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Aging of microplastics promotes their ingestion by marine zooplankton[☆]

Renske J.E. Vroom [a, b, *], Albert A. Koelmans [b, c], Ellen Besseling [b, c], Claudia Halsband [a]

[a] Akvaplan-niva, Fram Centre, N-9296 Tromsø, Norway
[b] Aquatic Ecology and Water Quality Management Group, Wageningen University & Research, P.O. Box 47, 6700 AA Wageningen, The Netherlands
[c] Wageningen Marine Research, P.O. Box 68, 1970 AB IJmuiden, The Netherlands

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ABSTRACT

Microplastics (<5 mm) are ubiquitous in the marine environment and are ingested by zooplankton with possible negative effects on survival, feeding, and fecundity. The majority of laboratory studies has used new and pristine microplastics to test their impacts, while aging processes such as weathering and biofouling alter the characteristics of plastic particles in the marine environment. We investigated zooplankton ingestion of polystyrene beads (15 and 30 μm) and fragments (≤30 μm), and tested the hypothesis that microplastics previously exposed to marine conditions (aged) are ingested at higher rates than pristine microplastics. Polystyrene beads were aged by soaking in natural local seawater for three weeks. Three zooplankton taxa ingested microplastics, excluding the copepod *Pseudocalanus* spp., but the proportions of individuals ingesting plastic and the number of particles ingested were taxon and life stage specific and dependent on plastic size. All stages of *Calanus finmarchicus* ingested polystyrene fragments. Aged microbeads were preferred over pristine ones by females of *Acartia longiremis* as well as juvenile copepodites CV and adults of *Calanus finmarchicus*. The preference for aged microplastics may be attributed to the formation of a biofilm. Such a coating, made up of natural microbes, may contain similar prey as the copepods feed on in the water column and secrete chemical exudates that aid chemo-detection and thus increase the attractiveness of the particles as food items. Much of the ingested plastic was, however, egested within a short time period (2—4 h) and the survival of adult *Calanus* females was not affected in an 11-day exposure. Negative effects of microplastics ingestion were thus limited. Our findings emphasize, however, that aging plays an important role in the transformation of microplastics at sea and ingestion by grazers, and should thus be considered in future microplastics ingestion studies and estimates of microplastics transfer into the marine food web.

1. Introduction

Microplastics, <5 mm in size, are omnipresent in marine environments (Andrady, 2011) and due to their minuteness, can be readily bioavailable to a range of aquatic organisms, and marine zooplankton in particular (Desforges et al., 2015). In situ, microplastics have been found inside filter-feeders such as copepods and euphausiids (Desforges et al., 2015), and planktivorous fishes (Boerger et al., 2010; Lusher et al., 2013; Bråte et al., 2016), demonstrating the potential for both direct feeding on microplastics as well as transfer up the food chain and possible biomagnification at the base of the marine food web. As grazers on primary producers (mainly microalgae), zooplankton represent an important link in energy transfer to higher trophic levels, including commercially important fish species. As zooplankton target prey in a size range similar to that of microplastics, it is important to study their ingestion of microplastics and potential adverse effects on the resilience of zooplankton communities. In the laboratory, ingestion of microplastics by copepods had been initially confirmed in early feeding studies of the 1970s and 80s (Ayukai, 1987; Wilson, 1973) and has recently been revisited in the context of plastic pollution for a variety of zooplankton taxa (Cole et al., 2013; Setälä et al., 2014). Whether zooplankton ingest microplastics or not depends on the size and feeding strategy of the organism and its preferred prey size range versus the microplastics size and shape encountered (Cole et al., 2013; Setälä et al., 2014). In the present study, we

focus on the marine copepods *Calanus finmarchicus* and *Acartia longiremis*: planktonic filter feeders between 1 and 4 mm in length, depending on species and stage. Copepods, abundant zooplankton in all world oceans, can ingest microplastics in the micro size range (<1 mm) (Cole et al., 2013; Setälä et al., 2014; Lee et al., 2013), which in turn may impact their health and/or that of higher trophic levels in their food chain. High concentrations of microplastics (several thousand per mL) negatively affected feeding rates of the copepod *Centropages typicus* (Cole et al., 2013) and survival and fecundity of *Tigriopus japonicus* (Lee et al., 2013). At a lower concentration (75 particles mL^{-1}), Cole et al. (2015) found energetic depletion and reduced reproductive output in the copepod *Calanus helgolandicus* feeding on 20 μm polystyrene (PS) beads. They concluded that microplastics competed with food items for ingestion and that the copepods did not select against non-nutritious particles. Food selectivity is, however, a trait that has been described for zooplankton and for calanoid copepods in particular (Leising et al., 2005). The level of selectivity appears to be dependent on both the physiology of the grazer ('hunger') and the abundance and properties of prey items, such as toxicity (Cowles et al., 1988; Kleppel, 1993). Surface characteristics play an essential role in particle detection, as many species are able to discriminate between inert and edible particles (DeMott, 1988a). Most laboratory studies on microplastics ingestion used laboratory grade microbeads, which are in a "pristine" state. This implies a smooth, sterile surface, and suspension in an anti-microbial agent. Microplastics in the marine environment, on the other hand, are exposed to seawater for extended periods of time. Due to this exposure, breakdown of plastic items takes place, creating irregularly shaped microplastics fragments. Consequently, fibres and fragments are found more frequently in the world's oceans than beads (Moore et al., 2001; Lusher et al., 2014; Thompson et al., 2004). Shape may influence microplastic ingestion as well as effects (Lambert et al., 2017): in the amphipod *Gammarus fossarum* fibres significantly reduced assimilation efficiency, whereas this effect was not found using beads (Blarer and Burkhardt-holm, 2016). On top of this, sea water exposure results in microplastic aging, altering particle surface characteristics from smooth and regular to brittle with cracks and rifts (Andrady, 2011). Furthermore, as plastic surfaces are hydrophobic, organic molecules will adsorb (Lobelle and Cunliffe, 2011). Together with the brittle, uneven surface, this creates a matrix to which microbes can attach, a process known as biofouling, or biofilm formation (Zettler et al., 2013). Adsorption of organic molecules occurs rapidly, within hours after microplastics enter the seawater, followed by colonization of microbes (Oberbeckmann et al., 2015). The first discovery of microbe-colonized microplastics in the marine environment dates back to samples collected in the 1970's in the Sargasso Sea (Carpenter and Smith, 1972). More recent findings report biofouled microplastics in a range of marine environments, such as the North Pacific Gyre and the North Atlantic ocean (Zettler et al., 2013; Oberbeckmann et al., 2015; Carson et al., 2013). The biofilms on these plastics contain a variety of organism groups, including diatoms and dinoflagellates (Oberbeckmann et al., 2015) and form diverse communities, which represent a nutritious coating around the - otherwise indigestible − microplastics. Microplastics may thus become attractive food items to grazing zooplankton, such as copepods. Consequently, biofilms may alter the interactions between microplastics and planktonic grazers, increasing ingestion rates of microplastics (Harrison et al., 2011). Many planktonic taxa, including calanoid copepods, possess chemo- and mechanoreceptors for prey detection, which assist in the decision to ingest or reject a given particle (Kleppel, 1993). Suggested mechanisms behind this are chemical cues emitted by particles (Poulet and Marsot, 1978) as well as their surface electrical charge and wettability (Gerritsen and Porter, 1982). The influence

of surface characteristics on microplastics ingestion has been studied for specific artificial coatings, for instance using microbeads inoculated with a marine bacterium (Powell and Berry, 1990), or soaked in algae (DeMott, 1988b). However, whether aging in natural sea water has any influence on microplastics ingestion has not been investigated to date. Lastly, the fate of microplastics post-ingestion remains unclear. While in some species microplastics can be retained in the gut for days (Cole et al., 2013) or translocate within the body if small enough (Browne et al., 2008), in others they are egested within hours (Cole et al., 2013; Kaposi et al., 2014). Data on egestion and accumulation of microplastics in prey organisms are crucial to assess the risks of bioaccumulation and trophic transfer through biomagnification.

Accordingly, we postulate that several factors pertaining to microplastics ingestion require more detailed investigation: 1. the role of particle size and shape, 2. the importance of microplastics aging (due to weathering and biofilm formation), 3. potential differences in the disposition to ingest microplastics between life stages of a given grazer species, and 4. differences in gut passage times between beads and fragments. We exposed zooplankton to PS particles of two different sizes (15 and 30 μm diameter) and shapes (beads and fragments) and studied their ingestion, egestion, accumulation and impact on grazer survival. We further investigated the role of plastic aging for ingestion in *Calanus finmarchicus* and *Acartia longiremis*, two abundant copepod species in the northern Atlantic Ocean, including differences between males, females and juvenile copepodite stages CV.

2. Materials and methods

2.1. Zooplankton collection

Zooplankton samples were taken on board of RV Hyas on May 19th and June 23rd, 2015 in the Norwegian Sea at Håkøybotn, Norway (69° 40′N, 18° 46′E). Vertical hauls were conducted with a plankton net (mesh size 180 μm) at a speed of 0.2 m s^{-1} from a depth of 35−45 m and contained mostly *Acartia longiremis*, *Pseudocalanus* spp. and decapod larvae (indet.). An additional sample was taken on June 26th in a deep water location (>100 m) in Balsfjord, Norway (69° 18′N 19° 12′E) to target *Calanus finmarchicus*. Plankton was kept in the laboratory at ambient temperature in 20 L plastic buckets provided with gentle aeration for up to a week until sorting with a dissecting stereoscope. Individuals of the same species or taxon were placed in new aerated buckets containing filtered seawater (60 μm) for up to three weeks prior to experiments. The plastic material of the buckets used is considered inert, and consumption of microplastics from bucket material due to abrasion is unlikely. No plastic particles other than fluorescently labelled beads used for experimental exposures (see below) have been observed in the experiments. Zooplankton was fed once a week with algae (Reed Mariculture, Shellfish Diet, 1800®) at 5000 cells mL^{-1}. The water in buckets was replaced regularly and dead zooplankton was removed. Only healthy-looking (intact and active) individuals were selected for use in experiments.

2.2. Seawater preparation

Seawater used prior to and during bioassays was supplied by the facility's tap system. Water was pumped from the fjord adjacent to the lab, mixing water from a layer at 60 m depth with surface water. This was subsequently filtered over a 60 μm filter and a 1 μm filter, to remove larger plankton and debris. Filtered seawater used in experiments is 1 μm filtered unless stated otherwise. Temperature of the incoming water was monitored daily and was on average 6.4 °C during our study.

2.3. Microplastics

Green fluorescent PS spheres of 15 and 30 µm diameter were purchased from Phosphorex, Inc. (USA). These were supplied in vials containing 1 mL 1% w:w microplastics suspended in deionized water containing surfactant and 2 mM sodium azide (antimicrobial agent). As this suspension was diluted at multiple orders of magnitude, solvent concentrations were always below 0.04 g L^{-1}. Thus, concentrations of sodium azide were kept far below the LC_{50} of *Daphnia* sp., a freshwater zooplankton (4.2 mg L^{-1}) (Johnson and Finley, 1980). PS contains styrene monomers, which can be moderately toxic to daphnids, but the total concentrations of microplastics used here were far lower than the styrene no effect concentration (NOEC) for daphnids (1.9 mg L^{-1}) (Cushman et al., 1997). Aged microbeads were created by pipetting 15 µm PS beads into a 1 L glass bottle containing filtered seawater (60 µm), which was placed on a rotating plankton wheel in the dark at ambient sea water temperatures (6.4 °C on average) for three weeks to enable biofilm formation (Lobelle and Cunliffe, 2011; Oberbeckmann et al., 2015). Afterwards, bottles were placed on a stirrer and subsequently vortexed to disperse aggregates. Aged microbeads were then filtered out using a vacuum pump and Whatman GF/C filters (1.2 µm pore size). Plastics were resuspended by gently spraying the filters with seawater (Fig. 1A).

Polystyrene fragments were prepared in a way similar to Hämer et al. (2014) (Hämer et al., 2014) by pulverisation of yellow fluorescent PS granules (Magic Pyramid Brücher & Partner KG). This resulted in irregularly shaped plastic fragments, which were suspended in filtered seawater and filtered over a 30 µm mesh. This provided us with a suitable fragment size range <30 µm (Fig. 1B and S1) to investigate the potential for ingestion. Microplastics concentrations were estimated by pipetting a subsample of 1 mL into a Sedgeweck rafter counting cell and subsequently using stereoscope camera software for sizing and quantification (Leica Application Suite (LAS) 4.5), averaging over ten subsequent counts.

We used microplastic concentrations in the range of 50—200 beads/fragments mL^{-1} in order to approach realistic environmental concentrations in laboratory exposures. Microplastics concentrations used in recent laboratory studies tend to be in the range of several thousands of beads per millilitre (e.g. Cole et al., 2013; Lee et al., 2013). However, the highest concentrations found in the natural environment for particles >333 µm are in the order of 0.1 particles mL^{-1} (Norén, 2007), whereas little is known about

in situ concentrations of microplastics within the size range used in our study.

## 2.4. Taxon-specific microplastics ingestion and role of plastic size

Three calanoid copepod species (*Calanus finmarchicus* (n = 18 individuals (ind.)), *Pseudocalanus* spp. (n = 27 ind.), *Acartia longiremis* (n = 27 ind.) and decapod larvae (indet.) (n = 5 ind.) were tested for microplastics ingestion. We applied two plastic treatments: 15 and 30 µm PS beads at concentrations of 224 and 28 particles mL^{-1} (each 0.41 mg L^{-1}), respectively, similar to concentrations used previously in zooplankton exposures (Cole et al., 2015). Prior to the experiment, organisms of each taxon were placed in filtered sea water (1 µm pore size) for 4 h to promote gut clearance. Subsequently, 5—10 individuals were added to a 0.5 L glass bottle containing either 15 or 30 µm PS beads. To ensure constant mixing of water, experimental bottles were placed on a plankton wheel rotating at 1 rpm. After 24 h, the organisms were removed from the bottles using a 120 µm sieve. Survival, the number of individuals containing plastic and the numbers of ingested plastic particles per individual were recorded.

2.5. Ingestion of microplastics fragments

To investigate the ingestion potential of microplastics fragments, females (n = 14 ind.), males (n = 9 ind.) and copepodites stage 5 (CV) (n = 3 ind.) of *C. finmarchicus* were exposed to PS fragments individually on 12-wells plates in 5 mL suspension of approximately 100 fragments mL^{-1} (but fragments <2 µm were below detection limit and thus not included in the counts). Following gut clearance, ingestion was studied after 4 h according to the procedure above.

2.6. Effect of aging on microplastics ingestion

Ingestion of pristine and aged microplastics was studied in *C. finmarchicus* CV (n = 200 ind.) and *A. longiremis* females (n = 100 ind.). We used 15 µm PS beads at concentrations of 100 particles mL^{-1} (0.19 mg L^{-1}) for *C. finmarchicus* and 200 particles mL^{-1} (0.38 mg L^{-1}) for *A. longiremis*. These concentrations accounted for the species-specific difference in plastic ingestion rate, while allowing the enumeration of microplastics in the gut after exposure. Healthy individuals were selected and placed in filtered
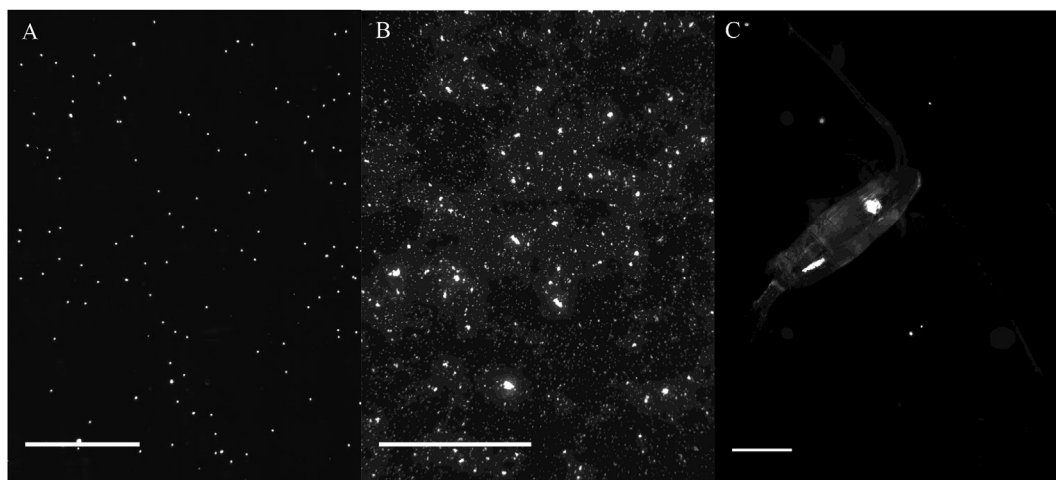
Fig. 1. Photographs of microplastics used in the experiments. A:15 µm microplastic beads, B: microplastic fragments <30 µm and C: a female *C. finmarchicus* with microplastic fragments in the foregut and a microplastics-filled fecal pellet in the hindgut. Scale bar (white line): 1 mm.

seawater 24 h prior to incubation to promote gut clearance. For each treatment 0.5 L glass bottles were used, with ten replicate bottles per treatment for *C. finmarchicus* and five for *A. longiremis*. Ten copepods were added to each bottle. Incubation duration was 4 h for *C. finmarchicus* and 24 h for *A. longiremis*, to account for the higher clearance rates of *C. finmarchicus*. Afterwards, copepods were placed individually in 12-well plates containing 4 mL filtered seawater to separate them from the medium and facilitate visualization. Survival, the occurrence of plastic ingestion and the number of ingested PS beads per individual were recorded.

2.7. Plastic ingestion by different copepod life stages

The above experiment was repeated with adult females of *C. finmarchicus* with the same approach (10 replicates with 10 individuals each, 15 μm PS beads; 4 h exposure), but lowered concentration of microplastics (50 particles mL^{-1}, equivalent to 0.095 mg L^{-1}), as females ingested many more particles than CVs and this impeded their enumeration in the gut. Due to an apparent difference between these two developmental stages, we aimed to add more data on stage-specific ingestion and additionally exposed twenty males and twenty females individually to pristine and aged plastics (15 μm PS beads) in 12-well plates in an incubation of 4 h. The presence and number of ingested PS beads was recorded for each copepod.

2.8. Egestion of microplastics

Gut passage of microplastics was measured for the *C. finmarchicus* CVs from the experiment above (n = 70 ind.), as well as copepods (all stages) that had ingested fragments (n = 17 ind.) upon their transfer to 12-well plates at 7 °C, immediately after assessing plastic ingestion (see above). After two to 4 h, plates were re-examined and the presence of plastic inside copepods was recorded. Faecal pellets sank to the bottom of the wells, preventing re-ingestion of microplastics. Individuals still containing plastics in their guts after 4 h were kept until complete gut depuration.

2.9. Effect of microplastics ingestion on survival

Healthy *C. finmarchicus* females (n = 176 ind.) were selected and placed individually in 12-well plates containing 4 mL filtered seawater. After one week of acclimation, survival of the copepods was examined. Surviving individuals were then randomly assigned to one of the following treatments: 15 μm PS beads at 50 particles mL^{-1} (1) or 500 particles mL^{-1} (2) or a control containing no microplastics (3). Treatments were applied once by replacing 1 mL of water from each well with new filtered seawater containing microplastics, or no microplastics for controls. An algae mixture (Shellfish Diet) was also added to each well at a concentration of 2.8 μg C L^{-1}. Copepods were exposed in the dark at a temperature of approximately 10 °C. Survival was recorded on days 3, 7 and 11. Ingestion of microplastics was assessed simultaneously by examining gut content and faecal pellets.

2.10. Visualization and imaging

A Leica M205FA fluorescence stereoscope equipped with green fluorescence filters (525−550 excitation, 440−470 nm emission) and a DFC3000 G greyscale camera and Leica Application Suite (LAS) 4.5 imaging software were used to visualize copepods and microplastics. Individual microplastics could be counted with high accuracy if present at low numbers (<30). Aggregations of >30 microplastics in suspension or within faecal pellets could not be enumerated.

2.11. Statistical analysis

IBM SPSS Statistics 19 was used for data analysis. For experiments carried out in bottles, means were calculated to account for bottle effects (within-treatment variation). The proportion of copepods ingesting plastics and average numbers of particles ingested were calculated per replicate bottle and differences between treatments were tested using student's *t*-test and ANOVA. For plate-incubations, occurrence of ingestion was compared for separate individuals using a binary logistic General Linear Model (GLM) and numbers ingested using student's *t*-test. Kruskal-Wallis tests were applied to test for differences in survival between treatments. The level of significance applied was α = 0.05 in all cases.

2.12. Method evaluation

In the course of this study, variation in microplastics concentration and incubation methods was introduced, which in some cases hampers the direct comparison between experiments. The reasoning behind this includes practical as well as logistic aspects, which will be elaborated on below. An overview of species and conditions used in all experiments can be found in the SI (table S1).

Concentration adjustments. Depending on copepod species as well as stage, different concentrations of microplastics were used. Although this complicates statistical comparisons, this was required in order to ensure microplastic ingestion by the copepods while simultaneously permitting the counting of individual microplastics in their guts.

Incubation methods. In our experiments, we used two different incubation methods: jointly in bottles (10 copepods per bottle) and individually in wells. In the first place, bottles were used as they could be placed on the rotating plankton wheel, allowing mixing and temperature adjustment. The advantage of using plates, however, was the possibility of decreasing the number of copepods needed, as individuals served as replicates, instead of bottles. Moreover, plates could directly be observed under the microscope, saving time and reducing handling stress to the copepods. Plate incubations were mainly used for pilot experiments which could illustrate and underline our main findings. In the microplastic effect experiment, the main reason for using plates was the reduction of handling stress: removing the copepods from the bottles and re-inserting them several times would severely influence copepod performance as well as cause the loss of individuals. Different results in bottles and plates could be due to microplastic availability: contrasting to bottles, plates were not rotating. Thus, sinking and settling of the PS particles was observed, for pristine as well as aged PS, beads and fragments. Additionally, microplastics encased in faecal pellets sink at an even higher rate than individual particles (Cole et al., 2016), and may therefore result in lower concentrations of available microplastics. Although microplastic resuspension by copepod movement was observed as well, microplastic availability in wells could have been lower than in bottles. However, in the microplastic effect study, we observed only a very slight decrease in the number of copepods with plastics in their guts over the study period, which counters a steep decline in microplastic availability over time using this method.

3. Results

3.1. Taxon-specific microplastics ingestion and role of plastic size

Ingestion of PS beads during a 24 h exposure was dependent on taxon as well as plastic size (Fig. 2). In *Acartia longiremis*, one adult female (n = 9 ind.) ingested one plastic bead of 15 μm, while none
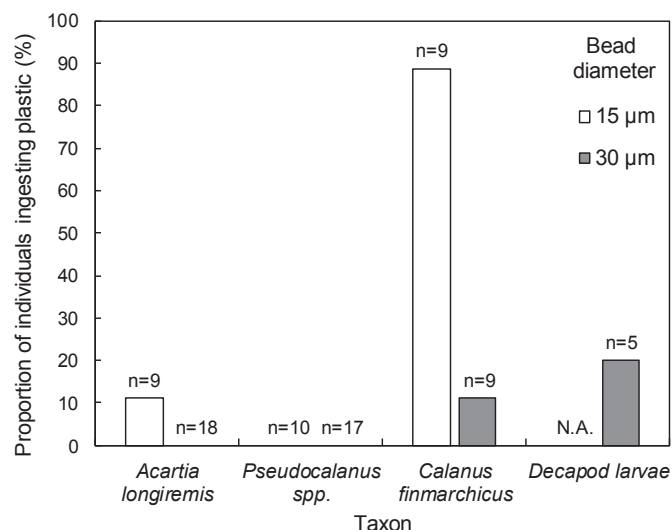
Fig. 2. Ingestion of polystyrene beads by different zooplankton taxa. Proportion of individuals feeding on 15 μm (224 mL^{-1}) and 30 μm (28 mL^{-1}) particles at a concentration of 0.42 mg L^{-1} after 24 h of incubation (n indicates the total number of individuals). Taxa on the x-axis are sorted from left to right by increasing body length.

ingested the 30 μm beads. *Pseudocalanus* spp. did not ingest beads of either size in the same exposure period. Of all taxa, *C. finmarchicus* CV ingested the most beads: 89% (n = 9 ind.) ingested 15 μm beads, ranging from two to four beads per individual. One *C. finmarchicus* (n = 9 ind.) ingested one 30 μm bead. Because of limited availability of individuals in the samples, decapod larvae (n = 5 ind.) were exposed to 30 μm beads only, where one individual ingested three beads.

3.2. Ingestion of microplastics fragments

All studied stages of *C. finmarchicus* ingested PS fragments (67% of CVs (n = 3 ind.), 71% of females (n = 14 ind.) and 56% of males (n = 9 ind.)) (Fig. 3B). The number of ingested fragments per copepod could not be counted, as the ingested particles were mostly very small and formed aggregates in the gut, making them

indistinguishable. When ingested, fragment aggregates filled 30–90% of the gut as estimated by visual observations, accumulating either in the front or the hind gut, or both (Fig. 1C).

3.3. Influence of aging on microplastics ingestion

Pristine as well as aged beads were ingested by both *C. finmarchicus* and *A. longiremis*. Aged microbeads were ingested by significantly higher proportions of copepods than pristine beads (Figs. 3A and 4) in *C. finmarchicus* CV (P = 0.007, student's *t*-test), females and males (P = 0.006; binary logistic GLM); as well as in *A. longiremis* females (P = 0.026; student's *t*-test). An exception was
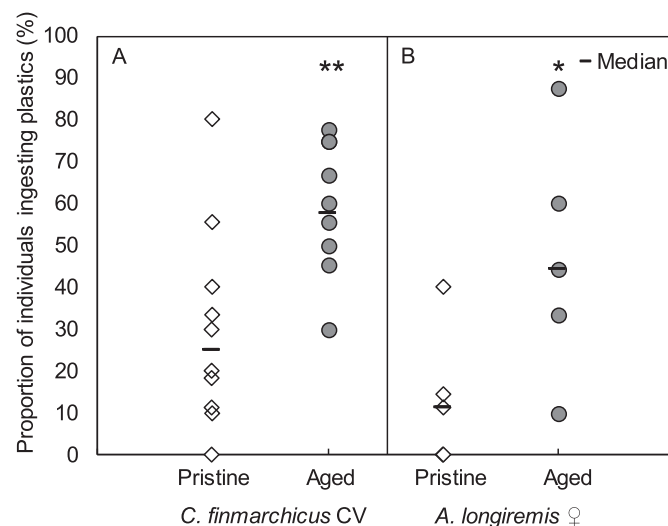
Fig. 4. Species-specific ingestion of pristine and aged plastic particles (15 μm diameter). Symbols represent values per replicate bottle, horizontal bars show median values. A: proportion of *C. finmarchicus* with PS beads in their gut after 4 h (10 bottles, 10 individuals per bottle; 100 particles mL^{-1}; 0.19 mg L^{-1}). B: proportion of *A. longiremis* with particles in their gut after 24 h (5 bottles, 10 individuals per bottle; 200 particles mL^{-1}; 0.38 mg L^{-1}). Symbols can overlap. Significant difference (increase of the fraction of copepods ingesting plastics) between pristine and aged beads are indicated by * (P < 0.05) or ** (P < 0.01). NB: results for copepodites CV are the same as presented in Fig. 3.
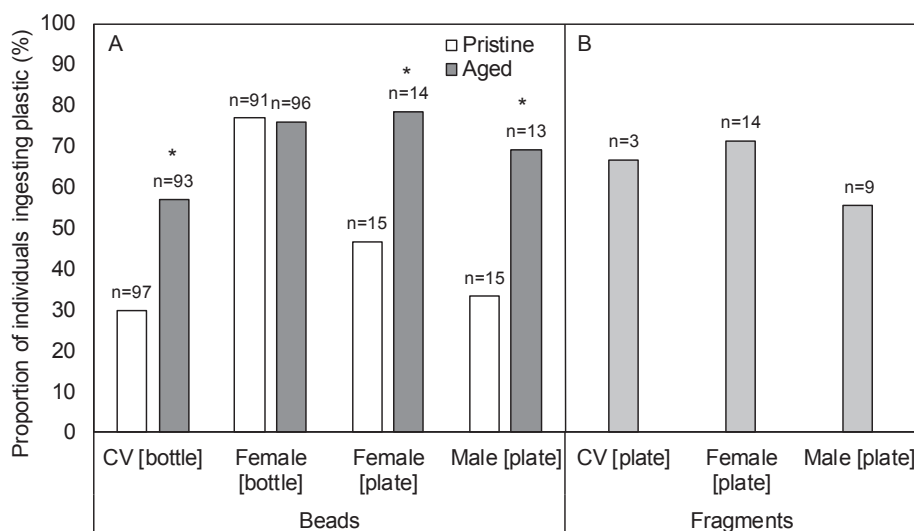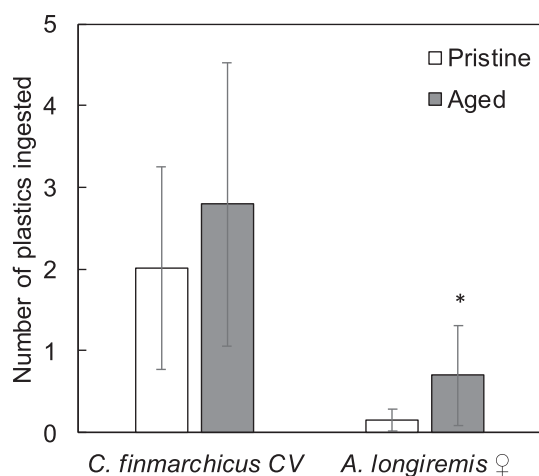

Fig. 3. Ingestion of microplastics by different life stages of *C. finmarchicus*. A: 15 μm polystyrene beads, pristine and aged, after 4 h of incubation (100 beads mL^{-1} for copepodites CV, 50 beads mL^{-1} for females and males). B: Ingestion of polystyrene fragments after 4 h of incubation (approximately 100 fragments mL^{-1}. Bottle or plate refer to the incubation method used. Significant increases in the fraction of individuals ingesting aged beads compared to pristine are indicated by * (P < 0.01).

observed in *C. finmarchicus* females incubated in bottles (n = 187 ind., 10 bottles per treatment), with only a subtle difference between pristine and aged particles (76 and 77%, respectively: P = 0.651; binary logistic GLM). The number of aged beads ingested was also higher than the number of pristine beads in both species (Fig. 5), but the difference was only statistically significant for *A. longiremis* females (P = 0.032), and *C. finmarchicus* males and females (7.4 aged particles versus 1.2 pristine particles, P < 0.0005; Mann-Whitney U).

3.4. Plastic ingestion by different copepod life stages

Plastic ingestion was studied for three different life stages of *C. finmarchicus*: juvenile CV, adult males and adult females (Fig. 3A). Females incubated in bottles ingested microplastics more frequently than CVs (P < 0.0005; ANOVA): only 30 and 56% of
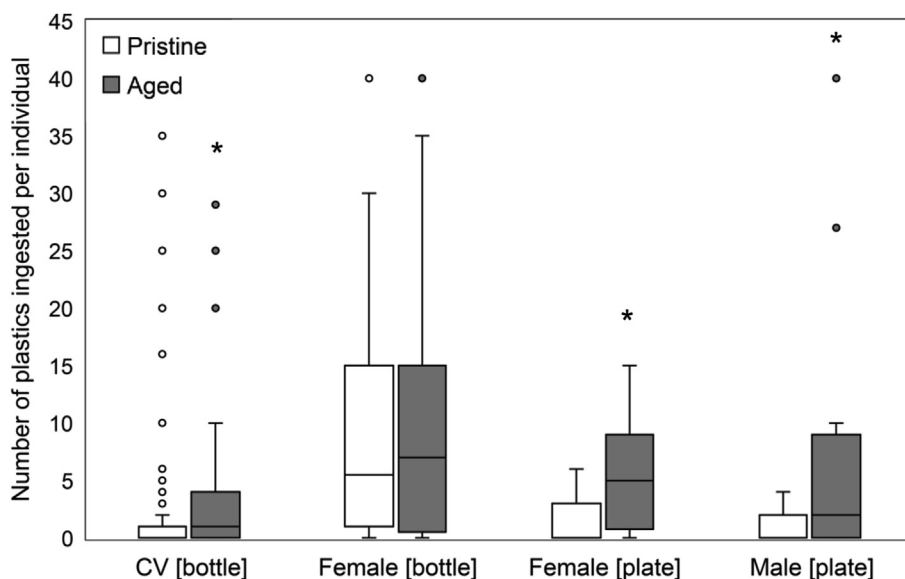
CVs, but 77 and 76% of females ingested pristine and aged beads, respectively, despite a two-fold higher concentration of microplastics in incubations with CVs (and thus higher chance of encounter). The number of beads ingested was not compared statistically between these stages as females ingested too many beads to enumerate them in the gut with the required precision. Sex-specific differences were, in contrast, not found: neither in proportion ingesting (P = 0.368; binary logistic GLM) nor in the number of microplastics ingested (P = 0.304; Mann Whitney U) (Fig. 6). The proportion of individuals ingesting fragments did not differ significantly between stages or sexes (P = 0.739; binary logistic GLM) in contrast to microbeads.

### 3.5. Microplastics egestion

The majority of copepods (94.3%) of *C. finmarchicus* CVs that had ingested plastics egested these within two to 4 h, while four individuals (5.7%) egested them overnight, i.e. within 18 h. No difference in gut passage time was observed between beads and fragments, or between copepod species.

3.6. Effect of microplastics on survival

During the short-term ($\leq$24 h) bottle incubations described above, a small number of individuals died (1.7% of *C. finmarchicus* CV (n = 191 ind.), 2.4% of *C. finmarchicus* females (n = 187 ind.), 6% of *A. longiremis* (n = 91 ind.)), but none of the experiments showed significant differences in survival between treatments and controls without plastics (Kruskal-Wallis, P > 0.05). No copepods died during short-term plate incubations, due to lower handling stress. In the long-term 11-day exposure experiment, microplastics did not affect survival of *C. finmarchicus* females (Fig. 7). During acclimation without plastics, 18 copepods died over 7 days, resulting in a mortality rate of 2.6 day^{-1} (n = 157 ind.). During exposure, 23 of 157 copepods died in total, resulting in a mortality rate of 0.64 day^{-1} in the control, 0.78 day^{-1} in the low plastic concentration and 0.78 day^{-1} in the high concentration treatment (n = 53 ind. per treatment). In the low concentration treatment, 88.7%, 85.7% and 81.3% of all copepods incubated had plastics in their guts on days 3,

Fig. 5. Average number of pristine and aged particles (15 μm diameter) ingested per copepod. *C. finmarchicus* CVs after 4 h of incubation (100 particles mL^{-1}; 0.19 mg L^{-1}). *A. longiremis*, after 24 h (200 particles mL^{-1}; 0.38 mg L^{-1}). Error bars represent 95% CI, * refers to a significantly higher number of aged beads ingested than pristine beads (P < 0.05).

Fig. 6. The number of 15 μm pristine and aged PS beads ingested by *C. finmarchicus* in different developmental stages (100 particles mL^{-1} for copepodites CV, 50 particles mL^{-1} for females; 0.095 mg L^{-1}). Bottle and plate refer to incubation method (see text for details). Numbers >30 particles (females incubated in bottles) are estimates, dots show outliers. Significant increases in the number of aged beads ingested compared to pristine beads are indicated by * (P < 0.001). NB: results for copepodites CV are the same as in Fig. 4.
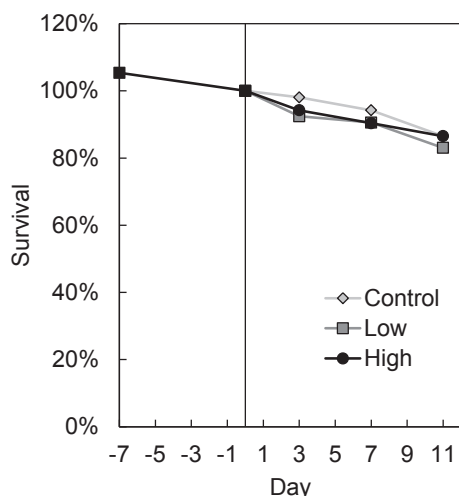
Fig. 7. Survival of female *C. finmarchicus* exposed to low (50 particles mL^{-1}), high (500 particles mL^{-1}) or no microplastics (control) over a period of 11 days. Acclimatization took place on day −7 to 0, after which plastics were added, as indicated by the vertical line.

7 and 11 respectively. For the high concentration, this was 88.5%, 87.8% and 87.2%.

4. Discussion

Our study confirmed the ingestion of polystyrene microplastics by different zooplankton species. The frequency and extent of ingestion were dependent on taxon, size and stage of the grazers, as well as the properties (aged or pristine, beads or fragments) of the microplastics. The impacts on copepod fitness and survival were limited, demonstrated by a high degree of microplastics egestion and low mortality, while food web implications may be more complex.

4.1. Species-specific ingestion

Both copepods and decapod larvae ingested microplastics, but the decapods ingested the larger ones (30 μm), while *Acartia longiremis* ingested only the smaller 15 μm particles. *Calanus finmarchicus* ingested both sizes, but was more prone to ingest the smaller particles. Differences in microplastics ingestion between taxa may be explained by differences in body size: adult *A. longiremis* were 0.9–1.3 mm in length and ingested less microplastics than *C. finmarchicus* adults and decapod larvae of 2–4 mm in length. Small copepods generally feed on smaller particles and at lower rates than large copepods (Wirtz, 2012), reflecting a smaller volume of water and therefore a lower number of particles passing over the feeding appendices per unit of time. Variation between species of similar size can be explained by differences in physiological status (e.g. hunger) and feeding rates. Low ingestion rates may be attributed to handling stress, but can also be a means of energy conservation when food concentrations are low. Furthermore, selective feeding may take place through the rejection or regurgitation of undesired particles (Ayukai, 1987; Leising et al., 2005; Donaghay and Small, 1979). Cole et al. (2013) studied several calanoid copepod species and all of them ingested beads, albeit with differences across stages and bead sizes. In their study, *Acartia clausi* females did not, or only partially ingest large beads >20 μm, but they did ingest the smallest ones of 7 μm. *A. longiremis* only ingested 15 μm beads in the present study, whereas ingestion of PS beads up to 59 μm has been observed in the congeneric species *A. tonsa* and *A. clausi* (Wilson, 1973; Cole et al., 2013). Microbead concentrations used in those studies were, however,

several orders of magnitude higher than those used here, resulting in elevated encounter rates. Surprisingly, one copepod species (*Pseudocalanus* spp.) did not ingest any microplastics at all. A complete lack of uptake of both 30 and 15 μm particles may reflect an enhanced ability to reject particles of low food quality: *Pseudocalanus* sp. feeding on a mixture of equally sized (12 μm) algae and PS beads ingested many more algal cells than beads, showing a high degree of selectivity compared to other copepods (DeMott, 1988a). If aging of the microplastics would have induced ingestion remains to be confirmed, since we offered only pristine beads, without a biofilm, to *Pseudocalanus*. Regarding decapod larvae, we only studied a small number, which limits our results. However, as one individual ingested microplastics, we can conclude that decapod larvae have the potential to ingest, and, therefore, can be affected by microplastics. Microplastic ingestion by decapods was found previously by Cole et al. (2013) when several decapod taxa ingested microplastics of 20.6 or 30.6 μm. Further studies are needed to investigate their feeding behaviour on microplastics (and whether particle characteristics such as size and shape play a role), as well as potential adverse effects.

Throughout this study, the number of individuals taking up microplastics as well as the number of particles ingested per individual was highly variable. High individual variability in feeding rates is common in copepods (Paffenhöfer, 1994) and has been found in a similar ingestion study with microbeads, where the copepod *A. tonsa* ingested between 0 and 90 beads within 15 min (Wilson, 1973). A reason for this variation could be heterogeneity in the physiological state of the copepods, as plankton collected in the field diverge in aspects such as body mass, genetic makeup and feeding history. Additionally, the composition of biofilm communities on the microplastics could vary resulting in differential attractiveness as food items.

4.2. The role of microplastic shape (beads and fragments)

Ingestion of plastic fragments and complete egestion in faecal pellets by *C. finmarchicus* was observed, although very small particles were not visible with the imaging we applied and might have remained in the gut or translocated to other tissues (Browne et al., 2008). We did not observe any difference in the gut passage time between beads and fragments, although the data on fragments were insufficient to compare the two shapes statistically. No physical harm to the copepods due to particle shape was observed. Potential microscopic damage to the gut epithelia, however, was not investigated and requires further studies at the tissue level. As ingestion of microplastics may be shape-dependent, a dose-response study investigating differences in ingestion of beads and fragments as a function of their concentrations is recommended.

4.3. The role of aging

We show that aged microplastics are ingested by more individuals and at higher rates than pristine plastics in two copepod species. This is important, as the use of pristine microplastics in the laboratory may lead to an underestimation of microplastic ingestion in the field, while accurate estimates are required for the parameterization of models (Koelmans et al., 2016). Both the proportion of individuals ingesting aged plastics and, in most cases, the number of aged particles consumed were consistently higher in all life stages and across species, with the exception of *C. finmarchicus* females incubated in bottles, where the difference was not statistically significant. A reason for this exception may be that females feed more indiscriminately in order to obtain and store energy for reproduction (Leising et al., 2005). This is refuted, however, as we observed a preference for aged beads by females in wells, and with

even lower microplastic concentration. Thus, the incubation method might have had an influence on these results, potentially due to lower microplastic availability in wells through sinking.

Both species studied (*C. finmarchicus* and *A. longiremis*) showed a preference for aged microbeads. A number of copepod species have the ability to distinguish between high and low quality food items, and choose natural over artificial particles (e.g. Harrison et al., 2011; Cole et al., 2016). Biofilms have been shown to grow on pristine plastic particles in seawater within hours (Zettler et al., 2013; Oberbeckmann et al., 2015). A preference for aged microplastics can therefore likely be attributed to biofouling. Biofilms can exude chemical cues and change the surface characteristics of microplastics (Lobelle and Cunliffe, 2011). Therefore, a biofilm may disguise the inert nature of plastic particles, thus creating particles more similar to food items than pristine plastics. Uptake of 'flavoured' and coated microbeads has been investigated previously: for instance, calanoid copepods *Eudiaptomus* spp. more readily ingested plastic beads soaked in algae solution than pristine ones (DeMott, 1988a); microbeads inoculated with a marine bacterium were ingested, passed through the gut and were egested in faecal pellets by *Eurytemora affinis*, whereas sterile beads were ingested but later partially regurgitated by the copepods (Poulet and Marsot, 1978). Recently, it has been speculated that dimethyl sulphite (DMS) exuded by biofilms on plastic debris may serve as an olfactory cue for sea birds and stimulates their foraging on plastics (Savoca et al., 2016). The same mechanisms may make microplastics attractive food items for grazing zooplankton. Another particle characteristic that changes as particles age is density. Particles that acquire a biofilm become denser and may therefore sink at an increased rate (Kooi et al., 2017). In the experiments carried out in plates, increased sinking of aged particles and, therefore, lower availability in the water phase may have influenced the results. In our bottle experiments, however, microplastics are constantly resuspended through the rotating motion. Yet we see an increased ingestion of aged particles in both bottle and plate incubations. This observation confirms that other mechanisms, such as the abovementioned 'flavour', must play a significant role.

4.4. Stage-specific differences

Another important finding is a stage-specific difference in the proportion of ingesting individuals and the number of particles ingested. All stages of *C. finmarchicus* studied here (CV, males, females) showed a preference for aged over pristine beads: adults ingested microplastics more frequently and at higher numbers than CVs, while both sexes ingested plastics at similar rates. This may be attributed to grazing rates, which increase as copepods develop, augmenting in body size and carbon content (Meyer et al., 2002). For instance, the congener *C. helgolandicus* feeding on algal cells increases its ingestion rate with developmental stage and body weight (Paffenhöfer, 1971). Although some of our results come from different incubation techniques and therefore need to be treated with caution, such differences need to be taken into consideration when quantifying microplastics ingestion for whole populations and subsequent estimates of biomagnification, and thus warrant further investigation across the total of 12 stages. In contrast to PS beads, no stage-specific differences were observed for PS fragments. This might be due higher fragment concentrations: 100 fragments mL^{-1} only included fragments >2 μm in diameter. Moreover, fragments were offered in a size range, whereas beads included only one specific size. Copepods are able to discriminate between particles of different sizes and avoid the ingestion of a specific size (Wilson, 1973). Thus, copepods may not have been able to select against PS fragments, therefore ingesting them at higher proportions than beads in the case of copodites CV.

4.5. Egestion

When *C. finmarchicus* ingested 15 μm beads, they were subsequently passed through the gut by peristaltic movements, compiled in the hindgut and excreted within a few hours. These observations are consistent with previous studies on other copepod species (Cole et al., 2013; Setälä et al., 2014) as well as planktonic larvae (Kaposi et al., 2014). Microplastics retention times were comparable to gut residence times of natural food in *Calanus* sp. in the laboratory as well as in situ (Simard et al., 1985; Tande and Båmstedt, 1985). Accumulation of microplastics in faecal pellets can have consequences for their fate in the water column: faeces packed with low-density microplastics have decreased density and sinking velocity and are more likely to disintegrate (Cole et al., 2016). This increases their retention time in upper water layers, along with their bioavailability to, for instance, coprophagic zooplankton species.

4.6. Mortality

In our study, microplastics did not impact survival during short and long term exposure, although microplastics were extensively ingested. In bottle incubations, more copepods died than in plates, probably due to higher handling stress. In a longer term (11-days) exposure, microplastics had no effect on the survival of *C. finmarchicus* females. A small number of dead copepods observed at the end of the experiment looked peculiar, with parts of the guts extruding from the bodies, but it was unclear if this was caused by the microplastics or by general deterioration of the remains. Our results agree with Cole et al. (2015). who found no impact on survival of *Calanus helgolandicus* exposed to 20 μm PS beads at 75 particles mL^{-1} in a 9-day experiment.

4.7. Food web implications

Although microplastics did not directly impact individual performance of copepods in our study, microplastic ingestion can possibly lead to sub-lethal effects which can have implications on food web dynamics. Although microplastics were not observed to cause physical damage, ingestion of microplastics might negatively influence feeding of zooplankton on algae cells, through satiation (Wright et al., 2013), or by causing a switch in feeding behaviour in an attempt to avoid plastic ingestion (Cole et al., 2015). Via this mode of action, food assimilation and thus energy for e.g. growth and reproduction could be reduced (Lee et al., 2013; Cole et al., 2015). As zooplankton are at the base of the marine food-web, they form an important source of energy for invertebrates and planktivorous fish. Possible impacts of microplastics on zooplankton fitness and/or population size can therefore propagate up the food chain and affect higher trophic organisms.

Despite the fact that microplastics were quickly egested by copepods in our study and accumulation was not observed, microplastics might accumulate in zooplankton consumers. Trophic transfer of microplastics from copepods to mysid shrimps has been confirmed (Setälä et al., 2014), but whether the microplastics accumulate in higher trophic levels or are egested has not yet been studied.

4.8. Conclusion & recommendations

Our findings show that aging and weathering of microplastics are important factors to take into account in laboratory studies investigating the dynamics and effects of plastic ingestion by marine biota. For the parameterization of ecosystem models aiming to project population and ecosystem impacts of microplastics, realistic estimates of their ingestion rates are essential (Koelmans et al.,

2016). The use of pristine microplastics in laboratory experiments could result in underestimations of microplastics ingestion rates in the field, where presumably most plastic particles are colonized by biofilms shortly after they enter the aquatic environment. Next to ingestion rates, ecotoxicological effects can depend on physico-chemical properties of the microplastics such as surface characteristics and shape (Lambert et al., 2017). We therefore recommend the use of aged microplastics particles in future assessments of microplastics ingestion. Soaking the microplastics for three weeks in natural seawater prior to exposure to the organism of interest should grant enough time for a biofilm to form. However, this should be confirmed by for instance scanning electron microscopy (SEM) (Zettler et al., 2013). On top of this, studying biofilm development and composition will provide insights into the mechanisms that drive grazing responses of zooplankton. Furthermore, potential adverse effects of biofouled microplastics should be assessed at the organism as well as the food web level, including the potential for trophic transfer. Long term exposures over several generations are needed to study the effect on population fitness, whereas feeding experiments of copepods to planktivores could reveal potential biomagnification. Besides altering uptake, aging of microplastics can influence microplastic behaviour in the water column and subsequent bioavailability, e.g. through increased sinking rates (Kooi et al., 2017; Kowalski et al., 2016) and a high degree of aggregation as observed in this study. A better understanding of the transformations, vertical distribution and transport of (aged) microplastics in marine environments is necessary in order to create accurately parameterized models to assess the risks of microplastics in the marine environment.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.08.088.

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