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The plastic Trojan horse: Biofilms increase microplastic uptake in marine filter feeders impacting microbial transfer and organism health

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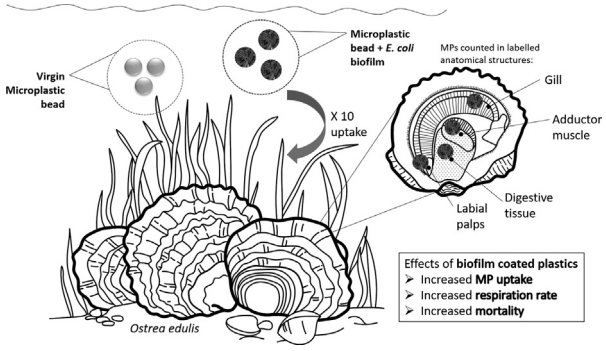
HIGHLIGHTS

- Microplastic (MP) pollution and its impact on marine organisms is a major concern.
- Microplastics are readily colonised with a biofilm in the marine environment.
- Filter-feeder uptake of microbially-coated MP was 10× greater than virgin MP uptake.
- Metabolic impacts were observed in filter-feeders exposed to microbially-coated MPs.
- Studies using virgin MP can underestimate the uptake and impact on marine organisms.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastic pollution has become a major source of concern, with a large body of literature surrounding the impacts of microplastic ingestion by biota. However, many of these studies utilise virgin microbeads, which are not reflective of environmental microplastics that are rapidly colonised with microbial communities (plastisphere) in marine ecosystems. It is a concern therefore that current evidence of the impacts of microplastics on biota are unrepresentative of the environmental microplastic pollution. In this study, uptake and bioaccumulation of both virgin and *Escherichia coli* coated microplastics, by European native oysters (*Ostrea edulis*) were compared, and the physiological responses of oysters to the exposure were investigated. The uptake of *E. coli* coated microplastics was found to be significantly higher than the uptake of virgin microplastics, with average concentrations of 42.3 ± 23.5 no. g^{-1} and 11.4 ± 0.6 no. g^{-1} microbeads found in oysters exposed to coated and virgin microplastics, respectively. This suggests that environmental microplastic uptake into the marine trophic web by benthic filter feeders may be greater than previously thought. The oxygen consumption and respiration rate of oysters exposed to *E. coli* coated microplastics increased significantly over time, whilst virgin microplastics did not produce any measurable significant physiological responses. However, less than 0.5% of the total amount of administered microbeads were retained by all oysters, suggesting a limited residence time within the organisms. Although microplastics did not bioaccumulate in oyster tissues in the short-term, microorganisms assimilated by the ingestion of coated microplastics may be transferred to higher trophic levels. This poses a risk, not only for wildlife, but also for food safety and human health. The capacity to carry pathogens and expose a wide range of organisms to them means microplastics may have an important role as vectors for disease.

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1. Introduction

Plastic production dramatically increased over the decades, from approximately 2 million Mt. of plastic produced annually in the 1950s, up to 350 million Mt. produced in 2017 (Geyer et al., 2017). As a direct consequence, plastic waste production has also increased over time. It is estimated that 79% of the 9.1 billion tons of plastics produced to date was deposited in landfills and the natural environment, and this is predicted to double by 2050 (Geyer et al., 2017). Moreover, indiscriminate disposal and accidental release of plastics contribute greatly to their heterogeneous distribution and uncontrolled accumulation in the environment. Plastic waste generated on land usually enters the aquatic environment intentionally, or more often, accidentally through waste mismanagement (Law, 2017). In 2010 alone, an estimated 4 to 12 million MT of plastic entered the marine environment and this is predicted to increase by an order of magnitude by 2025 (Jambeck et al., 2015). Once in the marine environment, plastics degrade into smaller pieces, rather than undergoing a straightforward process of mineralization. The entry of plastic debris in the marine environment and the effects of plastic contamination on biodiversity and human health have become a source of concern during the past decades (Secretariat of the Convention on Biological Diversity (SCBD), 2012). Of particular concern are microplastics, which are small pieces of plastics with size ranging between 100 nm and 5 mm, although the appropriateness of this definition is still debated (Frias and Nash, 2019). Microplastics are characterised by various shapes (e.g., spherical and fibres) and compositions (e.g., polystyrene, polypropylene and polyamide), and they are commonly classified as primary and secondary, based on their origin (Cole et al., 2011). Primary microplastics are manufactured on the microscale and found in cosmetics, personal-hygiene and cleaning products (Boucher and Friot, 2017). Secondary microplastics usually result from the fragmentation of larger debris by mechanical-, photo-, chemical-, thermal-, and biological- degradation (Efimova et al., 2018; Gewert et al., 2015). Microplastics can enter the marine environment through many different pathways, including riverine transport, sewage and wastewater effluents, direct release, and atmospheric deposition. The current number of microplastic particles floating on the ocean's surface ranges from 15 to 51 trillion (Van Sebille et al., 2015), and it is estimated that 490,000 t of buoyant microplastics entered the marine environment in 2010 alone (Burns and Boxall, 2018). It is widely accepted that once released, it is practically and economically infeasible to recapture marine microplastics for recycling or disposal. Consequently, microplastic debris is ubiquitous in the aquatic environment, found even in the most remote habitats, such as the deep-sea, mid-oceanic islands and polar ice caps (Peeken et al., 2018; Kane and Clare, 2019), with the highest concentrations usually observed in heavily industrialised and urbanised waters (Shahul Hamid et al., 2018).

The ingestion of microplastics by a wide variety of taxa poses a risk to wildlife and ecological processes (Galloway et al., 2017). Microplastics have been detected in different organs (e.g., liver, gut, muscle, and gills) of a wide range of organisms, including zooplankton, molluscs, crustaceans, fish and seabirds (Abbasi et al., 2018; Guzzetti et al., 2018). A large portion of microplastics suspended in the water column can be captured and directly ingested by planktonic and benthic organisms. Due to their sessile lifestyle, which inhibits avoidance behaviours, oysters and other bivalves are usually more vulnerable to contaminant accumulation, and hence considered ideal models for ecotoxicology. Recently, many studies have focused on the uptake of microplastics by bivalve molluscs because, as filter feeders, they process large volumes of water to capture particles (Canesi et al., 2012; Qu et al., 2018). Microplastics may also be ingested indirectly through trophic transfer, if predators consume contaminated prey (Nelms et al., 2018), although very little research is available on biomagnification of microplastics, with opposing viewpoints. The effects of ingested microplastics have been largely investigated on several taxa, revealing physical damages, metabolic disorder, growth inhibition, oxidative stress and genotoxicity, under laboratory conditions (Wright et al., 2013; Foley et al., 2018; Sussarellu et al., 2016). It has also been suggested that microplastics may have a further impact on food security (Hantoro et al., 2019), human health (Galloway, 2015), and socio-economic wellbeing (Beaumont et al., 2019) when accumulated in aquatic organisms of commercial interest.

However, to date the majority of studies investigating the impacts of microplastics ingestion by aquatic organisms have used virgin or uncolonised plastic particles, which do not reflect conditions in the environment accurately. Once in the aquatic environment, microplastics are colonised by a wide variety of microorganisms, forming multispecies biofilms characterised by surface-associated microbial cells enclosed in an extracellular polymeric substance matrix. The microbial communities coating plastic debris are collectively referred to as the 'plastisphere' (Jacquin et al., 2019). Biofilms differ in microbial composition and concentration compared to the surrounding medium and certain types of microplastic can also harbour specific taxa, found exclusively upon them (Zettler et al., 2013; Ogonowski et al., 2018). Recent studies have identified hazardous microorganisms and gut-associated pathogens usually found in sewage, colonising the surface of microplastics (Rodrigues et al., 2019). For example, *Escherichia coli,* a faecal indicator often used to assess water quality and wastewater contamination, with pathogenic strains of concern for both human health and antibiotic resistance. This bacteria is common and persistent in marine coastal waters (e.g. Craig et al., 2004; Leonard et al., 2018), particularly those subjected to WWTW effluent releases. It has been shown that marine biofilms on submerged surfaces are reservoirs for *E. coli* (Shikuma and Hadfield, 2010) and drug resistant *E. coli* colonise suspended marine particles in estuaries (Song et al., 2020). Therefore, microplastic ingestion may be considered a threat to aquatic organisms, not only because of their intrinsic toxicity, but also because of their possible role as vectors for diseases. By concentrating microbes and pathogens from the surrounding environment on their surfaces, microplastics have the potential to deliver a higher infectious dose to organisms that consume them.

The present study assessed and compared the uptake and bioaccumulation of both virgin and *E. coli* coated microplastics by the European flat oyster *Ostrea edulis*. The physiological responses of oysters to both virgin and biofilm coated microplastics were also measured and compared, by monitoring oxygen consumption, respiration rate, clearance rate, mortality rate and condition index. It is hypothesised that biofilm coated microplastics uptake by filter-feeders will be greater as they may not be recognized as inorganic matter.

2. Methods

2.1. Oyster conditioning

Ninety oysters, collected from the University of Portsmouth's pontoon in Langstone Harbour (50° 48′ 24.0″ N, 1° 01′ 19.4″ W), were transferred to the Institute of Marine Sciences, University of Portsmouth, where all the experiments were conducted. Oyster shells were gently scrubbed to remove associated epi-fauna and flora, and their length (mm), width (mm), depth (mm) and wet weight (g) were measured (Supplementary Table A1). All oysters were equally divided among 9 different 30 L tanks (10 oysters per tank). Environmental conditions were maintained by a Recirculating Aquarium System (RAS) (TMC Reef Skim 1500, TMC V2 Bio Fluidised Sand Bed Filter) as follows: Temperature (13 °C), salinity (34‰), pH (8) and Dissolved Oxygen (DO) (11 mg L^{-1}). Parameters were measured and adjusted daily, if required. In order to recover from sampling, translocation and scrubbing, the oysters were starved (depuration) and acclimated for 48 h to laboratory conditions.

2.2. Experimental design

The experimental design included three treatments (A, B, C) and three replicates per treatment (1, 2, 3), with oysters fed only on algal paste as a control (A), on algal paste and virgin microplastics (B), or

algal paste and microplastics inoculated with the bacteria *E. coli* (C). From day three, all oysters were fed a concentrated algal paste (Ocean Delight), diluted to 25 × 103 cells mL-1, until the end of the experiment (day fourteen). The paste was a mix of 5 of the most commonly used algal species in aquaculture: *Isochrysis galbana, Pavlova lutheri, Nannochloropsis oculata, Tetraselmis suecica, Thalassiosira pseudonana* (Helm and Bourne, 2004). The exposure to both virgin and *E. coli* coated microplastics began on day four and lasted ten days (until day fourteen). The microplastics used in this experiment were acrylic PolyMethyl MethAcrylate (PMMA) microspheres (diameter mean = 45 μm, min = 20.2 μm max = 76.8 μm) (Sigma, UK) (Supplementary Fig. A1), a primary microplastic commonly used in cosmetics as well as air blasting of boats hulls and machinery to remove rust and fouling. As a consequence they are commonly found in coastal waters, categorised as the second most abundant type of aquatic plastic pollution (Erni-Cassola et al., 2019). A preliminary study was carried out, in order to determine the amount of microbeads present in a known weight of PMMA. This involved the dilution of two known quantities of microplastics (2 and 5.5 mg) in MilliQ water, filtration on a filter-paper, staining with Nile Red and counting under a fluorescent microscope (Supplementary Fig. A1). Each concentration was replicated three times and the results were used to estimate the mean of microbeads contained in 1 mg of microplastics (~750 beads mg-1) (Supplementary Table A2). The microplastic concentration used in previous studies (Sussarellu et al., 2016) was increased by a factor of 10, in order to be able to detect any responses from the oysters during short term exposure events (ten days). A final concentration of 0.33 mg L-1 of microbeads was used in this experiment, with a total of 10 mg (~7500 microbeads) added daily to each 30 L tank of treatments B (virgin microplastics) and treatment C (*E. coli* coated microplastics). During the daily three-hour feeding period the system's pump was turned off, and oysters from treatments B and C were moved to six external tanks (30*L*) in order to avoid microplastic and microbial contamination of the RAS. Air stones were added to each tank in order to maintain an internal flow and prevent the sedimentation of algal cells and microplastics.

2.3. E. coli coating

Five hundred milligrams of virgin PMMA microbeads (Sigma, UK) were added to a 50 mL LB broth (Sigma, UK), immediately inoculated with *Escherichia coli* (NCTC12923), and incubated for 24 h at 37 °C in a shaking incubator (220 rpm). Colony forming units were estimated using nutrient agar (Oxoid) counts and estimated at 1.68×10^8 CFU/mL. The culture was then filtered using a sterile Grade 1, (Whatman) cellulose filter (pore size 11 μm) and the PMMA microbeads were allowed to air dry and collected into sterile containers.

2.4. Physiological responses

Oyster health and mortality were checked daily by regular inspections, and the mortality rate was calculated for each treatment. Physiological responses were assessed during the experiment by measuring respiration and clearance rates, which are key variables that control physiological conditions such as the growth of marine bivalves (Hutchinson and Hawkins, 1992; La Peyre et al., 2020; Hartwell et al., 1991; Casas et al., 2018), and post-experiment by calculating the condition index.

2.4.1. Respiration rate

A system consisting of nine respiration chambers (1 L), connected to peristaltic pumps through silicone air tubes, was used to assess oyster oxygen consumption and respiration rate on day three (pre-exposure), day nine (five days of exposure), and day fourteen (ten days of exposure). One randomly selected oyster from each tank (n = 9) was placed into individual respiration chambers filled with seawater previously aerated with compressed air for 20 min to reach oxygen saturation. Respiration chambers were placed in an enclosed water bath in the dark at the same constant temperature as the RAS (13 °C). The same nine oysters were used throughout the experiment. Over a six-hour period, the dissolved oxygen (DO) (mL L^{-1}) was measured hourly for the first three hours, and then every half an hour, by using a Fibox 4 Fibre-Optic Oxygen Meter and oxygen sensor spots individually glued to the inside of each chamber (PreSens; Germany) (Supplementary Table A3). The meter was calibrated to 100% O_2 saturation before measurements, using aerated seawater, and 0% O_2 saturation by using sodium thiosulphite in excess. The oxygen consumption (mL L^{-1}), which is the difference of oxygen concentration between the starting (T_0 = 0 min) and the ending (T_1 = 390 min) points of measuring, was calculated for each treatment on each day of measuring (Supplementary Table A4). Three extra empty respiration chambers (no oysters) were filled with seawater only (control A), with virgin microplastics + seawater (control B), and with coated microplastics + seawater (control C) respectively. The oxygen concentration inside the three empty chambers was measured and recorded in the same way as mentioned above, and the change of oxygen concentration inside each empty chamber over the six-hour period was used as a control in the assessment of the respiration rate (Supplementary Table A5). Respiration rate (mL O_2 h^{-1} g^{-1} AFDW) was calculated on each day of measuring by using oxygen consumption data (Bayne et al., 1985). Respiration rate measurements were then normalised to the organic weight of each oyster (the organism's mass without inorganic matter) using the Ash Free Dry Weight (AFDW) (Supplementary Table A6). In order to calculate the AFDW following the gravimetric method, the soft tissue of the nine oysters used for respiration measurement was dried at the end of the experiment at 80 °C for 48 h. The dried flesh was then placed in a furnace at 550 °C for 16 h and the resulting ash was weighed and used to calculate the AFDW (Supplementary Table A7) (Walne and Mann, 1975).

2.4.2. Clearance rate

Clearance rate, defined as the volume of water completely cleared of suspended particles per unit of time, was calculated on day three (pre-exposure), day nine (five days of exposure) and day fourteen (ten days of exposure) by measuring the consumption of a fixed ration of algal cells in a static system. Three randomly selected oysters from each tank (n = 27) were placed on elevated holding platforms contained in 1 L glass beakers filled with 800 mL of filtered seawater (40 μm). The same twenty-seven oysters were used throughout the experiment. Algal paste, diluted to 25×10^3 cells mL^{-1} concentration, was then added to each beaker, and 2 ml water samples were collected from each beaker immediately after the addition of algal paste (T_0) and again after 1 h (T_1). Algal settlement was prevented by using magnetic stirrers and placing the magnetic bars underneath the holding platform. The collected samples were fixed with 20 μL of glutaraldehyde (1%) and refrigerated for 30 min before being frozen in liquid nitrogen and stored at −80 °C until the following analysis (Vaulot et al., 1989). A flow-cytometer (Sysmex CyFlow Cube 8) was used to determine the exact concentration of algal cells in each sample. Nine extra empty beakers (no oysters), filled only with the holding platform and seawater were used as a control, in order to assess the algal settlement (Supplementary Table A8). The mean value of settled algal cells was used as a control in the assessment of the clearance rate. Clearance rate (L h^{-1} g^{-1} AFDW) was calculated on each day of measuring by using algal cells consumption data (Coughlan, 1969). Clearance rate measurements were then normalised to the oyster organic mass using the mean AFDW value (Supplementary Table A9).

2.4.3. Condition index

Condition index, a rapid measure of ecophysiological activity in bivalves and other mollusc species, was assessed for three oysters from each tank (n = 27) at the end of the experiment (day fifteen). Flesh (soft tissue) and shells were dried separately at 80 °C for 48 h before being weighed. Dry weight data were then used to calculate the condition index (Crosby and Gale, 1990) (Supplementary Table A10).

2.5. Microbead load in oyster tissue

Microplastic load in oyster tissues, an indicator of intake and bioaccumulation of microbeads, was estimated at the end of the experiment (day fifteen) for one oyster from each tank (n = 9), by digestion of oyster flesh and microscopy. Following 1 h of defrosting, gills & mantle, labial palps, digestive tissue & gonads, and adductor muscles were dissected and weighed. Tissues were separately and rapidly dissolved in 15 ml of 10% potassium hydroxide (KOH) and heated in a water bath (100 °C) for 15 min, stirring every 5 min. This method of tissue digestion did not modify the external structure of microbeads as the melting temperature of PMMA is 160 °C and the integrity of the bead structure was maintained (Supplmentary Fig. 1A). Samples were filtered using a glass Buchner Filter (Cole-Parmer) and filter papers (F1/KA4, Smith Filters, 11 μm), which were dried at room temperature afterwards. Filters were stained with 600 μL of Nile Red solution (10 μg ml^{-1}) and incubated in the dark at room temperature for 15 min (Erni-Cassola et al., 2017). The number of microbeads on each filter paper were counted under a fluorescent microscope (Leica DFC310 FX, Leica EL6000) and used to calculate the total microbead concentration (no. g^{-1}) in each oyster, and the microbead concentration in different tissues of each oyster (Supplementary Table A11). The percentage of microbeads retained by each oyster in different tissues was calculated using the approximate amount of microbeads added daily to each tank (~7500 microbeads) throughout the entire experiment, (Supplementary Table A12). PMMA microsphere characteristics were determined by spectroscopy; Raman spectra of the untreated MP PMMA beads in comparison to reference spectra were taken using a 532 nm laser and Renishaw InVia Qontor MicroRamen and WiRE software. PMMA microbead morphological summary statistics collected from Renishaw Particle Analysis software (n = 53) (Supplementary Fig. A1).

2.6. Statistical analyses

Physiological performance data (oxygen consumption, respiration rate, clearance rate and condition index) were first tested for normality and homogeneity of variance. No transformations were applied as the normality tests indicated normal distributions of data. A two-way ANOVA (analysis of variance) was performed to test how oxygen consumption, respiration and clearance rates varied between treatments (Factor: treatment; Levels: A – control; B - virgin microplastics; C - *E. coli* coated microplastics) and days of exposure to microplastics (Factor: exposure; Levels: pre-exposure – day 3; mid-exposure – day 9; post-exposure – day 14). Where a significant interaction between treatment and exposure was observed, simple main effects were investigated using one-way ANOVAs. Where significant main and simple main effects were identified, a *post-hoc* PAIRWISE test (Tukey's HSD test) was carried out. To determine whether there were significant differences in condition index between the three different treatments, a one-way ANOVA was performed. A two-way ANOVA was also applied to determine whether the concentration of microbeads varied between treatments (no microplastics, virgin microplastics, *E. coli* coated microplastics) and type of tissue (gills and mantle, labial palps, digestive tissue and gonads, and adductor muscle). A Johnson transformation was applied before carrying out the test, as the data showed a non-normal distribution. For all the tests mentioned above, statistical significance was accepted at $\alpha = 0.05$ (MINITAB® v.18).

3. Results

3.1. Mortality rate

Mortality rate was 0% for oysters fed on algal paste (treatment A) and exposed to virgin microplastics (MPs) (treatment B), and 3.3% for oysters exposed to *E. coli* coated MPs (treatment C), with one mortality after eleven days of exposure.

3.2. Respiration rate

A two-way ANOVA identified a significant interaction between the two factors (treatment × exposure) ($F = 13.90$; $p \leq 0.001$). After investigating simple main effects, *post-hoc* Tukey's tests found no significant increase in oxygen consumption after five days of exposure (day nine, mid-exposure) ($p > 0.22$). However, oyster oxygen consumption (mean ± SE) was significantly higher on day fourteen ($p \leq 0.001$), after ten days of exposure, than on days three and nine for every treatment (1.663 ± 0.04, 1.52 ± 0.029, and 2.523 ± 0.185 ml O2 L^{-1} for treatments A, B and C respectively) (Fig. 1a). No significant differences in oxygen consumption were found between oysters exposed to virgin MPs (treatment B) and the control (treatment A) on each day of measurements ($p > 0.563$). Therefore, the exposure to virgin MPs had no effects on oyster oxygen consumption. The oysters exposed to *E. coli* coated MPs (treatment C) consumed significantly more oxygen than oysters from the other treatments ($p \leq 0.001$) on both day nine (1.353 ± 0.028 ml O_2 L^{-1}) and day fourteen (2.523 ± 0.185 ml O_2 L^{-1}) (Fig. 2).

Significant differences in mean respiration rate were found between treatments ($F = 5.16$; $p = 0.017$) and between days of exposure ($F = 5.04$; $p = 0.018$). There was no significant interaction between the two factors (treatment × exposure) ($F = 1.10$; $p = 0.388$). There was no significant interaction between the two factors (treatment × exposure) ($F = 1.10$; $p = 0.388$). The *post-hoc* Tukey's test performed on the significant main effects identified no significant changes in oyster respiration rate after five days of exposure (day nine, mid-exposure) ($p = 0.977$). However, respiration rate was found significantly higher on day fourteen, after ten days of exposure, than on days nine ($p = 0.042$) and three ($p = 0.028$) for every treatment (0.247 ± 0.041, 0.334 ± 0.099 and 0.687 ± 0.231 ml O_2 h^{-1} g^{-1} for treatments A, B and C respectively) (Fig. 1b). Contrary to virgin MPs, which did not have significant effects on oyster respiration rate ($p = 0.838$), *E. coli* coated MPs had significant effects on oyster respiration ($p = 0.019$)

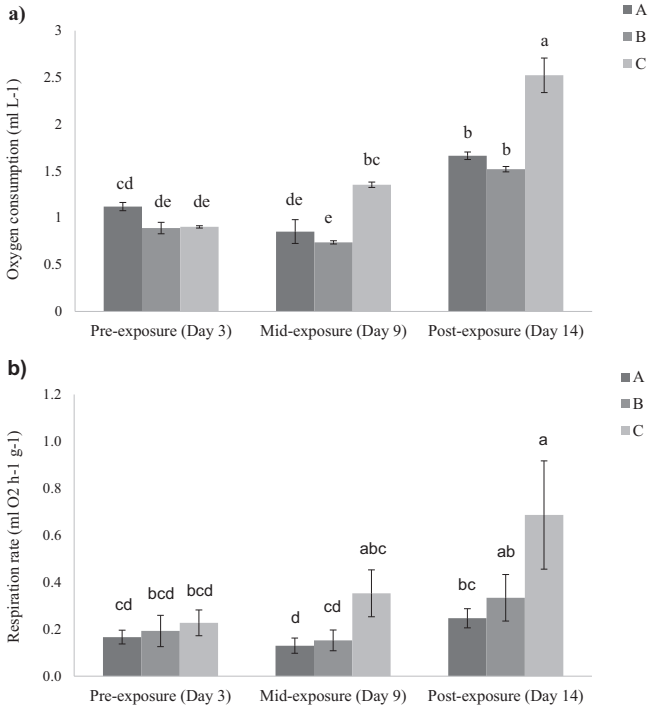

Fig. 1. Oxygen consumption (ml L^{-1}, mean ± SE) (a) and respiration rate (ml O_2 h^{-1} g^{-1} AFDW, mean ± SE) (b) of treatments A (control), B (virgin MPs) and C (*E. coli* coated MPs), before (day three), during (day nine) and after (day fourteen) the exposure to microplastics; means that share a letter (a–e) are not significantly different (two-way ANOVA, $p > 0.001$ (a), $p > 0.05$ (b)).

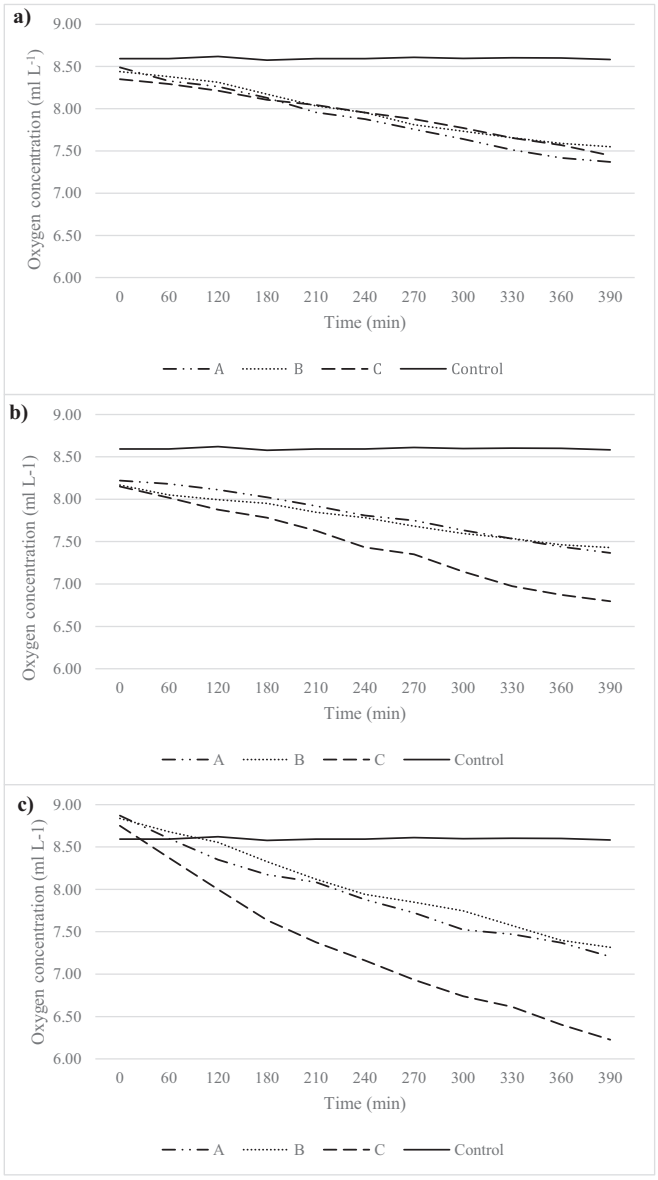

Fig. 2. Oxygen concentration (ml L^{-1}) of treatments A (control), B (virgin MPs) and C (*E. coli* coated MPs) recorded over a six-hour period, (a) before (day three), (b) during (day nine) and (c) after (day fourteen) the exposure to microplastics.

after only five days of exposure, with the highest values of respiration rate recorded for oysters exposed to coated MPs (treatment C) on both day nine (0.353 ± 0.100 ml O_2 h^{-1} g^{-1}) and day fourteen (0.687 ± 0.231 ml O_2 h^{-1} g^{-1}).

3.3. Clearance rate

There were no significant differences in mean clearance rate between treatments ($F = 1.77$; $p = 0.198$) and between days of exposure ($F = 1.79$; $p = 0.195$). No significant interaction was found between the two factors (treatment × exposure) ($F = 0.68$; $p = 0.615$). Therefore, ten days of exposure to both virgin (treatment B) and *E. coli* coated MPs (treatment C) did not have any significant effects on oyster clearance rate.

3.4. Condition index

No significant differences in the mean oyster condition index were found between treatments (One-way ANOVA; $F = 0.43$; $p = 0.670$). The mean values of condition index (mean ± SE) were: 2.331 ± 0.348 for oysters fed on algal paste (treatment A); 2.324 ± 0.265 for oysters exposed to virgin MPs (treatment B), and 1.986 ± 0.289 for oysters exposed to *E. coli* coated MPs (treatment C).

3.5. Microbeads load in oyster tissue

A two-way ANOVA identified significant differences in microbeads concentration (no. g^{-1}) between all treatments ($F = 19.40$; $p \leq 0.001$), with significantly higher concentrations of microbeads (mean ± SE) found in oysters exposed to *E. coli* coated MPs (treatment C) (42.360 ± 23.588 no. g^{-1}) compared to oysters exposed to virgin MPs (treatment B) (11.443 ± 0.4 no. g^{-1}) and the control (treatment A) (0.872 ± 0.317 no. g^{-1}). Considering the approximate amount of microbeads added daily to each tank (~7500 beads), the percentage of microbeads retained by the oysters exposed to virgin and *E. coli* coated MPs was 0.13% and 0.43% respectively (Fig. 3). The highest percent contribution to the total amount of microbeads found in oysters exposed to virgin MPs (treatment B) was from the digestive tissue and gonads (66.8%) (15.704 ± 2.120 no. g^{-1} microbeads), whilst the highest percent contribution to the total amount of microbeads found in oysters exposed to *E. coli* coated MPs (treatment C), was from the gills and mantle (47.2%) (57.277 ± 26.634 no. g^{-1} microbeads) (Fig. 3). Oysters exposed to *E. coli* coated MPs (treatment C) had the highest values of microbeads concentration in every group of tissues (Fig. 4a). However, no significant differences in microbeads concentration were found between different groups of tissues ($F = 0.45$; $p = 0.718$), and no significant interaction between the two factors (treatment × tissue) ($F = 1.14$; $p = 0.372$).

Considering the approximate amount of microbeads added daily to each tank (~7500 beads), the percentage of microbeads retained in the gills and mantle was 0.02% for oysters exposed to virgin MPs (treatment B) and 0.2% for oysters exposed to *E. coli* coated MPs (treatment C) (Fig. 4b). The percentage of microbeads retained in digestive tissue and gonads of oysters was 0.09% and 0.19% for the treatments containing virgin (treatment B) and coated MPs (treatment C), respectively. Low percentage of microbeads were retained in the adductor muscle of both treatments (0.01% and 0.02% for treatments B and C respectively), and in the labial palps of only oysters exposed to *E. coli* coated MPs (treatment C) (0.01%).

4. Discussion

The amount of microplastics ingested by bivalves is frequently low compared to concentrations in the environment. Previous studies have demonstrated that bivalves do not ingest all particles captured by the gills, (Rosa et al., 2018; Ward et al., 2019). Rather they select the particles whose physical characteristics resemble those of the food these organisms usually feed on (e.g., size and shape). Oysters have been found

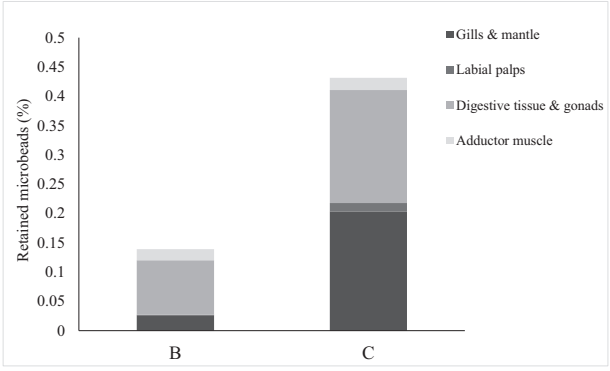

Fig. 3. Percentage of microbeads (%) retained by oysters exposed to virgin (treatment B) and *E. coli* coated (treatment C) microplastics in each group of tissues (gills & mantle, labial palps, digestive tissue & gonads, adductor muscle).

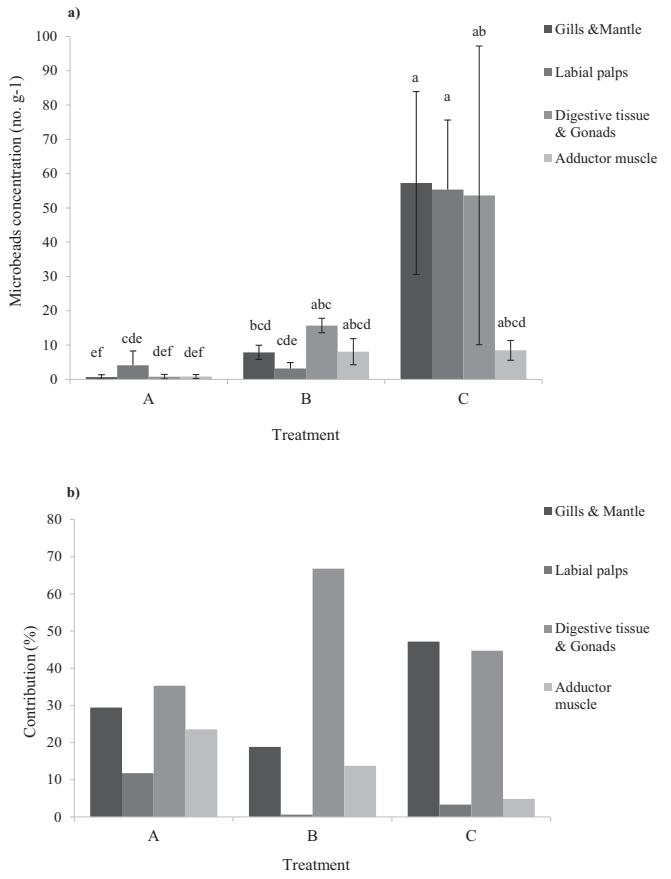

Fig. 4. Microbeads concentration (no. g^{-1}, mean ± SE) in four different groups of tissues (gills & mantle, labial palps, digestive tissue & gonads, adductor muscle) of treatments A (control), B (virgin MPs) and C (*E. coli* coated MPs) (a); means that share a letter (a–f) are not significantly different (two-way ANOVA, $p > 0.05$). Percentage of microbeads (%) retained in each group of tissues (gills & mantle, labial palps, digestive tissue & gonads, adductor muscle) of oysters exposed to virgin (B) and *E. coli* coated MPs (C) (b).

to be particularly selective, rejecting a higher proportion of microplastics. This might be due to the more complex heterorhabdic gill structure of oysters and their particle selection, which occurs in two sites (gills and labial palps) through bidirectional transport, (Ward et al., 1998). They firstly open the valves and the inflowing water slows as it enters the inhalant chamber, allowing the largest particles to settle onto the mantle being rejected (Yonge, 1926). The action of cilia makes the water flow between gill filaments and the remaining particles are trapped and deposited onto the gills, stimulating mucus production. Small and large particles are then separated, smaller particles on the basal groove of the gill demibranches and the larger on the gills' free margin. Whilst both sized particles move to the labial palps, the larger ones are eliminated onto the mantle. The small particles from the basal groove then enter the basal fold between the labial palps, experiencing little selection before ingestion. In contrast, the large particles from the free margin undergo more discrimination upon the labial palps, with the largest particles again falling onto the mantle (Yonge, 1926). Particles reaching the stomach enter the food sorting caecum and experience another selection based on mass, during which heavy particles move to the mid-gut to be expelled as faeces, whilst lighter particles re-enter the stomach's lumen and the digestive diverticula prior to digestion. All rejected particles are then expelled from the mantle as pseudofaeces via sudden valve contractions (Yonge, 1926). This mechanism of particle selection is mainly based on size and mass, although differences in capture efficiencies between algae of similar sizes might suggest an alternative nutrition-based selection process (Ward and Shumway, 2004). Capture efficiencies typically increase non-linearly with particle sizes over a 1 μm diameter, although the likelihood of ingestion decreases significantly with particles larger than 100 μm diameter, with a possible upper limit between 600 μm and 900 μm (Ward and Kach, 2009). *O. edulis* specifically can ingest particles between 0.6 μm and 363 μm diameter (Green, 2016).

This is congruent with the results of the current study, with less than 0.5% of the total amount of administered microbeads found in all oysters exposed to both virgin (0.13%) and *E. coli* coated (0.43%) microplastics (Fig. 3). However, the amount of microbeads ingested by oysters exposed to the *E. coli* coated microplastics (42.3 ± 23.5 no. g^{-1} microbeads) was significantly higher than the ones exposed to virgin microplastics (11.4 ± 0.6 no. g^{-1} microbeads), demonstrating that microplastics coated with biofilms are more likely to be ingested by primary consumers. The presence of a bacterial biofilm may inform that food is present and that a surface is neither toxic nor temporary. Organisms may also find microplastics carrying nutrient-rich biofilms more attractive than virgin ones, because of the higher nutritional characteristics, and they actively select them (Unabia and Hadfield, 1999). The percentage of microbeads retained in gills and mantle of oysters exposed to *E. coli* coated microplastics (0.2%) was ten times higher than oysters exposed to virgin microplastics (0.02%) (Fig. 4b) and confirms oysters' preference for biofilm coated particles in this study. The Water Framework Directive contains the requirement to assess designated shellfish waters according to *E. coli* levels present in sample populations. This classification is then broken-down into one of five categories (Class A, Long-term B, Class B, Class C and Prohibited) that then determines the areas where bivalves can be harvested from and if they are safe for human consumption. Therefore the potential for increased levels of *E.coli* contamination via concentration and uptake of MP is of concern for the shellfish industry and human health via shellfish consumption.

It has been suggested that microplastics can be translocated from the digestive tract into the haemolymph and other tissues, where they may bioaccumulate (Walkinshaw et al., 2020). The presence of microplastics has been observed in the circulatory system and different organs of crabs, bivalves and fish (Brennecke et al., 2015; Browne et al., 2008; Lu et al., 2016). Microplastic retention in soft-tissues of commercially important species may represent a substantial problem for aquaculture businesses, which sell them for human consumption (Carbery et al., 2018; Li et al., 2018; Teng et al., 2019). However, unlike other contaminants, such as organochlorines (Borgå et al., 2001) and mercury (Lavoie et al., 2013), microplastics do not appear to biomagnify. From previous studies, the presence of microbeads of different sizes (6- 500 μm in diameter) in both faeces and pseudofaeces of bivalves, suggests that these particles can be rejected, not only pre-ingestion, during the particle-selection process, but also after the ingestion, passing through the digestive system (Gonçalves et al., 2019). Therefore, microplastics might represent a more transitory contaminant with a limited residence time within organisms and this may explain why microplastics do not appear to biomagnify. Not translocating from the digestive tract into other tissues, it is much less likely that organisms at higher trophic levels will ingest significant amounts of microplastics through a carnivorous diet (Walkinshaw et al., 2020). Although the current study found microbeads in each group of tissues analysed, the high relative proportion of microbeads in the digestive tissue of oysters exposed to both virgin (66.8%) and *E. coli* coated microplastics (44.7%) supports the theory that microplastics are transitory (Fig. 3). Further analysis on microbead concentrations in faeces and pseudofaeces are needed to identify the pathway of microplastics through the digestive system of oysters and potential for bioaccumulation and biomagnification.

Despite several studies on bivalves having reported alterations in cell physiology, immune system responses, antioxidant capacity, reproductive function, behaviours, filtering activity and nutrient intake, metabolic-, respiration- and growth- rate following the exposure and retention of microplastics (Gardon et al., 2018; Green, 2016; Sussarellu et al., 2016), there is a growing set of evidence that the overall

exposure of aquatic organisms to the chemicals leaching from microplastics is negligible compared to other sources. Burns and Boxall (2018) suggest that the concentrations at which microplastics are toxic typically exceed those observed in the natural environment. This is confirmed by the present study, which did not report any significant differences in respiration rate, clearance rate and condition index between oysters exposed to virgin microplastics and the control. In contrast, the exposure to *E. coli* coated microplastics had clear effects on oyster respiration, with oxygen consumption increasing significantly over time (Fig. 2) and with significantly higher respiration rates compared to the other treatments (Fig. 1). The increases in oxygen consumption and respiration rates may be due to the immune system responses of oyster to the presence of *E. coli*. When recognized by bivalve haemocytes, *E. coli* can stimulate the production/release of antibacterial substances and additional haemocytes (Canesi et al., 2002). *E. coli* may therefore increase the metabolism and subsequently the oxygen demand and respiration rate of the infected oysters, as well as affecting their feeding behaviour and condition index. However, the increase in oxygen consumption and respiration rate, experienced by oysters exposed to biofilm coated microplastics, might also represent a physiological response to the higher microplastic uptake. As such, further investigations are needed to distinguish these interactions and effects of microplastic uptake on oyster health.

No significant differences in condition index and clearance rate were found between oysters exposed to *E. coli* coated microplastics and the control, although at the end of the experiment (after ten days of exposure), mortality was observed only in *E. coli* exposed oysters ($n = 1$), suggesting that a longer exposure to biofilm coated microplastics may lead to more substantial physiological responses.

Oysters and other bivalves are potentially at risk of bacterial (and possible pathogen) accumulation due to the large volumes of water they filter. However, the likelihood of being ingested is higher for bacteria attached to microplastics than freely suspended ones. For example, bacteria of the *Vibrio* genus are often found colonising the surface of microplastics at higher concentrations than the surrounding habitat (Kirstein et al., 2016). If microplastics carry pathogens and expose organisms that would not otherwise be exposed to these pathogens, microplastics will act as vectors for diseases (Silva et al., 2019; Jacquin et al., 2019).

Moreover, once ingested, freely suspended bacteria usually end up in the digestive tract where they are digested (McHenery and Birkbeck, 1985). In contrast, when attached to microplastics, bacteria are more likely to be rejected during the bivalves's particle selection, ending up in the mantle and promoting the infection of this tissue (Allam et al., 2013). Therefore, the attachment to microplastics could not only increase bacteria's likelihood of being captured, but also prevent bacteria from being digested and destroyed within the organism.

5. Conclusion

This study indicates that biofilms can make the surface of microplastics more attractive to benthic filter feeders, increasing the number of microbeads ingested. Future studies investigating the effects of environmental microplastic pollution on ecosystem health should consider that the presence of a biofilm can affect the uptake and organismal response to microplastics. Also, contrary to virgin microplastics, which did not induce any physiological responses, oysters exposed to biofilm coated microplastics showed an increase in oxygen consumption and respiration rate after only five days of exposure, suggesting an immune response of *O. edulis* to either the presence of *E. coli* or the higher microplastic uptake. The high concentration of microbeads found in the digestive tissue of oysters exposed to both virgin and *E. coli* coated microplastics suggests microplastics are transitory, with a limited residence time within organisms. However, whilst further research is needed to understand microplastic bioaccumulation and biomagnification pathways, pathogens assimilated by filter feeders may be transferred to higher trophic levels. The pathogen vector role of microplastics does pose a great risk, not only to aquatic wildlife, but also to food safety and human health, negatively impacting fisheries and aquaculture businesses. Therefore, studies that use virgin microplastics to investigate the effects on filter feeders, ignoring the presence of biofilms that are likely present in the natural environment, may lead to underestimation of the real risks microplastic pollution poses to wildlife and ecological processes, and the crucial role of microplastics as a vector for pathogen transmission.

CRediT authorship contribution statement

Monica Fabra performed the oyster exposure experiments. Luke Williams and Monica Fabra performed sample analysis. Joy Watts supplied microplastics coated with *E. coli*. Monica Fabra, Joanne Preston and Joy Watts designed the study. Monica Fabra, Joy Watts, Michelle Hale, Fay Couceiro and Joanne Preston wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149217.

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