Microplastic ingestion by zooplankton

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Abstract

Small plastic detritus, termed ‘microplastics’, are a widespread and ubiquitous contaminant of marine ecosystems across the globe. Ingestion of microplastics by marine biota, including mussels, worms, fish and seabirds, has been widely reported, but despite their vital ecological role in marine food-webs, the impact of microplastics on zooplankton remains under-researched. Here, we show that microplastics are ingested by, and may impact upon, zooplankton. We used bio-imaging techniques to document ingestion, egestion and adherence of microplastics in a range of zooplankton common to the northeast Atlantic, and employed feeding rate studies to determine the impact of plastic detritus on algal ingestion rates in copepods. Using fluorescence and coherent anti-Stokes Raman scattering (CARS) microscopy we identified that thirteen zooplankton taxa had the

capacity to ingest 1.7 – 30.6 µm polystyrene beads, with uptake varying by taxa, life-stage and bead-size. Post-ingestion, copepods egested faecal pellets laden with microplastics. We further observed microplastics adhered to the external carapace and appendages of exposed zooplankton. Exposure of the copepod *Centropages typicus* to natural assemblages of algae with and without microplastics showed that 7.3 µm microplastics (>4000 ml^{-1}) significantly decreased algal feeding. Our findings imply that marine microplastic debris can negatively impact upon zooplankton function and health.

Keywords

Plastic debris, microplastics, zooplankton, marine pollution, algal ingestion, coherent anti-Stokes Raman scattering microscopy, CARS

1. Introduction

It has been estimated that up to 10% of plastics produced globally enters our oceans, so it is of little surprise that plastic debris is now a pervasive and resilient pollutant of the marine environment [1, 2]. Larger plastic debris, such as monofilament line, plastic strapping and plastic bags, can entangle, garrotte, drown, or be eaten by an array of marine wildlife [3]. There is compelling evidence that microplastics – small plastic <5 mm in diameter – also negatively impact upon marine biota [4]. Microplastics consist of synthetic polymer products manufactured to be of a small size, such as exfoliates in cosmetics [5], and those items derived from the fragmentation of larger plastic debris, for example polyester fibres from fabrics [6], polyethylene fragments from plastic bags [7] and polystyrene particles from buoys and floats [8]. Typically, high-density plastics (e.g. polyvinyl chlorides, polyester) settle out of the water column, whilst low-density plastics (e.g. polyethylene, polystyrene) remain buoyant, although freshwater inputs, storms and biofilm formation may result

in vertical mixing [9, 10]. Floating plastic debris is susceptible to local and ocean currents resulting in higher-than-average waterborne microplastic concentrations in areas of confluence [11].

Microplastics are of environmental concern as their small size makes them available to a wide range of marine biota [12]. Microplastic ingestion has been demonstrated in marine organisms, including amphipods, lugworms and barnacles [4], mussels [13], decapod crustaceans [14], seabirds [15], and fish [16, 17]. Ingested microplastics might obstruct feeding appendages, aggregate and block the alimentary canal, limit the food intake of an organism or be translocated into the circulatory system [13, 14]. Further, microplastics may introduce toxicants to the organism: firstly, additives incorporated into a plastic during manufacture to improve its properties (e.g., phthalates for malleability and polybrominated diphenyl ethers (PDE) for heat resistance) might leach out of weathered plastic debris [18, 19]; secondly, the large surface area to volume ratio and hydrophobic properties of microplastics leave them susceptible to the accumulation of hydrophobic organic contaminants (HOCs) which could dissociate post-ingestion [20].

The extent to which microplastics are ingested and can impact upon zooplankton is uncertain. Zooplankton have a vital ecological role in marine ecosystems, both as primary consumers in the marine food web, and in the case of meroplankton, consisting of the juvenile life stage of numerous commercially important species. The widespread presence of small plastic debris in the water column makes interactions between zooplankton and microplastics highly likely; indeed, both small plastic debris and zooplankton >333 µm in diameter have been recurrently sampled together in sea surface trawls and by continuous plankton recorders [4, 11, 21, 22]. Zooplankton display a range of feeding modes, which vary by life-stage, species and prey availability [23]. Zooplankton can use a combination of chemo- and mechano-receptors to select prey, and their ability to preferentially feed on one species of algae over other algae, plastic beads or detritus has been demonstrated [24-26]. Laboratory experiments, in which latex beads were used to model algal ingestion, have shown that zooplankton have the potential to ingest small plastics [26-28]. Uptake of these small plastics likely

results from indiscriminate feeding modes (e.g. filter-feeding), by which prey with equivalent spherical diameters (ESD) <100 µm are non-selectively fed upon [23, 29].

Due to the complexities of sampling and extracting microplastics from the marine environment, existing studies have largely focussed on detritus >333 µm [1, 30]. However, there is evidence of very small microplastics (<100 µm) both in the benthos and water column. Sampling of shoreline, estuarine and harbour sediments has shown the presence of ~20 µm diameter fibrous polymers [4, 6, 31], and microplastic fibres, granules, films and polystyrene spheres ranging in size from 38 µm – 1 mm [32]. In the water column, sampling with a 80 µm mesh in Swedish coastal waters captured 100,000 times greater concentrations of microplastics than when using a 450 µm mesh, with a maximal concentration of 102,000 microplastics per m^3 sampled near a polyethylene production facility [33]. Sampling of microplastics in this size range is exceptional, as such there is currently insufficient data to determine realistic environmental concentrations of these particles.

Here, we investigate the ingestion of minute microplastics, ≤31 µm diameter, by a range of zooplankton species, and examine their impact on zooplankton function and feeding. To explore the hypothesis that zooplankton are capable of ingesting microplastics, 15 zooplankton taxa - representative of abundant mesozooplankton in northeast Atlantic coastal systems - were exposed to polystyrene spheres in the size range 7.3 – 30.6 µm suspended in natural seawater, then analysed using fluorescence microscopy. Using the copepod *Temora longicornis*, we explored where 0.4 – 3.8 µm microplastics accumulate, both internally and externally, using a novel bio-imaging technique: coherent anti-Stokes Raman scattering (CARS) microscopy. Finally, to test the hypothesis that microplastics negatively impact upon zooplankton feeding, we exposed the copepod *Centropages typicus* to natural assemblages of algae and polystyrene beads, using fluorometry and flow cytometry to quantify algal ingestion.

2. Materials and methods

2.1 Zooplankton sampling

Zooplankton sampling was conducted between November 2011 and October 2012 at Station L4 (50° 15'N, 4° 13'W), a coastal site located in the western English Channel 12 km south of Plymouth, UK [34, 35]. A 200 µm mesh was used to collect zooplankton via horizontal surface tows and vertical hauls. Collected zooplankton were held in 2 L of seawater within a coolbox, and transported to controlled-temperature facilities at Plymouth Marine Laboratory (Plymouth, UK). For all experimental procedures, we maintained the zooplankton at ambient sea-surface temperatures (ranging 10-17°C depending on sampling date). Specimens were hand-selected under a dissecting microscope within two hours of sampling, and then collectively held in 2 L of filtered seawater (0.22 µm Millipore filter) for 24 hours to allow full gut depuration. In all, fourteen mesozooplankton taxa (size: 0.2-20 mm), representative of the most commonly occurring zooplankton in the western English Channel and covering a range of life-stages and life-strategies, in addition to cultured *Oxyrrhis marina*, a heterotrophic dinoflagellate (size: 15-30 µm), were selected for microplastic ingestion studies (Table 1).

2.2 Natural seawater preparation

For the algal ingestion studies, natural seawater (5 L) was collected from the sea surface at station L4, passed through a 200 µm mesh into a polycarbonate carboy and returned to the laboratory within 2 hours. The seawater was further screened with a 100 µm mesh to ensure the removal of any grazing micrometazoans then stored in the dark for 24 hours at ambient sea-surface temperature to maintain the natural communities of algae at normal concentrations. Prior to experimental work, the seawater was mixed thoroughly by gentle inversion of the water in the carboy.

120

121 *2.3 Microplastics*

122 Exposures used commercial polystyrene spheres (SPHERO™ Spherotech). With global production
123 rates of 10.6 million tons in 2001, polystyrene is the fourth most commonly produced polymer in the
124 world and its presence as a constituent of marine debris is commonly reported [30, 36]. The bead
125 sizes used in each experiment (0.4-30.6 µm) were selected to be comparable with the prey size
126 range of the zooplankton exposed [23, 37].

127

128 *2.4 Microplastic ingestion by zooplankton*

129 To ascertain whether zooplankton ingest microplastics we conducted exposures using fluorescent
130 polystyrene beads, and used microscopy to assess uptake. Microplastic suspensions were made up
131 by pipetting 20 µl of 7.3, 20.6 or 30.6 µm diameter fluorescently-labelled (yellow fluorescence: 400-
132 500 nm excitation, 450-550 nm emission) polystyrene spheres into glass vials containing 20 ml of
133 filtered seawater (0.1% v/v: 3,000 beads ml^{-1} (7.3 µm); 2,240 beads ml^{-1} (20.6 µm); 635 beads ml^{-1}
134 (30.6 µm)), then mixed through repeated inversion. With larger zooplankton (e.g. copepods,
135 decapod larvae, chaetognaths), individual specimens were added directly to the vial (n = ≥6 per
136 exposure), and fitted to a rotating plankton wheel (<5 RPM) for 24-hours. For smaller zooplankton or
137 those with low-survivability in the laboratory (e.g. bivalve larvae, gelatinous holoplankton, *O.*
138 *marina*), individual specimens were exposed to microplastic suspensions in Petridishes (n = ≥6 per
139 exposure) at ambient sea temperature for 1 hour (with the exception of bivalve larvae which were
140 exposed for 24 hours using this method). Post-exposure, zooplankton were washed with filtered
141 seawater and transferred to Eppendorf tubes containing 1 ml of 4% formalin . Ingestion was
142 ascertained by viewing specimens at x40-400 magnification with an Olympus IMT2 inverted light
143 microscope with fluorescence to determine the presence of polystyrene beads (fluorescing yellow-

144 green) within the alimentary canal or body cavity of the zooplankton. To better understand the
145 interactions between zooplankton and microplastics, both live and preserved copepods and select
146 zooplankton specimens were viewed under the microscope for varying lengths of time to observe
147 the feeding process, ingestion, gut passage and egestion of polystyrene beads.

148

149 *2.5 Interactions between microplastics and copepods*

150 To explore the internal distribution and external adherence of microplastics in zooplankton, we
151 firstly exposed the copepod *Temora longicornis* to polystyrene beads and then employed CARS
152 microscopy (see below) to visualise their uptake. Microplastic suspensions were formulated by
153 adding 12 µl of 0.4, 1.7 or 3.8 µm diameter non-labelled polystyrene spheres to 24 ml of filtered
154 seawater (0.05% v/v: 1 x 10^6 beads ml^{-1} (0.4 µm), 380 x 10^3 beads ml^{-1} (1.7 µm), and 40 x 10^3 beads
155 ml^{-1} (3.8 µm)), which were mixed through inversion and sonication. Individual *T. longicornis* (*n* = ≥6
156 per exposure) were added to each vial, rotated at <5 RPM at ambient sea temperature for 24 hours.
157 Post-exposure, specimens were poured onto a 200 µm mesh suspended in filtered seawater (to
158 prevent damage to the copepods), washed gently, preserved in 4% formalin and then transferred to
159 the bio-imaging suite at the University of Exeter (Exeter, UK).

160

161 *2.6 Coherent anti-Stokes Raman scattering (CARS) microscopy*

162 CARS microscopy is a novel microscopy technique that provides label-free contrast, based on
163 vibrational spectroscopy [38] which has exceptional capability for locating polymer particles within
164 biological tissues with subcellular precision [39, 40]. CARS imaging was performed using a custom-
165 built microscopy system based on a commercial confocal laser-scanning microscope and a
166 synchronised dual-wavelength picosecond laser source. Laser excitation was provided by an optical
167 parametric oscillator (OPO) (Levante Emerald, APE, Berlin) pumped with a frequency doubled

168 Nd:Vandium picosecond oscillator (High-Q Laser Production GmbH). The pump laser generated a 6
169 ps, 76 MHz pulse train at 532 nm with adjustable output power up to 10 W. The OPO produced
170 collinear signal and idler beams with perfect temporal overlap and provided continuous tuning over
171 a range of wavelengths. The signal beam was used as the pump, ranging from 670 to 980 nm and
172 fundamental of Nd:Vandium (1064 nm) used as the Stokes beam. The maximum combined output
173 power of the pump and Stokes was approximately 1 W, which was attenuated to reduce the power
174 at the sample to between 15 - 30 mW. To improve the transmission of the near-IR excitation through
175 the commercial microscope (IX71 and FV300, Olympus UK) the galvanometer mirrors were replaced
176 with silver mirrors and the tube lens was replaced with a MgF2 coated lens. The collinear pump and
177 Stokes beams were directed onto the scanning confocal dichroic which was replaced by a silver
178 mirror with high reflectivity throughout the visible and NIR (21010, Chroma Technologies, USA). The
179 forward-CARS signal was collected by the air condenser, transmitted by the dichroic mirror and
180 directed onto a red-sensitive photomultiplier tube (R3896, Hamamatsu Photonic UK). The epi-CARS
181 signal was collected using the objective lens and separated from the pump and Stokes beams by a
182 long-wave pass dichroic mirror (z850rdc-xr, Chroma Technologies, USA) and directed onto a second
183 R3896 photomultiplier tube at the rear microscope port. The CARS signal was isolated at each
184 photodetector using a single band-pass filters centred at the anti-Stokes wavelengths. Imaging was
185 performed using either a 60X water immersion, or 20X air objective (UPlanS Apo, Olympus UK).

186

187 *2.7 Impact of microplastics on copepod feeding*

188 To determine whether microplastics negatively impact upon a copepod's ability to ingest natural
189 prey, we exposed the copepod *Centropages typicus* to natural assemblages of algae with and
190 without microplastics, and compared algal ingestion rates between treatments. In our initial
191 experiment, designed to identify the size of microplastic that would have the greatest impact on *C.*
192 *typicus* feeding, we exposed individual *C. typicus* specimens (n = ≥6 per exposure) to 23 ml of natural

seawater containing 0 or 23 µl of 7.3 or 20.6 µm fluorescent polystyrene beads (0.1% v/v), rotated at <5RPM for 24 hours. To quantify algal concentrations within the natural seawater pre- and post-exposure, we vacuum filtered the exposure media through a glass fibre filter, and then transferred the filter to 7 ml of acetone, held at 4°C in the dark for 24 hours. The chlorophyll levels within the acetone solution were measured using a Turner fluorometer. Since 7.3 µm microplastics had the most notable impact on *C. typicus* feeding, we conducted a further experiment to establish a dose-response relationship between microplastic concentration and food uptake. Microplastic suspensions consisted of 0, 2.5, 5, 10 or 20 µl additions of 7.3 µm fluorescent polystyrene beads in 23 ml of natural seawater. A 1.8 ml aliquot of natural seawater was taken from all vials at T_0 and fixed with 40 µl of 50% glutaraldehyde (4% final concentration), inverted for 2 minutes, refrigerated at 4°C for 30 minutes and subsequently snap-frozen in liquid nitrogen and stored in a -80°C freezer prior to analysis using analytical flow cytometry. Individual *C. typicus* (n = ≥6 per exposure) were added to experimental vials, while controls (with no copepod) were set up to determine natural growth or decline of algae over the exposure period. The vials were incubated on a rotating plankton wheel (5RPM) for 24 hours in the dark. Post-exposure (T_{24}), a further 1.8 ml aliquot was fixed (as with T_0). Flow cytometric analysis was carried out on thawed natural seawater samples using a BD Accuri C6 flow cytometer [41]. Particle abundance data was subsequently used to calculate the ingestion rates of algae by *C. typicus* [42].

2.8 Statistical analysis

Data was analysed using Microsoft Excel. Student's T-tests were used to compare experimental data with controls, with significant difference attributed where $P \leq 0.05$. Regression analysis was used to analyse the correlation between algal ingestion rates and microplastic concentration.

3. Results

3.1 Microplastic ingestion by zooplankton

The majority of zooplankton (13 of 15) exposed to polystyrene beads (7.3 – 30.6 µm) demonstrated the capacity to ingest microplastics (Table 1). Organisms exhibiting uptake included copepods (Fig. 1i + Fig. 1ii), bivalve larvae (Fig. 1iii) and decapod larvae (Fig. 1iv + Fig. 1v). Only two specimens – chaetognaths (*Parasagitta* sp.) and siphonophorae (Cnidaria) – showed no evidence of ingestion. All four species of copepods examined demonstrated some affinity for ingesting microplastics, with *Centropages typicus* and *Temora longicornis* able to consume 7.3, 20.6 and 30.6 µm polystyrene beads (Fig. 1ix). The other copepods showed evidence of size-based selectivity: *Acartia clausi* ingested 7.3 µm beads but ingested significantly less 20.6 and 30.6 µm beads, and *Calanus helgolandicus* showed significantly less affinity for 30.6 µm beads than for 7.3 µm beads. The decapod Brachyurans demonstrated variability in microplastic ingestion depending upon life-stage: brachyuran zoea showed no affinity for 20.6 µm beads, while the more-developed brachyuran megalopa readily ingested such beads. *Obelia* sp., Paguridae larvae and Porcellinidae (zoea) exhibited individual variability in their ability to ingest polystyrene beads, with less than half the exposed specimens in a cohort showing evidence of microplastic uptake.

Live observations of copepods, euphausids and doliolids found microplastics were ingested via filter-feeding. In copepods and euphausids, this process relied upon the rapid movement of the swimming legs and external appendages, which generated a feeding current that indiscriminately drew surrounding beads towards the organism. With doliolids, we observed the microplastics being drawn through the anterior siphon into their body cavity, where the polystyrene beads were entrapped and drawn towards the gut. *Oxyrrhis marina*, a single celled heterotrophic dinoflagellate, demonstrated a more direct method of ingestion, locating particles with their flagella and then engulfing the polystyrene beads. Post- ingestion, copepods typically aggregated beads within the anterior mid-gut, shifted them to the posterior mid-gut via peristaltic action (Fig. 1i + Fig. 1ii) and egested them within

densely-packed faecal pellets (Fig. 1vi + Fig. 1viii). Typically, microplastic-laden faecal pellets were egested within hours. In the absence of food, individual microplastic beads could remain in the intestinal tract of *C. helgolandicus* for up to 7 days (data not shown). During observations of both live and preserved zooplankton specimens, including copepods, decapod larvae and euphausids, microplastics often adhered to the specimens' external surfaces. In copepods that died during the exposure period, polystyrene beads would coat the carapace in vast numbers; similarly, beads were observed to cling to the shed carapace of a moulting *C. helgolandicus* copepodite. In live specimens, microplastics were found to concentrate between the external appendages of copepods, including the swimming legs, feeding apparatus, antennae and furca (Fig. 1vii).

3.2 Interactions between microplastics and copepods

CARS microscopy used a blend of transmitted light to capture the structure of the copepod, and Raman shifts of 2845 cm^{-1} (C-H) and 3050 cm^{-1} (aromatic C-H) to visualise the polystyrene (Fig. 2i). *Temora longicornis* ingested both 1.7 and 3.8 µm polystyrene beads; use of Z-stacking – in which 2D images at incremental focal plains are layered together to form a 3D image – confirmed that microplastics clumping in the posterior mid-gut were, indeed, internalised (Fig. 2ii; yellow dots), but sufficient resolution to identify microplastic translocation was not possible. CARS imaging confirmed that microplastics adhere to the external appendages of the zooplankton: polystyrene beads (0.4 – 3.8 µm) accumulated between the filamental hairs on appendages, including the furca (Fig. 2iii; blue dots), rear swimming legs (Fig. 2iii; red dots) and antennules, and between the segments of the carapace, particularly around the urosome and swimming legs.

3.3 Impact of microplastics on copepod feeding

Using chlorophyll concentration as a proxy for algal abundance, we identified that 7.3 µm microplastics had a significant impact on algal ingestion by the copepod *Centropages typicus* (data not shown) and identified a significant dose-response relationship between ingestion rates and the concentration of 7.3 µm polystyrene beads. Exposed to seawater – containing natural assemblages of algae – *C. typicus* ingested ~12 *Synechococcus* sp. ind^{-1} h^{-1} (Fig. 3i) and ~24 picoeukaryotes ind^{-1} h^{-1} (Fig. 3ii). These ingestion rates decreased when additionally exposed to ~4,000 microplastics ml^{-1}; this decrease was statistically significant at concentrations of ≥7,000 microplastics ml^{-1} (t-test: P ≤0.05). When considering all of the <20 µm ESD algal groups identified using flow cytometry – *Synechococcus sp.*, picoeukaryotes, nanoeukaryotes and cryptophytes – in combination (hereafter referred to as "total algae"), *C. typicus* presented total algal ingestion rates of ~34 algae ind^{-1} h^{-1} in the absence of microplastics. Total algal ingestion rates for *C. typicus* were significantly reduced with the addition of ≥4,000 microplastics ml^{-1} (t-test: P ≤0.05; Fig. 3iii). Furthermore, we identified a strong, logarithmic relationship (R^2 = 0.70, P ≤0.05) between the ingestion rate of total algae and microplastic concentration (Fig. 3iv).

4. Discussion

Our results show that a range of zooplankton common to the northeast Atlantic can ingest microplastics (1.4 – 30.6 µm diameter), with capacity for uptake varying between species, life-stage and microplastic size. Microplastics were indiscriminately ingested via filter-feeding and later egested in faecal pellets, typically within a matter of hours. Microplastics accumulated on the external surface of dead zooplankton, and were found trapped between the external appendages of live copepods. We visualised 1.7 and 3.8 µm polystyrene beads clustered within the alimentary canal and aggregated between the setae and joints of external appendages. Lastly, we demonstrated that the presence of 7.3 µm polystyrene beads could significantly reduce the algal ingestion rate of the copepod *Centropages typicus*, in a dose-response relationship.

We demonstrated that thirteen zooplankton taxa – including holoplankton, meroplankton and microzooplankton – have the capacity to ingest polystyrene beads in the absence of natural food. All four copepod species showed uptake of microplastics, with varying degrees of selectivity: *T. longicornis* and *C. typicus* ingested 7.3, 20.6 and 30.6 µm beads, while *A. clausi* and *C. helgolandicus* fed on 7.3 µm beads but less frequently ingested larger beads. Using CARS microscopy, we further identified that *T. longicornis* could ingest 1.7 and 3.8 µm microplastics, however, we found no evidence of 0.4 µm beads being ingested. Brachyuran larvae only ingested 20.6 µm polystyrene beads as megalopa (post-zoea larvae), with no uptake observed when in the earlier zoea stage. Microplastics were also ingested by the filter-feeding euphausids and doliolids, and *Oxyrrhis marina*, a heterotrophic dinoflagellete that ingests motile or immotile prey through engulfment via a non-permanent cytosome [43]. These findings corroborate the results of several previous studies, which documented the uptake of <100 µm microplastics by *Acartia tonsa* [28], *Calanus pacificus* adults, copepodites and nauplii [26, 44, 45], *Oxyrrhis marina* [46], ciliates [47, 48], echinoderm larvae [27] and salps [49].

We did not observe microplastic uptake in *Parasagitta* sp. (chaetognaths) following 1- or 24- hour exposures to 30.6 µm beads, or siphonophorae (Cnidaria) exposed to 20.6 µm plastics, possibly as a result of handling stress, or more likely because these zooplankton are raptorial predators and feed actively, so were not enticed to capture the immotile microplastics [37]. Furthermore, only 10-50% of *Obelia* sp., Paguridae larvae and Porcellinidae (zoea) specimens presented with polystyrene beads in their intestinal tracts post-exposure. As we also observed size-selective ingestion in *A. clausi* and *C. helgolandicus*, it is important to consider how microplastics may impact on different zooplankton feeding strategies. Zooplankton use both mechanoreception (i.e. detection of pressure disturbances within the water) and chemoreception (i.e. detection of infochemicals emitted by algal cells) to sense prey [29, 37]. As such, the clean immotile beads used in our algal-free experiments are less likely to be detected by exposed zooplankton, although it is possible that aged microplastics, that have developed bio-films during their residence within the marine environment [10], may generate a

316 chemosensory response; this effect was observed in the copepod *Eurytemora affinis* which more
317 readily ingested beads spiked with bacteria than when offered beads alone [50]. While some
318 copepods will continuously filter-feed regardless of prey availability, others (e.g. *C. pacificus*, *A.*
319 *tonsa*) can limit their movement and filter-feed at reduced rates to conserve energy when faced with
320 low food-concentrations [51, 52]. The presence of algae promotes greater uptake of microplastics in
321 the filter-feeding copepods *Calanus pacificus* [26] and *Eucalanus pileatus* CV copepodites [53];
322 notably, *A. clausi* only ingests 16 µm polystyrene beads in the presence of algae [24]. Some
323 zooplankton can ingest or reject prey upon capture, depending on surface characteristics and charge
324 of the particle, both echinoderm larvae and the copepods *A. clausi* and *E. pileatus* can reject plastic
325 beads that coalesced within their mouthparts [27, 53, 54]. The presence of microplastics may also
326 alter the behaviour of zooplankton, limiting their capacity to feed: in *Acartia tonsa* copepodites,
327 contact with 45 µm plastic beads caused the organisms to "jump", limiting time dedicated to feeding
328 bouts and reducing their clearance rates by 60% [55].

329 Post-ingestion, polystyrene beads were observed to coalesce within the mid-gut of copepods prior
330 to egestion. While gut-retention times of these microplastics were typically similar to natural food
331 items (i.e. egestion occurred within hours), a follow-up experiment found some *Calanus*
332 *helgolandicus* individuals retained microplastics for up to 7 days. Microplastics found in the marine
333 environment include fibres, granules and fragments manufactured from a range of polymers [30]; if
334 such irregularly-shaped and fibrous microplastics were ingested, they may become entangled within
335 the intestinal tract, potentially resulting in a non-biodegradable gut-blockage and greater gut-
336 retention times. Plastic fibres entangle within the intestinal tracts of Nephrops in this manner [14],
337 while fish [16, 17] and seabird dissections [15] have demonstrated that marine wildlife can retain a
338 range of plastic detritus within their stomachs near-indefinitely. Prolonged gut-retention times of
339 plastics and gut-blockages in zooplankton may limit the ability of these organisms to ingest and
340 digest food, and may pose a toxic risk. During manufacture, a suite of additives (e.g. plasticisers,
341 flame-retardants, anti-microbials) are added to plastics, and large surface area to volume ratio and

342 hydrophobic properties of microplastics make them particularly susceptible to the adherence of
343 waterborne contaminants (e.g. PCBs, DDT and PAHs) [19]. The leaching of additives and
344 disassociation of toxic contaminants post-ingestion has been modelled in polychaete worms [56] and
345 demonstrated in streaked shearwaters [57]. In zooplankton, as with other marine biota, these
346 contaminants might be considered endocrine-disruptors, carcinogenic or toxic, with repercussions
347 for growth, sexual development, fecundity, morbidity and mortality [58, 59]. Of further concern is
348 trophic-transfer: microplastics (and contaminants released from microplastics) within lower-trophic,
349 keystone organisms such as zooplankton may result in the trophic-transfer of these contaminants up
350 the food-chain, with the potential for bio-accumulation and therefore adverse health consequences
351 in higher trophic organisms.

352 Copepods that died during exposures, and shed moults of copepodites, were coated in microplastics
353 – presumably because of hydrophobic- or static-attractions between the negatively-charged
354 polystyrene (average zeta potential: -41.8 mV) and organic material – a process that acts to
355 concentrate microplastics from the surrounding seawater. Our observations of microplastic laden
356 faecal pellets egested by copepods provided no indication that passage through the alimentary canal
357 had any discernible impact on the microplastics. However, plastics may alter the density and
358 structural integrity of faecal pellets with potential repercussions on vertical carbon flux [60]. During
359 our studies, we also found microplastics were becoming trapped between the external appendages
360 and carapace segments of live copepods. We found that very small microplastics (0.4 – 3.8 µm)
361 became lodged between the filamental hairs and setae of the antennules, furca and the swimming
362 legs [29, 61]. As these appendages have key roles in copepod function and behaviour, this may have
363 repercussions for locomotion, ingestion, mating and mechanoreception, that may limit their ability
364 to detect prey, feed, reproduce and evade predators.

365 We found that the presence of 7.3 µm beads significantly reduced the amount of algae eaten by the
366 copepod *Centropages typicus*, whereas 20.6 µm beads showed no discernible impact on algal

consumption. This suggests *C. typicus* can preferentially feed upon algae over 20.6 µm beads (but could not differentiate between the algae and 7.3 µm beads), or, that only the smaller beads impact on copepod feeding (i.e. 7.3 µm beads are small enough to become entrapped between external appendages or be recurrently ingested). A similar finding has been observed with *Acartia clausi* and *Calanus pacificus* nauplii, which selectively fed upon small algae while avoiding larger beads, but could not discriminate between algae and beads of a similar size [24, 45, 54]. We found that a concentration of 4,000 beads ml^{-1} was enough to result in significantly reduced algal ingestion rates. This relationship reached saturation at concentrations of >5000 beads ml^{-1}. Two previous studies have found similar results, where the ingestion rates of the copepod *A. clausi* [24] and *C. pacificus* [45] were significantly reduced by the presence of beads of a similar size to the algae. A reduction in algal feeding may have severe consequences for copepods, as limited energy intake, in particular with species that have minimal lipid reserves (e.g. *Centropages, Acartia*), could result in decreased fecundity and growth, or increased mortality [24, 62]. We do not yet know whether 5000 particles ml^{-1} can be considered an environmentally relevant concentration for microplastics <10 µm in size. Perpetual fragmentation of plastic litter, coupled with the increasing popularity of household products containing microscopic plastic exfoliates [5], suggests marine plastic debris is becoming, on average, smaller over time [63]. However, due to the complexities of sampling and extraction, and in the absence of unified sampling methodologies, microplastics are still considered to be an under-researched fraction of marine litter, with no consistent data relating to plastic detritus <333 µm in diameter [1, 30, 64]. Further, we must consider that microplastics made of polymers other than polystyrene, potentially laden with chemical additives or adhered contaminants, could result in different interactions with zooplankton with variable impacts on function.

Our findings confirm that ingestion of marine microplastic debris by zooplankton in the ocean is feasible. Potential impacts include reduced function and health of the individual, trophic-transfer of contaminants to predators, and the egestion of faecal pellets containing microplastics. Better

knowledge of the extent of microplastic contamination of oceans waters is now a research imperative.

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Figure Legends

Figure 1: Microplastics of different sizes can be ingested, egested and adhere to a range of zooplankton, as visualised using fluorescence microscopy: (i) the copepod *Centropages typicus* containing 7.3 µm polystyrene (PS) beads (dorsal view); (ii) the copepod *Calanus helgolandicus* containing 20.6 µm PS beads (lateral view); (iii) a D-stage bivalve larvae containing 7.3 µm PS beads (dorsal view); (iv) a Brachyuran (decapod) larvae (zoea stage) containing 20.6 µm PS beads (lateral view); (v) a Porcellanid (decapod) larvae, containing 30.6 µm PS beads (lateral view); (vi) 30.6 µm PS beads in the posterior-gut of the copepod *Temora longicornis* during egestion, (vii) 1.4 µm PS beads trapped between the filamental hairs of the furca of *C. typicus*; (viii) a *T. longicornis* faecal pellet containing 30.6 µm PS beads; (ix) proportion of copepods (*Acartia clausi*, *Calanus helgolandicus*, *Centropages typicus* and *Temora longicornis*) with microplastics in their guts following 24 hours of exposure to 7.4, 20.6 and 30.6 µm polystyrene beads. ∗ denotes statistically significant ($P \leq 0.05$) lower consumption of larger beads compared with that of 7.3 µm beads. Scale bar (grey line): 100 µm.

Figure 2: Coherent anti-Stokes Raman scattering (CARS) microscopy: (i) Spontaneous [•] and stimulated [•] peaks for polystyrene beads, Raman shifts of 2845 cm^{-1} (C-H) and 3050 cm^{-1} (aromatic C-H) were used to visualise the polystyrene; (ii) 3.4 µm microplastics accumulated in the alimentary canal [ac] of the copepod *Temora longicornis* (yellow dots); beads further adhered to the exterior of the copepod's urosome [u], furca [f] and posterior swimming legs [sl] (blue dots); (ii) 3.4 µm microplastics (red dots) adhered to the external surface of the posterior swimming legs of *T. longicornis*. Scale bar [grey line]: 50 µm.

Figure 3: Exposure to increasing concentrations of microplastics in the copepod *Centropages typicus* (n = ≥5). Treatments comprise seawater containing natural assemblages of algae [A] with 4,000 [B], 7,000 [C], 11,000 [D] and 25,000 [E] 7.3 µm polystyrene beads per ml. ∗ denotes statistically significant ($P \leq 0.05$) lower ingestion rates (cells $individual^{-1}$ $hour^{-1}$) than in controls. Graphs show ingestion rates of: (i) *Synechococcus* sp.; (ii) Picoeukaroytes; (iii) all algae present; (iv) plot comparing positive *C. typicus* algal ingestion rates at differing microplastics concentrations - logarithmic regression: $R^2 = 0.70$ ($P \leq 0.05$).

Tables

Table 1: The capacity for a range of zooplankton to ingest microplastics, demonstrated using fluorescent microscopy.

Organism	Taxonomy	Microplastic	Exposure	Ingestion
Holoplankton (Copepods)				
Acartia clausi	Copepoda (Calanoida)	7.3	24	Yes
Acartia clausi	Copepoda (Calanoida)	20.6	24	No
Acartia clausi	Copepoda (Calanoida)	30.6	24	Partial
Calanus helgolandicus	Copepoda (Calanoida)	7.3	24	Yes
Calanus helgolandicus	Copepoda (Calanoida)	20.6	24	Yes
Calanus helgolandicus (juv.)	Copepoda (Calanoida)	20.6	24	Yes
Calanus helgolandicus	Copepoda (Calanoida)	30.6	24	Partial
Centropages typicus	Copepoda (Calanoida)	7.3	24	Yes
Centropages typicus	Copepoda (Calanoida)	20.6	24	Yes
Centropages typicus	Copepoda (Calanoida)	30.6	24	Yes
Temora longicornis	Copepoda (Calanoida)	7.3	24	Yes
Temora longicornis	Copepoda (Calanoida)	20.6	24	Yes
Temora longicornis	Copepoda (Calanoida)	30.6	24	Yes
Holoplankton (Other)				
Doliolidae	Tunicata	7.3	1	Yes
Euphausiidae	Euphausiacea	20.6	24	Yes
Parasagitta sp.	Chaetognatha	20.6	1	No
Parasagitta sp.	Chaetognatha	30.6	24	No
Obelia sp.	Cnidaria (Hydrozoa)	20.6	1	Partial
Siphonophorae	Cnidaria (Hydrozoa)	20.6	1	No
Meroplankton				
Bivalvia (larvae)	Mollusca	7.3	24	Yes
Brachyura (megalopa)	Decapoda	20.6	24	Yes
Brachyura (zoea)	Decapoda	20.6	24	No
Caridea (larvae)	Decapoda	20.6	24	Yes
Paguridae (larvae)	Decapoda	20.6	24	Partial
Porcellanidae (zoea)	Decapoda	30.6	24	Partial
Microzooplankton				
Oxyrrhis marina	Dinoflagellata	7.3	1	Yes

Microplastic uptake is based upon the number of individuals in a treatment (n = ≥6) that contained beads in their alimentary canals or body cavity following 1 or 24 hour exposures to either 7.3, 20.6 or 30.6 µm fluorescent polystyrene beads. ESD = Equivalent Spherical Diameter. Scoring system: Yes (>50%); Partial (<50%); No (0%).

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