



# Microplastic Consumption and Its Effect on Respiration Rate and Motility of *Calanus helgolandicus* From the Marmara Sea

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Consumption rates of polystyrene microplastics (beads of 6, 12, and 26  $\mu\text{m}$  diameter) and their effects on energy metabolism and motor activity of the copepod *Calanus helgolandicus* living in the Marmara Sea were investigated. All sizes of microplastic particles were actively consumed and excreted via fecal pellets, however, copepods displayed a significant preference for beads sized 6  $\mu\text{m}$ . In a mixture of algae and microplastics beads of 6  $\mu\text{m}$ , microplastics consumption rates linearly ( $r^2 = 0.78$ ,  $n = 154$ ) increased 800 times from  $50.8 \pm 17.3$  to  $8,612 \pm 5,972$  beads  $\text{ind}^{-1} \text{day}^{-1}$  with an increase in bead concentration from 10 to 44,000 beads  $\text{ml}^{-1}$ . The total and basal metabolic rates as well as time spent swimming for *C. helgolandicus*, decreased 1.7, 1.8 and about 3-fold, respectively after 7–8 days exposure to microplastic treatments, which was similar to the metabolism and activity of starving animals in filtered water. In copepods consuming microplastics, all vital parameters decreased on the first day of exposure, indicating either accelerated starvation, probably due to increased losses of energy and biological matter in the formation of fecal pellets and/or traumatic/toxic effects of the polystyrene beads on the copepods. Our data from laboratory experiments indicate that the presence of large concentrations of microplastics in water, even when mixed with algae, lowered energy metabolism levels of *C. helgolandicus*.

**Keywords:** microplastics, consumption, respiration, behavior, *Calanus*, Marmara Sea

## INTRODUCTION

Microplastics are already numerically one of the most abundant items present in the plankton and sediment of the marine environment (do Sul and Costa, 2014). Removal of microplastics from the marine environment poses one of the greatest challenges for the human race with almost no potential solutions as yet. These facts on microplastics coupled with their ubiquity and availability as false food items for the marine food chain call for dedicated studies on their impact to marine biota (Secretariat of the Convention on Biological Diversity, and Scientific and Technical Advisory Panel GEF, 2012).

Since the size of microplastics (either 1–1,000  $\mu\text{m}$  or 20–5,000  $\mu\text{m}$  particles of marine litter, Hanke et al., 2013 or Hartmann et al., 2019, respectively) may coincide with the sizes of phytoplankton cells, many zooplankton species are reported to consume them inadvertently (Huntley et al., 1983; Ayukai, 1987; Cole et al., 2013, 2015, 2016; Lee et al., 2013; Wright et al., 2013; Desforges et al., 2015; Ogonowski et al., 2016; Frydkjær et al., 2017; Scherer et al., 2017; Vroom et al., 2017; Gorokhova et al., 2018; Botterell et al., 2019; Coppock et al., 2019). Generally, zooplanktonic organisms display limited selectivity of food particles feeding upon any available particles of appropriate size. Wilson (1973), Paffenhöfer and Van Sant (1985), and Cole et al. (2013) have shown that many copepod species can ingest microplastics within a size range of 7–30  $\mu\text{m}$  (similar to sizes of their algal food). Smaller microplastics (e.g., 3.8  $\mu\text{m}$  as shown by Cole et al., 2013) can externally adhere to the posterior swimming legs of the copepod *Temora longicornis*. Uptake of microplastics by small zooplankton is very critical, since these lower trophic level species play a significant role in transferring such particles along the whole trophic chain in the marine ecosystem. Therefore, even some zooplankton species which are raptorial predators themselves would be vulnerable to microplastic pollution when their smaller sized prey is contaminated.

Cole et al. (2013) demonstrated that for several species of copepods, exposure to 7.3  $\mu\text{m}$  polystyrene beads at concentrations of 4,000–25,000 microplastics  $\text{ml}^{-1}$  significantly reduced the feeding rates on algae. This shows that microplastics can significantly affect the health of copepods and their suitability as food items for next trophic levels. It has been recorded that exposure of *Calanus helgolandicus* to microplastic particles sized 20  $\mu\text{m}$  even at low concentrations (75 beads  $\text{ml}^{-1}$ ) significantly reduced reproductive output, but there were no significant differences in respiration rates (Cole et al., 2015). We should note that *Calanus helgolandicus* species is not particularly suitable for studying the effects of diet on energy metabolism since it is naturally adapted to long-term starvation due to its ability to accumulate large lipid reserves (Lee et al., 1970). Indeed, in the experiments of Cole et al. (2015), the active metabolic rate for *C. helgolandicus* specimens after 10-day exposure to a plastic bead diet averaged 0.7  $\mu\text{L O}_2 \text{ ind}^{-1} \text{ day}^{-1}$  with no significant difference in comparison to controls feeding on microalgae. This indicates that further investigations with different experimental designs are needed to better understand the effects of microplastics on *Calanus*.

The Sea of Marmara, as well as the Black Sea connected to it by the Bosphorus Strait, is inhabited by the same year-round mass species of the genus *Calanus* (Isinibilir et al., 2009), which for a long time was attributed to *Calanus helgolandicus* var. *ponticus* (Jaschnov, 1955). Later, based on difference in prosome to urosome length relationships and on the occurrence of supernumerary pores on the ventral side of urosome segments in individuals from the Black Sea, the Mediterranean Sea and Atlantic Ocean populations, Fleminger and Hulsemann (1987) recognized the Black Sea population as a distinct species—*Calanus ponticus*. In 1991, a new name, *Calanus euxinus* was given to this species by Hulsemann (1991). Repeated

morphological comparisons of the Black Sea population with the Atlantic and Mediterranean (Yebra et al., 2011) and the Marmara Sea (Isinibilir et al., 2009) populations did not confirm the comparative morphological measurements by Fleminger and Hulsemann (1987). In addition, it should be taken into account that the difference in the number of urosomal supernumerary pores in Black Sea females may be due to the fundamentally lower salinity of this sea. Finally, by a series of molecular studies, it was shown that level of genetic differentiation between *Calanus helgolandicus* and *Calanus euxinus* is typical for conspecific calanoid copepod populations (Papadopoulos et al., 2005; Unal et al., 2006; Yebra et al., 2011; Figueroa et al., 2019) within European waters, despite very distinct geographic barriers/conditions prevail for the Black Sea. Therefore, the species of *Calanus* in the present study is referred to as *Calanus helgolandicus*, which has been referred to as *C. euxinus* or *C. ponticus* in different studies from the Marmara Sea or the Black Sea.

The aim of this work was (1) to test the ability of Marmara Sea *Calanus helgolandicus* to consume microplastic beads of three different sizes: 6, 12, and 26  $\mu\text{m}$ , more or less corresponding to the range of phytoplankton cell sizes they consume, and (2) monitoring changes in metabolic and motor activity rates for a microplastics diet with respect to well fed or starving individuals.

## MATERIALS AND METHODS

Zooplankton samples were collected at the beginning of April both in 2018 and 2019 with a closing Nansen net (opening diameter: 50 cm, mesh size: 200  $\mu\text{m}$ ) by vertical hauls from the bottom (150 m) to the surface at a station located (40°51,715N–28°57,901E) in the Marmara Sea (salinity 22 psu, temperature 10–15°C) near the Princes' Islands, off Istanbul, in Turkey. Approximately 2 h after collecting samples, healthy *Calanus helgolandicus* specimens were sorted individually at the laboratory, from the diluted sub-samples using a wide pipette. Selected copepods were placed in 1 liter volume vessels containing 0.45  $\mu\text{m}$  filtered sea water (20 psu salinity, temp. 17°C) for acclimation to the experimental conditions. Polystyrene microplastics (Fluoro-Max Red Dry Fluorescent Particles) of 6  $\mu\text{m}$  (Cat No 36-2), 12  $\mu\text{m}$  (Cat No 36-3), and 26  $\mu\text{m}$  (Cat No 36-5) were purchased from Thermo Scientific™.

Our research in April 2018 was focused on preliminary identification of the ability of adult *Calanus helgolandicus* females to consume microplastic beads along with natural phytoplankton. Further experiments aimed to understand possible preferences between microparticle sizes (6, 12, and 26  $\mu\text{m}$ ), coupled with measurement of consumption rates of microplastic beads fed in a mixture of monoalgal (*Rhodomonas salina*) cultured cells. In April 2019, our main research focused on the effect of 6  $\mu\text{m}$  microplastics consumption on *C. helgolandicus* in terms of total and basal energy metabolism and motor activity.

## Mono-Algal Culture

The original strain of the cryptomonad algae *Rhodomonas salina* (5–10  $\mu\text{m}$  size range) was provided by the Culture Collection

of Algae and Protozoa (CCAP), Scotland, UK. Initial microalgae cultures were inoculated in test tubes (30 ml) containing f/2 + Si medium previously sterilized at 121°C for 15 min. All sub-cultures were maintained at 23°C and salinity of 32 psu under a 12L:12D photoperiod. *R. salina* culture volume was continuously up-scaled from 30 ml test tubes to 250 ml Erlenmeyer flasks, followed by 1, 5, and 30 L culture containers in photobioreactors, continuously. Illumination was maintained at 200  $\mu\text{mol}/\text{m}^2/\text{s}$  at the culture surface. Population growth was determined daily by cell counting using Neubauer chambers under Leica DM100 microscope.

## Microplastic Uptake Experiments (for Consumption and Fecal Pellet Assessments)

To determine the rate of microplastic consumption, selected *Calanus helgolandicus* individuals (under Nikon SMZ-100 microscope) were placed in 1 L vessels containing 0.45  $\mu\text{m}$  filtered sea water of (20 psu, 17°C) for preliminary acclimation to the experimental conditions. The following day, actively swimming copepods were picked and individually placed in 10–20 ml cells/vessels for further investigation.

Four feeding (FEED) treatments (up to 5 days) were performed in experiments in April 2018 set up as follows:

- (A) To assess the selective consumption of beads of different sizes;
- (FEED1): Females were incubated for 3 days in seawater with a mixture of natural phytoplankton (dominated by *Skeletonema sp.*) collected in the upper layer of the Marmara Sea ( $\sim 5,000$  cells  $\text{ml}^{-1}$ ) and microplastics of diameter 6, 12, and 26  $\mu\text{m}$  (total 5,000 beads  $\text{ml}^{-1}$ ), one female per 10 ml vial, (16 replicates). The water and mixture of microplastics/phytoplankton were renewed and sediments were removed every second day. At the end of the 3rd day, the fecal pellets deposited were collected with a pipette, their number was counted. The number as well as the volume of microplastics contained in each pellet was also calculated taking into account the size of beads.
- (B) To evaluate the efficiency of microplastic consumption by adult females in the presence and absence of algae, four longer duration experiments (5–6 days) were performed (**Figure 1**):
- (FEED2): Incubation of females for 5 days with only *R. salina* ( $\sim 5,000$  cells  $\text{ml}^{-1}$ ), two individuals per 50 ml, (4 replicates);
- (FEED3) Incubation of females for 5 days with a mixture of *R. salina* ( $\sim 5,000$  cells  $\text{ml}^{-1}$ ) and microplastics of 6  $\mu\text{m}$  (5,000 beads  $\text{ml}^{-1}$ ), two females per 50 ml (4 replicates);
- (FEED4) Incubation of females for 5 days with microplastics of 6  $\mu\text{m}$  ( $\sim 5,000$  beads  $\text{ml}^{-1}$ ), two females per 50 ml (4 replicates).

Two feeding (FEED) treatments (up to 6 days) were performed in experiments in April 2019 set up as follows:

(FEED5): Incubation of females for 6 days with a mixture of *R. salina* ( $\sim 5,000$  cells  $\text{ml}^{-1}$ ) and microplastics of 6  $\mu\text{m}$  ( $\sim 5,000$  beads  $\text{ml}^{-1}$ ), one individual per 10 ml (10 replicates). The counting of the number of pellets, analysis of their contents, and the renewal of the concentration of the mixture of microplastics and algae along with seawater were performed at the end of each experimental day.

(FEED6): To assess relationship between microplastic concentration and the rate of its consumption (after the first “meeting” of copepods with microplastics), incubation of females for 7 h in water with *R. salina* (5,000 cells  $\text{ml}^{-1}$ ) and microplastics of 6  $\mu\text{m}$ , gradually increasing the microplastic concentration 2–5-fold hourly from 10 to 44,000 beads  $\text{ml}^{-1}$  (one female per 10 ml, 24 replicates). At the end of each hour, the number of pellets and beads contained in them was counted whilst the seawater and microplastics were replenished.

The concentration of *R. salina* of  $\sim 5,000$  cells  $\text{ml}^{-1}$  was typical for many experiments on copepods feeding (Meyer et al., 2002; Carotenuto et al., 2012; Helenius et al., 2019).

For short-term exposures of less than a day, the algae and microplastic concentration was monitored every hour in experimental vessels. In multi-day experiments, concentrations were determined at the end of each day. The experimental conditions did not allow us to use an incubation wheel, but vessels were often shaken during the experiment. It is known that adults *Calanus* could quickly sink (if they do not deliberately strive to the bottom) and, therefore, spend most of their time at the bottom, even in large vessels, enabling active copepods to filter microplastic beads from the bottom, especially in small 10 ml containers (Treatments FEED1, FEED5, FEED6). With frequent water changes and relatively low temperatures, copepods observed to be quite active even in a multi-day experiment (Treatment FEED5). Maintenance of required concentrations of microalgae and microplastics during experiments (considering bead sizes) were achieved by adding solutions of known concentration to the water determined by a standard method using a hemocytometer.

The microplastic consumption rate was calculated in accordance with number of beads in fecal pellets and the number of pellets per unit time: hourly in short-term and daily in longer-term experiments.

The number and size of pellets were counted under a dissection microscope (Zeiss Opton) at magnification of  $\times 16$ , and microplastic content in pellets were determined under a microscope with an increased magnification of  $\times 60$ –150. To simplify microplastic bead counting, pellets placed on a glass slide were crushed by a coverslip (**Figure 1**).

## Determination of Energy Metabolic Rates

Energy metabolic rates determined by measuring respiration (RESP) of copepods under different experimental set-ups. During all experiments, approximately 20–50 *C. helgolandicus* females were constantly kept in 1 liter vessels at a salinity 20 psu and temperature 17°C under each of 6 treatments (**Figure 2**) described below:



**FIGURE 1 | (A)** Photograph of an intact fecal pellet—450  $\mu\text{m}$  length and crushed **(B)** to count plastic beads of sizes 6 and 12  $\mu\text{m}$ .

(RESP1): Incubation for 3 days with only *Rhodomonas salina* (5,000 cells  $\text{ml}^{-1}$ );

(RESP2): Incubation for 3 days with a mixture of *R. salina* (5,000 cells  $\text{ml}^{-1}$ ) and microplastics size 6 and 12  $\mu\text{m}$  (overall concentration of 5,000 beads  $\text{ml}^{-1}$ );

(RESP3): Incubation for 8 days with microplastics size 6  $\mu\text{m}$  (5,000 beads  $\text{ml}^{-1}$ ) (6–12 replicates);

(RESP4): Incubation for 7 days in clean filtered water (starvation) (8–15 replicates);

(RESP5): Incubation for 3 days with microplastics size 12  $\mu\text{m}$  (5,000 beads  $\text{ml}^{-1}$ ) (6 replicates);

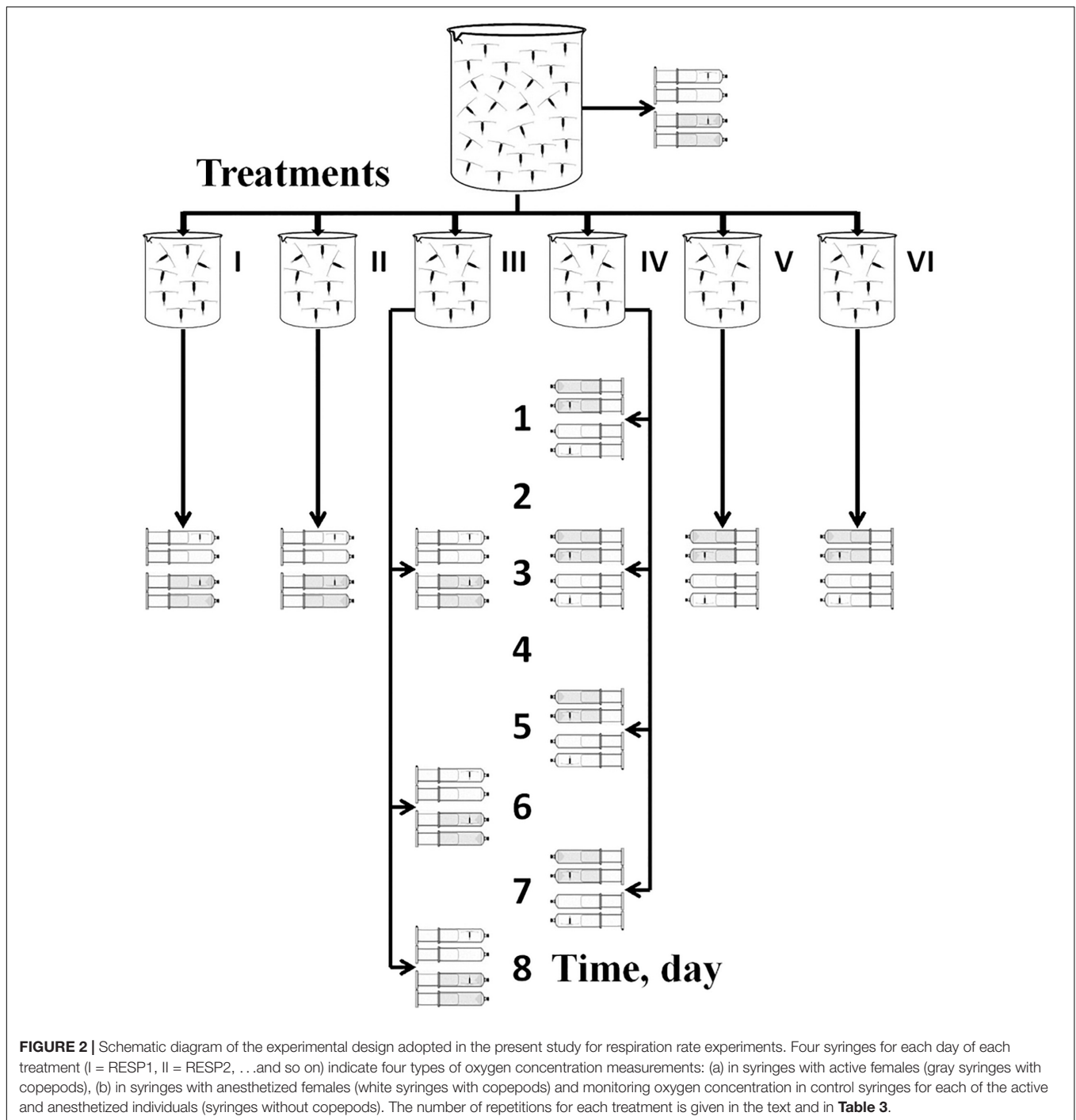
(RESP6): Incubation for 3 days with microplastics size 26  $\mu\text{m}$  (5,000 beads  $\text{ml}^{-1}$ ) (6 replicates).

In addition, a 1 day control experiment after catching specimens was performed (9 replicates). Microplastic and/or algal concentrations in water were replenished on a daily basis.

After the start of creating the specified conditions in the treatments, measurements were performed starting from the third day of incubation (except for the first day of starvation). After 3 days, measurements were taken every other day.

Total and basal energy metabolic rates were determined individually from respiration rates of active and anesthetized females at 20°C during 1–2 h of exposition in syringes, slightly higher than the temperature of the containers where copepod stock maintained before experiments (17°C, which is better for the maintenance of copepods). Thus, our results were comparable with numerous studies of active and anesthetized females of this species, performed by us earlier at a temperature of 20°C (Svetlichny and Umanskaya, 1991; Svetlichny and Hubareva, 2005; Svetlichny et al., 2010).

Respiration rates of females were determined individually, using the closed sealed chamber method, by all-glass experimental and control syringes (2.0 ml volume) used as



the respirometers. Females were gently transferred via pipette into an experimental syringe filled with sea water supplied from a protective sieve disc (mesh size 100  $\mu\text{m}$ ) at the confluent outlet. In order to obtain identical initial oxygen and seston content, control and experimental syringes were connected to a plastic tube with water gently pumped through and repeated several times. Syringes were then separated, closed with stoppers and placed into the chamber at a constant temperature of 20°C. Incubation periods were approx. 1 and 2 h for active and

anesthetized females, respectively. Following exposure, water samples from experimental and control syringes were transferred to the small measuring flow chamber of variable volumes (up to 0.3 ml) and connected to a luminescent dissolved oxygen sensor Hach LDO<sup>TM</sup> in order to measure concentrations of dissolved oxygen.

Oxygen consumption rates of copepods were calculated from differences between final oxygen content in experimental (with copepods) and control syringes (without animals) and expressed

as the amount of oxygen consumed per individual ( $\mu\text{gO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ ) or per wet weight ( $\mu\text{gO}_2 \text{ mg WW}^{-1} \text{ h}^{-1}$ ).

Copepods were anesthetized using magnesium chloride (Svetlichny et al., 2010; Svetlichny and Hubareva, 2014) at a concentration of ( $15 \text{ g l}^{-1}$ ). The same individuals used to calculate total energy metabolic rates were utilized. On average, complete immobilization of *C. helgolandicus* females occurred after 10 min. Immediately after immobilization, copepods were transferred to the experimental syringes containing filtered sea water with a twofold lower concentration of magnesium chloride, enough for copepods to remain immobile during the 2 h exposure period. During incubation, syringes with anesthetized animals were rotated every 10 min to avoid the development of  $\text{O}_2$  gradients within the water volume. After incubation, individuals were gently transferred to fresh seawater where length and width measurements were obtained. Only results of experiments in which the copepods did not mobilize during incubation but only recovered activity post-incubation were analyzed. Individuals subjected to anesthesia were not used in further experiments.

Body wet weight (WW, mg) was calculated as  $\text{WW} = \rho V$  where  $\rho$  is body density, taken to be  $1.05 \text{ mg mm}^{-3}$  and  $V$  is body volume ( $\text{mm}^{-3}$ ) which was calculated according to Svetlichny et al. (2012).

## Behavior Monitoring

Motor activity parameters of copepods (total duration of swimming, frequency of locomotor patterns) were determined by video recording at 60 fps with a Nikon 1V1 camera equipped with a long-focus lens (scale  $\sim 1:4$ ) under inactive artificial lighting. 5–10 individuals were placed in transparent glass aquaria ( $50 \times 30 \times 70 \text{ mm}$ ) with filtered sea water, containing microalgae, or microplastics, or a mixture of both. Several 1 min recordings were repeated within an hour under the given experimental conditions.

Frequency and kinematic parameters of mouth appendage movement were determined using high-speed (1,200 fps) filming with a Nikon 1V1 camera at higher magnification (scale  $\sim 1:1$ ) for shorter periods of time ( $\sim 5 \text{ s}$ ). Illumination aboard the vessel was provided via a narrow-beam 5 W led lamp. At least 10 replications for each individual were performed.

The following parameters of locomotor activity were determined in copepods; (a) feeding on microalgae *ad libitum* before starvation; (b) after 1 day of exposition on microplastics of  $6 \mu\text{m}$  alone and starvation in clean water; (c) after 7 days of exposition on microplastics of  $6 \mu\text{m}$  alone and starvation in clean water; (d) after the addition of microalgae at the end of the 7th day of starvation.

## Statistical Analyses

All data were tested for normality with the Shapiro-Wilk test, homogeneity of variances by Levene's test, and treated using one-way ANOVA. Means were compared by the two-tailed Student's *t*-test ( $p < 0.05$ ) and Duncan's Multiple Range Test (DMRT) using SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, United States). Duncan's Multiple Range test (DMRT) is a *post hoc* test to measure differences between pairs of means (which are shown as a, b, c, bc, d, cd, etc. in

relevant figures below). In this test, whilst same letters mean no significant difference, different letters indicate significant differences among pairs of means. Linear correlation was used to determine the relationship between proportion of the initial consumption rate and day, microplastic consumption rate and microplastic concentration in the water. Correlation coefficients ( $R^2$ ) and significance ( $P < 0.05$ ) were then calculated using regression analysis. Values presented in the figures and tables are means  $\pm$  standard deviations *SD*. All values presented as percentage were arc cosine transformed before performing any statistical test.

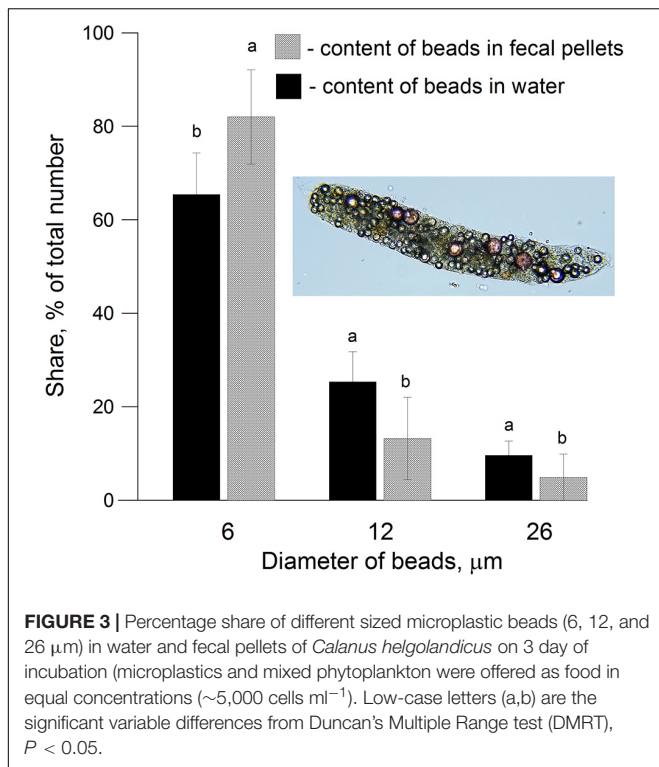
## RESULTS

### Microplastics Bead Consumption and Size Selectivity

In our investigations, almost immediately after the addition of microplastics to the experimental vials containing either natural phytoplankton (dominated by *Skeletonema* sp.) or *Rhodomonas salina* culture, adult females began to actively consume the microplastics, as evidenced by the large numbers of beads in the fecal pellets. No mortality of *Calanus helgolandicus* specimens was noted in all experiments, With the exception of two dead females after 6 days, no other mortalities of *Calanus helgolandicus* specimens were noted for all experiments.

In a 3 day feeding experiment (treatment FEED1), where *C. helgolandicus* specimens were exposed to natural phytoplankton and microplastics (diameters 6, 12, and 26  $\mu\text{m}$ ) in equal concentrations ( $\sim 5,000 \text{ cells/beads ml}^{-1}$ ), the average fecal pellet production rate (FPPR) reached 24 pellets  $\text{female}^{-1} \text{ day}^{-1}$ . Duncan's Multiple Range test (DMRT) showed that relative shares of microplastics of 6  $\mu\text{m}$  ( $82.0 \pm 10.1\%$  of total) were significantly higher (*t*-test,  $df = 45$ ,  $P < 0.05$ ,  $n = 16$ ) in fecal pellets (Figure 3) compared to their relative concentrations in the water ( $65.4 \pm 8.9\%$  of total). For the two other size groups (i.e., 12 and 26  $\mu\text{m}$ ), relative concentrations of microplastics in fecal pellets ( $13.2 \pm 8.7$  and  $4.9 \pm 4.5\%$ , respectively) were lower than those in water ( $25.3 \pm 6.4$  and  $9.5 \pm 3.1\%$ , respectively) indicating preferential feeding on 6  $\mu\text{m}$  sized beads by *C. helgolandicus*.

During 5 day experiments with females fed different diets: *Rhodomonas salina* only, a mixture of *R. salina* and 6  $\mu\text{m}$  microplastics and exclusively 6  $\mu\text{m}$  microplastics (treatments FEED2, FEED3, and FEED4), FPPR varied widely from  $6.0 \pm 2.0$  pellets  $\text{female}^{-1} \text{ day}^{-1}$  at the beginning of treatments FEED2, to  $36.7 \pm 17.6$  pellets  $\text{female}^{-1} \text{ day}^{-1}$  in treatment FEED3 (Table 1). In general, no significant difference was observed in the average FPPR between treatments for the entire experimental period, with the exception of Day 1, when the FPPR in treatments FEED3 and FEED4 proved significantly higher ( $P < 0.05$ ) than in FEED2. However, during these treatments, the number of microplastic beads decreased 5 and 8-fold in treatments FEED3 and FEED4 respectively, while microplastics consumption rates (MBCR) decreased by 3.7-fold during 4 days of exposure in treatment FEED3 and 11.5-fold during 5 days of exposure in treatment FEED4 (Table 1).

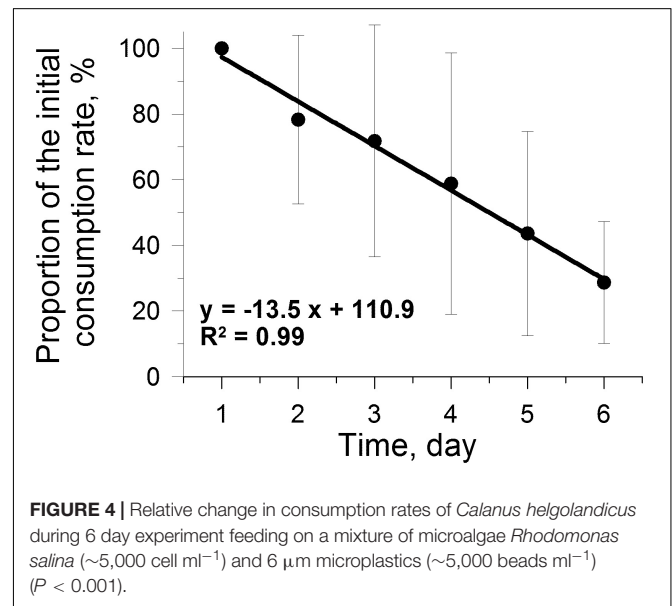


Fecal pellet sizes differed depending on copepod diet (Table 2) but displayed no definite relationship to numbers of beads within. Maximum pellets size (0.58 ± 0.11 length, 0.08 ± 0.01 mm width) was found for copepods feeding on pure microplastics (Treatment FEED4) which was significantly higher than pellets of individuals fed an algal diet (Treatment FEED2 and FEED3) ( $P < 0.05$ ).

In the experiment designed to assess the effect of microplastics on consumption rates in the long-term, the average microplastics consumption rate by individuals, expressed as a percentage of the initial consumption rate, significantly decreased by approximately 3-fold ( $R^2 = 0.9861$ ,  $P < 0.05$ ) over 6 days (Figure 4) of exposure to a mixture of microplastics (6 μm,

**TABLE 2 |** Fecal pellet sizes for *Calanus helgolandicus* females obtained after 24 h from different diet treatments (Mean ± Standard Deviation SD, low-case letters denote significant variable differences between means of different treatments using Duncan's Multiple Range test,  $P < 0.05$ ).

Treatments	Length, mm	Width, mm
FEED2	0.41 ± 0.08 <sup>b</sup>	0.06 ± 0.01 <sup>b</sup>
FEED3	0.45 ± 0.10 <sup>b</sup>	0.06 ± 0.01 <sup>b</sup>
FEED4	0.58 ± 0.11 <sup>a</sup>	0.08 ± 0.01 <sup>a</sup>

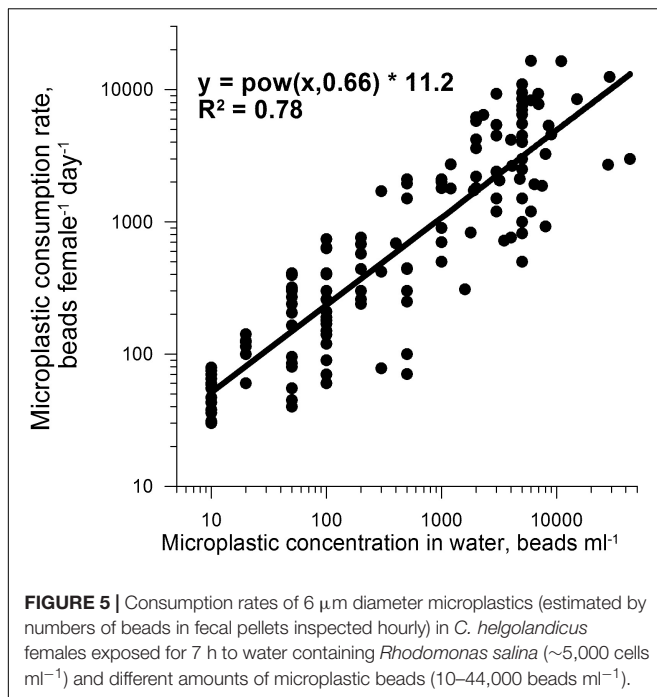


~5,000 beads ml<sup>-1</sup>) and *Rhodomonas salina* algae ~5,000 cells ml<sup>-1</sup> (Treatment FEED5).

During a 7 h experiment (designed to assess short term relationship between consumption rate and microplastics concentrations) in a medium containing *Rhodomonas salina* (5,000 cells ml<sup>-1</sup>) and 6 μm microplastic beads (Treatment FEED6), *C. helgolandicus* females showed a statistically significant increase ( $y = 11.2 \times 0.66$ ;  $R^2 = 0.78$ ,  $P < 0.001$ ,

**TABLE 1 |** Fecal pellet production rates (FPPR, pellets female<sup>-1</sup> day<sup>-1</sup>), microplastic beads numbers in one pellet (BP, beads × 10<sup>3</sup> pellet<sup>-1</sup>) and daily microplastics beads consumption rates (MBCR, beads × 10<sup>3</sup> female<sup>-1</sup> day<sup>-1</sup>) of *Calanus helgolandicus* females in the 5 day experiments with algae *Rhodomonas salina* and microplastics of 6 μm diameter (5,000 cells/beads ml<sup>-1</sup>) (values expressed in Mean ± Standard Deviation SD, low-case letters denote significant variable differences between means using Duncan's Multiple Range test,  $P < 0.05$ ).

Day	Treatments						
	(FEED2) Algae <i>R. salina</i>	(FEED3) Algae <i>R. salina</i> + microplastic beads			(FEED4) Microplastic beads		
	FPPR, pellets female <sup>-1</sup> day <sup>-1</sup>	FPPR, pellets female <sup>-1</sup> day <sup>-1</sup>	BP, beads × 10 <sup>3</sup> pellet <sup>-1</sup>	MBCR, beads × 10 <sup>3</sup> female <sup>-1</sup> day <sup>-1</sup>	FPPR, pellets female <sup>-1</sup> day <sup>-1</sup>	BP, Beads × 10 <sup>3</sup> pellet <sup>-1</sup>	MBCR, beads × 10 <sup>3</sup> female <sup>-1</sup> day <sup>-1</sup>
1	6.0 ± 2.0 <sup>c</sup>	19.5 ± 18.9 <sup>b</sup>	1.9 ± 0.7 <sup>a</sup>	27.9 ± 14.3 <sup>b</sup>	32.5 ± 12.8 <sup>a</sup>	1.6 ± 0.4 <sup>b</sup>	52.1 ± 13.4 <sup>a</sup>
2	10.3 ± 7.2 <sup>b</sup>	9.0 ± 5.6 <sup>b</sup>	2.0 ± 0.8	19.0 ± 7.1 <sup>b</sup>	16.8 ± 1.7 <sup>a</sup>	1.8 ± 0.3	30.4 ± 4.7 <sup>a</sup>
3	14.8 ± 8.5 <sup>b</sup>	10.3 ± 9.2 <sup>c</sup>	0.2 ± 0.007	2.8 ± 0.1 <sup>b</sup>	28.3 ± 14.1 <sup>a</sup>	0.2 ± 0.002	5.1 ± 0.06 <sup>a</sup>
4	30.3 ± 23.9 <sup>a</sup>	36.7 ± 17.6 <sup>a</sup>	0.2 ± 0.006	7.4 ± 0.2 <sup>a</sup>	18.4 ± 5.3 <sup>c</sup>	0.2 ± 0.001	3.4 ± 0.02 <sup>b</sup>
5	21.5 ± 9.3 <sup>c</sup>	31.8 ± 20.4 <sup>a</sup>	0.4 ± 0.3	16.5 ± 11.2 <sup>a</sup>	24.5 ± 14.1 <sup>b</sup>	0.2 ± 0.0005	4.5 ± 0.01 <sup>b</sup>

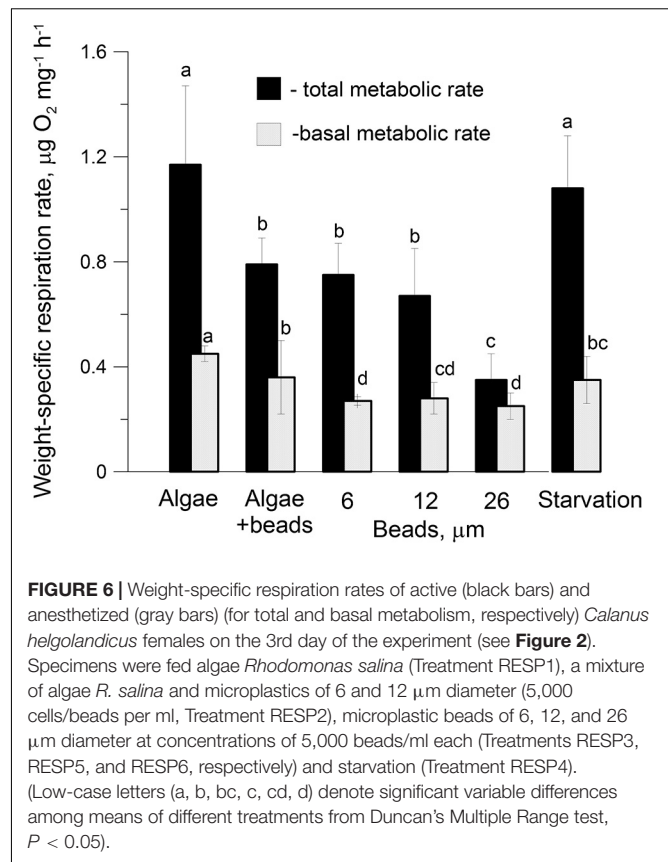


$n = 154$ ) in microplastic consumption rates from  $50.8 \pm 17.3$  to  $8,612 \pm 5,972$  beads  $\text{female}^{-1} \text{day}^{-1}$  as microplastic bead concentrations increased from 10 to 44,000 beads  $\text{ml}^{-1}$  (Figure 5).

## Effects of Microplastic Consumption on Energy Metabolism

Weight-specific respiration rates (WRR) of active and anesthetized females (for total and basal metabolism, respectively) exposed to a mixture of concentrated natural phytoplankton ( $\sim 5,000$  cells  $\text{ml}^{-1}$ ) immediately after selection in the laboratory were  $1.15 \pm 0.09$  and  $0.5 \pm 0.11$   $\mu\text{g O}_2 \text{mg}^{-1} \text{h}^{-1}$ , respectively. Corresponding WRR values obtained were very similar for the experimental group fed 3 days with the algae *Rhodomonas salina* in the same concentration (active females  $1.14 \pm 0.2$  and anesthetized females  $0.45 \pm 0.04$   $\mu\text{g O}_2 \text{mg}^{-1} \text{h}^{-1}$  (Figure 6, Treatment RESP1). Surprisingly, WRR of active females starved during 3 days was only slightly lower ( $1.08 \pm 0.2$   $\mu\text{g O}_2 \text{mg}^{-1} \text{h}^{-1}$ , Figure 6, Treatment RESP4) than those fed with natural phytoplankton or *R. salina*. Duncan's Multiple Range test (DMRT) showed that there was no significant difference between the WRR values of RESP1 (fed with *R. salina*) and RESP4 (starved) for the active metabolism.

However, in active females kept for 3 days in a mixture of algae and microplastics (Treatment RESP2), WRR values were 1.4-fold lower than in females consuming the algae diet only (Figure 6). In females that consumed only microplastics of 6 and 12  $\mu\text{m}$  (Treatments RIII and RV, respectively), in addition to a decrease in the WRR's of active individuals, the WRR's of anesthetized individuals also significantly ( $P < 0.05$  using DMRT) decreased by 1.7-fold. WRR's for both active and anesthetized individuals (for total and basal metabolism, respectively) were especially



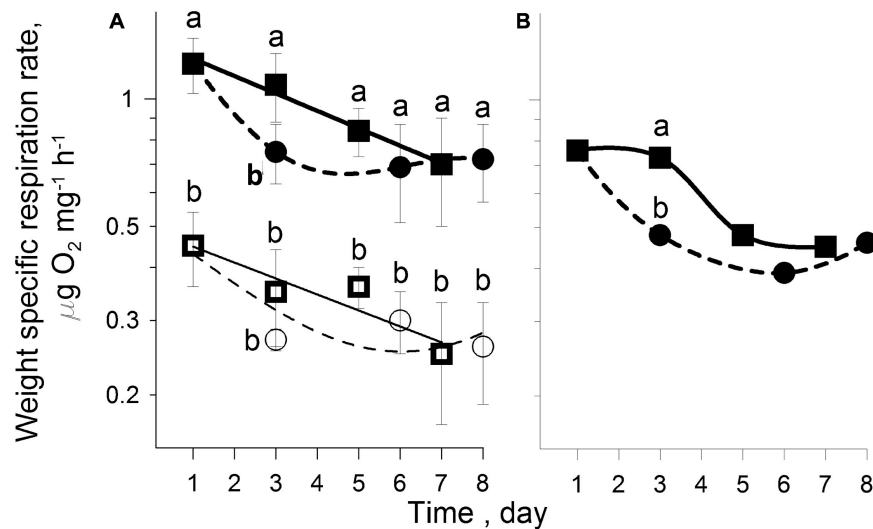
low in females consuming only microplastics beads of 26  $\mu\text{m}$  diameter ( $0.35 \pm 0.1$  and  $0.25 \pm 0.06$   $\mu\text{g O}_2 \text{ind}^{-1} \text{h}^{-1}$ , respectively) (Figure 6).

In an 8 day experiment with females fed a diet of 6  $\mu\text{m}$  microplastics (Treatment RESP3), the WRR of both active ( $1.21 \pm 0.18$   $\mu\text{g O}_2 \text{ind}^{-1} \text{h}^{-1}$ , indicating total metabolism) and anesthetized individuals ( $0.45 \pm 0.09$   $\mu\text{g O}_2 \text{ind}^{-1} \text{h}^{-1}$ , indicating basal metabolism) decreased significantly ( $p < 0.001$ ) by 1.6-fold to minimum levels by the third day, while the decline of the WRR of starving females was gradual over the course of 7 days (Treatment RESP4) (Figure 7A and Table 3). Metabolic scope of activity (SA, Figure 7B), characterizing energy expenditure of motor activity alone and defined as the difference between the WRR's of active and anesthetized individuals, decreased for both treatments from 0.76 to 0.45  $\mu\text{g O}_2 \text{ind}^{-1} \text{h}^{-1}$ , accounting for approximately the same share ( $\sim 63\%$ ) of total metabolic rates of active females. This amounted to approximately 1.8-fold of basal metabolic rates for immobilized individuals in both maintenance regimes.

## Effect of Microplastic Beads on Locomotory Activity

Unfortunately, we did not conduct long-term parallel experiments with algae-eating copepods, given our many old observations of copepod behavior and our control experiment on respiration rate of feeding females. For example, we showed





**FIGURE 7 | (A)** Temporal changes in active (●) and anesthetized (○) WRR of females that consumed 6 µm microplastics and active (■) and anesthetized (□) WRR of starved females (Treatments RESP3 and RESP4); **(B)**: Temporal changes in scope of activity of microplastic ingesting (●) and starved (■) females. [Low-case letters (a,b) denote significant variable differences between each active and anesthetized groups using Duncan's Multiple Range test,  $P < 0.05$ ].

**TABLE 3 |** Total and basal weight specific respiration rates (µg O<sub>2</sub> mg<sup>-1</sup> h<sup>-1</sup>) of active and anesthetized *Calanus helgolandicus* females respectively, exposed to starvation (except Day 1 of experiment) and microplastics [number of replications in parenthesis, Mean ± Standard Deviation SD; low-case letters (a,b,c) denote significant variable differences among each active and anesthetized groups using Duncan's Multiple Range test,  $P < 0.05$ ].

Days	Starvation		Consumption of microplastics	
	Active	Anesthetized	Active	Anesthetized
1	1.21 ± 0.18 (8) <sup>a</sup>	0.45 ± 0.09 (5) <sup>b</sup>	–	–
3	1.08 ± 0.2 (15) <sup>a</sup>	0.35 ± 0.09 (7) <sup>c</sup>	0.75 ± 0.12 (6) <sup>b</sup>	0.27 ± 0.016 (4) <sup>c</sup>
5	0.84 ± 0.11 (12) <sup>a</sup>	0.36 ± 0.04 (6) <sup>b</sup>	–	–
6	–	–	0.69 ± 0.18 (12) <sup>a</sup>	0.3 ± 0.05 (6) <sup>b</sup>
7	0.7 ± 0.2 (8) <sup>a</sup>	0.25 ± 0.08 (4) <sup>b</sup>	–	–
8	–	–	0.72 ± 0.15 (8) <sup>a</sup>	0.26 ± 0.07 (8) <sup>b</sup>

(Svetlichny and Yarkina, 1989) that in females of *C. helgolandicus*, the average daily swimming time is about 50–70%, and the frequency of the mouth appendages, equal to 40–50 Hz at 20°C, can be maintained in laboratory conditions for tens of days and depends only on the temperature of the water. So, despite lack of control group, we could safely assume that copepods feeding with algae would have similar result after 7 days. This is also confirmed by our new inclusion of data (not included in the Ms before) from measurements after the addition of microalgae at the end of 7th days of starvation (see end of section “Behaviour Monitoring” as well as **Figure 8**).

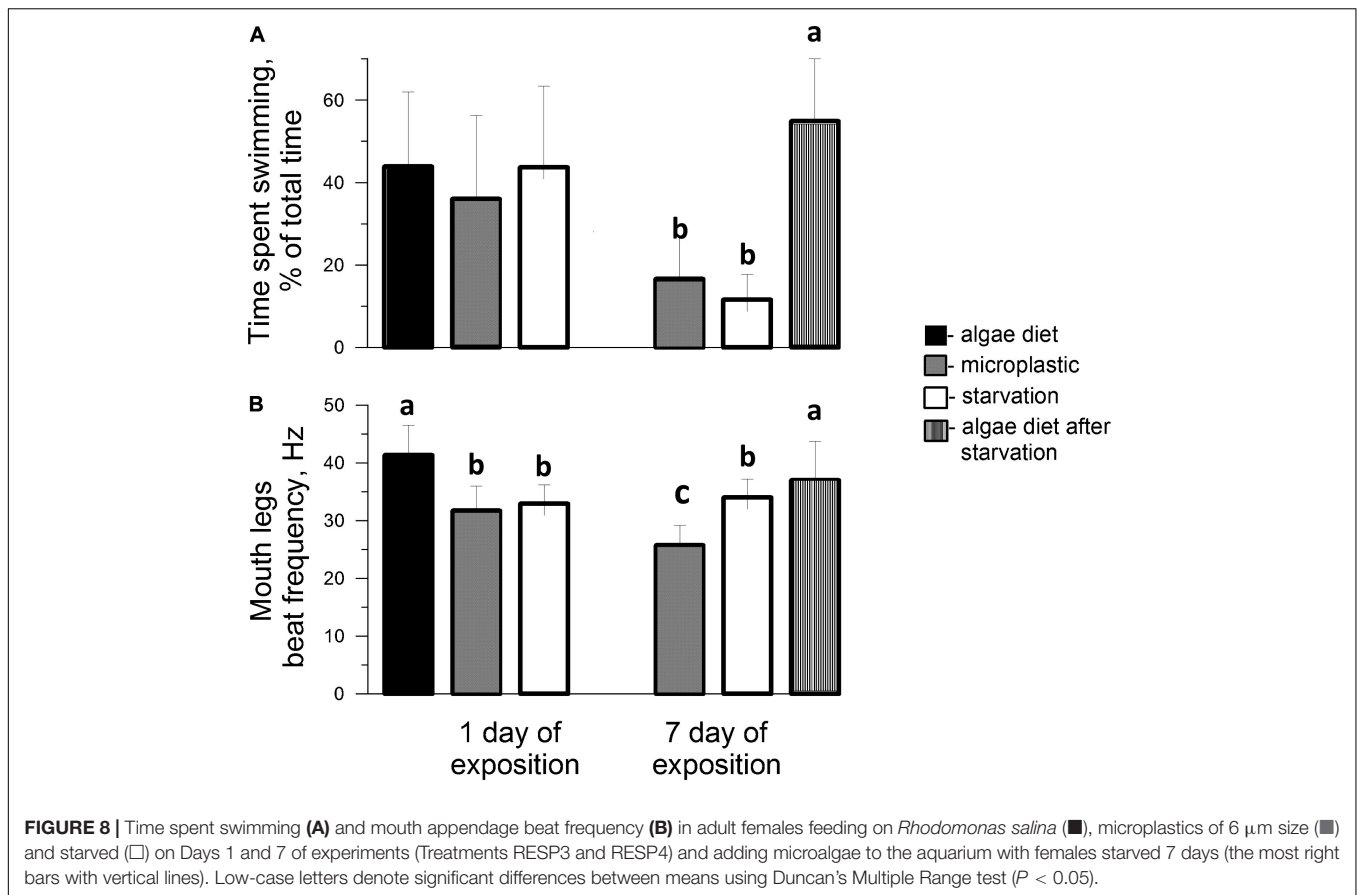
*Calanus helgolandicus* individuals were most active in water containing a natural mixture of planktonic algae or *Rhodomonas salina*. In some observations they were seen to continuously swim and feed in the water column for several seconds, but on average the time spent swimming was  $43.9 \pm 18.1\%$  of total observation time, the frequency of movement of the mouth

appendages was  $41.3 \pm 5.2$  Hz (**Figure 8**). At the end of the first day, the time spent for swimming remained approximately the same for both starved and fed with 6 microplastics of 6 µm groups, however, frequency of locomotion significantly decreased to  $31.7 \pm 4.3$  and  $32.9 \pm 3.3$  Hz ( $P < 0.05$ ) respectively for these groups. On the 7th day of fasting in clean water and water containing microplastics of 6 µm, the females were mainly observed at the bottom of the vessel, producing short intervals of mouth appendages movement. Time spent swimming decreased to 11.6 and 16.6% in starving and microplastic consumed females, respectively, however, the frequency of strokes by mouth limbs significantly decreased to  $25.7 \pm 3.5$  Hz only in females consuming microplastic ( $P < 0.05$ ). After the addition of microalgae to the aquarium, in which the behavior of females starving for 7 days was recorded, the frequency of their locomotion remained approximately the same ( $37.02 \pm 6.7$  Hz), however, the time spent swimming significantly ( $P < 0.05$ ) increased by almost five times up to  $54.9 \pm 15.1\%$ .

## DISCUSSION

### Microplastic Consumption

*Calanus helgolandicus* relates to calanoid copepods that feed on phytoplankton cells due to the water flow generated by their highly bristled mouth appendages (Cannon, 1928). Initially it was assumed that their action is like a primitive filtering device. Boyd (1976) proposed that the second maxillae of *C. helgolandicus*, generally behave as “leaky sieves,” retaining food particles larger than their intersetule distance. Nival and Nival (1976) concluded that individuals of *C. helgolandicus* were capable of consuming particles less than 5 µm in size. However, according to a review by Hansen et al. (1994), the optimal algal diet size for adult calanoid



copepods varies between 14 and 80 μm, while the minimum is 4–9 μm.

Studies, performed using high-speed movies of feeding calanoid copepods, showed that their modes of particle capture involve complex behaviors, quite unlike those attributed to passive filter-feeders. Calanoid copepods can collect, manipulate and reject individual particles (Koehl and Strickler, 1981; Price et al., 1983; Koehl, 1984; Price and Paffenhöfer, 1984; Cowles et al., 1988) and can perceive their motile prey remotely due to chemosensory abilities (Poulet and Marsot, 1978) and via hydromechanical signals using mechanoreception (Kjørboe, 2011; Gonçalves and Kjørboe, 2015).

Although calanoid copepods are able to discriminate between edible and inedible food, their ability to consume non-edible particles of the same size has long been known e.g., organically coated and non-coated polymer spheres (Poulet and Marsot, 1978; Vanderploeg et al., 1990) latex particles (Hansen et al., 1991), suspended silicon carbide powder (Sew et al., 2018), polystyrene spheres (DeMott, 1986, 1988), and various other types of microplastic debris (e.g., Cole et al., 2013, 2015, 2016; Setälä et al., 2014; Coppock et al., 2019). This may be due to the fact that non-food particles entering the water quickly become coated with bacteria (Rummel et al., 2017; Vroom et al., 2017; Rist and Hartmann, 2018) and may mimic the organic detritus they consume, or acquire the “smell” of algae, such as diatoms, which secrete mucus into the water. Specially flavored spheres were

actively consumed by copepods in the experiments of DeMott (1986, 1988).

Total amount of microplastic beads in the deposited pellets indicates the end point of all latent processes that can exert their influence on different time scales. Many previous studies have shown a high correlation between the rate of food intake and the rate of fecal pellet formation in copepods (Corner et al., 1972; Gamble, 1978; Paffenhöfer and Knowles, 1979; Ayukai and Nishizawa, 1986; Tsuda and Nemoto, 1990; Paffenhöfer et al., 1995). Therefore, defecation rate may be used to derive ingestion rate (e.g., Reeve and Walter, 1977; Båmstedt et al., 1999).

In our experiments adult *Calanus helgolandicus* consumed microplastics of sizes 6, 12, and 26 μm presented to them, regardless of the presence or absence of algae in the water. However, they displayed selective preference for the smallest particles of 6 microns. The proportion of 6 μm microplastics in fecal pellets was significantly higher (i.e., 1.25-fold) than in the water, while concentrations of 12 and 26 μm microplastics in pellets were almost half of those in water (Figure 3).

It is possible that since the small phytoplankton of the diatom complex (*Skeletonema costatum*) prevailed in the field in April (both in 2018 and 2019), copepods sampled for the experiment may have been already adapted to feeding on small particles. Indeed, the same diatom species (*Skeletonema costatum*) has been repeatedly used previously in laboratory cultivation of *Calanus helgolandicus* (e.g., Huskin et al., 2000; Rey et al., 2001).

Huntley et al. (1983) showed that closely related copepods of *Calanus pacificus* sp. consume polystyrene beads of similar sizes to diatoms and *Peridinium* algae. These and our findings are consistent with the conclusion of Cole et al. (2015) that *Calanus helgolandicus* were not able to differentiate between microplastic beads and algae of a similar size.

In our experiments, when 6  $\mu\text{m}$  microplastic beads were added to the water, the females immediately began to consume with the first fecal pellets appearing at the bottom of the vessel after 7 min. This corresponds to the maximum gut passage time for calanoid copepods (Mauchline, 1998).

During a 1 day exposure to a mixture of 6  $\mu\text{m}$  microplastics and *R. salina* of similar size (at a constant concentration of 5,000 cells  $\text{ml}^{-1}$ ), the consumption of microplastics by *C. helgolandicus* continuously increased from  $50.8 \pm 17.3$  to  $8,612 \pm 5,972$  beads  $\text{female}^{-1} \text{day}^{-1}$  with the increase in microplastic concentration from 10 to 44,000 beads  $\text{ml}^{-1}$  (Figure 5). The very large variability in number of fecal pellets deposited (average value  $26.3 \pm 13.4$  pellets  $\text{female}^{-1} \text{day}^{-1}$ ) did not enable determination of dependence of pellet production on the concentration of presented microplastics, however, numbers of beads in pellets significantly increased at a concentration range of 10–5,000 beads  $\text{ml}^{-1}$  from  $15.5 \pm 13.1$  to  $1449.9 \pm 867.8$  beads  $\text{pellet}^{-1}$ .

Considering that the volume of one 6  $\mu\text{m}$  microplastic bead is approximately  $113 \mu\text{m}^3$ , and the fecal pellet volume of *C. helgolandicus* females averaged approximately  $2.9 \times 10^6 \mu\text{m}^3$ , the volume fraction of microplastics in pellets, despite their huge abundance, averaged only 5.6%. This is probably why in our experiments no correlation was found between fecal pellet size and microplastics consumption rate. Close correlations of pellets volume were observed in *C. helgolandicus* from the western English Channel both at low microplastic concentrations of 75 beads  $\text{ml}^{-1}$  (Cole et al., 2016) and high concentrations of approx. 1,000 beads  $\text{ml}^{-1}$  (Coppock et al., 2019) up to  $1.7 \times 10^6$  and  $2.2 \times 10^6 \mu\text{m}^3$ , respectively, and was even higher than pellet volumes ( $1.4 \times 10^6 \mu\text{m}^3$ ) of *C. helgolandicus* during bloom conditions in the North Sea (Riser et al., 2003), but less than  $3.26 \times 10^6 \mu\text{m}^3$  observed in the closely related *Calanus pacificus*, which also consumed a natural diet (Uxue and Kaname, 1994). According to a review by Martens (1978), the largest pellets of *C. helgolandicus* can reach 650  $\mu\text{m}$  with a volume of about  $5 \times 10^6 \mu\text{m}^3$ .

In general, both the sizes and the production of pellets in *C. helgolandicus* in our experiments corresponded to the ranges previously known for this species feeding on a natural diet (Mauchlin, 1998; Huskin et al., 2000).

Summarizing the data obtained, it can be stated that, although at the beginning of *C. helgolandicus* exposure to a mixture of algae and microplastics of the 6  $\mu\text{m}$  preferred size, they actively began to consume it in proportion to the concentration in the water, during the next 5 days, the microplastic consumption rates decreased several fold identified as a decrease in the microplastic content of pellets (see Table 3). This was probably a consequence of the reaction of copepods to a high mean concentration of microplastics ( $\sim 5,000$  beads  $\text{ml}^{-1}$ ), since at low concentration ( $\sim 75$  beads  $\text{ml}^{-1}$ ) a similar effect was not observed (Cole et al., 2015).

The feasibility of studying effects of high concentrations of small (several microns in size) microplastics on marine organisms has been debated recently (e.g., Huvet et al., 2016; Lenz et al., 2016) in light of the understandable methodological difficulties in assessing actual concentrations at sea. Although the levels of larger microplastics ( $>100 \mu\text{m}$ –5 mm) from the marine environment are measured for many coastal areas, smaller microplastics of ( $<26 \mu\text{m}$ ) are not known and could be much higher than that larger microplastics. A wide range of experimental concentrations is therefore extremely important for predicting the consequences of the development of negative scenarios of plastic pollution. Small suspended particles originating both from natural and anthropogenic sources are a common occurrence in the marine (especially coastal) environment. For example, high concentrations of suspended sediments occur within the vicinity of dredging and reclamation works (Erftmeijer et al., 2012). Similarly, high levels of larger sized microplastics in the influent and effluent of wastewater treatment plants have been reported (Hidayaturrahman and Lee, 2019). In any case, it should be emphasized that experiments in laboratory conditions incorporating high microplastic concentrations are useful in order to explore the possible impacts at marine ecosystems levels.

## Respiration

*C. helgolandicus* belong to the group of cruising feeder calanoids, who spend most of their total daily energy budget on finding and extracting food particles along their routes (Kjørboe, 2011). This part of the energy budget is called metabolic scope of activity (SA) determined by the difference between the total respiratory rate of active and basal respiratory rate of anesthetized individuals (see Hochachka and Somero, 2002). A significant part of the energy supplied by food is also consumed in the digestion process referred to as specific dynamic action of food (SDA) (Kjørboe et al., 1985; Thor, 2002; Svetlichny and Hubareva, 2005). Together with the costs of maintaining the body (basal energy metabolism, BM), these components add up the total energy metabolism (TM), estimated by the respiration rate of active individuals. It is known that at maximum speed and duration of movement, the respiration rates of planktonic crustaceans can increase 3–7 times (Pavlova and Minkina, 1987; Svetlichny and Umanskaya, 1991; Buskey, 1998; Svetlichny and Hubareva, 2002). Therefore, their energy metabolism should be more sensitive to the motor activity of crustaceans than to the consumption and digestion of food.

In our experiments, weight specific rates (WRR) of active *C. helgolandicus* females well fed by a mixture of phytoplankton algae or starved during 1 day insignificantly varied within 1.14–1.21  $\mu\text{g O}_2 \text{mg}^{-1} \text{h}^{-1}$ , while the WRR of immobilized, anesthetized individuals was 2.3–2.6 times lower (0.45–0.5  $\mu\text{g O}_2 \text{mg}^{-1} \text{h}^{-1}$ ). In females starving for 3 days, the corresponding values decreased to  $1.08 \pm 0.2$  and  $0.35 \pm 0.09 \mu\text{g O}_2 \text{mg}^{-1} \text{h}^{-1}$ , respectively, however, the difference between TM and BM, which characterizes expenditure on motor activity, remained almost the same ( $0.73 \mu\text{g O}_2 \text{mg}^{-1} \text{h}^{-1}$ ), or 63% of TM. Assuming that a significant decrease in BM of starved, anesthetized females compared with active copepods is due to the cessation of digestive activity, i.e., SDA, their value should be about 9% of TM. A close

share in BM, SDA, and SA values of TM was found in females of this species undertaking almost continuous cruising swimming (Svetlichny and Hubareva, 2005).

In our 3 day respiratory experiment, on *C. helgolandicus* females feeding on a mixture of algae and microplastics or microplastics alone, TM, BM, and SA were even lower than for starved individuals. SA decreased especially significantly in individuals who consumed large microplastics of 26  $\mu\text{m}$  (Figure 7).

With longer exposure of females in a suspension of 6  $\mu\text{m}$  microplastics beads and starvation conditions (8 and 7 days, respectively), the levels of TM, BM, and SA decreased by  $\sim 1.8$ -fold compared to the initial levels to the same extent, however, the decrease in energy metabolism of individuals on a plastic diet in the initial days of the experiment was faster (Figure 8). One of the reasons for this, requiring further research of clarification, may be the additional expenditure of energy and loss of biological material by females consuming microplastics on the formation of a peritrophic membrane covering fecal pellets (Gauld, 1957), which consists of chitinous microfibrils and a ground substance containing acid mucopolysaccharides and proteins (Yoshikoshi and Ko, 1988). Indeed, FPPR in starved females (Treatment RESP4, not marked in results) did not exceed 2 pellets female<sup>-1</sup> day<sup>-1</sup>, while in females consuming microplastics it reached  $32.5 \pm 12.8$  pellets female<sup>-1</sup> day<sup>-1</sup> (Table 1). The decrease in energy metabolism during starvation in *C. helgolandicus*, like other species of this genus (Conover, 1962; Ikeda, 1971, 1977; Mayzaud, 1976), is a consequence of physiological adaptation to the experience of prolonged starvation due to the large number of reserve lipids (Lee et al., 1972).

In general, our data indicates that the presence of large concentrations of microplastics in water, even when mixed with algae, reduces the level of total energy metabolism due to a decrease in both basal and active metabolic rates of *C. helgolandicus*.

However, at low microplastic concentrations, this effect is not obvious. Thus, in experiments of Cole et al. (2015), females of *C. helgolandicus*, fed a mixture of phytoplankton and microplastics at a concentration of (75 beads ml<sup>-1</sup>), for almost 9 days at a constant temperature ( $\sim 18^\circ\text{C}$ ) TM levels were almost as high ( $\sim 0.7 \mu\text{L O}_2 \text{ ind}^{-1} \text{ h}^{-1} \approx 0.9 \mu\text{g ind}^{-1} \text{ h}^{-1}$ ) as in our experiments with females consuming a natural diet without the addition of microplastics (see Table 3).

## Behavior

The main types of behavior of copepods of the genus *Calanus* have long been described in the pioneering experimental works of Cannon, 1928; Lowndes, 1935; Hardy and Bainbridge, 1954 and were greatly developed later (e.g., Alcaraz et al., 1980) including some recent works (e.g., Kiørboe, 2011; Chen and Hwang, 2018).

The motor and nutritional activity of *C. helgolandicus* under natural conditions is associated with their daily diurnal vertical migrations, due to which, time spent swimming (T) varies widely from 15–20 to 90–95% depending on day and night time events (Alcaraz et al., 1980; Svetlichny and Yarkina, 1989; Kiørboe, 2011; Chen and Hwang, 2018). During the night-time grazing period, these copepods can maintain nearly continuous mouth

appendage locomotion for several minutes (up to an hour). Nevertheless, the average daily activity at a temperature of about  $20^\circ\text{C}$  is 60–70% (Kiørboe, 2011; Chen and Hwang, 2018). In large aquariums (up to 4 L) time spent undergoing routine locomotion in *C. helgolandicus* increased from 11 a.m. to 1 p.m. and again in the evening (Svetlichny and Umanskaya, 1991).

We examined copepod behavior in the afternoon. Periodic jumps when touching the bottom with furcal bristles and short-term ascent with subsequent descending (“hop and sink”), circular swimming in the water column or intermittent movement at the bottom (resting the head against the vessel wall), represented typical behavior of *C. helgolandicus* females in the specimens we observed in relatively small vessels (approx. 100 ml). In all these cases, the activity of the females was carried out due to a more or less prolonged series of metachronic strokes by the mouth appendages, accompanied by small abdominal strokes. Simultaneously together with the body propulsion, the mouth appendages generated a feeding current due to which food particles were concentrated on the second maxilla in the vicinity of the mouth opening in the form of a small batch, which was then sucked into the mouth opening. This type of feeding is evident by recording the behavior of attached copepods using high-speed video recording (Svetlichny and Hubareva, 2005: our observations). In *C. helgolandicus* females kept under laboratory conditions with sufficient food and water conditioning, the average daily locomotor activity is about 50–70% and the frequency of blows with oral appendages in the range of 40–50 Hz at  $20^\circ\text{C}$  can persist for tens of days and depends only on temperature waters (Svetlichny and Yarkina, 1989). In our present experiment with *C. helgolandicus* females feed algae *ad libitum*, the average time spent swimming and frequency of movement of the mouth appendages ( $43.9 \pm 18.1\%$  of total observation time and  $41.3 \pm 5.2$  Hz, respectively) corresponded to the usual activity level of this species at  $20^\circ\text{C}$  and remained so at the beginning of fasting or kept only in water with microplastics (Figure 8).

However, during the 7 day exposure to microplastics, the parameters of motor/feeding activities, i.e., time spent swimming and mouth appendage beat frequency, decreased 2.6 and 1.6-fold, respectively. Copepods which predominantly grazed at the bottom of the vessel consumed less microplastics, and in general their behavior corresponded to starved individuals (Figure 8). Nevertheless, the mouth appendage beat frequency ( $34 \pm 3.2$  Hz) of starving females was higher than for copepods which consumed microplastics ( $25.7 \pm 3.5$  Hz). This confirms our conclusion, made on the basis of respiration data, that with prolonged exposure to microplastics beads, *C. helgolandicus* display a more rapid adaptive decrease in energy metabolism response than occurs during starvation. The adaptive nature of the decrease in the activity of copepods is confirmed by the fact that after the addition of microalgae, the time spent on swimming/feeding, increased in them according to the principle of hypercompensation (Figure 8).

In conclusion, the trends we have identified for a gradual decrease in the rate of microplastic consumption, energy metabolism, and motor activity could be caused not only by accelerated starvation, but also by the traumatic effects of

microparticles on the copepods intestinal parenchyma, which is more pronounced when larger particles of microplastic are consumed. The likelihood of such an effect of microplastics should be verified in future experiments.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

## AUTHOR CONTRIBUTIONS

MI, AEK, and LS conceived and planned the experiments. MI, LS, TM, KE, ET, OD, and EY carried out the experiments. KE maintained the algal culture. MI, LS, KE, and AEK contributed to the interpretation of the results. LS took the lead in writing the

manuscript followed by extensive revision by AEK to achieve the final version. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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