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Unpalatable plastic: efficient taste-discrimination of microplastics in planktonic copepods

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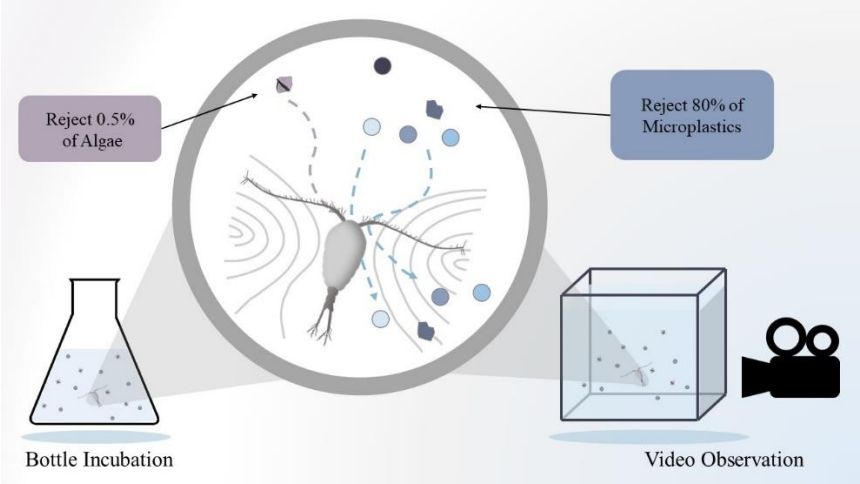
Abstract

Planktonic copepods are the most abundant animals in the ocean and key players in global biochemical processes. Recent modelling suggests that zooplankton ingestion of microplastics (MPs) can disrupt the biological carbon pump and accelerate a global loss of oceanic oxygen. Here we investigate the behavioural responses and ingestion rates of a model feeding-current generating copepod when exposed to microplastics of different characteristics by small-scale video observations and bottle incubations. We found that copepods rejected 80% of the microplastics after touching them with their mouths parts, in essence exhibiting a kind of taste discrimination. High rejection rates of microplastics were independent of polymer type, shape, presence of biofilms, or sorbed pollutant (pyrene), indicating that microplastics are unpalatable for feeding-current feeding copepods and that post-capture taste discrimination is a main sensorial mechanism in rejection of microplastics. In an ecological context, taking into account the behaviours of planktonic copepods and the concentrations of microplastics found in marine waters, our results suggest a low risk of microplastic ingestion by zooplankton and a low impact of microplastics on the vertical exportation of fecal pellets.

Keywords: Zooplankton, Copepods, Feeding Behavior, Microplastic, Taste Discrimination,

Synopsis: Feeding-current feeding copepods reject 80% of the captured microplastics by taste discrimination. This suggests that chemical signals from synthetic polymers make microplastics unpalatable to copepods.

TOC:



Introduction

Microplastics are ubiquitous pollutants in the marine environment [1,2]. Understanding the consequences of plastic pollution in marine ecosystems is therefore of major societal and scientific concern. Most biological oceanographic processes are directly linked to the presence and activities of planktonic organisms [3], which are exposed to microplastics in the water column. This makes zooplankton of particular interest in relation to potential global environmental impacts of microplastic pollution. It has been hypothesized that the ingestion of microplastics instead of organic prey by zooplankton may change the sinking velocity of their fecal pellets [4], consequently affecting the vertical transportation of carbon and weakening the marine biological carbon pump [5,6]. It is even predicted to accelerate global loss of ocean oxygen through reduced grazing on primary producers [7]. Among planktonic organisms, copepods are the most abundant animals in the ocean and the dominant zooplankton group [8,9]. Ingestion of microplastics by copepods is potentially the main route by which plankton-sized microplastics enter marine food webs and are transferred to higher trophic levels [10,11]. A better knowledge of the interactions between copepods and microplastics is therefore essential to understand the

62 fate and impacts of marine plastic pollution.

63 It is well documented that marine macro- and megafauna ingest plastic debris with more than
64 900 recorded cases of vertebrate species, including sea mammals, sea birds, marine turtles
65 and fishes, being entangled by, or having consumed plastics [12,13]. Additionally, laboratory
66 research has clearly demonstrated that zooplankton, including copepods, ingest plankton-sized
67 microplastics when exposed to high concentrations [10,11,14-16]. However, evidence of microplastic
68 consumption by copepods in the natural environment and its consequences is lacking. Several
69 field surveys have reported ingestion of microplastics by copepods [17-21]. However, for some of
70 these studies, the reported size of ingested microplastics is outside of the size-range of natural
71 prey, and in some cases, it is even larger than the mouth of the copepods, suggesting
72 entanglement or sample contamination rather than actual ingestion. In any case, these field
73 studies indicate a low occurrence of microplastic ingestion in natural zooplankton communities,
74 which contradicts the high probability of microplastic ingestion predicted from laboratory studies.
75 One reason for this mismatch in the findings of laboratory and field research may be the
76 methods used in the laboratory. Most laboratory research on the ingestion of microplastics by
77 zooplankton has been conducted using virgin spherical microplastics [10] whereas investigations
78 of zooplankton preferences for different shapes [4], aging states [22] and other characteristics of
79 microplastics are still lacking. In addition, most laboratory research involves only bottle
80 incubations that allow little insight the mechanisms of the interactions between different types
81 of microplastics and copepods. A practical tool to open the “black box” of bottle incubations
82 when investigating the feeding behavior of zooplankton is small-scale video observation. This
83 technique has successfully been applied to study selective feeding behavioral responses of

84 copepods to different species of harmful algae [23].

85 In this study, we investigated how different characteristics of microplastics affect the behavioral
86 responses and ingestion rates of the feeding-current feeder, *Temora longicornis*. *Temora*
87 species are distributed worldwide from coastal to oceanic waters [24]. Moreover, feeding-current
88 feeding is one of the three dominant feeding modes of planktonic copepods [25,26]. Through
89 modelling we represent the global distribution of the feeding-current feeding mode with the aim
90 of reflecting the relevance of this feeding mode in the world oceans [27,28]. Furthermore, we used
91 direct small-scale video observations and parallel bottle incubations to quantify the feeding
92 behavioral responses of copepods to diverse microplastic characteristics which are common in
93 marine environment and biota [29-31]: plastic polymer type (polystyrene (PS) vs polyethylene (PE)),
94 shape (irregular vs sphere), presence of biofilms (bio-fouled vs “clean” microspheres) and
95 sorption of organic pollutants (microspheres with sorbed pyrene vs “clean” microspheres). We
96 hypothesized that feeding-current generating copepods (i) do not discriminate between plastic
97 polymer types; (ii) show a higher rejection of microplastics when plastic particles are irregular
98 (different from normal prey which are typically of regular shape); (iii) have a higher ingestion of
99 bio-fouled microplastics than virgin microplastics; and (iv) show a higher rejection of
100 microplastics with sorbed chemicals pollutants. Our results will provide a better understanding
101 of how plastic properties and weathering processes influence the risk of microplastics to enter
102 the marine food webs via zooplankton.

103

104 **Materials and Methods**

105 **Spatial modelling**

106 To represent the global distribution of feeding-current feeders, the global ocean was discretized
107 into roughly 5000 polygons of similar area and feeding-current feeders were assumed to be
108 represented by the world most abundant genera: *Paracalanus*, *Pareucalanus*, *Parvocalanus*,
109 *Rhincalanus*, *Pseudocalanus*, *Calocalanus*, *Nannocalanus*, *Temora, Acartia*, *Calanus*,
110 *Centropages*, *Pleuromamma*, and *Euchaeta*. Observation-based estimates were derived
111 polygon-wise as community-weighted means using abundance observations [32], body length
112 data [33], and the procedure described by Brun et al. [34]. For model extrapolations we fitted
113 generalized additive models [35,36], assuming beta distribution and using average and range of
114 monthly sea surface temperature (derived from the HadISST1 product [37]) and average
115 chlorophyll a concentration (derived from http://www.globcolour.info/) as predictors.

116 **General experimental approach**

117 To test our hypothesis, we conducted four studies:

118 a) "Polymer type" (PS vs PE), where copepods (*T. longicornis*) were exposed to either (i)
119 algae and virgin, spherical PS microplastics or (ii) algae and virgin, spherical PE
120 microplastics.

121 b) "Shape" (spheres vs irregular fragments), where copepods were exposed to either (i)
122 algae and virgin, spherical PS microplastics or (ii) algae and virgin, irregular PS fragments.

123 c) "Biofilms" (bio-fouled vs "clean" microspheres), where copepods were exposed to either
124 (i) algae and spherical PE microplastics with biofilms or (ii) algae and virgin, spherical PE
125 microplastics without biofilms ("clean").

126 d) "Sorbed pollutants" (microplastics with sorbed pyrene vs microplastics without sorbed
127 pyrene) where copepods were exposed to either (i) algae and spherical PE microplastics with

sorbed pyrene, (ii) algae and spherical PE microplastics without sorbed pyrene, (iii) algae and spherical PS microplastics with sorbed pyrene or (iv) algae and spherical PS microplastics without sorbed pyrene.

In all experiments, copepods were exposed to a nominal prey:microplastic ratio of 1:1 with concentrations of 200 cells mL^{-1} and 200 MPs mL^{-1}. Measured experimental concentrations are shown in Table S1. Feeding behavioral responses of individual copepods, including prey detection, capture, handling, rejection and ingestion of prey and different microplastic types were examined by small-scale video observations, as described in details below. Feeding rates of copepods on the studied prey and microplastics were calculated from both video observations and bottle incubations as described below.

Experimental organisms

The culture of our model copepod species, *T. longicornis*, originates from samples from the Gullmars fjord (Sweden) and Øresund (Denmark) in 2016. The copepods were subsequently maintained in a continuous laboratory culture at the Technical University of Denmark. They are grown in 30 L tanks with filtered seawater (salinity= 30 psu) at 18℃ in the dark. Copepod cultures were fed a mixed diet consisting of cultured phytoplankton (*Heterocapsa steinii* (formerly known as *H. triquetra), Thalassiosira weissflogii, Rhodomonas salina*) and a heterotrophic dinoflagellate (*Oxyrrhis marina*). The phytoplankton and *O. marina* cultures were maintained in the lab [25].

The day before the experiments, healthy copepod females of similar size (prosome length approximately 740 µm) were sorted under a stereo microscope and kept overnight in glass beakers with 0.2 µm-filtered seawater. From the sorted copepod stock, we picked females for

both bottle incubations and video experiments.

The dinoflagellate *H. steinii* was the model prey used in the feeding experiments. The cultures of *H. steinii* were maintained in autoclaved 0.2 μm-filtered seawater with B1 medium at 16℃, 150 μmol photons m^{-2} s^{-1}, 12 h light: 12 h dark cycle, and a salinity of 30 psu. The size distribution and concentration of *H. steinii* were measured with a Beckman Multisizer Coulter Counter before the experiment. Only cultures in exponential growth phase were used as prey. The equivalent spherical diameter (ESD) of algal cell was approximately 20 μm on average.

Preparation of the different types of microplastics

Study 1: "Polymer type"

We used virgin spherical microplastics of the two different plastic polymers, PS and PE. 20 μm PS microspheres suspended in water with tween 80 were purchased from Degradex®. 20 μm PE microspheres were purchased from Cospheric® as powder. In both cases, the microplastics were suspended in a 0.01% tween 80 miliQ water solution to prepare the working suspensions.

Study 2: "Shape"

To obtain irregular microplastics, PS pellets (500 μm in diameter, purchased from Cospheric®) were frozen with liquid nitrogen and subsequently ground using an IKA A11 basic analytical mill. The resulting microplastic fragments were suspended in a 0.01% tween 80 solution. The suspension of microplastics was then filtered through nylon filters with 30 μm and 15 μm mesh sizes in sequence to obtain irregular microplastics with an average size of approximately 20 μm. Upon filtration, the fragments were collected and re-suspended in a 0.01% tween 80 solution. 20 μm PS microspheres were used as spherical microplastics to compare with irregular microplastics of the same size and polymer but with different shape.

Study 3: "Biofilms"

Seawater, containing natural microbial communities, was collected from a Danish estuary, Limfjorden, and filtered through 8 µm polycarbonate filters. To produce bio-fouled microplastics, 20 µm PE microspheres were added to 600 mL Pyrex bottles containing the 8 µm-filtered seawater, in a concentration of 50 MPs mL^{-1}. The bottles were placed in a plankton wheel at 1 rpm and incubated with the following conditions: temperature of 18 ℃, light intensity of 100 µmol photons m^{-2} s^{-1} and a photoperiod of 12 h light: 12 h dark cycle. B1 medium (1 mL L^{-1}) [38] was added to all the bottles to avoid nutrient depletion. After four weeks of incubation, the presence of a biofilm on the MPs was confirmed using DAPI (4', 6-Diamidino-2-Phenylindole, Hydrochloride) staining and examination under an epifluorescence microscope with UV light (Fig. 1).

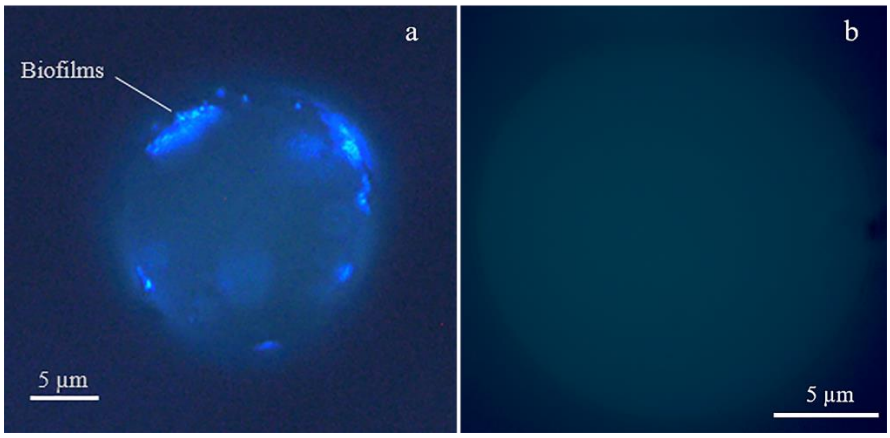


Figure 1. Epifluorescence microscope image of a bio-fouled PE microplastic stained with DAPI under UV illumination (a) and the same microplastic without biofilms (b). Bright blue fluorescent areas correspond to the DNA of biofouling microorganisms growing on the surface of the microplastic particle.

Study 4: "Sorbed pollutants"

To obtain microplastics with a sorbed hydrophobic pollutant, PE and PS microspheres

respectively were exposed to a pyrene solution. Pyrene powder was diluted in methanol to prepare a stock pyrene solution of 100 μg mL^{-1}. 10 mg of microplastics were added to acid-washed glass bottles (68 mL) with a pyrene solution of 50 μg L^{-1}. Additionally, 10 mg of microplastics were added to bottles with methanol alone as a control treatment. The bottles were incubated for 72 hours in a plankton wheel at 5 rpm at 18 ℃ in the dark. Upon incubation, we filtered the bottle contents through 5 μm polycarbonate filters, separating the microplastics (residue) from the pyrene solution (filtrate). A subsample of the collected microplastics was re-suspended in filtered seawater to be used in the bottle incubations and filming experiments, respectively. Another subsample was stored at -80 ℃ for later pyrene sorption analysis. The concentration of pyrene sorbed by the microplastics was measured in triplicates of microplastic samples with an Agilent 6890 gas chromatograph. Extraction was performed by adding 4 mL n-hexane: acetone (6:4) directly to the vials. The extraction time was 24 h. Chromatographic separation was achieved on an Agilent 6890 gas chromatograph equipped with a 60 m × 0.25 mm inside diameter × 0.25 μm film thickness DB-5ms column (Agilent Technologies). A 2 μL sample was injected in splitless mode with the sample inlet held at 300 °C. The oven was programmed to 70 °C, then 20 °C/min to 300 °C, and then 50 °C/min to 325 °C held for 10 min. Helium was used as carrier gas with a 1 mL/min constant flow. Detection was achieved on an Agilent 5975C triple-axis mass-selective detector operated in SIM mode with the MS source at 230 °C and the quadrupole at 150 °C.

The average concentration of sorbed pyrene in the PE microplastics was 0.059 ± 0.009 μg mg^{-1}. This value is the same order of magnitude as observed by Wang and Wang [39].

Particle sizes

The size (equivalent spherical diameter, ESD) and concentration of each type of microplastic in the prepared stock suspensions were measured using a Multisizer Coulter Counter. The average size of PE and PS microspheres was 20.5 ± 0.2 μm and 20.6 ± 0.5 μm, respectively. The average size of the obtained irregular microplastics was 20.6 ± 1.7 μm. *H. steinii* used for the experiment had an average ESD of 17 ± 0.4 μm.

Video observation

Before the video recordings, copepods were tethered to a needle from their dorsal surface [40]. Tethering does not affect the feeding selective behavior of *T. longicornis* [23,41]. The video observation was conducted in a thermo-constant filming room (at 16℃). A 10×10×10 cm^3 transparent container was placed between an infrared light and a high-speed camera (Phantom V210). In each treatment, 800 mL of the microplastic-alga suspension was added to the container and gently stirred by a magnetic stirrer. Then, a single tethered copepod was attached to a micromanipulator by the other edge of the tether immersed in the mixed particle suspension. Subsequently, the tethered copepod was adjusted to the center of screen field in focus. A 3-hour video recording (resolution: 1024×512 pixels; frame rate: 100 Hz) was started instantly after preparing the set up. Due to limited storage space on the camera, each video lasted for maximum 100s. Thus, with 28 recorded videos, a total of 3h were saved for analysis. All the experimental operations were conducted outside the filming room and the room was kept in darkness throughout the entire process to minimize any interruption. Three copepod females from each treatment were filmed separately.

The capture, ingestion and rejection events of *T. longicornis* were counted from the videos. The copepods beat their feeding appendages constantly to maintain the feeding current

(percentage of time beating = 99.7% ± 0.1%) and scan the surrounding water. When prey particles were drawn into their detection range, contractions of swimming appendages were observed, in many cases followed by a successful capture of the particle. A behavioral event was defined as “ingestion” when the captured particle was handled, tasted and finally eaten by the copepod (Movie S1 and S2). On the contrary, a behavioral event was defined as “rejection” when the particle was actively “kicked” away by the copepod after tasting (Movie S3). Although the used prey and microplastics have similar sizes, it was easy to visually distinguish dinoflagellates from microplastics in the video (Movie S1 and S2) due to their specific morphological characteristics.

Bottle incubation experiments

All the glassware used for these experiments was acid-washed with 10% HCl and rinsed three times with miliQ water. Experiments were conducted in triplicates in 600 mL Pyrex glass bottles with lids lined with polytetrafluoroethylene (PTFE) protection. Five copepod females were incubated in each bottle. For all the treatments, we prepared 3 initial bottles (time=0), 3 control bottles (without copepods) and 3 experimental bottles (with copepods). The bottles were first filled with 0.2 µm-filtered seawater (salinity = 30 psu), Aliquots of microplastics and algae working suspensions were added to each bottle to obtain the desired exposure concentrations for each treatment (200 MPs mL^{-1} and 200 cells mL^{-1}). Subsequently, the copepods were added to the experimental bottles. Finally, the bottles were filled up with filtered seawater, closed with a lid and wrapped in aluminum foil. The bottles were mounted on a plankton wheel (1 rpm) in a temperature-controlled incubation room at 16℃ for 24 hours.

At the beginning of the incubation (time= 0), for each treatment, 25 mL samples of microplastic-

alga mixture were collected from the three initial bottles to measure the precise concentration of microplastics and algae added (Table S1). After the 24 hours of incubation, 25 mL samples were collected from three experimental and three control bottles, respectively, to measure the final concentration of microplastics and algae. All 25 mL samples were immediately fixed with 1% of Lugol's solution and subsequently microplastics and algae were counted under an inverted microscope using Sedgewick-Rafter counting chambers. At the end of the experiment, copepods were examined under a stereomicroscope to verify that there was no mortality during the experiment. We did not observe mortality in any treatment. The ingestion and clearance rates were calculated according to Frost [42]. Selective feeding was evaluated using the electivity index (E) [43]. Electivity index of the particle type I (E_i) was calculated as:

$$E_i = \frac{Wi-(1/n)}{Wi+(1/n)}$$

with *n* as the total number of particle types in a given bottle (n=2), and the coefficient *Wi* as

$$W_i = \frac{Fi}{\sum Fi}$$

Where, Fi is the clearance rate of the particle type i, and ∑ Fi is the sum of clearance rates on all food types. The electivity index (E) ranges between -1 and +1, where 0 indicates no electivity (no selective grazing), negative values correspond to avoidance and positive values represent selection.

Statistical analysis

Statistical analysis was performed using IBM-SPSS v25. For each treatment, we statistically analyzed the significant differences between algae and microplastics ingestion and clearance rates. Furthermore, we tested the statistical differences in feeding rates on algae and microplastic types among the treatments. One-way ANOVA was applied followed by a pairwise

multiple comparison using Bonferroni test. In treatment (T7), of the incubation experiment, the number of replicates was only two due to the loss of one sample during the analysis, in this case, we used a t-test analysis to evaluate the difference between ingestion of algae and microplastics. Significant difference was determined at $P<0.05$ (Table S2).

Results

Global distribution of feeding-current feeding copepods

Our results show that feeding-current feeding copepods are commonly found across the global ocean, in particular in high and middle latitudes (Fig. 2a). In low latitudes, this feeding mode represent approximately the 40% of the copepods on average, while in some areas of high latitudes it reaches the 80% (Fig. 2b).

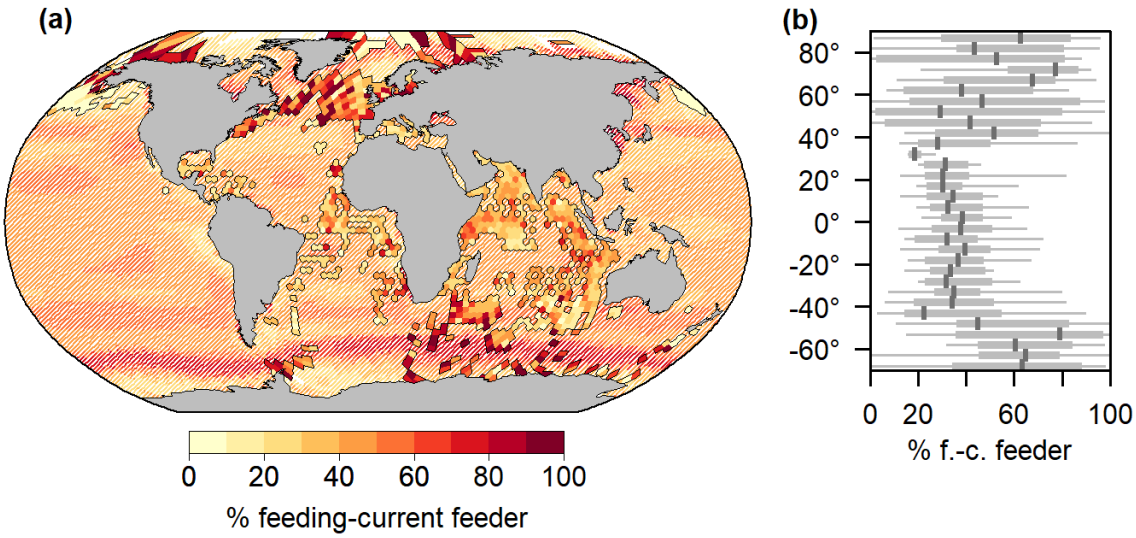


Figure 2. Global (a) and latitudinal (b) distribution of the fraction of feeding-current feeding copepods. Dashed areas represent model extrapolations and solid colors/latitudinal boxplots are observation-based estimates.

Video observations: capture rates, ingestion rates, and rejection percentages of algae

and microplastics

Video observations showed that the copepod *T. longicornis* did not differentiate between algae and microplastics before capture. Within each treatment, the capture rates for algae and microplastics were not significantly different ($P>0.05$, Fig. 3a and 3b). In addition, there were no significant differences in capture rates on algae between treatments ($P=0.167$). Capture rates on algae ranged from 2014 to 4423 cells ind.$^{-1}$ d^{-1} (Fig. 3a), and capture rates on microplastics ranged from 1445 to 5249 MPs ind.$^{-1}$ d^{-1} (Fig. 3b).

Overall, ingestion rates on algae or microplastics did not significantly differ across all treatments ($P=0.166$ and 0.184, respectively). The average ingestion rate on algae was 2995 cells ind.$^{-1}$ d^{-1} (Fig. 3c), which was 5 times higher than on microplastics (Fig. 3d) and similar to the capture rate of algae (Fig. 3a).

All examined copepods presented a significantly higher rejection rate of microplastics than algal cells. The averaged percentage of rejected algae and microplastics, considering all the treatments, was 0.5% ± 0.2% and 78.3% ± 3.2%, respectively (Fig. 3 e-i). Generally, an algal cell or a microplastic particle was captured and then handled by the copepod for approximately 120 ms before it was tasted. Afterwards, most of the algal cells and a few microplastic particles were ingested, whilst the majority of microplastics were spit out after being tasted for an average of approximately 500 ms.

We did not find significant differences in the rejection percentage of microplastics between polymer types ($P=0.304$). The average percent of rejection of virgin PS and PE microspheres was 71.9% ± 11.3% and 82.6% ± 4.4%, respectively (Fig. 3e). Similarly, the shape of microplastics did not affect the percentage of rejection ($P=0.964$). Compared to spherical PS,

irregular PS was only 0.5% more rejected by *T. longicornis* on average (Fig. 3f). The attachment of a biofilm and the sorption of pyrene did not change the proportion of rejected microplastics either. 82.5% ± 10.2% of bio-fouled PE microspheres were rejected by *T. longicornis*, which was very close to the ratio of clean PE microspheres (Fig. 3g). PE microspheres with pyrene, and to a lower degree PS with pyrene, appeared to be rejected less by *T. longicornis* than their control treatments. However, the differences were found not to be statistically significant (Fig. 3h and 3i).

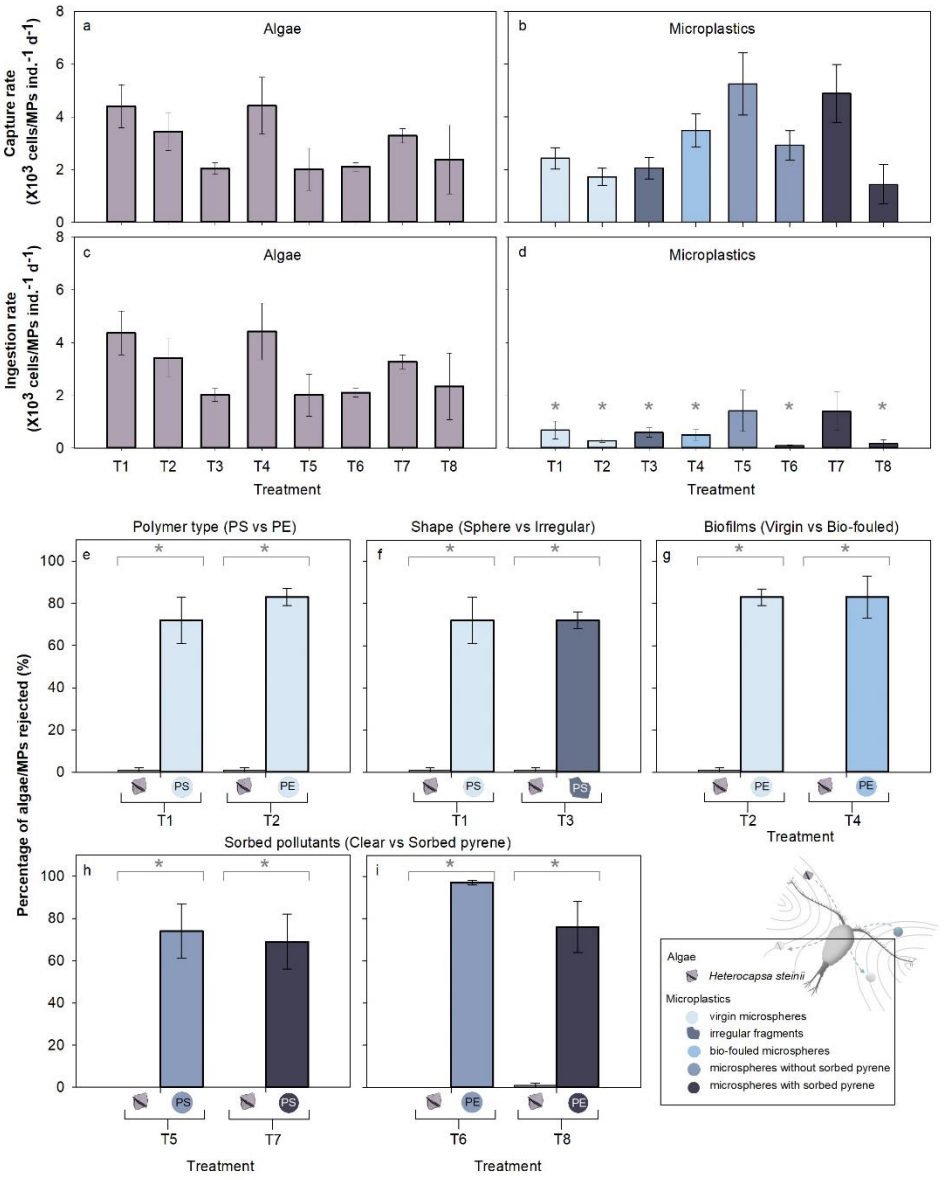


Figure 3. Feeding behaviors of *T. longicornis* on algae and microplastics in the different

326 treatments recorded by video camera. Capture rates of *T. longicornis* on *H. steinii* (a) and
327 microplastics (b). Ingestion rates of *T. longicornis* on *H. steinii* (c) and microplastics (d).
328 Percentage of *H. steinii* and microplastics that were rejected by *T. longicornis* when supplied
329 simultaneously (e-i). Comparison between treatments added with (e) virgin PS and PE
330 microspheres, (f) spherical and irregular PS, (g) virgin and bio-fouled PE microspheres, and (h-
331 i) clean and pyrene-polluted PS/PE microspheres. Error bars show standard errors (n=3). Note
332 that algae and microplastics were offered together in each treatment. Asterisks (*) represent a
333 statistically significant difference between algae and microplastic ingestion rate or percentage
334 of rejection within each treatment.
335
336 **Bottle incubations: ingestion and selection of algae and microplastics**
337 The daily ingestion and clearance rates of *T. longicornis* on algae and microplastics were also
338 calculated from 24-hour bottle incubations (Fig. 4a, 4b and Table S3). A significant decrease in
339 microplastic concentration only occurred in 7 out of 24 bottles. Besides, in all the treatments
340 the ingestion of algae was significantly different from the ingestion of microplastic. Ingestion
341 rates of algae (Fig. 4a) were 1.4-12.6 times higher than of microplastics (Fig. 4b) and had the
342 same order of magnitude as the rates of algae ingestion measured in video observation. In
343 general, *T. longicornis* presented a distinct preference for algae and largely avoided eating
344 microplastics when exposed to alga-microplastic mixtures. Overall, no significant differences
345 occurred between algae ingestion rates among all treatments with the exception of the
346 treatment with bio-fouled microplastics (T4). The low ingestion rate on algae in T4 leads to a
347 non-significant difference between microplastic and algae ingestion in that treatment. The

electivity index (E) of algae varied from 0.21 to 0.33 among treatments, with positive E values indicating selection. By contrast, the electivity index of microplastics varied from -0.49 to -1.00, with negative E values indicating avoidance (Fig. 4c).

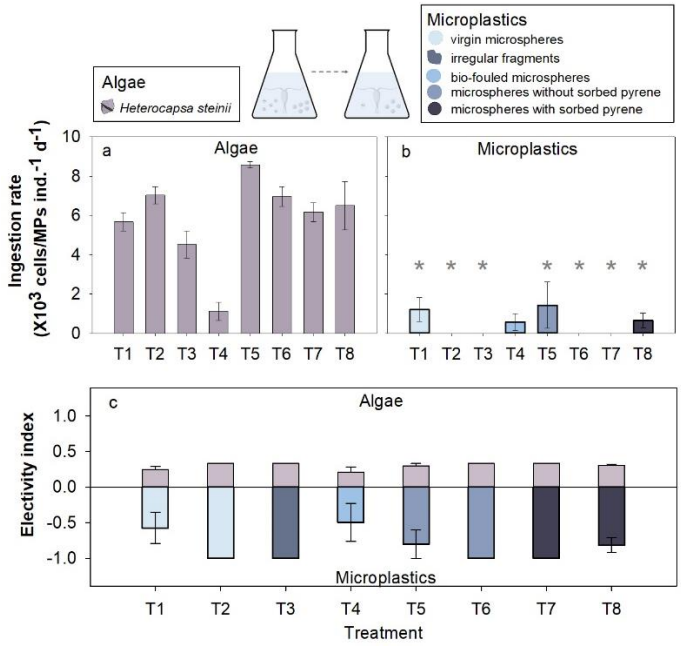


Figure 4. Feeding behaviors of *T. longicornis* on algae and microplastics in the different treatments recorded from bottle incubations. Ingestion rates of *T. longicornis* on *H. steinii* (a) and microplastics (b). Electivity index (c) of *T. longicornis* among mixtures of algae (top bars) and microplastics (bottom bars). Error bars show standard errors (n=3). Note that algae and microplastics were offered together in each treatment. Asterisks (*) represent a statistically significant difference between algae and microplastic ingestion rate within each treatment.

Discussion

Behavior and feeding rates of *T. longicornis* exposed to algae and microplastics

Current knowledge about the ingestion and effects of microplastics on copepods is largely based on bottle incubations [10,15,16,44,45]. Most studies use ingestion rates as the main parameter

363 to describe microplastic consumption and selection by copepods, but this is not sufficient to
364 reveal the underlying mechanisms. On one hand, copepods could either selectively graze on
365 plastic particles or indiscriminately ingest the particles with the natural prey. On the other hand,
366 copepods could either actively refuse microplastics or passively reduce microplastics intake by
367 physical impacts/interference of microplastics or by chemical toxicity associated with plastics
368 (leachates or sorbed pollutants) [46]. During a 'black box' bottle incubation, the different
369 mechanisms or processes might lead to similar overall ingestion rates. However, the detection,
370 handling and rejection rates of microplastics need to be evaluated to understand the impact on
371 the grazer. Here we did not observe any behavioral abnormalities (e.g. stop beating
372 appendages or no grazing movements), when *T. longicornis* was exposed to microplastics with
373 a similar sized microalga. We conclude that the lower ingestion rates of microplastics compared
374 to similar sized natural prey was due to an active selective behavior of the planktonic copepods.
375 Copepods have been shown to possess diverse sensors on their antennae, feeding
376 appendages, or body surfaces for detecting either hydro-mechanical or chemical signals
377 created by their prey [47]. The capacity of copepods for remote chemoreception is controversial
378 and discussed in literatures [48-51]. According to the calculation by Tiselius et al [51], distant
379 detection was only feasible for prey that is unusually large and leaking chemicals to the
380 environment, whilst it was more common to observe nearby or touch detection of prey cells
381 within a radius of around 10-50 µm. In the present study, the feeding response (i.e. ingestion
382 or rejection) of *T. longicornis* occurred only after capturing the alga or microplastic particle. This
383 demonstrates a nearby- or touch detection, which is similar to observations from previous
384 investigations [23,27]. In addition, the similar encounter and capture rates of algae and

385 microplastics (Fig. 3a and 3b) suggest that *T. longicornis* does not carry out any pre-capture
386 selection between algae and microplastics. Thus, all 20 μm microplastics and algae were
387 equally perceived and captured when they were very close to the antennae or feeding
388 appendages of *T. longicornis*.
389 The evaluation and selection of prey by the copepods occurred post-capture when prey touched
390 the setae on the feeding appendages and in the mouth. The duration of the subsequent
391 handling time is mainly caused by the position of the prey particle when it is first captured [52]. In
392 our study, the duration was variable but it did not show any statistically significant difference
393 between prey types. The handled particle was pushed into the mouth, tasted and either
394 ingested immediately by *T. longicornis* or spat out. In many cases, microplastics were handled,
395 tasted, and spat out several times until finally being pushed away by *T. longicornis.* Tasting was
396 therefore the main mechanism used by *T. longicornis* to discriminate microplastics from normal
397 prey.
398 Results from both video observation and bottle incubation further showed that algal ingestion
399 by the studied copepod was not impeded by the presence of microplastics at the studied
400 concentrations (≈ 200 MPs mL^{-1}). To evaluate if the used microplastic concentration can affect
401 the ingestion rates of the studied copepod, we compared our results with previous studies with
402 the same copepod species and type of alga but in absence of microplastics [27,53]. According to
403 those data, when *T. longicornis* was given dinoflagellate *H. steinii* as the sole food, the ingestion
404 rate increased linearly with the algal concentration. If we consider the initial algal concentration
405 (200 cells mL^{-1}) used here, the ingestion rates are estimated to be around 4500 cells $ind.^{-1}$ d^{-1},
406 which are very close to the ingestion rates measured in this study (Fig. 3c and 4a). Early studies

have similarly demonstrated that algal ingestion by other copepods, for example *Acartia clausi* and *Calanus pacificus*, were not affected by the presence of virgin plastic microspheres [54,55]. Similarly, fecal pellet production rates of arctic copepods, which are directly related to ingestion rates, were not affected by the presence of virgin microplastics at a concentration of 20 MPs mL^{-1} [16].

Effects of shape and polymer type on microplastic ingestion

The shape of microplastics is one of the characteristics that may regulate copepods' selective ingestion of microplastics. Botterell et al [11] further hypothesized that different feeding strategies of copepods might lead to different preferences for microplastic shapes. According to their experiments, feeding-current feeders ingested more fragments than fibers, suggesting differences in selectivity depending on microplastic shape. In the present study, we hypothesized that copepods will reject microspheres to a smaller extent than irregular fragments due to their similar shape to the natural prey. However, the ingestion rates of the feeding-current feeder *T. longicornis* on different shapes of microplastics showed no significant difference: upon capture, spherical and irregular PS particles were rejected in the same proportion (≈ 70%). Meanwhile, the algae offered together with microplastics were rarely rejected (≈ 1%).

The plastic polymer type is another factor that may affect the ingestion of microplastics by copepods [18]. Polymers differ in several physical and chemical characteristics, like hardness and density [56]. We measured the selection of two polymer types (PS vs PE) in this study, and found no significant difference between ingestion rates on virgin PS and PE microspheres. The similar high percentage of rejected microplastics by copepods (Fig. 3e) suggests that copepods

select similarly strictly against the two polymer types corroborating our hypothesis.

The high rejection rate of all types of microplastics tested in our experiments indicates that irregular shape and PE/PS polymers may not be crucial factors for *T. longicornis* to selectively reject a specific microplastic particle. The reasons for the high rejections may be that copepods dislike the chemical composition of virgin plastic or that virgin microplastics lack the organic signals, which help the copepods to recognize the particles as food. However, more studies on other microplastic physical characteristics (colors, additional shapes, other polymer types, etc.) could be very relevant to give a better overview of the effects of different microplastic types on zooplankton.

Effects of weathering on microplastic ingestion

Weathered microplastics are more bioavailable for marine organisms and potentially harmful for aquatic ecosystems due to their biofilm or absorbed pollutants [22,57-59]. When a primary microplastic enters the aquatic environment, bacteria quickly colonize the surface, and within the subsequent weeks, the dominant bacterial species could entirely change and create a new biofilm community depending on environmental conditions [60]. The organisms growing on the plastic surface release metabolic products, that can make microplastics smell and taste more like food particles [61]. For example, it was observed that microplastics with biofilm were preferred by some copepod species over virgin microplastics [22]. Another study showed that microplastics infused with dimethyl sulfide or dimethylsulfoniopropionate (DMS, DMSP), compounds that are naturally synthesized by marine phytoplankton, were ingested to a larger extent by *Calanus helgolandicus* and *Acartia tonsa* compared to clean microplastics [11]. Therefore, we hypothesized that bio-fouled microplastics would be ingested to a higher degree than clean

451 ('virgin') plastic particles in our experiment. We observed, however, that only a few bio-fouled
452 microplastics were ingested by *T. longicornis*. Based on both video observation and bottle
453 incubation, ingestion rates of bio-fouled microplastics were similar to "virgin" microplastics (Fig.
454 3 and 4). The bio-fouled microplastics were rejected at the same rates (≈80%) than virgin
455 microplastics and other types of microplastics (Fig. 3 e-i). This indicates that the presence of
456 biofilms did not promote the ingestion of microplastics in our study. Possibly the biofilms on our
457 microplastics had an organic signal that copepods cannot detect or biofilms were not thick
458 enough to completely inhibit the chemical signals of the synthetic polymers, which make
459 microplastics unpalatable to copepods. The ingestion and impact of bio-fouled microplastics is
460 of high interest for example due to their role as available surface for invasive species or for
461 antibiotic resistant bacteria [62,63]. Therefore, further studies are needed to evaluate the impact
462 of this biofilm coated microplastics.
463 Microplastics are potential vectors of harmful chemicals sorbed from the environment [64]. Since
464 plastics were reported to absorb high concentrations of PAHs (polycyclic aromatic
465 hydrocarbons) like pyrene [39,64] and copepods have the ability to avoid diesel oil in water [65], we
466 hypothesized that *T. longicornis* also has the ability to avoid plastic particles contaminated with
467 pyrene. However, no significant difference was observed between capture rates of pyrene-
468 contaminated microplastics and virgin microplastics. This indicates that either the concentration
469 of signals from pyrene-contaminated microplastics was not sufficient to stimulate the remote
470 chemosensitivity of copepods, or the compounds associated with our pyrene-contaminated
471 microplastics are not perceivable to *T. longicornis*.
472 Theoretically, aging of microplastics (biofilm formation and sorption of chemicals) could either

promote or impede microplastic ingestion by copepods. However, based on the high rejection rates of all types of microplastics observed in this study, the influence of microplastic aging appears limited for planktonic copepods with an efficient tasting-discrimination technique, like *T. longicornis*.

Ecological implications

Feeding behavior is a key trait to understand the entry of microplastics into marine food webs. Zooplankton, having an important trophic role in connecting primary producers and higher trophic levels, are considered one of the main vectors for small microplastics into marine food webs [66]. However, there is so far little evidence to support this hypothesis [67,68]. Approximately 11.500 copepod species are known worldwide [69] and they can be grouped into three main feeding modes: ambush feeders, cruising feeders and feeding-current feeders. Ambush feeders need a physical disturbance in the surrounding water to detect their prey. Cruising feeders swim and feed on the particles they encounter on their way. Feeding-current feeders, like *T. longicornis,* create a feeding current to draw and scan prey within their current. Active feeders (feeding-current feeders and cruising feeders) are one order of magnitude more efficient than ambush feeders at getting non-motile prey (e.g., diatoms) [25,26]. Since microplastics are non-motile and captured in the feeding current at the same rate as motile prey, feeding-current generating copepods are more susceptible to encounter and ingest microplastics than ambush feeding copepods. Therefore, feeding-current feeders may play a particularly important role in enabling microplastics to enter marine food webs. More importantly, feeding-current feeding copepods are the dominant zooplankton group in many oceanic areas, especially in the coastal and higher latitude areas of the northern hemisphere which normally contain high

densities of microplastics and other pollutants simutaneously (Fig. 2). Hence, foraging behavior of zooplankton is the key trait to understand the entry of microplastics into marine food webs. However, although filter feeding was hypothesized to be the riskier foraging behavior in terms of microplastic ingestion, our results indicate that feeding-current feeding copepods are very efficiently discriminating microplastics, reducing the risk of ingestion and the entry of microplastics into marine food webs. Recent field studies support the low risk of ingestion of microplastics by planktonic copepods [21,70,71].

Ingestion of microplastics by planktonic copepods in the natural environment is expected to be much lower than predicted from laboratory experiments.

Although data from laboratory experiments have shown a high degree of microplastic ingestion by copepods [14,67], the concentrations used were 4-5 orders of magnitude higher (10-1000 MPs mL^{-1}) than what is currently observed in marine surface waters (< 0.0001-0.01 MPs mL^{-1}) [72,73]. Consequently, the chance of encountering and capturing a microplastic particle by a copepod in the natural environment is much lower. As discussed above, we showed that copepods like *T. longicornis* can detect the plastics, evaluate their edibility and actively reject 80% of all captured microplastics. We observed that copepods can make mistakes in the selection of prey (20% in this study). The high ingestion of microplastics by copepods observed in laboratory experiments is likely an artefact due to the unrealistic high concentrations of microplastics used in the bioasays. Therefore, especially when exposed to low concentrations, the risk of microplastic ingestion by feeding-current feeding copepods appears to be minor. The selective behavior of feeding-current feeders minimizes the ingestion of microplastics and entry of microplastics in planktonic food webs. However, it is important to note that the entrance of

517 microplastics into marine food webs can still happen through organisms that do not have the
518 ability to discriminate between natural prey and microplastics or that use other mechanisms,
519 e.g. visual detection, to select their prey (e.g. fish larvae) [13,74].

520 *Can zooplankton ingestion of microplastics disrupt the biological carbon pump?*

521 The biological carbon (C) pump is the mechanism by which inorganic carbon fixed through
522 photosynthesis is exported out of the surface layer via biological processes. The biological C
523 pump is crucial for the sequestration of CO_2 and climate regulation [75]. Planktonic copepods are
524 keystone components of the biological C pump by ingestion of primary production, export of
525 particulate organic matter via fecal pellets and carcasses production, vertical migrations and
526 respiration during hibernation [76,77] (Fig. 5). The adverse biological effects of microplastics,
527 shown in laboratory studies, have raised concerns about the impact of microplastic pollution on
528 the C cycle [78].

529 Kvale et al [7] predicts that a physical effect of microplastic pollution via zooplankton negatively
530 affect the biological C pump and consequently the global ocean oxygenation. Kvale´s model
531 assumes that the ingestion and selection of microplastics by zooplankton is only driven by the
532 ratio of microplastics to natural food. However, this is not the case for planktonic copepods,
533 where foraging behavior and prey selection capability of copepods are key aspects that
534 determine the ingestion of microplastics as demonstrated here. Due to the capture mechanisms
535 (ambush feeder) and taste- discrimination (feeding-current feeders) of copepods, the ingestion
536 of microplastics is expected to be low and therefore also their impacts on planktonic copepods.
537 Grazing of zooplankton is not negatively affected by ingestion of virgin microplastics at *in situ*
538 concentrations of microplastics (0.0001-0.01 MPs mL^{-1}) [30,73]. In our studies, the ingestion rates

of copepods on algae were not affected by the presence of MPs and were similar to those in the absence of MPs [79]. Production and sinking rates of fecal pellets are also key processes in the biological C pump. Assuming that the presence of microplastics inside the fecal pellets increases their buoyancy, the pellets would be recycled in the water column reducing the C sequestration in the bottom waters. However, similar to grazing rates, fecal pellet production and sinking rates are not expected to be affected by ingestion of microplastics under natural conditions [16]. Therefore, it is unlikely that physical impacts of microplastics can disrupt the role planktonic copepods play in the global biological carbon cycle. Overall, our results indicate that, while there is a risk of entry of microplastics in the marine food webs, planktonic copepods are not expected to be a major entry route.

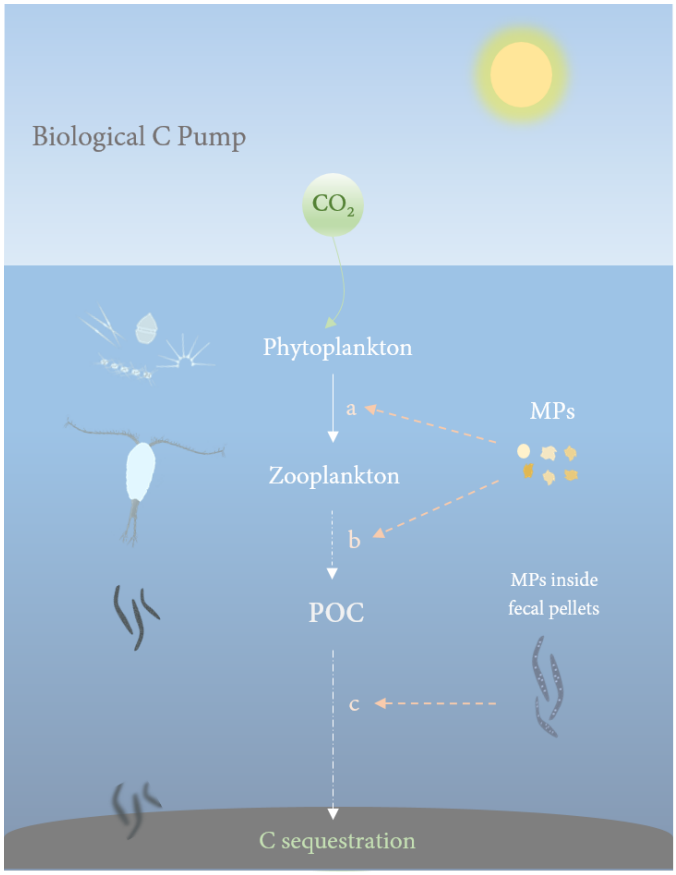


Figure 5. Simplified scheme of the key role of zooplankton in the “biological carbon (C) pump” and the potential impacts of microplastics (MPs) on the transfer and exportation of carbon:

552 decreased zooplankton grazing (a), reduced fecal pellet production (b), and lower sinking
553 velocity of fecal pellets containing ingested microplastics (c). POC (Particulate Organic
554 Carbon).

555

556 **Supporting information**: Additional tables and videos mentioned in the text.

557

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570

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