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Fiber ingestion by the bivalve *Perna perna* (Linnaeus, 1758): the importance of using concentrations, sizes, and thicknesses similar to the environment

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ABSTRACT

The ingestion of microplastics (particles smaller than 5 mm) by bivalves in the environment is already well established, but more laboratory studies are still needed to enable the assessment of the impacts of these particles in these organisms. In this sense, this study aimed to verify the ingestion of microplastics by bivalves in different experimental environments. For this, 60 specimens of the mussel *Perna perna* (Linnaeus, 1758) were exposed to different concentrations (25, 50, and 100 MPs.L^{-1}), sizes (1 to 2; 2.5 to 3.5; and ≥ 5 mm), and thicknesses (thick: 0.2 mm, polyethylene; and thin: 0.03 mm, polyester) during 24 h. The results showed that organisms prefer thin particles, and it was found that only one thick fiber was retained, while 23 thin particles were ingested. On the other hand, no significant differences in the uptake of fibers at different concentrations and sizes were identified, showing low concentrations of particles regardless of the treatment. We concluded, via this study, that mussels can ingest fibers of different sizes, thicknesses, and concentrations in the experimental environment, even over a short period of time (24 h). Moreover, it was observed that thickness probably was the main factor for fiber ingestion by mussels, with the thinner particles being preferentially ingested. Therefore, we suggest that new physiological studies employ fiber thickness as a preponderant factor for the ingestion of microplastics in bivalves and also that they choose sizes and concentrations similar to those found in the environment, enabling more robust and accurate results.

Keywords: Environmental Relevancy, Exposure, Microplastics, Mussels

Microplastics (<5 mm) are currently a ubiquitous issue worldwide, being identified in the most remote locations on the planet and in hundreds of species, including humans (Mishra et al., 2021; Thornton Hampton et al., 2022). Due to this growing degree of contamination over the world, microplastics have been widely studied over the last fifteen years (Mutuku et al., 2024). Studies have assessed their concentrations in aquatic, terrestrial, and atmospheric environments, as well as in biological compartments (Akanyange et al., 2022).

Among the latter, aquatic animals are the most studied, with evidence that microplastics adhered to phytoplankton and were then ingested by the most distinct groups of vertebrates and invertebrates, such as zooplankton, fish, turtles, mollusks, and marine mammals (Zantis et al., 2021; Nanthini devi et al., 2022). In addition to *in situ* studies, researchers have also been developing laboratory

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experiments under controlled conditions with these organisms to assess the ingested concentrations and the possible damage caused by this ingestion (Thomas et al., 2021).

These studies have evaluated three important stages of microplastic behavior: (1) bioavailability, which refers to the availability of particles in the medium and their capacity to adsorb or cross the biological membrane of an organism (Bradney et al., 2019); (2) bioaccumulation, that is, the net absorption of a contaminant by an organism (Miller et al., 2020); and (3) biomagnification, which shows the subsequent transfer of a contaminant through the food chain (Miller et al., 2020).

In general terms, these processes can cause serious issues in organisms, such as oxidative stress, physical effects, neurotoxicity, mortality, and problems in reproduction (Palmer and Herat, 2021; Li et al., 2022). The transfer of microplastics through the trophic chain has also been identified, which can cause more serious damage to organisms and even impact the health of human populations that feed on these organisms (Smith et al., 2018; De-la-Torre, 2020).

Due to this factor, bivalves are one of the main groups of organisms studied both in field collections and in laboratory experiments (Li et al., 2019a; Bom and Sá, 2021). These organisms, unlike other seafood (e.g. fish and crabs), are consumed whole, leading to the total transfer of the microplastics ingested by them, thus being a major source of these particles for humans (Ding et al., 2022). Moreover, their use in experiments is facilitated by their rapid adaptability in laboratory environments and also due to their capacity of ingesting various particle sizes (Ward et al., 2019b; Baroja et al., 2021).

By 2021, 68 published articles were identified using bivalves as target species for laboratory studies involving microplastics, mostly related to immunotoxicity, oxidative damage, genotoxicity, structural damage, and antioxidant capacity of organisms (Baroja et al., 2021). These articles showed various types of damage to organisms, from cellular, such as DNA damage and changes in enzymes, to different organs, such as abnormality in the gills and digestive gland (Alnajar et al., 2021; Choi et al., 2021; Li et al., 2022). High mortality rates of these organisms due to microplastics have also been observed (Phothakwanpracha et al., 2021).

In addition to these physiological responses, some studies have aimed to identify their rates of uptake, accumulation, and clearance of microplastics (Sendra et al., 2021). According to the authors, these processes are associated with the characteristics of plastics, such as shape, size, and type of polymer. However, an issue found in laboratory experiments with microplastics in bivalves is that normally the particle concentrations are not representative of the environment—usually many times greater than those found in water and/or sediment—and also do not represent the main shape identified in these matrices (Weis and Palmquist, 2021).

For example, Baroja et al. (2021) showed that experiments commonly use concentrations ranging from 10^4 to 10^6 MPs/L, whereas samples collected in the environment commonly present concentrations from 1 to 10^2 MPs/L., values also found by Kye et al. (2023) in a global review of microplastics in marine waters. These higher values employed in experiments can lead to more severe changes in the physiological functions of organisms, not representing what occurs in the natural environment.

The same occurs with shapes: Ward et al. (2019b) showed that pellets are marketed with uniform sizes/shapes and for this reason they are frequently employed in laboratory experiments. However, these particles are found in small percentages in the environment compared to fibers, which represent more than 85% of the total microplastics around the world (Carr 2017), in addition to not being the most commonly ingested shapes by organisms (Rebelein et al., 2021).

Finally, another factor to be considered in experiments is particle thickness, which can alter the ingestion of microplastics by organisms. However, to the best of the authors' knowledge, this factor has not yet been considered in research conducted in the laboratory or in environmental collections.

Thus, this study aimed to identify whether the thickness, sizes, and concentrations of fibers will influence their ingestion by organisms. The study presents two hypotheses: (1) thin particles would be more easily ingested by these organisms due to their selective ability to capture particles; and (2) the ingestion of particles by organisms would be more expressive in treatments with greater availability of smaller particles also due to the selectivity in particle size ingested by the mussel *Perna perna* (Linnaeus, 1758), as already identified for other species.

The mussel *Perna perna* was chosen due to its wide distribution on the Brazilian coast (Pierri et al., 2016) and also because it has been used as a bioindicator of microplastic contamination (Bom et al., 2022; Ribeiro et al., 2023). Moreover, this species of mussel has also been used in laboratory experiments evaluating possible physiological impacts caused by the ingestion of microplastics (Santana et al., 2018) and also to evaluate the effectiveness of the depuration technique for reducing these particles (Birnstiel et al., 2019). These studies showed that: (1) continuous exposure to microplastics did not cause physiological effects in mussels, possibly due to their abilities for long-term recovery and tolerance to stresses (Santana et al., 2018); (2) 93-h depuration reduced microplastic concentrations in wild and farmed organisms; and (3) fibers of different colors are eliminated differently (Birnstiel et al., 2019), results that provide important information regarding the contamination of microplastics by this species.

For the ingestion experiment, 60 adult organisms of the mussel *Perna perna* were collected with the aid of a metal spatula on a rocky shore located in Boi Island, Vitória, southeastern Brazil (for more information about the collection area, see Bom et al. (2022)). After collection, the organisms were kept in containers with seawater and immediately taken to the laboratory. Following procedures similar to Staichak et al. (2021), each organism was placed in a 1 L beaker with seawater collected in the same place and kept for 24 hours to acclimatize. To ensure greater realism with the environment, it was decided not to filter the water, allowing the bivalves to ingest both phytoplankton and MPs particles with only their concentrations/sizes/thicknesses being altered.

The beakers were kept aerated with the aid of BigAir aerators. Then, orange/yellow fibers were added to the beakers in various concentrations (25, 50, and 100 MPs.L-1), sizes (small: 1 to 2 mm; medium: 2.5 to 3.5; and large: ≥ 5 mm), and thickness (thick: 0.2 mm, polyethylene; and thin: 0.03 mm, polyester)—with three replicates for each treatment (concentration × size × thickness) and six control samples, totaling 60 samples. To obtain the particles used in the experiments, textile lines and nets were purchased and cut manually with the aid of scissors under a stereoscopic microscope equipped with a camera (Moticam Pro 252A), which made enabled verifying the exact sizes and thicknesses of the microplastics produced.

The choice of fiber characteristics employed in this study was due to: (1) orange/yellow colors are rarely found in environmental samples (Gago et al., 2018), including the study area where the mussels were collected (Bom et al., 2022); (2) sizes covered the wide range of microplastics found in mussels collected in wild/farmed areas (Li, J. et al., 2019; Bom and Sá, 2021); and (3) thicknesses commonly found—obtained by textile lines and nets—as they are the main sources of fibers from the oceans (Acharya et al., 2021; Sharma et al., 2024)

To facilitate the understanding of the results, the experiment was subdivided into two, which occurred simultaneously: Experiment A (thick fibers × concentrations × sizes) and Experiment B (thin fibers × concentrations × sizes). The experiment lasted 24 hours, after which the organisms were frozen at −20°C for further analysis. This time period was chosen for the experiment for two main reasons: (1) the mussels were not fed and (2) studies show that microplastics can be efficiently eliminated by pseudofeces or feces in a short period of time (Woods et al., 2018; Fernández and Albentosa, 2019), which would hinder observing the ingested particles.

The method of microplastics analysis was based on Patterson et al. (2019). In summary, the organisms were measured (total length) and weighed (total weight and soft tissue weight); the soft tissues were washed with filtered distilled water and placed in a 1 L beaker for the digestion of organic material. For this procedure, 100 mL of 10% Potassium Hydroxide (KOH) was added to the beakers, which were then covered with aluminum foil and placed in an oven at 50°C for 72 h. After digestion, the solution was filtered using a vacuum pump with glass fiber membrane filters with pore sizes of 1.2 µm to recover the plastic particles. Finally, each filter was placed in glass Petri dishes, covered, and dried at room temperature overnight for further analysis of the MPs.

The filters were observed under a Bel Photonics stereoscopic microscope equipped with a digital camera (Eurokam 12.0). Fibers were identified based on size and color, and only orange/yellow particles corresponding to the treatment specified sizes were counted, thus eliminating the risk of prior microplastics contamination in mussels. Concentrations were expressed as the number of particles per individual (MPs/ind).

Since the results for length, total weight, and soft tissue weight met the assumption of normality, ANOVA tests were performed to identify significant differences in these parameters between Experiments A and B. On the other hand, the number of fibers identified in the organisms did not meet the parametric assumptions of normality (Shapiro-Wilk tests) and homogeneity of variance (Levene tests); thus, Mann–Whitney tests were employed to verify possible differences between treatments.

As mentioned earlier, bivalves have been widely used in experimental studies involving microplastics (Li et al., 2019a; Baroja et al., 2021). However, as pointed by Burns and Boxall (2018), there "is a mismatch between the particle types, size ranges, and concentrations of microplastics used in laboratory tests and those measured in the environment". Moreover, according to these authors, "there is an urgent need for studies that address this mismatch by performing high quality and more holistic monitoring studies alongside more environmentally realistic effects studies." Therefore, it is necessary to identify several factors to make these studies representative of the environment, thus enabling observation of the impacts these particles cause on organisms at the concentrations currently found and enabling comparisons between experiments.

The first step in enabling comparisons in microplastic ingestion by bivalves in experiments is to evaluate possible significant differences in the size, total weight, and soft weight of the individuals used in the experiments. In the present study, the results showed that, the organisms in Experiment A showed a mean length of 56.40 ± 1.11 mm and weighted 16.83 ± 0.86 g in total and 4.85 ± 0.31 g in soft tissues, whereas the organisms in Experiment B measured 59.24 ± 1.05 mm, weighted 18.06 ± 0.99 g in total and 5.60 ± 0.34 g in soft tissues (Table 1). ANOVA tests showed no differences in any of these parameters, enabling comparisons between treatments. This is important because different sizes/weights of organisms have been shown to influence the concentrations of microplastics (Bonello et al., 2018; Patterson et al., 2019; Narmatha Sathish et al., 2020), possibly due to differences in capture, filtration, and clearance rates.

Table 1. Summary of means (± SE) of sizes, total weight, and soft tissue weight of organisms for each treatment.

	A	B	Control
Total length (mm)	56.40 ± 1.11	59.24 ± 1.05	62.83 ± 1.60
Total weight (g)	16.83 ± 0.86	18.06 ± 0.99	18.34 ± 1.60
Soft tissue weight (g)	4.85 ± 0.31	5.60 ± 0.34	4.75 ± 0.70

Another important factor considered is the characteristics of the microplastics used in the experiments, such as shapes, sizes, and color. Authors have already verified that pellets are commonly chosen in these studies (Palmer and Herat, 2021), even though they are not the most frequently identified shape in the environment (Burns and Boxall, 2018). Due to this factor, in the present study, we chose to use fibers, as they are the shape most representative of coastal and marine waters, as well as those found in bivalves (Acharya et al., 2021; Bom and Sá, 2021). The thickness of the fibers is also an important factor consider, as it can influence the ingestion capacity of organisms. However, to our knowledge, this factor has not been evaluated in previous studies, probably due to the limited use of these shapes in these works. In this study, two different thicknesses were chosen for the experiments (thick: 0.2 mm; and thin: 0.03 mm) to verify whether this parameter influences the ingestion of microplastics by bivalves.

The color of the microplastics is another relevant point, as it may lead to an overestimation of the results due to particles present before the experiment. In this study, yellow and orange colors were chosen precisely because they are rare in bivalve and surface water samples worldwide (Rezania et al., 2018; Ugwu et al., 2021), thus preventing this overestimation of the results. Moreover, fibers similar to those used in the experiments were found in the control samples. None of the six control organisms contained yellow or orange fibers, confirming that the particles found in the other organisms were in fact those introduced in the experimental approach.

Finally, selecting the concentrations of microplastics used in experiments is fundamental for understanding the number of particles ingested by organisms and their physiological consequences. As mentioned before, the concentrations used in laboratory studies are, in most cases, much higher than those found in coastal and marine environments (Burns and Boxall, 2018; Baroja et al., 2021), which does not enable accurate verification of what occurs in natural environments. Thus, the concentrations of fibers used in the present study were chosen because they are representative of environmental levels, being similar to those found in surface water samples from the same area (Bom et al., 2022b).

The two experiments showed that, in general, individuals are able to retain particles of the thicknesses used, but at different concentrations (Figure 1), with significant differences identified between them (Mann–Whitney; $p = 0.00528$; $U = 255$). In Experiment A, only one fiber particle was ingested (0.03 MPs/ind), whereas in Experiment B, 23 retained particles were identified (0.76 MPs/ind). Comparatively, the concentrations found in Experiment A were lower than those commonly found in bivalves collected from natural environments worldwide (Bom and Sá, 2021). On the other hand, the concentrations found in Experiment B are similar to those reported in several studies (Digka et al., 2018; Hermabessiere et al., 2019; Keisling et al., 2020), showing that the different thicknesses used in the experiments may be the predominant factor influencing these results. However, due to the lack of comparative studies, it is difficult to corroborate this finding.

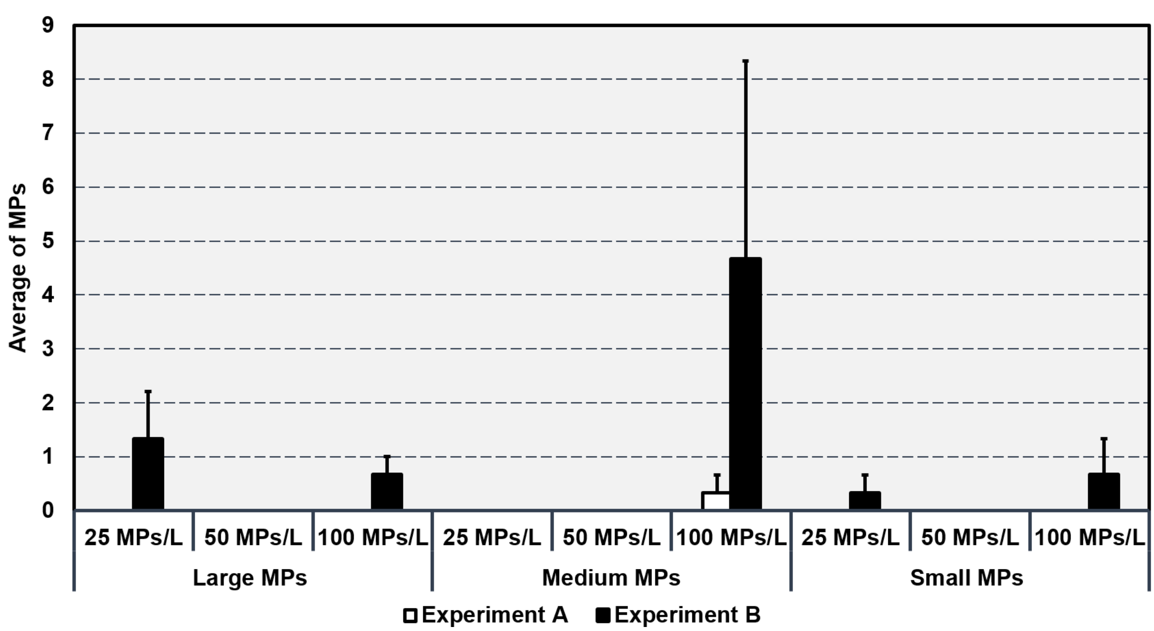

Figure 1. Box-plots of the concentrations of microplastics identified in mussels *Perna perna* in different experiments. The statistics observed in the boxplot are: thick continuous lines (median), thin continuous lines on top of box (third quartile); vertical line (maximum value disregarding outliers); and circles (outliers). Experiments A and B refer to different treatments, i.e., thin and thick fiber thicknesses, respectively.

Moreover, 3.7% and 33.3% of the organisms contained fibers in Experiment A and B, respectively. These low percentages contrast with those found of environmental studies, including the bivalve *Perna perna*, has been found to present microplastics in 75% to 100% of individuals (Santana et al., 2016; Bom et al., 2022b). This result may be explained by the fact that these organisms reject a high proportion of ingested microplastics via feces/pseudofeces (Ward et al., 2019a).

The duration of the experiments may have been another influential factor for the low assimilation of microplastics, as more hours might be required to identify particle accumulation. Staichak et al. (2021) showed that particle assimilation depends on exposure duration, with more particles identified in *P. perna* species during longer durations (48 h) compared to shorter periods (6 h). Similarly, Moreschi et al. (2020) found a strong correlation between the weight of microplastics filtered by bivalves and the exposure duration.

It should also be noted that one individual accounted for 52.17% of the fibers found in this experiment, retaining 12 particles. Apart from this individual, the remaining specimens retained mainly a single particle (6 org), with two and three fibers being found in one individual each. This discrepancy between maximum and minimum values may be related to the physiological characteristics of each organism, including filtration and egestion to feed and excrete. However, further studies on individual physiological rates are necessary to confirm this observation.

Notably, previous studies have reported variable microplastic concentrations among organisms analyzed in both field and laboratory settings. For example, a study conducted in China by Ding et al. (2021) identified variations ranging from 0 to 11 MPs/individual for the scallop *Chlamys farreri* (K. H. Jones & Preston, 1904) and from 0 to 16 MPs/individual for the clam *Ruditapes philippinarum* (A. Adams & Reeve, 1850). Similarly, Wang et al. (2023) showed, in an exposure experiment, that concentrations ranged from 0 to 8 MPs/individual and 2 to 11 MPs/individual in the species *Cyclina sinensis* (Gmelin, 1791) and *Mactra quadrangularis* Reeve, 1854, respectively, corroborating the notion that each individual ingests the particles differently.

Regarding the mean number of fibers found in the different treatments (concentrations × sizes) it was observed that the only particle retained in Experiment A was identified in the medium size of MPS under the high concentration treatment (Figure 2). In Experiment B, in turn, particles were identified in all three different size categories, mainly at concentrations of 25 and 100 MPs/L. The highest mean numbers in Experiment B were observed for the medium size at high concentration (4.66 MPs/individual) and for the large size at low concentration (1.33 MPs/individual) (Figure 2). Mann–Whitney tests suggested no significant differences in the ingestion of fibers of different sizes or concentrations in Experiment A. For Experiment B, these tests showed non-significant differences for most analyzed pairs, with the only exception being observed between the highest and medium concentrations ($p = 0.005$).

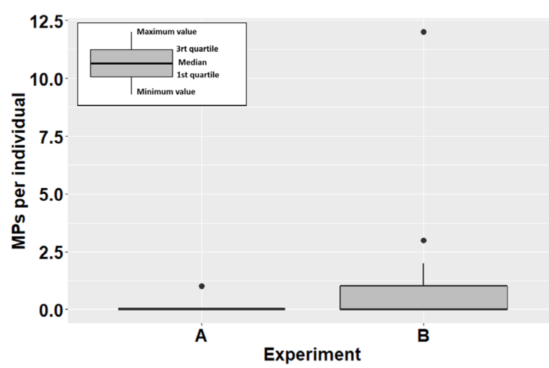

Figure 2. Mean ± Standard error of microplastics retained by the bivalve *Perna perna* in the different treatments. White and black bars represent Experiment A and B, respectively.

These results refute the main hypothesis of the present study, indicating that, in general, the ingestion of fibers by this bivalve species over 24 hours is independent of the fiber concentration in the surrounding medium. This finding contrasts with studies in natural environments, which have shown positive correlations between microplastic concentrations in organisms and in water (Wakkaf et al., 2020; Wang et al., 2021; Bom et al., 2022b).

Conversely, some studies have reported no relationships between microplastic concentrations in bivalves and those in the environment (Li et al., 2018; Coverton et al., 2019; Cho et al., 2021), suggesting that water concentration alone does not account for particle accumulation in organisms. A possible explanation for this lack of relationship is the role of biological processes involved in particle capture and rejection by bivalves, such as filtration, rejection, and selectivity rates. For example, Ward et al. (2019b) showed that filtration and egestion rates influence particle concentrations in organisms, noting that: (1) the proportion of rejected MPs increased with particle size; (2) higher proportion of rejected particles by their type (pellets > fibers); and (3) differential egestion of MPs was observed.

Comparisons between experimental and environmental collections are challenging since exposure duration to microplastics in environmental organisms is undefined, highlighting the need for further experiments. Experimental studies have found greater accumulation of microplastics in mussels exposed to higher concentrations (Li et al., 2019b; Phothakwanpracha et al., 2021); however, these experiments used concentrations exceeding those commonly found in surface seawater.

Finally, it was hypothesized that organisms would retain higher concentrations of the smaller fibers, as observed in previous experiments (Van Cauwenberghe et al., 2015; Ward et al., 2019b). This was not verified in the present study, which showed that organisms are capable of ingesting and retaining fiber of different sizes. This result is consistent with findings in samples from the natural environment, which indicate that bivalves can ingest a wide range of particle sizes—from particles smaller than 25 µm to larger than 5 mm (Teng et al., 2019; Zhu et al., 2019; Baechler et al., 2020). Ingestion of different sizes was also identified in a laboratory study using the mussel *Perna viridis* (Linnaeus, 1758)—a species similar to that used in the present study—in which small (<30 µm), medium (30–300 µm), and large (300–1000 µm) microplastics were found to be ingested (Phothakwanpracha et al., 2021).

The results presented in this study suggest that the use of fibers with different thicknesses influenced microplastic accumulation in mussels, suggesting that fiber thickness should be an important factor to be considered in future experimental and environmental studies. Moreover, it found that the ingestion of particles over a period of 24 hours was low, regardless of fiber size and concentration. These values are similar to those identified in several natural sampling studies worldwide, highlighting the importance of using concentrations, sizes, and shapes similar to those commonly found in the environment.

In conclusion, that mussels are capable of ingesting fibers of varying sizes and thicknesses when exposed to concentrations similar to those found in the environment, and this approach can be used in future studies to elucidate the physiological effects of microplastics on these organisms. Finally, we suggest that future studies employ longer exposure periods than those presented in the present study to better characterize microplastic elimination rates under these conditions.

Data availability statement

Data may be made available by contacting the corresponding author.

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Author contributions

F.C.B.: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing – original draft; Writing – original draft.

F.S.: Supervision; Validation; Project administration; Resources; Writing – original draft; Writing – review & editing.

Conflicts of interest

The authors declare no conflicts of interest.

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