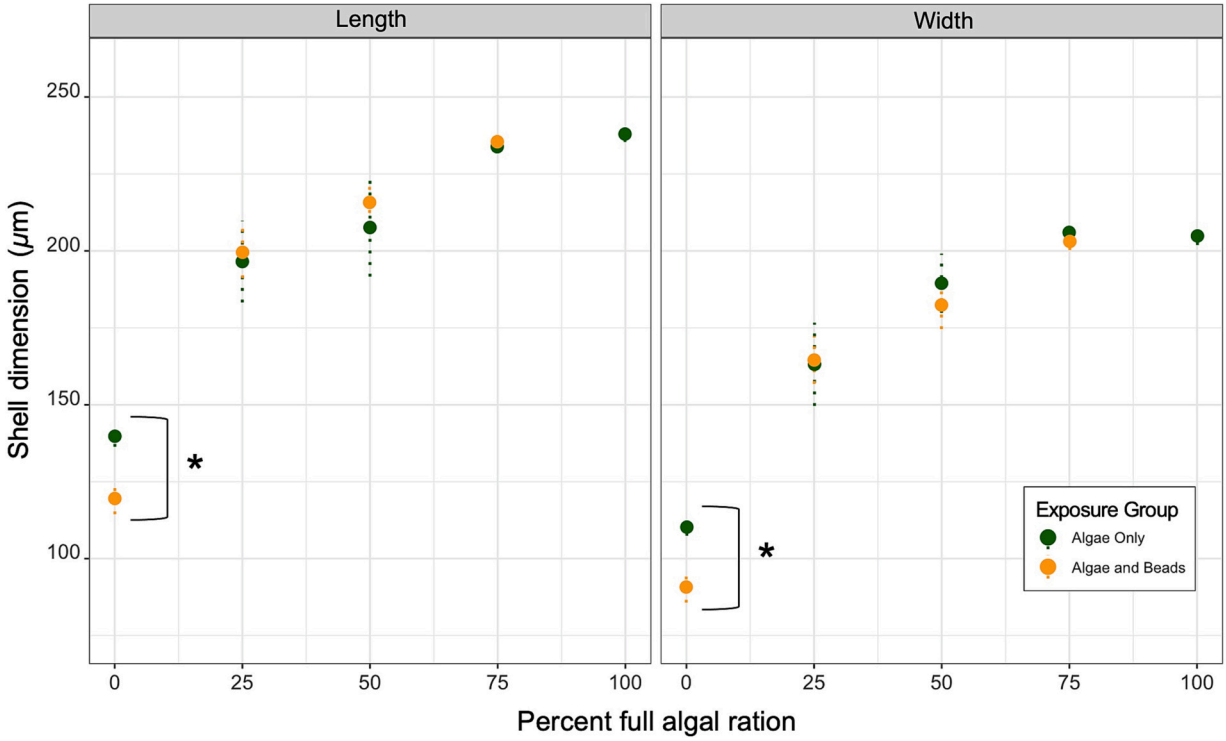

Fig. 4. Final shell lengths and widths of 14-day old mussel larvae fed on different rations of algae either with (Algae and Beads group) or without (Algae Only group) the addition of microplastic beads. Significant differences between measurements in the Algae Only and Algae and Beads groups are designated with a star (*) (Kruskal-Wallis and Dunn test, $p < 0.05$). The solid points are the average values for the replicates, and the dashed vertical lines on either side of the points show the standard errors.

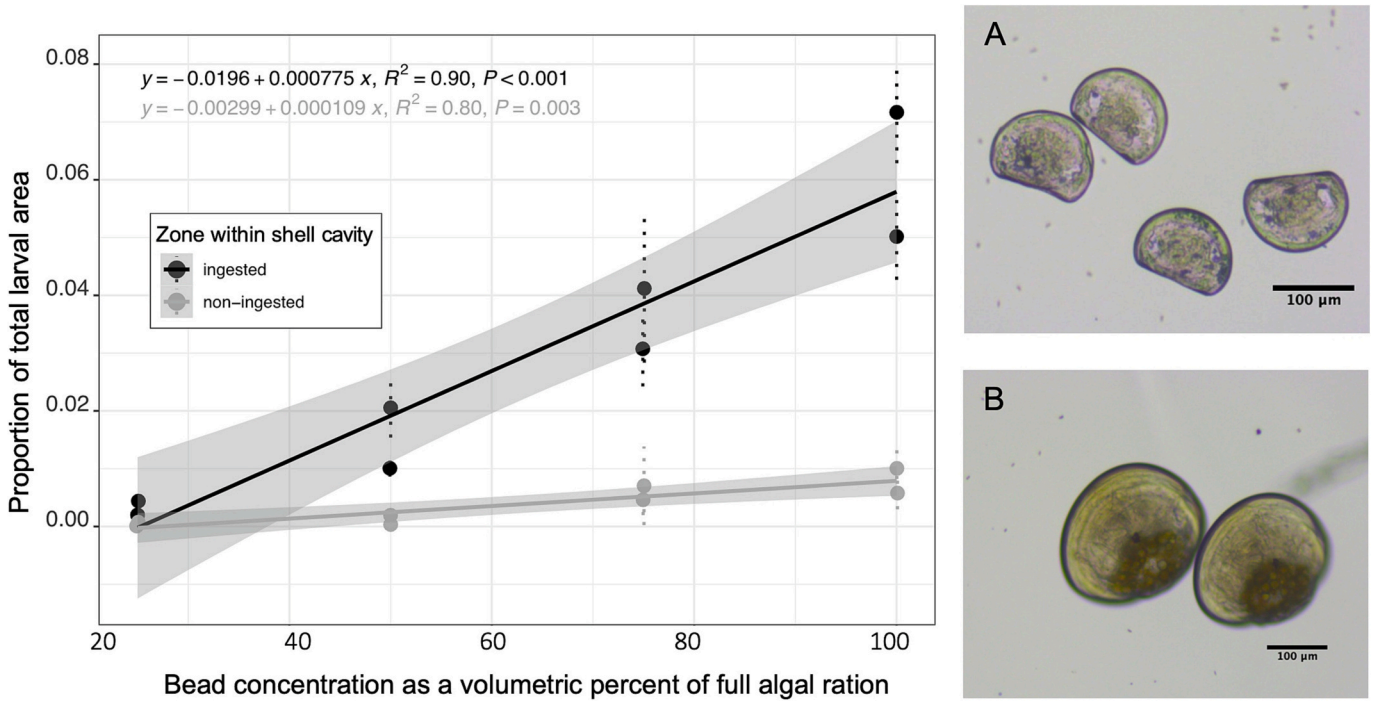

Fig. 5. The proportion of total larval shell area occupied by beads for 14-day old mussel larvae fed on different volumetric rations of 2 μm latex beads. Data points represent averages for flask replicates ($n = 2$) based on measurements of 15 random larvae sampled from each flask. The proportions are allocated to either: a) area within the body (ingested) or b) area within the shell cavity but non-ingested (zones detailed in Supplemental Fig. S3). Images of 14-day old larvae exposed to: A) 100% beads with 0% algae and B) 25% beads with 75% algae. The gray zones around the solid lines represent the 95% confidence intervals of the fitted models. The solid points are the averages of the measured larvae within the replicate, and the dashed vertical lines on either side of the points show the standard errors.

4. Discussion

4.1. Optimizing conditions for microplastic bead exposures

4.1.1. Dispersants

One of the greatest problems in exposure assays of marine organisms to microplastic suspensions is the lack of information on changes in particle concentrations during the exposure period. It is important to minimize settlement and agglomeration of particles over the duration of an experiment so that the reported dosages reflect the levels experienced by the organism throughout the trial period. This issue has been addressed in other studies through the use of aeration (Cole and Galloway, 2015), agitation on a rotary wheel (Beiras et al., 2018), and manual stirring (Weber et al., 2020) and by the addition of natural organic matter (NOM) to coat particles and reduce agglomeration (Fadare et al., 2019; Qiao et al., 2019). NOM coatings simulate organic coatings that would naturally cover microplastic particles in the environment (Gigault et al., 2021). Use of effective dispersants in studies with microplastics is especially important in saltwater exposures, as higher salinities are known to increase particle agglomeration (Shupe et al., 2021). NOM is not an ideal microplastic-coating because it: (1) can be composed of a variety of organic molecules and is not fully defined (Junaid and Wang, 2021), and (2) may contribute nutritionally to the diets of the exposed organisms (Amariei et al., 2022). NOM may also act as a food source for microorganisms, and may both impact the microbial flora of the microplastic coating and the microbiome of consumers (Fabra et al., 2021). For these reasons, we investigated alternative, chemically defined and nutritionally inert dispersants for microplastic beads. Additions of either methyl cellulose (2.5 and 5 $mg.L^{-1}$) or lignin sulfonate (5 $mg.L^{-1}$) to microplastic bead suspensions in seawater were successful in both maintaining concentrations of particles in the water, as well as minimizing agglomeration. Gum arabic neither maintained bead suspensions, nor reduced agglomeration.

Following the identification of methyl cellulose and lignin sulfonate as effective dispersants, a toxicity assay was conducted with these dispersants. Lignin sulfonate was found to be toxic to developing mussel larvae, while methyl cellulose was not. Lignin sulfonate is known to be toxic to fish at high concentrations; for example, a 48 h LC_{50} of 7300 $mg.L^{-1}$ was determined for rainbow trout exposed to lignin sulfonate (Roald, 1977). Additionally, lignin sulfonate has the potential to decrease the pH of solutions (Jones and Mitchley, 2001); however, in this study pH levels were found to be within acceptable levels, between 8.15 and 8.5, in the assay with mussel larvae. No dose-response or toxicity data for methyl cellulose could be found in the literature; however, Rosa et al. (2013) coated polystyrene (PS) microbeads with methyl cellulose to eliminate their surface charge in feeding studies with adult mussels and oysters without observed toxicity effects. Because it was not found to be toxic, and is highly effective in maintaining microplastic particles in suspension, methyl cellulose would likely be able to coat and act as a dispersant for other types of microplastics; however, more experimentation would be necessary to establish if it was effective in maintaining larger or denser particles, such as rubber tire particles, in suspension.

4.1.2. Antibiotics, agitation, and flask type

Addition of antibiotics to larval cultures significantly increased survival as well as shell growth in some culture vessel types. This is not surprising, as antibiotics have been reported to increase yields of cultured bivalve larvae (Prado et al., 2010). Antibiotics were added to minimize confounding factors in the larval microplastic exposures by reducing the effects of biofilms as a potential source of nutrients for the mussel larvae (Amariei et al., 2022).

Use of constant rotational movement of the flasks was found to be beneficial not only for keeping the microplastics in suspension, but for enhancing larval survival and growth. One study found rotation on an orbital shaker to be detrimental to larval culture (Beiras et al., 2018); however, their vessels were rotated at a higher rate than in this current study, which may be the cause of this discrepancy. Rotation has the potential to concentrate larvae and particles into the center of the vessel. For this reason, it was important to assess flasks with different bottom-shapes that could aid in minimizing this vortex effect. Therefore, flat-, baffled-, custom- (i.e. a more pronounced baffle) and dimpled-bottomed flasks were each evaluated for their impacts on particle suspension and larval culture. Assessment of particle suspension over 48 h in each flask type, revealed that the flasks performed similarly to each other. The only significant difference was that the dimple-bottomed flask retained a significantly higher percentage of its initial bead concentration at 24 h; which could be because the convex interior of the dimpled-bottom prevented the concentration of microplastic beads at the center of the bottom of the flasks.

The performance of larvae cultured in 125 mL flasks of different bottom-shapes, containing 50 mL of seawater, was compared both among the flask types, and to that of larvae cultured in 10 L culture vessels containing 5 L of seawater. Flask bottom types had similar effects on larval survival and growth, with the exception of baffled-bottomed flasks that had a negative effect on survival; therefore, the baffled-bottom flask was not selected for further experiments. With similar larval performances among other flask types, the improved performance of the dimple-bottom flask in maintaining particles in suspension resulted in its selection for larval culture experiments in this study.

Larval performance in flask cultures outperformed that of larvae cultured in the 10 L containers, as determined by both larval survival and growth. Other studies have cultured larval bivalves exposed to microplastics in small volumes of seawater, often due to a limited availability of microplastics for testing. Twelve-well plates, that hold several milliliters of seawater, have been commonly used for 48 h or less microplastic exposures (Bringer et al., 2020a; Bringer et al., 2020b; Franzellitti et al., 2019) and researchers studying longer exposures (> 72 h) have used volumes ranging from 0.25 to 1 L (Rist et al., 2019a; van der Schatte Olivier et al., 2020). Beiras et al. (2018) attempted to expose mussel larvae to microplastics in 10 mL of seawater but observed significantly reduced survival. This reduced larval survival may have been due to elevated surface area-to-volume ratios of small culture volumes, resulting in surface-associated bacteria having a more pronounced negative effect on larvae than in larger culture volumes. In our study, the addition of antibiotics also likely reduced negative effects of bacterial coatings of the culture vessel surfaces.

4.2. Adverse effects of microplastic beads on mussel larvae

Using optimized exposure conditions, the impacts of 2 μm ultrapure latex beads on larval *M. californianus* was determined in two different ways. Firstly, a traditional dose-response experiment was conducted in which all larvae were fed on full algal rations and the concentration of microplastic beads varied among treatments. The dose-response experiment showed that exposure to high concentrations of microplastic beads had negative impacts on larval mussel growth; however, interpretation of these results can be confounded by the effects of increased total (algal cells and beads) particle concentrations that result from additions of higher concentrations of beads.

A second experiment was carried out to assess the effects of microplastic beads while maintaining a constant total volume of suspended particles. This constant-volume experiment showed that bead effects depended on algal ration, suggesting that nutrient dilution by ingested beads in the larval gut is more pronounced with lower algal rations. Because bivalve larvae may naturally undergo periods of food scarcity in the environment, knowing the extent to which food availability alters microplastic impacts is necessary for the extrapolation of lab-based findings to real-world impacts. Our finding that the negative effects of microplastics are exacerbated at lower algal rations indicate that other studies that only exposed larvae to beads in combination with full algal rations, may have underestimated the full impacts of microplastics on

larval bivalves.

The experimental conditions of previous exposures of larval bivalves to microplastics are summarized in Table S1 and briefly discussed here in relation to the findings of this current study. In our first experiment, exposure to microplastic beads at increasing concentrations did not cause significant mortality. Similarly, mortality has not been observed in other larval bivalve exposures to PS microplastics when the algal ration was kept constant and surfactant-free microplastics were used (Bringer et al., 2020a; Capolupo et al., 2018). Additionally, we observed that only the larvae exposed to the highest concentration of beads, equivalent to 50% of the volume of the full algal ration, were significantly smaller than larvae fed on the full algal ration. Other studies have found that larval growth is impacted by the presence of PS beads; for example, Bringer et al. (2020a) found that oyster larvae exposed to surfactant-free micro PS beads (1–5 μm at 0.1–10 mg.L^{-1}) were smaller than unexposed larvae when fed identical algae rations. Additionally, Balbi et al. (2017) saw a significant decrease in shell size of mussel larvae following exposure to nano PS particles (50 nm at 0.001–20 mg.L^{-1}, surfactant-free).

Conflicting impacts of microplastic exposures on larval bivalve development have been reported. Capolupo et al. (2018), did not observe developmental abnormalities in mussel larvae exposed to up to 10,000 particles.mL^{-1} of 3-μm PS beads without surfactants. Malformed larvae were not observed in this current study either. In contrast, Rist et al. (2019a) reported a dose-dependent increase in malformations in larval *M. edulis* exposed to between 0.42 and 282 μg.L^{-1} of 2 μm PS over 15 days. Though the bead size and exposure duration in the study by Rist et al. (2019a) match conditions of this current study, their microplastic bead suspension contained Tween 20 and sodium azide, which may have had an adverse effect on the larvae. Like Rist et al. (2019a), Bringer et al. (2020b), reported negative impacts to larval bivalve development following exposure to microplastics even though their particles were surfactant-free (4–20 μm polyethylene at 0.1–10 mgL^{-1}). Certain plastic leachates and additives are known to impair the growth and development of larval bivalves (e Silva et al., 2016; Ke et al., 2019), and the various polymer types, coatings, and additives of microplastics used for exposures (Table S1) likely account for the different reported impacts on development. In all of these studies, as well as in our own dose-response exposure, algal rations were kept constant over the range of tested bead concentrations, resulting in a possible confounding effects of total particle concentrations on larval responses.

In the second experiment, total volumes of particles (beads plus algal cells) were kept constant for all bead exposures to study the larval effects of bead concentration, independent of total particle concentration effects. Reducing the algal ration decreased mussel larvae growth and survival. A further reduction in survival was evident when beads were added to a 25% algal ration or beads were fed to starved larvae. Addition of beads to higher algal rations had no effect on larval growth, as determined by final shell lengths and widths. Food limitation has been suggested as the dominant mechanism through which microplastics cause negative impacts on organisms (de Ruijter et al., 2020; Mehinto et al., 2022); for example, studies with the freshwater crustacean *Daphnia magna* showed that adverse effects of microplastics on growth and survival were exacerbated under low food conditions (Ogonowski et al., 2016; Schür et al., 2021). Additionally, the adverse effects of PS microbeads increased for adult mussels under limited food conditions (Shang et al., 2021; Wang et al., 2021). Shang et al. (2021) found that exposure to microplastics under a food shortage weakened the mussel's byssal threads and Wang et al. (2021) showed that exposure to microplastics induced oxidative stress for starved mussels. Rist et al. (2019a) assessed the acute uptake – over 4 h – of microplastics on larval bivalves under food-limited conditions. They used concentrations of 100%, 75%, and 50% of the algal volume replaced with beads and found that increased volume percentages of beads corresponded with increased bead uptake. Additionally, they confirmed that 2 μm plastic particles were actively filtered and ingested by mussel larvae; however, when assessing impacts on larval growth rate, they varied the concentration of beads but fed all larvae full algal rations (Rist et al., 2019a). Therefore, though uptake has been explored, no previous reported studies investigated the impacts of microplastics on growth and survival of larval bivalves under food limited conditions.

We observed increasing micro-bead ingestion following exposure of larval mussels to increasing volumetric proportions of beads in the diet. Ingestion of beads will result in nutritionally inert beads reducing the gut volume occupied by ingested algae, resulting in dilution of ingested food (Gray et al., 2015). Adult *Mytilus* have the ability to counteract the impacts of food dilution by increasing ingestion (Kiørboe et al., 1980). Larval mussels are also able to select and reject filtered particles (Gallager, 1988; Newell and Langdon, 1996), including microplastic beads (Rist et al., 2019a). Though feeding activity in bivalves decreases with lower food availability (Tenore and Dunstan, 1973), larval mussels in this study were seen to actively ingest 2 μm beads even without algae present. In the 100% bead exposures mussel larvae were observed to take up large quantities of microplastic beads. These were observed both in the gut and near the mouth. Particles at the entrance of the mouth could be either rejected or held for later ingestion (Newell and Langdon, 1996). Microplastic exposure can impact assimilation efficiencies and nutrient uptake in adult bivalves. Gardon et al. (2018) found that exposure to microplastics had a negative impact on food assimilation in adult pearl oysters (*Pinctada margaritifera*). Additionally, exposure to microplastics, especially under limited food conditions, impacts the energy budget of mussels (Wang et al., 2021). It is unknown if microplastic exposure impacts the energy budget of larval mussels as shown for adult mussels.

The spherical microplastics investigated in this study are not fully representative of environmental microplastic pollution. In surface waters, estimates of microplastics between 100 and 5000 μm are extremely low, around 1 particle per mL; however, plastic pollution can be heterogenous in surface waters, which may not be captured when extrapolating over large geographic areas (Lenz et al., 2016; Lindeque et al., 2020; Sutton et al., 2019). In our study, we conducted exposures with only 2 μm beads in concentrations up to 5.7×10^5 beads.mL^{-1}. This study's treatments were devised as percentages of the algal ration volume with the amount of microplastics added calculated based on the volumes of the algal rations. This was done to investigate potential replacement of food particles with a comparable volume of inorganic, non-nutrient, particles; therefore, the concentrations we used by particle count were above those currently measured in the environment. However, it is important to note that we used 2 μm beads and current monitoring methods do not typically identify particles <10 μm (Beiras and Schönemann, 2020). Traditional sampling nets have mesh sizes of around 300 μm (Lindeque et al., 2020; Sutton et al., 2019), which would be too large to capture the size of microplastics used in this study. In their 2020 study, Lindeque et al. found that the concentration of microplastics found in environmental samples is highly dependent on the size of the mesh used for sampling, with decreasing net mesh size drastically increasing the concentration of microplastics. They also postulated that, because of these sampling constraints, microplastic estimates in the environment are underestimated (Lindeque et al., 2020). Additionally, microplastics in the environment occur in a variety of shapes that also include fragments and fibers. Nonetheless, the results of this work could be similar to the effects of different types and shapes of microplastics within the size range that larvae can ingest. Nutrient dilution, due to microplastic particles occupying the gut volume, appeared to be a large driver of the impacts observed in this study and could occur with microplastics of different shapes. More research would be necessary to identify the residence time of microplastic particles of different shapes within the guts of larval bivalves. Future work would also benefit from comparisons of impacts of microplastics and natural inert particles of similar size, such as clay and cellulose particles. This would help establish if the observed impacts of this study on bivalve larval physiology are specific to microplastic particles or can be

produced by other nutritionally inert natural particles, such as clay and cellulose microparticles.

5. Conclusion

Measures should be taken to ensure that microplastic particles remain in suspension in order to maintain concentrations during challenge assays with suspension-feeders, such as bivalve larvae. In this study, a system was developed for exposing larval mussels to ultra-pure microplastic beads, that optimized conditions for both larval growth and maintenance of suspended particle concentrations. Both survival and growth of larval *M. californianus* were impacted by two-week exposure to 2 μm latex beads during early development. Impacts on survival were more severe under food limited conditions where the volume of the algal ration was replaced by equivalent volumes of beads in order to maintain constant total suspended particle volumes. Experiments that do not account for this may be underestimating the impacts of microplastics on larvae in the natural environment. Larvae were found to actively take up the 2 μm microplastic beads, both in the presence and absence of algae. A dose-dependent relationship was observed in which increasing concentrations of suspended beads resulted in higher proportions of the larval gut being occupied by beads. More research is needed to determine the extent to which nutrient uptake and energy allocation of larval bivalves is impacted by exposure to microplastic particles.

Funding sources

This research was funded by the National Science Foundation Growing Convergence Research Big Idea, 1935028.

Author contributions

Conceptualization, **CL, SH, MH**; investigation, analysis, manuscript writing, **BC;** methodology, review, and editing**, BC, CL, SH, MH;** supervision, project administration **CL;** funding acquisition, **SH** (PI). All authors have approved the published version of the manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Stacey Harper and Chris Langdon reports financial support was provided by National Science Foundation.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgment

The authors would like to acknowledge the NIEHS T32 Training Grant, ES07060. The authors would also like to thank the anonymous reviewers whose feedback has improved this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2023.151929.

References

Amariei, G., Rosal, R., Fernández-Piñas, F., Koelmans, A.A., 2022. Negative food dilution and positive biofilm carrier effects of microplastic ingestion by *D. magna* cause tipping points at the population level. Environ. Pollut. 294, 118622 https://doi.org/10.1016/j.envpol.2021.118622.

Athey, S.N., Albotra, S.D., Gordon, C.A., Monteleone, B., Seaton, P., Andrady, A.L., Taylor, A.R., Brander, S.M., 2020. Trophic transfer of microplastics in an estuarine food chain and the effects of a sorbed legacy pollutant. Limnol. Oceanogr. Lett. 5, 154–162. https://doi.org/10.1002/lol2.10130.

Azimi, P., Zhao, D., Pouzet, C., Crain, N.E., Stephens, B., 2016. Emissions of ultrafine particles and volatile organic compounds from commercially available desktop three-dimensional printers with multiple filaments. Environ. Sci. Technol. 50, 1260–1268. https://doi.org/10.1021/acs.est.5b04983.

Baechler, B.R., Granek, E.F., Hunter, M.V., Conn, K.E., 2020a. Microplastic concentrations in two Oregon bivalve species: spatial, temporal, and species variability. Limnol. Oceanogr. Lett. 5, 54–65. https://doi.org/10.1002/lol2.10124.

Baechler, B.R., Stienbarger, C.D., Horn, D.A., Joseph, J., Taylor, A.R., Granek, E.F., Brander, S.M., 2020b. Microplastic occurrence and effects in commercially harvested north American finfish and shellfish: current knowledge and future directions. Limnol. Oceanogr. Lett. 5, 113–136. https://doi.org/10.1002/lol2.10122.

Balbi, T., Camisassi, G., Montagna, M., Fabbri, R., Franzellitti, S., Carbone, C., Dawson, K., Canesi, L., 2017. Impact of cationic polystyrene nanoparticles (PS-NH2) on early embryo development of *Mytilus galloprovincialis*: effects on shell formation. Chemosphere 186, 1–9. https://doi.org/10.1016/j.chemosphere.2017.07.120.

Baldwin, B.S., Newell, R.I., 1995. Relative importance of different size food particles in the natural diet of oyster larvae. Mar. Ecol. Prog. Ser. 120, 135–145. https://doi.org/10.3354/meps120135.

Barnes, D.K., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. Philosoph. Trans. Royal Soc. B: Biol. Sci. 364, 1985–1998. https://doi.org/10.1098/rstb.2008.0205.

Batel, A., Borchert, F., Reinwald, H., Erdinger, L., Braunbeck, T., 2018. Microplastic accumulation patterns and transfer of benzo [a] pyrene to adult zebrafish (*Danio rerio*) gills and zebrafish embryos. Environ. Pollut. 235, 918–930. https://doi.org/10.1016/j.envpol.2018.01.028.

Beiras, R., Schönemann, A.M., 2020. Currently monitored microplastics pose negligible ecological risk to the global ocean. Sci. Rep. 10, 1–9. https://doi.org/10.1038/s41598-020-79304-z.

Beiras, R., Bellas, J., Cachot, J., Cormier, B., Cousin, X., Engwall, M., Gambardella, C., Garaventa, F., Keiter, S., Le Bihanic, F., 2018. Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton. J. Hazard. Mater. 360, 452–460. https://doi.org/10.1016/j.jhazmat.2018.07.101.

Borrelle, S.B., Ringma, J., Law, K.L., Monnahan, C.C., Lebreton, L., McGivern, A., Murphy, E., Jambeck, J., Leonard, G.H., Hilleary, M.A., 2020. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. Science 369, 1515–1518. https://doi.org/10.1126/science.aba3656.

Bringer, A., Cachot, J., Prunier, G., Dubillot, E., Clérandeau, C., Thomas, H., 2020a. Experimental ingestion of fluorescent microplastics by pacific oysters, *Crassostrea gigas*, and their effects on the behaviour and development at early stages. Chemosphere 254, 126793. https://doi.org/10.1016/j.chemosphere.2020.126793.

Bringer, A., Thomas, H., Prunier, G., Dubillot, E., Bossut, N., Churlaud, C., Clérandeau, C., Le Bihanic, F., Cachot, J., 2020b. High density polyethylene (HDPE) microplastics impair development and swimming activity of Pacific oyster D-larvae, *Crassostrea gigas*, depending on particle size. Environ. Pollut. 260, 113978 https://doi.org/10.1016/j.envpol.2020.113978.

Bringer, A., Cachot, J., Dubillot, E., Lalot, B., Thomas, H., 2021. Evidence of deleterious effects of microplastics from aquaculture materials on pediveliger larva settlement and oyster spat growth of Pacific oyster, *Crassostrea gigas*. Sci. Total Environ. 794, 148708 https://doi.org/10.1016/j.scitotenv.2021.148708.

Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C., 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). Environ. Sci. Technol. 42, 5026–5031. https://doi.org/10.1021/es800249a.

Capolupo, M., Franzellitti, S., Valbonesi, P., Lanzas, C.S., Fabbri, E., 2018. Uptake and transcriptional effects of polystyrene microplastics in larval stages of the Mediterranean mussel *Mytilus galloprovincialis*. Environ. Pollut. 241, 1038–1047. https://doi.org/10.1016/j.envpol.2018.06.035.

Carr, K.E., Smyth, S.H., McCullough, M.T., Morris, J.F., Moyes, S.M., 2012. Morphological aspects of interactions between microparticles and mammalian cells: intestinal uptake and onward movement. Prog. Histochem. Cytochem. 46, 185–252. https://doi.org/10.1016/j.proghi.2011.11.001.

Cole, M., Galloway, T.S., 2015. Ingestion of nanoplastics and microplastics by Pacific oyster larvae. Environ. Sci. Technol. 49, 14625–14632. https://doi.org/10.1021/acs.est.5b04099.

Cole, M., Liddle, C., Consolandi, G., Drago, C., Hird, C., Lindeque, P.K., Galloway, T.S., 2020. Microplastics, microfibers and nanoplastics cause variable sub-lethal responses in mussels (*Mytilus* spp.). Mar. Pollut. Bull. 160, 111552 https://doi.org/10.1016/j.marpolbul.2020.111552.

Costa, S., Coppola, F., Pretti, C., Intorre, L., Meucci, V., Soares, A.M., Freitas, R., Solé, M., 2020. The influence of climate change related factors on the response of two clam species to *diclofenac*. Ecotoxicol. Environ. Saf. 189, 109899 https://doi.org/10.1016/j.ecoenv.2019.109899.

Covernton, G.A., Collicutt, B., Gurney-Smith, H.J., Pearce, C.M., Dower, J.F., Ross, P.S., Dudas, S.E., 2019. Microplastics in bivalves and their habitat in relation to shellfish aquaculture proximity in coastal British Columbia, Canada. Aquac. Environ. Interact. 11, 357–374. https://doi.org/10.3354/aei00316.

Cózar, A., Echevarría, F., González-Gordillo, J.I., Irigoien, X., Úbeda, B., Hernández-León, S., Palma, Á.T., Navarro, S., García-de-Lomas, J., Ruiz, A., 2014. Plastic debris in the open ocean. Proc. Natl. Acad. Sci. 111, 10239–10244. https://doi.org/10.1073/pnas.1314705111.

Davidson, K., Dudas, S.E., 2016. Microplastic ingestion by wild and cultured Manila clams (*Venerupis philippinarum*) from Baynes sound, British Columbia. Arch. Environ. Contam. Toxicol. 71, 147–156. https://doi.org/10.1007/s00244-016-0286-4.
de Ruijter, V.N., Redondo-Hasselerharm, P.E., Gouin, T., Koelmans, A.A., 2020. Quality criteria for microplastic effect studies in the context of risk assessment: a critical review. Environ. Sci. Technol. 54, 11692–11705. https://doi.org/10.1021/acs.est.0c03057.
Deng, Y., Zhang, Y., Lemos, B., Ren, H., 2017. Tissue accumulation of microplastics in mice and biomarker responses suggest widespread health risks of exposure. Sci. Rep. 7, 46687. https://doi.org/10.1038/srep46687.
e Silva, P.P.G., Nobre, C.R., Resaffe, P., Pereira, C.D.S., Gusmão, F., 2016. Leachate from microplastics impairs larval development in brown mussels. Water Res. 106, 364–370. https://doi.org/10.1016/j.watres.2016.10.016.
Eriksen, M., Lebreton, L.C., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F., Ryan, P.G., Reisser, J., 2014. Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250, 000 tons afloat at sea. PLoS One 9, e111913. https://doi.org/10.1371/journal.pone.0111913.
Fabra, M., Williams, L., Watts, J.E., Hale, M.S., Couceiro, F., Preston, J., 2021. The plastic Trojan horse: biofilms increase microplastic uptake in marine filter feeders impacting microbial transfer and organism health. Sci. Total Environ. 797, 149217 https://doi.org/10.1016/j.scitotenv.2021.149217.
Fadare, O.O., Wan, B., Guo, L.-H., Xin, Y., Qin, W., Yang, Y., 2019. Humic acid alleviates the toxicity of polystyrene nanoplastic particles to *Daphnia magna*. Environ. Sci. Nano 6, 1466–1477. https://doi.org/10.1039/C8EN01457D.
Fendall, L.S., Sewell, M.A., 2009. Contributing to marine pollution by washing your face: microplastics in facial cleansers. Mar. Pollut. Bull. 58, 1225–1228. https://doi.org/10.1016/j.marpolbul.2009.04.025.
Fernández, B., Albentosa, M., 2019. Insights into the uptake, elimination and accumulation of microplastics in mussel. Environ. Pollut. 249, 321–329. https://doi.org/10.1016/j.envpol.2019.03.037.
Franzellitti, S., Capolupo, M., Wathsala, R.H., Valbonesi, P., Fabbri, E., 2019. The multixenobiotic resistance system as a possible protective response triggered by microplastic ingestion in Mediterranean mussels (*Mytilus galloprovincialis*): larvae and adult stages. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 219, 50–58. https://doi.org/10.1016/j.cbpc.2019.02.005.
Gallager, S.M., 1988. Visual observations of particle manipulation during feeding in larvae of a bivalve mollusc. Bull. Mar. Sci. 43, 344–365.
Gardon, T., Reisser, C.L., Soyez, C., Quillien, V., Le Moullac, G., 2018. Microplastics affect energy balance and gametogenesis in the pearl oyster *Pinctada margaritifera*. Environ. Sci. Technol. 52, 5277–5286. https://doi.org/10.1021/acs.est.8b00168.
Gigault, J., El Hadri, H., Nguyen, B., Grassl, B., Rowenczyk, L., Tufenkji, N., Feng, S., Wiesner, M., 2021. Nanoplastics are neither microplastics nor engineered nanoparticles. Nat. Nanotechnol. 16, 501–507. https://doi.org/10.1038/s41565-021-00886-4.
Gray, M.W., Kramer, S., Langdon, C., 2015. Particle processing and gut kinematics of planktotrophic bivalve larvae. Mar. Biol. 162, 2187–2201. https://doi.org/10.1007/s00227-015-2746-1.
Ishiwata, Y., Ohi, N., Obata, M., Taguchi, S., 2013. Carbon to volume relationship of *Isochrysis galbana* (*Prymnesiophyceae*) during cell divisions. Plankton Benthos Res. 8, 178–185. https://doi.org/10.3800/pbr.8.178.
Jeyavani, J., Sibiya, A., Bhavaniramya, S., Mahboob, S., Al-Ghanim, K.A., Nisa, Z.-U., Riaz, M.N., Nicoletti, M., Govindarajan, M., Vaseeharan, B., 2022. Toxicity evaluation of polypropylene microplastic on marine microcrustacean *Artemia salina*: an analysis of implications and vulnerability. Chemosphere 296, 133990. https://doi.org/10.1016/j.chemosphere.2022.133990.
Jones, D., Mitchley, M., 2001. A Reassessment of the Use of Ligno Sulphonate as an Additive for Unsealed Roads, ARRB Transport Research LTD Conference, 20th, 2001, Melbourne, Victoria, Australia, pp. 1–18.
Junaid, M., Wang, J., 2021. Interaction of nanoplastics with extracellular polymeric substances (EPS) in the aquatic environment: a special reference to eco-corona formation and associated impacts. Water Res. 201, 117319 https://doi.org/10.1016/j.watres.2021.117319.
Ke, A.-Y., Chen, J., Zhu, J., Wang, Y.-H., Hu, Y., Fan, Z.-L., Chen, M., Peng, P., Jiang, S.-W., Xu, X.-R., 2019. Impacts of leachates from single-use polyethylene plastic bags on the early development of clam *Meretrix meretrix* (Bivalvia: *Veneridae*). Mar. Pollut. Bull. 142, 54–57. https://doi.org/10.1016/j.marpolbul.2019.03.029.
Kiørboe, T., Mølenberg, F., Nøhr, O., 1980. Feeding, particle selection and carbon absorption in *Mytilus edulis* in different mixtures of algae and resuspended bottom material. Ophelia 19, 193–205. https://doi.org/10.1080/00785326.1980.10425516.
Lau, W.W., Shiran, Y., Bailey, R.M., Cook, E., Stuchtey, M.R., Koskella, J., Velis, C.A., Godfrey, L., Boucher, J., Murphy, M.B., 2020. Evaluating scenarios toward zero plastic pollution. Science 369, 1455–1461. https://doi.org/10.1126/science.aba9475.
Lenz, R., Enders, K., Nielsen, T.G., 2016. Microplastic exposure studies should be environmentally realistic. In: Proceedings of the National Academy of Sciences, 113, pp. E4121–E4122. https://doi.org/10.1073/pnas.1606615113.
Li, J., Lusher, A.L., Rotchell, J.M., Deudero, S., Turra, A., Bråte, I.L.N., Sun, C., Hossain, M.S., Li, Q., Kolandhasamy, P., 2019. Using mussel as a global bioindicator of coastal microplastic pollution. Environ. Pollut. 244, 522–533. https://doi.org/10.1016/j.envpol.2018.10.032.
Lindeque, P.K., Cole, M., Coppock, R.L., Lewis, C.N., Miller, R.Z., Watts, A.J., Wilson-McNeal, A., Wright, S.L., Galloway, T.S., 2020. Are we underestimating microplastic abundance in the marine environment? A comparison of microplastic capture with nets of different mesh-size. Environ. Pollut. 265, 114721 https://doi.org/10.1016/j.envpol.2020.114721.
Liu, J., Zhu, X., Teng, J., Zhao, J., Li, C., Shan, E., Zhang, C., Wang, Q., 2021. Pollution characteristics of microplastics in mollusks from the coastal area of Yantai, China. Bull. Environ. Contam. Toxicol. 107, 693–699. https://doi.org/10.1007/s00128-021-03276-7.
Loosanoff, V.L., Davis, H.C., Chanley, P.E., 1966. Dimensions and shapes of larvae of some marine bivalve mollusks. Malacologia 4, 351–435.
MacLeod, M., Arp, H.P.H., Tekman, M.B., Jahnke, A., 2021. The global threat from plastic pollution. Science 373, 61–65. https://doi.org/10.1126/science.abg5433.
Mankin, C., Huvard, A., 2020. Microfibers in *Mytilus* species (Mollusca, Bivalvia) from Southern California harbors, beaches, and supermarkets. Am. J. Undergrad. Res. 17, 35–44. https://doi.org/10.33697/ajur.2020.019.
Mehinto, A.C., Coffin, S., Koelmans, A.A., Brander, S.M., Wagner, M., Thornton Hampton, L.M., Burton, A.G., Miller, E., Gouin, T., Weisberg, S.B., 2022. Risk-based management framework for microplastics in aquatic ecosystems. Microplast. Nanoplast. 2, 1–10. https://doi.org/10.1186/s43591-022-00033-3.
Miller, M.E., Hamann, M., Kroon, F.J., 2020. Bioaccumulation and biomagnification of microplastics in marine organisms: a review and meta-analysis of current data. PLoS One 15, e0240792. https://doi.org/10.1371/journal.pone.0240792.
Newell, R.I., Langdon, C.J., 1996. Mechanisms and physiology of larval and adult feeding. In: The eastern oyster *Crassostrea virginica*, Maryland Sea Grant College, College Park, Maryland, pp. 185–229.
Ogonowski, M., Schür, C., Jarsén, Å., Gorokhova, E., 2016. The effects of natural and anthropogenic microparticles on individual fitness in *Daphnia magna*. PLoS One 11, e0155063. https://doi.org/10.1371/journal.pone.0155063.
Paul-Pont, I., Lacroix, C., Fernández, C.G., Hégaret, H., Lambert, C., Le Goïc, N., Frère, L., Cassone, A.-L., Sussarellu, R., Fabioux, C., 2016. Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: toxicity and influence on fluoranthene bioaccumulation. Environ. Pollut. 216, 724–737. https://doi.org/10.1016/j.envpol.2016.06.039.
Pepi, M., Focardi, S., 2021. Antibiotic-resistant bacteria in aquaculture and climate change: a challenge for health in the Mediterranean area. Int. J. Environ. Res. Public Health 18, 5723. https://doi.org/10.3390/ijerph18115723.
PlasticsEurope, E., EuPR, E., 2020. Plastics-the facts 2020. In: An Analysis of European Plastics Production, Demand and Waste Data, pp. 1–64.
Prado, S., Romalde, J.L., Barja, J.L., 2010. Review of probiotics for use in bivalve hatcheries. Vet. Microbiol. 145, 187–197. https://doi.org/10.1016/j.vetmic.2010.08.021.
Qiao, R., Lu, K., Deng, Y., Ren, H., Zhang, Y., 2019. Combined effects of polystyrene microplastics and natural organic matter on the accumulation and toxicity of copper in zebrafish. Sci. Total Environ. 682, 128–137. https://doi.org/10.1016/j.scitotenv.2019.05.163.
Rist, S., Baun, A., Almeda, R., Hartmann, N.B., 2019a. Ingestion and effects of micro-and nanoplastics in blue mussel (*Mytilus edulis*) larvae. Mar. Pollut. Bull. 140, 423–430. https://doi.org/10.1016/j.marpolbul.2019.01.069.
Rist, S., Steensgaard, I.M., Guven, O., Nielsen, T.G., Jensen, L.H., Møller, L.F., Hartmann, N.B., 2019b. The fate of microplastics during uptake and depuration phases in a blue mussel exposure system. Environ. Toxicol. Chem. 38, 99–105. https://doi.org/10.1002/etc.4285.
Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. PLoS One 10, e0146021. https://doi.org/10.1371/journal.pone.0146021.
Roald, S.O., 1977. Acute toxicity of lignosulphonates on rainbow trout (*Salmo gairdneri*). Bull. Environ. Contam. Toxicol. 17, 702–706. https://doi.org/10.1007/bf01685956.
Rochman, C.M., Brookson, C., Bikker, J., Djuric, N., Earn, A., Bucci, K., Athey, S., Huntington, A., McIlwraith, H., Munno, K., 2019. Rethinking microplastics as a diverse contaminant suite. Environ. Toxicol. Chem. 38, 703–711. https://doi.org/10.1002/etc.4371.
Rosa, M., Ward, J.E., Shumway, S.E., Wikfors, G.H., Pales-Espinosa, E., Allam, B., 2013. Effects of particle surface properties on feeding selectivity in the eastern oyster *Crassostrea virginica* and the blue mussel *Mytilus edulis*. J. Exp. Mar. Biol. Ecol. 446, 320–327. https://doi.org/10.1016/j.jembe.2013.05.011.
RStudio Team, 2020. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA. URL. http://www.rstudio.com/. URL.
Rullens, V., Lohrer, A.M., Townsend, M., Pilditch, C.A., 2019. Ecological mechanisms underpinning ecosystem service bundles in marine environments–a case study for shellfish. Front. Mar. Sci. 6, 1–16. https://doi.org/10.3389/fmars.2019.00409.
Rummel, C.D., Jahnke, A., Gorokhova, E., Kühnel, D., Schmitt-Jansen, M., 2017. Impacts of biofilm formation on the fate and potential effects of microplastic in the aquatic environment. Environ. Sci. Technol. Lett. 4, 258–267. https://doi.org/10.1021/acs.estlett.7b00164.
Salimi, A., Alavehzadeh, A., Ramezani, M., Pourahmad, J., 2022. Differences in sensitivity of human lymphocytes and fish lymphocytes to polyvinyl chloride microplastic toxicity. Toxicol. Ind. Health 38, 100–111. https://doi.org/10.1177/07482337211065832.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH image to image J: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.
Schür, C., Weil, C., Baum, M., Wallraff, J., Schreier, M., Oehlmann, J.R., Wagner, M., 2021. Incubation in wastewater reduces the multigenerational effects of microplastics in *Daphnia magna*. Environ. Sci. Technol. 55, 2491–2499 doi:10.1021%2Facs.est.0c07911.
Serra-Compte, A., Maulvault, A.L., Camacho, C., Alvarez-Munoz, D., Barceló, D., Rodriguez-Mozaz, S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*). Environ. Pollut. 236, 824–834. https://doi.org/10.1016/j.envpol.2018.02.018.
Shang, Y., Gu, H., Li, S., Chang, X., Sokolova, I., Fang, J.K., Wei, S., Chen, X., Hu, M., Huang, W., 2021. Microplastics and food shortage impair the byssal attachment of

thick-shelled mussel *Mytilus coruscus*. Mar. Environ. Res. 171, 105455 https://doi.org/10.1016/j.marenvres.2021.105455.

Shupe, H.J., Boenisch, K.M., Harper, B.J., Brander, S.M., Harper, S.L., 2021. Effect of nanoplastic type and surface chemistry on particle agglomeration over a salinity gradient. Environ. Toxicol. Chem. 40, 1820–1826 doi:10.1002%2Fetc.5030.

Summers, S., Henry, T., Gutierrez, T., 2018. Agglomeration of nano-and microplastic particles in seawater by autochthonous and de novo-produced sources of exopolymeric substances. Mar. Pollut. Bull. 130, 258–267. https://doi.org/10.1016/j.marpolbul.2018.03.039.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M.E.J., Le Goïc, N., Quillien, V., Mingant, C., Epelboin, Y., 2016. Oyster reproduction is affected by exposure to polystyrene microplastics. Proc. Natl. Acad. Sci. 113, 2430–2435. https://doi.org/10.1073/pnas.1519019113.

Sutton, R., Franz, A., Gilbreath, A., Lin, D., Miller, L., Sedlak, M., Wong, A., Box, C., Holleman, R., Munno, K., 2019. Understanding microplastic levels, pathways, and transport in the San Francisco Bay region. San Francisco Estuary Institute, SFEI-ASC Publication No. 950, 1–402.

Tallec, K., Huvet, A., Di Poi, C., González-Fernández, C., Lambert, C., Petton, B., Le Goïc, N., Berchel, M., Soudant, P., Paul-Pont, I., 2018. Nanoplastics impaired oyster free living stages, gametes and embryos. Environ. Pollut. 242, 1226–1235. https://doi.org/10.1016/j.envpol.2018.08.020.

Tenore, K., Dunstan, W., 1973. Comparison of feeding and biodeposition of three bivalves at different food levels. Mar. Biol. 21, 190–195. https://doi.org/10.1007/BF00355249.

USEPA, 2016. OCSPP 850.1055: bivalve acute toxicity test (embryo-larval). Ecological effects test guidelines. In: United States Environmental Protection Agency. Prevention, Pesticides and Toxic Substances, pp. 1–18.

Van Cauwenberghe, L., Janssen, C.R., 2014. Microplastics in bivalves cultured for human consumption. Environ. Pollut. 193, 65–70. https://doi.org/10.1016/j.envpol.2014.06.010.

Van Cauwenberghe, L., Claessens, M., Vandegehuchte, M.B., Janssen, C.R., 2015. Microplastics are taken up by mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) living in natural habitats. Environ. Pollut. 199, 10–17. https://doi.org/10.1016/j.envpol.2015.01.008.

van der Schatte Olivier, A., Jones, L., Vay, L.L., Christie, M., Wilson, J., Malham, S.K., 2020. A global review of the ecosystem services provided by bivalve aquaculture. Rev. Aquac. 12, 3–25. https://doi.org/10.1111/raq.12301.

Van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B.D., Van Franeker, J. A., Eriksen, M., Siegel, D., Galgani, F., Law, K.L., 2015. A global inventory of small floating plastic debris. Environ. Res. Lett. 10, 124006 https://doi.org/10.1088/1748-9326/10/12/124006.

Wang, S., Zhong, Z., Li, Z., Wang, X., Gu, H., Huang, W., Fang, J.K.-H., Shi, H., Hu, M., Wang, Y., 2021. Physiological effects of plastic particles on mussels are mediated by food presence. J. Hazard. Mater. 404, 124136 https://doi.org/10.1016/j.jhazmat.2020.124136.

Ward, J.E., Levinton, J.S., Shumway, S.E., 2003. Influence of diet on pre-ingestive particle processing in bivalves: I: transport velocities on the ctenidium. J. Exp. Mar. Biol. Ecol. 293, 129–149. https://doi.org/10.1016/S0022-0981(03)00218-1.

Ward, J.E., Rosa, M., Shumway, S.E., 2019. Capture, ingestion, and egestion of microplastics by suspension-feeding bivalves: a 40-year history. Anthropocene Coasts 2, 39–49. https://doi.org/10.1139/anc-2018-0027.

Watts, A.J., Urbina, M.A., Goodhead, R., Moger, J., Lewis, C., Galloway, T.S., 2016. Effect of microplastic on the gills of the shore crab *Carcinus maenas*. Environ. Sci. Technol. 50, 5364–5369. https://doi.org/10.1021/acs.est.6b01187.

Weber, A., Jeckel, N., Wagner, M., 2020. Combined effects of polystyrene microplastics and thermal stress on the freshwater mussel *Dreissena polymorpha*. Sci. Total Environ. 718, 137253 https://doi.org/10.1016/j.scitotenv.2020.137253.

Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492. https://doi.org/10.1016/j.envpol.2013.02.031.

Zhao, Y., Qiao, R., Zhang, S., Wang, G., 2021. Metabolomic profiling reveals the intestinal toxicity of different length of microplastic fibers on zebrafish (*Danio rerio*). J. Hazard. Mater. 403, 123663 https://doi.org/10.1016/j.jhazmat.2020.123663.