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Microplastics in Marine Ecosystems: Exposure, Ingestion, and Accumulation Dynamics in Seahorses

Universidade do Algarve

Faculdade de Ciências e Tecnologia

2025

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Master’s thesis in Marine Biology

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Universidade do Algarve

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Declaração de autoria de trabalho

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(Catarina Simas de Melo)

Acknowledgements

When I was presented with this research topic, I did not hesitate for a moment – I immediately accepted the challenge. Investigating a subject as the problem of plastics that, since I was very young, has interested me a lot, combined with an animal as fascinating as seahorses, seemed to me the ideal topic. I am truly grateful for the opportunity to embark on this journey of discovery regarding the intricate dynamics of seahorses in an environment increasingly influenced by anthropogenic activities.

Beyond all the academic and technical support provided during the development of this study, I would like to express my sincere gratitude to my family and friends for their moral support and encouragement, which have been invaluable throughout this academic path.

This study would not have been possible without the essential technical assistance provided by João and André during the experimental phase at Ramalhete Research Station. I am also thankful to Cristóvão, who generously offered his help on weekends and whenever needed. My supervisor, Jorge Palma, was an exceptional mentor, always patient and willing to clarify my doubts, ensuring I had the necessary support throughout this process. His readiness to assist and his constructive criticism have greatly contributed to the rigor and quality of this thesis, and his mentorship and guidance were truly indispensable.

A genuine thank you to my entire family, whom I have deep admiration for. I have been inspired by my father's curiosity and determination, and my mother, Raquel, and Hugo, have never wavered in their support of my dream of pursuing Marine Biology, or *"as Pescas"* as they like to call it. I would like to give a special acknowledgment to my grandfather, who ensured I had everything necessary to make this journey as smooth as possible. His constant encouragement to be critical and meticulous thorough in my writing reminded me that I was becoming a specialist in this field and with that came the responsibility to master every detail of it.

Throughout this process, I have not only gained knowledge but also developed new skills as an aspiring researcher. This has been a journey of personal growth, testing my ability to undertake this work independently and proving to myself that I am capable of seeing it through.

Resumo

O plástico tornou-se um elemento indispensável na vida humana, levando a um aumento exponencial da sua produção. No entanto, o seu consumo inconsciente e má gestão dos resíduos plásticos têm contribuído para que este se torne um dos principais poluentes nos ambientes marinhos costeiros. Estas partículas podem entrar no oceano através de diversas fontes, tanto terrestres (como sistemas de esgoto, vento e rios) como marítimas (como pesca e transporte marítimo). Uma vez no meio aquático, o plástico pode fragmentar-se em partículas menores, transformando-se em microplásticos (MPs) (< 5 mm), ou até nanopartículas (< 1 μm), afundando e acumulando-se nos sedimentos. Devido ao seu tamanho reduzido, os MPs podem ser ingeridos por uma grande diversidade de espécies marinhas, desde zooplâncton até grandes peixes, podendo causar impactos negativos no sistema digestivo, afetar a fisiologia dos organismos ou até levar à morte. A sua capacidade de bioacumulação e transferência entre diferentes níveis tróficos tem despoletado uma grande preocupação na comunidade científica, dado que podem representar riscos ecológicos.

Os cavalos-marinhos, pertencentes à família dos singnatídeos, habitam em águas pouco profundas e são predadores oportunistas que recorrem predominantemente a pistas visuais para caçar. O seu focinho tubular que potencia o mecanismo de sucção que utilizam para capturar pequenas presas planctónicas como anfípodes, decápodes, isópodes e misidáceos, torna-os também, especialmente vulneráveis à ingestão de microplásticos. No entanto, os estudos sobre este tema ainda são recentes e escassos. Devido à semelhança entre o tamanho dos MPs e o das presas naturais dos cavalos-marinhos, a ingestão pode ocorrer por uma falta de identificação dos MPs, enquanto potenciais presas. Muitas espécies de cavalos-marinhos enfrentam ameaças populacionais, pelo que compreender os impactos específicos dos MPs nestes organismos é crucial para o desenvolvimento de estratégias de conservação, tanto da espécie como do seu habitat.

A Ria Formosa é um sistema lagunar de baixa profundidade caracterizado por habitats de pradarias marinhas com uma grande biodiversidade. No entanto, tem vindo a sofrer de uma elevada pressão antropogénica devido às várias atividades que são desenvolvidas ao redor. Para além disso, são feitas descargas de esgotos neste ambiente e sendo que a sua hidrodinâmica é limitada por apenas algumas ligações ao oceano (sem total renovação da água), isto promove a retenção de poluentes o que o torna um ecossistema sensível. Aqui ocorrem espécies de elevada importância comercial e

ecológica, onde se incluem as duas espécies de cavalos-marinhos nativas europeias – *Hippocampus hippocampus* e *Hippocampus guttulatus* – as quais pela sua biologia característica são também elas bastante vulneráveis a distúrbios antropogénicos e alterações ecológicas.

Este estudo procurou: i) avaliar a presença, composição e características dos microplásticos em diferentes substratos da Ria Formosa, referenciados como habitats naturais para os cavalos-marinhos, e ii) analisar a ingestão de MPs pelo *H. guttulatus*, tanto em ambiente selvagem como em condições de cativeiro. Para tal, a exposição a MPs foi primeiramente avaliada através da análise de amostras dos substratos (erva marinha, *Caulerpa prolifera* e sedimento complexo), e de conteúdos gastrointestinais dos cavalos-marinhos selvagens. Posteriormente, realizou-se um estudo experimental em ambiente controlado para compreender a dinâmica de ingestão e acumulação de MPs. Para isso, consideraram-se dois tipos de alimentação (viva e congelada, individualmente fornecida em conjunto com uma mistura de MPs) para determinar a tipologia de ingestão (se acidental ou se voluntária), bem como o período de retenção (onde era apenas fornecido alimento sem MPs) para verificar possíveis processos de acumulação.

Os resultados da análise dos diferentes substratos indicaram uma concentração significativa de MPs na Ria Formosa, especialmente no substrato sedimentar, sugerindo um possível aumento na contaminação ao longo dos anos quando comparado com estudos anteriores. A comparação dos dados sugere que fatores como pressão antropogénica, atividades industriais, turísticas e piscatórias, e processos hidrodinâmicos podem ter influenciado esta tendência crescente na acumulação de MPs nos substratos costeiros. A elevada exposição aos MPs leva também a que haja uma maior ingestão por parte dos animais, facto que se verificou através da análise do conteúdo do trato gastrointestinal (GIT) dos cavalos-marinhos selvagens e que mostrou uma presença de MPs em todos os animais observados independentemente do sexo ou tamanho. No entanto, verificou-se uma correlação positiva entre a quantidade de MPs ingeridos e o comprimento total dos indivíduos. Estes resultados reforçam a hipótese de que a ingestão de MPs pode estar mais dependente da disponibilidade e do comportamento alimentar do que de características individuais, como a personalidade trófica. Em termos de tipos de MPs, as fibras foram os mais comuns em ambos estudos feitos em ambiente natural (substratos e animais), as quais são frequentemente associadas a produtos têxteis, redes de pesca e descargas de águas residuais, o que justifica a sua ocorrência na Ria Formosa onde a influência dessas fontes é significativa. Ao analisar a distribuição das cores,

os MPs pretos e azuis foram os mais comuns, tanto nas amostras de substrato como nos tratos gastrointestinais (GIT) dos cavalos-marinhos selvagens. No entanto, é admissível uma salvaguarda de erro nas classificações visuais e na diferenciação de cores devido a limitações no estereoscópio, e de potenciais processos de oxidação que podem ter ocorrido nos MPs observados. A nível de tamanhos, foi encontrada uma grande amplitude comprimentos, incluindo algumas fibras que se enquadraram na categoria mesoplásticos, mas que estariam enroladas e só quando esticadas atingiam esses tamanhos, o que justifica a sua ingestão pelos animais.

Durante o estudo experimental em cativeiro, todos os cavalos-marinhos consumiram microplásticos tendo-se verificado uma maior ingestão de MPs quando alimentados com alimento vivo comparativamente com o alimento congelado. Isto pode ser explicado pelo aumento da atividade na captura de presas vivas (que ressuspende os MPs, aumentando a sua probabilidade de ingestão acidental) ou pelo facto de que em modo caça não terem tanto tempo para distinguir entre uma presa e MP, sugerindo que alguns MPs podem ser consumidos voluntariamente, mas possivelmente confundidos por uma presa durante a ação de caça. Coincidentemente, e tal como no ambiente natural, as fibras foram também o grupo de MPs mais consumido, reforçando a ideia que pelo seu tamanho e forma, estas podem ser confundidas com o alimento, mas também pela sua menor massa serem mais sensíveis ao movimento e podem ser ingeridas acidentalmente como foi possível observar. Entre os fragmentos fornecidos, os únicos que não foram consumidos foram os de cor azul, o que levanta a hipótese da existência de algum tipo de seletividade com base na cor. Por fim, foi também possível verificar que existiu retenção e possível acumulação devido à exposição prolongada a MPs. Estes resultados destacam a necessidade urgente de reduzir a contaminação por MPs nos ecossistemas marinhos, dado o impacto ecológico que podem ter em espécies vulneráveis como os cavalos-marinhos. Para futuras investigações, sugere-se o uso de metodologias mais precisas para a análise dos microplásticos que permitam não só uma caracterização mais exata, mas também a identificação do material de forma a ser possível deduzir as suas origens. Melhorias experimentais poderiam incluir uma maior gama de cores de MPs e condições de luz mais próximas do habitat natural, permitindo uma análise mais precisa de preferência e possível seletividade na captura de MPs.

Palavras-chave: Poluição, Microplásticos, Cavalo-marinho, Monitorização, Ria Formosa, Conservação

Abstract

The increasing human activities have caused growing pressure on marine ecosystems, with microplastics (MPs) emerging as a new pollutant and causing concern among the scientific community due to their small size and potential to be ingested by various marine organisms and move up the food chain. Seahorses are particularly susceptible due to their suction-feeding mechanism. Ria Formosa, a biodiversity hotspot, faces major pollution challenges, including high human pressure and sewage discharges, increasing contaminants and risks for its marine fauna.

This study aimed to assess the exposure levels of seahorses to MPs by analyzing their presence across three dominant substrate types in the Ria Formosa, as well as their ingestion via gastrointestinal contents (GIT) of wild seahorses. Additionally, an experimental trial in captivity evaluated ingestion dynamics, determining whether MP consumption was voluntary or accidental, and if accumulation occurred. Results revealed a high concentration of MPs, predominantly in the sediment substrate, which contributes to greater ingestion by seahorses. MPs were found in all sampled seahorses, regardless of sex or size, with bigger individuals ingesting more MPs. Fibers were the predominant MP type in both the substrate and GIT, as well as the colours black and blue. Under experimental conditions, MP ingestion was higher with live food than frozen food, suggesting involuntary consumption during hunting (with increased movement), or voluntary due to failure to distinguish MPs from prey. While fibers were the most ingested, blue fragments were not consumed, raising the hypothesis of color selectivity. There was MP retention, suggesting possible accumulation over time.

These findings highlight the urgent need to mitigate MP contamination and improve methodologies for MP identification, ensuring a better understanding of their ecological impacts on vulnerable species like seahorses.

Keywords: Pollution, Microplastics, Seahorse, Monitoring, Ria Formosa, Conservation

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List of abbreviations, acronyms and symbols

A – Dragging transect sampling method

BW – Body weight

Cau – *Caulerpa prolifera* substrate

CF – Condition factor

Core – Replicated sediment sampling method

DD – Data deficient

Ev – Seagrass substrate

FC – Food consumed

fib – Fiber

fibB – Blue fibers

fibR – Red fibers

FL – Leftover food

FP – Food provided

frag – Fragment

fragB – Blue fragments

fragD – Black fragments

fragR – Red fragments

fragW – White fragments

GIT – Gastrointestinal tract

MPs – Microplastics

NT – Near threatened

PC – Plastic consumed

Pdig – Digested microplastics

PL – Leftover plastics

PP – Plastic provided

Sed – Complex sediment substrate

µ-FTIR – micro-Fourier Transform Infrared Spectroscopy

LDIR – Laser Direct Infrared

Introduction

Seahorses belong to the family Syngnathidae and share familial ties with pipefishes and seadragons (Foster & Vincent, 2004). Morphologically, seahorses are characterized by a horse-like head positioned at a right angle to an erect body, complemented by a long tubular snout devoid of teeth adapted for suction feeding (Foster & Vincent, 2004). Unlike typical fish, seahorses lack a differentiated stomach in their digestive tract and exhibit an exoskeleton stretched over bony plates, instead of scales, which is visible as rings encircling the trunk and tail, along with a prehensile tail (Foster & Vincent, 2004; Lourie et al., 2004). Their sedentary (almost stationary) lifestyle and slow mobility (Ahnesjö & Craig, 2011) render seahorses highly sensitive to fluctuations in local feeding resources, as their limited swimming capabilities restrict them from covering substantial distances in search of food (Foster & Vincent, 2004; Pierri et al., 2022; Lazic et al., 2023).

Seahorses have been claimed to be an indicator of environmental quality (Zhang & Vincent, 2019; Faleiro et al., 2015; Liu et al., 2021; Costa et al., 2023), primarily due to their habitats frequently being subjected to anthropogenic disturbances making them vulnerable to ecological changes, including habitat degradation, pollution (Zhang & Vincent, 2019), by-catch (Ahnesjö & Craig, 2011; Planas et al., 2023; Pierri et al., 2022) and target fisheries driven by overexploitation demand in Traditional Chinese Medicine markets (Vincent et al., 2011). As a result, many seahorse species face risks of population depletion, a trend that has been documented over recent decades (Foster & Vincent, 2004; Pierri et al., 2022; Spatafora et al., 2023).

Despite some seahorse species being classified as Vulnerable, Endangered, or even Critically Endangered by the IUCN Red List of Threatened Species, a considerable number remain classified as Data Deficient (DD) owing to insufficient information (Pollom et al., 2021). For instance, *Hippocampus guttulatus* is generally categorized as DD (Pollom, 2017a), reflecting gaps in global data on their distribution, population trends, and main threats (Planas et al., 2023). However, in the Mediterranean region, the species is classified as Near Threatened (NT), as datasets are available on population size and trends in that area (Pollom et al., 2017b). Although there has been a notable increase in scientific research attention in recent years, most studies focus on reproductive biology and physiological traits rather than broader population dynamics and species-specific distribution patterns (Lazic et al., 2023; Pierri et al., 2022; Segaran et al., 2023). Among the key factors influencing seahorse conservation are water quality and anthropogenic activities (Foster & Vincent, 2004; Zhang et al., 2016). Furthermore, significant research into the various threats faced by these species, such as microplastic contamination, is relatively recent (Segaran et al., 2023; Jinhui et al., 2019).

Microplastics represent a new type of environmental pollutant, with widespread prevalence in marine ecosystems (Liu et al., 2022; Boucher & Friot, 2017), having recently attracted significant attention from the scientific community. Research indicates that microplastics have been detected in a wide range of marine species, suggesting their ingestion by both larger fish and smaller aquatic organisms (Jinhui et al., 2019; Onay et al., 2023; Wright et al., 2013; Gall & Thompson, 2015).

Seahorses, in particular, have emerged as important subjects of research regarding microplastic ingestion (Onay et al., 2023). Their suction-feeding mechanism, which targets small planktonic prey, makes them particularly susceptible to ingesting microplastics (Jinhui et al., 2019). This ingestion can occur directly when microplastics are mistaken for prey (Ory et al., 2017) or involuntarily sucked in during feeding (Lazic et al., 2023). Additionally, indirect ingestion may occur through trophic transfer from contaminated prey species such as mysids, copepods, and amphipods (Palma et al., 2008; Desforges et al., 2015).

Despite growing research on microplastic pollution, its specific effects on seahorses remain poorly studied. This gap highlights the need for targeted studies to better monitor the abundance of microplastic pollution in seahorse habitats, assess exposure levels, and understand the mechanisms of ingestion, retention, and consequently, their physiological impacts (Jinhui et al., 2019).

This study aims to clarify the effects of microplastic ingestion on the physiological and ecological health of seahorses. It investigates the ingestion, exposure, and accumulation dynamics of microplastics (MPs) in *Hippocampus guttulatus*, a seahorse species native to the Ria Formosa lagoon, a temperate, seagrass-dominated ecosystem. It examines microplastic presence in habitat samples and in the gastrointestinal tracts of wild seahorses, as well as ingestion observed in controlled experiments. By addressing critical knowledge gaps on the specific impacts of microplastics on seahorses, this research provides valuable insights into the broader effects of microplastic pollution on marine biodiversity and informs conservation strategies for seahorses and their habitats.

Seahorses (genus *Hippocampus*)

This unique group of bony fish possesses a unique reproductive strategy that sets them apart from the others: the females transfer their eggs to a specialized abdominal pouch of the male, where fertilization and development occur, rendering males responsible for gestation (Ahnesjö & Craig, 2011; Foster & Vincent, 2004).

During the juvenile phase, they are characterized by proportionally larger heads and slimmer, more spikier bodies, with higher coronets, and they exhibit planktonic behavior immediately after birth (Foster & Vincent, 2004). Seahorses have no pelvic and caudal fins, yet retain a single dorsal fin for propulsion, accompanied by two small pectoral fins serving stabilization and steering functions, along with a reduced anal fin (Foster & Vincent, 2004).

Many syngnathid species inhabit shallow habitats, including lagoons, estuaries, and coastal marine waters (Ahnesjö & Craig, 2011; Spatafora et al., 2023), although habitat preferences can vary among species (Foster & Vincent, 2004). In the Ria Formosa, a temperate region characterized by seagrass beds, two species can be found: *Hippocampus hippocampus* (Linnaeus 1758), which is associated with structurally simpler habitats (like sandy areas), and *H. guttulatus* (Cuvier 1829), which shows a positive correlation with more complex areas that offer greater vegetation coverage, providing essential holdfasts for their prehensile tails (Foster & Vincent, 2004; Curtis & Vincent, 2005; Correia, 2022). Additionally, prey abundance is also a determinant in habitat selection, potentially leading to some variation in the distribution of the species (Pierri et al., 2022).

Seahorses are ambush predators that rely predominantly on visual cues to hunt for live, mobile prey (Palma et al., 2008; Foster & Vincent, 2004). Their diet includes small organisms that can be swallowed by their elongated snout, mainly amphipods, decapods, isopods, and mysids (Planas et al., 2023; Foster & Vincent, 2004; Lazic et al., 2023). When feeding, they remain motionless until prey approaches, at which point they use their tubular snout with a rapid and strong suction to draw in their food (Foster & Vincent, 2004). Occasionally, seahorses feed near the substrate, injecting water into it to resuspend prey (Foster & Vincent, 2004), but then their fast and powerful suction action can lead to accidental ingestion of non-prey items (Lazic et al., 2023).

Microplastics (MPs)

With rapid economic growth and the exponential increase in global plastic production since it became an indispensable material worldwide, plastic emerged as one of the major pollutants in coastal water environments as its unconscious consumption has caused many tons of plastic to be disposed annually (Barnes et al., 2009; Wright et al., 2013; Boucher & Friot, 2017). Plastics are made of synthetic organic polymers known for their durability, meaning they do not degrade but break into meso-, micro-, and nanoplastics (Barnes et al., 2009).

The definition of microplastics (MPs), while not universally agreed upon, can be described as "any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water" (Frias & Nash, 2019).

Microplastics are predominantly found in oceanic gyres, coastal areas, and their sediments (Wright et al., 2013; Barnes et al., 2009). The primary forms of microplastics identified in the literature are pellets, fragments, and fibers (Frias et al., 2018). These particles can enter the ocean through various sources, including land-based or maritime activities such as fisheries (Gao et al., 2023). Mismanaged plastic waste often becomes debris transported into the marine environment via rivers, drainage systems, sewer systems, or carried by wind (Rochman, 2018). Moreover, urban runoff waters contribute particles from tire abrasion during driving (Boucher & Friot, 2017), while sewage effluent introduces fibers from laundering synthetic textiles or microbeads from cosmetic products because even though larger debris is removed during sewage treatment processes, a significant fraction of microplastics eludes capture, as filters are generally not designed to retain such small particles (Boucher & Friot, 2017; Browne et al., 2011; de Falco et al., 2018).

While a considerable portion of plastics is buoyant enough to float in seawater, certain microplastics possess higher densities or are quickly accompanied by microbial biofilms, which alter their physicochemical properties and promote sinking (Ivar do Sul & Costa, 2014; Andrady, 2011). As a result, large quantities of microplastics accumulate on seabeds (Barnes et al., 2009; Frias et al., 2018).

Microplastics migrate, diffuse, and accumulate within aquatic systems, where they are then consumed by marine organisms, leading to dangerous effects on their digestive systems and, in some cases, resulting in mortality (Jinhui et al., 2019; Lusher et al., 2013; Gall & Thompson, 2015).

Beyond causing physical damage, microplastics have the potential to release harmful biological and chemical toxicants, acting as vectors for attached heavy metals and other pollutants that may disrupt the normal physiological functions of aquatic life and induce various toxic effects (Jinhui et al., 2019; Liu et al., 2022; Tang et al., 2021b). Due to the similarity in particle size to natural planktonic prey, microplastics are easily misidentified and ingested by aquatic animals (Ivar do Sul & Costa, 2014; Ory et al., 2017; Liu et al., 2022; Sun et al., 2019). Once these environmental pollutants enter the food chain, they migrate through it and bioaccumulate, leading to adverse ecological consequences (Gao et al., 2022; Tang et al., 2021b; Liu et al., 2022; Sun et al., 2019).

Ria Formosa

The Ria Formosa located in the southern coast of Portugal (Fig. 1) is a coastal lagoon system characterized by a triangular morphology, encompassing about 100 km^2 and 55 km in length, with a maximum width of six kilometers and an average depth of less than two meters (Cravo & Jacob, 2019). It has been classified as a Portuguese Natural Park since 1987 (Newton et al., 2022) and is designated as a Category III Protected Area, which includes Natural Monuments or Features (Beltrán et al., 2023). Ecologically, the lagoon is significantly important, supporting a remarkable diversity of habitats with high biodiversity, including sensitive seagrass meadows and important populations of seahorses (Moura et al., 2019; Beltrán et al., 2023). However, the systematic and illegal harvesting of high-value species, such as seahorses and sea-cucumber (primarily for foreign markets like Asia) has notably degraded its ecological status (Newton et al., 2022).

Figure 1: Location and geomorphology of Ria Formosa. (Adapted from Google Earth)

The lagoon is shielded from the direct impact of marine wave action by a barrier-island system, with extensive marsh area and tidal channels connected to the sea through inlets which guarantees daily renewal of water and nutrients with the tides (Moura et al., 2019; Veríssimo et al., 2019). Nonetheless, water circulation within the inner lagoon remains restricted, with only 70% of the water being exchanged daily with the Atlantic Ocean (Bebianno et al., 2019). This results in hazardous substances discharged from the land or the atmosphere accumulating within the lagoon, particularly in the areas characterized by blind ends (Bebianno et al., 2019, 2021). The Ria Formosa system receives effluent discharges from 28 domestic and industrial wastewater treatment plants, of which 12 discharge directly into the lagoon (Bebianno et al., 2019), resulting in its classification as a sensitive area regarding urban wastewater discharges (Veríssimo et al., 2019).

The Ria Formosa represents a complex economic and social-ecological system that provides valuable ecosystem services and benefits to the surrounding region (Bebianno et al., 2019, 2021). Its key economic activities include sand and salt extraction, agriculture, animal rearing, aquaculture, fisheries, food processing, golf, tourism, and real estate development (Bebianno et al., 2019). Thus, various substances and contaminants enter the lagoon through atmospheric deposition, river discharges, agriculture and road runoff, golf course drainage, sewage effluent, aquaculture waste, industrial effluent, and emissions from harbors and boats (Bebianno et al., 2019). Additionally, marine litter from fishing and tourism also contributes to environmental degradation (Bebianno et al., 2019). The increase in waste production and pollution has intensified the pressures on this fragile ecosystem (Bebianno et al., 2019).

Material and Methods

Task 1. Sample collection in the natural environment

1.1. Quantitative and qualitative analysis of inert components collected from in situ benthic and supra-benthic samples

Three sites within the Ria Formosa were selected for sample collection, each representing a distinct habitat: seagrass (“Ev”, location: 37°0'13.23"N, 7°49'6.29"W), complex sediment (“Sed”, location: 37°0'17.52"N, 7°48'40.23"W), and *Caulerpa prolifera* (“Cau”, location: 37°0'21.87"N, 7°48'46.46"W) (Fig. 2). At each location, one sample was collected by dragging a net over a 50-meter transect (“A”), capturing material from both the benthic and supra-benthic zones.

Additionally, three replicate samples ("Core") were taken from the benthic layer and the adjacent surface sediment to ensure a comprehensive assessment.

Figure 2: Sampling locations for substrate collection, each representing a distinct habitat, along the study area in Ria Formosa: seagrass ("Ev", 37°0'13.23"N, 7°49'6.29"W), complex sediment ("Sed", 37°0'17.52"N, 7°48'40.23"W), and *Caulerpa prolifera* ("Cau", 37°0'21.87"N, 7°48'46.46"W). (Source: Adapted from Google Earth)

For the drag sampling, a net measuring one meter in width and 50 cm in height was used. It featured a mesh size of 150 microns at the base to capture fine particles, while the sides and top had a coarser one-millimeter mesh to allow water flow. The net had a total length of 1.5 meters and was equipped with a removable 150-micron mesh bag at the terminal section for efficient sample collection. To establish the sampling path, divers laid a 50-meter transect tape along the seafloor.

The net was then dragged 1 meter to the side of this transect to minimize disturbance to the sample composition. Two divers guided the net along the designated path, ensuring continuous contact with the seafloor. Once collected, the sample was immediately transferred onboard and kept under appropriate conditions for transport.

Afterward, three replicated sediment samples were collected from the opposite side of the transect, at 15, 30, and 45 meters of the transect belt. For each replicate, a mesh bag measuring 50 cm in width and height with a one-millimeter mesh size was used. The net was placed over the sediment and pressed evenly to a depth of 2.5 cm, ensuring the collection of both sediment and surface material. The samples were securely closed with wire, transferred to individual bags, and kept in suitable conditions for transport.

In the laboratory, samples were initially stored frozen and later thawed as needed for processing. The processing protocol started by passing each sample through sieves with progressively smaller mesh sizes. This procedure facilitated the separation of organic material, inorganic debris, and plastics. The material retained on the larger sieves was examined visually for the presence of larger plastics, while the finer material collected on the final sieve (150 µm) was analyzed under a stereoscope (Zeiss Axiocam 208 color). Identified microplastics were carefully isolated and stored in Eppendorf tubes for further quantitative and qualitative analyses, as well as for detailed measurements. To document and measure the isolated microplastics, they were arranged in Petri dishes, photographed using the stereoscope with Zeiss Labscope software, and measured using ImageJ software to ensure precise dimensional analysis. Throughout all steps, all equipment was washed three times with distilled water before use to prevent external contamination.

1.2. Quantitative and qualitative analysis of inert components in the gastrointestinal tract contents of wild seahorses

Adult *H. guttulatus* (Fig. 3) were collected from a mixed sediment habitat (location: 36°59'15.49"N, 7°51'20.65"W), based on the fact the number of observed seahorses in the two other habitats (seagrass and *C. prolifera*) was residual and do not sustain a viable sample size for further analysis. A 50-meter transect belt was established to define the sampling area, where two divers manually collected seahorses along the transect line. Collected seahorses were placed in soft mesh bags to ensure safe transportation and prevent potential damage. Once an adequate number of individuals had been gathered, they were taken back to the boat and transferred to a large container filled with water previously collected at that same location and aerated to maintain adequate conditions (Fig. 4).

Figure 3: *Hippocampus guttulatus* collected for sampling.

Figure 4: Aerated container filled with site-collected seawater used to hold seahorses after capture, ensuring appropriate conditions.

For sample processing, three buckets were prepared: one containing an anesthetic solution (2-phenoxyethanol) at a proportion of 0.167mL/L, one filled with filtered seawater to eliminate any external contamination, and a third one filled with seawater for the recovery period post-anesthesia (Fig. 5). Seahorses were individually removed from the holding container, and data on sex, mesh bag number (corresponding to the gastrointestinal tract sample, or "GIT"), total length (as the sum of head length and total height) as described by Palma et al., 2017, and weight were recorded. Each individual was then placed in the anesthetic solution to reach a sedated state, allowing for careful handling.

Figure 5: Buckets used during sample processing.

Once anesthetized, a soft catheter (1 mm Ø) was carefully inserted through the mouth past the esophagus, and filtered water was slowly injected to perform a lavage of the digestive tract (Fig. 6). This procedure flushed the gut contents, which were either regurgitated or, in some cases, expelled through the anus and entered the filtered water bucket. The process was repeated twice to ensure thorough flushing of the digestive tract. The seahorse was then left briefly in the filtered water bucket to observe if any additional residues were expelled.

Figure 6: Gastrointestinal tract (GIT) lavage procedure being performed using a soft catheter inserted through the mouth to flush out microplastics.

The seahorses were then placed in a recovery bucket to regain their full physiological function, including free swimming. They were released back at their original location at least five minutes after full recovery had been identified. Meanwhile, the water used in the lavage process was passed through a 150-micron removable mesh sieve to collect any retained residues. Each sieve was designated for an individual seahorse, then folded and stored in separate plastic bags, which were subsequently placed in a refrigerated cooler to prevent degradation of the collected materials.

In the laboratory, the samples were frozen at -18°C until analysis. During processing, each mesh filter was unfolded and examined under a stereoscope (Zeiss Axiocam 208 color). Every identified microplastic particle was photographed using Zeiss Labscope software and subsequently measured with ImageJ to ensure accurate dimensional analysis. A quantitative and qualitative analysis of microplastics was also conducted. Throughout all steps, all equipment was thoroughly washed three times with distilled water before use to prevent any external contamination.

Task 2. Ingestion and retention of microplastics in captive *H. guttulatus*

The experimental procedure was designed as a two-round study, with each round consisting of two trials of one week each (one with live and one with frozen food). Each fish (n = 18) was individually allocated into each of the nine replicated tanks. All individuals were carefully selected from a captive broodstock based on their good physical condition, absence of pregnancy, and lack of disabilities. In the first round, five males and four females were selected, while in the second round, four males and five females were chosen, ensuring the sex evenness. On the first day of each round, prior to being placed in its respective tank, each seahorse was measured for total length as described above and weighed for body weight (BW (g)). The BW was used to determinate the amount of food provided to each individual, set at 1.5% BW d^{-1}. At the end of each round, the seahorses were re-measured and re-weighed allowing to calculate both initial and final Condition Factors ($CF = (\frac{wet\ weight\ (g)}{height^3\ (cm)}) \times 100$), to ensure that experiment did not induce any potential threat the survival and welfare of this animals.

Each tank (40L) was equipped with an artificial holdfast to allow the seahorses to attach themselves, air tubing for moderate aeration, constant water flow (80 L/h) and a filter covering the water outflow to prevent loss of microplastics and food particles (Fig. 7). Temperature and salinity were kept, respectively, at 19.7±0.1 °C and 37.6 ± 0.1‰.

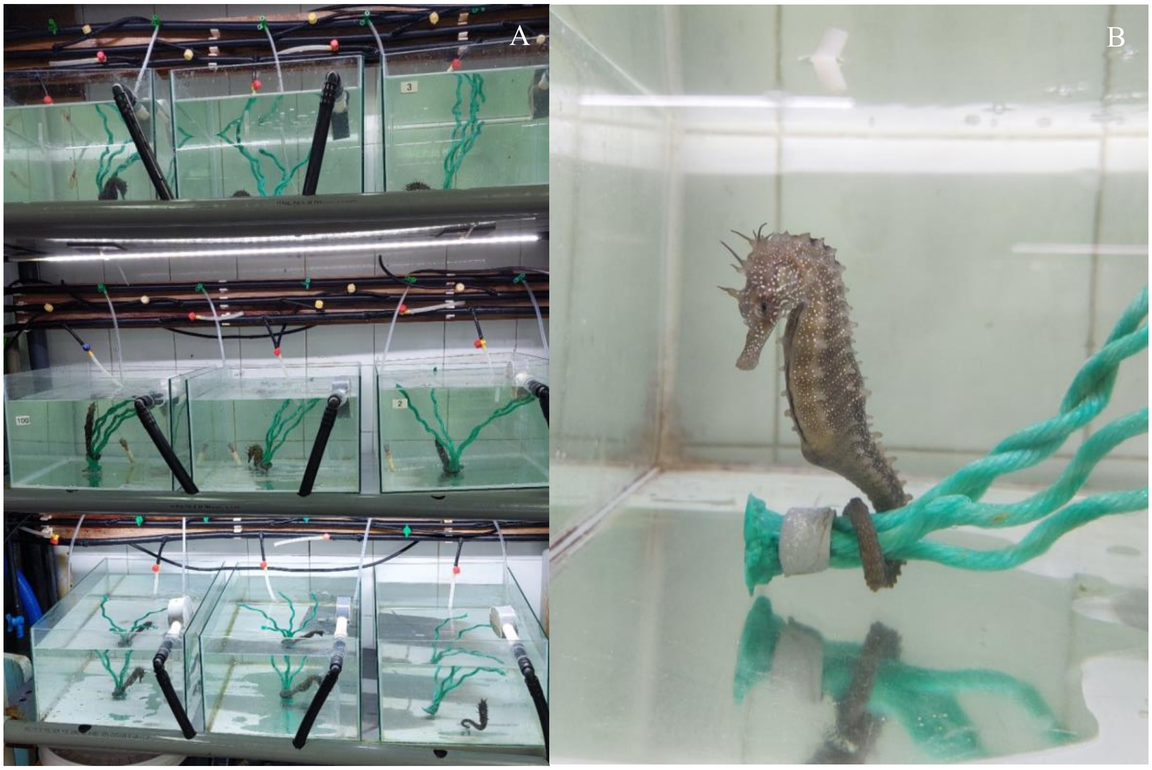

Figure 7: (A) Experimental setup with nine tanks used during the experiment. Each tank was equipped with an artificial holdfast (B), aeration, constant water flow, and an outflow filter to retain microplastics and food particles.

During the two-week experiment, seahorses were fed daily at 11:00 AM with a ration equivalent to 1.5% BW d^{-1}. The diet consisted of mysids (*Diamysis lagunaris*) and shrimp (*Palaemonetes varians*), with each food type corresponding to 0.75% of each individual's body weight. This amount of food provided (FP) was adjusted to be 50% less than the standard feeding amount (3% BW d^{-1} (Palma et al., 2012, 2017)) to ensure sub-*ad libitum* conditions. The food was captured daily using hand nets from the surrounding ponds of the Ramalhete Research Station near the Ria Formosa. Excess water was removed prior to weighing to ensure accurate wet weight measurements. A portion of the captured food was frozen and only defrosted immediately before weighing for use.

A mixture of microplastics was prepared and supplied along with the natural diets, with the microplastics pre-weighed to ensure that the amount provided (PP) to each animal was consistent. The mixture included small amounts of seven types of plastics (Fig. 8): black fragments (fragD, 2–3 mm), blue fragments (fragB, 2–3 mm), white fragments (fragW, 2–3 mm), red fragments (fragR, 1–4 mm), blue fibers (fibB, 4 mm) and red fibers (fibR, 4 mm). The total weight of microplastics added to each preparation was, on average, 81±12 microplastics per individual daily. These preparations were made the day before and stored in Petri dishes (Fig. 9) to facilitate mixing with the food the next day to be provided to each seahorse (Fig. 10). At the start of the experiment, nylon fragments (1–3 mm) were also include in the microplastic mix but due to their buoyancy characteristics which made them be unavailable to the seahorses, they were removed from further analysis.

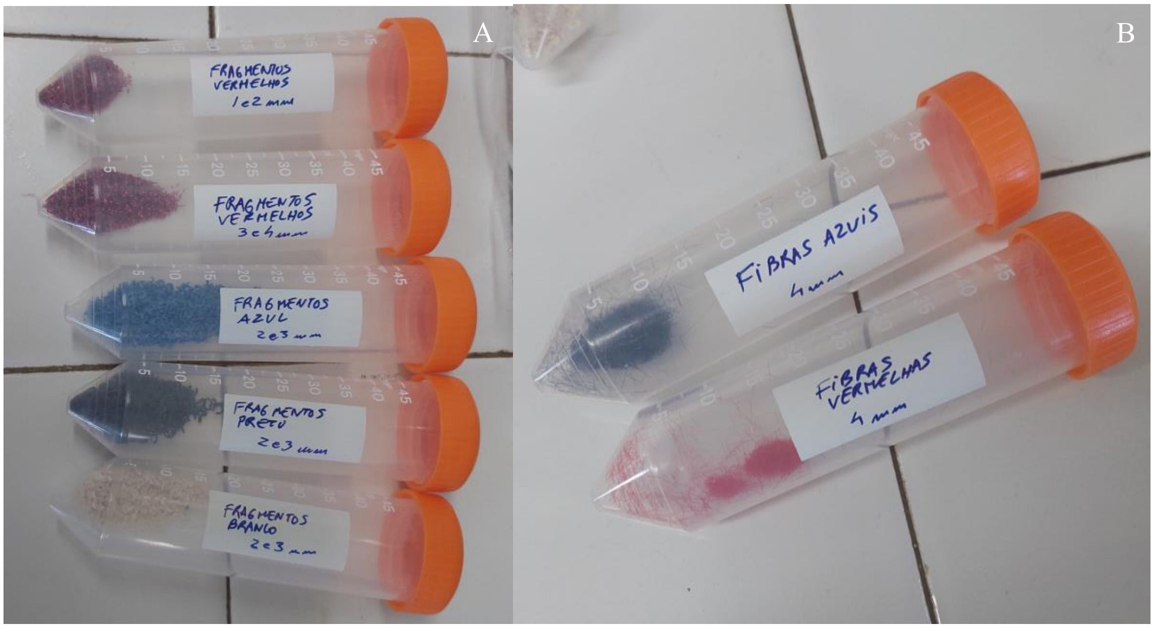

Figure 8: Microplastics used in the feeding experiment. (A) Falcon tubes containing the four types of fragments: red (fragR), blue (fragB), black (fragD), white (fragW). (B) Falcon tubes with the two types of fibers: blue (fibB) and red (fibR).

Figure 9: Daily preparation of microplastics for individual exposure. Microplastics were counted and placed in Petri dishes (average of 81 ± 12 MPs per individual per day) the day before use, to facilitate mixing with food prior to feeding.

Figure 10: Preparation of daily feedings. (A) Daily feeding portions for each of the 9 individuals, containing the mix of microplastics and food. (B) Detail of one feeding portion, showing the visible microplastics mixed with natural diet items.

The feeding schedule was organized as follows: In the first trial, live food mixed with microplastics was provided for five consecutive days, followed by two days of only live food. In the second trial, frozen food mixed with microplastics was provided for five consecutive days, followed by two days of only frozen food. The provision of a natural diet for a two-day period allowed for the verification of potential excretion of any microplastics accumulated during the five days of exposure. Upon the conclusion of the second trial, the second round was initiated, following the same feeding protocol.

On each of the five days when microplastics were provided, five hours after feeding, each tank was thoroughly cleaned to collect non-ingested food and microplastics. Cleaning was done by siphoning, and non-eaten food and microplastics were collected using a 500 μm removable mesh sieve. Microplastics and food were then manually separated with tweezers. The leftover food was weighed, and its wet weight was recorded as "food leftover" (FL), while the microplastics were dried in an oven at 50ºC for 24 hours, weighed as "plastics leftover" (PL), and stored in Eppendorf tubes. The FL values were used to calculate the "food consumed" ($FC = FP - FL$).

On the following morning, prior to the next feeding, each tank was cleaned again by siphoning to collect all feces. These samples which included the digested microplastics (Pdig) were preserved in Falcon tubes containing 70% ethanol until further analysis, which included quantifying and identifying the ingested and excreted microplastics.

Task 3. Statistical analysis

All statistical analyses were performed using the RStudio program (version 4.4.2). A p-value of 0.05 was considered to interpret statistical differences.

Descriptive statistics for all seahorse parameters measured were expressed as mean ± standard deviations. This approach was also applied to the length and concentration of microplastics (when averaged), as well as dietary assessments when applicable.

The Shapiro-Wilk test was performed to assess the normality of the data prior to proceeding with further statistical analyses. For the comparison of two variables, the Wilcoxon test was utilized for non-parametric data, while the t-test was applied for parametric data. When comparisons involved three or more variables, the Kruskal-Wallis test was used, followed by pairwise comparisons using the pairwise Wilcoxon test if significant differences were detected.

Associations between qualitative variables were examined using Pearson's Chi-squared test. To evaluate correlations between quantitative variables, Pearson's product-moment correlation was applied for normally distributed data, and Spearman's rank correlation was used for non-normal distributions.

Results

Task 1. Sample collection in the natural environment

1.1. Quantitative and qualitative analysis of inert components collected from in situ benthic and supra-benthic samples

A total of 337 microplastics (MPs) were collected from the three substrate samples. The sediment sample contained a significantly higher concentration of MPs (66%, 221 MPs) compared to the *Caulerpa* sample (26%, 88 MPs) and the seagrass sample (8%, 28 MPs) ($p < 0.05$; Fig. 11). The estimated MP concentration per substrate type was 232 MPs/m² for sediment, 91 MPs/m² for *Caulerpa*, and 23 MPs/m² for seagrass (Table 1).

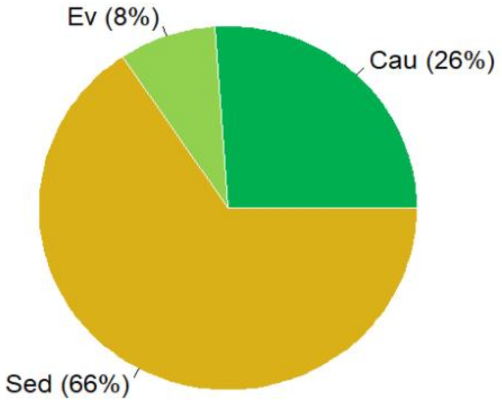

Figure 11: Proportion of microplastics (MPs) collected in the three different substrates: Sediment (Sed), *Caulerpa* (Cau), and Seagrass (Ev).

The collection method "Core" yielded a significantly higher number of MPs (77%, 258 MPs – 20% Cau, 5% Ev and 51% Sed), despite sampling a considerably smaller area (0.75 m² per substrate). In contrast, the dragging method ("A") retrieved a lesser quantity of MPs (23%, 79 MPs – 6% Cau, 3% Ev and 14% Sed) over a larger area of 50 m² per substrate ($p < 0.05$; Fig. 12).

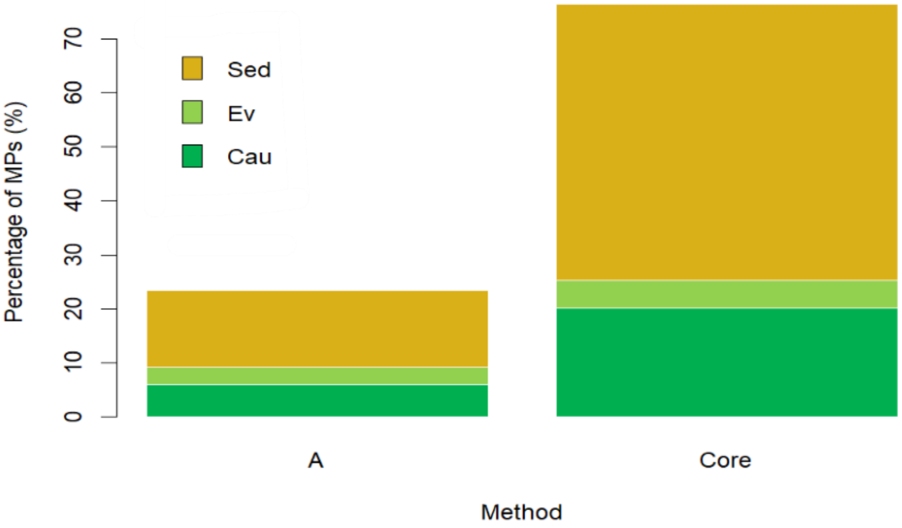

Figure 12: Comparison of the percentage of microplastics (MPs) collected using two sampling methods – drag sampling (A) and the three replicated substrate samples (Core) – each one across the three substrates (Sediment (yellow), Seagrass (light green), and *Caulerpa* (green)).

Two distinct types of MPs were identified: fibers (fib) and fragments (frag) (Fig. 13). Notably, fibers represented the predominant type of collected MPs (76% of the total, 255 MPs), while fragments comprised a smaller fraction (24%, 82 MPs) (Fig. 14). The estimated concentration (Table 1) of fibers is 131.5 in sediment, 87.1 in *Caulerpa*, and 22.9 fibers/m² in seagrass. For fragments, the concentrations are 100.1, 4, and 0 fragments/m², respectively.

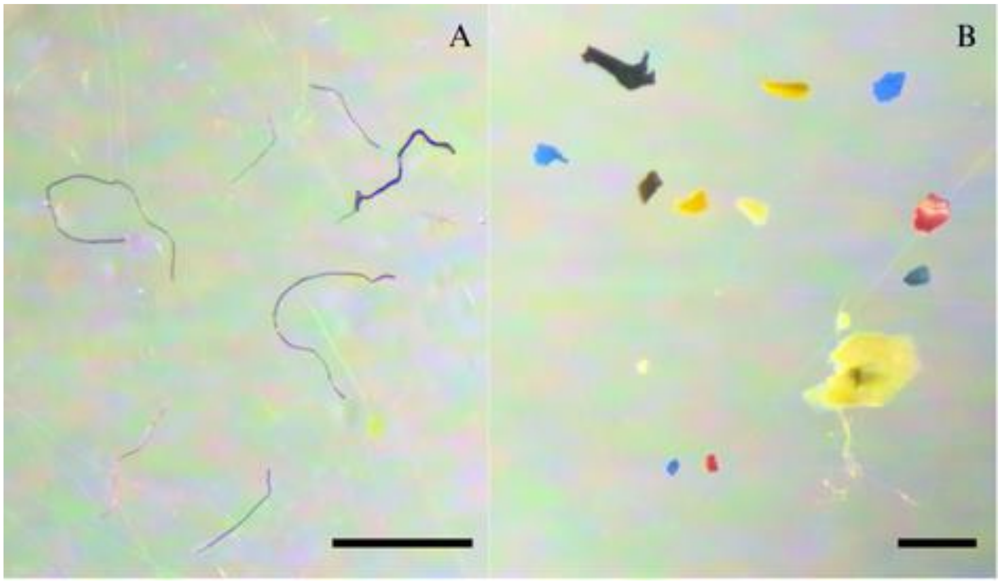

Figure 13: Two distinct types of microplastics (MPs) identified under a stereoscope: (A) fibers (fib), and (B) fragments (frag). The black scale bars represent one millimeter.

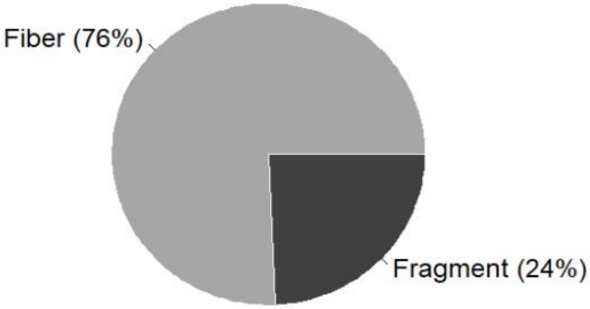

Figure 14: Proportion of microplastic (MP) types identified across the substrate samples: fibers (fib) and fragments (frag).

A significant relationship was observed between substrate type and microplastic (MP) type ($p < 0.05$; Fig. 15), as well as between collection method and MP type ($p < 0.05$; Fig. 16). Regarding substrate types, sediment contained the highest number of fragments (23%, 79 MPs), whereas *Caulerpa* and seagrass contained significantly fewer, with only three (1%, 3 MPs) and none (n=0), respectively. Fibers were present in all substrate types, following a similar pattern: 142 fibers in sediment (42%), 85 in *Caulerpa* (25%), and 28 in seagrass (9%). For collection methods, the estimated MP concentrations were markedly different. Method “A” yielded 0.5 ± 0.3 fibers/m^2 (22%, 75 MPs) and only 4 fragments (2%, 0.0 ± 0.0 fragments/m^2). In contrast, the “Core” method resulted in significantly higher values, with 80.0 ± 54.3 fibers/m^2 (53%, 180 MPs) and 34.7 ± 56.6 fragments/m^2 (23%, 78 MPs).

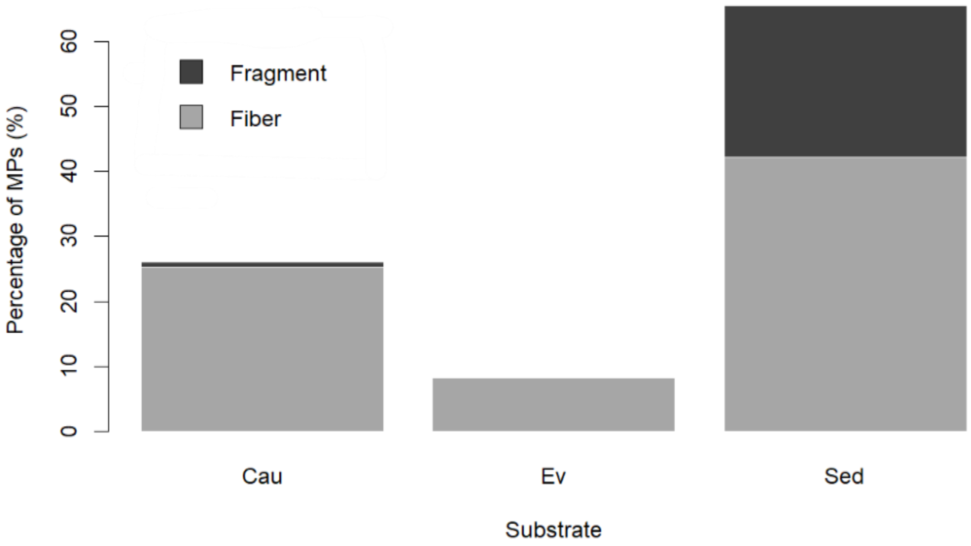

Figure 15: Percentage of microplastics (MPs) by type - fragment (dark grey) and fiber (grey) - across three substrates: *Caulerpa* (Cau), Seagrass (Ev), and Sediment (Sed).

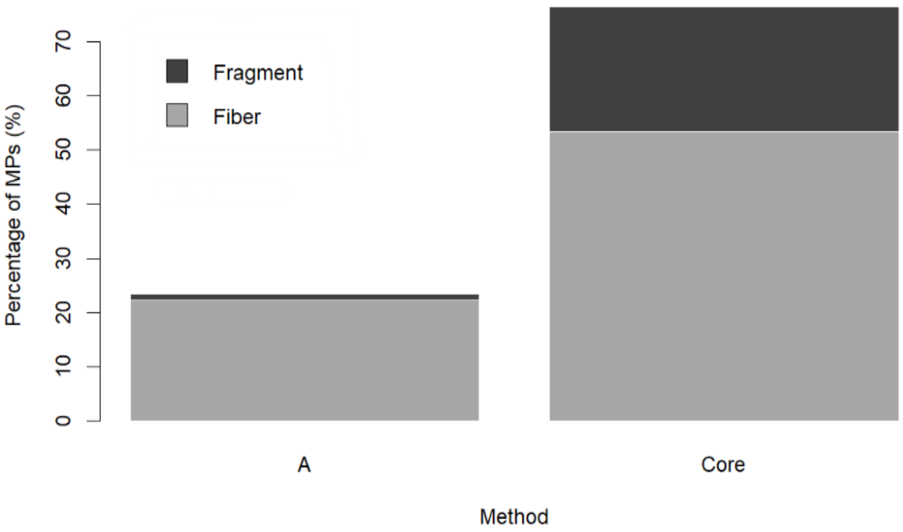

Figure 16: Percentage of microplastics (MPs) by type - fragment (dark grey) and fiber (grey) – per sampling method: drag sampling (A) and the three replicated substrate samples (Core).

In terms of colour distribution, eight different MP colours were identified (Fig. 17, 18), with black being the most abundant (44%, 149 MPs), followed by blue (37%, 124 MPs) and red (11%, 37 MPs). The remaining colours, including green (1%, 4 MPs), brown (0%, 1 MP), white (2%, 6 MPs), yellow (3%, 9 MPs), and transparent (2%, 7 MPs) MPs (Fig. 19), accounted for smaller percentages and were grouped into the "others" category (8%, 27 MPs). The estimated concentration across the three substrates was 50.5 ± 34.4 black MPs/m², 40.7 ± 43.2 blue MPs/m², and 13.4 ± 17.7 red MPs/m², while the "others" category accounted for 10.7 ± 14.1 MPs/m² (Table 1).

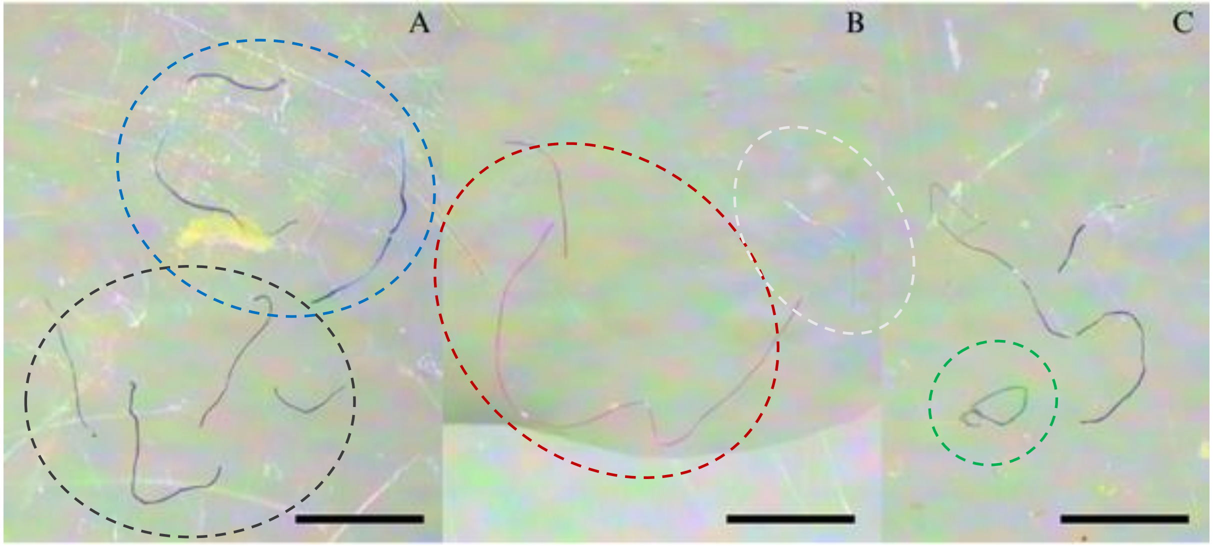

Figure 17: Fibers of different colours identified under the stereoscope. Dashed lines indicate colour groupings, with (A) showing black and blue fibers, (B) presenting red and transparent fibers, and (C) green fibers alongside other black fibers. The black scale bars represent one millimeter.

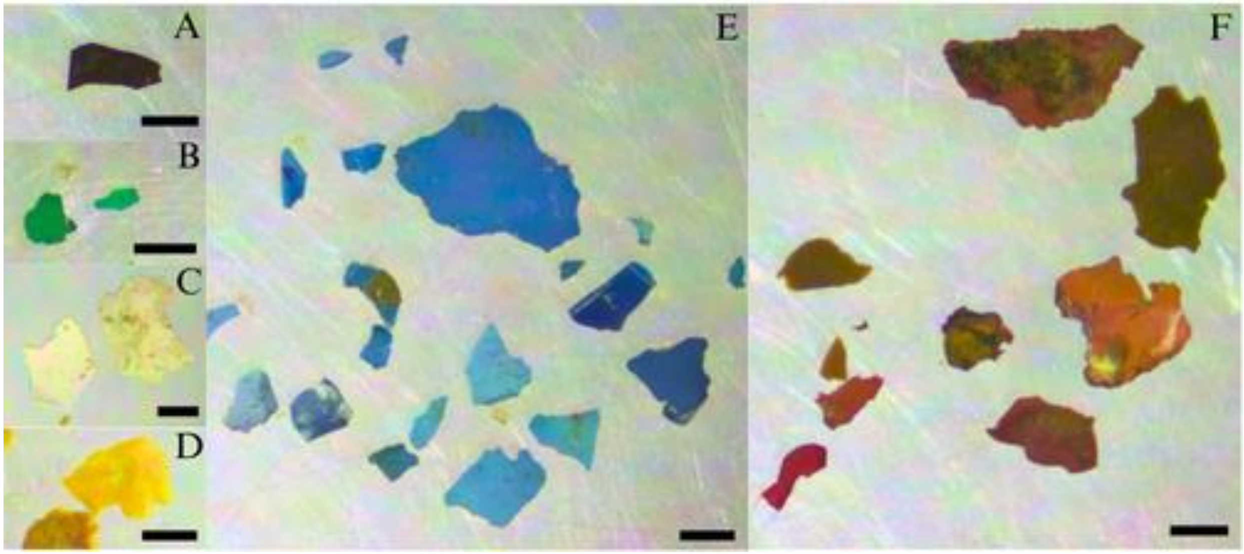

Figure 18: Fragments of different colours identified under the stereoscope: (A) black, (B) green, (C) white, (D) yellow, (E) blue, and (F) red fragments. The black scale bars represent one millimeter.

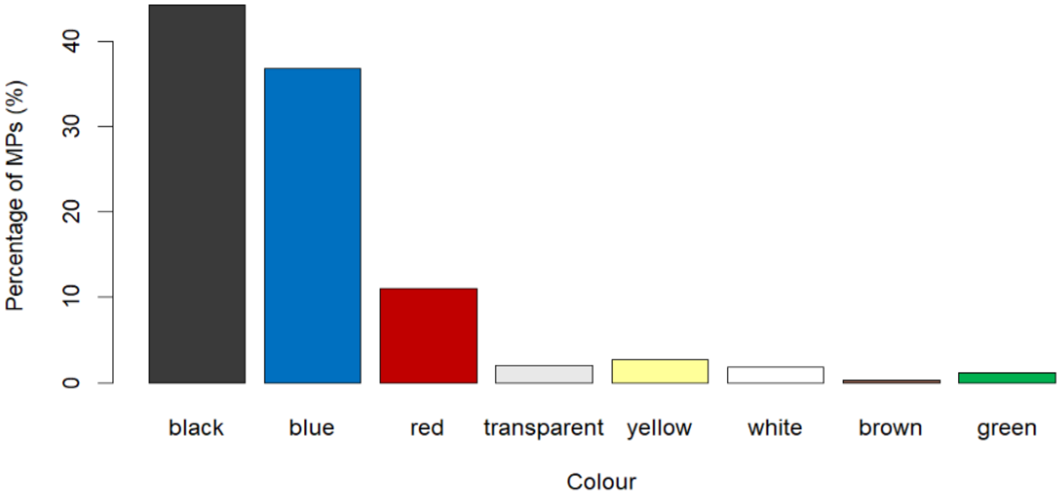

Figure 19: Percentage distribution of microplastics (MPs) colours identified across the substrate samples.

Significant relationships were also identified between MP colour and substrate type ($p < 0.05$; Fig. 20), as well as between MP colour and type ($p < 0.05$; Fig. 21). In the case of *Caulerpa*, black MPs represented the highest proportion (18%), followed by blue MPs (6%). Red and other colored MPs were present in lower proportions, each accounting for 1%. In seagrass samples, black MPs again predominated, comprising 5%, while blue MPs accounted for 2%. As with *Caulerpa*, red and other MPs remain at 1% each. Sediment analysis revealed a different distribution pattern: blue MPs were most prevalent at 29%, followed by black (21%), red (9%), and other colours (7%). When MP color was analyzed by type, fibers were predominantly black (43%), followed by blue (25%), red (5%), and other colors (3%). In contrast, fragments were mostly blue (approximately 12%), with red at around 6%, other colors at about 5%, and black being the least common at 1%.

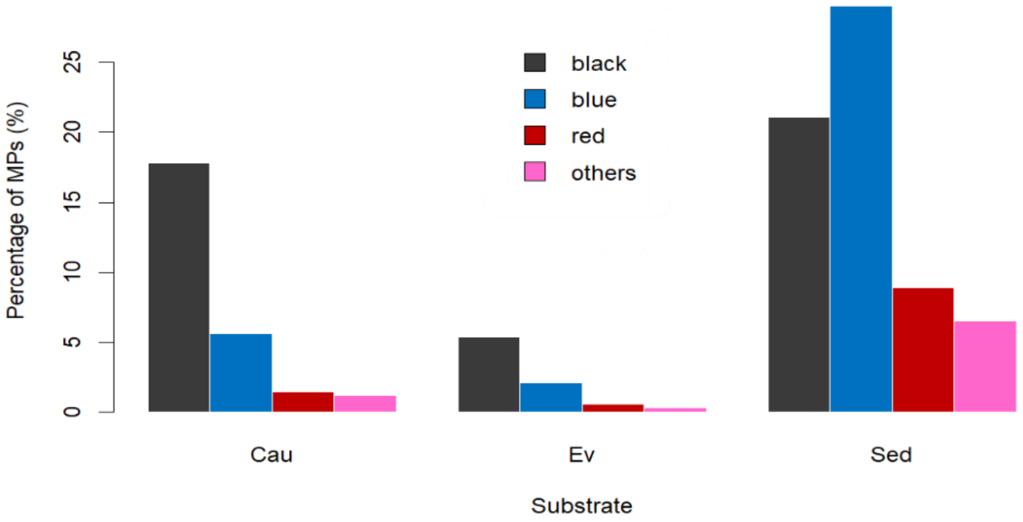

Figure 20: Percentage of microplastics (MPs) by colours (black, blue, red, and others) across three substrates: *Caulerpa* (Cau), seagrass (Ev), and sediment (Sed). The category "others", represented by the pink colour, includes transparent, yellow, white, brown, and green MPs.

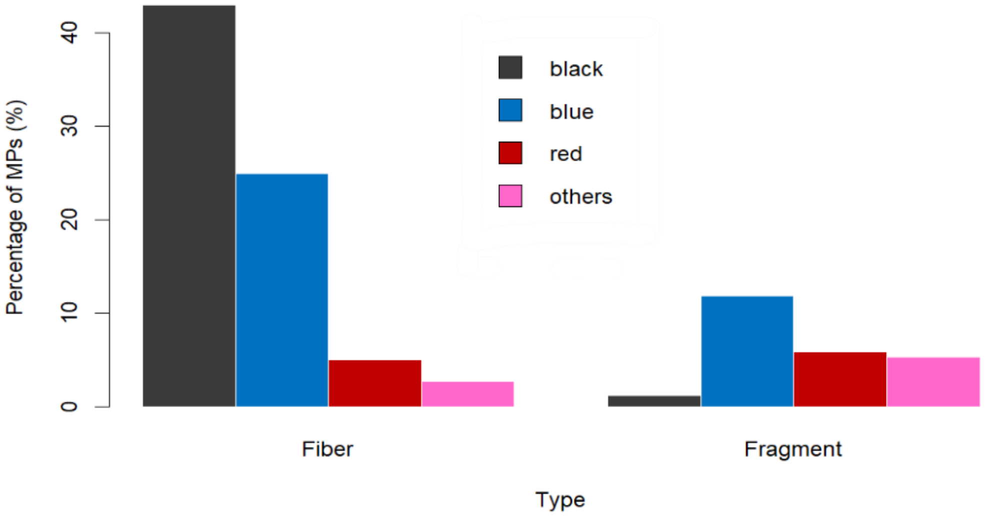

Figure 21: Percentage of microplastics (MPs) by colour (black, blue, red, and others) for two types (fiber and fragment). The category "others", represented by the pink colour, includes transparent, yellow, white, brown, and green MPs.

Table 1: Microplastic (MP) concentration per square meter across different categories: sampling method (drag sampling (A) and replicated substrate samples (Core)); microplastic type (fiber or fragment); and microplastic color (black, blue, red, and others (including transparent, yellow, white, brown, and green MPs)). Values are presented for the three substrate types (*Caulerpa* (Cau), seagrass (Ev), and sediment (Sed)), along with the mean (Mean) and corresponding standard deviation.

		Substrate			
		Cau	Ev	Sed	Mean
MPs		91.1	22.9	231.6	115.2 ± 106.4
Method	A	0.4	0.2	1	0.5 ± 0.4
	Core	90.7	22.7	230.7	114.7 ± 106.1
Type	Fiber	87.1	22.9	131.5	80.5 ± 54.6
	Fragment	4	0	100.1	34.7 ± 56.7
Colour	Black	56.4	13.5	81.5	50.5 ± 34.4
	Blue	22.7	9.3	90	40.7 ± 43.2
	Red	6.7	0	33.4	13.4 ± 17.7
	Others	5.3	0	26.7	10.7 ± 14.1

Regarding physical dimensions, fibers exhibited a significantly greater ($p < 0.05$) average length (2.8 ± 1.9 mm) compared to fragments (1.6 ± 1.6 mm). Size distribution analysis indicated that the majority of both microplastic (MP) types measured less than 4 mm in length (Fig. 22). Fibers were most frequently observed in the 1 – 2 mm size range, while fragments were predominantly concentrated in the 0 – 1 mm range.

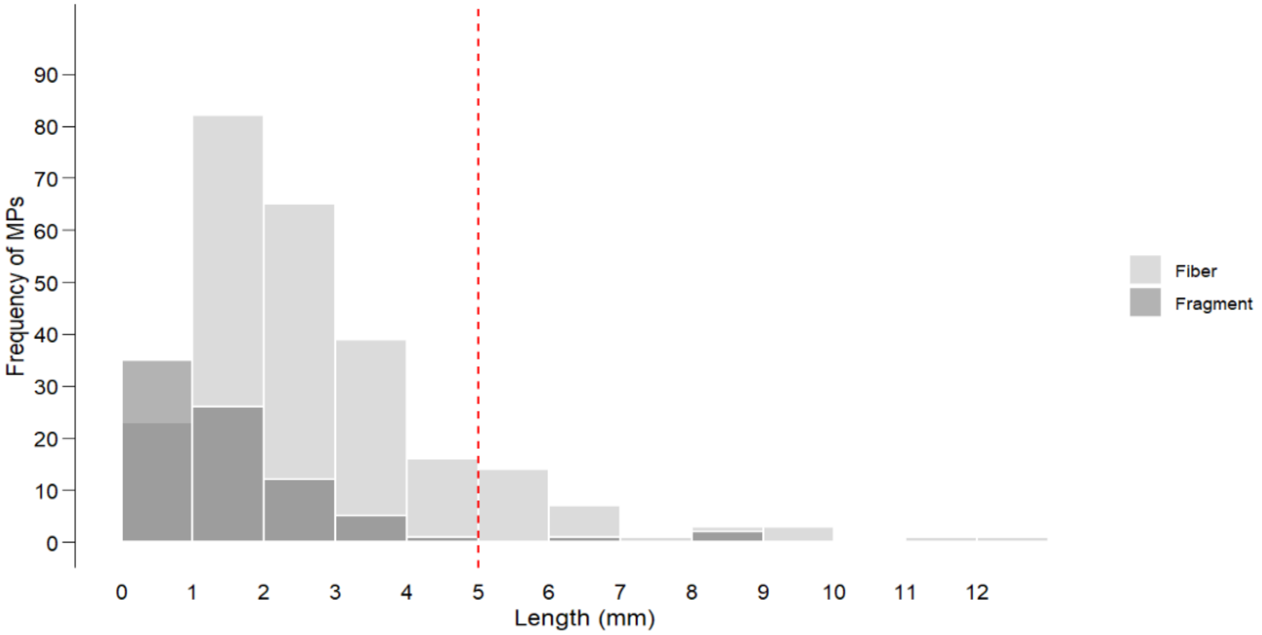

Figure 22: Frequency distribution of microplastic (MP) lengths, in millimeters (mm), by type: fiber (light grey) and fragment (dark grey). The red dashed line represents the threshold separating microplastics (<5 mm) from larger plastics (meso- and macroplastics).

1.2. Quantitative and qualitative analysis of inert components in the gastrointestinal tract contents of wild seahorses

A total of 40 individuals (18 females and 22 males) were collected for sampling. The mean total length was 16.9 ± 1.2 cm, while the mean weight was 11.6 ± 3.1 g. The mean CF was 0.4 ± 0.1.

MPs were present in all individuals examined, with a total of 291 MPs identified and an average of 7.3 ± 3.5 MPs per gastrointestinal tract (GIT), ranging from 1 to 17 MPs per individual. A statistically significant positive correlation ($p < 0.05$) was established between the number of MPs detected and the length of the individuals, while no significant differences were observed between males and females or between individuals regarding MP counts ($p > 0.05$).

Fibers constituted the predominant type of MP within the GIT (95%, 277 MPs), while fragments represented only 5% (14 MPs).

Five colours of MPs were identified: black, blue, red, transparent, and green (Fig. 23). Among these, black was the most abundant (56%, 164 MPs), followed by blue (35%, 101 MPs). The remaining colours had lower percentages (red: 4%, transparent: 3%, green: 2%) and were grouped into the “others” category for the purpose of statistical analysis.

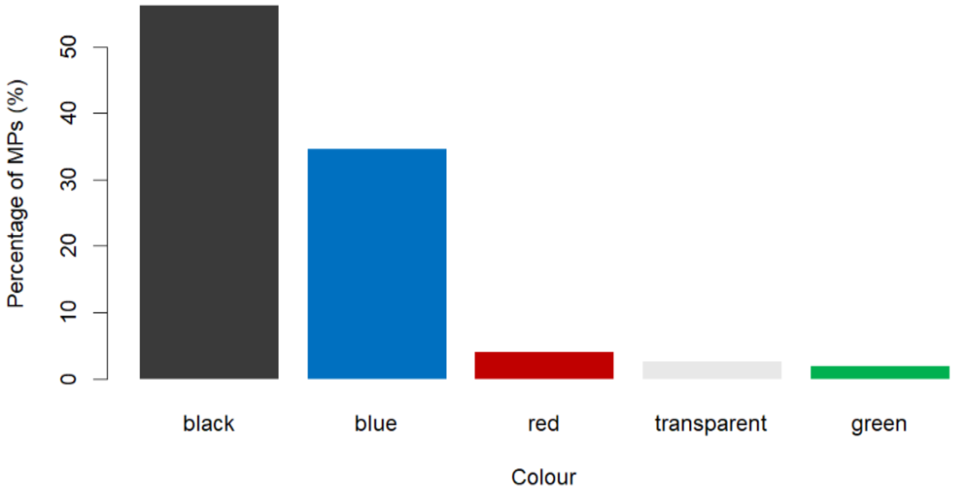

Figure 23: Percentage distribution of microplastics (MPs) found in seahorses’ gastrointestinal tract (GIT) by colour.

A significant relationship was noted between the type of MP and its colour ($p < 0.05$, Fig. 24). Fibers were predominantly black, comprising 54% of the total sample, followed by blue (35%), and a smaller fraction of the "others" (7%). In contrast, fragments were much less frequent with no blue fragments observed and both black and the other colours recorded at only 2% each.

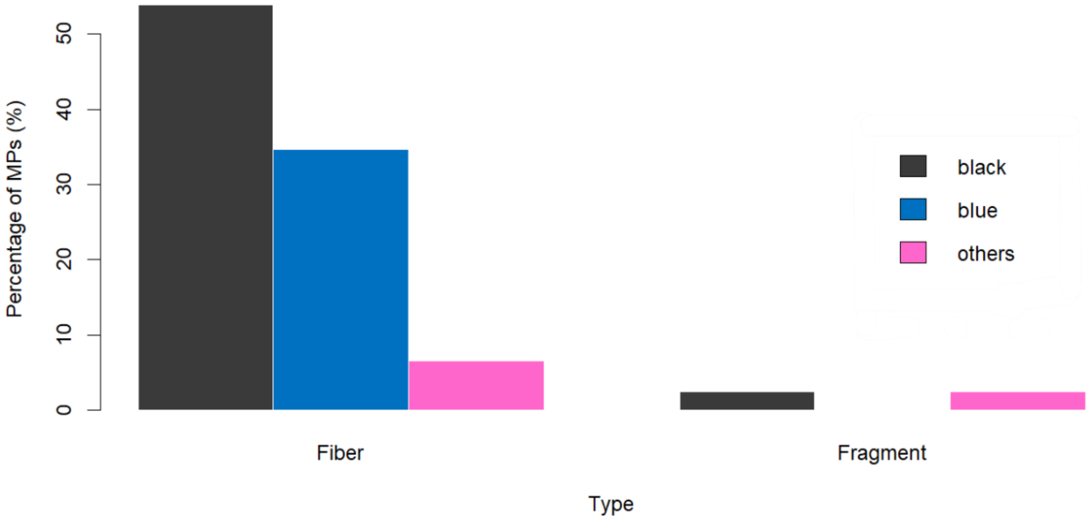

Figure 24: Percentage distribution of microplastics (MPs), found in seahorses' gastrointestinal tract (GIT), by colour (black, blue, and others) for two types (fiber and fragment). The category "others", represented by the pink colour, includes red, transparent, and green MPs.

The average length of MPs was 2.8 ± 3.6 mm, ranging from 0.1 to 40.0 mm, with the majority to be less than or equal to 6 mm in length (Fig. 25). Significant differences were observed between the types of MPs ($p < 0.05$), with the size range of 1 – 2 mm containing the highest number of MPs. No statistically significant differences in MP lengths were identified across different GITs ($p > 0.05$).

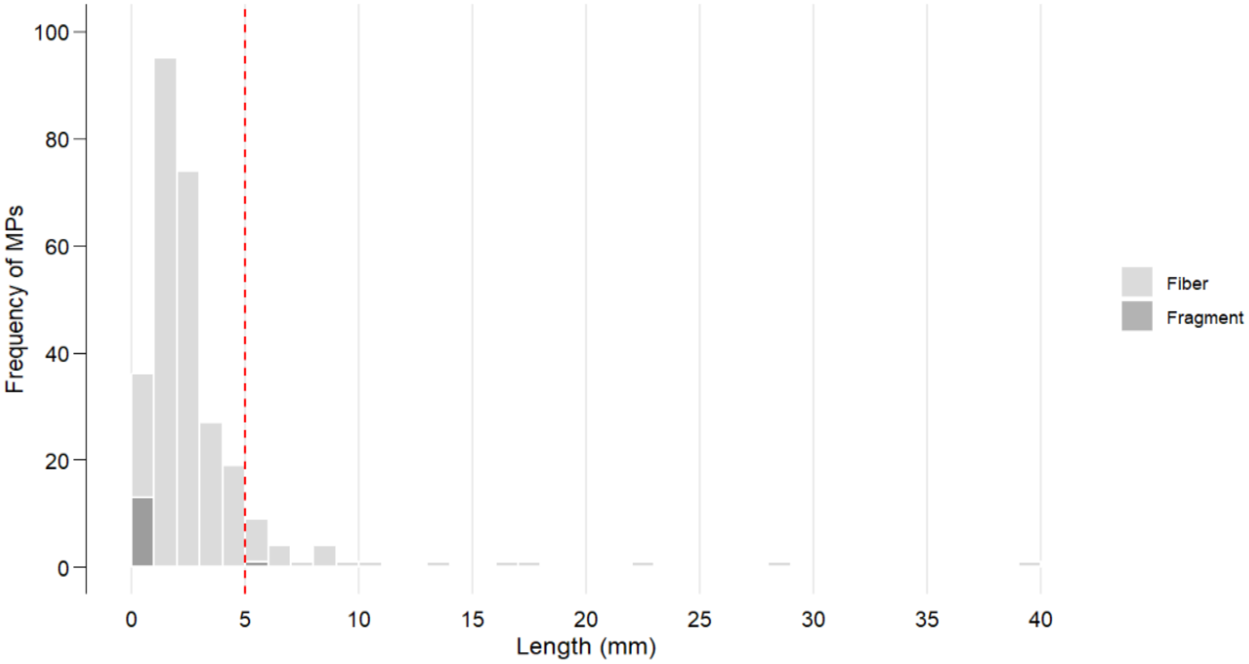

Figure 25: Frequency distribution of microplastic (MP) lengths, in millimeters (mm), by type: fiber (light grey) and fragment (grey). The red dashed line represents the threshold separating microplastics (<5 mm) from larger plastics (meso- and macroplastics).

Task 2. Ingestion and retention of microplastics in captive *H. guttulatus*

In this study, 18 individuals were used in the experimental trial. The average total length was 18.8 ± 1.7 cm, while the initial weight was 16.0 ± 4.0 g, decreasing slightly to 15.5 ± 4.5 g by the end of the experiment. The mean condition factor (CF) was 0.4 ± 0.1. Although a slight decrease was observed by the end of the study, the difference was not statistically significant ($p > 0.05$).

The proportion of food consumed (FC) was assessed in relation to the quantity provided (FP) for each individual. Average food consumption was 84.9 ± 14.8% for live food and increased to 93.1 ± 12.8% for frozen food, with the difference being statistically significant ($p < 0.05$; Fig. 26).

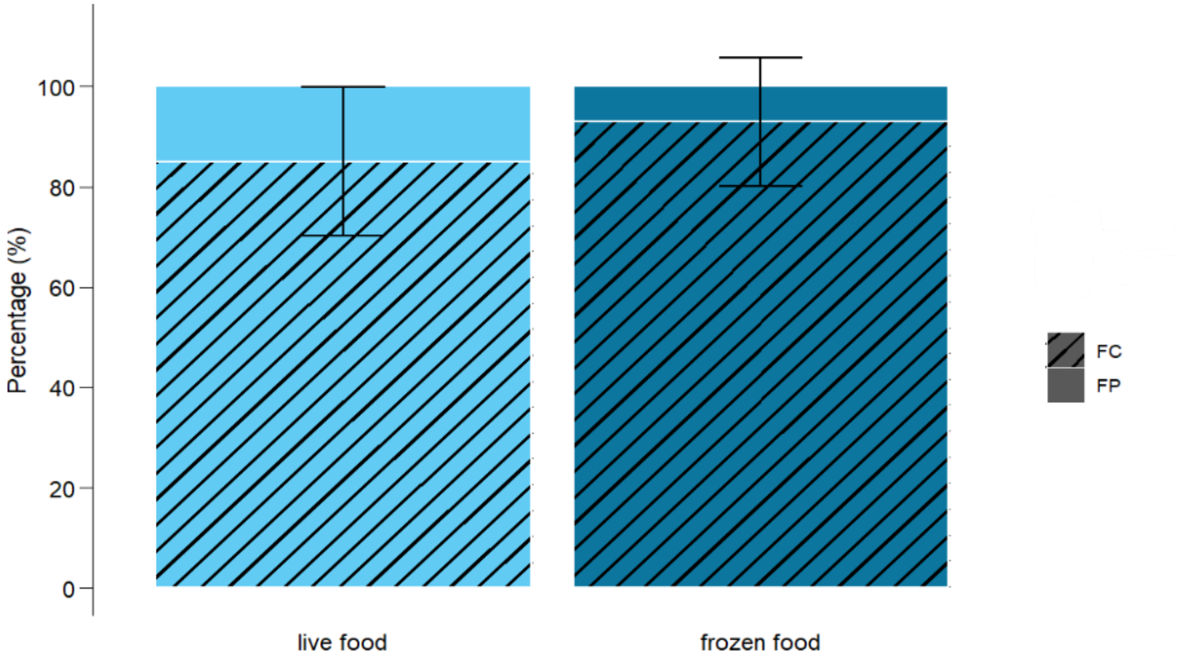

Figure 26: Percentage of food provided (FP; plain) and consumed (FC; stripes) by food treatment: live food (light blue), during the first week, and frozen food (dark blue), during the second week.

The daily average of each type of microplastic provided (PP) to each animal was 81.2 ± 11.5 MPs in total – 20.1 ± 2.7 fibB, 20.2 ± 3.2 fibR, 2.9 ± 0.4 fragB, 2.2 ± 0.4 fragD, 32.0 ± 4.1 fragR, and 3.8 ± 0.4 fragW. To estimate the total exposure over the 10-day experiment, these values were multiplied by 10.

The analysis of microplastic consumption (PC) revealed that some individuals, on certain days, reported an intake of microplastics that was lower than the quantity excreted from previously ingested microplastics. Consequently, these instances resulted in negative outcomes and were excluded from further analysis, being categorized as non-applicable (NA) to preserve the integrity of the statistical evaluation. Despite observing variations in PC, statistical analyses revealed no significant differences in the intake of microplastics between the two diets (live *versus* frozen food) ($p > 0.05$, Fig. 27), nor in the total PC among the 18 individuals ($p > 0.05$).

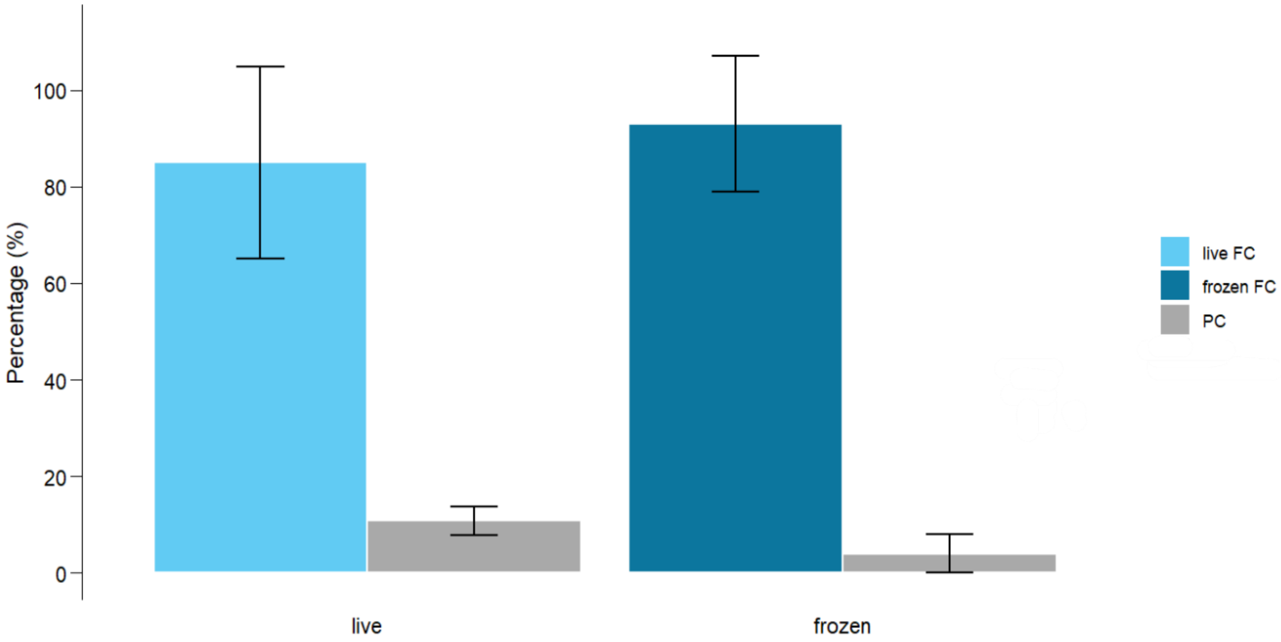

Figure 27: The proportion of food (FC) and plastic (PC; grey) consumed between the live (light blue) and frozen (dark blue) food treatments.

Regarding the excreted microplastics (Pdig), collected in the morning, their proportion was calculated relative to the amount of microplastics provided (PP). The average total microplastic consumption was 3.5 ± 3.5%, while the average per individual was 4.9 ± 1.4%. No statistically significant differences were observed ($p > 0.05$; Fig. 28).

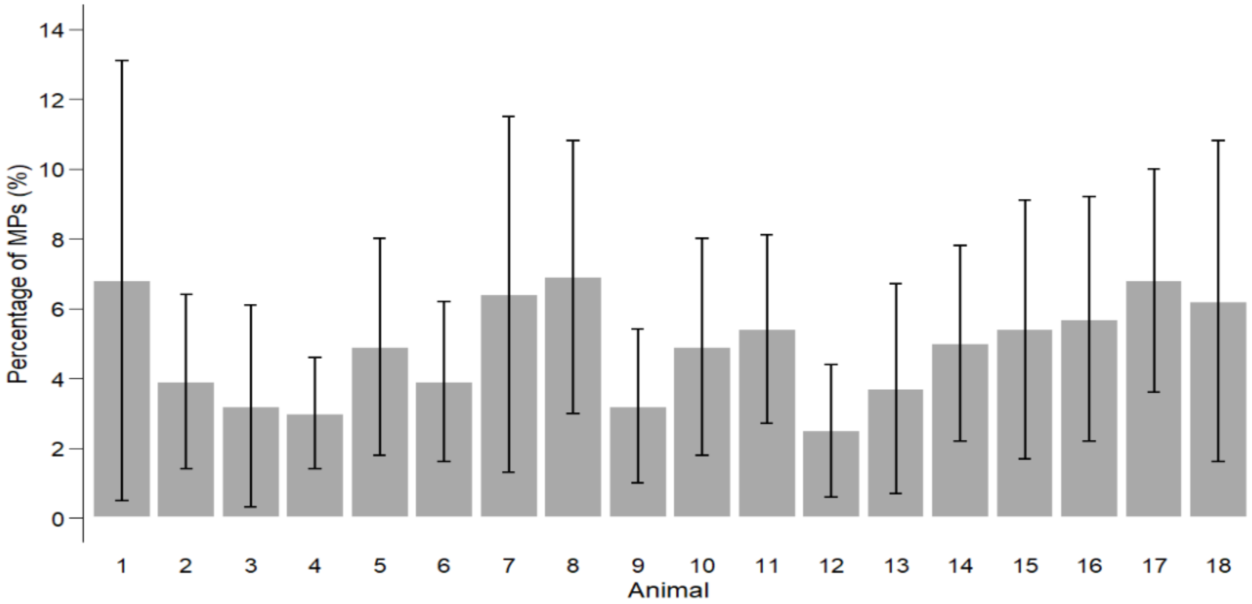

Figure 28: Percentage of MPs excreted (Pdig) in total by each animal.

The highest mean percentage of ingestion was observed for fibB (5.7 ± 2.5%), followed by fibR (5.5 ± 1.8%) and fragR (4.9 ± 1.8%). Lower ingestion percentages were recorded for fragD (2.6 ± 1.8%) and fragW (2.2 ± 1.8%), while fragB were not ingested by any individual (Fig. 29). Significant differences ($p < 0.05$) were observed among the types of microplastics consumed, with the exception of the fiber types (fibR and fibB) and between the black and white fragments (fragD and fragW). In addition, it was observed that most white fragments (fragW) retrieved from the fecal samples in the morning had changed to an orange hue (Fig. 30).

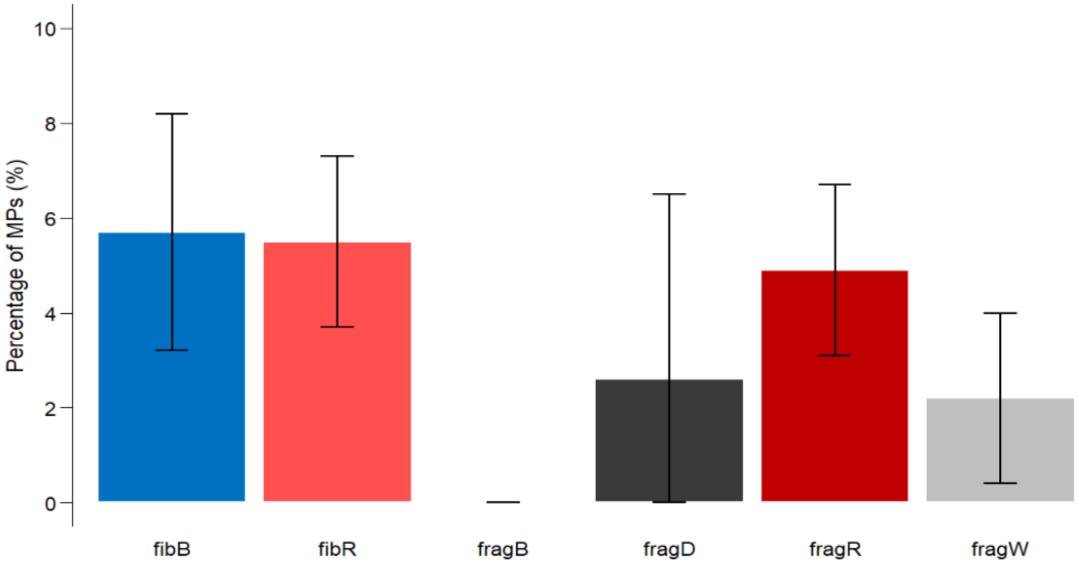

Figure 29: Percentage of MPs types (blue fiber (fibB), red fiber (fibR), blue fragment (fragB), black fragment (fragD), red fragment (fragR) and white fragment (fragW) excreted (Pdig) in total.

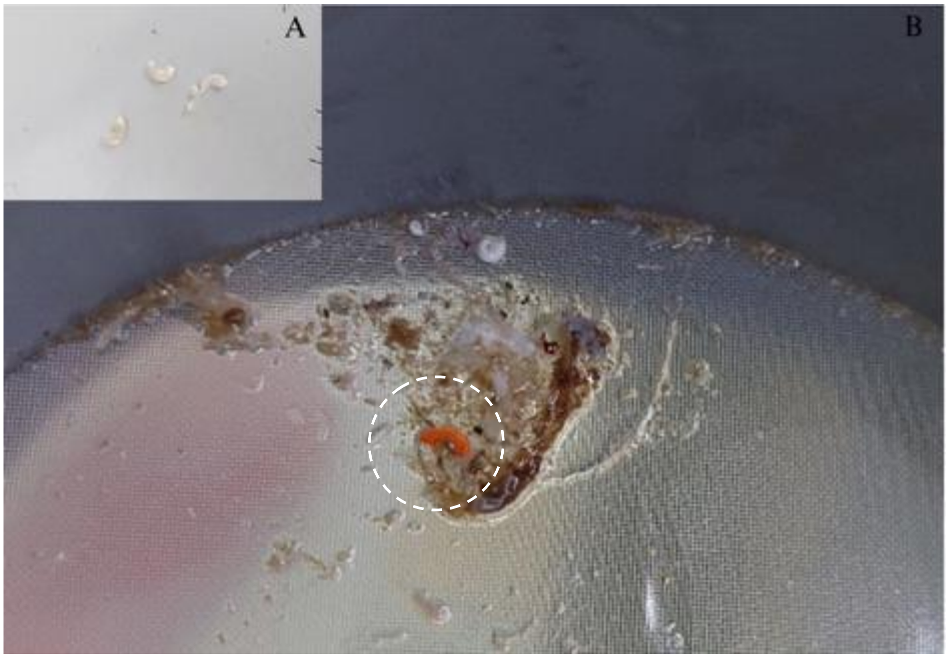

Figure 30: Fecal sample collected during the experiment. (A) White fragments prior to being provided to the seahorses and later (B) the same type of microplastic collected from feces (highlighted by the dashed white circle), showing an orange coloration after passage through the digestive tract.

Discussion

The proliferation and accumulation of macro- and microplastics on shorelines and sediments have intensified dramatically over recent decades (Barnes et al., 2009; Thompson et al., 2004). This alarming trend is closely associated with the growth of coastal populations alongside the escalation of various anthropogenic activities, including fisheries, aquaculture, energy production, maritime commerce, tourism, and recreational pursuits (Newton et al., 2022). Collectively, these activities are major contributors to marine pollution, which poses extensive implications for marine life (Newton et al., 2022).

Seahorses are unanimously considered as flagship species, granting them considerable relevance as bioindicators for accessing structural habitat impacts and biodiversity conservation (Liu et al., 2021; Vincent et al., 2011). Environmental hazards occurring in coastal waters, such as oil spills and chemical waste deposition pose a great threat to seahorses (Liu et al., 2021; Qin et al., 2020; Tang et al., 2021a). They have become a suitable model for assessing the possible risks associated with marine environmental contaminants because of their unique life history and widespread coastal distribution (Vincent et al., 2011). This study employed the long-snout seahorse as a model organism to evaluate microplastic pollution within the Ria Formosa region. The primary research questions addressed include the quantification of microplastics (MPs) in the surrounding environment, which illustrates the exposure levels experienced by seahorses; the presence and concentration of MPs within the gastrointestinal tracts (GITs) of wild seahorses, which reflect ingestion rates; and, under controlled conditions, the mechanisms of ingestion (whether voluntary, accidental, or both), the quantities ingested, and any evidence of accumulation.

A key factor influencing the concentration of plastic debris is anthropogenic pressure, as population growth coupled with increasing human activities (such as intensive fishing, recreational uses of marine environments, and demographic shifts favoring coastal migration) are predicted to further increase the influx of plastic waste into marine ecosystems (Newton et al., 2022; Bellas et al., 2016; Andrary, 2011). Once introduced into aquatic systems, microplastics may initially float but can subsequently sink and accumulate on substrates over time (Andrady, 2011; Barnes et al., 2009). Although Ria Formosa is designated as a biodiversity hotspot, it remains vulnerable to significant human pressures (Bebianno et al., 2021). Intense fishing and tourism activities contribute to pollution, with waste entering the lagoon through multiple pathways, including discharges from wastewater treatment plants (Bebianno et al., 2021). The lagoon's restricted hydrodynamics exacerbate pollutant entrapment, intensifying environmental stress on the ecosystem (Bebianno et al., 2021). A study on microplastics in coastal sediments from the Southern Portuguese shelf waters found that the overall percentage of microplastics per sediment volume was 0.24% (Frias et al., 2016). A comparison with our results suggests a potential increase in microplastic concentration over time. When assessing the presence of plastics across three different habitat substrates within the Ria Formosa, microplastics were detected in all sampled environments, with sediments showing the highest concentrations. This observation corroborates existing literature which reports sediment as a hotspot for MP accumulation (Bellas et al., 2016; Browne et al., 2011). The higher concentration of MPs in sediment may also be attributed to sampling efficiency, as the relative absence of vegetation and other obstructive elements permits superior retrieval effectiveness compared to algal or seagrass substrates.

Among the microplastic types observed, fibers emerged as the most abundant, supporting findings from other investigations that identify fibers as predominant constituents of marine microplastic pollution (Thompson et al., 2004; Browne et al., 2011; Zhao et al., 2018; Gao et al., 2023; Frias et al., 2016). The potential sources of these fiber materials include hygiene and cosmetic products, textiles, and material from the fishing industry (e.g., ropes and nets) (Andrady, 2011; Browne et al., 2011). Considering the Ria Formosa's association with municipal discharges and intensive fishing activities, it is not surprising that fibers represented the most frequently observed MPs. In Frias et al. (2016), 80.6% of the collected microplastics were identified as fibers, whereas Bebianno et al. (2023) reported a slightly lower proportion of 68%. Fragments were present in both studies but consistently represented a smaller share of the total microplastic composition – 19.4% and 29% respectively.

In terms of microplastic coloration, this study identified three main colours: black, blue, and red, with black being the most prevalent, followed by blue. However, when comparing these findings with other sediment studies, this trend does not appear to be entirely similar. Gao et al. (2023) reported that the majority of MPs found in sediments were blue (47%), followed by transparent (38%), black (10%), and red (4%). Similarly, Bebianno et al. (2023) found that most microplastics in sediments were either blue or transparent, with 59.2% of fragments being transparent or blue. Meanwhile, fibers, despite predominantly being transparent (37%), were also found in notable quantities in black, blue, and green. It is important to note that distinguishing microplastic colours occasionally presented challenges due to limitations in the stereoscope resolution and precision, which may have affected colour classification accuracy. Another important factor to consider is the potential chemical alterations, such as oxidation, which can lead to colour changes in MPs over time due to prolonged environmental exposure (Andrary, 2011; Younis & Elkady, 2024).

In terms of size classification, both mesoplastics (> 5 mm) and microplastics (< 5 mm) were detected, with the majority measuring less than 5 mm. The most prevalent size range was 0 – 1 mm. Fibers were most frequently observed in the 1 – 2 mm size range, whereas fragments were predominantly concentrated in the 0 – 1 mm range. The findings presented by Gao et al. (2023) align with our results, as 45% of the microplastics found in sediment in their study measured less than 1 mm, followed by the 1 – 2 mm category at 29%, with only 2% of plastics exceeding 5 mm. Similarly, Bebianno et al. (2023) reported that 98% of plastics found in sediment fell within the microplastic category (< 5 mm), with just 2% surpassing this threshold. It is imperative to consider that some fragments may have broken down during handling, potentially affecting both the size distribution analysis and the counting accuracy. Regarding the fibers, most exhibited varying degrees of folding or curling, thereby complicating efforts to measure them accurately, as sizing was conducted in sections rather than in a straightened form.

Upon a detailed analysis of different studies conducted on microplastic quantification in marine substrates, it becomes apparent that variations in methodologies used and discrepancies in result presentation pose substantial challenges for direct comparisons. These divergences can either mask or amplify trends in the abundance of marine plastic, thereby complicating efforts to assess contamination levels accurately. To advance the field, a concerted effort towards the standardization of both methodological and analytical techniques is imperative. Such standardization would enhance the comparability of research findings, ultimately facilitating a more robust and comprehensive understanding of microplastic pollution in marine substrates.

The escalating abundance of microplastic in marine environments has implications for their bioavailability and the subsequent exposure experienced by marine organisms (Vroom et al., 2017; Liu et al., 2022; Botterell et al., 2019). Smaller MPs are more easily encountered and ingested by both lower trophic organisms and higher trophic planktivorous species, which might ingest MPs passively during normal feeding behavior or through misidentification as natural prey (Wright et al., 2013; Vroom et al., 2017). Moreover, demersal species are particularly susceptible to the accumulation of MPs compared to pelagic species, posing a significant threat to bottom-dwelling aquatic organisms such as seahorses, in this region (Onay et al., 2023). This variability in exposure levels within their environment is closely tied to MP ingestion (Vroom et al., 2017).

Hippocampus guttulatus can be found across all three sampled habitats within the Ria Formosa, however, its abundance is much lower in seagrass meadows and *Caulerpa* beds compared to sediment substrates. For this reason, individuals sampled for MP ingestion analysis, based on gastrointestinal tract (GIT) content, were exclusively collected from sediment habitats. Sampling individuals from the other two habitats in statistically meaningful numbers would have required a substantially larger survey area or sampling of multiple identical sites, both of which were not feasible within the scope of this study. Nevertheless, this approach provides a representative insight into what might be expected in other habitats. Given that behavioral patterns are transversal across individuals inhabiting different environments, it is reasonable to assume that MP ingestion rates are proportional to their prevalence in each habitat. Thus, the collection of specimens from a single location can be interpreted as a case study – serving as a proxy for broader ecological realities encountered by these species in their natural environments.

To our knowledge, the presence of microplastics (MPs) in gastrointestinal tracts (GITs) of seahorses has not been previously studied in Ria Formosa, preventing any comparative analysis. In the present investigation, MPs were detected in the GITs of all sampled individuals, with no significant differences observed between males and females or among individuals ($p > 0.05$). However, a positive correlation was identified between the quantity of ingested MPs and the total length of the seahorses ($p < 0.05$), which has also been reported in other fish study (Boerger et al., 2010). In contrast, Onay et al. (2023) found a positive correlation between seahorse weight and MP consumption, a pattern that was not observed in our study. These findings lend support to the hypothesis that MP ingestion may depend on the combination of availability and the influence of the feeding behavior (Sun et al., 2019; Chen et al., 2022) of seahorses, characterized by suction feeding that might be less selective (Foster & Vincent, 2004; Lazic et al., 2023). While it is evident that seahorses can directly ingest MPs, it is also plausible that they may acquire these particles indirectly through contaminated prey or sediment – a behavior documented in others seahorse studies (Onay et al., 2023; Jinhui et al., 2019) and other demersal feeders that ingest sediment (where MPs accumulate) during feeding (McGoran et al., 2017). Despite our analysis in this task did not ascertain whether the MPs were consumed directly or via trophic transfer within the food web for wild seahorses, the experimental trial can reveal whether the ingestion is accidental or voluntary, providing clues about what happens in the natural environment.

Our results indicated that fibers constituted the predominant type of ingested MPs, consistent with exposure observations in this study and corroborated by previous research, which recognize fibers as the most likely type of MP ingested by fish inhabiting coastal waters, with reported values ranging from 67% to 97% (Onay et al., 2023; Sun et al., 2019; Bellas et al., 2016; Lusher et al., 2013; Ugwu et al., 2021; Gao et al., 2023).

Regarding colour of ingested MPs, black and blue were found to be the most common, which aligns with substrate findings from the environmental sampling. Notably, blue fragments were absent from the GIT contents. These results are consistent with other studies reporting black MPs as the predominant type found in the GITs of seahorses (61%) (Onay et al., 2023) as well as in other demersal fish species (with values between 45.4% and 76%) (Bellas et al., 2016; Lusher et al., 2013; McGoran et al., 2017). Additionally, some studies report a high occurrence of blue MPs, with proportions of 90% (Gao et al., 2023) and 28.1% (Ugwu et al., 2021).

In terms of size, most MPs detected in GITs were small to medium-sized (< 3 mm) , consistent with findings from numerous previous studies (Bellas et al., 2016; Gao et al., 2023; Lusher et al., 2013; Onay et al., 2023). Given that adult seahorses primarily feed on amphipods, decapods, isopods, and mysids (Foster & Vincent, 2004) , the relatively small MP size found in their GITs aligns with expected feeding behaviors, likely resulting from misidentification of MPs with small mysids or amphipods. Nevertheless, a diverse range of MP sizes, including mesoplastics, was recorded. This variability can be attributed to the presence of entangled fibers that, while easier to ingest, may exhibit larger dimensions upon measurement. Additionally, larger seahorses were observed to ingest bigger MPs, supporting previous studies that show a correlation between fish size and the size of ingested MPs. Larger fish tend to consume larger MPs (Sun et al., 2019), as the size of the seahorse's mouth plays a critical role in determining both the maximum and optimal prey size (Onay et al., 2023; Sun et al., 2019).

In the wild, microplastics (MPs) occurrence was observed across three substrate types in the Ria Formosa lagoon, and individuals were collected from one of these sites for gastrointestinal tract analysis. This approach provided an initial insight into the relationship between environmental MP exposure and ingestion under natural conditions. However, to better understand the specific drivers of MP ingestion, a controlled experimental framework was necessary to precisely monitor MP availability and exposure. Therefore, an experimental study was conducted in which seahorses were fed a daily mixture of food and MPs, enabling a more accurate assessment of ingestion patterns and influencing factors.

During the experimental phase of this study, no significant differences ($p < 0.05$) were observed in the condition factor (CF), indicating that the welfare of the seahorses was ensured throughout the duration of the trial. This stability in CF suggests that the behavior of the seahorses may mirror the natural patterns typically observed in the wild. Nevertheless, it is advisable to undertake a longer-term investigation to determine whether CF remains stable with prolonged exposure to microplastics. Extended exposure may potentially lead to increased ingestion and accumulation of microplastics, which could elevate physiological risks and ultimately result in a decline in the overall condition of the seahorses (Jinhui et al., 2019).

The rationale for offering live food followed by frozen food was to evaluate whether microplastic ingestion was accidental – due to reduced hunting behavior) – or voluntary, associated with active hunting behavior during live food consumption. The results showed that frozen food was more readily consumed, as it did not require seahorses to engage in hunting behaviors, unlike live food. This was particularly evident with shrimp, where seahorses often abandoned their attempts to catch live individuals after several failed tries. In contrast, frozen food settled at the bottom of the tank, making it easier for seahorses to consume via suction feeding. While frozen food was consumed in greater quantities, MP ingestion was higher when fed live food. One possible explanation is that the increased activity levels in the tank during attempts to capture live prey, leading to the resuspension of MPs, thereby elevating the likelihood of unintentional ingestion (Liu et al., 2022). Another hypothesis is that when fed inert food, seahorses consumed larger quantities of food due to the ease of capture but ingested fewer MPs because they had more time to differentiate between actual food and MPs. Conversely, when consuming live prey, the overall amount of food intake was lower due to greater capture difficulty, yet MP ingestion was higher, suggesting that some MPs may have been consumed voluntary, possibly mistaken for live prey during hunting attempts.

Regarding fiber ingestion, observations often revealed that they were sucked and ingested when seahorses intended to capture mysids, suggesting that accidental ingestion is the most plausible explanation for their presence. However, the high prevalence of fiber ingestion also raises the possibility that fibers resemble prey items (Boerger et al., 2010) more closely than fragments, potentially resulting in intentional ingestion due to mistaken identity. However, a contradictory finding to the involuntary ingestion emerged during the analysis of the types of MPs consumed. All types were ingested by at least one individual, with the exception of blue fragments (fragB), which could indicate a degree of selectivity in capture. Given that fragments are denser and thus less likely to resuspend due to seahorse movement, a hypothesis emerges: fibers may have been ingested accidentally, while fragments may have been consumed voluntarily, particularly considering that blue fragments (fragB) were also absent from the GITs of wild seahorses.

The decision to withhold MPs for two days between different diet types was to evaluate MP accumulation. Evidence of accumulation was observed in most cases, as MPs continued to appear in the feces during this interval. Similar findings have been reported in studies involving seahorses (Jinhui et al., 2019) and other fish species, where microplastic accumulation has been noted in gills, liver, and intestinal tissues (Okamoto et al., 2022). In the present study, it was observed that most white fragments (fragW) retrieved from fecal samples exhibited an orange hue, suggesting they had been ingested and excreted, and possibly remained longer in the gastrointestinal tract (GIT). Although reports on MP retention and excretion times in fish under laboratory conditions remain limited, previous studies suggest variability in excretion times (Okamoto et al., 2022). This range was from immediate excretion, with most MPs being expelled within the first four hours, to gradual excretion over 24 hours, and delayed excretion, where MPs remain in the GIT for over 16 hours (Okamoto et al., 2022). Such findings underscore the potential for MPs to persist in fish gastrointestinal tracts (GIT) for extended periods following ingestion (Okamoto et al., 2022). In our study, MPs were recovered only after 24 hours, thwarting the calculation of retention times but confirming their persistence within the GIT.

Comparing and integrating findings from the different components of this study, no significant differences ($p > 0.05$) in microplastic consumption were found among individuals, whether in wild or captive seahorses. This refutes the hypothesis that MP ingestion is linked to individual personality or behavioral traits (Chen et al., 2022), reinforcing that exposure to MPs and feeding mechanics are primary determinants in the consumption of such materials (Vroom et al., 2017; Jinhui et al., 2019; McGoran et al., 2017). Therefore, it is imperative to control microplastic pollution in marine environments to mitigate potential ecological risks to these fish (Sun et al., 2019; Jinhui et al., 2019; Onay et al., 2023). Although definitive conclusions on the reasons behind MP ingestion remain elusive, the findings support the hypothesis that seahorses exhibit a degree of selection during hunting, leading to unintentional MP consumption due to prey resemblance (Boerger et al., 2010; Ory et al., 2017). Moreover, some of the ingested MPs are likely accidental, as suction-feeding can unintentionally draw in smaller MPs suspended in the water column (Lusher et al., 2013).

Fibers emerged as the predominant MP type across all components of the study, including sediment substrates and gastrointestinal tract contents of both wild and captive seahorses. Their lightweight and diminutive size may facilitate accidental ingestion (Liu et al., 2022; Lusher et al., 2013) and could also lead to confusion with prey based on size and colour similarities (Liu et al., 2022; Boerger et al., 2010; Ory et al., 2017). To improve the MP characterization, methodologies such as micro-Fourier Transform Infrared Spectroscopy (µ-FTIR) or Laser Direct Infrared (LDIR) analysis could be employed. µ-FTIR is a fingerprinting technique that enables molecular-level characterization, allowing the identification and distinction of the different materials, to precisely identify polymer composition (Frias et al., 2014) and LDIR would allow for directly calculate the number of particles to analyze and determine the polymer type, abundance and size distribution (Liu et al., 2022). These approaches would facilitate the verification of identified materials as indeed plastics and enhance understanding of their potential sources.

In examining the coloration of MPs, black and blue were dominant in environmental sediment samples and wild seahorse GIT contents. However, in captivity, red and blue MPs were more prevalent. Previous studies have reported differences in colour preference between fish in laboratory conditions versus natural environments (Okamoto et al., 2022). Generally, vertebrates possess two types of photoreceptor cells: cones (enable colour vision during light conditions) and rods (do not distinguish among colours, are more sensitive under low-light conditions, meaning that they are used most heavily in the dark) (Okamoto et al., 2022). Bony fishes have four spectral cone types comprising alternating rows of double cones with red and green members and single blue and UV cones, allowing for colour recognition (Okamoto et al., 2022). Although Ria Formosa is a shallow-water environment, depth and visibility may still influence color perception, potentially affecting the detection and differentiation of certain colours, such as red, which is clearly visible under optimal lighting conditions, but may become indistinguishable in low visibility situations. This phenomenon was previously documented by Okamoto et al. (2022), who observed that under light conditions, some fish species exhibited a preference for red, yellow, and green MPs. However, during darker conditions, there was a notable decline in the ingestion of MPs, and the color preferences observed under brighter conditions dissipated. These findings underscore the critical role that ambient light and water clarity may play in the ability of marine organisms to differentiate between MPs and natural prey. Future experimental designs should incorporate fibers in a broader spectrum of colors (at least the same colours as fragments provided), to ensure a more comprehensive comparison between MP types and colors. Additionally, an alternative experiment could involve simulating the light conditions present in wild habitats, ensuring reliable comparisons between laboratory and field settings. This would help assess whether seahorses have distinct color perception and preferences when selecting prey or MPs.

Given the inherent sensitivity of studies involving MP analysis, minimizing external contamination is crucial. Ideally, the laboratory environment should be isolated, with restricted access during sample processing. Efforts to reduce plastic materials used in filtration, storage, and examination could include wearing only cotton clothing, using glass petri dishes, and selecting non-plastic filters for sample processing. Additionally, contamination control could be improved by exposing a Petri dish to laboratory air during sample processing to determine potential airborne MP deposition. Although all materials were cleaned with distilled water prior to use, these measures would enhance confidence in the accuracy of results, mitigating external contamination risks.

Considering the findings of this study, it became evident the complex relationship between microplastic exposure and ingestion processes, which are influenced by various factors, including habitat characteristics, environmental availability, and feeding behaviors. The existing literature has documented MP ingestion in different fish species, but seahorses appear to exhibit enhanced susceptibility, consuming MPs both intentionally and accidentally. Due to the limited research on this taxonomic group, further investigation is essential to better understand the specific impacts of MP ingestion on seahorse populations.

This study offered useful insights into the interactions between *H. guttulatus* and microplastics within both wild and controlled environments. However, additional studies are necessary to comprehensively assess the long-term physiological and ecological consequences of MP ingestion. This is particularly important as our findings indicated that MPs can be retained and are likely to accumulate overtime with increased exposure, raising significant concerns regarding potential health risks to seahorses.

Future research should focus on extended exposure trials that incorporate critical environmental factors, such as photic conditions, to elucidate their influence on MP ingestion (especially when it comes to colours) and retention rates. Additionally, refining analytical methodologies, such as µ-FTIR or LDIR, would enhance the identification and characterization of MPs, thereby improving our knowledge of their sources and broader environmental implications.

Given the widespread presence of MPs in marine ecosystems, it is crucial to implement robust mitigation strategies to reduce plastic pollution is a priority. Conservation initiatives must prioritize habitat protection, promote sustainable waste management practices, and advocate for policies designed to limit microplastic releases from urban and industrial sources. Addressing these challenges is essential for preserving the ecological integrity of marine ecosystems and ensuring the protection of vulnerable species, such as seahorses, from the risks associated with plastic contamination.

Conclusion

The increase in global plastic pollution in recent years has positioned microplastics as one of the most critical challenges for marine ecosystems, given their potential effects on aquatic organisms. While much research has been conducted on various marine species, studies focusing on seahorses remain limited, despite their role as sentinel species for environmental health due to their low mobility and sensitivity, making them valuable indicators of regional pollution levels and their impacts.

Although this study represents only a small fraction of microscopic plastic in this environment, the presence and exposure of MPs in Ria Formosa, with their direct impact on MP ingestion by *Hippocampus guttulatus*, are significant and concerning, reflecting the high levels of environmental contamination of this coastal ecosystem. Due to their suction-feeding behavior, often close to the sediment, it could be suggested that the ingestion of MPs could be involuntary. However, the experimental trial confirmed that the ingestion of MPs occurs both involuntarily and possibly due to confusion with natural prey, with fibers being the most frequently consumed MPs, both in the wild and in captivity, likely because of their size and morphology, which resemble natural prey. In addition, the prolonged retention of MPs in individuals suggests possible accumulation, which may cause physiological risks in the long term. Regarding colour, the absence of blue fragments ingestion in captivity proposes that there may be some colour selectivity, while the high consumption of red fragments in captive settings contrasts with their lower occurrence in the wild, possibly due to poor visibility and colour changes caused by environmental effects.

In conclusion, these results emphasize the urgent need for measures to reduce MP pollution, improve monitoring methodologies, and further investigation on the long-term effects of MP exposure on seahorses as well as their ingestion through the trophic chain. These findings and advancing research will not only deepen our understanding of MP dynamics but may also contribute to more effective management policies and conservation strategies, reinforcing the protection of seahorses and their habitat against the growing issue of plastic contamination.

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